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The role of progesterone in the biosynthesis of cortisol in human adrenal tissue.
E.H.D. Cameron, M.A. Beynon and K. Griffiths.

Corticosteroid synthesis in a clear cell adenoma: a time-based study.
E.H.D. Cameron and K. Griffiths.
J. Endocrinol. (1968), 41, 327-338.
(132) Ultramicrochemical studies on the site of formation of dehydroepiandrosterone sulphate in the adrenal cortex of the guinea-pig.
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In 'Advances in steroid biochemistry and pharmacology'.

(137) C₁₉-steroid biosynthesis in the human adrenal gland.
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(138) Ultramicrochemical and tissue culture studies of adrenal tissue.
In 'Tissue culture in medical research'. Eds. F. Jacoby, K.T. Rajan.

(139) The adrenal cortex.
K. Griffiths and E.H.D. Cameron.

(140) In vitro synthesis of steroids by a feminising adrenocortical carcinoma: effect of prolactin and other protein hormones.
Acta Endocr. (1976), 82, 561-571.
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Europ. J. Cancer (1972), 8, 131-137.

(154) Urinary aetiocholanolone in patients with early breast cancer from South East Scotland and South Wales.

(155) Circulating hormone concentrations in women with breast cancer.
Lancet (1976), 1, 1100-1102.

(156) Plasma-hormones during anaesthesia for breast surgery.
Lancet (1976), ii, 1416-1417.

(157) Circadian variation in urinary melatonin in clinically healthy women in Japan and the United States of America.
Experientia (1979), 35, 416-418.


C. Chronobiology: breast skin temperature rhythms and breast disease

(164) Breast skin temperature rhythms throughout the menstrual cycle.

(165) Prolactin and breast skin temperature rhythms in relation to breast cancer.
J. Endocrinol. (1980), 87, 28P-29P.

(166) Characterisation of breast skin temperature rhythms of women in relation to menstrual status.

(167) A fresh approach to breast cancer.

(168) Towards a chronopsy: Part III. Automatic monitoring of rectal, axillary and breast surface temperature and of wrist activity; effects of age and of ambulatory surgery followed by nosocomial infection.
Erna Halberg, Richard Fanning, Franz Halberg, Germaine Cornelissen, Douglas Wilson, Keith Griffiths and Hugh Simpson.
Chronobiologia (1981), 8, 253-269.

(169) A feasibility study for early detection of breast cancer using breast skin temperature rhythms.

(170) Bimodal age-frequency distribution of epitheliosis in cancer mastectomies. Relevance to Preneoplasia.
Cancer (1982), 50, 2417-2422.


D. Prolactin and breast cancer

(174) Response of plasma prolactin and growth hormone to insulin hypoglycaemia.
Lancet (1972), ii, 1283-1285.

(175) Basal prolactin blood levels in three strains of rat with differing incidence of 7,12-dimethylbenz(a)anthracene induced mammary tumours.

(176) Plasma prolactin in breast cancer.

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In 'Steroid receptors and hormone-dependent neoplasia'.
Eds. J.L. Wittliff & O. Dapunt, Masson Publ. USA Inc.

The binding of tamoxifen to oestrogen receptor proteins under equilibrium and non-equilibrium conditions.

The effect of tamoxifen and stilboestrol on plasma hormone levels in postmenopausal women with advanced breast cancer.

Biochemical effects of cytotoxic estrogens and cytotoxic antiestrogens.
In 'Cytotoxic estrogens in hormone receptor tumours',

The biochemistry of tamoxifen action.
In 'Advances in sex hormone research', Vol. 4

The biochemistry of tamoxifen therapy of breast cancer.

Cellular aspects of non-steroidal antiestrogen action.
Robert I. Nicholson, Peter Davies, Paul Daniel and K. Griffiths.
In 'XI. International Congress of Clinical Chemistry',
F. Steroid receptors and prognosis

(197) Endocrine aspects of primary breast cancer.  
K. Griffiths, P.V. Maynard, D.W. Wilson, Davies, P.  
I.C.I. Publ. Ltd. p.221-236.

(198) Relationships between oestrogen-receptor content and histological grade in human primary breast tumours.  

(199) Estrogen receptor assay in primary breast cancer and early recurrence of the disease.  

(200) Oestrogen receptors and prognosis in early breast cancer.  
Lancet (1979), i, 995-996.

(201) Clinical, pathological and biochemical aspects of oestrogen receptor in primary human breast cancer.  
Paul V. Maybard and Keith Griffiths.  
In 'Steroid receptor assays in human breast tumours'.  

(202) Relationship of oestrogen-receptor status to survival in breast cancer.  

(203) The relationship of oestradiol receptor (ER) and histological tumour differentiation with prognosis in human primary breast carcinoma.  
In 'Breast cancer. Experimental & Clinical aspects'.  
(204) Estrogen receptors in early breast cancer.
In 'Breast cancer. Experimental & Clinical aspects'.
Eds. H.T. Mourisden & T. Palshof, Pergamon Press,

(205) The prognostic value of estrogen receptor content of human breast tumours.
In 'Steroid receptors and hormone dependent neoplasia'.
Eds. J.L. Wittliff & O. Dapunt. Masson Publ. USA Inc.,

(206) Relationship between primary breast tumour receptor status and patient survival.
Cancer (1980), 46, 2765-2769.

(207) Estrogen receptors and breast cancer.
Environmental Health Perspectives (1981), 38, 143-146.

(208) Steroid receptors in early breast cancer: value in prognosis.

(209) Quantitative oestradiol receptor values in primary breast cancer and response of metastases to endocrine therapy.

(210) Oestrogen-receptor status and sites of metastasis in breast cancer.

(211) A prognostic index in primary breast cancer.
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(218) Development of statistical and analytical techniques for
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D.W. Wilson, S.J. Gaskell, D.R. Fahmy, B.G. Joyce,
G.V. Groom, K. Griffiths, K.W. Kemp, A.B.J. Nix and
R.J. Rowlands.
In 'Radioimmunoassays', Ed. Ch.A. Bizollon, Elsevier/
p.281-294.

(219) Analytical performance characteristics and chronobiological
considerations in quality control.
D.W. Wilson, A.B.J. Nix, R.J. Rowlands, K.W. Kemp and
K. Griffiths.
In 'Quality control in clinical endocrinology'.
Eds. D.W. Wilson, S.J. Gaskell and K.W. Kemp, Alpha Omega
Publishing Ltd., Cardiff, 1979, pp.5-18.

(220) Observations on principles of quality control appropriate
to clinical endocrinology.
K.W. Kemp, A.B.J. Nix, R.J. Rowlands, D.W. Wilson and
K. Griffiths.
In 'Quality control in clinical endocrinology'. Eds.
D.W. Wilson, S.J. Gaskell and K.W. Kemp, Alpha Omega
Publishing Ltd., Cardiff, 1979, p.136-141.
(221) Application of Cusum techniques to monitor error in hormone assays.
In 'Quality control in clinical endocrinology'. Eds.

(222) A computer program for monitoring and analysing internal quality control data.
In 'Quality control in clinical endocrinology'.

(223) Advantages of Cusum techniques for quality control in clinical chemistry.

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I. STUDIES ON THE FUNCTIONAL ZONATION OF THE ADRENAL CORTEX.

A. FUNCTIONAL ZONATION OF THE ADRENAL CORTEX.
STEROID SECRETION BY SLICES OF ZONA FASCICULATA AND ZONA RETICULARIS TISSUE OF THE HUMAN ADRENAL CORTEX

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STEROID SECRETION BY SLICES OF ZONA FASCICULATA AND ZONA RETICULARIS TISSUE OF THE HUMAN ADRENAL CORTEX

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We have shown (Grant, Symington & Duguid, 1957) that the administration of ACTH to human subjects prior to adrenalectomy results in an increased ability of the resected adrenal gland to hydroxylate steroids at the 11β-position (Fig. 1). Direct analysis of adrenal venous blood, collected during the operation, shows that the increased hydroxylation is accompanied by an increased secretion of cortisol (see Table). Furthermore, there appears to be a good correlation between the steroid 11β-hydroxylation, cortisol formation, and the degree of lipid depletion of the adrenal cortex observed histologically. Exposure to ACTH results in a lipid-depleted cortex in which many of the clear cells of the fasciculata
Fig. 2. Clear-cell-zone slice cut from adrenal cortex of gland removed surgically from human subject. (Haematoxylin and eosin.) \( \times 70. \)

Fig. 3. Compact-cell-zone slice cut from adrenal cortex of gland removed surgically from human subject. (H. & E.) \( \times 70. \)
zone have been replaced by, or have been transformed into compact cells, characteristic of the reticular zone (see Symington, p. 12). It thus appeared that steroid biosynthesis and secretion might be associated with the compact cell whereas the clear cell stores precursor, largely in the form of cholesterol esters.

In order to investigate this possibility, slices about 0.3 mm. thick were cut from cortical tissue of adrenal glands removed surgically from patients with carcinoma of the breast. The slices were cut in a direction parallel to the capsule, using a modified Stadie-Riggs wet tissue microtome and with the gland chilled to about 0° C. It was found possible, with practice, to distinguish roughly between fasciculata and reticularis tissues by the pronounced yellowish colour of the former and red-brown colour of the latter.

Since, however, the two tissues invaginate one another, more precise means of distinguishing slices of the different zones were sought. Because of the relatively large size of the fasciculata clear cells as compared with the reticularis compact cells, and because the amount of deoxyribonucleic acid (DNA) per cell is supposed to be constant, it might be expected that the DNA content of a given weight of fasciculata tissue would be less than that of reticularis tissue. Also the cholesterol content of fasciculata tissue should be higher and the nitrogen content lower than in the case of reticularis tissue. Determinations of DNA, cholesterol and total nitrogen were therefore used as an aid in the identification of the zones from which the slices had been cut. In addition small pieces were removed from the edges of the slices for histological examination. The histological appearance of tissue slices and the results of chemical analysis are shown in Figures 2, 3 and 4.

Rather to our surprise we found that homogenates prepared

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Blood flow (ml./min.)</th>
<th>Blood cortisol (µg./ml.)</th>
<th>Blood cortisol (µg./min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without ACTH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.25</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>2.3</td>
<td>3.4</td>
</tr>
<tr>
<td>100 i.u. ACTH/day for 4 days before operation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.8</td>
<td>3.2</td>
<td>5.8</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>3.8</td>
<td>7.6</td>
</tr>
</tbody>
</table>
from slices of both reticularis and fasciculata zones show an equal ability to effect 11β-hydroxylation in vitro. Hence the increased 11β-hydroxylation observed in ACTH-treated glands cannot be attributed solely to the greater number of compact cells in these glands. Moreover, when ACTH was administered, prior to the removal of the adrenal gland, homogenates of both zones showed an increase in 11β-hydroxylating activity. Thus the fasciculata zone cannot be regarded merely as a storage zone.

We next turned our attention to the short-term action of ACTH which might be investigated by exposing adrenocortical slices to this hormone in vitro. We used the technique of Saffran and Schally (1955), in which about 40 mg. slices are incubated in a Krebs-Ringer bicarbonate medium. A preliminary 1-hour incubation is allowed to wash preformed steroid out of the slices. This is followed by 2 hours' incubation without ACTH and finally 2 hours' with ACTH. After the second and third incubations the medium is collected for steroid analysis. The results are shown in Figure 5. It is well known that the steroid hormones, cortisol and corticosterone, are not stored in the gland. Moreover, the first pre-incubation period in this technique permits the ‘washing out’ of preformed steroids. We are thus safe in assuming that the values shown represent newly synthesized steroid and not a release of preformed steroid stored in the tissues.

We have thus reached the interesting conclusion that the fasciculata clear-cell zone is particularly sensitive to ACTH and that the reticularis compact-cell zone shows a relatively smaller response to this hormone. Part at least of the response of the compact-cell

![Fig. 4. Typical results of chemical analyses of fasciculata clear-cell-zone and reticularis compact-cell-zone slices. R = reticularis. F = fasciculata.](image-url)
zone to ACTH must be attributed to the clear cells which invariably contaminate the compact-cell-zone slices. Thus it may be that the compact- and clear-cell zones produce the steady 'baseline' amounts of cortisol and corticosterone required for 'normal' daily living, but on stress or ACTH administration the fasciculata cells come into action and are well provided with reserve precursor from which to synthesize increased amounts of steroid hormone.

These matters could be more readily investigated if we had relatively pure preparations of clear and compact cells. We are investigating the possibility of obtaining these by treating the adrenal tissue with trypsin and floating off the presumably less dense lipid-laden clear cells. Unfortunately, however, these cells seem especially easily damaged and whereas one can obtain compact cells with comparative ease, very few intact clear cells are found after treatment with trypsin.

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REFERENCES

A BIOCHEMICAL INVESTIGATION OF
THE FUNCTIONAL ZONATION OF
THE ADRENAL CORTEX IN MAN

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A Biochemical Investigation of the Functional Zonation of the Adrenal Cortex in Man

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SINCE THE early histologic studies of Harley (1) and Arnold (2), who described the division of the adrenal cortex into three distinct concentric layers of cells (an outer zona glomerulosa, central zona fasciculata and inner zona reticularis), much has been written concerning the physiologic role of these zones. The literature on this subject, up to early 1960, is reviewed in detail by Deane (3). It appeared to be well established by this date that the cells of the zona glomerulosa, in a number of species, including man, are the sole site of aldosterone biosynthesis. The biosynthetic role of the fascicular and reticular zones was, however, less clearly defined.

Information on the functional zonation of the adrenal cortex has been derived from investigations of glands from various species, although these glands are known to differ histologically (4, 5) and biochemically (6). Our interest has been predominantly in the function of the "clear" cells of the zona fasciculata and the "compact" cells of the zona reticularis in man (4, 7).

This paper presents the results of biochemical investigations designed to throw further light on these matters.

Materials and Methods

Adrenal glands. Glands were removed surgically from patients with metastatic mammary carcinoma and immediately placed in a container in crushed ice. Some patients received 40 units of ACTH (1.5 units/mg) intramuscularly twice daily for 4 days prior to operation.

Slices of adrenal tissue. The following operations were performed at about 0°C. Adhering fat and connective tissue were removed, and a representative cross section of the gland was removed for histologic examination. The rest of the gland was then slit lengthwise in a plane parallel with the flat surface, and blood clots, medulla and obvious metastatic tumor were removed and discarded. The cortex was then cut into flat pieces about 1 X 1.5 cm. From these, pieces about 0.3 mm thick were cut in a small Stadie-Riggs wet tissue microtome (8). With experience, slices containing mainly brownish reticular tissue could be separated from those of yellow fascicular tissue. Further identification of the predominant cell types in these slices was achieved by histologic and chemical examinations described later.

The slices were incubated whole or homogenized as described below.

Homogenates. Cell free homogenates (20% w/v) of slices were prepared in 0.25 M sucrose containing 0.12 M nicotinamide, using a vertical stroke homogenizer of 8 mm bore (9).
Chemical examination. The total nitrogen content of dialyzed portions of homogenates was determined by digestion with sulfuric acid and a mercury catalyst, followed by direct nesslerization. DNA was determined by the method of Paul (10), and free and ester cholesterol by the method of Sperry and Webb (11).

Incubations and determination of steroids. 11-Deoxycorticosterone (DOC, 60 \(\mu\)g in 10 \(\mu\)l propylene glycol) was incubated with homogenates (200 \(\mu\)l) of fascicular or reticular tissue and cofactor-buffer solution. This solution, conditions and controls were similar to those described by Brownie and Grant (12), but conducted on a smaller scale. DOC and corticosterone were extracted from the incubation mixtures and determined by the methods of Grant (13).

At least 3 samples, each 50–80 mg, of slices taken from the fascicular and from the reticular zones were incubated in separate vessels in 1.5 ml Krebs-Ringer bicarbonate glucose solution, as described by Saffran and Bayliss (14). For the final incubations 200 mU ACTH were added to each vessel. Medium removed after the preincubation period was discarded. After subsequent incubations the medium was retained for steroid determinations. One ml volumes were extracted with ethylene chloride. The dry residue from the extracts was dissolved in ethanol and optical densities were observed (14). Ethanolic solutions were then evaporated and the residue was chromatographed on paper with known amounts of cortisol, corticosterone and cortisone on adjacent strips. A benzene: methanol: water solvent system was used (15). Known steroids were located on the chromatograms under ultraviolet light and extracted by soaking the paper overnight in ethyl acetate: methanol (1:1, v/v). Corresponding areas on the chromatograms of material from the incubations were treated in the same way. The amount of reducing substances in the residues from the extracts was determined by the blue tetrazolium method of Mader and Buck (16).

Results

Separation and characterization of fascicular and reticular tissue for biochemical study. As previously stated, it was usually possible to separate slices according to their brown or yellow color. Histologic examination of random samples of these slices indicated that the brown slices were mainly reticular tissue ("compact"

![Fig. 1. Slice of adrenocortical reticular tissue—mainly "compact" cells. (H. & E. Magnification ×175.)](image-url)
cells), whereas yellow slices were mainly fascicular tissue ("clear" cells) (Fig. 1 and 2). In what follows, "compact" cell tissue will be regarded as synonymous with reticular zone and "clear" cell tissue with fascicular zone tissue.

Fig. 2. Slice of adrenocortical fascicular tissue—mainly "clear" cells. (H. & E. Magnification X200.)

Fig. 3. Section of adult human adrenal cortex showing the irregular reticular-fascicular border. (H. & E. Magnification X90.)
The normal folding contour of the gland and irregular reticular-fascicular border (Fig. 3) sometimes made it difficult to obtain satisfactory slices. Preparation of slices of fascicular tissue is also often difficult with glands removed from patients treated with ACTH before operation on account of the decreased width of the fascicular zone in such glands (Fig. 4).

The higher cell density and the lower stainable lipid content observed in sections of the reticular tissue suggested that it should contain more nitrogen and DNA and less cholesterol than equivalent wet weights of fascicular tissue. In all cases in which histologic examination of the slices indicated that efficient separation of fascicular and reticular zones had been achieved, the total nitrogen and DNA contents of the reticular zone slices were higher than those of the fascicular zone and the cholesterol content was lower (Table 1 and Fig. 5).

Fig. 5 illustrates the proportions of free and esterified cholesterol found in homogenates of the separated fascicular and reticular tissues. The amounts of free cholesterol are similar in both zones. The esterified cholesterol, which makes up the greater proportion of the total cholesterol in the tissues, is, however, higher in the fascicular zone and varies in amount on stimulation of the gland by

![Diagram](image-url)

**Fig. 4.** Diagrammatic representation of the effect of administered ACTH and of stress on the histologic appearance of the adrenal cortex in man.
ACTH administered before operation.

**DOC hydroxylation by homogenates of fascicular and reticular tissue.** Paper chromatographic investigations confirmed earlier observations (17) that, under the conditions of homogenate incubation used, corticosterone is the only detectable hydroxylation product of DOC. Typical quantitative results are shown in Table 2. The amount of DOC metabolized by the homogenates was, therefore, taken as a measure of 11β-“hydroxylase” activity. It may be seen in Fig. 6 that the

![Free Cholesterol vs Total Cholesterol](image)

**Fig. 5.** Cholesterol content of fascicular and reticular tissue separated from human adrenal cortex. Mean values and ranges found are shown for 5 experiments with glands removed (a) without prior treatment with ACTH, (b) after administration of 40 units ACTH in twice daily for 4 days.

Nature and relative amounts of the ultraviolet light absorbing lipids produced. The

*Table 1. DNA and nitrogen content of slices of an adrenal gland*

<table>
<thead>
<tr>
<th>Tissue slices</th>
<th>Mean DNA content* (µg/100 mg wet wt of slices)</th>
<th>Mean total nitrogen* content (mg/100 mg wet wt of slices)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fascicular (F)</td>
<td>82.0 ± 2.6 (6)</td>
<td>0.94 ± 0.04 (6)</td>
</tr>
<tr>
<td>Reticular (R)</td>
<td>113.0 ± 6.0 (6)</td>
<td>1.20 ± 0.01 (6)</td>
</tr>
</tbody>
</table>

* Standard errors are given, and the number of determinations is shown in parentheses.
paper chromatographic behavior of the ultraviolet light absorbing lipids secreted by the fascicular and reticular slices suggests that the substance present in greatest amount is cortisol. Smaller amounts of substances behaving like cortisone, corticosterone and 11β-hydroxyandrost-4-ene-3,17-dione were also detected. Further evidence in support of the identification of these substances was obtained from an investigation of their reactions with blue tetrazolium, alkaline m-dinitrobenzene (Zimmermann reaction) and from the spectra of their solutions in concentrated H₂SO₄ (18).

The separation and determination of cortisol and corticosterone indicated that there was a greater production of cortisol than of corticosterone in response to ACTH added in vitro (Fig. 9).

**Discussion**

In this investigation it has frequently been possible to obtain a good separation of the two principal types of cells found in the adrenal cortex in man. These are the "clear" cells, characteristic of the zona fasciculata, and the "compact" cells, characteristic of the zona reticularis (4).

Chemical, as distinct from histochemical, methods have now shown that the "clear" cells contain relatively more cholesteryl ester and less nitrogen and DNA than the "compact" cells measured on a wet tissue weight basis. The amount of free cholesterol in both cell types is approximately the same (0.5–0.6 mg/100 mg tissue). The greater proportion of cholesterol in both cell types is in the ester form, and it is this form which decreases in amount on stimulation with ACTH. It may be noted that cholesteryl ester decreases in both the "clear" and "compact" cells. The amount of free cholesterol remains unchanged (Fig. 5). From the data available it is impossible to say whether the free cholesterol is

![Fig. 6. Hydroxylation of DOC by homogenates of fascicular and reticular tissue removed from human adrenal cortex. The mean values and ranges are shown for 5 experiments.](image)

![Fig. 7. Effect of ACTH on rates of ultraviolet absorbing lipid formation by slices of fascicular and reticular tissue from human adrenal cortex. Mean values and ranges are shown (a) for 6 experiments in which ACTH was not administered before operation, (b) for a control experiment in which ACTH was not administered in vivo nor added in vitro, (c) for 2 experiments in which 40 units ACTH was administered im twice daily for 4 days before operation.](image)
derived from reserves of ester cholesterol and is thus involved in corticosteroid biosynthesis, or whether it is merely a structural component of the cells, as suggested by Andersen and Sperry (19). It would seem reasonable to regard cholesterol ester as the precursor of corticosteroids. In vitro experiments in which cholesterol has been used as a precursor of steroid hormones do not preclude the possibility of preliminary esterification. Esterification might prevent oxidation of the Δ4-3β-hydroxy group prior to removal of the side chain by the C20-22 desmolase, thus avoiding the formation of cholest-4-en-3-one, which is known to be toxic (20) and to inhibit cholesterol biosynthesis (21).

Homogenates prepared from slices with a preponderance of "clear" cells show similar steroid 11β-"hydroxylase" activity to those from slices with a preponderance of "compact" cells. The similarity is observed whether the enzyme activity is based on the wet weight or the nitrogen content of the tissue samples taken. This result was unexpected in view of an earlier observation (17) that the 11β-"hydroxylase" activity of whole adrenocortical homogenates appeared to be directly proportional to the number of "compact" cells in the cortex. It must now be concluded that 11β-"hydroxylase" is not confined to the "compact" cell, and that the increased 11β-"hydroxylase" activity, observed in homogenates of adrenal glands from ACTH-treated patients (17), cannot be explained simply on the basis of the increased number of compact cells in these glands. It is possible that the increased activity may be attributed to the "compact" cells newly formed, under the influence of ACTH, from "clear" cells at the fascicular-reticular border (Fig. 3). The present technique of separating the zones of cells by slicing by hand would be inadequate to distinguish a zone of such active "compact" cells. Improved techniques, which should make this possible, are at present under investigation.

More mitochondria are observed in "compact" than in "clear" cells (22), and

---

**Table 2. 11β-Hydroxylation of DOC**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>DOC metabolized during the incubation (μg)</th>
<th>Rate of metabolism of DOC (μg DOC/mg N/hr)</th>
<th>Corticosterone formed during incubation (μg)</th>
<th>Rate of 11β-hydroxylation (μg corticosterone /mg N/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.2</td>
<td>70</td>
<td>25.0</td>
<td>72.5</td>
</tr>
<tr>
<td>2</td>
<td>24.7</td>
<td>71</td>
<td>26.0</td>
<td>73.8</td>
</tr>
</tbody>
</table>

---

**Fig. 8. Effect of ACTH on rates of ultraviolet absorbing lipid formation by slices of fascicular and reticular tissue from the human adrenal cortex.** ACTH was not administered before removal of the glands. Mean values and ranges for 3 observations are shown in Expt. 1 based on nitrogen content and in Expt. 2 based on DNA content of tissue.
it is known that $11\beta$-"hydroxylase" is a mitochondrial enzyme (23). Obviously, however, from the present results, $11\beta$-"hydroxylase" activity cannot be related to the number of mitochondria in the cell. Indeed, it is possible that not all "compact" cell mitochondria are involved in $11\beta$-hydroxylation.

The effect of ACTH on the formation of steroids by tissue slices containing "clear" and "compact" cells has been studied. Although both types of preparation respond, there is a significantly greater response with slices containing "clear" cells. Whether or not the response observed with "compact" cell tissue is due to the unavoidable presence of some "clear" cells in the slices cannot be decided at present.

Comparison of the in vitro activity of "clear" and "compact" cells from glands removed after ACTH administration is difficult, since the narrow zona fasciculata of such glands (Fig. 4) does not readily provide slices containing mainly "clear" cells. In experiments in which satisfactory slices were obtained [Fig. 7(c)], the biosynthetic activity of the "clear" cells without ACTH in vitro was significantly greater than usual, whereas that of the "compact" cells was about the same as usual. These observations suggest that it is the "clear" cells which show the greater response to ACTH in vivo.

The separation and determination of steroids formed by the slices shows that the most pronounced steroidogenic effect of ACTH added in vitro is on the formation of cortisol. The identification of $11\beta$-hydroxyandrost-4-ene-3,17-dione as a product of both "clear" and "compact"

Table 3. Ultraviolet absorbing lipid formation by fascicular tissue for periods up to six hours

<table>
<thead>
<tr>
<th>Medium</th>
<th>Incubation period</th>
<th>UV absorbing lipid formed (µg apparent cortisol)/100 mg tissue/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRB*</td>
<td>30 Min Pre-incubation</td>
<td>7.0</td>
</tr>
<tr>
<td>KRB</td>
<td>0–2 hr</td>
<td>7.0</td>
</tr>
<tr>
<td>KRB + ACTH</td>
<td>2–4 hr</td>
<td>25.0</td>
</tr>
<tr>
<td>KRB + ACTH</td>
<td>4–6 hr</td>
<td>24.5</td>
</tr>
</tbody>
</table>

* KRB = Krebs-Ringer bicarbonate glucose solution.
cells is not in keeping with those zonation theories which attribute adrenal androgen production to the reticular zone alone (24, 25). Deane (3) has, however, postulated that both zones are capable of androgen biosynthesis. The formation of testosterone from progesterone in vitro by both “clear” and “compact” cell zones, recently demonstrated in our laboratories (26), supports our present findings that both zones can contribute to adrenal androgen formation.

The investigations now reported establish unequivocally that both “clear” cell (fascicular) and “compact” cell (reticular) zones are able to synthesize and secrete $C_{19}$ and $C_{21}$ adrenal steroid hormones other than aldosterone.

In the light of present knowledge, our views on the functional zonation of the adrenal cortex in man are as follows. Aldosterone is formed in the zona glomerulosa cells. With regard to the biosynthesis of other $C_{21}$ and of $C_{19}$ adrenocortical steroids, the zona fasciculata and zona reticularis should be regarded as one functional unit. An earlier hypothesis from our group envisaged the “compact” cells of the zona reticularis as responsible for the biogenesis of steroid hormones and the “clear” cells of the zona fasciculata as a store of precursor of steroid hormone (4, 7). This hypothesis must now be modified to include the view that “clear” as well as “compact” cell zones contribute to the daily basal secretion of cortisol and adrenal androgens. The proportional contributions of the two zones are unknown. The observations of Oftad et al. (27), however, suggest that the contribution of cortisol from the “clear” cells may be considerable, and that adrenal androgens may come largely from the “compact” cells.

We consider that the principal effect of ACTH on steroid biosynthesis occurs in the “clear” cells. Observations on the rat (28–32), and the increased enzyme activity in cells of the fascicular-reticular border in man (33), suggest that it is these cells which are most affected. As stated above (see Fig. 4), adrenal glands removed after ACTH administration have very narrow “clear” cell zones. The “clear” cell tissue slices obtained from such glands are thus likely to have a high proportion of “active” cells of the border zone and it is not surprising, therefore, that their steroid biosynthetic activity is found to be high in vitro [Fig. 7(c)].

It is possible that the functioning of the “clear” cells of the zona fasciculata in man is a safety mechanism designed to provide additional cortical hormone in emergency. If the emergency is prolonged, the functioning of the “clear” cells may give time for the cortex to increase enzymes and structures required to meet the continued demand for cortical hormone. The changes in RNA and phospholipid (34) and increased uptake of amino acid (35, 36) by the adrenal cortex in response to ACTH would be in keeping with this theory.

Acknowledgments

The authors are grateful to the Scottish Hospital Endowments Research Trust for a grant from which the expenses of this work were defrayed. They are indebted to surgeons of Edinburgh and Glasgow hospitals for their cooperation in providing the adrenal glands for investigation. They also wish to thank Mr. J. Jacobson, Mr. D. Hay, Jr. and Mr. T. Parker for skilled technical assistance. The helpful advice of Dr. J. Paul in connection with DNA determinations is also gratefully acknowledged.

References

2. Arnold, J., Virchow Arch. 35: 64, 1866.
6. Grant, J. K.: In Comparative Biochemistry,
3β-HYDROXYSTEROID DEHYDROGENASE IN THE ADRENAL GLAND AND PLACENTA

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(Received 27 July 1964)

SUMMARY

3β-Hydroxysteroid dehydrogenase activity was studied histochemically in human, monkey, and rat adrenal glands and in human placentae. Tissue sections were incubated separately with each of the following substrates: (1) 3β-hydroxypregn-5-en-20-one (pregnenolone); (2) sodium 3β-sulphoxypregn-5-en-20-one (pregnenolonesulphate); (3) 3β-acetoxypregn-5-en-20 one (pregnenolone acetate); (4) 3β,16α-dihydroxyprogren-5-en-20-one (16α-hydroxyprogrenolone); (5) 3β,17α-dihydroxyprogren-5-en-20-one (17α-hydroxyprogrenolone); (6) ammonium 3β-sulphoxy-17α-hydroxyprogren-5-en-20-one (17α-hydroxyprogrenolone ammonium sulphate); (7) 3β-hydroxyandrost-5-en-17-one (DHA); (8) 3β-sulphoxyandrost-5-en-17-one (DHA sulphate); (9) 3β-acetoxyandrost-5-en-17-one (DHA acetate); (10) androst-5-ene-3β, 17β-diol (androstenediol).

The histochemical results obtained with pregnenolone and DHA as substrates resemble those described by other workers. Using pregnenolone sulphate and 17α-hydroxyprogrenolone sulphate, a strong histochemical reaction with diformazan deposition was found in the zona fasciculata of the adrenals of all species and in the placental syntrophoblast. With DHA sulphate an extremely weak histochemical reaction was obtained with the adrenal zona fasciculata, monofomazan only being deposited. The syntrophoblast, however, showed intense 3β-hydroxysteroid dehydrogenase activity when incubated with DHA sulphate. These results accord with recent findings regarding the secretion and metabolism of 3β-sulphoxysteroids.

A strong histochemical reaction was also obtained in both adrenal and placental tissues using 17α-hydroxyprogrenolone, 16α-hydroxyprogrenolone, androstenediol, pregnenolone acetate, and DHA acetate. These steroids have not previously been described as substrates for the histochemical demonstration of 3β-hydroxysteroid dehydrogenase in the adrenal or placenta.
INTRODUCTION

In recent years the histochemical localization of 3β-hydroxysteroid dehydrogenase activity has been well studied in the adrenal cortex (Wattenberg, 1958; Levy, Deane & Rubin, 1959; Cavallerò & Chiappino, 1961; Dawson, Pryse-Davies & Snape, 1961) and placenta (Goldberg, Jones & Turner, 1963). This enzyme catalyses the conversion of a steroid Δ⁴-3β-hydroxy group to a Δ⁴-3-oxogroup and the reduced nicotinamide-adenine dinucleotide (NADH₂) formed in the reaction can be coupled to the reduction of a tetrazolium salt by NADH₂: lipoamide oxidoreductase. Pregnenolone and DHA have been used as substrates for this histochemical reaction by most workers but 17α-hydroxyprogrenolone (Baillie & Griffiths, 1964a), 16α-hydroxyprogrenolone, pregnenediol and androstenediol (Baillie & Griffiths, 1965) have been shown to be satisfactory as substrates and in the foetal mouse testis enzyme-substrate specificity has been sufficiently pronounced to suggest the existence of more than one 3β-hydroxysteroid dehydrogenase (Baillie & Griffiths, 1964b). The recent demonstration of sulphokinase activity in adrenal tissue (Cohn, Mulrow & Dunne, 1963; Sneddon & Marrian, 1963; Wallace & Lieberman, 1963) directed attention towards 3β-sulphoxysteroids, and it has been shown in the Leydig cells of the testis that pregnenolone sulphate and 17α-hydroxyprogrenolone sulphate are better utilized in the histochemical demonstration of 3β-hydroxysteroid dehydrogenase activity than the free steroids. A poor reaction only was obtained with DHA sulphate (Baillie & Griffiths, 1965).

In the light of these observations, it was decided to investigate the histochemical reactivity of the adrenal cortex and placenta with particular respect to the sulphates of pregnenolone, 17α-hydroxyprogrenolone and DHA.

MATERIAL AND METHODS

Adrenal glands were obtained from two women operated on for mammary carcinoma, and from two rhesus monkeys and two rats; pieces of placental tissue of about 1 cm.³ were secured from four placentae removed at Caesarean section at term for obstetric reasons not related to placental function. In addition, placental tissue from a normally delivered woman was studied.

Each tissue sample was immediately frozen on solid carbon dioxide, sectioned at 15 μ in a cryostat maintained at −20°, and the sections attached to clean dry glass slides by transient thawing. Before incubation, the slides were brought to room temperature and dried in air. Sections from each specimen were incubated separately at 37° with the steroid listed below for 30 min. in the medium described by Wattenberg (1958) buffered at pH 7 with either phosphate or phthallate. The final concentration of steroid was 0-1 mm. Nitro-BT (2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene)-ditetrazolium chloride) (L. Light and Co.) was employed as a final electron acceptor. The steroids, dissolved in propylene glycol, were: (1) pregnenolone, (2) pregnenolone sodium sulphate, (3) pregnenolone acetate, (4) 16α-hydroxyprogrenolone, (5) 17α-hydroxyprogrenolone, (6) 17α-hydroxyprogrenolone ammonium sulphate; (7) DHA, (8) DHA sodium sulphate, (9) DHA acetate, and (10) androstenediol.
Preparation of 3β-sulphoxy-17α-hydroxy pregn-5-en-20-one, ammonium salt (17α-hydroxy pregnenolone ammonium sulphate)

17α-Hydroxy pregnenolone ammonium sulphate was prepared by the method of Sobel & Spoerri (1941). Solvents were removed under reduced pressure and the solid residue was triturated with 15 ml. chloroform in which the sulphating reagent is insoluble. The resulting slurry was filtered on a sintered glass funnel and the residue washed repeatedly with warm chloroform. The chloroform solution (approx. 250 ml.) was then shaken vigorously with 1 vol. of 2N-NH₄OH and after two further extractions with 1 vol. of 2N-NH₄OH, the combined ammoniacal layers were washed with 100 ml. chloroform. The 17α-hydroxy pregnenolone ammonium sulphate was extracted from the aqueous medium by the procedure of Edwards, Kellie & Wade (1953). The ether-ethanol extract was evaporated to dryness in a rotary evaporator and inorganic salts were removed by treating the residue with warm ethanol and filtering. The ethanolic solution was concentrated in vacuo and the 17α-hydroxy pregnenolone ammonium sulphate allowed to crystallize out at low temperature. A small amount (200 μg.) of the crystalline material was chromatographed on 3 g. of alumina. No 17α-hydroxy pregnenolone could be detected in the appropriate fraction with the sulphuric acid-ethanol reagent of Oertel & Eik-Nes (1959) in conditions which would normally detect 2 μg. A further 100 μg. of the residue was chromatographed on Whatman No. 1 paper in the ‘alkaline system’ of Schneider & Lewbart (1958). A single rather streaked spot was detected by the methylene-blue test (Crepy & Judas, 1960) for sulphate esters.

Examination of the sulphate ester by the Oertel & Eik-Nes (1959) reaction gave an assay of 102.8% on the basis of the formula 17α-hydroxy pregnenolone ammonium sulphate.

RESULTS

(1) Adrenal tissue

Similar histochemical results were obtained with the adrenal glands from the three species studied. Using the conventional substrates pregnenolone and DHA, 3β-hydroxysteroid dehydrogenase activity was localized by a heavy blue diformazan deposit in the zona fasciculata of the adrenal cortex (Pl. 1, fig. 1). Only a faintly positive reaction was observed in the zona glomerulosa and no significant formazan deposition was seen in the zona reticularis. A similar result was observed with 17α-hydroxy pregnenolone.

The results obtained with the sulphates are of particular interest. The diformazan deposition after incubation with pregnenolone sulphate (Pl. 1, fig. 2) and with 17α-hydroxy pregnenolone sulphate (Pl. 1, fig. 3) is very similar to that seen with the free steroids. In contrast however (Pl. 1, fig. 4), DHA sulphate was not utilized to any extent and only a slight pink monoformazan deposit could be detected in the zona fasciculata. Heavy diformazan deposition was also observed in the zona fasciculata after incubation with 16α-hydroxy pregnenolone (Pl. 2, fig. 5), androstenediol (Pl. 2, fig. 6) and the 3β-acetoxyl derivatives of pregnenolone (Pl. 2, fig. 7) and DHA (Pl. 2, fig. 8).
(2) Placental tissue

The chorionic villi of the human placenta at term are covered with a single layer of syntrophoblast although occasional cytotrophoblast cells can be recognized after a careful search, particularly on the placental septae.

Using pregnenolone, 17α-hydroxyprogrenolone, and DHA as substrates, intense 3β-hydroxysteroid dehydrogenase activity was seen in the syntrophoblast. The cytoplasm was coloured deep pink due to monoformazan deposition and contained abundant dark blue minute diformazan deposits.

Pregnenolone sulphate (Pl. 3, fig. 9), 17α-hydroxyprogrenolone sulphate (Pl. 3, fig. 10) and DHA sulphate (Pl. 3, fig. 11) were better utilized than the free steroids in that diformazan deposition occurred more rapidly in the syntrophoblast. With each steroid sulphate intense mono- and diformazan deposition was found in the trophoblast. Similar results were obtained with 16α-hydroxyprogrenolone (Pl. 3, fig. 12). Incubation with androstenediol resulted in little diformazan deposition although monoformazan could be detected in the syntrophoblast (Pl. 3, fig. 13). Use of the 3β-acetoxy derivatives of pregnenolone and DHA (Pl. 3, fig. 14) lead to heavy mono- and diformazan deposition in the syntrophoblast.

DISCUSSION

Several groups of workers have demonstrated histochemically that 3β-hydroxysteroid dehydrogenase in the adrenal cortex of various animals can utilize the substrate pregnenolone and DHA (Wattenberg, 1958; Levy et al. 1959; Cavallero & Chiappino, 1961; Dawson et al. 1961) and the histochemical distribution in the adrenal cortices of rat, monkey, and man, described in the present report is in accord with earlier descriptions. Histochemically the greatest activity of the 3β-hydroxysteroid dehydrogenase is found in the zona fasciculata, and this has been confirmed biochemically with DHA as substrate (Cameron, Magrini & Grant, 1964; Grant, 1964). Our observations indicate that the same histochemical distribution is obtained with 17α-hydroxyprogrenolone, androstenediol and 16α-hydroxyprogrenolone as substrates.

Recently evidence has been obtained that DHA is secreted by the human adrenal cortex, mainly as the sulphate ester (Baillie, 1960; Vande Wiele, McDonald, Bollé & Lieberman, 1963) and sulphokinase activity has also been demonstrated in human adrenal tissue (Cohn et al. 1963; Wallace & Lieberman, 1963). DHA sulphate gave only a faintly positive histochemical reaction when used as substrate for the enzyme 3β-hydroxysteroid dehydrogenase in the adrenal cortices of rat, monkey and man. A strong reaction in the zona fasciculata was, however, obtained when the sulphates of pregnenolone and 17α-hydroxyprogrenolone were used. This strong reaction was obtained even in a medium containing phosphate ions, known to inhibit sulphatase activity (Roy, 1957; Burstein & Dorfman, 1963). While the precise metabolic role of the steroid sulphates in steroid hormone biosynthesis remains unknown, several reports of the direct metabolism of steroid sulphates have recently appeared. Of especial interest are the conversions of (a) pregnenolone-3H sulphate-35S to 17α-hydroxyprogrenolone-3H sulphate-35S in vitro using homo-
3β-ol dehydrogenase in adrenal and placenta

3β-ol dehydrogenase is an enzyme that catalyzes the conversion of 3β-hydroxysteroid dehydrogenase to 3β-hydroxy-5α-steroids in the presence of NAD. This enzyme is involved in the biosynthesis of pregnenolone from cholesterol. In the adrenal gland, the enzyme is responsible for the conversion of cholesterol to pregnenolone, while in the placenta, it plays a role in the conversion of cholesterol to progesterone.

The enzyme is also involved in the metabolism of estrogen. In the adrenal gland, it is involved in the conversion of estrogen to androgens. In the placenta, it is involved in the conversion of estrogen to progesterone.

The enzyme is found in the cytoplasm of steroidogenic cells. It is composed of a single polypeptide chain and is activated by the binding of NAD to its substrate binding site. The enzyme is also inhibited by the binding of NADH to its product binding site.

The enzyme is regulated by a number of factors, including the concentration of NAD and NADH, the pH of the cell, and the presence of other enzymes in the cell. The enzyme is also regulated by the presence of other steroids, such as progesterone and androgens.

In conclusion, 3β-ol dehydrogenase is a key enzyme in the biosynthesis of steroids. Its activity is regulated by a number of factors, and it plays a crucial role in the metabolism of steroids in the adrenal gland and placenta.

The authors are indebted to Professor Goligher, University Department of Surgery, Leeds General Infirmary, for the adrenal glands; and to Dr D. McKay Hart, Snr., Director of Obstetrics and Gynaecology, Stobhill Hospital, Glasgow for placenta: one of us (E.H.D.C.) is indebted to I.C.I. for a personal grant.

REFERENCES


3β-ol dehydrogenase in adrenal and placenta


DESCRIPTION OF PLATE

PLATE 1

Fig. 1. 3β-Hydroxysteroid dehydrogenase in the zona fasciculata of the human adrenal cortex using DHA as substrate. (x 130.)

Fig. 2. Intense diformazan deposition with pregnenolone sulphate as substrate in the zona fasciculata of the monkey adrenal cortex. (x 90.)

Fig. 3. Diformazan deposition in the zona fasciculata of the human adrenal cortex after incubation with 17α-hydroxyprogrenolone sulphate. (x 180.)

Fig. 4. Monoformazan in the zona fasciculata of the monkey adrenal cortex after incubation with DHA sulphate. (x 90.)

PLATE 2

Fig. 5. Deposition of diformazan in the zona fasciculata of the human adrenal cortex after incubation with 16α-hydroxyprogrenolone. (x 90.)

Fig. 6. Deposition of diformazan in the zona fasciculata of the human adrenal cortex after incubation with androstenediol as substrate. (x 90.)

Fig. 7. Diformazan deposition in the monkey adrenal cortex after incubation with pregnenolone acetate. (x 90.)

Fig. 8. Diformazan distribution in the human adrenal cortex after incubation with DHA acetate. (x 130.)

PLATE 3

Fig. 9. Diformazan deposition in the syntrophoblast after incubation of human placental tissue with pregnenolone sulphate. (x 90.)

Fig. 10. 3β-Hydroxysteroid dehydrogenase activity in the syntrophoblast of human placental tissue after incubation with 17α-hydroxyprogrenolone sulphate. (x 90.)

Fig. 11. 3β-Hydroxysteroid dehydrogenase activity in the syntrophoblast of human placental tissue after incubation with DHA sulphate. (x 90.)

Fig. 12. Formazan deposition in the syntrophoblast of human placental tissue after incubation with 16α-hydroxyprogrenolone. (x 90.)

Fig. 13. A few diformazan particles in the syntrophoblast of human placental tissue after incubation with androstenediol. (x 280.)

Fig. 14. Deposition of mono- and diformazan in the syntrophoblast of human placental tissue after incubation with DHA acetate. (x 90.)
METABOLISM OF TESTOSTERONE BY ADRENAL TISSUE OF THE GOLDEN HAMSTER AND IDENTIFICATION OF 19-HYDROXYLATED STEROIDS

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Earlier studies (Griffiths, 1963) have shown that the adrenal gland of the golden hamster possesses an active 19-hydroxylating enzyme system. This was demonstrated by measuring the conversion of 11-deoxycorticosterone (DOC) to 19-hydroxy DOC. The ability of this enzyme system to hydroxylate C_{19}-steroids has now been investigated.

Adrenal glands (300 mg.) from twelve male hamsters were homogenized in 500 μl.

Table 1. Summary of evidence for the identification of steroids from the incubated adrenal tissue of the golden hamster

For key to solvent systems see Griffiths, Grant & Symington, (1964).

<table>
<thead>
<tr>
<th>Material (esterified substrate)</th>
<th>Chemical reaction</th>
<th>Chromatographic mobility identical with that of</th>
<th>Solvent systems (d.p.m./μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iv</td>
<td>Acetylation</td>
<td>19-Hydroxytestosterone</td>
<td>PB 11, PG/T, B 10, ET 19, F/C 8587</td>
</tr>
<tr>
<td></td>
<td>Alkali treatment</td>
<td>19-Nortestosterone</td>
<td>PB 11 8450</td>
</tr>
<tr>
<td>vi</td>
<td>Acetylation</td>
<td>11β-Hydroxytestosterone</td>
<td>B 10, F/C 8259</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>Adrenosterone</td>
<td>PB 21 4856</td>
</tr>
<tr>
<td></td>
<td>Oxidation and reduction</td>
<td>11β-Hydroxytestosterone diacetate</td>
<td>F/Cyc, P 10 5040</td>
</tr>
<tr>
<td>vii</td>
<td>Reduction</td>
<td>19-Hydroxytestosterone diacetate</td>
<td>F/Cyc, P 10 4556</td>
</tr>
<tr>
<td></td>
<td>Reduction and acetylation</td>
<td>19-Hydroxytestosterone acetate</td>
<td>F/Cyc, P 10, PG/T 5217</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>11β-Hydroxyandrostenedione</td>
<td>PB 21, PB 11, PG/T, B 10 11578</td>
</tr>
<tr>
<td></td>
<td>Alkali treatment</td>
<td>19-Norandrostenedione</td>
<td>PB 21, PB 11, PG/T, B 10 11578</td>
</tr>
<tr>
<td>ix</td>
<td>Reduction</td>
<td>11β-Hydroxyandrostenedione</td>
<td>B 10 11567</td>
</tr>
<tr>
<td></td>
<td>Reduction and acetylation</td>
<td>11β-Hydroxyandrostenedione acetate</td>
<td>F/Cyc, P 10 11567</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>Adrenosterone</td>
<td>PG/T, F/Cyc, B, PB 21 11050</td>
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<tr>
<td></td>
<td>Oxidation and reduction</td>
<td>11-Oxotestosterone acetate</td>
<td>F/Hex 11050</td>
</tr>
<tr>
<td>x</td>
<td>Oxidation</td>
<td>Testosterone</td>
<td>PB 21, P 10 358</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Androstenedione</td>
<td>PB 10 358</td>
</tr>
<tr>
<td></td>
<td>Reduction</td>
<td>Adrenosterone</td>
<td>PG/T, PB 21, PB 11 358</td>
</tr>
<tr>
<td></td>
<td>Reduction and acetylation</td>
<td>11-Oxotestosterone acetate</td>
<td>PB 21, PB 11 375</td>
</tr>
</tbody>
</table>
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0.25 m-sucrose containing 0.12 m-nicotinamide, as described earlier (Griffiths, 1963). The homogenate (450 µl.) was incubated with 3 µc [4-14C]testosterone (sp. ac. 8.8 µc/µmole) for 60 min. at 37° in 900 µl. medium containing 677 µmole tris buffer, pH 7.4, 675 µmole potassium chloride, 135 µmole potassium fumarate, 2.94 µmole NADP, 49.7 µmole MgSO₄, 14.7 µmole ATP, 22.5 µmole glucose-6-phosphate (G6P) and 1.0 Kornberg unit of G6P-dehydrogenase. Procedures for the isolation of the neutral steroid fraction, paper chromatography systems and steroid estimation and identification were those described by Griffiths, Grant & Whyte (1963) except that radioactivity was measured by liquid scintillation counter. 19-Norsteroids were prepared according to Meyer (1955).

Table 2. Radioactivity of substances isolated from incubations of adrenal tissue of golden hamster as a percentage of total radioactivity in the neutral fraction

<table>
<thead>
<tr>
<th>Metabolites (in order of decreasing polarity)</th>
<th>% of total radioactivity</th>
<th>Rₚ value in solvent system</th>
<th>Metabolites (in order of decreasing polarity)</th>
<th>% of total radioactivity</th>
<th>Rₚ value in solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin material (i)</td>
<td>10-6</td>
<td>0</td>
<td>19-Hydroxyandrostenedione (vii)</td>
<td>13-5</td>
<td>0.18</td>
</tr>
<tr>
<td>Unknown (ii)</td>
<td>2-4</td>
<td>0</td>
<td>Unknown (viii)</td>
<td>23-8</td>
<td>0.28</td>
</tr>
<tr>
<td>Unknown (iii)</td>
<td>2-8</td>
<td>0</td>
<td>11β-Hydroxyandrostenedione (ix)</td>
<td>7.7</td>
<td>0.51</td>
</tr>
<tr>
<td>19-Hydroxytestosterone (iv)</td>
<td>17-9</td>
<td>0.05</td>
<td>Testosterone (x)</td>
<td>1.0</td>
<td>0.67</td>
</tr>
<tr>
<td>Unknown (v)</td>
<td>2-1</td>
<td>0.09</td>
<td>Adrenosterone (xi)</td>
<td>7.1</td>
<td>0.75</td>
</tr>
<tr>
<td>11β-Hydroxytestosterone (vi)</td>
<td>0-5</td>
<td>0.11</td>
<td>Unknown (xii)</td>
<td>9-7</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Evidence was obtained for the identification of 19-hydroxytestosterone (iv), 11β-hydroxytestosterone (vi), 19-hydroxyandrostenedione (vii), 11β-hydroxyandrostenedione (ix) and adrenosterone (xi) as metabolites of testosterone (x) (Table 1). The proportions of the various metabolites are shown in Table 2. Ninety per cent of the incubated radioactivity appeared in the neutral steroid fraction. The identity of metabolite (viii) has not yet been completely established. However, it is not a 6α-, 6β-, 11β-, 16α or 19-hydroxylated androstenedione or testosterone. Paper chromatographic results suggest that this metabolite is a 16-oxo-17-hydroxysteroid.

We are grateful to Dr M. Ehrenstein, University of Pennsylvania Hospital, Philadelphia, for the 19-hydroxysteroids.

REFERENCES

DETERMINATION OF THE 11β-HYDROXYLASE ACTIVITY IN MICROGRAM SAMPLES OF TISSUE; ITS QUANTITATIVE HISTOLOGICAL DISTRIBUTION IN THE RAT ADRENAL, AND THE INFLUENCE OF CORTICOTROPHIN

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SUMMARY

Recent biochemical studies on the functional zonation of the human adrenal cortex suggest that one of the earlier principal effects of corticotrophin (ACTH) on steroid biosynthesis occurs at the fasciculo-reticular border. This report deals with procedures for the study of the 11β-hydroxylase activity in microgram amounts of adrenal tissue and with the distribution of this activity throughout the adrenal cortex of the rat. ACTH administration is shown to stimulate the 11β-hydroxylase activity at the fasciculo-reticular border.

INTRODUCTION

Recent biochemical studies on the functional zonation of the human adrenal cortex (Griffiths, Grant & Symington, 1963) support the concept that one of the earlier principal effects of corticotrophin (ACTH) on steroid biosynthesis occurs in the clear cells at the fasciculo-reticular border. Observations that the activity of certain enzymes at this border increases after administration of ACTH, glucose-6-phosphate dehydrogenase (G6PD) in man (Studzinski, Symington & Grant, 1962) and G6PD and 6-phosphogluconic acid dehydrogenase (6PGD) in the rat (Greenberg & Glick, 1960) add further support to the view that there is an active border zone in certain adrenal cortices.

Since an increased activity of steroid 11β-hydroxylase has been demonstrated in homogenates of the human adrenal after ACTH administration (Grant, Symington & Duguid, 1957) which may have been due to an increased activity at the fasciculo- reticular border (Griffiths et al. 1963), it was decided that a quantitative study of the histological distribution of this enzyme in the adrenal cortex would be of interest.

The present report deals with procedures to study the 11β-hydroxylase activity in microgram amounts of adrenal tissue, the distribution of this activity throughout the adrenal cortex of the rat, and the effect of ACTH.

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MATERIALS AND METHODS

Animals

Adrenal glands were obtained from male albino rats (Swiss white strain) weighing about 250 g. and approximately 3-4 months old. They were killed by a single blow on the head at 1.30 p.m. ACTH (Organon), 25 i.u./kg. body wt. in 0.5 ml. 0.9% NaCl solution, was administered s.c. to some of the animals 3 hr. before they were killed. Control rats received a subcutaneous injection of 0.5 ml. saline.

Adrenal tissue

The adrenals were quickly removed, frozen in solid carbon dioxide and transferred to filter paper saturated with saline in a glass vial standing in a cryostat at -15°. Cylinders of tissue were bored out from the glands and fresh-frozen microtome sections (diameter, 2 mm.; 16 μ thick; volume, 0.5 μl.) were then prepared as described previously (Grunbaum, Geary & Glick, 1956). One section was brushed flat on to a glass slide for histological examination, and the next two sections were placed in glass-stoppered reaction tubes (30 mm. long, 4 mm. internal diameter) for enzyme assay. This sequence was followed through the entire cortex and into the medulla of the adrenal gland. Sections on the slide were stained with haematoxylin and Sudan IV for histological examination.

Conditions of incubation

The series of reaction tubes, standing in holes drilled from an aluminium block (to maintain the contents of the tube at a low temperature outside the cryostat), were removed from the cryostat and 10 μl. of 0.25 m-sucrose containing 0.12 m-nicotinamide (Handler & Klein, 1942) were added. To each tube was also added 800 μg. 11-deoxycorticosterone (DOC), dissolved in 150 μl. ethanol. In experiments in which [1,2-3H]DOC was used, 800 μg. steroid of specific activity 70-5 mCi/μg. were added. The procedure for addition of DOC to the reaction tubes is described in a later section. Using a dispensing self-adjusting pipette (Glick, 1961, p. 74), 15 μl. of a medium containing the following constituents were added to the reaction tubes: 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS), pH 7.4, 1.13 μmole; potassium chloride, 1.125 μmole; magnesium sulphate, 82.9 μmole; potassium fumarate, 225 μmole; ATP (disodium salt), 24.65 μmole; glucose-6-phosphate (G6P) (dipotassium salt), 375 μmole; NADP (monosodium salt), 4.9 μmole; G6P-dehydrogenase, 0.02 Kornberg unit.

The tissue was immediately disintegrated in the medium by rapid vibration or 'buzzing' (Glick, 1961, 1962), and the tubes were placed in a water bath at 37° for 30 min.

Addition of DOC to the reaction tube

A cathetometer and a micropipetting device were used (Glick, 1961, pp. 188, 181, respectively). The pipette consisted of drawn-out capillary tubing, bent at the tip and ground to a point with a dental grinding disk. The pipette is siliconed by dipping in and running through a 5% solution of a silicone oil in chloroform, and
Determination of $11\beta$-hydroxylase activity

after drying and rinsing, it is calibrated with a solution of Rose Bengal (absorption max. 552 mμ) (Glick, 1961). The coefficient of variation of 10 replicate volumes at the 150 mμ level was 1-1%.

Extraction of steroids

Benzene : chloroform (6:1 v/v), 100 μl., was added to the reaction tubes to stop the reaction. In experiments in which [1,2-3H]DOC was used, 20 μg. of non-radioactive corticosterone were added in the benzene : chloroform mixture.

Medium and solvent were mixed with a rotating stainless steel wire bent in the shape of a figure 8. The mixture was centrifuged, and the solvent transferred to a 1-0 ml. conical centrifuge tube (Misco, California). The medium was extracted twice more with 100 μl. benzene : chloroform and the combined solvent mixture evaporated. The residue was partitioned between 200 μl. methanol : water (7:3, v/v) and 200 μl. petrol ether, (b.p. 60–80°) The petrol ether was removed and discarded, and the methanolic solution evaporated in vacuo.

After extraction with benzene : chloroform, the aqueous phase containing the protein was dried in vacuo, and the residue taken up in 1-0 ml. of \(\pi\text{-}\text{NaOH}\) for protein-nitrogen (Pn) analysis using the bromsulphalein method of Nayyar & Glick (1954).

Assay of $11\beta$-hydroxylase

The enzymic activity was measured by three separate procedures:

(a) A major portion of the dried methanolic extract was transferred to fluorimeter cuvettes and the corticosterone present in the extract determined by a fluorimetric procedure described later. For this simple procedure, use is made of the fact that DOC, in amounts as high as 800 mg., has no effect on the fluorescence of corticosterone present in a ratio of 20:1 (w/w) respectively. Also for this procedure, measured amounts of corticosterone (150 mg.) were added to reaction tubes containing incubation medium and two tissue sections, and these were taken through the extraction procedure to check steroid recoveries.

(b) The methanolic extract was chromatographed on Hyflo supercel columns using the solvent system benzene : hexane (7:3, v/v) as mobile phase and methanol : water (7:3, v/v) as stationary phase. Sufficient Hyflo supercel (1 g./1-0 ml.) was mixed with the stationary phase and a slurry prepared by addition of the mobile phase. The slurry was packed into columns (45 mm. long, 3-8 mm. in diameter) with a small perforated steel disk packer (Howard & Martin, 1950). The elution pattern (Fig. 1) shows that corticosterone was recovered in the fraction corresponding to 1-4–2-8 ml. of the eluate. The dried eluate was taken up in 100 μl. ethanol, 80 μl. were transferred to a glass tube microcuvette, 30 mm. long with inner and outer diameters of 3 and 5 mm. (Glick & von Redlich, 1963), and the ethanol was removed in vacuo. The fluorescence reagent (water : H₂SO₄ : ethanol, 25:75:10, by vol.) was added in 60 μl. portions at 30 sec. intervals to each tube and ‘buzzed’ immediately. The tubes were spun and the fluorescence read 50 min. after addition of the acid-ethanol reagent. Conditions for determination of the fluorescence are described in a later section.

Also, as in extraction (a) controls were set up. The mean recovery of corticosterone added to incubation medium and tissue samples taken from eleven regions through the adrenal cortex was 88-7% ± 0-83 (s.d.).
(c) In some experiments, 56.4 mμC [1,2-3H]DOC were added to the reaction mixture. In these experiments, 20 μg. non-radioactive corticosterone contained in the first 100 μl. of the benzene:chloroform mixture were added to the incubate. The dried methanolic extracts were transferred in methylene chloride to chromatoplates, spread with Merck silica-gel-G containing a phosphor (H 913, Levy West Laboratories, Harlow), and run in the solvent system chloroform:methanol:water (188:12:1, by vol.).

Fig. 1. Separation of 400 mμg. of [1,2-3H]DOC and corticosterone on HyflosuperCel columns (length, 45 mm., diameter, 3.8 mm.) using the solvent system benzene:hexane (7:3, v/v); methanol:water (7:3, v/v); 0.2 ml. fractions were collected. Aliquots of each fraction were taken for counting and determination of fluorescence (×—×), as described in text, p. 4. CPM = radioactivity in counts/min. in the DOC (●——●) fractions.

Corticosterone was recovered by scraping off the silica-gel, transferring it to a small glass tube (drawn out at the bottom and plugged at the tip with glass wool) and eluting the steroid with 3.0 ml. methanol. Recoveries of corticosterone from the plate were approximately 80%.

The methanol was evaporated and the steroid concentration determined by its selective absorption at 240 mμ in 2.0 ml. ethanol using the 0.5 ml. microcells of the Unicam SP 500 spectrophotometer. Samples were also removed for counting with a Packard Tri-Carb scintillation spectrometer and for determination of corticosterone by fluorescence.

**Determination of corticosterone by fluorescence**

The conditions under which corticosterone fluoresces and the amount of corticosterone recovered from HyflosuperCel columns (as in b) were determined using the Aminco-Bowman spectrophotofluorometer with a cuvette adapter for the small tubes (Glick & von Redlich, 1963). The fluorescence of corticosterone, determined directly after extraction (as in a) and after thin-layer chromatography (as in c), was measured with a Locarte fluorimeter.

Fluorescence was developed by adding the reagent (water:H₂SO₄:ethanol, 25:75:10, by vol.) to the dry steroid and leaving the mixture for 50 min. at room temperature. Extra pure acid (Merck) was used in all determinations. These conditions were determined by experiments shown in Figs. 2 and 3, which demonstrate
the effect of time and concentration of aqueous sulphuric acid on the development of corticosterone fluorescence, and the difference between the use of aqueous and ethanolic sulphuric acid.

Fluorescence was measured at 530 mμ in an Aminco–Bowman spectrophotofluorometer with an activation wavelength of 475 mμ; \( \frac{1}{16} \) in. slits were used in the incident and fluorescent beams with \( \frac{1}{8} \) in. slits on each side to reduce scatter, and a \( \frac{3}{16} \) in. slit was placed in front of the photomultiplier. Narrow waveband (primary 5-0 mμ, secondary 5-5 mμ), all-dielectric filters (Barr & Stroud, Ltd. Glasgow) transmitting light at 475 and 530 mμ were used with the Locarte fluorimeter.

**RESULTS**

The conditions for incubation were based on those described by Brownie & Grant (1954). The substrate concentration and duration of incubation were established after experiments (Fig. 4) in which samples of rat adrenal homogenates, containing amounts of Pn equivalent to two tissue sections were incubated as described in the text.

It was also found that ‘buzzing’ the tissue before incubation, in contrast to merely incubating slices, resulted in a 2–3-fold increase in enzyme activity. For these experiments, whole adrenals were frozen, mounted on a microtome and sectioned until the medulla was reached. These sections were discarded. Further sections were then obtained containing all types of adrenal tissue, which were used for the incubations.
No decrease in enzyme activity was observed when half of an adrenal gland was kept frozen for 60 min., homogenized, and 10 μl. samples incubated, compared with the homogenate prepared directly from the other half and left standing at 4° for 60 min.

Fig. 4. Effect of substrate concentration (A) and time of incubation (B) on the 11β-hydroxylase activity of 10 μl. samples of rat adrenal homogenates (prepared from adrenal glands of different animals) containing amounts of tissue protein nitrogen (Pn) equivalent to two tissue sections. Ten μl. homogenate were incubated with 15 μl. reaction medium, as described in the text, p. 2.

Since DOC in amounts as high as 800 μg. has no effect on the fluorescence of corticosterone present in a ratio of 20:1 (w/w) respectively, for the simple 11β-hydroxylase assay, the whole of the incubation extract was subjected to the acid conditions for fluorescence development. Figure 5 shows the quantitative histological
Determination of $11\beta$-hydroxylase activity through the rat adrenal cortex in control animals and animals treated with ACTH. The profile of $11\beta$-hydroxylase distribution shows a peak at the region of the fasciculo-reticular border in the adrenal glands from ACTH-treated rats.

**Fig. 5.** Quantitative histochemical distribution of $11\beta$-hydroxylase activity, based on tissue protein nitrogen (Pn), throughout the rat adrenal cortex, determined by procedure (a) (see text, p. 3). Vertical bars represent ranges of activity determined at various regions in the cortex (four expts.). Upper diagram: controls (0.5 ml. 0.9% NaCl solution injected s.c. 3 hr. before killing. Lower diagram: ACTH-treated animals (25 i.u./kg. in 0.5 ml. saline, injected s.c. 3 hr. before killing). Regions marked G, F, R and M denote respectively zona glomerulosa, zona fasciculata, zona reticularis and medulla; mixed zones are designated by both letters.

Procedure (b) for the assay of $11\beta$-hydroxylase was used to confirm that the activity at the border zone after ACTH did in fact represent corticosterone formation. The corticosterone formed was measured after purification by partition chromatography on Hyflosuperce columns (Fig. 1).

The lower limit for estimation by this procedure was approximately 2.0 mg. corticosterone. Experiments in which tissue slices were incubated without the steroid, showed that the 'blank' fluorescence is the same for each type of adrenal tissue, and that these readings were only 2–3 times higher than the fluorescent...
reagent blank. Therefore, high fluorescence readings, associated with fasciculo-reticular border zone tissue, are not due to extractable endogenous material fluorescing under these conditions. The quantitative distribution of 11β-hydroxylase determined by this procedure (Fig. 6) was similar to that shown in Fig. 5.

Radioisotope dilution, followed by isolation on thin-layer chromatoplates (procedure c) was also used to study 11β-hydroxylase activity in the rat adrenal cortex since certain endogenous materials in rat tissue enhance corticosterone fluorescence without themselves fluorescing under these acid conditions. The results are shown in Fig. 7. The increase in 11β-hydroxylase activity at the fasciculo-reticular border was confirmed.

**DISCUSSION**

A variety of proportions of sulphuric acid to ethanol have been used in various laboratories to develop fluorescence from corticosteroids, and the measurement of the fluorescence has been made at different intervals after addition of the acid solution to the steroid. The lack of general agreement on optimal conditions has been pointed out by Braunsberg & James (1960) who concluded that empirical tests are required for each compound. The optimal conditions they found for the measurement of the fluorescence of corticosterone and cortisol are similar to those reported in the present study. Subsequently, for analysis of cortisol in human plasma, Braunsberg & James (1962) employed 75% ethanolic sulphuric acid and read the fluorescence after 6 min.

In their procedure for the determination of corticosterone and cortisol in sub-
Determination of 11β-hydroxylase activity

milligram amounts of adrenal tissue, Glick, von Redlich & Levine (1964) used 65% ethanolic acid and measured the fluorescence without waiting. Different brands of sulphuric acid were found to give widely different blank fluorescences. Steenburg & Thomasson (1964) obtained maximum fluorescence intensity for cortisol with 75%

![Graph](image_url)

Fig. 7. Quantitative histochemical distribution of 11β-hydroxylase activity based on tissue protein nitrogen (Pn), in the rat adrenal cortex, determined by procedure (c) (see text, p. 4). Vertical bars represent ranges of activity determined at various regions in the cortex (two expts.); upper diagram, controls, and lower diagram, ACTH-treated animals, the same as in Fig. 5. Regions marked G, F, R and M, denote respectively zona glomerulosa, zona fasciculata, zona reticularis and medulla; mixed zones are designated by both letters.

Ethanolic sulphuric acid, and allowed exactly 15 min. between addition of the acid and measurement of the fluorescence. Neither Glick et al. (1964) nor Steenburg & Thomasson (1964) tried aqueous acid as employed in this investigation.

Early work on human adrenal glands (Grant et al. 1957) had shown that the 11β-hydroxylase activity of homogenates of the whole gland appeared to be directly proportional to the number of compact cells in the reticular zone in the cortex. Later experiments (Griffiths et al. 1963), however, failed to show any difference between homogenates from slices of fascicular and reticular tissue to produce 11β-hydroxylation of DOC. It had to be concluded that the increased 11β-hydroxylase activity in homogenates of adrenal glands from ACTH-treated patients (Grant et al. 1957)
could not be explained simply by the increased number of compact cells in these glands. The increased activity was attributed therefore to the compact cells, newly formed under the influence of ACTH, from the clear cells of the fascicular zone at the fasciculo-reticular border (Griffiths et al. 1963).

The results reported in this paper offer further evidence that, in the rat, one of the effects of ACTH administration is the stimulation of 11β-hydroxylase activity at the fasciculo-reticular border. The rat has an adrenal cortex with a well-defined fascicular and reticular zone comparable to that found in man (Symington, 1960). These experiments would, therefore, further support the concept of an active fasciculo-reticular border region proposed in current theories concerning the functional zonation of the adrenal cortex (Griffiths et al. 1963). This concept is also supported by other findings which demonstrate the stimulant effect of ACTH on G6PD and 6PGD (Greenberg & Glick, 1960; Studzinski et al. 1962) at the fasciculo-reticular border of the adrenal cortices of rat and man. Peak concentrations of other physiologically active constituents (phenolsulphatase, Glick & Stecklein, 1956; succinic dehydrogenase, Glick & Greenberg, 1958; biotin, Ferguson & Glick, 1961), in the fasciculo-reticular border region of the rat adrenal, that were not influenced by ACTH treatment, have been reported in earlier quantitative histochemical studies.

ACTH appears to have a number of effects: it stimulates the secretion of cortisol from slices of human fascicular tissue incubated in Krebs–Ringer bicarbonate glucose solution (Griffiths et al. 1963), and it increases steroid biosynthesis in the rat adrenal (Sheppard, Swenson & Mowles, 1963). This may be due to the effect of ACTH on the stage in the steroid biosynthetic process between cholesterol and pregnenolone (Stone & Hechter, 1954), and may be the principal mechanism for the provision of additional cortical hormone in an emergency. ACTH also appears to stimulate the activity of specific enzymes which may be directly or indirectly involved in the biosynthesis of adrenal steroids, namely that of G6PD and 6PGD, which provide NADPH for steroid hydroxylations, and that of the 11β-hydroxylase which is involved in the final reaction of the biosynthetic chain for adrenocortical hormone synthesis.

However, certain discrepancies in the literature remain to be clarified. Thus, Hilf & Burnett (1964) found that ACTH caused an increase in G6PD activity in the regenerating rat adrenal, although they failed to observe this effect in the normal gland in an earlier study (Hilf, Burnett & Borman, 1962). Kuhn & Kissane (1964) also failed to find an effect of ACTH on G6PD or 6PGD in the normal rat adrenal, but they reported that salt deprivation elicited an increase of G6PD activity in the glomerular zone, and treatment with cortisone decreased the activities of both G6PD and 6PGD in the fascicular and reticular zones. No obvious explanation of these inconsistencies is apparent at this time.

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REFERENCES


Oestrogen Biosynthesis in vitro by Adrenal Tissue from the Golden Hamster

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There is little direct evidence to show that the normal adrenal cortex synthesizes oestrogens from C19 steroids. Oestrogens have been found to be synthesized from testosterone in the ovary (Baggett, Engel, Savard & Dorfman, 1956; Wotiz, Davis, Lemon & Gut, 1958), the testis (Baggett et al. 1959), the placenta (Ryan, 1959) and once in a human adrenal carcinoma (Baggett et al. 1959). Engel (1962) stated that, although there was a substantial amount of indirect evidence to suggest that the normal adrenal cortex secretes oestrogen, there was no direct proof of this, and Engel & Dimoline (1963) failed to obtain convincing evidence for the aromatization of C19 steroids by adrenal tissue from patients with metastatic carcinoma of the breast. He suggested that an extra-adrenal conversion of C19 steroids into oestrogen (West, Damast, Sarro, Phillips & Pearson, 1956) may be at least as important as oestrogen secretion by the cortex. Recent investigations with the adrenal gland of the golden hamster (Griffiths, 1963; Griffiths & Giles, 1965), demonstrating an active 19-hydroxylating enzyme system in this tissue, suggested that this gland may be of use in studying oestrogen synthesis by normal adrenal tissue. The ability of the golden hamster adrenal gland to aromatize testosterone is now described.

Adrenal glands (113 mg.) from 12 male hamsters were homogenized in 1-0 ml. of 0-25 m sucrose containing nicotinamide (0-12 n) in a Philpot & Stanier (1956) homogenizer. The homogenate (0-9 ml.) was incubated with 27-8 μmole of [1,2-3H]testosterone (specific activity 1-41 μC/μmole) for 60 min. at 37° in 1-0 ml. of a medium containing tris buffer, pH 7-4, KCl, fumarate, NADP, MgSO4, ATP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (Griffiths & Giles, 1965). The purity of the incubated testosterone was checked by radiochromatogram scanning. The reaction was stopped by adding 3-0 ml. of ethanol. Carrier steroids (300 μg. of each in 0-5 ml. of ethanol) were added to the incubation mixture: 17α-oestrone [3-hydroxyoestr-1,3,5(10)-triene-17-one], 16α-hydroxyoestrone [3,16β-dihydroxyoestr-1,3,5(10)-triene-17-one], oestradiol-17β [oestratriene-3β,16α,17β-diol], oestradiol-17α [oestratriene-3β,16β,17β-diol], 11β-hydroxyoestrone [3,11β-dihydroxyoestr-1,3,5(10)-triene-17-one] and 11β-hydroxyoestradiol-17β [oestratriene-3α,11β,17β-triol]. After mixing, steroids were extracted with 30 vol. of acetone and the phenolic steroid fraction was isolated as described by Griffiths, Grant, Browning, Whyte & Sharp (1966).

All chromatography was performed on glass plates (20 cm. × 20 cm.) spread with thin layers of Merck silica gel HF254/366. Oestrogens and their derivatives can be detected on this material either by absorption or fluorescence by using short-wave (254 nm) or long-wave (366 nm) u.v. light. The phenolic steroid fraction was chromatographed on four thin-layer plates with solvent system A (cyclohexane-ethyl acetate, 11:9, v/v). Bands of steroid were eluted with ether. Steroids with Rf values corresponding to oestrone (Rf 0-68), oestradiol-17α (Rf 0-57) and oestradiol-17β (Rf 0-50) were eluted and re-run in solvent system A. 16α-Hydroxyoestrone (Rf 0-37), 11β-hydroxyoestrone (Rf 0-30) and 11β-hydroxyoestradiol-17β (Rf 0-08) were eluted and re-run in the more polar solvent system B (benzene-ethyl acetate, 9:11, v/v).

Portions of the steroids were then rechromatographed on thin layers directly or after derivative formation (Griffiths, Grant & Whyte, 1965) and specific activities measured. Oestrogen methyl ethers were prepared as described by Brown (1955). For specific activity determinations, oestrogens and their derivatives were determined by a Kober reaction (Brown, 1955), and the radioactivity in steroid residues was measured with a Packard Tri-Carb liquid-scintillation spectrometer. Phosphor in the thin-layer chromatograms did not interfere with scintillation counting nor with the Kober reaction.

Evidence for the formation of oestrone, oestradiol-17β and oestradiol-17α from testosterone in vitro by hamster adrenal tissue is presented in Table 1. The percentage conversion from the precursor is low. No radioactivity was found to be associated with the carrier 11β-hydroxyoestrone after it had been converted into the 3-methyl ether or to the acetate. Only suggestive evidence was obtained for the formation of either 11β-hydroxyoestradiol-17β or a 16-oxygenated
Table 1. Evidence for the identification of the oestrogens isolated from the incubation of testosterone with hamster adrenal tissue

Experimental details are given in the text. The percentages of total radioactivity incubated represent minimal values since tritium may be lost from the 1,2-position in the A-ring by the aromatization reaction.

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>Chemical reaction</th>
<th>Chromatographic mobility identical with that of</th>
<th>Solvent system (Rf)</th>
<th>Specific activity (disintegrations/min./µmole)</th>
<th>Percentage of total radioactivity incubated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrone</td>
<td>—</td>
<td>Oestrone</td>
<td>A (0-68)</td>
<td>8885</td>
<td>0-011</td>
</tr>
<tr>
<td>Oestradiol-17â</td>
<td>Reduction</td>
<td>Oestradiol-17â</td>
<td>A (0-50)</td>
<td>8640</td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17â</td>
<td>Methylation</td>
<td>Oestrone 3-methyl ether</td>
<td>A (0-88)</td>
<td>8281</td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17â</td>
<td>Oxidation</td>
<td>Oestrone</td>
<td>A (0-50)</td>
<td>51437</td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17â</td>
<td>Methylation</td>
<td>Oestradiol-17â 3-methyl ether</td>
<td>A (0-69)</td>
<td>52826</td>
<td></td>
</tr>
<tr>
<td>11â-Hydroxy-oestradiol-17â</td>
<td>—</td>
<td>11â-Hydroxyoestradiol-17â</td>
<td>A (0-08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16â-Hydroxy-oestrone/16-oxo-oestradiol</td>
<td>—</td>
<td>16â-Hydroxyoestrone</td>
<td>B (0-50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17â</td>
<td>Reduction</td>
<td>16-Epioestriol</td>
<td>A (0-28)</td>
<td>6377</td>
<td></td>
</tr>
</tbody>
</table>

Oestrogen. Recovery of these carrier steroids was low after initial purification on thin-layer chromatography. The specific activity of 11â-hydroxy-oestradiol-17â diacetate only, run in solvent system B (Rf 0-88), was determined. Alkaline treatment in the fractionation procedure results in the isomerization of 16â-hydroxyoestrone to 16-oxo-oestradiol-17â [3,17â -dihydroxyoestra-1,3,5(10)-trien-16-one] (Fishman, 1960), and these steroids are not resolved in solvent system A or B. The yield of the resultant carrier 16-oxo-oestradiol-17â was low and the specific activity of one derivative, 16-eioestriol [oestra-1,3,5(10)-trien-3,16â,17â-triol], was determined (Table 1).

This work was submitted by J. R. L. as part of a Thesis for the degree of B.Sc. (Honours) in Biochemistry in the Department of Biochemistry, University of Glasgow.


It is ten years since Haynes & Berthet (1957) proposed a theory to explain how the adrenocorticotrophic hormone (ACTH) stimulates the adrenal cortex to increase its output of steroids. Despite continuous investigations since then, at the molecular and the whole gland level, our understanding is still far from complete. A number of excellent reviews have appeared (Yates & Urquhart, 1962; Hilf, 1965; Pastan, 1966). It is, however, our opinion that certain aspects of the matter may not have received adequate attention. The work of Professor Symington and his colleagues (Dobbie, Mackay & Symington, 1967) reveals how unsatisfactory our knowledge of the anatomy of the adrenal cortex in man has been. As a result of this work, we are only now beginning to understand how constituents of the blood plasma, including corticotrophin, are transported to the adrenocortical cells. Without this knowledge, we can never hope to know how the concentration of corticotrophin to which the cells are exposed varies within the adrenal gland. In considering the action of corticotrophin very few investigators have taken into account the fact that the adrenal cortex contains a variety of tissues in its different zones. Mention may have been made of the fact that the zona glomerulosa is little influenced by corticotrophin, but possible differences of effect on cells of the fascicular and reticular zones have been largely ignored. Because of its convenient size and ready availability, the adrenal gland of the ox has been studied extensively. Its morphology is, however, quite different from that in man, the species in which we might be expected to be most interested (Symington, 1960). The human adrenal gland, which is also of a very convenient size for study, has been the subject of most of our biochemical investigations. It was for a time fairly readily available in a fresh and, if free from secondary tumour deposits, in a presumed normal state, at surgical adrenalectomy for the treatment of carcinoma of the breast. This treatment is, however, much less frequently resorted to at the present day, and this most valuable material is now rarely available. Finally, the recent availability of synthetic polypeptides with corticotrophin-like activity and having shorter lengths of the sequences of amino acids found in the naturally
occurring corticotrophins, offer the interesting prospect of studying the biochemical activities of different parts of the molecule in some detail. On various occasions in the past, activities of corticotrophin preparations from pituitaries have been suggested to be due to contaminants of the preparations. With the synthetic material, such possibilities can be eliminated. Our biochemical investigations have been concerned mainly with the site of action of corticotrophin within the adrenal cortex in man and the action of synthetic corticotrophin.

The problem is complicated by the dichotomy of action of corticotrophin which is most evident when we consider the effects of the hormone administered to man. There is an almost immediate increase in adrenal blood flow, cortisol secretion and cortisol:corticosterone ratio. A slower, prolonged tropic effect on the adrenal gland is also later observed if stimulation is continued. We were able to study the rapid effects of ACTH during cannulation of adrenal veins of patients with breast cancer undergoing adrenalectomy (Grant, Forrest & Symington, 1957). Administration of as little as 10 units of ACTH intravenously doubled or trebled the rate of blood flow, increased the cortisol secretion, measured in μg per min, and increased the cortisol:corticosterone ratio from about 3 to about 10, as compared with the pre-ACTH values. There was no evidence from a number of similar observations that the abnormal conditions of collection of adrenal venous blood provoke maximal stimulation of the gland by endogenous ACTH (Yates & Urquhart, 1962). The conditions for collection of the blood during animal surgery may constitute a greater stress. Professor Symington’s histological and histochemical investigations of the glands subsequently removed showed no evidence of increased ACTH action, but this would not be expected to appear for some time. The expected morphological and histochemical changes were seen in glands removed after exposure to 80 units of ACTH administered daily for 4 days prior to surgery. But, even in these cases, further stimulation could be obtained on administration of more ACTH during cannulation of the adrenal vein.

Symington, Duguid & Davidson (1956) were among the first to observe the increase in adrenocortical RNA on administration of ACTH to human subjects. The role of RNA in protein synthesis was just beginning to be understood at this time and the significance of this effect of ACTH was not at first appreciated. In subsequent investigations (Grant, Symington & Duguid, 1957) growth of the adrenal cortex (increased thickness of cortex), altered morphology and increased steroid 11β-hydroxylation, in response to prolonged administration of ACTH to the patients before adrenalectomy, were demonstrated. Others, notably Nugent, Eik-Nes, Samuels &
Tyler (1959), observed that prolonged stimulation with ACTH renders the adrenal cortex more sensitive to the hormone, so that, in order to maintain a constant rate of cortisol secretion, amounts of ACTH administered must be continually decreased, a point which we shall return to in the next section.

**SITE OF ACTION OF CORTICOTROPHIN WITHIN THE ADRENAL GLAND**

Working with fresh human adrenal tissue, we were anxious to know if the large clear cells of the zona fasciculata and the more compact cells of the zona reticularis differed in their response to corticotrophin. A good separation of tissues containing the different cells was obtained by means of a small Stadie Riggs wet tissue microtome. With these preparations, we were surprised to find that only the clear cells of the zona fasciculata respond to corticotrophin in vitro with an increased production of adrenocortical steroids (Fig. 1). Such in vitro experiments may, of course, only give some indication of the ‘short term’ action of corticotrophins. The compact cells of the zona reticularis are undoubtedly influenced in the ‘long term’ by increased concentrations of corticotrophins as indicated by the changes in, for example, RNA content already referred to, and by the increasing sensitivity of the cortical output of steroids to prolonged stimulation.
Compared with the clear cells, the compact cells are deficient in cholesterol esters, the precursors of the steroid hormones. They may depend on the plasma for the supply of these precursors. This offers an explanation of their failure to respond to corticotrophins in vitro which has not yet been explored thoroughly.

Fig. 2. Effect of Corticotrophin Organon in vivo on the cholesterol content of different zones of adrenal glands surgically removed from human subjects.

If the patient received 80 units of natural corticotrophin daily for 4 days prior to the second stage of a bilateral adrenalectomy, severe lipid depletion was observed histologically and biochemically (Fig. 2, Griffiths, Grant & Symington, 1963). In these circumstances it was difficult to obtain satisfactory slices of the fascicular zone tissue, which was greatly decreased in amount. Results of some experiments with tissue slices obtained under these circumstances suggested that greater steroid production was occurring in the slices which contained cells from both zones. However, the
slicing technique was not sufficiently good at the time to investigate this matter in detail.

The next contribution was made by Dr Studzinsky, working in our laboratory (Studzinsky, Symington & Grant, 1962). He found that of three NADP-linked dehydrogenases, glucose 6-phosphate, 6-phosphogluconic acid and isocitric dehydrogenase, in the fresh adrenal cortex of human subjects only the first showed greater activity or increased amount in vitro when corticosterone was administered (Fig. 3). The increase in dehydrogenation did not parallel the increase in weight of the gland. On studying the distribution of dehydrogenation of glucose 6-phosphate throughout the gland, the reason becomes evident. In the case of what might be called the ‘resting’ adrenal gland, removed without prior administration of corticosterone, there is a uniform distribution throughout the gland. If, however,
the gland had been exposed to endogenous corticotrophin, so that the histological appearance of the cortex is similar to that seen following severe stress, then the glucose 6-phosphate dehydrogenase activity shows a sharp rise in the region where the fascicular and reticular zones meet. The enzyme activity is also invariably higher in the stimulated than in the unstimulated reticular zone (Fig. 4).

![Graph](image-url)

**Fig. 4.** Effect of Corticotrophin Organon *in vivo* on the distribution observed *in vitro* of glucose 6-phosphate dehydrogenation in the adrenal cortex in man *in vitro*. ●, After ACTH; ○, without ACTH.

It was obvious that a more sophisticated method was needed to dissect the different zones of the adrenal cortex for biochemical study. The histochemical techniques of Grunbaum, Geary & Glick (1956) appeared to be the methods of choice. Unfortunately, at this time human material was extremely scarce and further evidence of special biosynthetic activity at the fascicular–reticular border had to be obtained with rat adrenal glands. These were rapidly removed and frozen in solid CO₂. Cylinders of frozen tissue, 2 mm in diameter, were then drilled out from the gland in the cryostat. Sections were cut from these at 16 μ. Some were stained with sudan for lipid and used to indicate the position, within the cortex, of other sections which were used to investigate the site of production of the rat adrenocortical hormone corticosterone. This sequence was followed through the entire cortex. Deoxycorticosterone was used as substrate in the incubations of the tissue sections and the production of hormone observed in two ways, by measuring the fluorescence due to the corticosterone
formed or by an isotopic procedure. These experiments showed that corticotrophin administration resulted in a sharp increase in hormone synthesis at the fascicular–reticular border (Fig. 5, Griffiths & Glick, 1966). Of course, it cannot be presumed that the same observation will be made in man.

**CONCLUSION**

It is our speculative conclusion, from these observations, that synthesis of new steroid biosynthetic enzymes, under the influence of rising concentrations of corticotrophin, occurs in the clear cells of the fascicular–reticular border. These cells, being endowed with an abundance of steroid hormone precursor in the form of cholesterol esters, show particularly marked biosynthetic activity. It is also possible that these cells are so located in the cortex that they are exposed to the highest concentrations of corticotrophin.
THE ACTION OF $\beta^{1-24}$ SYNTHETIC CORTICOTROPHIN ON THE ADRENAL CORTEX

A number of polypeptides share with natural corticotrophin the ability to stimulate the adrenal cortex to secrete steroid hormones. One of these, a tetracosapeptide, having the same sequence of amino acids as the $N$-terminal 24 amino acids of natural corticotrophins, was synthesized by Kappeler & Schwyzer (1961). This substance, now referred to as 'Synacthen', was made available to us by CIBA Laboratories in 1963. In collaboration with A. P. M. Forrest of the Surgical Unit, Royal Infirmary, Cardiff, it was of interest to investigate the action of this substance on the adrenal cortex of patients undergoing adrenalectomy for the treatment of carcinoma of the breast. The majority of the patients had already had their pituitary function abolished or greatly diminished by prior implantations of yttrium and were receiving 37-5 mg of cortisone daily. By the use of Synacthen, a pure substance of known composition, it might safely be assumed that changes observed in the adrenal cortex are not due to contamination by substances such as vasopressin or growth hormone, which may have been present in preparations of natural corticotrophins used in earlier investigations. As in the earlier investigations, the corticotrophins were administered before the second operation of a two-stage bilateral adrenalectomy. In this way, the first gland removed acts as a control for the corticotrophin-treated gland. Response to corticotrophin was judged by the change in weight and histological appearance of the glands removed, and by the increase in urinary total 17-hydroxycorticosteroids, which were measured by the method of Few (1961) slightly modified. Intramuscular administration of Synacthen was not always satisfactory. The most consistent results were obtained by the intravenous administration of 25 U (0-25 mg) of Synacthen intravenously over 3 h, three times daily for 4 or 5 days, before the second adrenalectomy. For comparison, 40 U of Organon corticotrophin were administered intramuscularly, twice daily, to two patients for 6 days prior to the second operation. The results are shown in Table 1. It is interesting to note that marked increases in gland weight are obtained with Synacthen as well as with the preparation of natural corticotrophin (Organon). Dr J. Dobbie examined sections of the glands and reported increase in thickness of cortex and almost complete lipid depletion in most cases after Synacthen administration. The search for an adrenal growth factor other than ACTH (Jailer, 1960) is thus probably unnecessary. Indeed, attempts to simulate the increases in adrenal RNA obtained with ACTH by a variety of substances including growth hormone have met with little success (Bransome & Reddy, 1961; Sigel & Dowling,
ADRENAL EFFECTS OF ACTH

1964). It is an open question whether the increases in RNA which follow corticotrophin administration are associated with steroid production per se, or tissue hyperplasia, or both. The rapid increase in corticosteroid production observed on corticotrophin administration suggests that in the early stages of adrenal stimulation, at least, the two processes may not be associated.

Table 1. The effects of administered corticotrophins on the adrenal glands of human subjects

<table>
<thead>
<tr>
<th>Corticotrophin</th>
<th>Weight of glands (g)</th>
<th>Excretion of urinary total 17-hydroxy-corticosteroids (mg/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Patient with intact pituitary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 units Synacthen i.m.—t.d.s.—for 4 d</td>
<td>5.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Patient 4 months after yttrium implant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 units Synacthen i.v.—t.d.s.—for 5 d</td>
<td>2.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Patient 7 months after yttrium implant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.v. as before, but for 4 d</td>
<td>2.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Patient 4 months after yttrium implant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 units Synacthen i.m.—t.d.s.—for 6 d</td>
<td>2.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Patient 3 months after yttrium implant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 units Corticotrophin Organon i.m.—b.d.—for 6 d</td>
<td>2.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>

IN VITRO BIOSYNTHESIS

Using the severely atrophic and Synacthen-stimulated glands removed from the patients, steroid biosynthesis was investigated in vitro with [4-14C]progesterone as substrate. Homogenized adrenal tissue was incubated with NADP, ATP, glucose 6-phosphate and its dehydrogenase under conditions similar to those described by Lawrence & Griffiths (1966). The products were identified and measured quantitatively by the procedures described by Griffiths, Grant & Whyte (1963), the investigation being limited to the study of the formation of cortisol, corticosterone and 16α-hydroxyprogesterone. The last substance is of unknown physiological significance but is regularly formed in good yield in similar in vitro studies. Typical results obtained with Synacthen, shown in Fig. 6, are similar to those found with natural corticotrophin.

CONCLUSIONS REGARDING SYNACTHEN

Synacthen, synthetic β1-24-corticotrophin, and natural animal corticotrophin, administered in vivo, have similar effects in increasing the weight and causing histological changes in human adrenal glands, which have
become atrophic as a result of hypophyseal destruction by yttrium implants. Glands stimulated in vivo by the corticotrophins show similar increases in steroid biosynthetic capacity in vitro.

![bar chart]

Fig. 6. Effect of $^{131}$I synthetic corticotrophin (Synacthen Ciba) in vivo on the steroid biosynthetic capacity in vitro of the adrenal cortex of hypophysectomized subjects. The results represented by the unshaded columns were obtained without, by the black columns with, Synacthen.

ACKNOWLEDGEMENTS

The authors are indebted to Professor A. P. M. Forrest and his staff at the Surgical Unit, Royal Infirmary, Cardiff, and to surgeons at Glasgow Hospitals for their splendid co-operation. The earlier parts of the work were done during the receipt of generous financial support from the Scottish Hospital Endowments Research Trust. Thanks are due to Organon Laboratories Ltd. and Ciba Laboratories Ltd. for gifts of corticotrophin and Synacthen.

REFERENCES


**COMMENT**

El-Shaboury: With regard to the ‘slow action’ of ACTH outlined by Dr Grant I would like to draw attention to reports some years ago that ACTH had an immediate action on the adrenals, causing a rapid rise in
cortisol concentration in the blood, and a slower action maintaining the increased steroid output. It was postulated at that time that the slower action of ACTH was the result of activation of enzyme systems responsible for steroid biosynthesis. Would Dr Grant throw some light on the significance of the increase in glucose 6-phosphate dehydrogenase that he found during ACTH administration, and say whether there was evidence that the slower action of ACTH was due to activation of enzymes as postulated some years ago.

**Grant:** The increases in enzyme activity which were observed in the adrenal cortex were brought about by administration of 80 units of ACTH daily for 4 days before removal of the second adrenal in a two-stage adrenalectomy. Such ACTH administration caused increase in the growth of the gland, lipid depletion and increase in RNA, and these changes must be looked upon as part of the slow trophic action of ACTH. The very prompt increase in output of cortisol, the change in the cortisol/corticosterone ratio, and the increase in adrenal blood flow are examples of the rapid immediate action of ACTH.

**Ewart:** With regard to the mode of action of ACTH, could an adrenal preparation maximally stimulated by NADPH show increased steroidogenesis on addition of ACTH?

**Grant:** Priming with NADPH did not limit the effects of other substances but, despite Herculean efforts in many laboratories, one does not really understand what corticotrophin is doing. Certainly one could stimulate with NADPH and then obtain further stimulation with, for example, cyclic AMP.

**Symington:** There are two other factors that one should consider when discussing ACTH action. These are the vitamin A and corticosteroid content of the cells. These are possibly higher in the adrenocortical cells than in any other. Both substances influence membrane stability.

**Kitay:** One additional site of action of ACTH on the adrenal cortex that has been studied in my laboratory is the regulation of the production of tetrahydro metabolites by the rat adrenal gland. Following hypophysectomy the production of tetrahydrocorticosterone is increased, correlated with enhanced adrenal steroid reductase activity. These changes are reversed by ACTH replacement. In other words total steroid production falls after hypophysectomy but the decrement in blue tetrazolium-reactive compounds is much slower than the fall in fluorescent steroids. Although this may well not be the major effect of ACTH it is of interest that an increase in corticosterone secretion is accomplished by inhibition rather than stimulation.
A COMPARISON OF THE CONVERSION IN VITRO OF PREGNENOLONE SULPHATE AND PREGNENOLONE TO STEROID HORMONES BY TISSUE FROM A CLEAR CELL ADENOMA OF THE ADRENAL GLAND

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SUMMARY

Tissue from a clear cell adenoma of the adrenal gland was incubated simultaneously with [7α-3H]pregnenolone sulphate and [4-14C]pregnenolone in Krebs-Ringer bicarbonate-glucose solution. Evidence was obtained for the conversion of pregnenolone sulphate to cortisol and corticosterone. The differences in the ability of the tissue to convert the sulphate and the free steroid to corticosteroids and androgens are discussed in relation to theories on the functional zonation of the human adrenal cortex.

INTRODUCTION

It is now well established that dehydroepiandrosterone sulphate (DHA sulphate) is secreted by the human adrenal gland (Baulieu, 1962; Wieland, Levy, Katz & Hirschmann, 1963). Evidence is also available (Calvin, VandeWiele & Lieberman, 1963) that DHA sulphate can be formed in vivo from pregnenolone sulphate without loss of the sulphate ester group. Furthermore, Roberts, Bandi, Calvin, Drucker & Lieberman (1964) reported the formation of DHA sulphate from cholesterol sulphate which had been infused into the splenic artery of a woman with an adrenocortical carcinoma and, more recently, Shimizu (1966) demonstrated the conversion of 20α-hydroxycholesterol to pregnenolone sulphate by human adrenal slices in vitro. There is, therefore, good evidence that steroid sulphates may be involved in the biosynthesis of the adrenal hormones, but as yet little is known about the enzymes which regulate the biosynthetic pathways shown in Fig. 1(a). Killinger & Solomon, (1965) have reported that pregnenolone sulphate was not metabolized by normal adrenal tissue in vitro.

The present investigation deals with the biosynthesis in vitro of various steroid hormones from [4-14C]pregnenolone and [7α-3H]pregnenolone sulphate by tissue from a clear cell adenoma of the adrenal gland.
MATERIALS AND METHODS

Clinical information and histological features of the tumour

The tumour was removed from the right side of a 36-yr.-old woman who had been referred to Cardiff Royal Infirmary with clinical signs and symptoms of Cushing's syndrome. Change of appearance, buffalo hump, weight increase and the appearance of striae, low back ache and 1 year of abnormal scanty menstrual bleeding were found at examination. There was no hirsutism. The pituitary fossa was normal. Perirenal insufflation and an aortogram showed a mass in the region of the right adrenal. At laparotomy, an encapsulated tumour weighing 67 g. was removed with the adjoining 'normal' adrenal. The tumour was identified by Professor Symington as an adrenal clear cell adenoma. The large central yellow mass of the tumour consisted exclusively of clear cells; this was the tissue incubated. Darker areas at the periphery showed a mixture of clear and compact cells, with areas of necrosis and myeloid metaplasia. No mitotic activity was observed in the sections examined. The nuclei were vesicular with a moderate degree of pleomorphism. Sections from the adjoining 'normal' adrenal showed marked cortical atrophy.

Preparation of tissue and conditions of incubation

The tumour was chilled to about 0° immediately on removal from the patient and kept at this temperature until prepared for incubation 30 min. later.

Part of the tumour was finely chopped with a razor blade, and 2 g. of the minced tissue was incubated simultaneously with 83·4 mmol each of [4-14C]pregnenolone (26·92 µc/mmol) and [7α-3H]pregnenolone sulphate (151·9 µc/mmol) in 24·0 ml. Krebs-Ringer bicarbonate-glucose medium for 2 hr. at 37° and shaken in a 95% oxygen:5% carbon dioxide mixture.

Radioactive steroids used as precursors in the incubation were checked for purity by diluting samples with carrier material and chromatography on thin layer and paper in a number of solvent systems. Pregnenolone sulphate was also solvolysed and chromatographed. Radioactive material on paper and thin layers was measured using a Packard Model 7201 Radiochromatogram Scanner.

Extraction and preliminary separation of steroids in the incubated mixture

After incubation, the mixture was diluted with 10 ml. acetone, and with 300 µg. each of the following non-radioactive carriers: pregnenolone, 17α-hydroxypregnenolone, DHA, androst-5-ene-3β,17β-diol (androstenediol), testosterone, androstenedione, 11β-hydroxyandrostenedione, progesterone, 17α-hydroxyprogesterone, cortisol, corticosterone, pregnenolone sulphate, 17α-hydroxypregnenolone sulphate, DHA sulphate and testosterone sulphate. The mixture was then homogenized in a Silverson mixer with 5 vol. acetone and filtered. The residue was washed twice with the same volume of acetone.

The pooled acetone extract and washings were taken almost to dryness under reduced pressure; the material in the aqueous residue was distributed between aqueous methanol and light petroleum. The aqueous methanol was reduced to a
small volume, water was added and the free steroids extracted with $3 \times 30$ ml ether. Steroid sulphonates were extracted from the aqueous residue by the method of Thomas & Bulbrook (1964).

### Chromatographic separation of steroids

Thin-layer chromatography on silica gel was used to isolate the individual free steroids. The procedures for the preparation of plates and elution of steroids were similar to those described by Griffiths, Grant, Browning, Whyte & Sharp (1966) except that Merck silica gel H254/366 was used and that ethyl acetate was used to elute the $\Delta^4$-3$\beta$-hydroxysteroids from the silica gel. Also only $2$ ml water were used in the extraction procedure. Steroid sulphonates were eluted by adding $4$ ml ethyl acetate to the silica gel, mixing thoroughly, adding $2$ ml saturated NaCl solution and shaking vigorously. The upper ethyl acetate layer was removed after centrifugation.

Radioactive substances on thin-layer plates, $5$ cm wide, were detected by a Packard radiochromatogram scanner (Model 7201). The $\Delta^4$-3-oxo and the $\Delta^4$-3$\beta$-hydroxysteroids were detected on thin-layer plates by use of u.v. light of $254$ and $350$ m$\mu$ respectively (using the Universal u.v. lamp, Camag, Muttenz, Switzerland).

The following solvent systems were used: (I) chloroform:acetone (185:15), (II) chloroform:ethanol (19:1), (III) toluene:ethyl acetate: methanol (85:85:30), (IV) cyclohexane:ethanol (9:1), (V) benzene:methanol (17:3), (VI) benzene:ethyl acetate (9:1), (VII) tertiary butanol:ethyl acetate:5N-NH$_2$OH (41:50:20), (VIII), hexane: ethyl acetate (1:1). Solvent proportions are by volume.

### Identification of steroids

Steroids were measured after elution from thin layers of silica gel by procedures described (Griffiths, Grant, Browning, Cunningham & Barr, 1966). The procedures for reduction, oxidation, saponification and acetylation have been described by Griffiths, Grant & Whyte (1963b). Steroid sulphonates were solvolysed according to Burstein & Lieberman (1958a). Radioactivity was measured with a Nuclear Chicago liquid scintillation spectrometer (Model 6860). Counting conditions were such that $^{14}$C and $^3$H could be determined simultaneously with counting efficiencies in Channels I and II respectively of $17\%$ and $54\%$ for $^{14}$C and $40\%$ and $0\%$ for $^3$H. The absolute quantities of $^3$H were calculated using the standard equations (Okita, Kabora, Richardson & LeRoy, 1957), and those for $^{14}$C from direct readings in Channel II. Radioactivity in steroid sulphonate fractions was determined by dissolving the residue in the vial in $1-0$ ml methanol and adding $9-0$ ml scintillator. Quenching was determined using the external standard.

### Neutral steroid fraction

The neutral steroid fraction was applied as $3$ in. long lines on three thin-layer plates and was chromatographed in solvent system I. Bands of steroids corresponding in chromatographic mobility to progesterone ($R_F$ 0·74), androstenedione ($R_F$ 0·60) pregnenolone ($R_F$ 0·48), DHA ($R_F$ 0·44), 17$\alpha$-hydroxyprogesterone ($R_F$ 0·30), testosterone ($R_F$ 0·27), 17$\alpha$-hydroxypregnenolone ($R_F$ 0·23), androstenediol ($R_F$ 0·22), 11$\beta$-hydroxyandrostenedione ($R_F$ 0·15), and corticosterone (origin) and cortisol (origin) were located. The steroids were eluted from these bands and 17$\alpha$-hydroxy-
Table 1. Identification of steroids isolated from the incubation of [4-14C]pregnenolone and [7α-3H]pregnenolone sulphate with tissue from an adrenal clear cell adenoma

<table>
<thead>
<tr>
<th>Compound isolated</th>
<th>Derivative formed</th>
<th>Radioactivity ran with authentic steroid in solvent system</th>
<th>Specific activity (disintegrations/min./m mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14C</td>
<td>3H</td>
</tr>
<tr>
<td>Progesterone</td>
<td>20β-Hydroxyprog-4-en-3-one</td>
<td>I:IV</td>
<td>16-8</td>
</tr>
<tr>
<td></td>
<td>20β-Acetoxyprog-4-en-3-one</td>
<td>V</td>
<td>17-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>17-6</td>
</tr>
<tr>
<td>Androstenedione</td>
<td></td>
<td>I:IV</td>
<td>5-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIII</td>
<td>4-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI</td>
<td>4-8</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>3β-Acetoxyprog-5-en-20-one</td>
<td>I:V</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>20/β-Acetoxyprog-4-en-3-one</td>
<td>VIII</td>
<td>263</td>
</tr>
<tr>
<td>DHA</td>
<td>3β-Acetoxyandrost-5-en-17-one</td>
<td>I:V</td>
<td>1-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td>17α,20β-Dihydroxyprog-4-en-3-one</td>
<td>IV</td>
<td>1-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I:II</td>
<td>76-0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>75-0</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td>I:II</td>
<td>1-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>1-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>1-3</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenolone</td>
<td>3β-Acetoxy-17α-hydroxy-pregnenolone-4-en-20-one</td>
<td>I:II</td>
<td>7-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>7-3</td>
</tr>
<tr>
<td>Androstenediol</td>
<td></td>
<td>I:II</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>0</td>
</tr>
<tr>
<td>11β-Hydroxyandrostenedione</td>
<td>11β-Hydroxytestosterone</td>
<td>I:V</td>
<td>81-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>81-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>77-4</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>11β,20β,21-Trihydroxyprog-4-en-3-one</td>
<td>I:III</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>417</td>
</tr>
<tr>
<td></td>
<td>11β-Hydroxy-21-acetoxy-androst-4-en-3-one</td>
<td>III</td>
<td>448</td>
</tr>
<tr>
<td>Cortisol</td>
<td>Adrenosterone</td>
<td>I:III</td>
<td>2197</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>2297</td>
</tr>
<tr>
<td></td>
<td>11β,17α-Dihydroxy-21-acetoxyprog-4-ene-5,20-dione</td>
<td>III</td>
<td>2310</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>3β-Acetoxyprog-5-en-20-one</td>
<td>VII</td>
<td>29-7</td>
</tr>
<tr>
<td></td>
<td>Pregnenolone</td>
<td>VIII</td>
<td>52-5</td>
</tr>
<tr>
<td></td>
<td>Pregen-5-ene-3β,20β-diol</td>
<td>VIII</td>
<td>22-2</td>
</tr>
<tr>
<td>17α-Hydroxypregnenolone sulphate</td>
<td>3β-Acetoxy-17α-hydroxyprog-5-en-20-one</td>
<td>VII</td>
<td>3-8</td>
</tr>
<tr>
<td></td>
<td>17α-Hydroxyprogrenolone</td>
<td>VIII</td>
<td>3-5</td>
</tr>
</tbody>
</table>
Steroid biosynthesis by adrenal adenoma tissue

Table 1 (cont.)

<table>
<thead>
<tr>
<th>Compound isolated</th>
<th>Derivative formed</th>
<th>Radioactivity run with authentic steroid in solvent system</th>
<th>Specific activity (disintegrations/min./mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VII and VIII</td>
<td>$^{14}C$ and $^2H$</td>
</tr>
<tr>
<td>Androstenediol sulphate</td>
<td>Androstenediol diacetate</td>
<td>IV</td>
<td>0 and 0</td>
</tr>
<tr>
<td></td>
<td>Androstenediol</td>
<td>VIII</td>
<td>0 and 0</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>3α-Acetoxyandrost-5-ene-17-one</td>
<td>VI</td>
<td>4.2 and</td>
</tr>
<tr>
<td></td>
<td>DHA</td>
<td>VIII</td>
<td>4.3 and 23.0</td>
</tr>
<tr>
<td></td>
<td>Androstenediol</td>
<td>VIII</td>
<td>4.3 and 22.1</td>
</tr>
<tr>
<td>Testosterone sulphate</td>
<td>Testosterone acetate</td>
<td>VII</td>
<td>0 and 5.4</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>VIII</td>
<td>0 and 4.0</td>
</tr>
<tr>
<td></td>
<td>Androstenedione</td>
<td>IV</td>
<td>0 and 3.9</td>
</tr>
</tbody>
</table>

pregnenolone and androstenediol separated in solvent system II. Cortisol and corticosterone were isolated in system III.

Fractions containing androstenedione, progesterone, 17α-hydroxyprogesterone and 11β-hydroxyandrostenedione fractions were acetylated to facilitate the removal of any alcoholic impurities. Androstenedione and progesterone were then isolated after chromatography in solvent system IV, 17α-hydroxyprogesterone in system II, and 11β-hydroxyandrostenedione in system V. Pregnenolone and DHA fractions were acetylated, the acetates chromatographed in system VI, eluted, saponified and the parent alcohols chromatographed in system V.

Androstenediol and 17α-hydroxypregnenolone were purified by acetylation, chromatography in system IV and saponification; corticosterone by acetylation, chromatography in system III and saponification. Portions of the individual steroids were re-chromatographed directly or after the formation of derivatives as described by Griffiths et al. (1963b). Specific activities were then determined after elution from the plates.

**Steroid sulphate fraction**

The conjugate fraction was applied as 3 in. long lines on two thin-layer plates and was chromatographed in system VII described by Pierrepont, Griffiths, Grant & Stewart (1966). The sulphates of pregnenolone, 17α-hydroxypregnenolone, androstenediol, DHA, and testosterone sulphate are not resolved ($R_p$ 0.35). Material from the sulphate zone was eluted, counted and solvolysed. This procedure cleaves the sulphates of neutral and phenolic steroids (Sneddon & Marrian, 1963) but is without effect on phosphates (Burstein & Lieberman, 1958b) and glucosiduronic acids (Jacobsohn & Lieberman, 1962). Androstenediol (300 µg.) was added to the solvolysed extract. Steroids obtained had the same chromatographic mobility as pregnenolone, 17α-hydroxypregnenolone, androstenediol, DHA and testosterone. These were then acetylated, chromatographed and treated as indicated in Table 1.
RESULTS

Evidence (Table 1) was obtained for the formation from [4-14C]pregnenolone of the following free steroids: 17α-hydroxyprogrenolone, DHA, progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, 11β-hydroxyandrostenedione, corticosterone and cortisol. Some evidence was also obtained for the formation from pregnenolone of the sulphates of pregnenolone, 17α-hydroxyprogrenolone and DHA. Furthermore, proof is given for the formation of the same free steroids from [7α-3H]-pregnenolone sulphate.

Table 2 shows the distribution of radioactivity among the metabolites formed, and Fig. 1(b) the percentage of incubated steroids converted to metabolites.

Table 2. Radioactivity of steroids incubated and found after [4-14C]pregnenolone and [7α-3H]pregnenolone sulphate were incubated simultaneously with tissue from an adrenal clear cell adenoma

<table>
<thead>
<tr>
<th>Steroid isolated</th>
<th>Found after incubation (mCi)</th>
<th>% radioactivity found in isolated steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14C</td>
<td>3H</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>116.3</td>
<td>3284.0</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenolone</td>
<td>2.9</td>
<td>49.1</td>
</tr>
<tr>
<td>DHA</td>
<td>0.5</td>
<td>9.8</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7.4</td>
<td>111.5</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>31.1</td>
<td>298.9</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>168.6</td>
<td>186.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>846.6</td>
<td>1209.3</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>2.3</td>
<td>23.7</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>11β-Hydroxyandrostenedione</td>
<td>35.9</td>
<td>79.6</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>9.8</td>
<td>1836.0</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenolone sulphate</td>
<td>1.5</td>
<td>5.8</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>2.0</td>
<td>10.6</td>
</tr>
<tr>
<td>Androstenediol sulphate</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Testosterone sulphate</td>
<td>0.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

DISCUSSION

The investigations of Calvin et al. (1963), Calvin & Lieberman (1963) and of Roberts et al. (1964) have established that the steroid sulphates of Δ5-3β-hydroxysteroids undergo metabolic reactions analogous to those of the corresponding free alcohols (Fig. 1(a)). It seems probable that the relatively large amount of DHA sulphate synthesized and secreted by the human adrenal gland (Wieland et al. 1963; VandeWiele, MacDonald, Bolté & Lieberman, 1963) originates from pregnenolone sulphate formed from cholesterol sulphate. However, the extent to which the sulphates are involved in the biosynthesis of the steroid hormones is not known. Baulieu & Dray (1963), Siiteri & MacDonald (1963) and Bolté, Mancuso, Erickson, Wiqvist & Diczfalusy (1964) have reported on the importance of DHA sulphate as a precursor of oestrogens in the foeto-placental unit, and in vitro studies of Morato, Lemus & Gual (1965) have shown that DHA sulphate, when incubated with a placental microsomal fraction, was a better precursor of oestrogens than DHA. However,
Steroid biosynthesis by adrenal adenoma tissue

(a)

Cholesterol → 20α-Hydroxycholesterol → Cholesterol sulphate

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Percentage Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone sulphate</td>
<td>0.07</td>
</tr>
<tr>
<td>17α-Hydroxy-pregnenolone sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Cholesterol → Pregnenolone

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Percentage Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Hydroxy-pregnenolone</td>
<td>0.02</td>
</tr>
<tr>
<td>17α-Hydroxy-progesterone</td>
<td>0.08</td>
</tr>
<tr>
<td>Androstenedione (A)</td>
<td>0.05</td>
</tr>
<tr>
<td>Testosterone (T)</td>
<td>0.05</td>
</tr>
<tr>
<td>11β-Hydroxy-androstenedione (11β/A)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Cortisol → Corticosterone (B)

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Percentage Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-Hydroxy-androstenedione (11β/A)</td>
<td>0.05</td>
</tr>
<tr>
<td>Testosterone (T)</td>
<td>0.05</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Killinger & Solomon (1965) were unable to demonstrate any metabolism of pregnenolone sulphate by normal adrenal tissue.

In the studies now reported, the capacity of adrenal tissue to synthesize steroid hormones from [7α-3H]pregnenolone sulphate and [4-14C]pregnenolone has been compared. Conflicting evidence tends to arise from studies in vitro in which mixtures
of cell types are incubated, and from the use of preparations containing broken cells. Results are often difficult to interpret in terms of physiological adrenal function. In the present studies, whole cells were used to avoid problems associated with the use of additional cofactors, and the incubated tissue consisted exclusively of the fascicular clear cell type (Symington, 1960).

Furthermore, no attempt has been made in these studies to allow for the endogenous steroid either present in or secreted by the tissue during the incubation. Conversion figures must therefore represent minimum values, although cortisol is most probably the major endogenous steroid secreted (Griffiths, Grant & Symington, 1963a) and the source of the major error. These are, however, comparative studies.

The results show that both steroids were well metabolized by the tumour tissue, 14·5 and 5·2% respectively, of pregnenolone sulphate and pregnenolone remained at the end of the incubation period. Furthermore, 11% of the pregnenolone sulphate was converted to cortisol and corticosterone (Fig. 1(b)) although 43·2% of the pregnenolone was transformed to these corticosteroids. Pregnenolone sulphate is metabolized to steroid hormones, but the precise metabolic role of the steroid sulphates in hormone biosynthesis remains unknown. The results provide no indication as to which of the following pathways for the synthesis of cortisol from pregnenolone sulphate function:

\[(a) \rightarrow 17\alpha\text{-}\text{hydroxy pregnenolone sulphate} \rightarrow 17\alpha\text{-}\text{hydroxy pregnenolone},\]

\[(b) \rightarrow \text{pregnenolone} \rightarrow 17\alpha\text{-}\text{hydroxy pregnenolone}, \text{or}\]

\[(c) \rightarrow \text{pregnenolone} \rightarrow \text{progesterone} \rightarrow 17\alpha\text{-}\text{hydroxy progesterone}.\]

Earlier histochemical observations of Baillie & Griffiths (1965) on mouse testicular tissue had suggested that, under certain conditions, 17α-hydroxypregnenolone sulphate may be a better substrate for 3β-hydroxysteroid dehydrogenase than 17α-hydroxyprogrenolone.

Twice as much pregnenolone was transformed to C₁₉-D₄-3-oxosteroids, testosterone, androstenedione and 11β-hydroxyandrostenedione (1·7%) than was pregnenolone sulphate (0·8%), although the difference may not be significant. However, of real interest is the small degree of conversion of pregnenolone sulphate to DHA sulphate (0·08%) and to DHA (0·08%). The sulphating capacity of human adrenal tissue in vitro is well established (Wallace & Lieberman, 1963; Cohn, Mulrow & Dunne, 1963), but in our studies only 0·6% of the pregnenolone was transformed into the steroid sulphates isolated, and only 0·09% to DHA sulphate.

This small degree of conversion of pregnenolone and pregnenolone sulphate to DHA sulphate still leaves the problem of the biosynthesis of DHA sulphate unanswered. Although the normal adrenal secretes relatively large amounts of DHA sulphate, the cells from which this steroid originates are yet to be identified. Griffiths et al. (1963a) showed that the clear cells and the compact cells of the adrenal cortex have qualitatively similar biosynthetic activities, and that both types of cell synthesize and secrete corticosteroids and 11β-hydroxyandrostenedione. It has also been shown (Ward & Grant, 1963) that tissue from a clear cell adenoma converts progesterone to testosterone in vitro. However, the ‘clear’ cell of an adenoma may not behave biochemically like the clear cell of the zona fasciculata. It is of interest in this context that a histochemical study of the 3β-hydroxysteroid dehydrogenase of the human adrenal cortex (Baillie, Cameron, Griffiths & Hart, 1965) showed that, although the sulphate of pregnenolone and 17α-hydroxy pregnenolone gave a positive reaction
for this enzyme in the clear cells, DHA sulphate did not, suggesting that DHA sulphate was not metabolized by the adrenal cortex. It may be that the compact cell is responsible for DHA sulphate synthesis, and further studies using the ultramicrochemical techniques described previously (Griffiths & Glick, 1966) are currently being undertaken in this laboratory.

Also of interest was the formation of testosterone sulphate. Dixon, Phillips & Kase (1965) have described the formation of testosterone sulphate from progesterone in vitro by virilizing adrenal tumour tissue but did not refer to the cell type incubated.

These studies provide therefore some indication of the extent to which pregnenolone and its sulphate are converted into steroids secreted by adrenal tissue. However, as stated earlier, in these experiments no consideration was given to the endogenous steroid secretion. Further time-based incubation studies with this tissue in which this endogenous secretion is measured are now being completed. These show conclusively that the biosynthetic pathway to cortisol from pregnenolone, involving 17α-hydroxyprogrenolonolone is the major pathway, and that only a small amount of cortisol is formed via pregnenolone → progesterone → 17α-hydroxyprogesterone. These results will be the subject of a further communication.

The authors gratefully acknowledge the generous financial support of the Tenovus organization in Cardiff.

We also wish to thank Professor T. Symington, Institute of Pathology, Royal Infirmary, Glasgow, for the identification of the tumour investigated, and Dr C. L. Hewitt and Dr G. F. Woods, Organon Laboratories Ltd. for steroid sulphates.

We are grateful to Professor A. P. M. Forrest, Surgical Unit and Dr J. Picton Thomas, Medical Unit, Cardiff Royal Infirmary, for allowing us to investigate this patient and providing the tissue.

REFERENCES


K. Griffiths, D. Cunningham and E. H. D. Cameron


THE ROLE OF PROGESTERONE IN THE BIOSYNTHESIS OF CORTISOL IN HUMAN ADRENAL TISSUE

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SUMMARY

The ability of cells from the zona fasciculata and the zona reticularis of the human adrenal cortex to transform labelled pregnenolone and progesterone to cortisol in vitro was investigated. Examination of the $^3$H:14C ratios of 16α-hydroxyprogesterone, 17α-hydroxyprogesterone, 11-deoxycorticosterone and cortisol formed during incubations in vitro suggested that the role of progesterone in the transformation of pregnenolone to cortisol might be a relatively minor one.

An attempt was subsequently made to estimate the relative importance of the biosynthetic pathway to cortisol by way of progesterone in hyperplastic adrenal tissue by a mathematical approach.

INTRODUCTION

Although the classical route for the synthesis of cortisol from pregnenolone is generally thought to involve progesterone (Hechter & Pincus, 1954), it is known that a second pathway via 17α-hydroxyprogrenolone is also present in human adrenal tissue (Lipsett & Hokfelt, 1961; Mulrow & Cohn, 1961) (see Fig. 1). Weliky & Engel (1962, 1963) concluded from incubation studies with hyperplastic and neoplastic human adrenal tissue that pregnenolone was converted to cortisol, apparently by the pathway involving 17α-hydroxyprogrenolone and 17α-hydroxyprogesterone, but not to progesterone or corticosterone. Progesterone incubated simultaneously was transformed into cortisol. However, although their results are widely quoted, these authors drew erroneous conclusions from their investigations (Cameron, 1966; Kumari & Goldzieher, 1966). Furthermore, Ward & Grant (1963) failed to isolate cortisol from an incubation of hyperplastic adrenal tissue with labelled progesterone.

A further study of the role of progesterone in the synthesis of cortisol therefore seemed necessary. After preliminary investigations to measure the ability of the fascicular and reticular tissue to metabolize pregnenolone and progesterone, it was decided to obtain a more quantitative assessment of the importance of progesterone in the biosynthesis of cortisol by a mathematical approach based on the in vivo studies of Kopin (1963).
Adrenal glands. (a) The first gland was taken from a 56-yr.-old woman (E.R.) with breast cancer. Before operation, she had no steroid, corticotrophin (ACTH) or pituitary implant therapy. The gland received at the laboratory approximately 8 hr. after removal, was of normal appearance. (b) The second gland was a hyperplastic adrenal which was received 1 hr. after its removal from an 11-yr.-old boy (D.H.) with Cushing’s syndrome.

Pieces of both glands, and of separated slices of fascicular and reticular tissue, were fixed in 10% neutral formalin for histological examination.

Preparation of slices. Slices of fascicular and reticular tissue were prepared from the first gland (E.R.) as described by Griffiths, Grant & Symington (1963). Tissue (150 mg.) from each zone was then finely chopped with a safety razor blade. A chopped mixture of fascicular and reticular tissue (780 mg.) was prepared from the hyperplastic gland, which was impossible to section.

Incubation, extraction and fractionation of steroids. The procedures used were similar to those described by Griffiths, Grant, Browning, Whyte & Sharp (1966). The chopped fascicular (150 mg.) and reticular (150 mg.) preparations were separately incubated with a mixture of 92.4 mμmoles of [7α-3H]pregnenolone (sp.act. 122.6 mc/mM) and 23.1 mμmoles each of [4-14C]progesterone (sp.act. 26.1 mc/mM) and of unlabelled 17α-hydroxyprogrenenolone. The mixed tissue (780 mg.) was incubated with 44.0 mμmoles of [7α-3H]pregnenolone (sp.act. 432 mc/mM) and 27.7 mμmoles each of [4-14C]progesterone (sp.act. 38.7 mc/mM) and unlabelled 17α-hydroxyprogrenenolone. The sliced tissue was incubated in 4 ml. Krebs-Ringer bicarbonate glucose medium, the mixed tissue in 12 ml. for 3 hr. at 37° in 95% O₂:5% CO₂.

Incubations were terminated by the addition of 20 ml. acetone. Carrier steroids (300 μg. of each), cortisol, 17α-hydroxyprogesterone, 16α-hydroxyprogesterone and 11-deoxycorticosterone (DOC) were then added to both the fascicular and reticular incubations: similar quantities of these steroids together with pregnenolone, 17α-hydroxyprogrenenolone, progesterone, corticosterone, 11-deoxycortisol and cortisone were added to the hyperplastic tissue incubation mixture.

Carrier steroids were extracted and purified as previously described (Cameron & Grant, 1967).

Chromatography, identification and quantitative measurement of steroids. The procedures used were similar to those described in earlier publications for derivative formation, Δ⁴-3-oxosteroid determination (Griffiths, Grant & Whyte, 1963) and for measurement of Δ⁵-3β-hydroxysteroids (Griffiths, Grant, Browning, Cunningham & Barr, 1966).

Chromatography was performed on thin layers of Merck silica gel-G containing a phosphor (H913, Levy West Labs., Harlow) or on Merck silica gel HF₂₅₄₉₆₆. The solvent systems used were as follows: (I) cyclohexane:ethyl acetate (90:110), (II) benzene:methanol (170:30), (III) chloroform:methanol:water (187:12:1), (IV) chloroform:acetone (185:15), (V) benzene:ethyl acetate (180:20), (VI) benzene:hexane:ethanol (140:50:10). All solvent proportions are by volume.

Radioactivity was measured with Packard Tri-Carb or Nuclear Chicago Mark I Liquid Scintillation Spectrometers. The observation that the specific activities of a
Cortisol synthesis in man

steroid and two of its derivatives differed by not more than 10% was taken as satisfactory evidence for radiochemical purity. The mean of the three specific activities was used to calculate the percentage conversion from the original radioactive steroid incubated.

RESULTS

Counting of cells in haematoxylin- and eosin-stained sections showed that the material taken to represent the zona fasciculata contained approximately 96% fascicular type ‘clear’ cells and that taken to represent the zona reticularis contained approximately 85% of reticular-type ‘compact’ cells. The specific activities of the steroids isolated and of their derivatives are given in Table 1. Total and percentage conversions are shown in Table 2.

It should be noted that: (a) the conversion of pregnenolone to cortisol, 17α-hydroxyprogesterone, 16α-hydroxyprogesterone and DOC was significantly higher in fascicular than in reticular tissue, especially in the case of the conversion of pregnenolone to cortisol (conversion of 117.3 mμc to cortisol from 11,381 mμc [7α-3H]pregnenolone in the incubation of fascicular tissue and a corresponding conversion of 18.5 mμc to cortisol in the incubation of reticular tissue); (b) the conversion of [4-14C]progesterone (604 mμc incubated) to the metabolites isolated did not appear to be significantly different in the separated zones; (c) when fascicular tissue was incubated the 3H:14C ratios of cortisol (9.09) and 17α-OH-progesterone (7.61) were very much higher than those of 16α-OH-progesterone (1.97) and of DOC (0.33).

Similarly, when reticular tissue was incubated there was a highly significant difference between the 3H:14C ratios of cortisol (1.96) and 17α-hydroxyprogesterone (3.22) on the one hand and DOC (0.09) on the other; (d) when hyperplastic adrenal tissue was incubated, the 3H:14C ratios of the 17α-hydroxycorticosteroids (e.g. cortisol: 36.61) and the 17-deoxycorticosteroids (e.g. corticosterone: 2.83) were again entirely different.

DISCUSSION

Wattenberg (1958) described a histochemical reaction for the localization of 3β-hydroxysteroid dehydrogenase activity in the adrenal cortex. The distribution of this activity indicated that the conversion of Δ5-3β-hydroxysteroids to Δ5-3-oxosteroids takes place in the zona fasciculata, particularly in the outer regions. Cameron & Grant (1967) found that this histochemical pattern could not be supported by biochemical evidence in equine adrenal tissue and the present results show that in the human adrenal cortex, Δ5-3β-hydroxysteroid dehydrogenation takes place in the compact cell of the reticular zone. Thus this histochemical reaction only demonstrates those areas in a tissue section of high activity and a negative histochemical reaction need not necessarily mean that the enzyme is absent.

Furthermore, although this preliminary examination of the ability of the separated zones to utilize [7α-3H]pregnenolone and [4-14C]progesterone showed that both zones metabolized progesterone in the same way (see Table 2), the metabolism of pregnenolone was quite different. Comparatively little 3H was associated with the 17-deoxycorticosteroid DOC compared with the 17α-hydroxycorticosteroids, 17α-hydroxyprogesterone and cortisol. Thus there is evidence that little pregnenolone is converted to cortisol via progesterone in the adrenal gland. These results complement and con-
Table 1. Identification of steroids isolated from the simultaneous incubation of [4-¹⁴C]progesterone and [7α-⁵H]pregnenolone with human adrenal tissue

<table>
<thead>
<tr>
<th>Material investigated and chemical reaction</th>
<th>Product mobility identical with that of:</th>
<th>Solvent system</th>
<th>Specific activities (mCi/m mole x 10⁶)</th>
<th>Fascicular tissue</th>
<th>Reticular tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>Cortisol</td>
<td>III</td>
<td>15,163</td>
<td>1,707</td>
<td>849</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Cortisol acetate</td>
<td>III</td>
<td>13,659</td>
<td>1,597</td>
<td>839</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Adrenosterone</td>
<td>II</td>
<td>14,609</td>
<td>1,697</td>
<td>878</td>
</tr>
<tr>
<td>17α-OH progesterone</td>
<td>17α-OH progesterone</td>
<td>II</td>
<td>9,933</td>
<td>4,758</td>
<td>1,479</td>
</tr>
<tr>
<td>Reduction</td>
<td>17α,20β-Dihydroxyprogren-4-en-3-one</td>
<td>I</td>
<td>10,608</td>
<td>4,903</td>
<td>1,440</td>
</tr>
<tr>
<td>Acetylation</td>
<td>17α-Hydroxy-20β-acetoxyprogren-4-en-3-one</td>
<td>I</td>
<td>9,787</td>
<td>4,474</td>
<td>1,466</td>
</tr>
<tr>
<td>16α-OH progesterone</td>
<td>16α-OH progesterone</td>
<td>III</td>
<td>2,730</td>
<td>1,490</td>
<td>1,204</td>
</tr>
<tr>
<td>Acetylation</td>
<td>16α-OH progesterone acetate</td>
<td>VI</td>
<td>2,553</td>
<td>1,512</td>
<td>1,101</td>
</tr>
<tr>
<td>Oxidation</td>
<td>16α-Oxoprogesterone</td>
<td>I</td>
<td>1,325</td>
<td>1,101</td>
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<tr>
<td>Reduction</td>
<td>16α,20β-Dihydroxyprogren-4-en-3-one</td>
<td>III</td>
<td>2,488</td>
<td>1,488</td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>DOC</td>
<td>II</td>
<td>6,577</td>
<td>499</td>
<td>6,168</td>
</tr>
<tr>
<td>Acetylation</td>
<td>DOC acetate</td>
<td>VI</td>
<td>2,205</td>
<td>6,015</td>
<td></td>
</tr>
<tr>
<td>Reduction</td>
<td>20αβ,21-Dihydroxyprogren-4-en-3-one</td>
<td>I</td>
<td>2,400</td>
<td>5,561</td>
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<td>Acetylation</td>
<td>20αβ,21-Diacetoxyprogren-4-en-3-one</td>
<td>I</td>
<td>7,294</td>
<td>548</td>
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<tr>
<td>DOC</td>
<td>DOC</td>
<td>I</td>
<td>94-86</td>
<td>29-55</td>
<td></td>
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<tr>
<td>Acetylation</td>
<td>DOC acetate</td>
<td>I</td>
<td>88-92</td>
<td>29-28</td>
<td></td>
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<tr>
<td>Reduction</td>
<td>20αβ,21-Dihydroxyprogren-4-en-3-one</td>
<td>II</td>
<td>93-02</td>
<td>29-77</td>
<td></td>
</tr>
<tr>
<td>Corticosterone</td>
<td>Corticosterone</td>
<td>III</td>
<td>82-66</td>
<td>29-97</td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>Corticosterone acetate</td>
<td>III</td>
<td>85-77</td>
<td>32-03</td>
<td></td>
</tr>
<tr>
<td>Reduction</td>
<td>11β, 20αβ,21-Trihydroxyprogren-4-en-3-one</td>
<td>III</td>
<td>89-59</td>
<td>30-39</td>
<td></td>
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<tr>
<td>11-Deoxycorticosterone</td>
<td>11-Deoxycorticosterone</td>
<td>III</td>
<td>340-7</td>
<td>9-91</td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>11-Deoxycorticosterone acetate</td>
<td>III</td>
<td>359-1</td>
<td>9-28</td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>Androstenedione</td>
<td>II</td>
<td>331-6</td>
<td>8-65</td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td>Cortisol</td>
<td>III</td>
<td>271-3</td>
<td>7-84</td>
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<tr>
<td>Acetylation</td>
<td>Cortisol acetate</td>
<td>III</td>
<td>288-7</td>
<td>7-79</td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>Adrenosterone</td>
<td>II</td>
<td>285-2</td>
<td>7-69</td>
<td></td>
</tr>
<tr>
<td>Cortisone</td>
<td>Cortisone</td>
<td>III</td>
<td>19-82</td>
<td>6-64</td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>Cortisone acetate</td>
<td>III</td>
<td>18-06</td>
<td>6-64</td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>Adrenosterone</td>
<td>II</td>
<td>19-91</td>
<td>6-68</td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>Pregnenolone</td>
<td>IV</td>
<td>30-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>Pregnenolone acetate</td>
<td>IV</td>
<td>28-06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction</td>
<td>Pregn-5-ene-3β,20β-diol</td>
<td>IV</td>
<td>28-02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-OH progrenolone</td>
<td>17α-OH progrenolone</td>
<td>IV</td>
<td>151-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>17α-Hydroxyprogrenolone acetate</td>
<td>V</td>
<td>157-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>Progesterone</td>
<td>IV</td>
<td>38-58</td>
<td>2-99</td>
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<tr>
<td>Reduction</td>
<td>20β-Hydroxyprogren-4-en-3-one</td>
<td>IV</td>
<td>34-23</td>
<td>2-82</td>
<td></td>
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<tr>
<td>Acetylation</td>
<td>20β-Acetoxyprogren-4-en-3-one</td>
<td>I</td>
<td>34-83</td>
<td>2-73</td>
<td></td>
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</table>
Cortisol synthesis in man

Table 1 (cont.)

<table>
<thead>
<tr>
<th>Steroid isolated</th>
<th>Fascicular and reticular tissue</th>
<th>²H</th>
<th>¹⁴C</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-OH progesterone</td>
<td>I</td>
<td>183-2</td>
<td>2-82</td>
</tr>
<tr>
<td>Reduction</td>
<td>I</td>
<td>183-7</td>
<td>2-89</td>
</tr>
<tr>
<td>Acetylation</td>
<td>I</td>
<td>176-2</td>
<td>2-85</td>
</tr>
<tr>
<td>16α-OH progesterone</td>
<td>III</td>
<td>15-90</td>
<td>3-09</td>
</tr>
<tr>
<td>Acetylation</td>
<td>III</td>
<td>15-86</td>
<td>3-28</td>
</tr>
<tr>
<td>Reduction</td>
<td>III</td>
<td>17-03</td>
<td>3-26</td>
</tr>
</tbody>
</table>

Table 2. Radioactivity associated with steroids isolated from the incubations (mCi)

(Percentages of the radioactivity incubated are shown in parentheses)

<table>
<thead>
<tr>
<th>Steroid isolated</th>
<th>Fascicular tissue*</th>
<th>Reticular tissue*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>²H:¹⁴C</td>
<td>²H:¹⁴C</td>
</tr>
<tr>
<td>Cortisol</td>
<td>³H:¹⁴C</td>
<td>³H:¹⁴C</td>
</tr>
<tr>
<td>17α-OH progesterone</td>
<td>272-4 (1-43)</td>
<td>—</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1396 (7-34)</td>
<td>—</td>
</tr>
<tr>
<td>17α-OH progesterone</td>
<td>333-1 (1-75)</td>
<td>27-2 (2-54)</td>
</tr>
<tr>
<td>16α-OH progesterone</td>
<td>1646 (8-66)</td>
<td>24-7 (2-30)</td>
</tr>
<tr>
<td>DOC</td>
<td>147-8 (0-78)</td>
<td>29-4 (2-74)</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>838-7 (4-41)</td>
<td>268-4 (25-04)</td>
</tr>
<tr>
<td>11-Deoxycorticisol</td>
<td>745-7 (3-92)</td>
<td>263-5 (24-58)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>2981 (15-88)</td>
<td>77-9 (7-27)</td>
</tr>
<tr>
<td>Cortisone</td>
<td>2336 (12-29)</td>
<td>63-8 (5-95)</td>
</tr>
<tr>
<td></td>
<td>160-4 (0-84)</td>
<td>5-4 (0-50)</td>
</tr>
</tbody>
</table>

Fascicular and reticular tissue†

Table 2. Radioactivity associated with steroids isolated from the incubations (mCi)

* Incubated with 11,331 mCi [7α-³H]pregnenolone and 604 mCi [4-¹⁴C]progesterone.
† Incubated with 19,010 mCi [7α-³H]pregnenolone and 1,072 mCi [4-¹⁴C]progesterone.

firm those of Lipsett & Hökfelt (1961), Mulrow, Cohn & Kuljian (1962) and Weliky & Engel (1962, 1963), who demonstrated that 17α-hydroxyprogrenolone is transformed to cortisol, thereby indicating that two pathways may exist for the formation of the adrenal hormone (Fig. 1). However, these authors were unable to provide information on the relative importance of the various routes to cortisol from pregnenolone. It thus seemed obvious that a much more detailed analysis of the metabolites of pregnenolone and progesterone in vitro was necessary, and it was decided to re-examine the role of progesterone in cortisol synthesis in the highly active tissue of a hyperplastic adrenal. Moreover, it was thought to be of considerable interest at this stage to devise a new method for the assessment of the relative magnitudes of alternative metabolic pathways in experiments in vitro, since the control of the relative amounts of cortisol, aldosterone and adrenal androgens (e.g. DHA sulphate) secreted by
adrenal cells must be exercised through the metabolism of one or more of the intermediates, pregnenolone, 17α-hydroxypregnenolone and progesterone.

Figure 2 shows a diagram similar to one described by Kopin (1963) as a convergent metabolic pathway, where all the products are derived from a single precursor. One of the products, $C_2$, is common to both intermediates $B_1$ and $B_2$. The equation

$$f_{B_2C_2} = \frac{[^3\text{H}]_{C_2}}{[^1\text{C}]_{C_2}}$$

gives the fraction of $C_2$ derived from $B_2$ in this simple system, assuming that precursor $A$ is labelled with isotope $^3\text{H}$ and intermediate $B_2$ with isotope $^{1\text{C}}$. It is important to realize that this equation was applicable only in vivo where the isotopically labelled precursor and intermediate could be completely metabolized to their end-products which were collected and analysed as urinary extracts. The superficial resemblance of the theoretical system of Kopin to the known pathways of pregnenolone metabolism in adult human adrenal tissue is apparent (Fig. 1). Admittedly, the situation with regard to steroid metabolic pathways in vitro is vastly more complicated but it seemed that at least a semi-quantitative assessment of the relative magnitude of alternative metabolic pathways from pregnenolone could be made, hence clarifying the role of progesterone in cortisol synthesis. In an in vitro system of adrenal steroid biosynthesis the corresponding so-called 'products' $C_2$ and $C_3$ would be 17α-hydroxyprogesterone and DOC. These steroids are not products in such a system since they are extensively further metabolized—17α-hydroxyprogesterone to 11-deoxy cortisol, cortisol and cortisone, and DOC to corticosterone. In Kopin's
system, $C_2$ and $C_3$ are 'sinks' in the sense that the isotopes $^3\text{H}$ and $^{14}\text{C}$ collect at these points and can be used for analysis. If we substitute $(C_2 + \Sigma C_2)$ and $(C_3 + \Sigma C_3)$ to represent $(C_2 + \text{all its metabolites})$ and $(C_3 + \text{all its metabolites})$ respectively then $(C_2 + \Sigma C_2)$ and $(C_3 + \Sigma C_3)$ become the isotope 'sinks' (see Fig. 3).

For really accurate assessment of the alternative pathways it would be necessary for all the $^3\text{H}$ and $^{14}\text{C}$ labels to pass beyond the $17\alpha$-hydroxyprogesterone and DOC 'gateways'. This is impossible in an in vitro system, but if the incubation of tissues is sufficiently prolonged, an approximation to the ideal situation would be achieved.

\[
J_{B_2(C_2+\Sigma C_2)} = \frac{[^3\text{H}]}{[^{14}\text{C}]}_{C_2+\Sigma C_2} / \frac{[^3\text{H}]}{[^{14}\text{C}]}_{C_3+\Sigma C_3}
\]

Fig. 3. ‘Convergent metabolic pathway’ adapted for the situation in which $C_1$, $C_2$ and $C_3$ are not end-products. The equation gives the fraction of $C_2 + \Sigma C_2$ derived from $B_2$ provided that $A$, $B_1$ and $B_2$ are completely metabolized.

A second source of error would arise if $17\alpha$-hydroxyprogesterone and DOC had common products or if there was a third major pathway of cortisol synthesis, say by way of $17\alpha,21$-dihydroxyprogrenonelone. As yet there is no evidence that either situation is true in the adult human adrenal cortex. Thus the equation

\[
J_{B_2(C_2+\Sigma C_2)} = \frac{[^3\text{H}]}{[^{14}\text{C}]}_{C_2+\Sigma C_2} / \frac{[^3\text{H}]}{[^{14}\text{C}]}_{C_3+\Sigma C_3}
\]

(see Fig. 3) should give a reasonable estimate of the proportion of $(C_2 + \Sigma C_2)$ derived from $B_2$ in an in-vitro system such as that found in incubations of human adrenal tissue. When the appropriate values are substituted in this equation it is found that approximately 7% of $17\alpha$-hydroxyprogesterone + $11\text{-deoxycortisol} + \text{cortisol} + \text{cortisone}$ was formed from pregnenolone via progesterone in the incubation of hyperplastic adrenal tissue. This shows that the classical route for cortisol biosynthesis via progesterone is in fact a minor one and indicates that the only major pathway is that involving $17\alpha$-hydroxyprogrenonelone in this tissue. Further work is necessary to prove the latter point since the experimental approach outlined in this paper only allows an assessment of the role of progesterone as an intermediate in the transformation of pregnenolone to cortisol.
The authors wish to express their gratitude to Professor A. P. M. Forrest, Department of Surgery, Royal Infirmary, Cardiff, for supplying the tissues used in this investigation and to Professor T. Symington and his staff, Department of Pathology, Royal Infirmary, Glasgow, for the histological data. Dr I. J. Kopin, Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Md., U.S.A., provided constructive criticism and encouragement. One of us (E.H.D.C.) received generous financial support from Imperial Chemical Industries for part of this work undertaken in the Department of Steroid Biochemistry, Royal Infirmary, Glasgow.

The authors gratefully acknowledge the generous financial support of the Tenovus organization in Cardiff.

REFERENCES
CORTICOSTEROID SYNTHESIS IN A CLEAR CELL ADENOMA: A TIME-BASED STUDY

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Tenovus Institute for Cancer Research, The Welsh National School of Medicine, Heath, Cardiff, CF4 4XX

(Received 6 October 1967)

SUMMARY

The removal of a large clear cell adenoma (67 g.) from a patient in the Cardiff Royal Infirmary presented an opportunity to investigate the relative importance of alternative metabolic pathways to cortisol in adrenal tissue consisting primarily of a single cell type. Experiments were performed in which chopped tumour tissue was incubated simultaneously with [4-14C]-pregnenolone and [7α-3H]progesterone for periods of time ranging from 5 to 120 min.

The conversion of these precursors to a variety of corticosteroids and intermediates was determined by radioisotope dilution techniques. From the results obtained, it was concluded that progesterone played only a minor role in the transformation of pregnenolone to cortisol in this tissue. It appeared that a large proportion of any progesterone formed from pregnenolone is rapidly metabolized to the 17-deoxycorticosteroids.

INTRODUCTION

Much useful information is provided by the incubation in vitro of radioactive steroids with steroid hormone-secreting tissues. This information may take the form of the identity of the steroid produced, or possibly the nature of the biosynthetic pathways used by the tissue to effect such production. However, fundamental assumptions are often made which require examination.

It is assumed that observed conversion patterns and the relative proportions of the measured end-products reflect the secretory pattern in vivo. This need not be the case since incubated cells are confronted with an alien environment, and presented with precursors in a way and in relative concentrations not found in vivo. The extrapolation of results in vitro to the in vivo state should ideally be supported by experimental evidence in vivo, although this is not often possible in human endocrine studies.

Interpretation of conversion figures can be difficult, especially when obtained from a single incubation. Low figures may mean either that a biosynthetic intermediate is not made in large amounts, or that the compound is rapidly metabolized, and that little remains at the end of the incubation period. For this reason the present investigation deals with five incubations terminated at times varying from 5 to 120 min. in
order to obtain a series of 'profiles' of the metabolism of [4-14C]pregnenolone and [7α-3H]progesterone in vitro with adrenal tissue.

The isolation of reasonable yields of endocrine tissue consisting of cells of a single cell type is not easy and many investigations have had to be undertaken on adrenal, ovarian and testicular tissues which contain mixtures of cells, probably with different biosynthetic functions. Some success has been achieved by using a Stadie–Riggs wet-tissue microtome (Stadie & Riggs, 1944) for the separation of the zona fasciculata and zona reticularis of the adrenal cortex in man (Griffiths, Grant & Symington, 1963; Cameron, Beynon & Griffiths, 1968) and from horse (Cameron & Grant, 1967). More refined ultramicro techniques have also been used for the separation of the two zones in the rat cortex (Griffiths & Glick, 1966).

However, good yields of adrenal tissue of one cell type are available only from the clear cell adenoma. The cells closely resemble the clear cell of the fascicular zone (Plate, figs. 2, 3) histologically, and respond to corticotrophin (ACTH) stimulation in vitro in a manner similar to that of slices of fascicular tissue (Griffiths et al. 1963). It seems reasonable to assume that the cells are functionally similar and such tissue has been used in the investigations now described.

In a previous publication Cameron et al. (1968) described an investigation of metabolic pathways involved in the biosynthesis of cortisol from pregnenolone in hyperplastic adrenal tissue. They concluded that less than 10% of the [7α-3H]pregnenolone incubated in vitro had been transformed to cortisol via progesterone. The present paper deals with the results of groups of five experiments with tissue from a clear cell adrenal adenoma incubated simultaneously with [4-14C]pregnenolone and [7α-3H]progesterone. The purpose in performing these experiments was twofold: (1) to take advantage of a single cell type to perform a series of time-based studies and observe the variation of conversion figures with respect to time. (2) To attempt an assessment of the relative magnitude of alternative metabolic pathways to cortisol from pregnenolone in tissue closely resembling that of the zona fasciculata of the normal adrenal gland.

MATERIALS AND METHODS

Adrenal tissue. A well encapsulated adrenal tumour (67 g.) was removed from a 35-yr.-old woman with Cushing's syndrome (Pl., fig. 1). The tumour was placed in a polythene bag and transported to the laboratory on crushed ice. Experimental work on the tissue began less than 1 hr. after its removal from the patient.

Histology. Representative pieces of all tissue to be incubated were fixed in 10% neutral formalin for histological examination by Professor T. Symington, Institute of Pathology, Royal Infirmary, Glasgow.

Preparation of tissue for biochemical investigation. For the incubations a single pool of tissue was prepared. Only material with a bright yellow appearance and firm texture was used and when sufficient had been obtained it was finely chopped with a safety razor blade and thoroughly mixed. Portions (5 x 2 g.) were then weighed out.

Incubation, extraction and fractionation of steroids. The procedures used were similar to those described by Griffiths, Grant, Browning, Whyte & Sharp (1966). Each 2 g. portion of chopped tumour tissue was incubated with a mixture of steroids comprising 83.5 mμmole [4-14C]pregnenolone (2245 mμc), 4.8 mμmole [7α-3H]progesterone
(10,809 mμc) and 4.8 mμmole unlabelled 17α-hydroxypregnenolone in 25 ml. Krebs-Ringer bicarbonate-glucose medium at 37° in an atmosphere of 95% O₂:5% CO₂. It was reasoned that the object of the experiments was to investigate the metabolism of pregnenolone. In order to approximate as closely as possible the criteria required by Kopin’s formula (Kopin, 1963; Cameron et al. 1968), it was necessary to regard the labelled progesterone as a tracer intermediate rather than simply a substrate. Thus [4-14C]pregnenolone was chosen since it had the lower specific activity. However, the mass of the [7α-3H]progesterone added could not be disregarded in terms of tissue:steroid ratio. Therefore, so as to avoid creating an initial ‘imbalance’ in the two major pathways to cortisol from pregnenolone, a quantity of non-labelled 17α-hydroxypregnenolone equimolar to the [7α-3H]progesterone was also added to each incubation.

Incubations were performed for periods of 5, 10, 20, 60 and 120 min. respectively and terminated by the addition of 40 ml. acetone. Carrier steroids (300 μg. of each) were then added to each mixture and included progesterone, 16α-hydroxyprogesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycorticosterone (DOC), 11-deoxycortisol, corticosterone, cortisol and cortisone. (Pregnenolone, 16α-hydroxy-pregnenolone, dehydroepiandrosterone, Δ₄-androstenedione and 11β-hydroxyandrostenedione were also added and the results relating to these steroids will be published elsewhere (Cameron, Beynon & Griffiths, in preparation)).

Mixtures were homogenized with a Silverson homogenizer and acetone extracts dried and partitioned between 80% aqueous methanol and light petroleum (b.p. 80–100°) as described previously (Cameron et al. 1968). The methanolic extracts were taken almost dryness in a rotary evaporator, diluted with water and the steroids extracted with chloroform.

Chromatography. All chromatography was performed on thin layers of Merck silica gel HF₄₂₅₄₃₆₃. The solvent systems used were as follows: (I) cyclohexane:ethyl acetate (90:110), (II) benzene:methanol (170:30), (III) chloroform:methanol:water (187:12:1), (IV) chloroform:acetone (185:15), (V) cyclohexane:ethanol (180:20), (VI) benzene:ethyl acetate (180:20). All solvent proportions are by volume.

Elution of steroids from thin-layer chromatograms was as described elsewhere (Griffiths et al. 1966) with the modification that 2 ml. water was routinely used to deactivate the silica gel (Cameron et al. 1968) and ethyl acetate to extract the steroids. The Δ₄-3-oxosteroids were visualized on thin layers by light at 254 mμ.

Identification and quantitative measurement of steroids. A preliminary separation of steroids into four ‘regions’ was achieved in system I. Region 1, containing non-polar steroids, was eluted and rechromatographed in system IV in order to isolate progesterone. Region 2 was rechromatographed in system II in order to isolate 17α-hydroxyprogesterone and 17α-hydroxypregnenolone. Region 3, containing DOC and 11-deoxycortisol and region 4 containing 16α-hydroxyprogesterone, corticosterone, cortisone and cortisol were re-run in system IV.

Individual steroids were then eluted and re-chromatographed directly or after formation of derivatives by acetylation, oxidation, reduction and saponification procedures (Griffiths, Grant & Whyte, 1963). For specific activity determinations, the mass of the Δ₄-3-oxosteroids was measured by making use of their selective absorption at 240 mμ. Blank areas of silica gel from each chromatogram were taken.
through the elution procedure to provide analytical blanks for optical measurements.

Radioactivity was measured with a Nuclear Chicago Mark I Liquid Scintillation Spectrometer. The observation of specific activities of a steroid and two of its derivatives differing by not more than 10% was taken as satisfactory evidence for radiocchemical purity of the steroid concerned. However, one derivative only was prepared in the case of progesterone and 17α-hydroxy pregnenolone. The mean of the specific activities was used to calculate the percentage conversion from the original radioactive steroid incubated.

Determination of endogenous production of steroids. When whole cells of the adrenal gland are incubated in a suitable medium such as Krebs–Ringer bicarbonate solution, they synthesize and secrete steroids such as cortisol, corticosterone, cortisone etc. from endogenous cholesterol (e.g. Griffiths, Grant & Symington, 1963) regardless of whether exogenous precursors have been added to the medium. If any steroid in an incubation with a radioactive steroid precursor should be produced endogenously in amounts that are large enough to alter significantly conversion values obtained by radioisotope dilution analysis, then the principles of this analysis do not hold and correction must be made.

In order to perform this correction, the experimental results of the fifth (120 min.) incubation of the present series were combined in simultaneous equations with those from a sixth incubation in which tumour tissue (2 g.) was incubated with [4-14C]-pregnenolone (and [7α-3H]cholesterol sulphate) for a similar period. Larger amounts of carrier steroids, cortisol (1 mg.), corticosterone (1 mg.) and cortisone (0.5 mg.), were added to the sixth incubation, enabling the following equations to be solved:

true % conversion = \( \frac{\text{S.A.}_\text{obs}.(5) \times [\text{mmole added (5)} + \text{mmole endog.}]}{\text{mμc incubated}} \times 100 \),

true % conversion = \( \frac{\text{S.A.}_\text{obs}.(6) \times [\text{mmole added (6)} + \text{mmole endog.}]}{\text{mμc incubated}} \times 100 \),

where \( \text{S.A.}_\text{obs}.(5) \) = specific activity (mμc/mmole) of a steroid isolated from the fifth incubation, \( \text{S.A.}_\text{obs}.(6) \) = specific activity (mμc/mmole) of the steroid isolated from the sixth incubation, mmole added (5) = mmole of the steroid added to the fifth incubation, mmole added (6) = mmole of the steroid added to the sixth incubation, the values for the endogenous production of cortisol, cortisone and corticosterone calculated for the fifth incubation were extrapolated back to give approximate values for endogenous production during the shorter time-intervals.

Assessment of relative magnitude of alternative metabolic pathways. In a previous publication (Cameron et al. 1968) a mathematical formula was derived for a semi-quantitative assessment of alternative metabolic pathways in vitro by a method adapted from that described by Kopin (1963) for studies in vivo. It was shown that if the sum of the percentage conversions to 17α-hydroxyprogesterone + 11-deoxy cortisol + cortisol + cortisone is \( C_2 + \Sigma C_2 \) (Text-fig. 1) and that to DOC + corticosterone is \( C_3 + \Sigma C_3 \), then the fraction of \( C_2 + \Sigma C_2 \) which is derived from pregnenolone via progesterone is approximately

\[
J_B C_2 = \frac{\text{[14C]}_{3H}^{3H} C_3 + \Sigma C_3}{\text{[14C]}_{3H}^{3H} C_2 + \Sigma C_2}.
\]
RESULTS

Examination of haematoxylin- and eosin-stained sections (Plate, fig. 3) showed that all material used for biochemical experiments appeared to contain only fascicular-type cells. No trace of reticular-type cells was found.

Table 1 shows the steroid derivatives formed and the solvent systems used to purify them. Abbreviations employed in the Text-figures and succeeding tables are also shown in Table 1. Evidence for the radiochemical purity of each steroid is given in Table 2. Table 3 shows the percentage conversions of \([4,\text{14C}]\text{pregnenolone and [7a-3H]}\text{progesterone to C}_2 + \Sigma C_2 \text{ and C}_3 + \Sigma C_3 \text{ and the values of } f_{B3}C_4 \text{ derived therefrom.}

Text-figs. 2–5 illustrate the conversions of \([4,\text{14C}]\text{pregnenolone and [7a-3H]}\text{progesterone to each individual steroid isolated as functions of time. The curves for corticosterone, cortisol and cortisone are corrected for the endogenous production of these compounds. For the transformation of [4,\text{14C}]\text{pregnenolone to DOC the peak labelling time appeared to be about 5 min. (Text-fig. 3). A similar peak labelling time was observed for the transformation of [7a-3H]}\text{progesterone to DOC (Text-fig. 5) but the difference in magnitude of conversion was enormous. Almost 30\% of the 3H-label was found in DOC after only 5 min. of incubation. However, the 3H content of the DOC fell rapidly over the first hour and at the end of this time only 2.3\% of the 3H-label remained in this intermediate.

A curious reversal in peak labelling times was revealed with 17\(\alpha\)-hydroxyprogesterone and 11-deoxycorticisol (Text-fig. 2). With the \(\text{14C-label, 11-deoxycorticisol reached its maximum isotopic content before 17\(\alpha\)-hydroxyprogesterone and the \(\text{14C level of 11-deoxycorticisol never exceeded that of 17\(\alpha\)-hydroxyprogesterone in any one

Text-fig. 1. Pathways of corticosteroid biosynthesis showing relationship of the steroids to the components of the formula (see above) derived from that of Kopin (1963).

Cholesterol

\[\text{[H]Pregnenolone}\]

17\(\alpha\)-OH pregnenolone

DHA

[16\(\alpha\)-OH progesterone]

\[\text{[H]Progesterone (B}_2\text{)}\]

17\(\alpha\)-OH progesterone (\(C_3\))

DOC (\(C_3\))

11-Deoxycorticisol

Cortisol

Corticosterone (\(\Sigma C_2\))

Text-fig. 1. Pathways of corticosteroid biosynthesis showing relationship of the steroids to the components of the formula (see above) derived from that of Kopin (1963).
incubation. The $^{14}$C maximum of 17α-hydroxyprogrenenolone is, on the other hand, virtually coincident with that of 11-deoxycortisol. The complete reverse was true of the $^3$H-label found in these steroids. The curves for cortisol and corticosterone (Text-fig. 4) show that [4-$^{14}$C]pregnenolone was transformed to cortisol at a much higher rate than to corticosterone and that the reverse was true of the transformation of [7α-$^3$H]progesterone. It should also be noted that the $^3$H content of corticosterone fell over the second hour of incubation. This observation may also be reflected in the $[3^H]$ values for $C_9 + \Sigma C_3$ (Table 3) which reach a maximum at 10 min., falling to the 5 min. level at the end of the 2 hr. incubation period.

Table 1. Formation and chromatography of derivatives of steroids isolated from incubations of tumour tissue with [4-$^{14}$C]pregnenolone, [7α-$^3$H]progesterone and 17α-hydroxyprogrenenolone

<table>
<thead>
<tr>
<th>Material investigated and chemical reaction</th>
<th>Product mobility identical with that of:</th>
<th>Abbreviation</th>
<th>Solvent system</th>
</tr>
</thead>
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<tr>
<td>Progesterone</td>
<td>Progesterone</td>
<td>Prog.</td>
<td>IV</td>
</tr>
<tr>
<td>Reduction</td>
<td>20β-Hydroxyprogren-4-en-3-one</td>
<td>20-P</td>
<td>IV</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenenolone</td>
<td>17α-Hydroxyprogrenenolone</td>
<td>17-P</td>
<td>II</td>
</tr>
<tr>
<td>Acetylation</td>
<td>17α-Acetoxyprogrenenolone</td>
<td>17-P</td>
<td>II</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>17α-Hydroxyprogesterone</td>
<td>17-P</td>
<td>II</td>
</tr>
<tr>
<td>Reduction</td>
<td>17αz,20β-Dihydroxyprogren-4-en-3-one</td>
<td>17,20-P</td>
<td>I</td>
</tr>
<tr>
<td>Reduction and acetylation</td>
<td>17α-Hydroxy-20β-acetoxyprogren-4-en-3-one</td>
<td>17,20-PAc</td>
<td>IV</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>11-Deoxycortisol</td>
<td>S</td>
<td>III</td>
</tr>
<tr>
<td>Reduction</td>
<td>11-Deoxycortisol acetate</td>
<td>SAc</td>
<td>III</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Androst-4-ene-3,17-dione</td>
<td>Δ4-A</td>
<td>V</td>
</tr>
<tr>
<td>Cortisol</td>
<td>Cortisol</td>
<td>F</td>
<td>III</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Cortisol acetate</td>
<td>FAc</td>
<td>III</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Androst-4-ene-3,11,17-trione</td>
<td>11-oxo-Δ4-A</td>
<td>IV</td>
</tr>
<tr>
<td>Cortisone</td>
<td>Cortisone</td>
<td>E</td>
<td>III</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Cortisone acetate</td>
<td>EAc</td>
<td>III</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Androst-4-ene-3,11,17-trione</td>
<td>11-oxo-Δ4-A</td>
<td>IV</td>
</tr>
<tr>
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<td>IV</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>11-Deoxycorticosterone</td>
<td>DOC</td>
<td>I</td>
</tr>
<tr>
<td>Reduction</td>
<td>11-Deoxycorticosterone acetate</td>
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<td>I</td>
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<td>20-DOC</td>
<td>IV</td>
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<tr>
<td>Reduction and acetylation</td>
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<td>20-DOCAc</td>
<td>IV</td>
</tr>
<tr>
<td>11β,20β,21-Trihydroxyprogren-4-en-3-one</td>
<td>11β,20β,21-Trihydroxyprogren-4-en-3-one</td>
<td>20-DOCdiAc</td>
<td>I</td>
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<tr>
<td>Corticosterone</td>
<td>Corticosterone</td>
<td>B</td>
<td>III</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Corticosterone acetate</td>
<td>BAc</td>
<td>III</td>
</tr>
<tr>
<td>Reduction</td>
<td>11β,20β,21-Trihydroxyprogren-4-en-3-one</td>
<td>20-B</td>
<td>III</td>
</tr>
<tr>
<td>16α-Hydroxyprogesterone</td>
<td>16α-Hydroxyprogesterone</td>
<td>16-P</td>
<td>III</td>
</tr>
<tr>
<td>Acetylation</td>
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</tr>
<tr>
<td>Reduction</td>
<td>16α,20β-Dihydroxyprogren-4-en-3-one</td>
<td>16,20-P</td>
<td>III</td>
</tr>
<tr>
<td>Acetylation and reduction</td>
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<td>IV</td>
</tr>
<tr>
<td>Reduction and acetylation</td>
<td>16α,20β-Diacetoxyprogren-4-en-3-one</td>
<td>16,20-PdiAc</td>
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</table>
Table 2. Identification of steroids isolated from simultaneous incubations of \([4,14C]\) pregnenolone, \([7\alpha,3H]\) progesterone and \(17\alpha\)-hydroxyprogrenolone with tumour tissue

<table>
<thead>
<tr>
<th>Steroid or derivative</th>
<th>(^{14}C)</th>
<th>(^{3}H)</th>
<th>(^{14}C,^{3}H)†</th>
<th>(^{14}C)</th>
<th>(^{3}H)</th>
<th>(^{14}C,^{3}H)</th>
<th>(^{14}C)</th>
<th>(^{3}H)</th>
<th>(^{14}C,^{3}H)</th>
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<td>5 min. (1)*</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prog.</td>
<td>136</td>
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<td>1:7:9</td>
<td>210</td>
<td>2,045</td>
<td>1:3:0</td>
<td>156</td>
<td>2,441</td>
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<tr>
<td>20-P</td>
<td>130</td>
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<td></td>
<td>214</td>
<td>3,093</td>
<td></td>
<td>164</td>
<td>2,525</td>
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<td>17-P</td>
<td>379</td>
<td>831</td>
<td></td>
<td>481</td>
<td>658</td>
<td></td>
<td>540</td>
<td>521</td>
<td></td>
</tr>
<tr>
<td>17,20-P</td>
<td>384</td>
<td>835</td>
<td>2:2:1</td>
<td>461</td>
<td>627</td>
<td>3:5:1</td>
<td>525</td>
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<td>S</td>
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<td>346</td>
<td>877</td>
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<td>SA6</td>
<td>178</td>
<td>1,071</td>
<td>1:1:3</td>
<td>374</td>
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<td>1:3:1</td>
<td>348</td>
<td>932</td>
<td>1:8:1</td>
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<tr>
<td>(\Delta^\alpha) A</td>
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<td>1,121</td>
<td></td>
<td>337</td>
<td>1,384</td>
<td></td>
<td>339</td>
<td>1,212</td>
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<tr>
<td>F</td>
<td>96-5</td>
<td>463</td>
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<td>861</td>
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<td>668</td>
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<tr>
<td>11-oxo-(\Delta^\alpha) A</td>
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<td>729</td>
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<td>26-3</td>
<td>75-2</td>
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<td>67-6</td>
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<td>80-8</td>
<td></td>
<td>71-2</td>
<td>122</td>
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<td>—</td>
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<td>1:6:1</td>
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<td>55-2</td>
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<td>14-9</td>
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<td>3,070</td>
<td>1:39</td>
<td>51-3</td>
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<td>1:20</td>
<td>93-8</td>
<td>8,706</td>
<td>1:17</td>
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<td>95-0</td>
<td>9,031</td>
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<td>12-8</td>
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<td></td>
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<tr>
<td>16,20-P</td>
<td>6-6</td>
<td>475</td>
<td></td>
<td>11-5</td>
<td>589</td>
<td></td>
<td>13-9</td>
<td>564</td>
<td></td>
</tr>
<tr>
<td>16,20-PdIA6</td>
<td>6-7</td>
<td>502</td>
<td>1:16</td>
<td>11-5</td>
<td>608</td>
<td>1:10</td>
<td>13-0</td>
<td>564</td>
<td>1:4</td>
</tr>
<tr>
<td>16,20-PdIA6</td>
<td>6-5</td>
<td>—</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>—</td>
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<td></td>
</tr>
</tbody>
</table>

* Incubation number.
† Ratio of % conversions from the radioactive steroids incubated. Conversions are calculated from the mean specific activities by the principles of radioisotope dilution analysis.
Table 3. Assessment of the relative contribution of the pathway, pregnenolone → progesterone → 17α-hydroxyprogesterone to the biosynthesis of cortisol in adrenal tumour tissue in vitro

<table>
<thead>
<tr>
<th>Incubation no.</th>
<th>Time (min.)</th>
<th>C₁+ΣC₂</th>
<th>C₁+ΣC₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>¹⁴C (%)*</td>
<td>³H (%)*</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>11-6</td>
<td>8-6</td>
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</tr>
<tr>
<td>3</td>
<td>20</td>
<td>30-6</td>
<td>11-6</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>60-6</td>
<td>16-6</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>71-6</td>
<td>20-8</td>
</tr>
</tbody>
</table>

* Sum of % conversions to 17-P+S+F+E
† Sum of % conversions to B, E and F corrected for endogenous production.
‡ Fraction of 17-P+S+F+E derived from pregnenolone via progesterone.

Text-fig. 2. Percentage conversion of [4-¹⁴C]pregnenolone to 17α-hydroxyprogesterone (▲—▲), 11-deoxycortic (＋——＋), 16α-hydroxyprogesterone (●—●) and progesterone (▼—▼) as functions of time.

Text-fig. 3. Percentage conversion of [4-¹⁴C]pregnenolone to cortisol, cortisone, corticosterone, 11-deoxycorticosterone and 17α-hydroxypregnenolone as functions of time. Symbols used are as follows: cortisol (●—●), cortisone (Δ—Δ), corticosterone (×—×), 11-deoxycorticosterone, (○—○) and 17α-hydroxypregnenolone (▼—▼).
As mentioned above, the sixth 'monitoring' incubation was performed with [4-14C]pregnenolone as one of its substrates. At the end of the 2 hr. incubation period carrier steroids were added, the diluted radioactive metabolites isolated and their specific activities determined in the usual manner. Cortisol, corticosterone and cortisone had mean specific activities for 14C of 844, 57.9 and 239 disintegrations/min./mμmole respectively. When the appropriate substitutions were performed in simultaneous equations (1) and (2), it was calculated that over the 2 hr. period approximately

830 mμmole (300 μg.) cortisol, 56 mμmole (19 μg.) corticosterone and 293 mμmole (106 μg.) cortisone had been formed. As stated above, these figures were then extrapolated back to give values for endogenous production during the shorter time-intervals.

**DISCUSSION**

Although it cannot be certain that the 'clear cell' of the adrenal adenoma is biochemically identical with that of the fascicular zone, there are many close resemblances...
which have already been described. However, this question is not so important as the assumption that all the cells incubated were of the same type. This assumption was supported by histological examination of random pieces taken from the tissue to be incubated, and care was taken to ensure that the pool of chopped tissue was homogeneous.

During incubation in vitro of human adrenal cells, steroids are secreted into the medium, particularly cortisol, corticosterone and cortisone (Griffiths, Grant & Symington, 1963). If reverse isotope dilution analysis is to be accurate, the contribution of the tissue to the total mass of each steroid isolated must either be negligible or measured. The technique of using a sixth incubation to 'monitor' the fifth of the current series may be criticized on the grounds that the production of each steroid need not necessarily be identical in any two separate incubations, especially those involving whole cells. However, in a pair of incubations from a homogeneous pool of clear cell adenoma tissue, the production of steroid from identical substrates ([4-14C]pregnenolone) must be very similar. Furthermore, the cortisol:corticosterone ratio of 15:1 and mean values of 300 μg. and 20 μg. for cortisol and corticosterone respectively are compatible with previous findings (K. Griffiths, J. K. Grant & T. Symington (1960), unpublished observations; Griffiths, Grant & Symington, 1963). It is also clear that steroid production over a period of 2 hr. is unlikely to be linear and the points on the corrected curves are near-approximations, although substantial improvements on uncorrected ones. The curves for steroids other than cortisol, corticosterone and cortisone were uncorrected since the quantities of these intermediates released into the medium are negligible.

Text-fig. 1 shows the major pathways known to be involved in adrenal steroid biosynthesis and it is self-evident that there are three main points of divergence in the scheme. (1) Pregnenolone may be metabolized either by 17α-hydroxylation to 17α-hydroxyprogrenolone or by 3β-hydroxysteroid dehydrogenation and Δ5-Δ4-isomserism to progesterone. (2) 17α-Hydroxyprogrenolone may be metabolized either by C-21 desmolase to DHA or by 3β-hydroxysteroid dehydrogenase and Δ5-Δ4-isomserase to 17α-hydroxyprogesterone. (3) Progesterone may be metabolized by 21-hydroxylase to DOC or by 17α-hydroxylase to 17α-hydroxyprogesterone. The final column in Table 3 shows the fraction of 17α-hydroxyprogesterone + 11-deoxy- Cortisol + Cortisol + Cortisone derived from pregnenolone via progesterone. These figures, particularly for those incubations 1–3, are a first approximation since the criteria for the validity of the formula cannot strictly be met in any in vitro system (Cameron et al. 1968). Nevertheless, it is apparent that less than 10% of the 17-hydroxylated steroids are formed via progesterone.

Table 2 shows that the 14C:3H ratios of the progesterone metabolites 16α-hydroxyprogesterone, DOC and corticosterone are extremely low compared with 17α-hydroxyprogesterone, etc., i.e. at the pregnenolone point of divergence, a very small proportion has been metabolized by the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomserase route. Text-figure 2 also shows that the conversion of [4-14C]pregnenolone to [14C]progesterone never exceeded 4.1%.

It must therefore be concluded that most of the pregnenolone is metabolized by way of 17α-hydroxyprogrenolone to 17α-hydroxyprogesterone and the small amount which reaches progesterone is rapidly transformed to the 17-deoxycorticosteroids.
Furthermore, from the fact that \( C_3 + \Sigma C_3 \) reaches a maximum value after 10 min. and the curves for corticosterone have maxima at about 60 min., it is probable that further metabolism of corticosterone occurred.

The formation and subsequent transformation of 17α-hydroxyprogrenolone would appear to hold the key to the major pathways of adrenal steroid biosynthesis. On the one hand 17α-hydroxyprogrenolone appears to be a major precursor of cortisol (Lipsett & Hökfelt, 1961; Mulrow & Cohn, 1961; Mulrow, Cohn & Kuljian, 1962; Weliky & Engel, 1962, 1963), and on the other of DHA. DHA sulphate is a major adrenal secretory product (Wieland, Levy, Katz & Hirschmann, 1963) although it has yet to be proved that DHA itself is the major precursor of DHA sulphate in the adrenal. If DHA is such a precursor then the metabolism of 17α-hydroxyprogrenolone may be directly responsible for the magnitude and sign of the urinary discriminant function described by Bulbrook, Hayward, Spicer & Thomas (1962) in relation to prognosis for breast cancer patients. A high desmolase relative to 3β-hydroxy-steroid dehydrogenase/Δ5,Δ4-isomerase activity would result in high 11-deoxy-17-oxosteroids relative to 17-hydroxycorticosteroids and would be associated with a (+)ve discriminant and a good prognosis. Conversely a low desmolase relative to 3β-hydroxy-steroid dehydrogenase/Δ5,Δ4-isomerase would be associated with a (−)ve discriminant and a poor prognosis. In all this, the effect of ACTH is of paramount importance since it is likely that the trophic hormone has a specific effect on steroid-metabolizing enzymes in the adrenal other than the classical one of increasing the transformation of cholesterol to pregnenolone (Stone & Hechter, 1954). Kass, Hechter, Macchi & Mou (1954) showed that prolonged administration of ACTH to rabbits had the effect of increasing 17-hydroxylase activity in their adrenals. More recently Fevold (1967) showed that this effect seems to have a substrate specificity favouring 17-hydroxylation of pregnenolone rather than progesterone. It has thus been established that progesterone plays but a minor role in the transformation of pregnenolone to cortisol in the fascicular-like clear cell of the adrenal adenoma. The implication is that the major pathway is pregnenolone → 17α-hydroxyprogrenolone → 17α-hydroxyprogesterone → 11-deoxy cortisol → cortisol. This point still remains to be proved since it is equally possible to postulate further hydroxylation of 17α-hydroxyprogrenolone before dehydrogenation at C-3. Indeed the reversal of the peak \(^{14}C\) labelling of 11-deoxy cortisol and 17α-hydroxyprogesterone (Text-fig. 2) suggests that such a pathway via 17α,21-dihydroxyprogrenolone exists and the question is now under active investigation. Furthermore, urinary metabolites of these possible intermediates have been isolated from the urine of newborn infants (Mitchell, 1967), who seem to have an adrenal deficient in 3β-hydroxy-steroid dehydrogenase activity (Lanman & Silverman, 1957).

The authors wish to record their appreciation of generous financial support from the Tenovus Organisation. They also wish to thank Professor A. P. M. Forrest, Department of Surgery, Royal Infirmary, Cardiff, for supplying the tissue used in this work and to Dr J. Picton Thomas, Medical Unit, Royal Infirmary, Cardiff for allowing them to study his patient. Professor T. Symington and his Staff, Department of Pathology, Royal Infirmary, Glasgow very kindly performed the histology and photomicrography of the tumour. Mr Ralph Marshall and his Staff, Department of Medical
Illustration, Royal Infirmary, Cardiff photographed the tumour and provided the photomicrograph of the normal adrenal gland.

REFERENCES


DESCRIPTION OF PLATE

Fig. 1. Well encapsulated adrenal tumour immediately after removal from patient. It weighed 67 g. and was approximately 5 cm. in diameter.

Fig. 2. Photomicrograph of a normal adrenal gland section showing capsule (C), zona fasciculata (ZF) zona reticularis (ZR) and medulla (M). The zona glomerulosa is not visible in this section. Haematoxylin and eosin. (× 60.)

Fig. 3. Typical photomicrograph of sections taken from representative pieces of tumour tissue incubated. Note that all cells appear to be of the fascicular type (see fig. 2). Haematoxylin and eosin. (× 190.)
ULTRAMICROCHEMICAL STUDIES
ON THE SITE OF FORMATION OF DEHYDROEPIANDROSTERONE SULPHATE IN THE ADRENAL CORTEX OF THE GUINEA-PIG

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SUMMARY

Although dehydroepiandrosterone sulphate has been recognized as one of the major secretory products of the human adrenal gland neither its biosynthetic pathway nor its site of formation within the cortex have been extensively studied. Ultramicrochemical techniques, which relate enzymic activity to well-defined groups of cells, have been used in the work now described to study the sulphation of dehydroepiandrosterone in the zones of the guinea-pig adrenal cortex. It has been shown that dehydroepiandrosterone sulphokinase activity resides only in the compact cell of the zona reticularis.

INTRODUCTION

It is now well established that dehydroepiandrosterone sulphate (DHA sulphate) is not only secreted by the human adrenal cortex (Baulieu, 1962; Wieland, Levy, Katz & Hirschmann, 1963) but that it is also one of the major secretory products of the gland (Vande Wiele, MacDonald, Bollé & Lieberman, 1963). However, the site of formation of DHA sulphate in the adrenal cortex is still unknown, and further investigations are also required to elucidate the pathways by which it is synthesized.

The ability of human adrenal tissue to convert DHA to its sulphate in vitro was demonstrated by a number of workers (Adams, 1963; Wallace & Lieberman, 1963; Cohn, Mulrow & Dunne, 1963) but the question of the significance of this finding was raised by the experiments of Lieberman and his colleagues (Calvin, Vande Wiele & Lieberman, 1964; Calvin & Lieberman, 1964; Roberts, Bandi, Calvin, Drucker & Lieberman, 1964), who showed that DHA sulphate can be synthesized from both cholesterol sulphate and from pregnenolone sulphate (see Fig. 1) without the loss of the sulphate group. On the other hand, studies with ‘human adrenal tumour slices’ (Lebeau, Alberga & Baulieu, 1964) suggested that the principal route for DHA sulphate synthesis involves DHA.

In most of the previous studies, little attention has been directed to the histology of the adrenal tissue investigated. In vitro, Griffiths, Cunningham & Cameron (1968) found only 0-09 and 0-08% conversion of pregnenolone and pregnenolone sulphate
respectively, to DHA sulphate by tissue from a clear cell human adrenal adenoma removed from a patient with Cushing's syndrome, and it was suggested that the clear cell of the zona fasciculata of the cortex may not normally synthesize and secrete DHA sulphate. This has now been studied with ultramicrochemical techniques (Griffiths & Glick, 1966), which relate enzyme activity to well-defined cell types, using the guinea-pig adrenal cortex in the absence of readily available human adrenal tissue.

**MATERIALS AND METHODS**

*Animals.* Adrenal glands were obtained from male albino guinea-pigs weighing about 800–1000 g. and approximately 9 months old. They were killed with an overdose of pentobarbitone sodium at 14.00 hr.

*Adrenal glands.* The adrenals were quickly removed, stripped free of fat and frozen in solid carbon dioxide. Cylinders of tissue were bored out of the glands and fresh frozen microtome sections (diameter, 2.5 mm., 16 μ thick, volume, 0.079 μl.) were prepared in a cryostat at −15° as described previously (Grunbaum, Geary & Glick, 1956). One section was brushed flat on to a glass slide for histological examination and the next three sections were placed in a glass-stoppered reaction tube (30 mm. long, 4 mm. internal diameter), containing 10 μl. of 0.25 m-sucrose and 0.12 m-nicotinamide solution (Handler & Klein, 1942) for enzyme study. This sequence was followed through the entire cortex and into the medulla of the gland. Sections on the slide were stained first with Sudan IV, and then with haematoxylin.

*Conditions of incubation.* To each reaction tube was added \( [7α-3H] \text{DHA} \) (48.96 ng., sp.act. 500 mc/mM) in 170 nl. ethanol. The procedure for this addition has been described by Griffiths & Glick (1966).

Radioactive steroids used as precursors in the incubation were checked for purity by diluting the samples with carrier material, and chromatography on thin layer and paper in a number of solvent systems. Radioactive material on paper and thin layer was measured with a Packard Model 7201 Radiochromatogram Scanner.

The incubation medium (15 μl.) consisting of phosphate buffer, pH 7.0, containing 0.025 m-magnesium sulphate, 0.02 m-potassium sulphate and 0.01 m-ATP (Pulkkinen, 1966) was then added. During these additions the tubes were maintained at a low
temperature by standing in holes drilled in an aluminium block which had been previously cooled to \(-15^\circ\), and had held the tubes in the cryostat.

Disintegration of the tissue in the medium was immediately effected by rapid vibration (Glick, 1961, 1962) and the tubes were placed in a water bath at 37° for 1 hr. The reaction was then stopped by the addition of ethyl acetate (100 \(\mu\)l.).

**Extraction and identification of steroids.** Non-radioactive DHA sulphate (100 \(\mu\)g. in 50 \(\mu\)l. ethanol) was added to each incubation tube followed by 50 \(\mu\)l. of a 25\% (w/v) sodium chloride solution. Medium and solvent were mixed with a rotating stainless-steel wire bent in the shape of a figure 8 and after centrifugation, the organic phase was transferred to a 1·0 ml. conical centrifuge tube. The aqueous phase was extracted twice more with 100 \(\mu\)l. ethyl acetate, and the combined extracts from each incubation were dried *in vacuo*. The DHA sulphate formed in each incubation tube was then determined by the following procedures: purification and identification of free steroids and their derivatives was carried out by thin-layer chromatography on silica gel (Merck silica gel HF254/366). The plates were prepared and the steroids eluted as described by Griffiths, Grant, Browning, Whyte & Sharp (1966). Sulphates were detected on, and eluted from, the plates by the procedure of Griffiths *et al.* (1968). Acetylation, reduction and saponification were carried out according to Griffiths, Grant & Whyte (1963). Steroids were measured after elution from thin layers by the procedure described by Griffiths, Grant, Browning, Cunningham & Barr (1966).

The series of residues from the incubations were chromatographed in the solvent system tert.-butyl alcohol:ethyl acetate:5 n-NH\(_4\)OH (41:50:20, v/v) (Pierrepoint, Griffiths, Grant & Stewart, 1966) and the DHA sulphate (\(R_f\) 0·35) located. Solvolysis (Burstein & Lieberman, 1958) of the DHA sulphate residues after elution yielded free DHA. DHA was then acetylated, chromatographed in the system benzene:ethyl acetate (9:1, v/v), eluted and the specific activity determined. Radioactivity was measured with a Nuclear Chicago liquid scintillation spectrometer (Model 6860). The remainder of the DHA acetate was then saponified, chromatographed in hexane: ethyl acetate (1:1, v/v), eluted and the specific activity again determined. In early experiments, the DHA residues were reduced and the specific activity of the third derivative obtained. It was found that the initial procedure purified the DHA sufficiently and that the specific activity of the reduced DHA did not differ from those of the acetate and the free compound. For these experiments therefore the percentage conversion of DHA to DHA sulphate in each incubation is based on the mean specific activity of the DHA and its acetate. The observation that these two specific activities did not differ by more than 10\% was taken as satisfactory evidence for radiochemical purity.

**RESULTS**

The quantitative histological distribution of a DHA sulphating enzyme system in the guinea-pig adrenal cortex is shown in Fig. 2. In all experiments the activity was found to be associated with the compact cells of the zona reticularis. Only one gland was sectioned into the medulla, and as Fig. 2 shows, the amount of DHA sulphate formed from DHA decreased as this region was entered.

The possibility that DHA sulphate may be metabolized further to sulphated forms of androst-5-ene-3\(\beta\),17\(\beta\)-diol (androstenediol) was checked by addition of carrier non-
radioactive androstenediol to the solvolyzed extracts from two of the incubations. Negligible amounts of radioactivity were associated with this carrier steroid. In these experiments, no attempt was made to study the formation of further hydroxylated products of DHA sulphate in the different zones.

The protein nitrogen (Pn) content of the tissue in each tube was determined by the bromsulphalein method of Nayyar & Glick (1954) and the weight (ng.) of DHA sulphate produced/5 μg. Pn/hr. calculated for each incubation.

![Graph](image)

**Fig. 2.** The quantitative histochemical distribution of a DHA sulphating enzyme system based on tissue protein nitrogen (Pn) throughout the guinea-pig adrenal cortex. Vertical bars represent ranges of activity determined in various regions in the cortex (4 expts). Regions marked G, F, R and M denote respectively zona glomerulosa, zona fasciculata, zona reticularis and medulla; mixed zones are designated by both letters.

**DISCUSSION**

Since the early histological studies of Harley (1858) and Arnold (1866), who described the three concentric layers of cells of the adrenal cortex, an outer zona glomerulosa, a central zona fasciculata and an inner zona reticularis, much has been written about the physiological role of these zones. It is now generally accepted that in the ox and rat adrenal cortex the cells of the zona glomerulosa are the site of aldosterone biosynthesis (Ayres, Garrod, Tait & Tait, 1958; Stachenko & Giroud, 1962). The investigations of Griffiths, Grant & Symington (1963) on human adrenal tissue appeared to indicate that there was qualitatively little difference between the biosynthetic activity of the clear cell of the zona fasciculata and the compact cell of the zona reticularis. Slices of tissue from both zones, incubated in Krebs–Ringer bicarbonate medium, were shown to secrete cortisol, corticosterone and 11β-hydroxyandrostenedione. Quantitative differences were, however, suggested by the finding that only cholesterol-laden clear cells responded to corticotrophin *in vitro*, with the secretion of more steroid.

The biosynthesis of cortisol and 11β-hydroxyandrostenedione by both types of
Site of formation of DHA sulphate

Ultramicrochemical techniques (Grunbaum et al. 1956; Griffiths & Glick, 1966) allow the study of enzyme activity in well-defined groups of cells from the rat adrenal cortex. Our results indicate that in the guinea-pig adrenal cortex, which has a differentiated z. fasciculata and z. reticularis, the sulphation of DHA, which can be formed from 17α-hydroxyprogrenolone by homogenates of this adrenal (Lipsett & Hofkelt, 1961; Trout et al. 1967), occurred only in the compact cells of the reticular zone. This suggests that DHA sulphate may be synthesized and secreted only by the compact cell, in agreement with the studies of Ofstad, Lamvik, Stoa & Emberland (1961), who reported that in a 47-year-old male who suffered from bronchiectasis, pulmonary fibrosis and renal amyloidosis, a post-mortem examination of the adrenal gland showed amyloid degeneration of the zona reticularis. Urinary steroid analysis in the final period of his life showed abnormally low C₁₉-steroid excretion, but a normal level of 17-hydroxycorticosteroids. Although these findings implied that C₁₉-steroids were synthesized by the cells of the zona reticularis, they were made before it was known that the human adrenal gland secretes substantial amounts of DHA sulphate. It is now suggested that there are qualitative differences in steroid biosynthesis between clear cells and compact cells. However, this may be due merely to differences in the degree of corticotrophin stimulation, and further work is required to elucidate how corticotrophin controls the formation of DHA sulphate and cortisol in well-identified adrenal cells. Such investigations may clarify the control of testosterone synthesis by the adrenal. Ward & Grant (1963) described the conversion of progesterone to testosterone by the clear cell of an adrenal adenoma removed from a patient with Cushing’s syndrome but were unable to provide evidence that the compact cell formed testosterone. Recent studies, however, by Cameron, Beynon & Griffiths (1968) and Cameron & Griffiths (1968) suggest that little progesterone may be formed in the human adrenal cortex, and that the progesterone synthesized is rapidly converted to corticosterone. Unpublished investigations (T. Jones & K. Griffiths) show that both the clear cell and the compact cell of the guinea-pig adrenal cortex convert DHA sulphate to testosterone.

It is well known that there are major differences in both histology and physiological activity between adrenal glands from different species. The demonstration that sulphation of DHA occurs only in the compact cell of the guinea-pig adrenal does not necessarily indicate that the same would be true of the human adrenal gland. However, in studies on adrenals from patients with carcinoma of the breast, similar results were obtained. Furthermore, little is known about the biosynthesis of DHA sulphate in any adrenal tissue. Some investigators (Calvin et al. 1963; Calvin & Lieberman, 1964; Roberts et al. 1964) suggest that cholesterol sulphate may be directly converted to DHA sulphate (Fig. 1). Shimizu (1966) has demonstrated the formation of pregnenolone sulphate from 20α-hydroxycholesterol by human adrenal slices (Fig. 1). On the other hand, Lebeau et al. (1964), who incubated human adrenal tumour slices, showed sulphation of DHA to be the final stage in the biosynthesis of the sulphate. The incubation studies of these workers were, however, conducted with tissue which was not histologically identified. Studies of Griffiths et al. (1968) showed
a very low conversion of pregnenolone sulphate (0-09%) and pregnenolone (0-08%) to DHA sulphate by tissue from a human clear cell adrenal adenoma.

There is an increasing interest in the adrenal gland of patients with breast cancer and in the relationship between urinary 17-hydroxycorticosteroids and aetiocholanolone, and prognosis (Bulbrook, 1965). It seems that the prognosis is related to the adrenal biosynthesis and secretion of cortisol and DHA (Deshpande, Jensen, Bulbrook & Douuss, 1967). Investigations on the control of the biosynthesis of these compounds in well-defined cells of the adrenal are therefore indicated, and ultramicrochemical studies on the human adrenal will be the subject of a further report.

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REFERENCES


FURTHER STUDIES ON THE RELATIONSHIP BETWEEN C₁₉- AND C₂₁-STEROID SYNTHESIS IN THE HUMAN ADRENAL GLAND


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SUMMARY

As part of a continuing study of adrenal steroids in relation to breast cancer, various experiments were performed in order to study relationships between androgen and corticosteroid biosynthesis. Chopped tumour tissue from a 'mixed cell' adrenal adenoma (7.4 g.) removed from a patient in Cardiff Royal Infirmary was incubated with [4-¹⁴C]pregnenolone and [7α-³H]17α-hydroxypregnenolone for periods of time ranging from 30 to 120 min. The results of this work suggest that 17α-hydroxyprogesterone may not be an obligatory intermediate in androgen or cortisol synthesis. Evidence from further experiments with 'normal' human adrenal tissue removed from breast cancer patients using previously established ultramicrochemical techniques indicates that dehydroepiandrosterone (DHA) sulphokinase enzyme system is confined to the zona reticularis of the gland. The conversion of [7α-³H]DHA sulphate, [7α-³H]androstenedione and [7α-³H]testosterone to oestrogens and their conjugates by adrenal homogenates was also investigated. Conversions were extremely low from all precursors.

INTRODUCTION

The production of androgens or their precursors has assumed major significance in the study of hormones in relation to breast cancer. Bulbrook, Hayward & Thomas (1964) and Hayward & Bulbrook (1968) have linked urinary 11-deoxy-17-oxosteroid and 17-hydroxycorticosteroid excretion in a statistical equation which permits discrimination between patients with Stage II of the disease, who will or will not respond to adrenalectomy or hypophysectomy after mastectomy. It is important therefore to study androgen as compared with corticosteroid synthesis in the human adrenal cortex since it is quantitatively the major site of C₁₁-steroid production.

The human adrenal cortex synthesizes and secretes 11β-hydroxyandrostenedione (Cooper, Touchstone, Roberts, Blakemore & Rosenthal, 1958; Lombardo & Hudson, 1959; Griffiths, Grant & Symington, 1963a), dehydroepiandrosterone and androstenedione (Cohn & Mulrow, 1963). Cohn & Mulrow (1963) reviewed the earlier work
on transformation of radioactive substrates to androgens by adrenal tissue. They also investigated the effect of corticotrophin (ACTH), on adrenal androgen production in vitro, and showed that if adrenal tissue is incubated in Krebs–Ringer bicarbonate-glucose medium, addition of ACTH increases 11β-hydroxyandrostenedione release but does not appear to stimulate dehydroepiandrosterone or androstenedione release. Baulieu (1962) and Wieland, Levy, Katz & Hirschmann (1963) also demonstrated the importance of dehydroepiandrosterone sulphate (DHA sulphate) as an adrenal secretory product in vivo. Previously, Cameron, Beynon & Griffiths (1968) showed, by mathematical analysis of percentage conversions of labelled pregnenolone and progesterone to corticosteroids and their intermediates in hyperplastic adrenal tissue, that progesterone is not a quantitatively important intermediate in cortisol formation. Thus the route involving 17α-hydroxypregnenolone is likely to account for more than 90% of the hormone. Confirmatory evidence was subsequently obtained (Cameron & Griffiths, 1968) in a similar fashion from a series of timed incubation studies on a ‘clear cell’ adenoma in which observations on peak labelling times of 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and 11-deoxycortisol also indicated that 17α-hydroxyprogesterone was not an obligatory intermediate in the conversion of 17α-hydroxypregnenolone to cortisol (see Fig. 1). 17α-Hydroxypregnenolone may also serve as a precursor of DHA and DHA sulphate. Recently, Griffiths, Cunningham & Cameron (1968) found very low conversions of [4-14C]pregnenolone and [7α-3H]pregnenolone sulphate to DHA and DHA sulphate (~0.1%) by tissue from a human adrenal ‘clear cell’ adenoma. This could be interpreted as evidence that the ‘clear cell’ of the zona fasciculata in the normal cortex, which closely resembles that of the tumour, may be unable to synthesize DHA sulphate.

The present investigation includes work on the site of formation of DHA sulphate in the cortex and on the biosynthetic pathways involved in C19-steroid synthesis and their relative importance.

---

**Diagram:**

```
Cholesterol

\[
\text{Pregnenolone} \rightarrow \text{Progesterone} \rightarrow 17\alpha\text{-OH-Progesterone}
\]

\[
17\alpha\text{-OH-Pregnenolone} \rightarrow 17\alpha,21\text{-OH-Pregnenolone} \rightarrow 11\text{-Deoxy-cortisol}
\]

\[
DHA \rightarrow \text{Androstenedione} \rightarrow \text{Cortisol}
\]

DHA sulphate \[11\beta\text{-OH-Androstenedione}\]

---

**Fig. 1.** Pathways of C19- and C21-steroid biosynthesis in the human adrenal gland.
Androgen biosynthesis in human adrenals

**MATERIALS AND METHODS**

**Adrenal tumour tissue**

(a) The tumour (7.4 g.) was removed from the right side of a 15-yr.-old girl with Cushing's syndrome. (b) 'Normal' adrenal glands were obtained from women with breast cancer. Tissues were routinely placed in polythene bags and transported to the laboratory on crushed ice. Experimental work on tissues started within 60 min. of their removal.

**Histology.** Representative pieces of tumour tissue to be incubated were fixed in 10% neutral formalin for histological examination by Professor T. Symington, Institute of Pathology, Royal Infirmary, Glasgow.

**Preparation and incubation of tissues**

**Adrenal tumour tissue.** This tissue was finely chopped with a safety razor blade (pieces > 1 mm.) and thoroughly mixed. Portions (5 x 1 g.) were weighed out for incubation.

(i) A portion was incubated with 83.5 n-mole [4-14C]pregnenolone (2.2 μc), 4.8 n-mole [7α-3H]progesterone (10.8 μc) and 4.8 n-mole unlabelled 17α-hydroxy-pregnenolone for 120 min. in 12 ml. Krebs–Ringer bicarbonate-glucose medium at 37° in an atmosphere of 95% O₂:5% CO₂. Incubations were terminated by addition of 20 ml. acetone.

(ii) Three portions were incubated with a mixture of steroids comprising 83.5 n-mole [4-14C]pregnenolone (2.2 μc), 1.8 n-mole [7α-3H]17α-hydroxypregnenolone (8.0 μc) and 1.8 n-mole unlabelled progesterone under identical conditions to (i) except that incubations were for 30, 60 and 120 min.

(iii) A portion was incubated with equimolar amounts (62.0 n-mole) of [4-14C]DHA (2.7 μc), [7α-3H]17α-hydroxypregnenolone (8.0 μc) and unlabelled 17α-hydroxyprogesterone for 120 min. again under the same conditions as (i).

Unlabelled 17α-hydroxypregnenolone was added in an equimolar amount to the [7α-3H]progesterone in incubation (i), permitting [4-14C]pregnenolone metabolism to proceed in an unbiased fashion along the alternative metabolic pathways shown in Fig. 1. Similar reasons applied with respect to the addition of unlabelled progesterone in incubation (ii) and of unlabelled 17α-hydroxyprogesterone in incubation (iii) with respect to the metabolism of [7α-3H]17α-hydroxypregnenolone.

'Normal' adrenal tissue. Although fascicular and reticular tissue of the human adrenal gland have been separated (Griffiths et al. 1963a) this was not possible with the adrenals obtained, and preparations of mixed fascicular and reticular tissue were therefore used.

Finely chopped tissue (1 g.) from each of three adrenals was homogenized in 5.0 ml. of 0.25 M-sucrose containing nicotinamide (0.12 M) in a Philpot & Stanier (1956) homogenizer. The homogenates (4.2 ml.) were incubated with 73.5 n-moles of [7α-3H]DHA sulphate (52 μc), or [7α-3H]testosterone (15 μc) or [7α-3H]androstenedione (75-7 μc) for 60 min. at 37° in 4.2 ml. tris buffer medium (pH 7.4) containing various cofactors described previously (Lawrence & Griffiths, 1966).
Ultramicrochemical investigations

Pieces of tissue from the tail region (Dobbie & Symington, 1966) of the adrenal gland were frozen in solid carbon dioxide for ultramicrochemical studies, and placed in a cryostat at —15°. Cylinders of tissue, from capsule through cortex to capsule, were bored out of the glands, and freshly frozen microtome sections (2.5 mm. diam. 16 μ thick, vol. 0.07 μl.) were prepared in a cryostat as described by Grunbaum, Geary & Glick (1956). One section was brushed flat on to a glass slide for histological examination and the next three sections were placed in a glass-stoppered reaction tube for enzyme study. This sequence was followed through the entire cortex and into the medulla of the gland. [7α-3H]DHA (0.049 μg., 500 μc/μmole) was incubated with the tissue and the steroids extracted and analysed by procedures described previously (Jones & Griffiths, 1968). After chromatography of the carrier DHA sulphate in solvent system VIII and eluting and solvolysing the residue, the percentage conversion of DHA to DHA sulphate in each incubation was calculated from the mean specific activity of the DHA and its acetate.

Fractionation of steroids

After the addition of acetone to the incubation, various non-radioactive carrier steroids were added to the mixtures.

Adrenal tumour tissue. (i) Carrier cortisol and corticosterone (1000 μg. of each) were added to the mixture in ethanol. The mixture was homogenized in a Silverson mixer and filtered. The residue was then washed twice with 30 ml. acetone and the pooled extract and washings dried under reduced pressure. Steroids were then extracted and fractionated as described (Cameron & Griffiths, 1968; Griffiths et al. 1968).

(ii) The following carrier steroids (500 μg. of each) were added to each of the three incubations: pregnenolone, progesterone, DOC, corticosterone, 11-deoxycortisol, cortisol, DHA, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, androst-5-ene-3β-17β-diol (androstenediol), androstenedione, 11β-hydroxyandrostenedione and the sulphates of 17α-hydroxy pregnenolone, DHA and androstenediol (3-monosulphate).

(iii) Cortisol, DHA, 17α-hydroxy pregnenolone, 17α-hydroxyprogesterone, androstenediol, androstenedione, testosterone, 11β-hydroxyandrostenedione and the sulphates of 17α-hydroxy pregnenolone, DHA and androstenediol (3-monosulphate) were added in 300 μg. amounts. Steroids from incubations (ii) and (iii) were separated as for incubation (i).

'Normal' adrenal tissue. Carrier oestrone, oestradiol-17β, oestriol and 11β-hydroxy oestradiol-17β (500 μg.) were added to incubation mixtures and the phenolic fractions isolated (Lawrence & Griffiths, 1966). The conjugated steroid fraction was chromatographed in solvent system VIII and the band of sulphates (Rf 0.35) eluted, solvolysed and 500 μg. carrier oestrone, oestradiol-17β and oestriol were added to the solvolysis extract.

Chromatography, isolation and measurement of steroids. Thin-layer chromatography on Merck silica gel HF254/366 was used to isolate the individual steroids in the various fractions. Steroids and their derivatives were detected and extracted from silica gel by procedures previously described (Fahmy, Griffiths, Turnbull & Symington, 1968).
The following solvent systems were used, the proportions being by volume: (I) chloroform : acetone (185:15); (II) cyclohexane : ethyl acetate (140:60); (III) benzene : ethyl acetate (180:20); (IV) cyclohexane : ethanol (180:20); (V) hexane : ethyl acetate (100:100); (VI) cyclohexane : ethyl acetate (90:110); (VII) chloroform : methanol; (VIII) tert.-butyl alcohol : ethyl acetate : 5n-NH₄OH (41:50:20); (IX) benzene : methanol (170:30).

Steroids were measured after elution, and derivatives prepared, by procedures already established (Griffiths, Grant & Whyte, 19636; Griffiths, Grant, Browning, Cunningham & Barr, 1966). Radioactivity was measured by a Nuclear Chicago liquid scintillation spectrometer (Model 6860). The observation that the specific activities of a steroid and two of its derivatives differed by not more than 10% was taken as satisfactory evidence for radiochemical purity. The mean of the specific activities was used to calculate the percentage conversion from the original steroid incubated.

RESULTS

Incubation of [4-14C]pregnenolone and [7α-3H]progesterone with adrenal tumour tissue gave conversions of 34-2% and 20-6% to cortisol respectively together with 1-56% and 47-6% conversions to corticosterone. Proof of radiochemical purity of the two steroids isolated is given in Table 1.

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent system</th>
<th>Specific activities (disintegrations/min./n-mole)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>VII</td>
<td>813</td>
<td>625</td>
</tr>
<tr>
<td>Cortisol acetate</td>
<td>VII</td>
<td>821</td>
<td>598</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>IX</td>
<td>845</td>
<td>632</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>VII</td>
<td>1893</td>
<td>28-9</td>
</tr>
<tr>
<td>Corticosterone acetate</td>
<td>VII</td>
<td>1837</td>
<td>28-9</td>
</tr>
<tr>
<td>11β,20α,21-Trihydroxy-pregn-4-en-3-one</td>
<td>VII</td>
<td>1764</td>
<td>28-9</td>
</tr>
</tbody>
</table>

Evidence for the identification of steroids isolated from the incubation of tumour tissue with [7α-3H]17α-hydroxyprogrenolone and [4-14C]pregnenolone (incubation (ii)) is given in Table 2. The table shows the derivatives formed, the solvent systems used for their purification and the specific activities of the compounds studied. The percentage conversions of the steroid precursors to the various metabolites are shown graphically in Figs. 2–5. None of these graphs is corrected for endogenous production of steroids by the tissue.

Of particular interest is the rapid formation of 17α-hydroxyprogrenolone from pregnenolone (Fig. 3). After 30 min. incubation only 8-5% of the [4-14C]pregnenolone remained. The concentration of two compounds only, cortisol and 11β-hydroxy-androstenedione, continued to increase throughout the incubation. Only small yields of corticosterone were obtained from pregnenolone. DHA sulphate formed in this incubation (in contrast to earlier studies with a clear cell adenoma (Griffiths et al. 1968)) appears to undergo further metabolism by the tissue (Figs. 2, 4). The results
would also suggest that DHA, once formed, is metabolized either to DHA sulphate, or to androstenedione and thence to 11β-hydroxyandrostenedione.

Similarly, this is suggested from the results of the experiment in which the tumour tissue was incubated (incubation (iii)) with [4-14C]DHA and [7α-3H]17α-hydroxy-pregnenolone (Table 3). Evidence for identification of steroids and the distribution

Table 2. Evidence for the identification of metabolites formed by incubating tumour tissue with [4-14C]pregnenolone and [7α-3H]17α-hydroxy-pregnenolone for varying time-intervals

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent system</th>
<th>30 min. incubation</th>
<th>60 min. incubation</th>
<th>120 min. incubation</th>
<th>Specific activities (disintegrations/min./n-mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14C</td>
<td>3H</td>
<td>14C</td>
<td>3H</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>I</td>
<td>272</td>
<td>0</td>
<td>222</td>
<td>0</td>
</tr>
<tr>
<td>Preg-5-one-3β,20β-diol</td>
<td>I</td>
<td>284</td>
<td>0</td>
<td>242</td>
<td>0</td>
</tr>
<tr>
<td>Pregnenolone acetate</td>
<td>I</td>
<td>266</td>
<td>0</td>
<td>220</td>
<td>0</td>
</tr>
<tr>
<td>17α-Hydroxy-pregnenolone</td>
<td>I</td>
<td>861</td>
<td>1839</td>
<td>462</td>
<td>924</td>
</tr>
<tr>
<td>17α-Hydroxy-pregnenolone acetate</td>
<td>III</td>
<td>952</td>
<td>2015</td>
<td>409</td>
<td>977</td>
</tr>
<tr>
<td>Cortisol</td>
<td>VII</td>
<td>669</td>
<td>3097</td>
<td>1013</td>
<td>3992</td>
</tr>
<tr>
<td>Cortisol acetate</td>
<td>VII</td>
<td>692</td>
<td>3186</td>
<td>1109</td>
<td>4281</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>IX</td>
<td>689</td>
<td>3121</td>
<td>1110</td>
<td>4286</td>
</tr>
<tr>
<td>DHA</td>
<td>V</td>
<td>150</td>
<td>521</td>
<td>86-0</td>
<td>283</td>
</tr>
<tr>
<td>DHA acetate</td>
<td>III</td>
<td>159</td>
<td>515</td>
<td>95-0</td>
<td>286</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>V</td>
<td>157</td>
<td>520</td>
<td>98-3</td>
<td>274</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>VIII</td>
<td>201</td>
<td>745</td>
<td>330</td>
<td>1119</td>
</tr>
<tr>
<td>DHA</td>
<td>V</td>
<td>189</td>
<td>826</td>
<td>322</td>
<td>1115</td>
</tr>
<tr>
<td>DHA acetate</td>
<td>III</td>
<td>189</td>
<td>744</td>
<td>329</td>
<td>1095</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>V</td>
<td>99-1</td>
<td>494</td>
<td>134</td>
<td>538</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I</td>
<td>104-0</td>
<td>518</td>
<td>146</td>
<td>594</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>II</td>
<td>95-7</td>
<td>486</td>
<td>137</td>
<td>591</td>
</tr>
<tr>
<td>11β-Hydroxyandrostenedione</td>
<td>I</td>
<td>71-1</td>
<td>370</td>
<td>155</td>
<td>671</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>I</td>
<td>66-6</td>
<td>347</td>
<td>146</td>
<td>659</td>
</tr>
<tr>
<td>11β-Hydroxytestosterone</td>
<td>VII</td>
<td>68-8</td>
<td>365</td>
<td>159</td>
<td>683</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>IX</td>
<td>211</td>
<td>521</td>
<td>248</td>
<td>545</td>
</tr>
<tr>
<td>17α,20β-Dihydroxy-preg-4-en-3-one</td>
<td>VI</td>
<td>225</td>
<td>561</td>
<td>228</td>
<td>496</td>
</tr>
<tr>
<td>17α-Hydroxy-20β-acetoxy-preg-4-en-3-one</td>
<td>I</td>
<td>203</td>
<td>509</td>
<td>247</td>
<td>537</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>VII</td>
<td>457</td>
<td>1826</td>
<td>326</td>
<td>1084</td>
</tr>
<tr>
<td>11-Deoxycorticosterone acetate</td>
<td>VII</td>
<td>453</td>
<td>1752</td>
<td>332</td>
<td>1042</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>IV</td>
<td>437</td>
<td>1745</td>
<td>314</td>
<td>1034</td>
</tr>
<tr>
<td>Cortisone</td>
<td>VII</td>
<td>23-6</td>
<td>104</td>
<td>44-0</td>
<td>180</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>VII</td>
<td>21-5</td>
<td>98-1</td>
<td>41-2</td>
<td>171</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>I</td>
<td>23-1</td>
<td>102</td>
<td>44-9</td>
<td>177</td>
</tr>
<tr>
<td>DOC</td>
<td>VI</td>
<td>36-7</td>
<td>0</td>
<td>20-0</td>
<td>0</td>
</tr>
<tr>
<td>DOC acetate</td>
<td>VI</td>
<td>37-5</td>
<td>0</td>
<td>18-9</td>
<td>0</td>
</tr>
<tr>
<td>20β,21-Dihydroxy-preg-4-en-3-one</td>
<td>I</td>
<td>37-2</td>
<td>0</td>
<td>20-3</td>
<td>0</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>VII</td>
<td>58-4</td>
<td>0</td>
<td>71-0</td>
<td>0</td>
</tr>
<tr>
<td>Corticosterone acetate</td>
<td>VII</td>
<td>61-1</td>
<td>0</td>
<td>67-6</td>
<td>0</td>
</tr>
<tr>
<td>11β,20β,21-Trihydroxy-preg-4-en-3-one</td>
<td>VII</td>
<td>61-3</td>
<td>0</td>
<td>71-6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values too low for accurate determination.
Androgen biosynthesis in human adrenals of radioactivity among the identified metabolites is also given. DHA was metabolized to DHA sulphate (19.8%) and to androstenedione (9.06%). 11β-hydroxyandrostenedione (22.73%) and testosterone (0.06%). Only 1.79% of the incubated DHA remained at the end of the 2 hr. incubation.

Large amounts of the incubated [4-14C]DHA (incubation (iii); Table 3) could not be accounted for in the metabolites isolated and characterized. Three experiments in which DHA sulphate, testosterone and androstenedione were incubated separately with 'normal' adrenal homogenates, were set up to establish the possible capacity of the tissue to form oestrogens. Table 4 shows the specific activities of the carrier oestrogens and their derivatives, and the percentage conversions of the precursor to the oestrogen (phenolic steroids containing insignificant quantities of radioactivity are not included in the Table). The specific activities are low, and in most cases, only two values were obtained since extensive chromatography was necessary to isolate and purify the oestrogens. Recoveries were poor. However, some evidence is presented
to indicate that the normal human adrenal possesses the capacity to synthesize oestrogens from C₁₉-steroids although it is obvious that the activity of these enzyme systems is low. There is no evidence of extensive oestrogen sulphate formation.

![Diagram](image)

**Fig. 3.** Percentage conversion of [4⁻¹⁴C]pregnenolone to 17α-hydroxypregnenolone (■—■), 17α-hydroxyprogesterone (x—x), 11-deoxycortisol (+—+), corticosterone (○—○) and cortisol (□—□) as functions of time.

The quantitative histological distribution of a DHA sulphating enzyme system in the human adrenal cortex is shown in Fig. 6. The distribution pattern closely resembles that described for the guinea-pig (Jones & Griffiths, 1968) in that the activity of the enzyme system appears to be associated with the compact cell of the zona reticularis.

The possible further metabolism of DHA sulphate to the sulphated forms of androstenediol was checked by the addition of non-radioactive androstenediol to the solvolyzed extracts of the incubation. Negligible amounts of radioactivity were associated with androstenediol.

**DISCUSSION**

The conversion of [4⁻¹⁴C]pregnenolone and [7α⁻³H]progesterone to cortisol and corticosterone in the yields shown (Table 1) confirm earlier observations (Cameron *et al.* 1968; Cameron & Griffiths, 1968) that relatively small amounts of pregnenolone
Androgen biosynthesis in human adrenals

are converted to progesterone in human adrenal tissue, and any progesterone synthesized tends to be transformed to the 17-deoxycorticosteroids. It was of interest that the pattern of steroid metabolism observed with this tumour tissue, consisting primarily of ‘compact cells’, was very similar to that determined earlier in studies with a ‘clear cell’ adrenal adenoma (Cameron & Griffiths, 1968).

![Graph](image)

**Fig. 4.** Percentage conversion of [7a-3H]17a-hydroxypregnenolone (■—■) to DHA (○—○), DHA sulphate (●—●), androstenedione (▲—▲) and 11β-hydroxyandrostenedione (△—△) as functions of time.

The similarities between the metabolic activity of the two types of tumour are exemplified by the rapid formation of 17α-hydroxypregnenolone from [4-14C]pregnenolone, and its equally rapid disappearance (Table 2; Fig. 3). The next ‘classical’ intermediate, 17α-hydroxyprogesterone, peaks subsequently, suggesting a logical sequence of reactions: pregnenolone → 17α-hydroxypregnenolone → 17α-hydroxyprogesterone. However, the demonstration of a peak labelling time for 14C in 11-deoxycortisol occurring before that of 17α-hydroxyprogesterone (Fig. 3), again provides some evidence for at least partial involvement of 17α,21-dihydroxypregnenolone in the synthesis of 11-deoxycortisol from 17α-hydroxypregnenolone. It is noteworthy in this
respect, that neither the $^{14}$C nor the $^3$H content of 17α-hydroxyprogesterone ever exceeded that of 11-deoxycortisol.

A comparison of the metabolism of [$7\alpha$-$^3$H]17α-hydroxypregnenolone in the presence of added [4-$^{14}$C]pregnenolone and unlabelled progesterone (tumour incubation

![Graph](image)

Fig. 5. Percentage conversion of [$7\alpha$-$^3$H]17α-hydroxypregnenolone ($\triangle$---$\triangle$) to 17α-hydroxyprogesterone ($\times$---$\times$), 11-deoxycortisol ($++$) and cortisol ($\square$---$\square$) as functions of time.

ii), and in the presence of [4-$^{14}$C]DHA and unlabelled 17α-hydroxyprogesterone (tumour incubation iii) provides a curious paradox. Conversions to all the $^3$H-labelled metabolites isolated were very similar with the exception of cortisol and 11β-hydroxyandrostenedione. However, the conversion of 17α-hydroxypregnenolone to these compounds was approximately halved in the presence of DHA and 17α-hydroxyprogesterone. Since the conversion of pregnenolone to 17α-hydroxypregnenolone is extremely rapid, and the role of progesterone in adrenal androgen synthesis from pregnenolone is probably a minor one, this suggests that the difference must be
Androgen biosynthesis in human adrenals due to the relatively large quantities (62 n-mole) of DHA and 17α-hydroxyprogesterone present in the incubation. The 17α-hydroxyprogesterone present may have exerted a mass effect on the conversion of 17α-hydroxypregnenolone to cortisol, although the low-3H-labelling of 17α-hydroxyprogesterone during incubation (ii) would not support this view. The relatively large quantity of DHA sulphate formed from

Table 3. Evidence for the identification of metabolites formed by incubating tumour tissue with [4-14C]DHA and [7α-3H]17α-hydroxyprogrenenolone

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent system</th>
<th>Specific activities (disintegrations/min./n-mole)</th>
<th>mC formed</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Hydroxyprogrenenolone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxy-3β-acetoxy-Pregnenolone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>VI</td>
<td>404 &lt; 0</td>
<td>162 &lt; 0</td>
<td>2-02 &lt; 0</td>
</tr>
<tr>
<td>17α,20β-Dihydroxyprogren-4-en-3-one</td>
<td>I</td>
<td>401 &lt; 0</td>
<td>162 &lt; 0</td>
<td>2-02 &lt; 0</td>
</tr>
<tr>
<td>17α-Hydroxy-20β-acetoxy-pregen-4-en-3-one</td>
<td>VI</td>
<td>380 &lt; 0</td>
<td>162 &lt; 0</td>
<td>2-02 &lt; 0</td>
</tr>
<tr>
<td>DHA</td>
<td>V</td>
<td>138 &lt; 100</td>
<td>66-5 &lt; 49-5</td>
<td>0-83 &lt; 1-81</td>
</tr>
<tr>
<td>DHA acetate</td>
<td>III</td>
<td>147 &lt; 110</td>
<td>66-5 &lt; 49-5</td>
<td>0-83 &lt; 1-81</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>V</td>
<td>144 &lt; 109</td>
<td>66-5 &lt; 49-5</td>
<td>0-83 &lt; 1-81</td>
</tr>
<tr>
<td>Androstenediol diacetate</td>
<td>III</td>
<td>0 &lt; 0</td>
<td>0 &lt; 0</td>
<td>0 &lt; 0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I</td>
<td>460 &lt; 530</td>
<td>213 &lt; 253</td>
<td>2-66 &lt; 9-25</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I</td>
<td>448 &lt; 554</td>
<td>213 &lt; 253</td>
<td>2-66 &lt; 9-25</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>II</td>
<td>444 &lt; 528</td>
<td>213 &lt; 253</td>
<td>2-66 &lt; 9-25</td>
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<tr>
<td>Testosterone acetate</td>
<td>II</td>
<td>0 &lt; 3-4</td>
<td>0 &lt; 1-64</td>
<td>0 &lt; 0-06</td>
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<tr>
<td>Androstenedione</td>
<td>I</td>
<td>0 &lt; 3-3</td>
<td>0 &lt; 1-64</td>
<td>0 &lt; 0-06</td>
</tr>
<tr>
<td>11β-Hydroxyandrostenedione</td>
<td>I</td>
<td>901 &lt; 1396</td>
<td>409 &lt; 636</td>
<td>5-12 &lt; 23-23</td>
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<tr>
<td>11β-Hydroxytestosterone</td>
<td>VII</td>
<td>926 &lt; 1453</td>
<td>409 &lt; 636</td>
<td>5-12 &lt; 23-23</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>I</td>
<td>921 &lt; 1436</td>
<td>409 &lt; 636</td>
<td>5-12 &lt; 23-23</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenenolone-sulphate</td>
<td>VIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxyprogrenolone</td>
<td>II</td>
<td>72-0 &lt; 0</td>
<td>28 &lt; 0</td>
<td>0-35 &lt; 0</td>
</tr>
<tr>
<td>17α-Hydroxy-3β-acetoxy-pregnenolone</td>
<td>II</td>
<td>68-9 &lt; 0</td>
<td>28 &lt; 0</td>
<td>0-35 &lt; 0</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>VIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHA acetate</td>
<td>III</td>
<td>1161 &lt; 1116</td>
<td>544 &lt; 542</td>
<td>6-8 &lt; 19-8</td>
</tr>
<tr>
<td>Androstenediol diacetate</td>
<td>III</td>
<td>1177 &lt; 1201</td>
<td>544 &lt; 542</td>
<td>6-8 &lt; 19-8</td>
</tr>
<tr>
<td>Androstenediol-sulphate</td>
<td>VIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androstenediol</td>
<td>I</td>
<td>0 &lt; 3-8</td>
<td>0 &lt; 1-90</td>
<td>0 &lt; 0-07</td>
</tr>
<tr>
<td>Androstenediol acetate</td>
<td>II</td>
<td>0 &lt; 4-2</td>
<td>0 &lt; 1-90</td>
<td>0 &lt; 0-07</td>
</tr>
<tr>
<td>Cortisol</td>
<td>VII</td>
<td>3951 &lt; 0</td>
<td>1519 &lt; 0</td>
<td>18-98 &lt; 0</td>
</tr>
<tr>
<td>Cortisol acetate</td>
<td>VII</td>
<td>3987 &lt; 0</td>
<td>1519 &lt; 0</td>
<td>18-98 &lt; 0</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>I</td>
<td>4292 &lt; 0</td>
<td>1519 &lt; 0</td>
<td>18-98 &lt; 0</td>
</tr>
</tbody>
</table>

DHA (~ 20%) may have inhibited 21-hydroxylation although not 11β-hydroxylation, since 25% of the incubated [4-14C]DHA was converted to 11β-hydroxyandrostenedione. The 3H:14C ratios of DHA (1:2-2), DHA sulphate (1:2-9), androstenedione (1:3-5) and 11β-hydroxyandrostenedione (1:4-5) suggest that little 'leakage' of the 3H-label into adrenal androgens occurs through 17α-hydroxyprogesterone.
Table 4. Some evidence for the identification of oestrogens formed from the incubation of various C<sub>19</sub>-steroids with human 'normal' adrenal homogenates

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent system</th>
<th>DHA-sulphate</th>
<th>Testosterone</th>
<th>Androstenedione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol-17β</td>
<td>VI</td>
<td>0</td>
<td>6·5</td>
<td>0</td>
</tr>
<tr>
<td>Oestradiol-17β diacetate</td>
<td>II</td>
<td>0</td>
<td>6·7 (0·036)</td>
<td>0</td>
</tr>
<tr>
<td>Oestradiol-17β-3-methyl ether</td>
<td>II</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Oestrone sulphate</td>
<td>VIII</td>
<td>4·0</td>
<td>0</td>
<td>4·8</td>
</tr>
<tr>
<td>Oestrone</td>
<td>VI</td>
<td>4·1</td>
<td>0</td>
<td>4·5 (0·005)</td>
</tr>
<tr>
<td>Oestrone acetate</td>
<td>II</td>
<td>4·4 (0·007)</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Oestrone-3-methyl ether</td>
<td>II</td>
<td>4·4</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Oestradiol-17β sulphate</td>
<td>VIII</td>
<td>—*</td>
<td>0</td>
<td>3·8</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>VI</td>
<td>6·1</td>
<td>0</td>
<td>3·8 (0·004)</td>
</tr>
<tr>
<td>Oestradiol-17β diacetate</td>
<td>II</td>
<td>5·8 (0·010)</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Oestradiol-17β-3-methyl ether</td>
<td>II</td>
<td>5·8 (0·010)</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

* Sufficient mass not available for accurate determination of specific activity.

Fig. 6. The quantitative histochemical distribution of DHA sulphating enzyme system based on tissue protein nitrogen (Pn) throughout the human adrenal cortex. Vertical bars represent ranges of activity determined in various regions in the cortex (three expts). Regions marked G, F and R denote respectively zona glomerulosa, zona fasciculata and zona reticularis; mixed zones are designated by both letters.
Androgen biosynthesis in human adrenals

These results are contrary to those of Cohn (1965) who suggested that the pathway 17α-hydroxyprogrenolone → 17α-hydroxyprogesterone → androstenedione was important in androgen biosynthesis in an adrenal adenoma (histologically undefined) although the evidence presented was unconvincing and the interpretation ignored the possibility of 17α,21-dihydroxyprogrenolone as an intermediate in DHA formation (Pasqualini, Lafoscade & Jayle, 1964).

Much of the 14C-label from the [4-14C]DHA incubated remained unaccounted for, and further work is necessary to establish the pathways of DHA metabolism in human adrenal tissue. However, of interest was the conversion of the DHA to DHA sulphate and also to androstenedione and 11β-hydroxyandrostenedione, in approximately similar yields under the conditions of incubation in which no cofactors were used. Time-course studies (Figs. 2, 5) also suggest that DHA once formed from 17α-hydroxyprogrenolone is metabolized either to DHA sulphate or to androstenedione and thence to 11β-hydroxyandrostenedione. The results also indicate that DHA sulphate undergoes further metabolism in human adrenal tissue.

The ability of human adrenal tissue to convert DHA to its sulphate in vitro has been demonstrated by a number of workers (Adams, 1963; Wallace & Lieberman, 1963; Cohn, Mulrow & Dunne, 1963) but the significance of the finding was questioned by Lieberman and his colleagues (Calvin, Vande Wiele & Lieberman, 1963; Roberts, Bandi, Calvin, Drucker & Lieberman, 1964) who suggested that DHA sulphate may be synthesized from cholesterol sulphate, via pregnenolone sulphate and 17α-hydroxyprogrenolone sulphate without loss of the sulphate group. The studies now reported provide evidence to support the view that DHA sulphate is synthesized from DHA formed in turn from pregnenolone via 17α-hydroxyprogrenolone. Such a view was expressed by Lebeau, Alberga & Baulieu (1964) using human adrenal tumour slices, again histologically undefined.

However, the formation of DHA sulphate (~8% after 60 min.) from pregnenolone by this tumour consisting primarily of 'compact cells' is of particular interest in comparison to earlier studies with a 'clear cell' adenoma (Griffiths et al. 1968). Only small yields of DHA sulphate were then formed from pregnenolone (0.09%) and pregnenolone sulphate (0.08%) and it was suggested that the 'clear cell' of the zona fasciculata may not normally synthesize DHA sulphate. Ultramicrochemical studies (Jones & Griffiths, 1968) confirmed that the DHA sulphokinase activity resides in the compact cell of the zona reticularis of the guinea-pig, and our further experiments (Fig. 6) indicate that a similar pattern occurs in the human adrenal gland. However, unlike the guinea-pig adrenal cortex, a clear separation between fascicular and reticular tissue is, in the human adrenal, difficult to obtain because of the grossly indented boundary between the zones. DHA sulphokinase activity measured in the tissue designated 'fascicular tissue' is probably due to columns of 'compact cells' often found in the zona fasciculata of the human adrenal cortex (Symington, Currie, O'Donnell, Grant, Oastler & Whyte, 1958). The pattern strongly suggests, however, that the enzymic activity increases in the presence of increasing amounts of tissue from the zona reticularis. The dip in the observed activity in the middle of the gland may well reflect the presence of muscular tissue or blood vessels in this area of the 'tail region' which does not contain medullary tissue (Dobbie & Symington, 1966). Control of androgen and corticosteroid production in the human adrenal cortex is a
complex problem and the possibility that DHA sulphate production as well as the action of ACTH may exert some regulatory effect on cortisol biosynthesis is worthy of further investigation. Such studies may help to explain in biochemical terms the place of the urinary 'discriminant' (Bulbrook et al. 1964) in the aetiology of breast cancer.

In the relationship between the adrenal cortex and the development of breast tumours there has always remained the suggestion that the cortex is responsible for oestrogen formation, a belief that is responsible for adrenalectomy in the treatment of breast cancer. In a recent publication, Goldzieher & Boyd (1967) reviewed the evidence for secretion of oestrogens by the human adrenal gland. This evidence is largely indirect and consists of various demonstrations of oestrogen in the urine of gonadectomized subjects (Dao, 1953; Diczfalusy, Notter, Edsmyr & Westman, 1959) some of whom had been stimulated with ACTH. Synthesis of oestrogens in vitro from radioactive steroid precursors by feminizing adrenocortical carcinoma tissue (Baggett, Engel, Balderas, Lanman, Savard & Dorfman, 1959) has also been reported. In recent studies, Lawrence & Griffiths (1966) were able to demonstrate oestrogen formation from [1,2-3H]testosterone in vitro in 'normal' adrenal tissue from the golden hamster. The present study now provides some evidence that 'normal' human adrenal tissue possesses the enzyme systems to synthesize oestrogen from C19-steroids such as DHA sulphate, androstenedione and testosterone. The activity of these systems is extremely low, and it may be more reasonable to accept suggestions that 'adrenal oestrogens' excreted in the urine of gonadectomized patients arise from peripheral metabolism of compounds such as testosterone (West, Damast, Sarro & Pearson, 1956; Ahmad & Morse, 1965) or androstenedione (Baulieu & Dray, 1963) which may originate from the adrenal gland.

The fact that substantial amounts of radioactivity were not isolated in the conjugated oestrogen fractions precludes the possibility that inability to demonstrate oestrogen synthesis in earlier adrenal studies in vitro was due to the failure to investigate the oestrogen sulphates.

The authors wish to record their appreciation of generous financial support from the Tenovus Organization. They also wish to express their gratitude to Professor A. P. M. Forrest, Department of Surgery, Royal Infirmary, Cardiff for supplying the tissue used in this work and to Dr J. Picton Thomas, Medical Unit, Royal Infirmary, Cardiff for allowing them to study the patient with the adrenal adenoma. Professor T. Symington and his Staff, Department of Pathology, Royal Infirmary, Glasgow very kindly performed the histology of the tumour. Mr Ralph Marshall and his Staff, Department of Medical Illustration, Royal Infirmary, Cardiff provided the photographs of the figures used in the text.

REFERENCES

Androgen biosynthesis in human adrenals


Steroid Biosynthesis by Cultures of Normal Human Adrenal Tissue

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Received November 27, 1969

Summary  The metabolism of $[^{14}N] 17a$-hydroxypregnenolone (3$eta$,17a-dihydroxyprog-5-en-20-one) by fascicular and reticular tissue, separated from a human adrenal gland, and maintained in organ culture, has been studied. The percentage conversions to three metabolites, cortisol, DHA (dehydroyepiandrosterone) and DHA sulphate were measured. Cortisol was produced by both zones but an increased production under the influence of ACTH only occurred in the fascicular tissue. DHA and DHA sulphate were produced mainly by the reticular tissue and there was a marked stimulation of their synthesis in this tissue under the influence of ACTH.

Assessment of steroid biosynthesis by adrenal tissue maintained in culture systems has thus far been confined to tumour tissue grown in monolayers (Stoller, Buonassisi & Sato, 1964; Kowal & Fiedler, 1968; Neville, Anderson, McCormick & Webb, 1968). No report has yet been made on the organ culture of normal adult human adrenal tissue although Block, Romney, Klein, Lippiello, Cooper & Goldring (1965) have succeeded in maintaining normal foetal human adrenal tissue in organ culture. Jones & Griffiths (1968) and Cameron, Jones, Jones, Anderson & Griffiths (1969) have shown that there is a qualitative difference in steroid production between the zona fasciculata and the zona reticularis of the adrenal cortex of both the guinea pig and human glands in that the sulphation of DHA takes place only in the compact cell of the zona reticularis. This work was carried out using an ultramicrochemical technique. In an attempt to continue the
study of the biosynthetic activity of the small amounts of normal human adrenal tissue available, it was decided to investigate the metabolism of 17α-hydroxy pregnenolone by separated fascicular and reticular cells maintained in long term organ culture under conditions which are sometimes considered to be more physiological than the established conditions employed in the usual short term *in vitro* incubation techniques.

**MATERIALS AND METHODS**

Normal adrenal tissue was obtained from a patient undergoing adrenalectomy for mammary carcinoma and was transported to the laboratory in a sterile container surrounded by crushed ice. In the laboratory, all work on the tissue was carried out in a Microflow tissue culture cabinet. The gland was stripped free of fat, and unfolded to reveal the dark brown reticular zone. This was scraped away gently, to give tissue consisting mainly of the compact cells of the zona reticularis. The light yellow coloured fascicular tissue was then removed in a similar manner. Four explant cultures of tissue from each zone were then established separately in organ culture dishes (Falcon Plastics), in Eagle's minimum essential medium fortified with 10% calf serum, 292 μg./ml. glutamine, 200 units/ml. penicillin and 100 μg./ml. streptomycin. Explants were cultured on lens paper rafts supported by stainless steel grids, a modification of the Trowell (1954, 1959) technique.

After two days the medium was changed, the fresh medium introduced containing 1 μC [7α-3H] 17α-hydroxy pregnenolone (19,900 μC/mM). Long-acting ACTH (Organon, corticotrophin Zn) was added to two fascicular and two reticular cultures (0.1 i.u.). After four more days, the media were collected for analysis.
and the tissue allowed to wash in fresh medium for another four day period. The medium for the final four days again contained \( [7\alpha-^3\text{H}] 17\alpha\)-hydroxyprogrenolone, and ACTH was added to the same cultures as for days 3-7. Media from identical cultures were then pooled and all were analysed for labelled cortisol, DHA and DHA sulphate after the addition of 500 \( \mu \text{g.} \) each of non-labelled carrier steroids. The tissue was dried on filter paper and weighed.

The two neutral steroids were extracted with ether, and the DHA sulphate with ethyl acetate after saturation of the media with ammonium sulphate. Table 1 shows the derivatives formed and the thin layer systems used for purification. Thin layer solvent systems used for these studies were as follows:-

I  Chloroform: methanol: water (187:12:1);
II  Cyclohexane; ethyl acetate (7:3);
III  Hexane: ethyl acetate (1:1);
IV  Benzene: ethyl acetate (9:1);
V  Cyclohexane: ethyl acetate (9:11);
VI  tert-butanol: ethyl acetate: 5 N NH\(_4\)OH (41:50:20).

Derivatives were prepared as described previously (Griffiths, Grant & Whyte, 1963) and the DHA sulphate solvolysed by the method of Burstein & Lieberman (1958). The specific activities of the steroids and their derivatives were measured after elution from thin layer plates by the procedures of Griffiths, Grant, Browning, Cunningham & Barr (1966), and the mean values used to determine percentage conversions. Radioactivity was determined using a Nuclear Chicago Liquid Scintillation Spectrometer (Model 6860).

**RESULTS**

Table 1 shows the evidence for the identification of steroids isolated from the incubation of \( [7\alpha-^3\text{H}] 17\alpha\)-hydroxy-
pregnenolone with the cultured tissues. The percentage conversion of $[7\alpha^{3}H]$17α-hydroxyprogrenolone to the three hormones investigated are also represented diagrammatically in fig. 1.

<table>
<thead>
<tr>
<th>Steroid and Derivative</th>
<th>Solvent System used for Puri-</th>
<th>Specific Activities (dpm/μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fication</td>
<td>1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>I</td>
<td>27.0</td>
</tr>
<tr>
<td>Cortisol acetate</td>
<td>I</td>
<td>26.7</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>II</td>
<td>28.8</td>
</tr>
<tr>
<td>DHA</td>
<td>III</td>
<td>11.4</td>
</tr>
<tr>
<td>DHA acetate</td>
<td>IV</td>
<td>12.4</td>
</tr>
<tr>
<td>Androst-5-ene-3β,17β-diol V</td>
<td>12.6</td>
<td>10.6</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>VI</td>
<td>21.5</td>
</tr>
<tr>
<td>DHA acetate</td>
<td>III</td>
<td>21.4</td>
</tr>
<tr>
<td>Androst-5-ene-3β,17β-diol V</td>
<td>21.8</td>
<td>8.5</td>
</tr>
</tbody>
</table>

The culture flasks, numbered 1-8 were set up as follows, the type of tissue being given and in parentheses the period of culture in days: 1, fascicular (3-7); 2, fascicular (11-15); 3, fascicular and ACTH (3-7); 4, fascicular and ACTH (11-15); 5, reticular (3-7); 6, reticular (11-15); 7, reticular and ACTH (3-7); 8, reticular and ACTH (11-15). The tissue was found to be less active during days 11-15 than during days 3-7, but the pattern of cortisol, DHA and DHA sulphate formation was maintained. No attempt was made to measure the production of these steroids from endogenous precursors.
DISCUSSION

Earlier studies (Griffiths, Grant & Symington, 1963) showed that both the fascicular and reticular tissue of the human adrenal cortex synthesized and secreted cortisol, corticosterone and 11β-hydroxyandrostenedione. However, only the clear cells of the zona fasciculata responded to corticotrophin in vitro with an increased synthesis of cortisol. Ultramicrochemical techniques employed by Jones & Griffiths (1968) and Cameron et al (1969) provided a more refined procedure for the separation of fascicular and reticular tissue, and experiments on the sul-
phation of DHA, in the guinea pig and human adrenal tissue, showed a qualitative difference between the zones. The sulphation of DHA was confined to the compact cells of the zona reticularis. A similar pattern has been provided by these organ culture studies, the formation of small amounts of DHA sulphate by the fascicular tissue probably being due to contaminating compact cells. It is of interest to note that in tissue culture, the pattern of DHA synthesis was similar to that of DHA sulphate, suggesting that DHA production may occur predominantly in reticular tissue.

Cortisol was synthesized from \( \textit{7a-}^{3} \text{H}\) \textit{17a-hydroxypregnenolone} by both zones, although no attempt was made to measure the production of cortisol from endogenous precursors. It is probably reasonable to assume however, that the presence of substantial amounts of cholesterol in the fascicular tissue and its metabolism by the tissue may have diminished the uptake of the radioactive precursor from the medium. The total amount of cortisol synthesized by the fascicular tissue may have been greater therefore, than that produced by the reticular tissue, although the incorporation of label into cortisol was greater in the reticular tissue. The results reported here show that the action of corticotrophin is not confined to the one zone.

Whereas conversion of \( \textit{7a-}^{3} \text{H}\) \textit{17a-hydroxypregnenolone} to cortisol doubled in the fascicular tissue under the influence of corticotrophin, there was no stimulation of the reticular tissue to synthesize more cortisol. This contrasts with the effect on DHA and DHA sulphate production, which was stimulated in the reticular tissue. Unpublished results from this laboratory have shown that the in vivo administration of corticotrophin does not stimulate the in vitro conversion of DHA to DHA sulphate in the guinea pig. The increase in the synthesis
of DHA sulphate observed here after corticotrophin stimulation may have been due to the increased production of its immediate precursor, DHA, from the \([\Delta^1 \text{H}]\) 17\(\alpha\)-hydroxyprogrenolone.

These results provide further evidence therefore that DHA sulphate synthesis and its control by corticotrophin is confined to the compact cell of the zona reticularis of the human adrenal cortex. Moreover, the study emphasizes the importance of working with well defined groups of cells when investigating biosynthetic pathways and the control mechanisms involved.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the generous financial support of the Tenovus Organisation.

REFERENCES

5. Griffiths, K., Grant, J.K., & Symington, T. J.clin.Endocr.Metab. 23, 776 (1963)
7. Jones, T. & Griffiths, K. J.Endocr. 42, 559 (1968)

ERRATUM

Volume 37, No. 3 (1969), in the Communication "Fluorescence Study of Interactions Between Valyl-tRNA Synthetase and Valine-Specific tRNAs from E. Coli," by C. Helene, F. Brun, and M. Yaniv, pp. 393-398, the relation, p. 394, should read

\[ I_F = I_M \frac{d}{d_0} \frac{1 - 10^{-d}}{1 - 10^{-d_0}} \] instead of \[ I_F = I_M \frac{d_0}{d} (1-10^{-d}). \]
STEROID SYNTHESIS IN A HUMAN VIRILISING ADRENAL CARCINOMA - SOME UNUSUAL FEATURES

By
E. H. D. Cameron, J. Hammerstein, D. Jones, S. Morris and K. Griffiths

ABSTRACT

A patient presenting at the Universitäts-Frauenklinik, Berlin with a long history of increasing hirsutism and virilism provided an opportunity to study an unusual form of adrenal hyperfunction. It was found that she behaved abnormally with respect to the dexamethasone suppression test since her highly elevated 17-oxosteroid output further increased by approximately 400% with 8 mg/day dosage. Her basal 17-hydroxycorticosteroid output was elevated but not grossly so, nor did she exhibit the usual signs of Cushing's syndrome of weight increase and florid complexion.

At operation a «tennis ball» size adrenal carcinoma was removed from the left side. Incubation studies were performed on sliced tumour tissue with substrates [1-14C] acetate, [7α-3H] pregnenolone, [4-14C] progesterone and [4-14C] androst-4-ene-3,17-dione. The frozen incubation mixtures were subsequently flown to Cardiff for steroid analysis by reverse radioisotope dilution techniques.

The combined urinary and incubation results suggested that, in vivo, the tumour secreted DHA and not DHA sulphate and that there appeared to be a considerable deficiency of DHA-3β-hydroxysteroid dehydrogenase/A4-isomerase and of DHA-3β-sulphokinase activities although the steroid-11β-hydroxylase, 17α-hydroxylase and 21-hydroxylase activities seemed unimpaired.
Since the discovery by Baulieu (1962) that dehydroepiandrosterone sulphate (DHA sulphate) was secreted by an adrenal carcinoma and also by the normal cortex (Wieland et al. 1963) interest has centred on the biosynthetic route by which this conjugate is made. It is also accepted that a grossly elevated 17-oxygensteroid output in the form of DHA sulphate (often accompanied by a relatively normal 17-hydroxycorticosteroid output) is diagnostic for adrenal carcinoma (Baulieu et al. 1967). The assumption has been that this high 17-oxygensteroid excretion results from secretion of DHA in the form of its sulpho-conjugate, and pre-operative investigation (Hammerstein 1968) of the conjugated urinary 17-oxygensteroid fraction from our patient indicated that the tumour presently under study was secreting either DHA and/or its sulphate.

Griffiths et al. (1968) found previously that cells from a clear-cell adenoma seem to have little capacity for the conversion of \([7\alpha-3H]\)pregnenolone sulphate and \([4-14C]\)pregnenolone to DHA sulphate (\(\sim 0.1\%\)). However, it was then shown (Cameron et al. 1969) that tissue from an adrenal tumour containing a mixture of «clear» and «compact» cells converted 19.8\% of \([4-14C]\) DHA, 6.8\% of \([7\alpha-3H]\) 17\alpha-hydroxypregnenolone and 5.2\% of \([4-14C]\) pregnenolone substrates to DHA sulphate. Furthermore, ultramicrochemical studies also indicated that in human as in guinea pig (Jones & Griffiths 1968) adrenals, the highest DHA sulphokinase activity was to be found in the «compact» cells of the zona reticularis. It was considered, therefore, that a complete study of the steroid biosynthetic pathways in a tumour suspected of producing large quantities of DHA or DHA sulphate would be of some interest.

**MATERIALS AND METHODS**

*Investigation of urinary hormones*

As previously described (Hammerstein 1968), urinary 17-oxygensteroids were extracted by the method of Dreker et al. (1952) and determined by the colour reaction of Zimmermann et al. (1952); 17-hydroxycorticosteroids were measured by the method of Few (1961), the three classical oestrogens by the method of Brown (1955) with values adjusted to 100\% recovery by means of radioactive internal standards, and pregnanediol by the method of Klopper et al. (1955) with quantitation of the end extract by gas-liquid chromatography (Hammerstein & Zielske 1968). Gonadotrophins were extracted by the method of Johnsen (1958) and determined by the bioassay technique of Klinefelter et al. (1943).

*Histology*

Representative pieces of the tumour were placed in 10\% neutral formalin for subsequent histological examination.

*Preparation of tissue for biochemical investigation*

Tissue from the tumour was sliced by means of a Stadie-Riggs hand microtome at
0.5 mm thickness just after its removal. Slices were then equally distributed among their respective incubation vessels in five portions of ~ 1.25 g/vessel.

**Incubation, extraction and fractionation of steroids**

Each incubation was carried out in 5 ml Krebs-Ringer bicarbonate-glucose medium (pH 7.4) at 37°C in 95% O₂: 5% CO₂ for 3 hours. The substrates used were purified by thin-layer chromatography and are shown in Table 1.

Incubations were terminated by rapid freezing. The still frozen mixtures were then transported by air from Berlin to Cardiff surrounded by solid CO₂ in a vacuum flask. Upon thawing, the mixtures were each diluted with 10 ml of acetone.

The following steroids (500 µg of each, specific activity ~ 4.4 X 10⁶ dpm/mg) were added to incubations 1 and 2; [7α-³H] DHA, [7α-³H] DHA sulphate, [1,2-³H] cortisol, [1,2-³H] corticosterone, and [7α-³H] 11β-hydroxyandrost-4-ene-3,17-dione prepared by incubation of rat adrenal mitochondria with [7α-³H] androst-4-ene-3,17-dione (upon isolation of the product, its purity was established by isotopic dilution techniques as described below). A «control» aliquot of the ³H-labelled mixture of steroids was also prepared. The [7α-³H] pregnenolone and [4-¹⁴C] progesterone incubation mixtures (incubations 3 and 4) were combined and the carrier steroids (500 µg of each) listed in Table 3 were added in ethanol. Similarly 500 µg of each of the steroids listed in Table 4 was added to incubation 5.

Procedures used for the extraction and partition of steroids from incubations 1, 2 and 3 + 4 were similar to those of Cameron & Grant (1967), but for incubation 5 the method of partition was that of Fahmy et al. (1968).

**Chromatography, identification and quantitative measurement of steroids**

Thin-layer chromatography on silica gel was used to isolate individual steroids. The procedures involved were those of Griffiths et al. (1966) as modified by Griffiths et al. (1968). The following solvent systems were employed: (I) Cyclohexane: ethyl acetate (90:110); (II) Chloroform: acetone (185:15); (III) Chloroform:methanol: water (187:12:1); (IV) Cyclohexane: ethyl acetate (140:60); (V) Cyclohexane: ethanol (180:20); (VI) Benzene: ethyl acetate (180:20); (VII) Tertiary butanol: ethyl acetate: 5 N NH₄OH (82:100:40); (VIII) Hexane: ethyl acetate (100:100), (IX) Benzene: ethyl acetate (140:60). Solvent proportions are by volume.

| Table 1. Substrates incubated with portions of virilising adrenal carcinoma. |
|---------------------------------|-----------------|-----------------|
| Incubation No.                | Substrate       | DPM X 10⁻⁶  |
| 1                              | [1-¹⁴C] acetate  | 111             |
| 2                              | [1-¹⁴C] acetate + 1 IU ACTH | 111             |
| 3                              | [7α-³H] pregnenolone | 9.30            |
| 4                              | [4-¹⁴C] progesterone | 3.32            |
| 5                              | [4-¹⁴C] androst-4-ene-3,17-dione | 4.89            |

Spec. activity (mCi/mM) 29 29 454 21.7 34.8
The specific activities of individual steroids and their derivatives (Griffiths et al. 1963) were determined by use of Nuclear Chicago Mark I Scintillation Spectrometer, the mass of \( \Delta^4 \)-3-oxosteroids being measured after elution by means of their selective absorption at 240 m\( \mu \) and that of \( \Delta^4 \)-\( \beta \)-hydroxy-steroids by use of a sulphuric acid/ethanol reagent (Oertel & Eik-Nes 1959). The observation of specific activities of a steroid and two of its derivatives differing by not more than 10\% was taken as satisfactory evidence for radiochemical homogeneity of the steroid concerned. However, one derivative was prepared in the case of 17\( \alpha \)-hydroxyprogrenenolone (incubations 3 + 4), testosterone and 19-hydroxytestosterone (incubation 5). The mean of the specific activities was used to calculate the percentage conversion from the original radioactive substrate incubated.

**RESULTS**

**Case report**

The patient (41 years) presented at the Endocrine Division of the Womens Hospital, Free University of Berlin in 1966 complaining of rapidly progressing hirsutism and substantial weight loss during the previous few weeks. Menstruation started when she was 14 years old. She underwent surgery in 1943 and 1951 for ovarian cysts resulting in the removal of the left ovary and resection of the other. Nonetheless, the menstrual cycle had been regular until the last year, when it became somewhat irregular with intervals between 24 and 30 days — a blood flow of 4–5 day's duration, which had always been slight, became still less in recent times. In 1956 and 1961 the patient became pregnant, both pregnancies resulting in miscarriages despite an intense desire for children. Her hirsutism which started with puberty, developed slowly during the first 15 years, although acne and alopecia were not noticed before the patient was 31 years old. Only three years later daily shaving was necessary. Concurrently decreasing volume of her breasts and increasing size of feet were also noticed. Muscular mass and strength as well as the skin were found to be of male type. The clitoris was only slightly enlarged and the voice female in pitch. The uterus was of normal size and shape as confirmed by laparoscopy. The vaginal smear was of the androgenised type. Two endometrial biopsies taken at day 15 and 27 of a 28 day long cycle both showed an endometrium in the proliferative state. Accordingly, the basal body temperature charts failed to show any thermogenic effect during three consecutive cycles. X-ray tomography after establishment of a retropneumoperitoneum revealed a spherical tumour on the left side of tennis ball size. On the basis of an arteriogram of the renal and adrenal vessels, the tumour was thought to be malignant. The diagnosis of an adrenal adenoma was made on the basis of the high-grade virilization, the X-ray findings, the elevation of the 17-oxosteroid excretion > 100 mg/day and its lack of response to ACTH and metopyrone. At surgery (1966) a well encapsulated tumour originating from the left adrenal was found. During the days following surgery the daily steroid excretion returned to
normal (17-oxosteroids varying since then between 4.9 and 15.7 mg and the 17-hydroxycorticosteroids between 4.9 and 20.7 mg, respectively). Concurrently the signs of hirsutism and virilism began to regress and normal female appearance has been restored during the 12 months after operation. The facial hair growth is still heavy, however, and it is not influenced by antiandrogen treatment with cyproterone acetate (Hammerstein & Cupcea 1969). Recurrence of the tumour has not become detectable so far.

Pathology and Histology
A well encapsulated tumour was found weighing ~ 200 g. The cut surface appeared medullary in type and was light grey-brown in colour with some fresh focal haemorrhages. In the marginal regions some bright yellowish elements were present probably of the original cortex. Histologically, the tumour mainly consisted of polygonal elements with bright cytoplasm. Nuclei were homogeneous in structure and rich in chromatin. Cells were arranged in solid cords, contained much brownish lipophilic pigments and were almost entirely »compact« in type. Connective tissue was generally distributed throughout the tumour which also had an extensive capillary network. The centre of the tumour showed fresh haemorrhages. Parts of the tumour seemed highly differentiated while other areas showed polymorphism of cells and nuclei. The original adrenal tissue was infiltrated by the tumour which had also invaded a vein. The sum of the evidence described led to a diagnosis of a well differentiated solid adrenal carcinoma.

Biochemistry
The urinary 17-oxosteroid and 17-hydroxycorticosteroid excretion values measured during the period of operation are shown in Fig. 1. It can be observed that although the patient reacted to ACTH and metopyrone as subjects with autonomous adrenal tumours are expected to do, she responded to dexamethasone paradoxically with a fourfold increase of the urinary 17-oxosteroid excretion to values above 400 mg/day. The 17-hydroxycorticosteroid values approximately doubled to about 20 mg/day but in contrast the pregnanediol and oestrogens (not included in Fig. 1) did not change to any major extent in this test. Urinary analyses performed prior to operation over days 4–7 of the menstrual cycle gave the following mean values per day (Hammerstein 1968): 17-oxosteroids, 114 mg; 17-hydroxycorticosteroids, 16.2 mg; oestrogens, 7.4 μg; pregnanediol, 1.8 mg. Gonadotrophins were 96 MUU, a value which is remarkably high in the presence of the extremely elevated 17-oxosteroids and a regular though anovulatory menstrual cycle.

When the partition of extracts from incubations 1 and 2 [1-14C] acetate was complete, it was found that 14C radioactivity was distributed as follows, Incubation 1: 80°–100°C b. p. light petroleum, 0.82 %; 80 % aqueous metha-
Results of urinary steroid analyses.

Fig. 1.

nol, 0.28%  
steroid conjugate« (extracted from aqueous residue containing 20% w/v (NH₄)₂SO₄ with ethyl acetate), 0.69% 20% (NH₄)₂SO₄ aq. residue, 7.9%. **Incubation 2**: Light petroleum, 1.10% 80% aqueous methanol, 0.32%  
steroid conjugates«, 0.35% 20% (NH₄)₂SO₄ aq. residue, 7.8%. Of the results for the steroids isolated from these two incubations only the specific activities for DHA (and its derivatives) are listed in Table 2 since this was the only steroid in which significant quantities of ¹⁴C were detected. No significant differences in the specific activities with respect to ³H were found in cortisol, corticosterone, DHA sulphate or 11β-hydroxyandrost-4-ene-3,17-dione isolated from incubations 1 and 2 and from the «control», indicating that substantial endogenous production of these steroids did not occur *in vitro* and that the presence of ACTH had no apparent effect. However, it should be noted that the specific activity of the DHA isolated from the incubations was approximately half that of the «control» showing that the portions of tumour tissue contained or had synthesized about 500 µg of DHA by the end of the 3 h incubation period.

Evidence for the identification of steroids isolated from the incubation of tumour tissue with [7α-³H] pregnenolone and [4-¹⁴C] progesterone (incubations 3 + 4), and with [4-¹⁴C] androst-4-ene-3,17-dione (incubation 5) is given in Tables 3 and 4, respectively. Tables 3 and 4 show the derivatives formed, the
<table>
<thead>
<tr>
<th>Steroid or derivative</th>
<th>Solvent system</th>
<th>Incubation 1 ([$^1$-$^14$C] acetate)</th>
<th>Incubation 2 ([$^1$-$^14$C] acetate + 1 IU ACTH)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androst-5-ene-3,17β-diol</td>
<td>II</td>
<td>$3^H$ 11.0</td>
<td>26.8 (0.15)</td>
<td>20.1</td>
</tr>
<tr>
<td>DHA acetate</td>
<td>VI</td>
<td>$4^C$ 24.7</td>
<td>27.0 (0.16)</td>
<td>20.0</td>
</tr>
<tr>
<td>DHA</td>
<td>I</td>
<td>$3^H$ 10.3</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>$4^C$ 24.0</td>
<td>24.2</td>
<td>19.6</td>
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</tr>
</tbody>
</table>

Identification of DHA isolated from the incubation of human adrenal tumour tissue with [$^1$-$^14$C] acetate.
Table 3.
Identification of steroids isolated from the incubation of human adrenal tumour tissue with \([7\alpha-^3H]\) pregnenolone and \([4-^14C]\) progesterone.
Percentage radioactivity found in the steroids isolated is also shown.

<table>
<thead>
<tr>
<th>Material investigated and chemical reaction</th>
<th>Product mobility identical with that of:</th>
<th>Solvent system</th>
<th>Specific activities (pN/mμmole)</th>
<th>(^{3}H)</th>
<th>(^{14}C)</th>
<th>% Radioactivity found in isolated steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
<td>Pregnenolone</td>
<td>II</td>
<td>420</td>
<td>*</td>
<td>-</td>
<td>7.28</td>
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<tr>
<td>Acetylation</td>
<td>Pregnenoloneacetate</td>
<td>IV</td>
<td>423</td>
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<td>-</td>
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<tr>
<td>Reduction</td>
<td>Pregn-5-ene-3β,20β-diol</td>
<td>II</td>
<td>443</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>17(\alpha)-Hydroxypregnenolone</td>
<td>17(\alpha)-hydroxypregnenolone</td>
<td>II</td>
<td>11.2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>3β-acetoxy-17(\alpha)-hydroxy-preg-5-en-20-one</td>
<td>VI</td>
<td>11.7</td>
<td>-</td>
<td>-</td>
<td>0.18</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Progesterone</td>
<td>II</td>
<td>4.8</td>
<td>15.8</td>
<td>-</td>
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<tr>
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<td>20β-hydroxypregn-4-en-3-one</td>
<td>II</td>
<td>4.4</td>
<td>15.7</td>
<td>0.08</td>
<td>0.76</td>
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<td>Reduction and acetylation</td>
<td>20β-acetoxypregn-4-en-3-one</td>
<td>V</td>
<td>4.5</td>
<td>15.9</td>
<td>-</td>
<td></td>
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<tr>
<td>17(\alpha)-Hydroxyprogesterone</td>
<td>17(\alpha)-hydroxyprogesterone</td>
<td>II</td>
<td>-</td>
<td>5.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Reduction</td>
<td>17(\alpha)20β-dihydroxy-pregn-4-en-3-one</td>
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<td>-</td>
<td>5.5</td>
<td>-</td>
<td>0.25</td>
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<td>17(\alpha)-hydroxy-20β-acetoxy-pregn-4-en-3-one</td>
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<td>-</td>
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<tr>
<td>11-deoxycorticosterone</td>
<td>11-deoxycorticosterone</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>17(\alpha),21-dihydroxypregnenolone</td>
<td>17(\alpha),21-dihydroxypregnenolone</td>
<td>III</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Material investigated and chemical reaction</td>
<td>Product mobility identical with that of:</td>
<td>Solvent system</td>
<td>Specific activities (dpm/mumole)</td>
<td>% Radioactivity found in isolated steroids</td>
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<td></td>
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<tr>
<td>Corticosterone</td>
<td>Corticosterone</td>
<td>III</td>
<td>59.2</td>
<td>129</td>
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<tr>
<td>Acetylation</td>
<td>Corticosterone acetate</td>
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<td>56.4</td>
<td>127</td>
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<tr>
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<td>11β,20β,21-trihydroxypregn-4-en-3-one</td>
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<td>I</td>
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<td>Androst-4-ene-3,11,17-trione</td>
<td>I</td>
<td>1.48</td>
<td>0.02</td>
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<td>Reduction</td>
<td>Oxidation, reduction and acetylation</td>
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<td>1.45</td>
<td>-</td>
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</tr>
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<td>Cortisol</td>
<td>Cortisol</td>
<td>III</td>
<td>-</td>
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<td>Acetylation</td>
<td>Cortisol acetate</td>
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<td>Oxidation</td>
<td>Androst-4-ene-3,11,17-trione</td>
<td>I</td>
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<tr>
<td>Cortisone</td>
<td>Cortisone</td>
<td>III</td>
<td>-</td>
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<td>-</td>
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<td>I</td>
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<tr>
<td>DHA</td>
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<td>VIII</td>
<td>1356</td>
<td>-</td>
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<td>DHA acetate</td>
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<td>1245</td>
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<tr>
<td>Reduction</td>
<td>Androst-5-ene-3β,17β-diol</td>
<td>VIII</td>
<td>1267</td>
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<tr>
<td>Material investigated and chemical reaction</td>
<td>Product mobility identical with that of:</td>
<td>Solvent system</td>
<td>Specific activities (dpm/mumole)</td>
<td>% Radioactivity found in isolated steroids</td>
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<tr>
<td>Androst-4-ene-3,17-dione</td>
<td>Androst-4-ene-3,17-dione</td>
<td>II</td>
<td>10.8</td>
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<tr>
<td>Reduction</td>
<td>Testosterone</td>
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<td>10.8</td>
<td>1.6</td>
<td></td>
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<tr>
<td>Reduction and acetylation</td>
<td>Testosterone acetate</td>
<td>IV</td>
<td>9.8</td>
<td>1.7</td>
<td></td>
<td></td>
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<tr>
<td>11β-hydroxyandrost-4-ene-3,17-dione</td>
<td>11β-hydroxyandrost-4-ene-3,17-dione</td>
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<td>81.4</td>
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<td>Androst-4-ene-3,11,17-trione</td>
<td>II</td>
<td>81.9</td>
<td>251</td>
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<tr>
<td>DHA sulphate</td>
<td>DHA</td>
<td>VII</td>
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<td>Solvolysis (S)</td>
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<td>DHA acetate</td>
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<td>29.2</td>
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<tr>
<td>S and reduction</td>
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<td>VII</td>
<td>32.0</td>
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<tr>
<td>S, reduction and acetylation</td>
<td>3β,17β-diacetoxyandrost-5-ene</td>
<td>VI</td>
<td>34.2</td>
<td>-</td>
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</table>

* Radioactivity not significantly above background.
Table 4.
Identification of steroids isolated from the incubation of human adrenal tumour tissue with [4-\(^{14}\)C] androst-4-ene-3,17-dione. Percentage radioactivity found in the steroids isolated is also given.

<table>
<thead>
<tr>
<th>Material investigated and chemical reaction</th>
<th>Product mobility identical with that of:</th>
<th>Solvent system</th>
<th>Specific activities (dpm (^{14})C/m(\mu)mole)</th>
<th>% Radioactivity found in isolated steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androst-4-ene-3,17-dione</td>
<td>Androst-4-ene-3,17-dione</td>
<td>II</td>
<td>15.9</td>
<td>0.35</td>
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<tr>
<td>Reduction</td>
<td>Testosterone</td>
<td>II</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>Reduction and acetylation</td>
<td>Testosterone acetate</td>
<td>IV</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>Testosterone</td>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Testosterone acetate</td>
<td>IV</td>
<td>2.75</td>
<td>0.06</td>
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<td>Androst-4-ene-3,17-dione</td>
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<td>11(\beta)-hydroxyandrost-4-ene-3,17-dione</td>
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<td>2609</td>
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<td>11(\beta)-hydroxytestosterone</td>
<td>III</td>
<td>2823</td>
<td>55.7</td>
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<tr>
<td>Oxidation</td>
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<td>II</td>
<td>2818</td>
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<td>19-hydroxyandrost-4-ene-3,17-dione</td>
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<td>IX</td>
<td>2.63</td>
<td></td>
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</tbody>
</table>
solvent systems used for their purification, the specific activities of the compounds studied, and the percentage conversions of the radioactive steroid precursors to the various metabolites.

**DISCUSSION**

Substantial conversion of [4-14C] androst-4-ene-3,17-dione to 11β-hydroxy-androst-4-ene-3,17-dione (55.7 %) and of [4-14C] progesterone to cortisol (23.6 %) and corticosterone (5.49 %) (see Tables 3 and 4) shows that the 11β-, 17α- and 21-hydroxylase systems in the tumour cells were able to function efficiently (Fig. 2). Indeed 19-hydroxylation of the androst-4-ene-3,17-dione was shown to have occurred by the isolation of 19-hydroxyandrost-4-ene-3,17-dione (0.38 %) and 19-hydroxytestosterone (0.05 %), although no oestrogen or oestrogen conjugate formation could be detected. This finding is unusual in human adrenal tissue since 19-hydroxylation is normally found only in non-fatty adrenal glands, for example bovine (Hayano & Dorfman 1955) and hamster (Griffiths & Giles 1963). It was also interesting to note that progesterone was converted much more efficiently to cortisol than to corticosterone, the opposite trend having been found previously with hyperplastic adrenal tissue (Cameron et al. 1968) and with a clear cell adenoma (Cameron 1968).
Acta endocr. 65, 1

& Griffiths 1968). However, [1-14C] acetate and [7α-3H] pregnenolone were converted to Δ4-3-oxosteroids only in small amounts, and in both instances the major metabolite was DHA, a pattern which is compatible with the urinary excretion values for the 17-oxosteroids. The relatively high urinary excretion of 17-hydroxycorticosteroids may have been due to an increased action of the residual adrenal glands in response to the stress of the presence of the tumour. In contrast, under similar incubation conditions to the present series of experiments [7α-3H] DHA was converted to androst-4-ene-3,17-dione (27.5 %), 11β-hydroxyandrost-4-ene-3,17-dione (22.5 %), testosterone (0.058 %) and epi-testosterone (0.016 %) by adrenal tissue from patients with breast cancer (Jones & Griffiths, unpublished data). Figures for conversions to DHA (incubations 1, 2 and 3) must be regarded as under-estimates in view of the difference in 3H specific activities between the DHA isolated from incubations 1 and 2, and from the «control». Since the figures for the DHA synthesized in incubations 1 and 2 are approximately half that of the control, it must be concluded that the portions of the tumour tissue incubated contained or had synthesized about 500 µg of DHA at the end of the 3 h incubation period. Conversion figures for DHA in the three incubations should, therefore, be approximately doubled. It is also likely in view of the evidence that the tumour secreted DHA and not DHA sulphate in situ and that DHA sulphokinase as well as the 3β-hydroxysteroid dehydrogenase-isomerase systems had almost been eliminated in the de-differentiation processes of tumour development.

Although it has been suggested that an initial effect of dexamethasone can be to raise the blood concentration of ACTH (James et al. 1965) by affecting the hypothalamic-pituitary axis, and it might be thought that this should explain the dramatic increase in 17-oxosteroid output, it is difficult to see how such a mechanism would operate in this particular case in the light of the non-stimulation of this output by exogenous ACTH (Fig. 1). Furthermore, the observed effect of dexamethasone was not a transient one but steadily increased with continued administration over a period of six days. No significant effect of ACTH was observed in vitro and indeed no real clue was obtained from this evidence which would explain the dramatic increase of 17-oxosteroid output in vivo during the dexamethasone suppression test. It is possible that the tumour stored the DHA which it synthesized, and administration of the glucocorticoid operated some sort of release mechanism for DHA secretion which in normal adrenal tissue from the zona reticularis would be released as DHA sulphate (Jones & Griffiths 1968; Cameron et al. 1969). Against this is the fact that no apparent product inhibition of the conversion of [7α-3H] pregnenolone to DHA was observed even with the relatively high concentration of the C19-steroid measured. That inhibition by DHA of 11β- and 21-hydroxylation as previously reported by Sharma et al. (1963) and Sharma &
Dorfman (1964) occurred is possible, although [4-14C] progesterone was efficiently converted to cortisol. Furthermore, it cannot be ruled out that the dexamethasone could have had a «permissive» role in the excretion of the DHA, by some action either on the liver or the kidney. Indeed mobilisation of DHA or other 17-oxosteroids stored in adipose or other tissues is yet another possibility. That the paradoxical response of the 17-oxosteroid excretion to dexamethasone is not specific for that steroid is apparent from the observation of Finkelstein (1958), who had similar results with a patient receiving cortisone. Others (Rivera et al. 1967; Mahesh & Greenblatt 1968) have also observed an anomalous increase in 17-oxosteroid output in response to dexamethasone administration in patients with Cushing’s syndrome, and recently a similar effect on 17-hydroxycorticosteroid output has been reported on a number of occasions (Brooks et al. 1966; Rose et al. 1969; French et al. 1969).

ACKNOWLEDGMENTS

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REFERENCES


Hammerstein J.: Trans. 3rd Meeting Int. Study Group for Steroid Horm. (1968) 323.


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IN VITRO SYNTHESIS OF STEROIDS
BY A FEMINISING ADRENOCORTICAL CARCINOMA:
EFFECT OF PROLACTIN
AND OTHER PROTEIN HORMONES

By
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H. Roberts¹), W. R. Butt²) and K. Griffiths

ABSTRACT
The study describes the effects of ACTH, prolactin and other protein hormones on the synthesis and secretion of steroid hormones by tissue from a feminising adrenocortical carcinoma removed from a post-menopausal female. Steroid production by the tissue was determined by high resolution-mass fragmentography and by radioimmunoassay. Prolactin and ACTH stimulated the synthesis of oestrogens by the tissue whereas GH, LH and ACTH were more effective than prolactin in stimulating androgen synthesis. The effect of protein hormones, other than ACTH, on adenylate cyclase activity of this tumour tissue indicated a lack of specificity of the membrane receptor sites.

Most adrenocortical carcinomas produce endocrine symptoms. Those associated with Cushing's syndrome and virilization are the most common (Symington & Jeffries 1962; Symington 1969) although carcinomas giving rise to primary aldosteronism (Conn et al. 1964), hypoglycaemia (Williams 1960) and feminisation (Gabrilove et al. 1965) have also been reported. Tumours of the adrenal cortex
causing feminisation are characterised by iso-sexual precocity in the pre-pubertal female and feminisation in the pre-pubertal (Wilkins 1948) and adult male (Gabriolove et al. 1965). Such feminising tumours are rare and Gabriolove et al. (1965) reviewed the 53 tumours, carcinomas and adenomas, which had previously been reported in the adult male to that date. Reports of feminising adrenocortical carcinomas in adult females are even more rare, presumably because of the difficulties in recognising the clinical manifestations of the condition, and only Procopé (1970) and Mathur et al. (1973) have described such patients. Urinary steroid analyses (Procopé 1970) and studies in vivo and in vitro (Mathur et al. 1973) were undertaken with these cases of feminising carcinoma in post-menopausal women.

The present report describes some studies in vitro on tissue from a feminising adrenocortical carcinoma also removed from a post-menopausal female.

**EXPERIMENTAL**

**Case report**

The patient (66 years old in 1974) underwent an operation in 1965 for a prolapsed vaginal wall. She experienced vaginal bleeding in August, 1972 and was given a course of oestrogens for one month. In October, a D & C was performed but no abnormality was found although bleeding continued intermittently over the subsequent 5–6 weeks. Cervical cytology in January 1973 showed some evidence of early malignant change and a hysterectomy and oophorectomy was performed in February. Histological examination indicated endometrial cystic hyperplasia with mitotic figures in stroma and epithelium, but no malignancy. On March 5, the following serum hormone values were found: progesterone, 0.7 ng/ml, oestradiol-17β, 800 pg/ml and FSH < 1 mU/ml. Repeat analyses in May showed oestradiol-17β, 820 pg/ml and FSH < 1 mU/ml. Plasma androstenedione concentration was approximately 30 times normal levels (140–280 nmol/l) and the testosterone concentration 11–17 nmol/l. An adrenal tumour was suspected and on admission to the Queen Elizabeth Hospital, Birmingham in July, 24 h urinary steroid values were found to be as follows: 17-oxosteroids 40 mg, 17-oxogenic steroids 6 mg and total oestrogens 760 μg. A dexamethasone suppression test (0.5 mg x 4, daily for 2 days, 2.0 mg x 4 daily for 2 days) failed to influence the concentration of plasma oestradiol and the FSH level remained less than 1 mU/ml. The plasma cortisol decreased from 30 μg/100 ml to 12 μg by the end of the test. An ACTH (tetracosactin, 0.5 mg b. d. over 4 days) stimulation test resulted in an increase in the plasma cortisol concentration from 20 to 40 μg/100 ml whereas the plasma oestradiol level on the control day was 1960 pg/ml and this decreased to 1060 pg by the fourth day of the ACTH administration. An aortogram showed the presence of a large tumour in the area of the left adrenal which was removed in August 1973. After operation, the plasma oestradiol concentration fell within 3 days to the normal post-menopausal level. Three weeks after the operation, the LH had risen to 5 mU/ml, the FSH to 31 mU/ml and the cortisol concentration in the plasma was in the normal range.

Histological examination of the tumour confirmed that it was an adrenal carcinoma which had been removed.
MATERIALS AND METHODS

The plasma and urinary steroid and protein hormone concentrations were determined at the Birmingham and Midland Hospital for Women (Dr. W. R. Butt).

Tissue culture
A portion of the adrenal tumour was transported to Cardiff in sterilised containers on ice. Tissue was cut into 1 mm³ explants under aseptic conditions for culture. Explants were cultured on lens paper rafts supported by stainless steel grids (Trowell 1959) in TC199 medium fortified with 10 % calf serum, penicillin 200 units/ml and streptomycin, 100 μg/ml, in organ culture dishes (Falcon Plastics Ltd., California, USA). Cultures were pre-incubated at 37°C in an atmosphere of 95 % air: 5 % CO₂ for 24 h. After this period, medium was removed, discarded and replaced with fresh medium (control cultures) or medium in which either ACTH (Synacthen®, β₁-2₄corticotrophin, Ciba Ltd), insulin (Weddel Pharmaceuticals Ltd), equine LH (Calbiochem Ltd), ovine prolactin (WHO, 2nd Int. Standard) or human GH (MRC 69/46) were dissolved. All cultures were prepared in triplicate and were incubated for 3 days. Medium was then removed and stored at −15°C for steroid analysis. Tissue from each culture was carefully blotted dry with tissue paper and weighed.

Steroid extraction
Steroids were extracted from the culture medium with ethyl acetate (3 x 4 vols.), while extraction from the original non-incubated tumour tissue was effected by the procedure described previously (Millington et al. 1974).

Analysis of culture medium by radioimmunoassay
Oestradiol-17β was determined in the medium by a specific radioimmunoassay using an antibody raised against oestradiol-6-(0-carboxymethyl) oxime-BSA. The cross-reaction of this serum with oestrone was of the order of 1 %. Oestradiol was measured using an antiserum against oestradiol-11-hemisuccinyl-BSA after alumina thin-layer chromatography (Cowley et al. 1976). Adrenocorticosteroids were determined using the competitive protein binding assay described by Murphy (1967) and a dog plasma as the source of the binding protein.

Analysis of steroids by combined gas chromatography-high resolution mass fragmentography
Aliquots of the culture medium from selected incubations were extracted with ethyl acetate. The dried steroid extracts were dissolved in ethanol, measured volumes transferred to 1 ml screw-cap vials and the ethanol removed. Bis (trimethylsilyl) acetamide (50 μl) and light petroleum (b. p. 60–80°C) (20 μl) were added and the tubes left overnight at 20°C. Combined gas liquid chromatography (GLC) high resolution-mass fragmentography was carried out as previously described in detail (Millington 1975) using a Varian-MAT 731 mass spectrometer. The assay for each steroid derivative was then conveniently set up by monitoring the intensity of the molecular-ion peak on a potentiometric recorder at a resolution of 10 000 (10 % valley). Under these conditions, the recorder response varied linearly with sample concentration in the range 0.1–10 ng/μl and the detection limit was approximately 3 pg/μl for the oestrogens and 25 pg/μl for the C₁₉-steroids (Millington 1975). The two parameters of GLC retention time and
(a) Mass spectrum of oestrone trimethylsilyl ether

(b) Mass fragmentograms – m/e 342 · 202 (oestrone-TMS-ether)

Control incubation
(1 µl = 0.90 mg tissue)

Incubation with prolactin
(1 µl = 0.67 mg tissue)

Fig. 1.
Principles of high resolution – mass fragmentography for the analysis of steroids in the culture medium.
high resolution molecular ion detection were sufficient to detect and quantify the components regardless of the complexity of the steroid mixture and the presence of overlapping GLC peaks.

Illustrated in Fig. 1 are the principles of high resolution-mass fragmentography (HRMF) as they apply to oestrone. Fig. 1 (a) shows the mass spectrum of oestrone-3-trimethylsilyl ether, obtained during routine combined gas chromatography and mass spectrometry. Clearly, the molecular ion at m/e 342.202 is ideal for specific ion monitoring and high resolution-mass fragmentograms showing the presence of oestrone in the culture medium extracts of control and prolactin containing incubations of the adrenal tumour explants are represented in Fig. 1 (b). Peak heights were recorded in arbitrary units and related to those of standard steroid solutions obtained from similar fragmentograms. Other steroids were assayed in turn by tuning the mass spectrometer to detect the appropriate molecular ions and repeating the procedure described for oestrone.

The m/e values for high resolution monitoring of the molecular ions obtained from the various steroids investigated by this technique and also the GLC retention data have been given previously (Millington 1975). Peak heights were determined for all steroids isolated from the culture medium and related to the peak height of steroid standards from similar fragmentograms.

Measurement of adenylate cyclase activity

Adrenal tumour tissue was minced finely and a 10% homogenate prepared in 0.25 M sucrose using 10 strokes of a Philpot & Stanier (1956) homogeniser. The homogenate was filtered through cheesecloth before use in the assay. All procedures were carried out at 0°C. Adenylate cyclase activity in the presence and absence of various protein hormone preparations was determined by the procedure established in these laboratories by Goldner & Boyns (1973) using [8-14C]ATP and cyclic [3H]AMP as tracer. The hormone preparations used were: ovine prolactin (WHO 2nd Int. Std.); β1-24corticotrophin (Synacthen®, Ciba Laboratories); HLH (IRC-2-10.12.64); HFSH (CPDS-6-Butt); HTSH (DE-32-3-Hartree); HCS (MRC, human placental lactogen 70/194); insulin (crystalline porcine insulin 23 IU/mg, Eli Lilly).

Normal adrenal studies

Tissue from a 'normal' adrenal gland removed from a patient with breast cancer was cut into 1 mm³ explants and cultured as described earlier. It was not possible to attempt to separate the tissue from the fascicular and reticular zones of the cortex and the explants would therefore be composed of mixed cortical cells. With the limited tissue available, only the effect of β1-24corticotrophin and prolactin on steroid secretion was studied.

RESULTS

A relatively specific radioimmunoassay for oestradiol-17β which had been found quite adequate for the routine determination of this hormone in plasma (Cameron & Jones 1972) was used to assess the rate of secretion of oestradiol-17β by the cultured tumour explants. Table 1 shows the values obtained. The results suggested that in vitro, neither GH nor LH at the concentrations used, effectively
Table 1.
Determination of steroids synthesised by adrenal tumour tissue in culture.

<table>
<thead>
<tr>
<th></th>
<th>RIA determination (pg/mg wet wt. tissue)</th>
<th>GLC-mass fragmentography (pg/mg wet wt. tissue)</th>
<th>Protein binding assay (ng/mg wet wt. tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestradiol</td>
<td>Androstenedione</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>Control</td>
<td>44</td>
<td>590</td>
<td>9</td>
</tr>
<tr>
<td>LH (5 µg)</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH (50 µg)</td>
<td>33</td>
<td>2320</td>
<td>15</td>
</tr>
<tr>
<td>GH (2.5 µg)</td>
<td>57</td>
<td>2910</td>
<td>25</td>
</tr>
<tr>
<td>α1-24ACTH (1.0 µg)</td>
<td>204</td>
<td>1730</td>
<td>150</td>
</tr>
<tr>
<td>Prolactin (5.0 µg)</td>
<td>134</td>
<td>1310</td>
<td>50</td>
</tr>
<tr>
<td>Insulin (0.08 mU) + ACTH (1.0 µg)</td>
<td>152</td>
<td>8460</td>
<td></td>
</tr>
<tr>
<td>Insulin (0.08 mU) + Prolactin (1.0 µg)</td>
<td>303</td>
<td>260</td>
<td>1120</td>
</tr>
</tbody>
</table>

Table 2.
Endogenous concentrations of steroids in adrenal tumour tissue.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Steroid concentration pg/mg wet wt. tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol-17β</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Oestrone</td>
<td>45</td>
</tr>
<tr>
<td>DHA</td>
<td>100</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Androsterone</td>
<td>27</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>17</td>
</tr>
<tr>
<td>Testosterone</td>
<td>35</td>
</tr>
</tbody>
</table>
stimulated the synthesis and secretion of oestradiol-17β by the adrenal tumour tissue. ACTH and prolactin however, with insulin appearing to influence the action of both these hormones, stimulated the output of oestradiol-17β. Values obtained for the endogenous oestradiol-17β concentration of the tumour tissue determined by high resolution GLC-mass fragmentography (Table 2), indicated that steroid was being actively synthesised during the three-day culture period.

Of particular interest however, was the concentration of endogenous oestrone (45 pg/mg tissue) compared with the concentration of oestradiol-17β and oestriol (<3 pg/mg tissue of both oestrogens). It was therefore obvious that with a cross-reactivity of oestrone of 1% in the oestradiol-17β radioimmunoassay, further analysis was necessary to determine the oestradiol-17β and oestrone concentration present in the culture medium. Values of the oestradiol-17β and oestrone concentrations in the medium, determined by high resolution GLC-mass fragmentography are shown in Table 1. The tumour tissue actively synthesised and secreted oestrone as well as oestradiol-17β into the medium and the results indicated again that ACTH and prolactin stimulated this biosynthetic activity. The results again demonstrated the influence of insulin on the secretory activity of the tumour tissue.

In similar manner, Table 1 shows the results obtained after the analysis of the androstenedione content of culture medium by both radioimmunoassay and high resolution mass fragmentography. These figures suggest that LH and GH, as well as ACTH and prolactin, were capable of influencing the synthesis and secretion of androstenedione by tissue explants.

Analysis of media from selected incubations indicated that no DNA sulphate was present in the media from the tumour tissue cultures both before and after incubation with either ACTH or prolactin.

Another series of analyses to investigate the effect of various protein hormones on the secretion of 11-hydroxycorticosteroids into the culture medium are also given in Table 1. Analysis of the 11-hydroxycorticosteroid concentration of the medium by the corticosteroid binding globulin method similar to that described by Murphy (1967) tended to indicate that in vitro, LH and prolactin exerted a greater effect on the tissue than did ACTH.

The effect of various protein hormones on the adenylate cyclase activity of tumour homogenates was assessed by a double isotope procedure, using [14C]ATP as enzyme substrate (Table 3). Prolactin, LH and FSH were all more effective than ACTH in stimulating the activity of the enzyme. When insulin was added to the tumour homogenate, adenylate cyclase activity was reduced when compared to control incubations. Studies have previously been performed in which adenylate cyclase activity has been measured in normal guinea pig tissue in the presence of several hormone preparations. In most experiments only β1-24 corticotrophin stimulated enzyme activity (Golder & Boyns 1973) although in one case LH also resulted in stimulation (Golder, unpublished data).
Table 3.

<table>
<thead>
<tr>
<th>Hormone added</th>
<th>Concentration</th>
<th>Enzyme activity in tumour tissue (Base-line control activity is 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta^{1-24}$ACTH</td>
<td>10.0 ng/ml</td>
<td>102.4</td>
</tr>
<tr>
<td>$\beta^{1-24}$ACTH</td>
<td>10.0 µg/ml</td>
<td>113.4*</td>
</tr>
<tr>
<td>Insulin</td>
<td>400.0 pg/ml</td>
<td>81.8*</td>
</tr>
<tr>
<td>HLH</td>
<td>1.5 µg/ml</td>
<td>135.4*</td>
</tr>
<tr>
<td>HFSH</td>
<td>1.5 µg/ml</td>
<td>143.3*</td>
</tr>
<tr>
<td>HTSH</td>
<td>1.5 µg/ml</td>
<td>101.7</td>
</tr>
<tr>
<td>HCS</td>
<td>1.5 µg/ml</td>
<td>145.4*</td>
</tr>
<tr>
<td>Prolactin</td>
<td>1.5 µg/ml</td>
<td>148.5*</td>
</tr>
<tr>
<td>Prolactin</td>
<td>60.0 µg/ml</td>
<td>149.1*</td>
</tr>
</tbody>
</table>

* Significance of increase or decrease in enzyme activity was determined by the Wilcoxon's Rank sum test -- $P < 0.05$.

Results from the studies on the steroid synthesis and secretion by the 'normal' adrenal tissue in culture are given in Table 4. The data indicates that DNA sulphate, DHA and androstenedione were present in the culture medium after 3 days incubation. $\beta^{1-24}$Corticotrophin stimulated the tissue to increase the output of all three steroids and also promoted oestradiol and oestrone synthesis. Prolactin also stimulated this tissue but not with the same effect as corticotrophin.

Table 4.

Determination of steroids synthesised by normal adrenal tissue in culture.

<table>
<thead>
<tr>
<th></th>
<th>O$_{22}$</th>
<th>O$_{21}$</th>
<th>DHA</th>
<th>A</th>
<th>DHAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>2.2</td>
<td>9.6</td>
</tr>
<tr>
<td>$\alpha^{1-24}$ACTH (1.0 µg)</td>
<td>30</td>
<td>130</td>
<td>3000</td>
<td>36.0</td>
<td>26.6</td>
</tr>
<tr>
<td>Prolactin (1.0 µg)</td>
<td>10</td>
<td>10</td>
<td>200</td>
<td>3.4</td>
<td>18.8</td>
</tr>
</tbody>
</table>
DISCUSSION

Feminising adrenocortical carcinomas are rare and there are few steroid biosynthetic studies on such tissue. Oestrogen biosynthesis in vitro from both C21- and C19-steroid precursors has been shown (Baggett et al. 1959; Gabrilove et al. 1965; Rose et al. 1969) and recent investigations (Mathur et al. 1973) demonstrated the conversion of radioactive acetate to oestrogens, oestrone being the predominant steroid formed.

It is interesting that in this present report, the concentration of endogenous oestrone relative to oestradiol-17β and oestradiol was found to be high (45 pg/mg wet wt. tissue). Furthermore, the tissue culture studies clearly indicate that the tumour tissue was actively synthesising oestrone and oestradiol-17β and these experiments relate well to the high urinary oestrogen excretion and plasma oestradiol-17β concentration observed in this patient. Although oestrone was the principal urinary oestrogen found in this patient, and also in a case reported by Procope (1970), this is not a consistent pattern associated with all feminising adrenocortical carcinomas (Harrison et al. 1966; Gabrilove et al. 1970). Similarly, active synthesis of androstenedione by the tissue in culture was also noted and this again was consistent with the elevated androstenedione and testosterone concentrations determined in the plasma of the patient. The clinical manifestations obviously were dependent on the balance of androgens and oestrogens produced, rather than on any absolute values.

Tissue culture studies also provided data on the effect of various protein hormones on steroid biosynthesis in vitro. During the clinical investigations, a dexamethasone suppression test was without effect on plasma oestradiol whereas an ACTH stimulation test caused a decrease in plasma oestradiol. The plasma cortisol was however suppressed to 50% of its control concentration after dexamethasone administration and was elevated from 20 to 40 μg/100 ml after administration of tetracosactin to the patient. In vitro, ACTH appeared to have little effect of the secretion of 11-hydroxycorticosteroids, whereas LH, GH and prolactin were found to stimulate the output of these steroids from the tissue. Androstenedione secretion was shown to be responsive in vitro to LH, GH and ACTH and, furthermore, ACTH produced the greatest effect on the synthesis of oestradiol-17β. Again oestrone synthesis was stimulated more by prolactin than by ACTH. The results suggested that insulin could modify the effect of the protein hormone on this tumour tissue thereby affecting steroid synthesis. This responsiveness of the steroid biosynthetic activity of this tumour tissue to various protein hormones related well to the apparent complete lack of specificity of the adenylate cyclase enzyme system to these protein hormones. Compared to HLH, HFSH, HCS and prolactin, ACTH had a limited stimulatory effect on this enzyme system. In relation to these observations, a recent report
of Pittaway et al. (1973) described the first in vitro demonstration of gonadotrophin sensitive steroidogenesis in a human adrenal cortical adenoma.

In normal guinea pig adrenal homogenates, β1-24ACTH was shown to specifically stimulate adenylate cyclase activity (Golder & Boyns 1973). Insulin alone had no significant effect on the enzyme activity although it did have a modulating effect on the stimulation produced by low doses of ACTH. A similar effect has also been demonstrated by Illiano & Cuatrecasas (1972) in a study of glucagon and epinephrine action on liver and fat cells. Moreover, although the adenylate cyclase enzyme system of the normal rat adrenal is responsive only to ACTH, experiments with a transplantable rat adrenal tumour indicated that several other hormones including LH, FSH and TSH increased cyclic-AMP synthesis (Schorr & Ney 1971; Schorr et al. 1971).

β1-24Corticotrophin stimulated the normal adrenal tissue to synthesise oestrogens and certain C19-steroids, but DHA sulphate in particular. Addition of prolactin also resulted in an increased output of steroids although not so effectively as corticotrophin, an effect similar to that described previously by this group (Boyns et al. 1972).

The value of high resolution-mass fragmentography is evident from these studies. The consistently lower values for oestradiol-17β and androstenedione obtained compared with those determined by radioimmunoassay reflect the greater specificity and reliability of the former technique, especially when applied to previously uninvestigated systems.

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REFERENCES


Millington D. S.: J. Steroid Biochem. 6 (1975) 239.

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I. INTRODUCTION

Since the initial demonstration (Bloch, 1945) that progesterone was synthesized in vivo from isotopically labelled cholesterol administered to a pregnant woman, the biosynthesis of steroid hormones, especially those of the human adrenal cortex, has provided a fascinating problem to numerous groups of investigators. Interest has often been directed towards the human adrenal cortex, although sufficient tissue has rarely been available for intensive biochemical study, and much of our knowledge of steroid synthesis has been derived from investigations on tissues from many animal species. The basis from which most of this knowledge has developed was provided by retrograde perfusion studies on the ox adrenal gland carried out by Pincus and his colleagues at the Worcester Foundation (Hetchter et al., 1951; Hechter and Pincus, 1954). Such investigations on the adrenal glands of other animals has helped to build up a comprehensive picture of the complex pattern of pathways for steroid biosynthesis, although the marked species differences in morphology and biochemistry of the glands have not always been carefully considered, and results from these studies have occasionally been extrapolated uncritically to the human
adrenal cortex. Furthermore, little attention has been directed to the zonation of the human adrenal cortex, or the possible differences in function of its various cell types.

Thus it is now appropriate that with the current interest in the human adrenal gland in relation to the aetiology of breast cancer (Bulbrook, 1969), there should be a re-assessment of the various pathways for steroid biosynthesis. In this, possible differences between the various cell types should be recognized and earlier theories on the functional zonation of the adrenal cortex brought up to date in the light of recent findings.

Because of the earlier histological studies of Harley (1958), Arnold (1866) and Gottschau (1883) attention was drawn to the division of the adrenal cortex

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**Fig. 1.** Photomicrograph of a human adrenal gland, showing capsule (c), zona fasciculata (zf) zona reticularis (zr) and medulla (m). The zona glomerulosa is not visible in this section. Haematoxylin and eosin (x 60).
into three layers of cells, the zona glomerulosa, the zona fasciculata and the zona reticularis. It was later shown that, whereas these three ‘classical’ zones were present in the adrenals of many animals there were important differences between species (Symington, 1960). Although prominent in the adrenal glands of ruminants, the zona glomerulosa is ill-defined in the human adrenal cortex, and is confined to scattered islets of cells situated below the capsule. In view of the focal nature of this zone in the human adrenal, the cells of the adjacent zona fasciculata often lie next to the capsule. The zona fasciculata appears to constitute most of the cortex (Fig. 1) and is composed of columns of large cholesterol laden cells, which appear vacuolated after paraffin sectioning and staining procedures. They have been referred to as clear cells (Symington, 1960) because of this vacuolated nature of the cytoplasm.

The adjacent zona reticularis is located next to the central medulla and is composed of small non-vacuolated compact cells which are arranged in alveoli separated by prominent thin-walled sinuses (Symington, 1962). The compact cells contain little cholesterol, and electron microscopic examination of these cells has shown few lipid globules (Carr, 1961; Mackay, 1969a,b).

These three ‘classical’ zones are not characteristic of all species, and histological studies have shown that the adrenals of certain animals have no zona fasciculata and contain little lipid. The tissue in these glands, between the zona glomerulosa and the medulla, consists of small compact-type cells. Glands such as these, found in ruminants and certain hibernators (e.g. golden hamster) have been termed ‘nonfatty’ in contrast to the ‘fatty’ adrenal with its three zones seen for example in rat, guinea pig and dog as well as man. More recently the elegant work of Dobbie and Symington (1966) and Dobbie (1969) has shown that the infra-structure of the cortex, and its relation to the medulla and central vein, is more complex than our simple description would suggest.

II. FUNCTIONAL ZONATION OF THE ADRENAL CORTEX

Over the years, there have been a number of theories attempting to correlate the histological structure of the fatty adrenal gland with function, most of them based on histological and histochemical studies. Gottschau (1883) introduced a concept, later elaborated by Bennett (1940) of cell migration. It was suggested that cortical cells form in the subcapsular region, and migrate centripetally to the zona fasciculata, which was designated the ‘secretory zone’. The clear cell of the fascicular tissue therefore synthesized and secreted all the steroid hormones of the adrenal gland. Cells then degenerated in the zona reticularis, and were removed by the extensive vascular channels of this region of the cortex.

There was little experimental evidence for this, however, and it was superseded by the ‘zonation theory’, the stimulus for which was provided by Swann
(1940). From studies showing that atrophy of the fascicular and reticular tissue, but not the glomerular tissue, occurred after hypophysectomy, Swann suggested a division of the secretory function of the cortex between the zona glomerulosa on the one hand and the zona fasciculata and zona reticularis on the other, with an unequal dependence upon the pituitary. The theory was extended by the histochemical studies of Deane, Greep and Chester Jones and most of this work was extensively reviewed by Deane (1962). It was suggested that the zona glomerulosa was the site for synthesis and secretion of the mineralocorticoid, and it has now been well substantiated that aldosterone is formed in this zone (Ayres et al., 1958a,b; Giroud et al., 1958).

Biosynthetic pathways involved in aldosterone formation have recently been discussed in detail (Raman et al., 1965; Grant, 1968) and in view of this, it is considered unnecessary to make further comment at this stage. Deane and her colleagues considered the zona fasciculata to be the site of formation of the adrenocorticosteroids, and because of the hyperplasia of the zona reticularis observed in the cortex of patients with adrenogenital syndrome (Blackman, 1946), it was suggested that adrenal androgens, and probably estrogens were synthesized by the cells of this zone. Each cell-type had a particular role to play in the overall capacity of the adrenal cortex to synthesize and secrete the various adrenal steroid hormones.

However, Yoffey's histochemical investigations (1953, 1955) on the localization of cholesterol, and acid and alkaline phosphatases in the adrenal cortex of the rat renewed interest in the zona reticularis and led him to believe that this zone might be the active site for adrenocorticosteroid synthesis. He considered the zona fasciculata merely to be a storage area for cholesterol.

The importance of the compact cell of the human adrenal cortex in the synthesis of adrenocorticosteroids was further emphasized by the studies of Symington and his colleagues. It was seen (Symington et al., 1955) that the most significant histological change in the cortex after ACTH administration occurred at the border region between the zona fasciculata and zona reticularis. Here the clear cells adjacent to the reticular tissue became depleted of lipid and acquired the histological characteristics of compact cells. Grant et al. (1957b) also related this increase in the number of compact cells after ACTH administration to an increased activity of the 11β-hydroxylase enzyme system of the whole adrenal, and it appeared that the site of the 11β-hydroxylase, and therefore adrenocorticosteroid synthesis, was the compact cell.

Griffiths et al. (1963), using a micro Stadie-Riggs (1944) wet tissue microtome obtained reasonably homogeneous samples of fascicular and reticular tissue from the adrenal cortices removed from patients with breast cancer. Experimental evidence from the incubations of these preparations, provided proof that cells from both zones possess the 11β-hydroxylase enzyme system. In fact there was no significant difference in the ability of homogenates of fascicular and
reticular tissue to effect steroid 11β-hydroxylation. Furthermore, when tissue slices from either zone were incubated in Krebs-Ringer bicarbonate-glucose medium for two-hour incubation periods, first in the absence, then in the presence of ACTH, it was also shown that both types of cell possessed the capacity to synthesize and secrete cortisol and corticosterone (Fig. 2). At the same time, these investigations demonstrated that only the clear cell responds well to ACTH under these in vitro conditions. It appeared then that one of the effects of ACTH was probably directed towards the mobilization of the cholesterol ester stored in the clear cell. This is consistent with the observations of Symington et al. (1955), which were concerned with the clear cell at the fascicular-reticular border region, and its subsequent change to the compact cell type after prolonged ACTH stimulation. It was also concluded that the increased '11β-hydroxylase' activity, observed in homogenates of adrenal glands from ACTH-treated patients (Grant et al., 1957b) was attributable to these stimulated cells at the border region.

In these early studies (Griffiths, 1960; Griffiths et al., 1963) it was also observed that 11β-hydroxyandrostenedione was synthesized and secreted by the cells of both fascicular and reticular tissue. Furthermore 11β-hydroxyandrostenedione, together with androstenedione and dehydroepiandrosterone (DHA) were shown to be present in small amounts in human adrenal vein blood (Lombardo et al., 1959; Hirschmann et al., 1960; Short, 1960). The former was
generally considered to be the major C19-steroid constituent of the adrenal vein blood and its synthesis by both clear and compact cells was contrary to the earlier zonation theories which attributed adrenal androgen production solely to the zona reticularis.

The clear and compact cells therefore synthesize and secrete in vitro, both C21- and C19-steroids. The steroid biosynthetic capacity of these cells appeared similar, and in view of this, it was suggested (Griffiths et al., 1963) that the fascicular and reticular zones should be considered as a single functional unit, the histological appearance of the cell-type merely reflecting the degree of activity. Histological study of the compact cell suggests that with the exception of aldosterone, this cell is actively synthesizing and secreting adrenal steroids for 'everyday' metabolic purposes. In conditions of stress, the cholesterol in the clear cells adjacent to the zona reticularis supplies the precursor for the steroid required immediately. There is evidence (Borkowski et al., 1967) that approximately 80% of the steroid hormones leaving the adrenal are formed from precursors, presumably cholesterol or its esters, circulating in the plasma. The compact cell may well derive its precursor cholesterol from this source, but it is difficult to decide whether the clear cell is merely a storage site for cholesterol ester (Symington, 1962), or indeed synthesizes steroid hormone at a relatively lower rate as suggested by the in vitro secretion studies of Griffiths et al. (1963). Ofstad et al. (1961) reported that in a 47-year-old male who suffered from bronchiectasis, pulmonary fibrosis and renal amyloidosis, a post-mortem examination of the adrenal gland showed amyloid degeneration of the zona reticularis. Urinary steroid analysis in the final period of his life showed abnormally low C19-steroid excretion, but a normal level of 17-hydroxycorticosteroids. This suggests that the contribution of the zona fasciculata to cortisol synthesis was considerable. Indirectly, the results also implied that the C19-steroids were synthesized by the zona reticularis, although the observations were made before it was known that the human adrenal gland secretes substantial amounts of DHA sulphate (Baulieu, 1962; Wieland et al., 1963; Vande Wiele et al., 1963; Gurpide et al., 1963).

These investigations concerned with the functional zonation of the human adrenal cortex correlate well with some of the established facts derived from in vivo studies in man. The synthesis and secretion, principally of cortisol, but also of smaller amounts of corticosterone by the slices of fascicular and reticular tissue is consistent with the cortisol:corticosterone ratios found in human adrenal venous blood (Bush, 1953; Grant et al., 1957a).

There is an increased secretion of cortisol in the adrenal vein blood of patients undergoing adrenalectomy for breast cancer, into whom ACTH was infused during the operation (Grant et al., 1957a), which is compatible with the observed effect of ACTH in vitro.

Studies on the vasculature of the human adrenal cortex (Dobbie and
Symington, 1966; Dobbie, 1969) provide some of the most convincing evidence in support of this zonation theory. Further support comes from the use of an ultramicrochemical technique (Grunbaum et al., 1956) in which cylinders of tissue (2 mm diam.) are bored from frozen adrenals. Fresh-frozen microtome sections (16 μm) were cut from the bore, one section placed on a slide for histology, and the next two placed in glass stoppered reaction tubes (30 mm long, 4 mm int. diam.) for enzyme assay. This sequence is followed through the entire cortex and into the medulla of the gland (Fig. 3). Using this technique, Greenberg and Glick (1960) demonstrated increased glucose-6-phosphate dehydrogenase (G6PD) activity after ACTH administration in the fascicular-reticular region of the rat cortex. A similar increase of G6PD was observed in human adrenal tissue (Studzinski et al., 1962), although they used a less refined procedure. Griffiths and Glick (1966) also showed by use of the ultramicrochemical technique, that the 'profile' of 11β-hydroxylase distribution had a peak of activity at the fascicular-reticular border in adrenals from ACTH-treated rats (Fig. 4). This technique has not yet been applied to the study of 11β-hydroxylase activity in the human cortex.

Endogenous precursors in both the clear and compact cell are thus efficiently converted to cortisol, corticosterone and 11β-hydroxyandrostenedione in vitro.
To establish whether the biosynthetic pathways for the production of these compounds are similar in both types of cell is much more difficult. In their studies of steroid biosynthesis in 'adrenal homogenates, minces or slices', many investigators have shown an apparent lack of appreciation of the fact that they were dealing with mixed cell populations. Few histology reports have appeared with biosynthetic studies involving adrenal adenomas. However, the identity of particular enzyme systems in human adrenal tissue has been established by such investigations, and further consideration should now be given to the assessment of the activity of the various alternative biosynthetic pathways that exist.
III. STEROID BIOSYNTHETIC PATHWAYS

The ‘classical pathway’ for the synthesis of cortisol in the human adrenal cortex is that based on the ox adrenal perfusion studies of Pincus and his colleagues (Hechter and Pincus, 1954; Hechter, 1958). Cholesterol, considered to be the principal precursor of steroid hormones, is converted by a series of reactions to pregnenolone. Pregnenolone is then converted to progesterone in two stages, first by oxidation of the 3β-hydroxyl group to the 3-oxo group, involving the 3β-hydroxysteroid dehydrogenase enzyme system (3βOHSD), and secondly by isomerization of the Δ⁵-3-oxo- to the Δ⁴-3-oxo-structure, requiring an isomerase enzyme system (Fig. 5). Progesterone can be hydroxylated in a well established and definite series of reactions, 17α-, 21-, and 11β-hydroxylations to give rise to cortisol, but it has also been shown that hydroxylation at C-21 may not precede 11β-hydroxylation to yield corticosterone (Fig. 6). Cortisol is quantitatively the principal glucocorticoid synthesized by the human adrenal cortex, although smaller amounts of corticosterone are also formed. In some species, however, (mouse, rabbit and rat) corticosterone is the only glucocorticoid secreted by the adrenal. It was therefore generally accepted until the early 1960s, that in the scheme for the biosynthesis of the adrenocorticosteroids, progesterone represented the branching point in the formation of either the 17α-hydroxycorticosteroids or the 17-deoxycorticosteroids.
The C_{19}-steroids, androstenedione and 11β-hydroxyandrostenedione, were considered to be formed from 17α-hydroxyprogesterone (Fig. 6). Testosterone, and the estrogens if secreted by the human adrenal, would be synthesized from androstenedione.

However, evidence was soon available that progesterone does not represent the only branching point in adrenocorticosteroid synthesis, and that pregnenolone may be at least as important. The secretion of 3β,17α-dihydroxyprog-5-en-20-one (17α-hydroxypregnenolone) by the dog adrenal (Carstensen et al., 1959) indicated that pregnenolone could be hydroxylated at C-17 before conversion of the Δ^5-3β-hydroxyl group to the Δ^4-3-oxo structure. Cox (1960) had also suggested that the reaction sequence pregnenolone→17α-hydroxyprogrenolone→17α-hydroxyprogesterone could account for the small rise in urinary 5β-pregnane-3α,20β-diol excreted by some patients with adrenal hyperplasia compared to the amounts of 5β-pregnane-3α,17α,20α-triol, pregn-5-ene-3α,17α,20α-triol and DHA. Furthermore, it was soon demonstrated in human adrenal tissue, that pregnenolone was converted to 17α-hydroxypregnenolone (Lipsett and Hökfelt, 1961) and to DHA (Goldstein et al., 1960; Solomon et al., 1960). The studies of Lipsett and Hökfelt (1961), Mulrow et al. (1962), Weliky and Engel (1962; 1963) also established the conversion of 17α-hydroxypregnenolone to cortisol and suggested the possible importance of this alternative pathway in normal tissue. At the same time, the conversion of pregnenolone to DHA provided a further alternative pathway to androstenedione and other C_{19}-steroids (Fig. 7), the metabolism of pregnenolone, 17α-hydroxypregnenolone and DHA being
Fig. 7. Alternative pathways for steroid biosynthesis—role of 3βOHS-isomerase enzyme systems.
effected to some extent by the activity of the corresponding 3βOHSD enzyme systems.

IV. 3βOHSD—ISOMERASE ENZYME SYSTEMS

With regard to the functional zonation theory, on a number of occasions it was shown by means of histochemical techniques that the enzyme 3βOHSD was absent or had little activity in the zona reticularis (Wattenberg, 1958; Cavallero and Chiappino, 1961; Dawson et al., 1961). Such findings are inconsistent with the observation that steroid hormones are synthesized from endogenous precursors by slices of reticular tissue (Griffiths et al., 1963), and further biochemical investigations using separated fascicular and reticular slices of equine (Cameron et al., 1964; Cameron and Grant, 1967) and of human (Cameron et al., 1968) adrenal tissue have provided evidence that both zones do indeed possess substantial 3βOHSD activity. It was observed, however, that the activity of the enzyme was somewhat higher in the zona fasciculata, although it remains doubtful whether the difference should produce such a marked effect when studied histochemically. Having accepted that such histochemical evidence may be of use for the delineation of areas of a tissue section with high concentration of enzyme, Baille et al. (1965) studied various Δ⁵-3β-hydroxysteroids to test the value of the reaction in assessing the possible metabolism of a substrate by the tissue. In human, monkey and rat adrenal sections, identical results were obtained—heavy di-formazan deposition in the zona fasciculata with little reaction in the zona reticularis with DHA, DHA acetate, pregnenolone and its acetate and sulphate, 17α-hydroxypregnenolone, 17α-hydroxy-pregnenolone sulphate, 16α-hydroxy-pregnenolone and androst-5-ene-3β,17β-diol. The interesting finding was that DHA sulphate, recently shown to be a major secretory product of the human adrenal cortex (Baulieu, 1962; Gurpide et al., 1963) gave no reaction. This might suggest that once formed, DHA sulphate is not further metabolized to any major extent by human adrenal tissue and this question will be considered later in greater detail.

In recent years there have been various reports on the Δ⁵-Δ⁴-steroid isomerases which catalyse the conversion of Δ⁵-3-oxosteroids, the products of the action of 3βOHSD (Figs 5 and 7) to Δ⁵-3-oxosteroids.

As yet, there has been no definitive work on the isomerase enzyme(s) of human adrenal tissue, although some of the findings from the investigation of other animal adrenal tissue (Ewald et al., 1964a,b; Krüskemper et al., 1964; Cheatum et al., 1967; Neville and Engel, 1968a,b) and with Pseudomonas testosteroni preparations (Neville and Engel, 1968c) may have important implications for the human tissue. Initially, it seemed probable that bovine adrenal cell preparations were capable of the isomerization of C₂₃- and
C₁₉-Δ⁵-3-oxosteroids and separate enzymes controlled the reactions. There was, however, a lack of specificity of these enzymes and both types of steroid appeared to be substrates for the same enzyme (Ewald et al., 1964a; Kruskemper et al., 1964). Later, Neville and Engel (1968a,b) examined various isomerase preparations with respect to competition for binding sites, and determined maximum reaction velocities for conversion of C₂₁- and C₁₉-steroid substrates in the same incubation. The results appeared to support the concept of a single steroid-isomerase, although the authors agreed that their preparations, in common with those of other workers, were neither truly soluble nor homogeneous, and that kinetic methods involving particulate systems might not distinguish between a single enzyme with low substrate specificity and multiple enzymes each reacting with a single substrate. It remains to be seen whether similar results are obtained with human tissue, and if so whether the rates of conversion of such steroids as pregnenolone to progesterone and 17α-hydroxy-pregnenolone to 17α-hydroxyprogesterone are related to the control of these alternative pathways. There is, however, some evidence for possible substrate specificity of the 3βOHSD-isomerase enzyme systems from incubation experiments with human hyperplastic and neoplastic adrenal tissue (Weliky and Engel, 1963; Webb et al., 1969). Also of interest with regard to the 3βOHSD-isomerase enzyme system is that, whereas it was once considered irreversible, recent studies of Ward and Engel (1964, 1966a,b) using a sheep adrenal microsomal preparation, showed the reverse reaction may occur under certain experimental conditions (Fig. 8).

**V. CORTISOL SYNTHESIS IN THE HUMAN ADRENAL GLAND**

In the ‘classical’ literature therefore, adrenal steroid synthesis has been considered to follow the pathway shown in Fig. 9, with cortisol synthesized primarily by the route involving progesterone, and the pathway via 17α-hydroxy-pregnenolone, referred to as the ‘alternative pathway’, remaining of unknown
significance. The problem of the relative importance of the two pathways was difficult to assess.

Lipsett and Hökfelt (1961) had established that 17α-hydroxypregnenolone was converted to cortisol by human adrenal slices and it is of interest to note that they found little evidence for its conversion to DHA. Weliky and Engel (1962) then compared the metabolism of [7α-3H]17α-hydroxypregnenolone and [4-14C]progesterone by slices of a human adrenal tumour removed from a six-year-old girl with Cushing's syndrome, and confirmed that both substrates were readily converted to cortisol. Unfortunately, equimolar amounts of

![Diagram of alternative pathways for cortisol synthesis from pregnenolone.]

substrate were not used in the experiment, 5.46 μg [7α-3H]17α-hydroxypregnenolone and 35.6 μg [4-14C]progesterone being incubated. In terms of radioactivity 17α-hydroxypregnenolone was efficiently converted to cortisol (63%), whereas the conversion of progesterone was considerably less (17%). When the calculation is however based upon mass, the ratio of conversion of 17α-hydroxy-pregnenolone and progesterone to cortisol is in fact 1:1.76, emphasizing the difficulties involved in setting up such experiments, and the care required with the interpretation of results (Cameron, 1966; Kumari and Goldzieher, 1966). These experiments also provided no information with regard to the transformation of pregnenolone to either 17α-hydroxy-pregnenolone or progesterone.

A further experiment which compared the ability of slices of hyperplastic adrenal tissue to metabolize 2.2 μg [7α-3H]pregnenolone and 37 μg [4-14C]-progesterone (Weliky and Engel, 1963) showed that progesterone was converted to the 17-deoxycorticosteroids, DOC, corticosterone and 16α-hydroxyprogester-
terone as well as to the 17-hydroxycorticosteroids, 17α-hydroxyprogesterone, 11-deoxycortisol and cortisol (Fig. 9). Pregnenolone, on the other hand, was not converted to progesterone or to any of the 17-deoxycorticosteroids investigated. Thus although 17α-hydroxypregnenolone was converted to 17α-hydroxyprogesterone, a reaction involving a 3βOHSD-isomerase enzyme system, the corresponding conversion of pregnenolone to progesterone did not occur (Fig. 7). This therefore suggested the presence of a substrate specific 3βOHSD and/or Δ⁵-Δ⁴-isomerase in this particular human tissue.

Ward and Grant (1963) also incubated [4-¹⁴C]progesterone with slices of hyperplastic adrenal tissue and with tissue from a ‘clear cell’ adenoma. Various labelled metabolites were isolated including corticosterone, but there was an unexplained failure to isolate 11-deoxycortisol or cortisol despite the formation of 17α-hydroxyprogesterone.

### Table 1. Percentage radioactivity associated with steroids isolated from the simultaneous incubation of [7α-³H]pregnenolone and [4-¹⁴C]progesterone with separated ‘clear’ and ‘compact’ cell tissue from a human adrenal gland.

<table>
<thead>
<tr>
<th>Steroid isolated</th>
<th>‘Clear’ Cells</th>
<th>‘Compact’ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%³H</td>
<td>%¹⁴C</td>
</tr>
<tr>
<td>DOC</td>
<td>0.18</td>
<td>10.20</td>
</tr>
<tr>
<td>16α-OH-progesterone</td>
<td>0.20</td>
<td>1.94</td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>0.79</td>
<td>1.95</td>
</tr>
<tr>
<td>cortisol</td>
<td>1.04</td>
<td>2.14</td>
</tr>
</tbody>
</table>

The precise role of progesterone in the biosynthesis of cortisol was equivocal and studies in this Institute have been directed towards a means of assessing the relative activity of the alternative pathways. In a preliminary series of experiments, Cameron et al. (1968) incubated [7α-³H]pregnenolone and [4-¹⁴C]progesterone with minced preparations of separated fascicular and reticular tissue of an adrenal from a patient with breast cancer. In this incubation 92.4 nmoles [7α-³H]pregnenolone and 23.1 nmoles each of [4-¹⁴C]progesterone and unlabelled 17α-hydroxypregnenolone were added, and the tissue and steroids shaken in Krebs-Ringer bicarbonate-glucose medium without cofactors. This therefore avoided creating an initial ‘imbalance’ in the two major pathways to cortisol from pregnenolone. DOC, 16α-hydroxyprogesterone, 17α-hydroxyprogesterone and cortisol were isolated as ‘representatives’ of 17-deoxy- and 17-hydroxycorticosteroids. Results are shown in Table 1. The ³H:¹⁴C ratio was considerably higher in the 17-hydroxycorticosteroids, especially when fascicular tissue was incubated indicating an active alternative pathway independent of
progesterone in these cells. At the same time a minced preparation of mixed fascicular-reticular tissue from a hyperplastic adrenal was incubated in a similar manner, to try to establish a method for the semi-quantitative assessment of the role of progesterone in cortisol synthesis. Conversion figures are given in Table 2.

**Table 2.** Conversions of [7α-3H]pregnenolone and [4-14C]progesterone by cells from a hyperplastic adrenal gland.

<table>
<thead>
<tr>
<th>Steroid isolated</th>
<th>%3H</th>
<th>%14C</th>
<th>3H/14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pregnenolone</td>
<td>1.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-OH-pregnenolone</td>
<td>7.34</td>
<td>2.54</td>
<td>0.689</td>
</tr>
<tr>
<td>progesterone</td>
<td>1.75</td>
<td>2.74</td>
<td>0.285</td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>8.66</td>
<td>2.30</td>
<td>3.765</td>
</tr>
<tr>
<td>16α-OH-progesterone</td>
<td>0.78</td>
<td>0.68</td>
<td>1.06</td>
</tr>
<tr>
<td>DOC</td>
<td>4.41</td>
<td>25.04</td>
<td>0.176</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>3.92</td>
<td>24.58</td>
<td>0.159</td>
</tr>
<tr>
<td>11-deoxycortisol</td>
<td>15.68</td>
<td>7.27</td>
<td>2.157</td>
</tr>
<tr>
<td>cortisol</td>
<td>12.29</td>
<td>5.95</td>
<td>2.066</td>
</tr>
<tr>
<td>cortisone</td>
<td>0.84</td>
<td>0.50</td>
<td>1.680</td>
</tr>
</tbody>
</table>

Three main points should be noted:

(i) The relatively low conversions of pregnenolone to progesterone and its metabolites 16α-hydroxyprogesterone, DOC and corticosterone contrasting with the corresponding conversions to the 17α-hydroxycorticosteroids, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol and cortisol.

(ii) In contrast to the metabolism of pregnenolone, the progesterone was mainly converted to the 17-deoxycorticosteroids, DOC and corticosterone with less transformation to cortisol and its intermediates.

(iii) The distinctly higher 3H:14C ratios of the 17α-hydroxycorticosteroids compared with the 17-deoxycorticosteroids again showed that the role of progesterone could be a relatively minor one.

A concept for the determination of the relative importance of alternative metabolic pathways (Kopin, 1963) suggested a possible means of approach to the problem of assessing the relative contribution of the two principal metabolic routes to cortisol. One of Kopin’s systems is shown in Fig. 10.

The similarity between Fig. 10, and Fig. 9, showing the alternative pathways is apparent. A[14C] would represent pregnenolone, B1 and B2 [3H] 17α-hydroxypregnenolone and progesterone respectively, and C1, C2 and C3 DHA, 17α-hydroxyprogesterone and DOC. Since these last three compounds are not end-products, however, the analogy breaks down. We therefore adapted the
mathematics to fit the circumstances and Fig. 11 shows how this was done. Thus, \((C_1 + \Sigma C_1)\), \((C_2 + \Sigma C_2)\) and \((C_3 + \Sigma C_3)\) are the isotope sinks at the three outlets from the alternative routes from A. Since the analogy between the situations pertaining to the ideal state and the actually observed state are not identical, as they can never be in a biological system, use of the formula in Fig. 11

\[
f_{B_2C_2} = \frac{[^{14}C]_{C_2}}{[^{3}H]_{C_1}}
\]

**Fig. 10.** Convergent metabolic pathway (Kopin, 1963). The equation gives the fraction of \(C_2\) derived from \(B_2\) provided \(C_1\), \(C_2\) and \(C_3\) are end-products and \(A\), \(B_1\) and \(B_2\) are completely metabolized.

\[
f_{B_2(\Sigma C_1 + \Sigma C_2 + \Sigma C_3)} = \frac{[^{14}C]_{\Sigma C_3}}{[^{3}H]_{C_3 + \Sigma C_3}}
\]

**Fig. 11.** Convergent metabolic pathway adapted for the situation in which \(C_1\), \(C_2\) and \(C_3\) are not end-products. The equation gives the fraction of \((C_2 + \Sigma C_2)\) derived from \(B_2\) provided that \(A\), \(B_1\) and \(B_2\) are completely metabolized.

must give an approximation only. When the appropriate values for 17α-hydroxyprogesterone + 11-deoxycortisol + cortisol + cortisone \((C_2 + \Sigma C_2)\) and for DOC + corticosterone \((C_3 + \Sigma C_3)\) were substituted in the equation, it was calculated that approximately 7% of the 17-hydroxycorticosteroids was formed from pregnenolone via progesterone in the incubation of mixed cell hyperplastic adrenal tissue referred to earlier. In this tissue, therefore, the role of progesterone in cortisol synthesis is but a minor one, and one can infer that the
route involving 17α-hydroxyprogrenolone may be the major pathway to the hormone.

It is well recognized, therefore, that much useful information is provided by in vitro incubation studies; and the care required in the interpretation of the results from such studies; especially with mixed cell preparations of adrenal tissue, is becoming apparent. However, to obtain sufficient normal human adrenal tissue of one-cell type for an extensive study of alternative pathways by the procedure described is difficult. Use has to be made of tumours such as the 'clear' cell adenoma consisting of cells resembling histologically the clear cell of the normal zona fasciculata. It is reasoned that tissue so similar in appearance to the normal cells might also show similar biochemical properties. Recently, such a

Table 3. Assessment of the relative contribution of the pathway, pregnenolone → progesterone → 17α-hydroxyprogesterone to the biosynthesis of cortisol in clear cell adenoma tissue in vitro.

\[\left(\frac{C_2 + \Sigma C_2}{C_3 + \Sigma C_3}\right) - \text{sum of percentage conversions to 17α-hydroxyprogesterone + 11-deoxycorticisol + cortisol + cortisone} ; (C_3 + \Sigma C_3) - \text{sum of percentage conversions to 11-deoxycorticosterone + corticosterone} ; f_{B2C2} - \text{fraction of (C_2 + \Sigma C_2) derived from pregnenolone via progesterone.}\]

<table>
<thead>
<tr>
<th>Incub. No.</th>
<th>C_2 + \Sigma C_2^{14}C</th>
<th>C_2 + \Sigma C_2^{3}H</th>
<th>C_3 + \Sigma C_3^{14}C</th>
<th>C_3 + \Sigma C_3^{3}H</th>
<th>f_{B2C2} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>11.5</td>
<td>8.6</td>
<td>1.7</td>
<td>39.1</td>
<td>3.2</td>
</tr>
<tr>
<td>2.</td>
<td>20.9</td>
<td>11.1</td>
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<td>3.</td>
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<td>4.</td>
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<td>5.</td>
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clear cell tumour weighing 67 g was used (a) to perform a series of time-based studies to observe the variation of conversion figures with time and (b) to assess the relative activity of the alternative metabolic pathways of the pure 'clear' cell of the adenoma using the equation described (Cameron and Griffiths, 1968).

The substrates incubated were 83.5 nmole [4-{14}C]pregnenolone, and 4.8 nmole of both [7α-{3}H]progesterone and unlabelled 17α-hydroxyprogrenolone. Steroids were incubated with 2 g portions of minced tissue in Krebs-Ringer bicarbonate-glucose without cofactors for various timed intervals. To accommodate the criteria required by Kopin's formula, it was necessary to regard the labelled progesterone as a tracer intermediate rather than simply a substrate and therefore to add a minimum mass of this compound. The unlabelled 17α-hydroxyprogrenolone again avoids the initial 'imbalance' in the two pathways. Table 3 illustrates the application of the Kopin procedure to the results of the
The percentage conversion of $[4-^{14}C]$pregnenolone and $[7α-^{3}H]$progesterone to $(C_2 + \Sigma C_2)$ and $(C_3 + \Sigma C_3)$ and the values of $f_{B_2}(C_2 + \Sigma C_2)$ derived therefrom are also shown. The figures for incubations 1-3 are a first approximation since the criteria for the validity of the formula cannot be strictly met in any in vitro system. Nevertheless, even as an assessment of the relative activities of the pathways, it is apparent that less than 10% of the 17-hydroxylated steroids were formed via progesterone. The doubts expressed by previous workers (Eichhorn and Hechter, 1957; Berliner et al., 1958; Mulrow and Cohn, 1961; Ward and Grant, 1963) that progesterone is much concerned in the synthesis of cortisol would appear to be substantiated. In a graphical representation, Fig. 12 shows the dramatic increase in the quantity of $^{14}C$ found
Fig. 13. Conversions of [7α-3H]progesterone by tissue from a clear cell adenoma to cortisol (F), corticosterone (B) 11-deoxycorticosterone (DOC) and cortisone (E) as functions of time.
Fig. 14. Conversions of [7α-3H]progesterone (Prog.) by tissue from a clear cell adenoma to 16α-hydroxyprogesterone (16-P), 11-deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17-P) as functions of time.
in cortisol contrasting with that in corticosterone. DOC was labelled rapidly, but equally rapidly lost the label in the formation of products. Fig. 13 shows the transformation of \([7\alpha-^3\text{H}]\) progesterone to the corticosteroids. Here the opposite picture from the previous one is seen—rapid labelling of DOC which was then transformed in substantial quantity to corticosterone. Cortisol labelling was relatively much less. Figure 14 shows the labelling of intermediates from progesterone. Note that the peak for 17α-hydroxyprogesterone preceded that of 11-deoxycortisol. Contrast this picture of the intermediates of pregnenolone, however, (Fig. 15) and note that the peak for 11-deoxycortisol preceded that of 17α-hydroxyprogesterone. This unexpected finding could be explained if the \(^{14}\text{C}\) from pregnenolone was able to ‘by-pass’ 17α-hydroxyprogesterone on its way to 11-deoxycortisol.
The proposed intermediate, 17α,21-dihydroxypregnenolone has been shown (Pasqualini et al., 1964) to be transformed not only to 11-deoxycortisol and cortisol, but also to DHA. The amount of 17α,21-dihydroxypregnenolone formed by human adrenal tissue has yet to be established, but the evidence already obtained suggests that yet another pathway exists to the quantitatively most important adrenal steroids cortisol and DHA sulphate. Furthermore, a second series of time-based incubations (Cameron et al., 1969) was performed on tissue from an adenoma which contained a mixture of ‘clear’ and ‘compact’ cell types, and the results were again plotted graphically. The substrates used were [4-14C]pregnenolone and [7α-3H]17α-hydroxypregnenolone. Figure 16 shows the rapid labelling of cortisol from pregnenolone, the poor conversion to corticosterone, and again the 11-deoxycortisol peak preceding that of 17α-hydroxyprogesterone. A similar picture with regard to cortisol formation and the reversal of the peak labelling time of 17α-hydroxyprogesterone and

![Figure 16](image-url)
11-deoxycortisol is seen in Fig. 17. However, a recent experiment (Cameron, 1969) in which mixed adrenal tissue from a patient with breast cancer, and also hyperplastic adrenal tissue were incubated with [7α-3H] 17α-hydroxyprogrenenolone and [4,14C] progesterone in a series of timed incubations failed to find labelled 17α,21-dihydroxyprogrenolone in any significant quantity, although cortisol formation was efficient. Further studies on these branching points would therefore be of interest.

VI. C19-STEROID SYNTHESIS

Figure 18 is concerned primarily with the conversion of [4,14C] pregnenolone to C19-steroids by this mixed cell adenoma. Of interest is the sequential
labelling of DHA, androstenedione and 11β-hydroxyandrostenedione, and of DHA and DHA sulphate. Similar sequences can also be observed in Fig. 19 for the conversion of the [7α-3H]17α-hydroxypregnenolone to the C19-steroids. The results would suggest that DHA once formed, is metabolized either to DHA

![Graph](image)

**Fig. 18.** Conversions of [4-14C]pregnenolone (Preg.) by tissue from a mixed cell adenoma to 11β-hydroxyandrostenedione (11-A), DHA sulphate (DS), androstenedione (A), 17α-hydroxypregnenolone (17-Preg.) and DHA (D) as functions of time.

sulphate, or to androstenedione and thence to 11β-hydroxyandrostenedione (Fig. 20).

DHA sulphate was generally considered to be an end product of the metabolism of DHA, and excreted in the urine. The observation that it was secreted by the human adrenal cortex (Baulieu, 1962; Vande Wiele et al., 1963; Wieland et al., 1963) was followed by the production rate studies of Gurpide et al. (1963), establishing that the sulphate was in fact a major secretory product.
The ability of human adrenal tissue to convert DHA to its sulphate in vitro was demonstrated by various workers (Cohn et al., 1963; Wallace and Lieberman, 1963; Adams, 1964), but the significance of this finding was in doubt after experiments of Lieberman and his colleagues (Roberts et al., 1964) who demonstrated that when [7α-3H]cholesterol-[35S]sulphate was injected into the blood supply of a massive adrenal carcinoma, 20% of the radioactivity recovered in the urine was associated with DHA sulphate, the major metabolite, bearing 3H and 35S-labels in the same ratio as the injected precursor. The intermediate steps (Fig. 21) in such a synthesis have also been investigated by the same group and the in vivo conversion of injected [7α-3H]pregnenolone-[35S]sulphate to [7α-3H]DHA-[35S]sulphate (Calvin et al., 1963) and in vitro conversion of [7α-3H]pregnenolone-[35S]sulphate to [7α-3H]17α-hydroxy-

![Graph](image-url)
pregnenolone-[35S] sulphate (Calvin and Lieberman, 1964) have been demonstrated. In all these experiments, yields of labelled DHA sulphate were low and interpretation difficult (Calvin and Lieberman, 1966), and the relative importance of these pathways has yet to be assessed by rigorous kinetic experiments using well defined adrenal tissue of pure cell type.

In further experiments with the ‘clear’ cell adrenal tumour described earlier, the metabolism of [4-14C]pregnenolone and [7α-3H]pregnenolone sulphate was studied (Griffiths et al., 1968). The conversion figures from pregnenolone and pregnenolone sulphate to cortisol were 37.7% and 9.5% respectively, and to DHA sulphate 0.09% and 0.08%. The conversion to the sulphate was low, and the significance of the pregnenolone sulphate—DHA sulphate pathway was questioned. However, with the ‘mixed’ cell adenoma (Cameron, et al., 1969) the corresponding figure for [4-14C]pregnenolone conversion to cortisol was 42.7% and to DHA sulphate 5.3%. It can be seen that the cortisol formation was of the same order, but that DHA sulphate synthesis was substantially increased implying that there may be some relation between the presence of ‘compact’ type cells and DHA sulphate formation. Further application of the ultramicrochemical technique to the guinea pig (Jones and Griffiths, 1968) and human (Cameron et al., 1969) adrenal cortices indicated that the DHA sulphating enzyme systems appeared to be associated with the compact cell. The quantitative histological distribution of the enzyme system in the cortex is shown in Figs 22 and 23. The histology of the tissue studied is therefore of particular importance. Conclusions from experiments such as those of Lebeau et al. (1964) stating that the principal route for DHA sulphate synthesis involves DHA in ‘human adrenal tumour slices’ are difficult to assess. The observation that synthesis of DHA sulphate may be confined to the compact cell is of course in agreement with the earlier work of Ofstad et al. (1961), who studied a patient with an adrenal gland containing degenerate zona reticularis referred to earlier. Furthermore, organ culture studies of fascicular and reticular tissue obtained from a patient with breast cancer have provided further evidence that DHA

![Fig. 20. Synthesis and metabolism of DHA.](image-url)
Fig. 21. Synthesis of steroid sulphates from cholesterol.
Fig. 22. The quantitative histochemical distribution of a DHA sulphating enzyme system based on tissue protein nitrogen (Pn) throughout the guinea pig adrenal cortex. Vertical bars represent ranges of activity determined in various regions in the cortex (4 expts.) Regions marked G, F, R and M denote respectively zona glomerulosa, zona fasciculata, zona reticularis and medulla; mixed zones are denoted by both letters.

Fig. 23. The quantitative histochemical distribution of a DHA sulphating enzyme system based on protein-nitrogen (Pn) throughout the human adrenal cortex. Regions marked G, F, R and M denote the zones of the gland as described in Fig. 22.
sulphate formation is the concern of the compact cell of the zona reticularis (Jones et al., 1969). It would appear that the main route for the formation of DHA sulphate in the human adrenal is probably via 17α-hydroxyprogrenolone and DHA (Cameron et al., 1969).

The production of androgens or their precursors by the human adrenal gland has assumed major significance in the study of hormones in relation to breast cancer (Bulbrook, 1969), and it would seem that further investigations into the control of DHA sulphate synthesis and secretion would be of particular value. Results from studies on the synthesis of androstenedione, 11β-hydroxyandrostenedione and testosterone are still equivocal. The experiments with the mixed cell adenoma (Figs 18 and 19) would suggest the C19-steroid biosynthesis occurs via pregnenolone-17α-hydroxyprogrenolone-DHA-androstenedione-11β-hydroxyandrostenedione (Fig. 24), which would be in accord with the conclusion of Cohn and Mulrow (1963) who investigated various normal, hyperplastic and neoplastic adrenal tissues. They concluded that androstenedione was synthesized by way of DHA rather than from progesterone via 17α-hydroxyprogesterone, although further experiments on the sequence of reactions 17α-hydroxyprogrenolone-17α-hydroxyprogesterone-androstenedione were necessary. Such an experiment (Cohn, 1965) with an ‘adenoma’ from a 35-year-old female with Cushing’s syndrome, incubating [7α-3H]17α-hydroxyprogrenolone and [4-14C]17α-hydroxyprogesterone indicated that in this tissue, 17α-hydroxyprogesterone was an important intermediate in the biosynthesis of androstenedione from 17α-hydroxyprogrenolone.

In an experiment in which [4-14C]DHA and [7α-3H]17α-hydroxyprogrenolone were incubated with minced tissue from the ‘mixed’ cell adenoma, however (Cameron et al., 1969), DHA sulphate (19.8%), androstenedione (9.1%), 11β-hydroxyandrostenedione (22.7%) and testosterone (0.06%) were formed from DHA. Only 1.79% of the DHA remained at the end of the two hour incubation. The $^3$H:14C ratios of the metabolites suggested that little
STEROID BIOSYNTHETIC PATHWAYS

'leakage' of the $^3$H-label into adrenal C$_{19}$-steroids occurred through 17α-hydroxyprogesterone. Further complexity arises from studies of Ward and Grant (1963) in which slices from a 'clear' cell adenoma were incubated with [4-$^{14}$C]progesterone. In these experiments, 30.7% of the progesterone was converted to 11β-hydroxyandrostenedione, although in a similar incubation with slices of mixed fascicular-reticular tissue from hyperplastic adrenals approximately 4.3% was converted. 17α-Hydroxyprogesterone was formed in both incubations, but no labelled 11-deoxycortisol or cortisol was detected. It is obvious that the pathways by which C$_{19}$-steroids are synthesized in the normal clear and compact cell are still not well understood. Indeed, Axelrod and Goldzieher (1968) recently incubated a brei of normal adrenal gland removed quickly from a young male killed accidentally, and showed conversion of [4-$^{14}$C]pregnenolone to progesterone (51.2%), corticosterone (11.9%), cortisol (10.3%) and androstenedione (2.25%). No 11β-hydroxyandrostenedione, DHA or testosterone were formed. The biosynthetic pattern differed therefore from previous reports of adrenal tissue studies, and further emphasizes the care required in the interpretation of all in vitro experiments.

Evidence for testosterone secretion by the normal human adrenal cortex has until recently been indirect. It was established that testosterone could be synthesized from progesterone by homogenates of adrenals from patients with breast cancer (Ichii et al., 1962; Kase and Kowal, 1962). Ward and Grant (1963) described the conversion of progesterone to testosterone by a 'clear' cell adrenal adenoma removed from a patient with Cushing's syndrome, but they were unable to provide definitive evidence that the compact cell formed testosterone. Later studies (Griffiths et al., 1968) showed the conversion of pregnenolone to testosterone and testosterone sulphate by minced tissue from a 'clear' cell adenoma, but in low yields. Dixon et al. (1965) have also demonstrated the formation of testosterone sulphate from progesterone in vitro by virilizing adrenal tumour tissue, but no reference to cell type was given. In spite of the fact that the adrenal has now been shown to secrete testosterone into the bloodstream (Baird et al., 1969), the possibility should not be ignored that the 'androgenic capacity' of the gland may be related to extra-adrenal metabolism of testosterone precursors, DHA sulphate, DHA or androstenedione.

Further work from this laboratory, in which [7α-$^3$H]DHA and [4-$^{14}$C]17α-hydroxyprogesterone were incubated with minced adrenal tissue, failed to show significant $^{14}$C-labelling of androstenedione and testosterone. DHA was converted to these compounds, and also to epi-testosterone, probably neither considered in most of the earlier incubation studies, nor completely separated from the isolated testosterone. An increase in epi-testosterone excretion in the urine after ACTH administration to castrated males has been demonstrated (Tamm et al., 1966), and its formation consistent with the observation of Wilson et al. (1964) that at least part of the epi-testosterone is
formed in the adrenal. It is of interest that Blaquier et al. (1967) described the formation of epi-testosterone from testosterone but not from DHA or androstenedione by human adrenal minces.

VII. ADRENAL ESTROGENS

Direct evidence for estrone and estradiol-17β secretion by the human adrenal has again, only recently been obtained (Baird et al., 1969). In the relationship between the adrenal cortex and the development of breast cancer, there had always remained the suggestion that the cortex might be responsible for estrogen formation, although previous evidence for this had been mainly indirect (Goldzieher and Boyd, 1967). It consisted principally of estrogen determinations in urine of gonadectomized subjects (Dao, 1953; Diczfalussy et al., 1959), some after ACTH stimulation although it is probable that some of the estrogen in the urine of such patients originated from the peripheral metabolism of C₁₉-steroids secreted by the adrenal (West et al., 1956; Baulieu and Dray, 1963; Ahmad and Morse, 1965). Evidence for the biosynthesis of estrogen from testosterone by \textit{in vitro} experiments with adrenal tissue has been obtained from studies with feminizing adrenocortical carcinoma tissue (Baggett et al., 1959) with the formation of estrone (0.5%) and estradiol-17β (0.3%). Since 19-hydroxylation of either androstenedione or possibly testosterone is considered an obligatory step before removal of the angular C₁₉-methyl group, Engel and Dimoline (1963) incubated human adrenal tissue with 190H-androstenedione but failed to obtain convincing evidence of aromatization. However, some evidence has recently been obtained for the \textit{in vitro} conversion of such C₁₉-steroids as DHA sulphate, androstenedione and testosterone to estrogen in adrenal tissue from patients with breast cancer, although the activity of the enzyme system is extremely low (Cameron et al., 1969). The observation that little radioactivity was isolated in the estrogen conjugates precluded the possibility that earlier failure to demonstrate estrogen synthesis had been due to sulphate formation.

VIII. METABOLISM OF CHOLESTEROL

A feature of most of the work relating to steroid hormone biosynthesis concerns the key role of pregnenolone as an obligatory intermediate. However, in considering the biosynthesis of the C₁₉-steroids, it is necessary to take the possibility of other pathways into account—pathways which do not include pregnenolone as such an intermediate. Dorfman and Ungar (1965) have suggested a pathway for C₁₉-steroid synthesis, cholesterol—17α,20α-dihydroxycholesterol—DHA, (Fig. 25) thus by-passing pregnenolone. Gual et al. (1962)
compared the conversion of \([4-^{14}C]\)cholesterol and \([7\alpha-^{3}H]\)pregnenolone to DHA by an adrenal adenoma. The yield from cholesterol was almost three times higher than that from the pregnenolone. They also isolated an unidentified compound which contained \(^3\)H but no \(^{14}\)C. Thus it was argued that \([4-^{14}C]\)cholesterol was not converted to pregnenolone, and this provided additional evidence that the \([^{14}C]\)DHA must have been formed by a route independent of pregnenolone. As will be seen later, this may or may not be the case if the question of the metabolism of exogenous and endogenous precursors is taken into consideration. Jungmann (1968), working with rat ovarian, testicular and adrenal homogenates fortified with NADPH, has shown quite conclusively that a pathway from cholesterol to DHA independent of pregnenolone does exist by the isolation of the predicted fragment \([^{14}C]\)2-methylheptan-6-one from incubations with \([26-^{14}C]\)cholesterol. \([7\alpha-^{3}H]\)Cholesterol was incubated with similar preparations and \([^{3}H]\)DHA and \([^{3}H]\)pregnenolone were isolated in every case. Again, from the \([26-^{14}C]\)cholesterol incubations he was able to isolate the six-carbon fragment \([^{14}C]\)isocaproic acid found previously (Staple et al., 1956; Ichii et al., 1963; Shimizu, 1966). Others (Constantopoulos et al., 1966) believe the six-carbon fragment to be isocaproaldehyde. This demonstrated that the two separate routes to the steroid hormones were in operation simultaneously under his experimental conditions. In similar experiments Shimizu (1964) was unable to detect \(C_{19}\)-steroid formation with homogenates of normal human adrenal or with rat adrenal or testes to which had been added the co-factors NAD\(^+\) and NADP\(^+\). Shimizu (1966) was, however, able to demonstrate similar conversions of \(20\alpha\)-hydroxycholesterol and \(17\alpha, 20\alpha\)-dihydroxycholesterol to DHA and cortisol by slices of normal adrenal tissue. Probably the whole cell preparations were better able to maintain a useful concentration of reduced co-factors than were the homogenates, and it is known that NADPH is essential for the biosynthesis of isocaproic acid from cholesterol (Ichii et al., 1965). Jungmann (1968) points out that his studies do not permit a comparison of the relative importance of two routes to the \(C_{19}\)-steroids, but

\[
\text{Cholesterol} \quad \rightarrow \quad 20\alpha\text{OH-Cholesterol} \quad \rightarrow \quad 17\alpha, 20\alpha\text{OH-Cholesterol} \quad \rightarrow \quad \text{DHA}
\]

\[
\text{20\alpha, 22OH-Cholesterol} \quad \rightarrow \quad \text{Pregnenolone} \quad \rightarrow \quad 17\alpha\text{OH-Pregnenolone}
\]

Fig. 25. Pathways showing routes from cholesterol to DHA sulphate.
suggests that under 'normal' conditions the main pathway may well be C\textsubscript{27}-sterol–C\textsubscript{21}-steroid–C\textsubscript{19}-steroid, that the alternative direct route does exist and may increase in importance under maximal stimulation or in pathologically damaged tissue.

Stone and Hechter (1954) demonstrated that the rate limiting step in corticosteroid synthesis was the degradation of the cholesterol side-chain and that this step was controlled by ACTH. However, it was some time before the intermediate steps by which cholesterol is transformed to pregnenolone were elucidated. The work of Solomon \textit{et al.} (1956) and Shimizu \textit{et al.} (1962) has suggested that cholesterol undergoes sequential hydroxylation at the 20α- and 22-positions before cleavage occurs between C-20 and C-22 to give pregnenolone and isocaproic acid (Ichit \textit{et al.}, 1963). The situation with respect to side-chain cleavage is still rather confused. Much work has been performed on rat or bovine adrenal mitochondrial preparations, but the adrenal has been investigated mainly by Shimizu and his colleagues and this work has been reviewed elsewhere (Shimizu, 1966).

In addition to the conversion of cholesterol to pregnenolone via 20α-hydroxysterol and 20α,22δ-dihydroxycholesterol (Solomon \textit{et al.}, 1956; Shimizu \textit{et al.}, 1962) the results indicated that a further pathway to the hormones may exist which involves the formation of 17α-hydroxy pregnenolone via 20α-hydroxycholesterol and 17α,20α-dihydroxycholesterol thus again by-passing pregnenolone. However, one or two pieces of contradictory evidence have marred this appealing proposal. Burstein \textit{et al.} (1969) has recently reported that 22R-hydroxycholesterol is formed at a rate 10 times that of 20α-hydroxycholesterol, and indeed this finding does not fit very well with the proposed route from cholesterol to pregnenolone. Shimizu (1964) has reported a number of experiments in which he used the substrate '17α,20α-dihydroxycholesterol'. Burstein \textit{et al.} (1968) have since pointed out that the structure assigned to this compound was wrong and that the synthesis used by Shimizu (1964) would result in the formation of 17α,20β-dihydroxy-20-isocholesterol. Thus it may well be argued that the work involving the use of this substrate (Shimizu, 1964; Shimizu, 1965; Shimizu \textit{et al.}, 1965) should be reassessed. Burstein \textit{et al.} (1968) also showed that the correctly synthesized 17α,20α-dihydroxycholesterol was more efficiently metabolized to 17α-hydroxy pregnenolone than the 20β-isomer by acetone powder preparations of guinea pig adrenal tissue. The formation of the corresponding Δ\textsubscript{4}-3-oxosteroid from 17α,20α-dihydroxycholesterol and from 20α-hydroxycholesterol by human adrenal slices was also demonstrated on a subsequent occasion (Shimizu, 1966). Formation of C\textsubscript{27}-steroids with a Δ\textsubscript{4}-3-oxosteroid structure immediately suggests the possibility of direct formation of progesterone or even 17α-hydroxyprogesterone. This has been investigated by the incubation of [7α-3H] 20α-hydroxycholesterol-4-en-3-one with human adrenal slices (Shimizu, 1966). Only the starting material was recovered
unchanged in each case indicating that metabolism of such compounds in the human adrenal gland is of little importance.

In consideration of these various pathways of cholesterol metabolism, it must be remembered that points of interchange may occur; e.g. Shimizu (1966) has described the conversion of 20α-hydroxycholesterol to pregnenolone sulphate by human adrenal slices. Thus the physiological route from cholesterol to pregnenolone sulphate may not include cholesterol sulphonate and the C₂₇-conjugate may be synthesized for an entirely different purpose (Fig. 25). However, evidence has yet to be produced that the classical conversion of cholesterol to pregnenolone is not the main route from cholesterol to the hormones and it is unlikely that the synthesis of 17α-hydroxy pregnenolone from pregnenolone has any serious competition from pathways involving hydroxylated derivatives of cholesterol.

IX. GENERAL CONSIDERATIONS

It is a disturbing fact that although much time and effort has been expended in the analysis of products from radioactive precursors, uncertainty remains as to whether the exogenous precursors added in vitro, or infused into an organ, behave in a similar manner to endogenous substrates. Yudaev and Droujinina (1967) described experiments in which rat adrenal tissue was incubated with [4-¹⁴C]progesterone with and without the presence of ACTH. In the absence of ACTH, the specific activity of the corticosteroids synthesized was about ten times lower than the progesterone present in the tissue. Stimulation of the synthesis of corticosteroids by ACTH was accompanied by a further lowering of the specific activity of the corticosteroids. The authors stated that such results cannot be explained by the formation of endogenous progesterone or by the dilution of labelled [4-¹⁴C]progesterone since the specific activity of the progesterone was not significantly altered and proposed that, following addition of ACTH, the greater part of the pregnenolone found is converted into corticosteroids by a route not involving progesterone. Investigations of Tejero-Lamarca and Oriol-Bosch (1967, 1969) are also of interest. Their technique involved the pre-incubation of guinea pig adrenal slices for 3 x 1 h with 100 μC portions of [1-¹⁴C]acetate. The slices were then rinsed, incubated with either [7α-³H]-pregnenolone or [7α-³H]progesterone for 15 minutes, rinsed again and finally incubated for 3 x 1 h periods in fresh medium. Steroids from the final three incubation periods and from the tissue at the end of the incubation were diluted with 100 μg of various carriers and analysed. The results indicated that the incorporation of the total quantity of ¹⁴C label into the steroids isolated was relatively constant and the label accumulated in end products such as cortisol, but that the incorporation of the ³H label decreased dramatically with time and
there was no accumulation of the $^3$H label in the end products. Similar results were obtained with homogenates which precludes the possibility of cell membrane effects being responsible for the differences in metabolism of the two labels. These authors also observed that the $^3$H/$^{14}$C ratio of the cortisol and corticosterone isolated from all incubation periods with both [7α-$^3$H]pregnenolone and [7α-$^3$H]progesterone was lower than their precursors. They interpret these results as possibly being due to the fact that both the labelling substrates were metabolized in different compartments from the pre-labelled ones (Fig. 26).

![Diagram](image)

Fig. 26. Diagrammatic representation of tissue compartmentalization (Tejero-Lamarca and Oriol-Bosch, 1969). (A—complete biosynthetic unit in which enzymes and substrates are organized. B—soluble enzymes and enzymes in smaller biosynthetic units. C—barrier preventing the $^3$H-labelled substrates from penetrating the organized compartment).

26). Again, if valid, these experiments illustrate the care required in interpretation of experimental work *in vitro*.

In considering pathways for the synthesis of steroid hormones, often ignored is the question of the availability of the steroid substrate, the form it takes, ester, free or conjugate, and whether it is synthesized endogenously or is brought into the cell from the plasma. Experimental design frequently disregards the fact that exogenous cholesterol in the *in vivo* state is presented to the cell in a much more acceptable form than the aqueous solution of even fine suspension used *in vitro*. The literature on the transformation of cholesterol to steroid hormones in the adrenal is vast and traps for the unwary lie in the enormous differences between animal species.
Dailey et al. (1960) have demonstrated by gas-liquid chromatographic analysis that the major cholesterol ester in man is the oleate (~50%) with a small proportion of linoleate (~3%) in sharp contrast to the plasma composition which has proportions of oleate and linoleate (~30%), (Swell et al. 1960; Riley and Nunn, 1960). Subsequently Riley (1963) showed in an extensive study of the fatty acid pattern of human adrenal cholesterol esters under various conditions, that stimulation of the gland with ACTH resulted in a fall in the total weight of adrenal cholesterol ester but that the relative proportion of each ester did not alter significantly. Thus it seems possible that at the cell membrane there may be a selective uptake of cholesterol esters, or that the cell utilizes them selectively (Riley, 1963). Once inside the cell cholesterol esters may aggregate in the lipid droplets before being distributed to the various parts of the cell including the mitochondria where side-chain cleavage in known to occur. In the rat, there is evidence that the cholesterol used for steroid hormone synthesis is predominantly the free sterol stored in the mitochondria (Ichii and Kobayashi, 1966; Ichii et al., 1967). Again, in the rat, it has been shown that the transformation of cholesterol ester to cholesterol is stimulated by ACTH (Davis and Garren, 1966). A similar observation has been made in human adrenal tissue (Griffiths et al., 1963). Addition of cholesterol to adrenal homogenates does not however increase steroid production (Peron and Koritz, 1960), suggesting either that the enzyme is saturated by the endogenous sterol or that the exogenous material is physically incapable of entering the biochemical pathway. Borkowski et al. (1967) have demonstrated, in a very elegant study, that two distinct pools of cholesterol exist in the human adrenal gland but that one acts as a source of steroid hormones. Furthermore, it was shown that approximately 80% of the hormone output of the adrenals in man was derived from cholesterol originating in the plasma.

Despite difficulties of interpretation, one may derive a great deal of information from experiments in vitro. It is not unreasonable that opinions vary on experimental design, and indeed information obtained from diverse approaches then becomes available for assessment of the whole picture. The work of this group has been concerned with the incubation in Krebs-Ringer bicarbonate-glucose of whole cell preparations without co-factor addition. There are numerous reports in the literature describing the addition of completely arbitrary amounts of co-factors, but it can be argued that enzyme systems in the intact cell should be allowed to produce their own ‘natural’ concentration of co-factor throughout the experiment.

There are other variables in experimental design, for example, the approach to the study of the study of alternate biosynthetic pathways by time-sequence incubations.

Vinson (1966) described a series of experiments on minced rat adrenal tissue in which equimolar labelled pregnenolone and progesterone were used simul-
taneously as precursors. Three incubations were set up and portions of the media removed at various time intervals. The abstracted portions were replaced with fresh medium containing no radioactive precursors. Steroids were extracted from each portion separately and identified by their chromatographic mobility and by the mobility of the corresponding acetates. It was assumed that since the peaks obtained by radioactivity scanning measurements for 180H-DOC, corticosterone, DOC and progesterone were symmetrical, they were radiochemically pure. Percentage conversions from the original substrates were determined by radioactivity measurement after elution of metabolites from the final paper chromatogram taking into account the dilution procedures used throughout the incubation period and the behaviour of identical or similar steroids extracted from buffer (Vinson and Rankin, 1965). The sequence of labelling times showed that in the rat, the pathway to corticosterone seemed to include progesterone. The procedure for the identification of steroids is not very adequate. It may be argued that to measure the quantity of radioactive metabolites in a medium containing a whole-cell preparation is of dubious value since the steroid-producing cell would not normally be expected to secrete intermediates in significant quantities. Despite such criticism which might also be levelled at subsequent work on human adrenal tissue (Whitehouse and Vinson, 1968), similar results were obtained to those of Cameron and Griffiths (1968) and Cameron et al. (1969). Whitehouse and Vinson (1968), however, found no evidence of the anomalous inter-position of the sequence of 11-deoxycortisol and 17αOH-progesterone arising from a labelled pregnenolone precursor. Characterization of steroid by recrystallization following the addition of carrier was performed on a ‘random’ basis.

The field covered by the title ‘Steroid biosynthetic pathways in the human adrenal’ is enormous. A great deal must, of necessity, be omitted, but an excellent complementary review by Grant (1968) would however be of particular interest to new investigators.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the generous financial support of the Tenovus Organisation. They would also like to thank Mr. Ralph Marshall and his staff, Department of Medical Illustrations, Royal Infirmary, Cardiff for providing the figures and tables, and also Miss Jacqueline Taylor and Mrs. Margaret Lewis for typing the manuscript. They are grateful to the following for their kind permission to reproduce some of the diagrams: the Journal of Endocrinology, Figs 4, 22 and 23; Alpha Omega Alpha Publishing, Caerphilly, Figs 6 and 12-19; Harvey Lectures, Fig. 3. Finally they thank Prof. A. P. M. Forrest, Department of Surgery, Royal Infirmary, Cardiff, for providing the tissue for the work performed at the Institute. His encouragement and support throughout have been of inestimable value.
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Ultramicrochemical and Tissue Culture Studies of Adrenal Tissue

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SUMMARY

An ultramicrochemical technique is described which has been used to determine the site of sulphation of dehydroepiandrosterone (DHA) within the adrenal cortex of man and guinea pig. It was shown that sulphation is confined to the zona reticularis in both cases. The metabolism of \([7\alpha-\text{3H}]17\alpha\text{-hydroxypregnenolone}\) by fascicular and reticular tissue, separated from a human adrenal gland, and maintained in organ culture, has been studied. The percentage conversions to three metabolites, cortisol, DHA and DHA sulphate were measured. Cortisol was produced by both zones but an increased production under the influence of ACTH only occurred in the fascicular tissue. DHA and DHA sulphate were produced mainly by the reticular tissue and there was a marked stimulation of their synthesis in this tissue under the influence of ACTH.

Preliminary results on the effect of prolactin on androgen production by guinea pig adrenal tissue and the separated zones of a human adrenal gland maintained in organ culture are presented. Prolactin stimulated the production of (1) testosterone in the guinea pig adrenal gland and (2) both testosterone and androstenedione in the human tissue.

The human adrenal cortex, as first described by Harley (1858) and Arnold (1866) is histologically divided into three concentric zones or layers of cells. The outer zone the zona glomerulosa is not always continuous and may occur as scattered islets of cells beneath the capsule. The adjacent zone, the zona fasciculata, constitutes a large proportion of the cortex and consists of cords of cholesterol laden cells. The inner zone, the zona reticularis, adjacent to the central medulla consists of eosinophilic cells containing little cholesterol.

Several theories have been advanced for the functions of these histologically defined zones, most of them based on histological and histochemical studies.

The first theory by Gottschau (1883), later elaborated by Bennett (1940) suggested a system of cell migration whereby cortical cells were formed in the subcapsular region and migrated centripetally to the zona fasciculata, which was designated the 'secretory zone'. The steroid hormones were considered to be synthesized and secreted by the fascicular cells, which subsequently degenerated in the zona reticularis, and were removed by the extensive vascular channels of this region of the cortex.
This theory was superseded by the 'zonation theory' of Swann (1940) who suggested a division of the secretory function of the cortex between the zona glomerulosa on the one hand and the zona fasciculata and zona reticularis on the other. Aldosterone has since been shown to be synthesized exclusively by the zona glomerulosa (Ayres, Garrod, Tait and Tait, 1958) A review by Deane (1962) describes histochemical studies by Deane, Greep and Chester Jones which considerably extended this 'zonation theory'. It was suggested that the mineralocorticoid was synthesized and secreted by the zona glomerulosa and that the adrenocorticosteroids were formed in the zona fasciculata. The observation of Blackman (1946) that adrenogenital syndrome was associated with hyperplasia of the zona reticularis and accompanied by increased androgen excretion led to the belief that the adrenal androgens and probably oestrogens, were synthesized by the cells of this zone.

Interest in the zona reticularis as an active zone was renewed when Symington and his colleagues (Symington, Currie, Curran and Davidson, 1955; Symington, Currie, O'Donnell, Grant, Oastler and Whyte, 1958) showed that the administration of adrenocorticotrophic hormone (ACTH) caused a significant histological change at the border region between the zona fasciculata and the zona reticularis. Administration of ACTH resulted in the fascicular cells adjacent to the reticular tissue becoming depleted of lipid and acquiring the histochemical characteristics of the reticular cells. This widening of the zona reticularis was found to be accompanied by an increase in the activity of the 11β-hydroxylase enzyme system of homogenates prepared from the whole gland (Grand, Symington and Duguid, 1957) and it appeared that the site of the 11β-hydroxylase and therefore adrenocortico-steroid synthesis, was the reticular zone. The two zones were therefore considered to function as one unit, the histological appearance of which varied with the degree of activity of the gland.

Modification of this theory became necessary when Griffiths, Grant and Symington (1963) obtained reasonably homogeneous slices of fascicular and reticular tissue from human adrenal cortices and showed that both cell types had the capacity in vitro to synthesize cortisol, corticosterone and 11β-hydroxyandrostenedione. The zona fasciculata could no longer be considered merely a storage zone, but an actively synthesizing tissue which contributed, in the resting gland, to the production of both C21—and C19—steroids.

Observations by Ofstad, Lamvick, Stoå and Emberland (1961) did not however fit this theory. They found that a patient with a normal zona fasciculata but a degenerated zona reticularis excreted normal amounts of 17-hydroxycorticosteroids but abnormally low amounts of C19—steroids, even after ACTH stimulation. Further evidence for a relationship between the zona reticularis and adrenal androgen production was obtained by Griffiths, Cunningham and Cameron (1968) when an adenoma consisting almost entirely of fascicular type cells, incubated in vitro, failed to convert both pregnenolone and pregnenolone sulphate to dehydroepiandrosterone sulphate (DHA sulphate), which is now known to be the main C19—steroid produced by the human adrenal gland.
Unequivocal evidence for the functions of the two major zones in the adrenal cortex can only be obtained through the study of completely separated zona fasciculata and zona reticularis cells. This paper describes how an ultra-microchemical technique (Grunbaum, Geary and Glick, 1965) and organ culture were employed to investigate some of the biosynthetic activities of the zona fasciculata and the zona reticularis from both human and guinea pig adrenal glands.

Ultramicrochemical Studies This technique was used to investigate the conversion of DHA to DHA sulphate.

Cylinders of tissue (2.5 mm diameter) were removed from the frozen gland by means of a borer attached to a slow revolving electric motor (Fig. 1). The borer with the tissue intact was then transferred to a microtome and serial sections, 16 µm thick cut. The first section was placed on a microscope slide and kept for histological study, the next three sections were placed in a small tube containing 10 µl of a sucrose and nicotinamide solution for the enzyme assay; the next section was again kept for histology-this pattern being repeated through the cortex and into the medulla. The sections on the slide were stained with Sudan IV and haematoxylin, the Sudan giving a strong red staining
of the cholesterol laden fascicular cells, while the haematoxylin stained all nuclei blue. The enzymic activity of the tissue in each tube was later related to the histology of the sections on the slide.

Addition of labelled steroid precursors to the sections in the tubes was effected by means of a cathetometer and micropipetting device (Fig. 2). The pipette was made by drawing out capillary glass tubing, bending the end and grinding the tip to a point by means of a dental grinding disc. For pipetting the cross wires of the cathetometer were focussed on the tip of the pipette. The cathometer was then raised through a predetermined distance and the solution of labelled steroid drawn up by means of a mouthpiece until its level coincided with the cross wires. The volume corresponding to this length of the pipette had been determined spectrophotometrically using a dye with a high extinction coefficient. Treatment of the pipette with silicone ensured complete discharge into each tube. The steroid precursor was added in about 150 nl ethanol and after the addition of the incubation medium (15 μl) (phosphate buffer, pH 7.0, 1/15 M containing K₂SO₄, 0.02 M; MgSO₄ 0.025 M and ATP 0.01 M) the tissue was homogenised by holding the tube against a fast revolving bent pin.

Fig. 2. Ultramicro-pipetting device and cathetometer
The enzymic activity was determined after the incubation by diluting the radioactive products with non-radioactive carrier steroid and measuring their subsequent specific activity after purification using thin layer chromatography. The specific activity of derivatives were also measured and providing that the values obtained did not differ by more than 10%, the mean was used to calculate the weight of steroid formed from the added substrate.

In order to be able to make a direct comparison of the enzymic activity in each incubation the protein nitrogen content of the tissue remaining in each tube was measured by the bromsulphalein method of Nayyar and Glick (1954).

Figure 3 shows the pattern of formation of DHA sulphate in the guinea pig adrenal gland. The rate of production of DHA sulphate has been plotted against distance from the surface of the gland. It can be seen that sulphation of DHA occurred in the zona reticularis only.

![Fig. 3. The quantitative histochemical distribution of a DHA sulphating enzyme system based on tissue protein nitrogen (Pn) throughout the guinea-pig adrenal cortex. Vertical bars represent ranges of activity determined in various regions in the cortex (6 expts). Regions marked G, F, R and M denote respectively zona glomerulosa, zona fasciculata, zona reticularis, and medulla; mixed zones are designated by both letters.](image)

A similar pattern was obtained in the human gland (Fig. 4). The border between the zona fasciculata and zona reticularis is not as well defined in the human as in the guinea pig resulting in a wide zone of mixed tissue. It would appear, however, that DHA sulphokinase activity is only found in the presence of reticular cells.
Organ Culture Studies

All experiments using the ultramicrochemical technique involved a 1 hr incubation of tissue homogenates in a suitable incubation medium. In an attempt to reproduce physiological conditions more closely some biosynthetic pathways operating in separated zona fasciculata and zona reticularis cells maintained in organ culture over a period of 14 days have been studied.

Explants of fascicular and reticular cells from a gland removed from a patient with breast cancer were separated as well as could be judged by eye and 4 cultures of each cell type set up in Eagle’s Minimum Essential Medium fortified with 10% Calf Serum, 292 μg/ml glutamine 200 units/ml Penicillin and 100 μg/ml Streptomycin. The atmosphere was 95% oxygen/5% CO2. After 2 days the media were changed, the fresh media containing 1 μCi of [7α-3H] 17α-hydroxypregnenolone; 0.1 i.u. of ACTH was added to two of the zona fasciculate and two zona reticularis cultures. Four days later the media were collected and the tissues washed in fresh media for a further period of 4 days. The media for the final 4 day period again contained [7α-3H] 17α-hydroxy-pregnenolone, and in some ACTH as for days 3-7, the ACTH being added to the same cultures. At the end of the culture period media from identical cultures were pooled and all were analysed for labelled cortisol, DHA and DHA sulphate. The tissues were dried on filter paper and weighed.
Figure 5 shows the results obtained and bearing in mind our previous results on the formation of DHA sulphate in the zona reticularis the production of a small amount of this steroid by what we designated zona fasciculata tissue may well have been due to the presence of some zona reticularis cells. Interestingly the pattern for DHA formation is similar to that for DHA sulphate suggesting that this too may be produced by the zona reticularis cells only. In our ultramicro in vitro incubations we have failed to show any direct effect of ACTH on the conversion of DHA to DHA sulphate. Thus the marked effect of ACTH on DHA sulphate production observed in the organ culture experiment may be due to the increased production of its immediate precursor, DHA, from the 17α-hydroxyprogrenolone incubated.

**STERIOD SYNTHESIS IN ORGAN CULTURE**

<table>
<thead>
<tr>
<th>Days 3-7</th>
<th>Days 11-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 5.** The conversion of [7α-3H] 17α-hydroxyprogrenolone to DHA sulphate, DHA and cortisol by control and ACTH treated adrenal tissue maintained separately in tissue culture over a period of 14 days. The unshaded columns represent the conversions during days 3-7 and the shaded columns the conversions during days 11-15. The results are based on 1 mg of tissue.

The work of Griffiths *et al.* (1963) and of Cameron and Grant (1967) has shown that both zones synthesize cortisol but that the zona fasciculata is more efficient than the zona reticularis in this respect. When interpreting the results for cortisol production in organ culture it must be borne in mind that no attempt was made to measure the production of cortisol from endogenous precursors. The apparent lack of production of substantial quantities of labelled cortisol from the exogenous [7α-3H] 17α-hydroxyprogrenolone by the zona fasciculata may have been due to the presence of unlabelled endogenous precursors.
Of interest here, however, was the effect of ACTH. Whereas the production of labelled cortisol doubled in the zona fasciculata cells under the influence of ACTH there was no observed effect in the zona reticularis tissue. This contrasts with the observations for DHA and DHA sulphate.

The importance of working with well defined groups of cells when investigating steroid biosynthetic pathways and the control mechanisms involved is thus emphasized.

The role of adrenal androgens in the maintenance of prostatic function has always been considered to be relatively minor. Robinson and Thomas (1971) have shown, however, that blood testosterone levels in patients suffering from prostatic cancer, and on oestrogen therapy, rises over a period of 6 months after orchidectomy. Pituitary ablation will again lower these levels to negligible amounts. Since oestrogen administration is known to increase prolactin secretion it was considered of interest to study the effect of prolactin on androgen synthesis by the adrenal gland.

Two organ culture experiments were carried out to study the effect of \( \alpha^{1-24} \) ACTH (1 \( \mu \)g/ml: 0.1 I.U./ml) and ovine prolactin (230 \( \mu \)g/ml: 5.0 I.U./ml) on the secretion into the organ culture medium of testosterone and adrostenedione by (1) mixed fascicular and reticular tissue of guinea pig adrenal glands and (2) separated fascicular and reticular tissue from a human adrenal gland. The cultures were set up as before and maintained for 2 periods of 3 days in the case of the guinea pig adrenal and for 1 period of 3 days in the case of the human gland. The steroid concentrations in the media collected were measured by gas liquid chromatography.

Figure 6 shows that the mixed tissue of the guinea pig adrenal gland was stimulated by ACTH to secrete increased amounts of both testosterone and androstenedione whereas the higher doses of prolactin only increased the secretion of testosterone.

<table>
<thead>
<tr>
<th>Period</th>
<th>Hormone</th>
<th>Testosterone (pg/mg tissue)</th>
<th>Androstenedione (pg/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>39</td>
<td>160</td>
</tr>
<tr>
<td>1</td>
<td>ACTH (0.10 I.U.)</td>
<td>63</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.50 I.U.)</td>
<td>95</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.05 I.U.)</td>
<td>31</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>ACTH (0.10 I.U.)</td>
<td>11</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.50 I.U.)</td>
<td>133</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.05 I.U.)</td>
<td>35</td>
<td>70</td>
</tr>
</tbody>
</table>

Fig. 6 The effect of ovine prolactin (2nd IS ovine prolactin) and ACTH on androgen production by guinea pig adrenal in organ culture.
The separated tissues of the human adrenal gland again showed a quantitative difference in steroid secretion between the two zones (Fig. 7). Prolactin but not ACTH increased the output of androstenedione and testosterone from the fascicular tissue whereas the reticular zone responded to both protein hormones with a marked increase in testosterone output and a more moderate increase in androstenedione secretion.

These results are of a preliminary nature and more work is necessary to confirm these observations. They do however indicate that the increased testosterone levels observed by Robinson and Thomas (1971) could be of adrenal origin and that the relapse of some of these patients may be due to the stimulation of the adrenal by prolactin, to produce androgenic steroids.

| STEROID PRODUCTION BY HUMAN ADRENAL IN ORGAN CULTURE |
|-----------------|-----------------|-----------------|------------------|
| TISSUE          | HORMONE ADDED   | Androstenedione (ng/mg tissue) | Testosterone (ng/mg tissue) | 11OHCs (ng/mg tissue) |
| Fasciculata     | Control         | 7.3             | 269              | 150              |
|                 | ACTH (10 U)     | 9.2             | 246              | 610              |
|                 | PROLACTIN(5 U)  | 12.4            | 707              | 6.70             |
| Reticularepisodens | Control       | 5.5             | 396              | 280              |
|                 | ACTH (10 U)     | 17.0            | 1828             | 720              |
|                 | PROLACTIN(5 U)  | 21.0            | 1801             | 390              |

Fig. 7. The effect of ovine prolactin (2nd IS ovine prolactin) and ACTH on androgen production by the separated zones of the human adrenal cortex in organ culture.

REFERENCES


C19-STEROID BIOSYNTHESIS IN THE HUMAN ADRENAL GLAND

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Recent in-vivo studies made by Deshpande, Jensen, Carson, Bulbrook & Douoss (1970) concerned with the synthesis of androgens by the human adrenal gland have again emphasized the equivocal nature of the results available on this subject. The present communication describes how results contrary to those obtained in vivo were derived from investigations in vitro: this prompts us to suggest that further studies are necessary on the control of androgen production. Evidence has been obtained (Ichii, Forchielli, Cassidy, Rosoff & Dorfman, 1962; Kase & Kowal, 1962; Ward & Grant, 1963) for the presence in various pathological types of human adrenal tissue of the enzyme systems necessary for the conversion of progesterone to adrenal androgens, while Deshpande et al. (1970) showed that in vivo, 17α-hydroxyprogesterone rather than dehydroepiandrosterone (DHA) is the more important precursor of androstenedione. On the other hand Cohn & Mulrow (1963) showed in both hyperplastic and neoplastic adrenal tissue that the immediate precursor of androstenedione was DHA. Our own studies in which adrenal tissue from a patient with breast cancer was incubated with equimolar amounts of DHA and 17α-hydroxyprogesterone provide further support for the importance of this pathway.

The tissue (1.48 g) was finely chopped, mixed and incubated with equimolar amounts (53.3 nmol) of [7α-3H]DHA (4.42 μCi) and [4-14C]17α-hydroxyprogesterone (2.00 μCi) in Krebs–Ringer bicarbonate–glucose medium (19 ml) for 1 h at 37 °C. The reaction was stopped with acetone containing 500 μg each of androstenedione, 11β-hydroxyandrostenedione, testosterone and epitestosterone. Steroids were extracted, purified by thin-layer chromatography, and derivatives prepared as described previously (Cameron & Griffiths, 1968; Griffiths, Cunningham & Cameron, 1968). Testosterone and epitestosterone were separated by chromatography on alumina (Woelm) in the solvent system cyclohexane:ethyl acetate (9:11, v/v). All steroids and derivatives were measured by their absorption at 240 nm and the radioactivity measured by a Nuclear Chicago liquid scintillation spectrometer. Table 1 shows the evidence for the radiochemical purity of the steroids investigated and the percentage conversion of the substrate to these metabolites.

The extensive metabolism of DHA compared with the relatively low conversion of 17α-hydroxyprogesterone to androstenedione, 11β-hydroxyandrostenedione, testosterone and epitestosterone by this adrenal tissue provides further evidence from in-vitro studies to support the suggestion (Cohn & Mulrow, 1963) that 17α-hydroxy-
progesterone is of minor importance in adrenal androgen biosynthesis. It is also in accord with the observation (Cameron, Jones, Jones, Anderson & Griffiths, 1969) that in a mixed cell adenoma, incubated with [4-14C]DHA and [7α-3H]17α-hydroxy-pregnenolone, there was little ‘leakage’ of the tritium label into adrenal androgens through 17α-hydroxyprogesterone. However, these results are at variance with the in-vivo results of Deshpande et al. (1970) and might indicate that the controlling influence of the probably high levels of corticotrophin (ACTH) present during the experiments in vivo of Deshpande would be worthy of investigation in vitro. Incubations, similar to the one described above, in the presence and absence of ACTH should prove useful.

Table 1. Evidence for the identification of metabolites formed by incubating adrenal tissue from a patient with breast cancer with [7α-3H]dehydroepiandrosterone and [4-14C]17α-hydroxyprogesterone

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Specific activities (d.p.m./nmol)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>1547</td>
<td>27-50</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1557</td>
<td>1-19</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>1531</td>
<td>1-20</td>
</tr>
<tr>
<td>11β-Hydroxyandrostenedione</td>
<td>1320</td>
<td>22-50</td>
</tr>
<tr>
<td>11β-Hydroxytestosterone</td>
<td>1315</td>
<td>1-32</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>1369</td>
<td>1-32</td>
</tr>
<tr>
<td>Testosterone</td>
<td>3-20</td>
<td>0-01</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>3-19</td>
<td>0-01</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>3-47</td>
<td>0-01</td>
</tr>
<tr>
<td>Epitesterone</td>
<td>0-87</td>
<td>0-00</td>
</tr>
<tr>
<td>Epitesterone acetate</td>
<td>0-86</td>
<td>0-00</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0-85</td>
<td>0-00</td>
</tr>
</tbody>
</table>

The formation of epitesterone by this human adrenal tissue is also intriguing. Epitesterone formation from testosterone has been reported by Blaquier, Dorfman & Forchielli (1967) but they failed to demonstrate its formation from either DHA or androstenedione. An earlier report from this laboratory (Jones, Cameron, Griffiths & Forrest, 1970) described its formation from pregnenolone by normal human adrenal tissue. The significance of epitesterone formation and the pathway by which it is synthesized remain to be determined.

REFERENCES

I. Introduction

Over the past two or three decades the adrenal cortex has been the subject of more study than any other type of endocrine gland. It is an extremely complex tissue and, in order to understand the mechanisms by which its growth and function are controlled, some knowledge of the anatomy, biochemistry and physiology of the adrenal gland is necessary. A knowledge of the steroid biochemistry of the various types of cell found within the human adrenal cortex can be of considerable value to the pathologist when attempting to interpret the histological appearance of various endocrinologically abnormal adrenal tissues, and much has been learnt from the close association of...
between pathologist and the steroid biochemist on the relationship between cell type and function. A reasonable understanding of the clinical picture associated with the various abnormalities of adrenal function can only follow an appreciation of the biochemical aspects of the adrenal cortex. Some excellent reviews exist, describing the work that has been done, but it is hoped that the following simplified account, which directs attention to some of the essential relationships between cell structure and function, will be of value to the enquiring student.

II. Morphology and Vasculature

The human adrenal glands, each weighing approximately 3.0–6.5 g and located close to the cranial poles of the kidneys, are composed of two separate and distinct endocrine tissues, the adrenal cortex and the adrenal medulla. Although of different embryological origin these two tissues have anatomically become closely associated in man and higher animals with the cortex encapsulating the medulla. In mammals both the cortex and medulla are retained within a common adrenal capsule. In most experimental animals, the adrenal gland is ovoid or spherical with the medulla symmetrical surrounded by cortex except medially where it often reaches the surface of the gland. The human adrenal is a folded structure with an irregular shape and the medulla is limited to the medial two-thirds of the gland. From the relatively flattened anterior faces of its glands the adrenal veins emerge. On the posterior surface, however there is a ridgelike elevation, the crest, which gives the adrenal gland a triangular structure. Recent studies of Dobbie and Symington (1966) have shown that the gland may be divided into three distinct morphological regions, the head, body and tail, which differ from each other in relative contents of cortex and medulla and in vascular patterns (Fig. 1).

Early histological studies in the latter part of the nineteenth century drew attention to the division of the adrenal cortex into three layers of cells, the zona glomerulosa, the zona fasciculata and the zona reticularis. It was shown later that, whereas these three zones were present in the adrenals of many animals, important species differences do exist. The ruminants, for example, have no zona fasciculata (see review by Symington, 1962). The zona glomerulosa, prominent in the glands of rat and sheep, is often ill-defined in the human adrenal cortex and may be confined to scattered islets of cells situated immediately below the capsule. Thus histological sections of the human adrenal often show the zona fasciculata lying next to the capsule.
Fig. 1. Diagrammatic representation of a left adrenal gland from which areas have been removed to show the course of the central vein (CV) enveloped by the cortical cuff (CF). The anterior surface of the adrenal is shown uppermost. M—medulla; C—cortex. (Dobbie and Symington (1966)—reproduced by courtesy of the Journal of Endocrinology.)

Fig. 2. Electron micrograph of tissue from the zona glomerulosa. X 34,590. Micrograph kindly supplied by Dr. A. M. Mackay, Chester Beatty Research Institute, London.
Cells of the zona glomerulosa have a relatively large nucleus and a small cytoplasmic volume. In comparison with the cells of the zona fasciculata, the cytoplasm contains little lipid material and gives a relatively strong positive stain for RNA. Although all adrenal cells have many mitochondria, those of the zona glomerulosa have fewer than the cells of the zona reticularis. Mitochondria of the zona glomerulosa are elongated with a cristate internal structure (Fig. 2) similar to that found in mitochondria from other tissues of the body, but differing, however, from the mitochondria of the zona reticularis. These mitochondria appear to have few internal membranous structures (Fig. 3). The zona fasciculata is usually the widest zone in the cortex and is composed of columns of large yellow, cholesterol containing cells, which appear vacuolated after paraffin embedding, sectioning and staining procedures (Fig. 4). These cells have therefore been referred to as clear cells or spongiocytes because of their histological appearance. The adjacent zona reticularis is located next to the central medulla and is composed of small, non-vacuolated, compact cells arranged in alveoli which are separated by thin-
walled sinuses. The compact cells are more basophilic and are rich in ribonucleoprotein and enzymes and contain relatively little cholesterol.

Fig. 4. Photomicrograph of the human adrenal cortex showing the capsule (c), zona fasciculata (zf), zona reticularis (zr) and medulla (m). The zona glomerulosa cannot be clearly distinguished in this section. Haematoxylin and eosin (×60).

The human adrenal gland has a very rich vascular supply terminating in numerous arterioles. Although most of these ramify over the capsule and eventually divide within the gland to form a narrow subcapsular plexus, some branches pass into the gland, traverse the cortex and supply the medulla directly (see also p. 233). Capillaries from the subcapsular plexus pass down through the columns of cells in the zona fasciculata to a rich plexus located in the zona reticularis (Fig. 5). From the cortico-medullary junction the blood drains into the central vein by small venules which pass between chromaffin
cells of the medulla and enter the medullary veins by passing between the prominent longitudinal muscle bundles which are a feature of the vein wall. It would appear that these longitudinal

![Diagram of adrenal gland circulation](image)

Fig. 5. The two types of circulation present in the adrenal gland are shown diagrammatically. In the “tail” regions of the gland (A) which are purely cortical (see fig. 1), the plexus reticularis is drained by vessels lying in the alar raphe.

Where the cortex and medulla are in juxtaposition (B), a type of portal system is found in which the blood is eventually collected in venous sinuses which may then pass between the muscle pillars of the veins. (Dobbie and Symington, 1966—reproduced by courtesy of the Journal of Endocrinology.)

muscle bundles regulate the blood flow through the cortico-medullary “vascular dam” and hence through the zona reticularis and inner zona fasciculata. Such a mechanism may well provide the system whereby the controlled uptake of ACTH (corticotrophin) by the various cells is exercised. The right central vein flows directly into the inferior vena cava but on the left side it emerges from the gland as the suprarenal vein and may join the inferior phrenic vein before draining into the left renal vein.

The structure of the adrenal cortex has also been extensively studied in pre-viable infants of less than 20 weeks' gestation, in stillbirths and in children dying in the neonatal period. The gland of the pre-viable fetus of 18 weeks consists of a narrow “adult cortex” in which the cells are small and contain a densely staining nucleus which occupies most of the cell. The “fetal zone” is the most prominent region of the cortex. The cells are large, have a dense nucleus and eosinophilic cytoplasm, and closely resemble the compact cells of the normal adult cortex. The width of the fetal zone accounts for
the disproportionately large size of the fetal gland until the time of birth. At this period the fetal zone occupies 80% of the cortex. After birth, however, it undergoes rapid involution, resulting in a marked decrease in the size of the gland. During the neonatal period, the adult zone increases in size, differentiates into zona fasciculata and zona glomerulosa, and occupies a larger proportion of the cortex. Reports suggest that the zona reticularis develops during the first year.

III. Adrenal Steroids

Early endocrinologists were concerned with attempts to prepare extracts of adrenal tissue capable of maintaining the life of an adrenalectomized animal. Most of this work, however, was directed to the study of water-soluble material but with the isolation of oestrone from urine in 1929, and its subsequent characterization came the realization that the steroid hormones were lipids. Lipid extracts of adrenal tissue were then found to be effective in maintaining life in adrenalectomized animals. In the late 1930s, American and Swiss groups of investigators succeeded in isolating about 30 steroids from bovine adrenal extracts, although it has since been shown that only some of these compounds are actively secreted by the human adrenal. Most of them were either intermediates in the biosynthesis of the secretory products or metabolites derived from them.

It is now established that certain steroids are major secretory products of the human adrenal gland, and that others are secreted in such small quantities that it would seem probable that their significance is relatively minor. A compound is generally considered to be a secretory product when it has been shown to be present in higher concentration in adrenal venous blood than in the arterial blood supplying the gland.

Cortisol and corticosterone are the two principal glucocorticoids secreted by the human adrenal. It is interesting that both these steroid hormones are not invariably synthesized by adrenals of all species and certain animals—rat, mouse and rabbit for example—secrete only corticosterone. The significance of this species variation is not yet known. Glucocorticoids are essential for the maintenance of life, play a role in the regulation of protein synthesis, promote gluconeogenesis and suppress inflammation by their effect on connective tissue. They also have an important role to play in the control of renal blood flow and of water and salt retention. The control of electrolyte balance by the kidney is, however, the principal concern of aldosterone, the main mineralocorticoid secreted by the adrenal.
These three steroids are referred to as the adrenocorticosteroids and are secreted only by the adrenal gland. The amounts of these compounds secreted daily, and the plasma levels are shown in Table 1.

A recent surprising discovery was that dehydroepiandrosterone sulphate (DHA sulphate) is a major secretory product of the human adrenal gland and is synthesized in an amount similar to that of cortisol. The significance of the secretion of such large amounts of this conjugated steroid is as yet unknown. It has no established metabolic role, yet because of the low rate of clearance of this compound by the kidney, is one of the most abundant of the plasma steroids (Table 1). It is interesting that DHA sulphate is synthesized by the adrenal gland since conjugation of steroids with a sulphuric or glucosiduronic acid residue is fundamentally one of the functions of the liver in the metabolic processes concerned with reduction and deactivation of steroid hormones.

Table 1. Some normal steroid levels in peripheral plasma. There are often variations in the reported levels of plasma steroids depending upon the methodology of the different laboratories and the appropriate references are given below. Normal adult daily excretion of total 17-hydroxycorticosteroids in the urine range from 5-21 mg for men and 4-17 mg for women. These values vary with age. The urinary total 17-oxosteroid excretion varies enormously with age and sex: male (aged 25 years), 7-23 mg/24 h; female (aged 25 years), 5-17 mg/24 h.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Approximate secretion rate (mg/24 h) in adult</th>
<th>Peripheral plasma concentration (Range in µg/100 ml)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>25</td>
<td>3-20</td>
<td>(1)</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>2-4</td>
<td>0-13-2-3</td>
<td>(1)</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0-04-0-20</td>
<td>0-005-0-017</td>
<td>(1)</td>
</tr>
<tr>
<td>DHA</td>
<td>0-35-0-85</td>
<td>0-40-1-70</td>
<td>(2)</td>
</tr>
<tr>
<td>DHA Sulphate</td>
<td>10-248</td>
<td>12-392</td>
<td>(3)</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0-003-0-185</td>
<td>0-030-0-170</td>
<td>(4)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0-020-0-070</td>
<td>0-300-1-200</td>
<td>(5)</td>
</tr>
<tr>
<td>11β-Hydroxyandrostenedione</td>
<td>0-090-0-280</td>
<td>0-070-0-300</td>
<td>(6)</td>
</tr>
</tbody>
</table>

There is also evidence that the normal human adrenal gland synthesizes and secretes trace amounts of oestrone, one of the oestrogens secreted in comparatively large amounts by the normal ovary. At the same time, there is some capacity to synthesize and secrete androstenedione and testosterone, especially the former steroid, in the normal human adrenal, although again in small amounts. Androstenedione and testosterone are the normal secretory products of the testis.

Some of the steroids that are intermediates in the series of reactions concerned with the biosynthesis of these products, to be discussed in detail later, are also secreted by the gland but their levels in the adrenal venous effluent are sufficiently low to suggest that they have little physiological importance. However, the control of the synthesis and secretion of these steroids by the normal adrenal cells can be changed in pathological conditions resulting in hormonal disturbances.

IV. Steroid Nomenclature

Steroids, like other lipids, are soluble in fat solvents, and relatively insoluble in water unless conjugated or highly oxygenated, and are generally considered as part of the unsaponifiable portion of tissue lipid extracts. They have a wide range of biological activity and include the adrenocorticosteroids, the male and female sex hormones loosely referred to respectively as the androgens and oestrogens, bile acids, animal and plant sterols, substances called cardiac glycosides, sapogenins and also certain alkaloids. In order to understand the many and varied inter-relationships of the steroid hormones to both their precursors and their metabolic products, a basic knowledge of steroid chemistry is essential.

These steroids have in common a carbon-ring structure, a perhydrocyclopentanophenanthrene nucleus, containing 17 carbon atoms, shown in Fig. 6, which illustrates the agreed numbering system for the various carbon atoms together with the lettering of the rings.
In most naturally occurring steroids, either one or two methyl (CH₃—) groups, referred to as the angular methyl groups, are attached to carbon atoms 10 and 13 and in others an aliphatic side-chain is attached to carbon atom 17. In order to provide a reasonable basis for the classification of steroids, they can be considered derived from certain basic saturated parent hydrocarbons (Fig. 7). Thus oestrane is the parent compound of the C₁₈-steroids or oestrogens, whereas the C₁₉-steroids or androgens, with an extra angular methyl group attached to C-10 can be considered derivatives of androstane. In like manner, pregnane is related to the C₂₁-steroids such as the adrenocorticosteroids and progesterone, cholane to the bile acids and cholestan to the C₂₇-sterols such as cholesterol.

As a general rule, when steroid structures are written down, the carbon atom numbers are not inserted, only the skeleton nucleus being shown and a line is drawn to indicate the presence of an angular methyl group (Fig. 8). The great diversity of the naturally occurring steroids is due to the attachment of substituents to the carbon nucleus and/or to the presence also of a double bond in the nucleus as seen in the structure of cortisol, the C₂₁-steroid hormone synthesized in the adrenal gland (Fig. 8).

Although shown as a planar structure, the steroid molecule is more complicated when its conformation, or the arrangement of the various atoms in space, is considered. For example, cyclohexane rings such as those of the A, B and C rings of the steroid nucleus, can exist in two forms, the "boat" or "chair" conformations (Fig. 9). The boat forms are normally unstable and Fig. 9 illustrates a perspective
view of a steroid molecule showing the more stable characteristic chair-like rings and the relatively planar structure.

The angular methyl groups project above the plane of the molecule in all naturally known steroids. If other substituents such as hydroxyl groups attached to the steroid nucleus project in the same direction as the angular methyl groups, then they are described as cis or β-oriented and on the flat representation of the steroid molecule, drawn as a solid line (C — OH) (Fig. 8). If the substituent group projects below the plane of the molecule and hence opposite to the angular methyl groups, then they are trans or α-oriented and the valency bond drawn as a dashed line (C — OH) (Fig. 8). In cortisol, therefore, the 11-hydroxyl group is β-, whereas the 17-hydroxyl group is an α-substituent.

The stereochemistry of the substituents, therefore, is comparatively easy, although the situation with regard to the stereochemistry of the nucleus is more complicated. The steroid nucleus contains 6 asymmetric carbon atoms at positions 5, 8, 9, 10, 13 and 14, and therefore 2⁶ or 64 possible stereoisomers could exist (Fig. 10). In nature, however, only the A-B ring junction produces stereoisomers, the remainder having an identical arrangement in all natural compounds (Fig. 10). The A-B ring junction exists in two forms, the α, trans or the β, cis orientated (Fig. 10), the hydrogen atom at carbon 5 conse-
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quently projecting either below or above the plane of the molecule respectively.

A number of steroids possess a carbonyl group (C = O) at carbon 20. Reduction of this group during normal metabolic reactions produces another asymmetric carbon atom, and two possible stereoisomers can therefore exist (Fig. 11).

Steroid nomenclature can be extremely complex and a recent report from the Commission of the International Union of Pure and Applied Chemistry (IUPAC) should be studied to ascertain the complete set of rules. However, as a simple guide, the names of steroids are based on the parent structure to which are added various prefixes and suffixes which show the nature of the attached substituent. The carbon atom to which the substituent is attached is indicated by the number as illustrated in the examples given in Fig. 12. The pregnanediol and pregnanetriol shown, C₂₁-steroids, have structures defined as 5α-pregnane-3α, 20α-diol and 5β-pregnane-3α, 17α, 20α-triol respectively (Fig. 12a, b). The stereochemistry of carbon atom 5 is indicated by the prefix 5α or 5β related to the parent name. The C₁₉-steroids of the androstane series, actio-
cholanolone and 11-oxoaetiocholanolone are further examples which indicate that in the presence of a carbonyl group, the hydroxyl group becomes a prefix with the carbonyl the suffix. Note the an becomes ane when preceding the consonant (Fig. 12d), and that

unsaturation in the nucleus is indicated by the suffix en or ene (Fig. 12e, f). The position of the double bond is given by the use of the lower number of the carbon atoms between which the double bond is positioned unless the double bond lies between two carbon atoms not consecutively numbered when both are given (Fig. 12g). The A-ring of the oestrogens is of course a benzene ring.

V. Steroid Biosynthesis

The period between 1929 and 1939 was an exciting period in the development of steroid biochemistry. In 1929, the isolation of
Oestrone was reported from two independent laboratories, that of Doisy and his colleagues in the USA, and by Butenandt in Germany. In the subsequent ten years, most of the major steroid hormones were isolated and characterized, and it became obvious to many investigators that there was a close relationship between the chemical structure of these hormones and that of cholesterol. The possibility that cholesterol could be the precursor of the steroid hormones was considered, and the experiments of Sayers in 1944 showing that administration to rats of a pituitary extract containing ACTH caused a decrease in the adrenal cholesterol concentration provided support for this theory. The structure, growth and secretory activity of the zona fasciculata and zona reticularis, although not the zona glomerulosa, are now known to be regulated by ACTH (see also chapter 25), and it appears therefore that a stress reaction, promoting increased ACTH secretion, would result in the stimulation of the adrenal cortex with the consequent synthesis of cortisol and corticosterone from the cholesterol and cholesterol esters stored in the clear cells of the zona fasciculata. These glucocorticoids appear to be essential to support the biological responsiveness of the body to stress.

The advent of 14C-labelled acetate and cholesterol allowed further studies to develop on the biosynthesis of the adrenal steroids. Much of our knowledge of these complex biosynthetic patterns was derived from the perfusion through bovine adrenal glands of these labelled compounds and also of labelled products formed from them. This work of Pincus and his group at the Worcester Foundation, USA confirmed that cholesterol was an essential precursor of the steroid hormones (see review by Hechter and Pincus, 1954) and laid the foundations for our understanding of the complexities of these synthetic reactions.

A. BIOSYNTHESIS OF CHOLESTEROL

The elucidation of the sequence of reactions involved in the biosynthesis of cholesterol has provided one of the classical bio-

\[
\text{CH}_3 \quad \text{CH}_2 = \text{C} - \text{CH} = \text{CH}_2 \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \\
\text{CH}_3 \quad \text{C} \quad \text{C} = \text{C} = \text{C} = \text{C} = \text{C} = \text{C} = \text{C} = \text{C}
\]

A. isoprene (\(\text{C}_5\text{H}_{10}\))

B. isoprene unit structure

Fig. 13. The isoprene molecule.

chemical problems of our time. Much of the basic work was performed when the use of radioactive tracers in biology was in its
infancy and consequently the practical difficulties encountered in the analytical procedures were great. Cholesterol, like all steroids, is related chemically and biochemically to the terpenoids which are built up from C5-units called isoprene units in a "head to tail" sequence (Fig. 13). Indeed the terpenoids, which are compounds of plant origin, seem to have identical pathways for the synthesis of the
C₅-units to that found for cholesterol biosynthesis in say, the liver or adrenal gland. These steps require the condensation of C₅-units (acetate) to provide a C₀-unit (mevalonate) followed by the loss of a single carbon atom as CO₂ to leave the C₂-unit (isoprene). This rather complicated sequence of enzymic reactions, beginning with the condensation of acetyl- and acetoacetyl-Coenzyme A, is shown in Fig. 14.

\[
\begin{align*}
\text{CH}_3\text{C} & \equiv \text{CH}_2\text{C}-\text{CH}_2\text{C}-\text{O} - \text{(PP)} \\
\text{IPP} & \\
\text{CH}_2\text{C} & \equiv \text{CH}_2\text{C}-\text{CH}_2\text{C}-\text{O} - \text{(PP)} \\
\text{DMAPP} & \\
\text{CH}_2\text{C} & \equiv \text{CH}_2\text{C}-\text{CH}_2\text{C}-\text{O} - \text{(PP)} \\
\text{geranyl pyrophosphate} &
\end{align*}
\]

Fig. 15. Stages in cholesterol synthesis (C₅-unit synthesis).

In isopentenylpyrophosphate (IPP) we have achieved the synthesis of the basic C₇-unit which can now be used to build up larger units in multiples of 5-carbon atoms (Fig. 15). Thus, a C₁₅-unit (farnesyl pyrophosphate) can be synthesized and condensation of two of these C₁₅-units is thought to result in the C₃₀-unit squalene (Fig. 16A). The structure of squalene may be written in a slightly different manner (Fig. 16B) which shows that it can have a somewhat “steroidal” appearance and, by means of experiments involving the use of ¹⁴C-acetate labelled at either the methyl or carboxyl position, the origin of the various carbon-atoms has been established (Fig. 17). When the
same exercise was applied to the completed cholesterol molecule, the results were similar.

Following the complex electron transfer sequence that actually "closes" the ring system one elimination and four migration reactions occur to give the first sterol lanosterol. These are migration of H-atoms from C-17 to C-20 and C-13 to C-17 together with CH₃-groups from C-14 to C-13 and C-8 to C-14, and elimination of the H-atom on C-9 (Fig. 18).

**B. CHOLESTEROL METABOLISM**

Cholesterol is the precursor of all the steroids manufactured by the human body. In order to achieve the synthesis of the steroid hormones, the side-chain of the cholesterol molecule must be degraded from eight carbon atoms down to two, i.e. from the C₂₉-sterol to the
C_{21}-steroid, pregnenolone. It is now accepted that a number of distinct pathways, some simple some extremely complex, are implicated in this conversion. Firstly, the side-chain cleavage occurs in the mitochondria of the adrenal cells and involves oxidation by molecular oxygen through the mediation of a recently identified haemprotein termed cytochrome P-450. Studies during the past few years have revealed that mitochondria of adrenal tissue possess an electron transport system, distinct from the classical respiratory chain, that is concerned with hydroxylation of steroids. The system involves sequential interaction between NADPH and a flavoprotein, with cytochrome P-450, the terminal oxidase, responsible for oxygen activation and steroid binding. Secondly, the major route from cholesterol to the hormones includes hydroxylation steps at C-20 and C-22. There is some dispute as to which process takes place first but the sequence of events that is accepted at the present time is given in Fig. 19. Some evidence presented recently suggests that hydroxylation at C-17 may precede the direct formation of 17α-hydroxypregnenolone or even DHA but, even if these pathways do exist, they are certainly of minor quantitative importance.

It was demonstrated during the early studies at the Worcester Foundation that the cholesterol side-chain splitting reactions were rate-limiting in adrenocorticosteroid synthesis and that ACTH controlled the conversion of cholesterol to pregnenolone. This will be discussed later in the section on ACTH action.

C. PREGNENOLONE METABOLISM

Adrenocorticosteroid synthesis

In much of the literature on adrenal steroid biosynthesis, and in many textbooks, the pathways considered to be involved in the
Fig. 20. Synthesis of cortisol and corticosterone.
formation of cortisol and corticosterone from pregnenolone are those shown marked (a) in Fig. 20, involving progesterone as an essential intermediate.

The first step, the conversion of pregnenolone to progesterone involves two enzymic systems present in microsomal preparations of adrenal tissue, a 3β-hydroxysteroid dehydrogenase, oxidizing the 3β-hydroxyl group to a 3-oxo-group in the presence of NAD as a hydrogen acceptor, and an isomerase catalysing the transfer of the double bond from ring B (5–6 position) to ring A (4–5 position) (Fig. 21).

Progesterone can then be metabolized by a series of hydroxylation reactions, in the sequence 17α, 21 and 11β, to form cortisol (Fig. 20). Introduction of a 21-hydroxy group into progesterone to yield DOC appears to preclude subsequent 17-hydroxylation. 11β-hydroxylation of DOC gives rise to corticosterone. The 17α- and 21-Hydroxylase enzyme systems are associated with the microsomal preparations of the adrenal tissue, the 11β-hydroxylase with the mitochondria, all three reactions requiring molecular oxygen and NADPH.

These pathways just described form the "classical" routes for cortisol and corticosterone synthesis. More recent studies, however, now indicate that there is an alternative pathway to cortisol (Fig. 20) which involves 17α-hydroxylation of pregnenolone to form 17α-hydroxyprogrenolone. The 3β-hydroxysteroid dehydrogenase/isomerase enzyme system then converts this 3β-hydroxy-intermediate to the 3-oxosteroid, 17α-hydroxyprogesterone. This alternative pathway is now considered to be the principal one by which cortisol is synthesized in the adult human adrenal gland, and the major proportion of any progesterone formed is converted into corticosterone.

Aldosterone, isolated in 1953, is synthesized and secreted by the zona glomerulosa. Its synthesis requires a mitochondrial 18-hydroxylase and an 18-hydroxysteroid dehydrogenase enzyme system (Fig. 22) and it would seem likely that both these systems are confined to the zona glomerulosa. There has been some considerable controversy as to whether progesterone, DOC or corticosterone is the
steroid that is hydroxylated at carbon-18, although recent studies with human tissue suggest that corticosterone may well be the immediate precursor of the 18-oxosteroids as shown in Fig. 22.

![Fig. 22. Aldosterone biosynthesis from corticosterone.]

Aldosterone exists in solution mainly as a form referred to as the 18,20-hemiacetal, also illustrated.

**D. ANDROGEN AND OESTROGEN SYNTHESIS**

The available evidence suggests that the normal human adrenal synthesizes and secretes only trace amounts of testosterone, the hormone produced in relatively large amounts by the testis. Furthermore, little if any of the oestrogenic hormones oestrone or oestradiol-17β are secreted, although it has been known for some time that enzymes similar to those capable of androgen and oestrogen synthesis

![Fig. 23. Testosterone synthesis.](image)

in the testis or ovary are present in limited amount in the normal adrenal. However, it would seem that any disturbance of these controlled biosynthetic pathways in various pathological conditions can result in increased secretion of androgen and oestrogen. Many adrenal tumours secrete large amounts of either androgen or oestrogen.

C.M.S. 3—7
Early studies with testicular tissue indicated that C$_{19}$-steroids were formed from 17a-hydroxyprogesterone, androstenedione being formed first and subsequently being reduced to testosterone (Fig. 23). A similar pathway by which 17a-hydroxyprogesterone can be metabolized is available in the adrenal cells, but at the same time the sidechain can be removed from 17a-hydroxyprogrenolone to give a C$_{19}$-steroid with a 3β-hydroxyl group—the compound dehydro-

\[
\text{DHA SULPHATE} \rightarrow \text{DHA} \rightarrow \text{ANDROSTENEDIONE} \rightarrow \text{TESTOSTERONE}
\]

\[
17\alpha\text{-HYDROXYPREGNENOLONE} \rightarrow 17\alpha\text{-HYDROXYPROGESTERONE}
\]

\[
\text{PREGNENOLONE} \rightarrow \text{PROGESTERONE}
\]

Fig. 25. Androgen synthesis.

epiandrosterone (DHA). DHA is converted to androstenedione by a 3β-hydroxysteroid dehydrogenase/isomerase enzyme system and by another pathway can be sulphated to give rise to DHA sulphate

(Fig. 24), one of the major secretory products of the human adrenal gland. It has still to be established whether the 17a-hydroxyprogrenolone → 17a-hydroxyprogesterone → androstenedione pathway or that involving DHA is the principal route involved in androgen synthesis in the adrenal gland. Although androstenedione is secreted
by the human adrenal gland, only trace amounts of testosterone are formed and the formation from androstenedione of 11β-hydroxyandrostenedione which is also secreted in small amounts, may well be the preferred pathway of metabolism, thereby forming some mechanism by which the synthesis of testosterone by the adrenal is controlled (Fig. 25).

Oestrogens are synthesized from C19-steroids via the reactions shown in Fig. 26. The enzymes concerned in promoting these reactions are present in human adrenal tissue but have little activity compared with the enzyme systems present in oestrogen-secreting tissues such as the ovary and placenta.

VI. Functional Zonation of the Human Adrenal Gland

Since the early histological studies in the latter part of the nineteenth century, when attention was first drawn to the division of the adrenal cortex into different layers of cells, there have been numerous theories attempting to correlate this histological structure with function. One early theory suggested that cortical cells are formed in the subcapsular region from which they migrate centripetally into the zona fasciculata. The latter was designated the “secretory zone”, the clear cell synthesizing and secreting all the steroid hormones of the adrenal gland. Cells then moved to the zona reticularis where they degenerated to be removed by the extensive vascular channels of that region of the cortex.

This "migration theory" was replaced by the "zonation theory", the stimulus for which was provided by the observation that atrophy of the fascicular and reticular tissue, but not glomerular tissue, occurred after hypophysectomy. It was suggested that there was a division of the secretory function of the cortex between the zona glomerulosa on the one hand, and the zona fasciculata and zona reticularis on the other, with an unequal dependence on the pituitary. It has now been well substantiated that the cells of the zona glomerulosa are responsible for the synthesis and secretion of aldosterone, the mineralocorticoid, and that ACTH has only a minor effect on aldosterone synthesis and secretion. From histochemical studies, it was considered that the zona fasciculata was the site of formation of the adrenocorticosteroids, cortisol and corticosterone, and that the cells of the zona reticularis synthesized the androgens, each cell type thereby playing some role in the overall capacity of the cortex to synthesize and secrete the various steroid hormones.

Following from this theory, the possibility that the compact cells of the zona reticularis of the human adrenal cortex may be important
in the synthesis of cortisol and corticosterone was suggested from studies on the action of ACTH in the adrenal gland (see review by Symington, 1962). The most significant histological changes seen in the cortex after administration of ACTH occur at the border region between the zona fasciculata and zona reticularis. The clear cells adjacent to the reticular tissue become depleted of lipid, and acquire the histochemical characteristics of compact cells (Fig. 27). It appeared that the stimulated gland had more compact cells than the “resting” gland, and experiments with steroid biosynthetic enzyme systems in these stimulated glands indicated that they were more active than the unstimulated tissues. This led to the presumption that the compact cells could be responsible for the synthesis and secretion of all adrenal steroids and the clear cell was merely a relatively inactive cell storing cholesterol. Further studies with separated tissue from the zona fasciculata and zona reticularis indicated that both types of cells possessed a similar steroid biosynthetic capacity, being capable of synthesizing and secreting cortisol, corticosterone and also the C_{19}-steroid 11β-hydroxyandrostenedione. It is interesting, however, that recent observations have indicated that the sulphation of DHA occurs only in the compact cell, and that it is primarily the clear cell that responds to ACTH under in vitro conditions, to synthesize more cortisol and corticosterone from the stored cholesterol ester.

It has been suggested, therefore, that the fascicular and reticular zones should be considered as a single functional unit, the histo-
logical appearance of the cell-type merely reflecting the degree of activity. It would appear that with the exception of aldosterone, the compact cell actively synthesizes and secretes adrenal steroids during normal non-stressful activity. A large proportion (~60%) of the steroid hormones leaving the adrenal are formed from precursors, presumably cholesterol or its esters circulating in the plasma, and the compact cell probably derives most of its precursor material from this source. Following ACTH stimulation (conditions of stress) the cholesterol in the clear cells adjacent to the zona reticularis provides precursor for immediate steroid hormone formation. Although the clear cell is the principal storage site for cholesterol, it may also contribute to the overall hormone production of the gland, but at a relatively lower rate. Such concepts correlate well with morphological studies on the adrenal gland which indicate that, because of the peculiar vasculature of the cortex, ACTH enters the gland and passes through the blood vessels of the zona fasciculata to the border zone and eventually into the zona reticularis. The blood flow (Fig. 5) controlled by the musculature of the venous system probably directs the effective action of ACTH to the cells of the border zone. The significance of the synthesis of DHA sulphate by the zona reticularis must remain a problem for further studies.

VII. Adrenal Steroid Metabolites

Although the identities of the various steroids secreted by the adrenal gland have been known for some considerable time, methods for the routine determination of the individual compounds have only been developed recently. A great deal of our information on adrenal function has been derived from the study of the urinary metabolites of these adrenal steroids, and it is necessary to describe briefly the compounds that are involved. Cortisol is metabolized in the liver, and to a lesser extent in other extra-adrenal tissues, in various ways which are shown in Fig. 28. Reduction of the double bond and of the carbonyl group in ring-A results in the formation of a number of isomers of tetrahydrocortisol (a), which together with the subsequent oxidation of the 11β-hydroxyl group produces similar isomers of tetrahydrocortisone (b) (Fig. 28) of which the 3α, 5β isomer is the principal metabolite of cortisol found in urine. Further reduction of the C-20 carbonyl group (c) gives rise to the cortols (from tetrahydrocortisol) and the cortolones (from tetrahydrocortisone). These various reactions occur sequentially and all of these products appear in the urine in different proportions. These metabolites, therefore, together with all the 17α-hydroxy steroid biosynthetic intermediates
Fig. 28. Metabolism of cortisol (liver).

Most of the C₁₉-steroids synthesized and secreted by the adrenal gland, DHA sulphate, 11β-hydroxyandrostenedione and androstenedione are eventually excreted in the urine and can be determined together as a group of steroids all possessing a 17-oxo group, by a colorimetric procedure referred to as the Zimmermann reaction. This group of steroids is termed “the 17-oxosteroids” or in the older literature as “the 17-ketosteroids”. A small proportion (approximately 10%) of the tetrahydrocortisone and tetrahydrocortisol is degraded in the liver (Fig. 28d) to such compounds as 11-oxaetio-
cholanolone (Fig. 12d) which would thus be determined as one of the 17-oxosteroid group.

VIII. CONTROL OF STEROID SECRETION

Glucocorticoid secretion is controlled by the concentration of ACTH in circulating plasma and a complicated chain of interactions is involved in the regulation of both ACTH and cortisol synthesis and release. Figure 30 shows how the mechanism works. Stress of any kind, which includes emotional upset, infection, hypoglycaemia or injury acts on the central nervous system inducing a stress reaction which is transmitted by neural pathways to the hypothalamus. This organ has the ability to synthesize under appropriate stimuli a series of substances known as releasing factors (see chapter 24 for a full account). Under the stimulation of stress, synthesis of a compound known as corticotrophin releasing factor (CRF) is greatly increased, CRF acting on the anterior lobe of the pituitary gland to cause secretion of ACTH into the bloodstream. The raised plasma concentration of ACTH subsequently stimulates the cells of the adrenal cortex to synthesize more cortisol. Finally, cortisol passes back via the bloodstream to the hypothalamus and depresses the synthesis of CRF. Thus the circle is complete with delicately balanced concentrations of both ACTH and cortisol mutually controlling the other's plasma levels. This is known as a negative feedback system.

It is interesting that more recent studies indicate that ACTH may have some controlling influence on the hypothalamic release of CRF and that cortisol may also directly affect the synthesis and secretion of ACTH by the pituitary. These mechanisms are referred to as the "short feedback" controls.

ACTH is a straight chain polypeptide comprising 38 amino acids. Until recently the only available source of ACTH was the pituitary glands of animals and highly purified preparations are widely avail-
gland into the bloodstream, and the available evidence suggests that this release may be necessary for increased steroid synthesis and secretion to occur.

B. LONG-TERM ACTION OF ACTH

This description is used to include effects of ACTH stimulation that are not manifest within minutes but take perhaps several hours or even days to become apparent. Figure 4 shows the typical histological appearance of the adrenal cortex with its two major zones, the zona fasciculata and the zona reticularis. Under the prolonged influence of ACTH, changes in the histological appearance of the zona fasciculata cells at the border region with the zona reticularis become apparent. Initially lipid disappears in a characteristic localized fashion (focal depletion) but eventually in cases of longer stress or ACTH administration the cells at the border region all become depleted of lipid (Fig. 27) and become indistinguishable from the compact cells of the zona reticularis. If the stimulus is not removed, the process continues until all the zona fasciculata cells become compact in type. The morphological change from clear to compact in type is accompanied by other changes at the subcellular level. The moving border zone seems to be marked by high steroid 11β-hydroxylase activity as well as 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase activities. The new compact cells show increased histochemical staining for alkaline and acid phosphatase together with greatly increased RNA concentration and content of mitochondria and lysosomes. In fact, they become indistinguishable from normal compact cells of the zona reticularis. When the stress or source of ACTH is removed, the gland reverts to normal but this process again starts at the original border region so that from a completely depleted gland returning to normal we can observe an interesting intermediate stage in which two regions of compact cells are separated by the regenerating clear cells of the new zona fasciculata (Symington, 1969).

The possible effect of ACTH on the synthesis and secretion of aldosterone has for a long time been a subject of considerable interest. It seems that ACTH can stimulate aldosterone secretion by the zona glomerulosa, but only for a restricted period of time. Increased plasma potassium levels can also promote aldosterone synthesis and release although the electrolytes probably play only a minor role in the direct control of hormone production. There is now considerable evidence to indicate that the primary mechanism concerned with the regulation of aldosterone production and thereby sodium balance is associated with the kidney. A compound renin is
released from the kidney when the renal perfusion pressure falls. Renin promotes the formation of angiotensin II from angiotensinogen produced by the liver which has a direct action on the zona glomerulosa to increase aldosterone secretion. This results in an increase in sodium and water retention with a concomitant restoration of renal perfusion. Angiotensin II also appears to have an effect on the renal tubules.

IX. DISORDERS OF THE ADRENAL CORTEX

A. HYPOCORTICALISM

In 1855 Addison described a condition due to tuberculosis of the adrenal cortex. Tuberculosis was a common endemic disease in the United Kingdom at that time but with the advent of antibiotic treatment for the disease its incidence has happily been much reduced and classical Addison’s disease of tuberculous origin is now also uncommon. However, recent investigations in human endo-

<table>
<thead>
<tr>
<th>Table 2. Causes of hypocorticalism.</th>
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<tbody>
<tr>
<td><strong>Primary Adrenal</strong></td>
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<tr>
<td>(a) Adults</td>
</tr>
<tr>
<td>1. Addison’s disease</td>
</tr>
<tr>
<td>2. Adrenal haemorrhage (Waterhouse-Friderichsen syndrome)</td>
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<tr>
<td>3. Contralateral adrenal atrophy (Cushing’s syndrome due to neoplasm)</td>
</tr>
<tr>
<td>(b) Children</td>
</tr>
<tr>
<td>1. Addison’s disease</td>
</tr>
<tr>
<td>2. Adrenal haemorrhage (Waterhouse-Friderichsen syndrome)</td>
</tr>
<tr>
<td>3. Congenital lesions:</td>
</tr>
<tr>
<td>(i) Congenital adrenal hypoplasia—cytomegalic or karyomegalic type</td>
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<tr>
<td>(ii) Congenital adrenal hyperplasia (adrenogenital syndrome)</td>
</tr>
<tr>
<td>(iii) Congenital lipoid hyperplasia</td>
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<td>(iv) Adrenal cyst</td>
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<table>
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<tr>
<th><strong>Primary Hypothalamic-Pituitary</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Adults</td>
</tr>
<tr>
<td>1. Simmonds’ disease</td>
</tr>
<tr>
<td>2. Sheehan’s syndrome</td>
</tr>
<tr>
<td>3. Iatrogenic (steroid-induced)</td>
</tr>
<tr>
<td>(b) Children</td>
</tr>
<tr>
<td>1. Structural—encephalic, cyclopic</td>
</tr>
<tr>
<td>2. Congenital adrenal hypoplasia—encephalic type</td>
</tr>
</tbody>
</table>

(see Symington, 1969).
crinology have revealed that hypocorticalism has a number of other origins (see Table 2). Sudden withdrawal of corticosteroid therapy after prolonged treatment may produce the symptoms of adrenal insufficiency (iatrogenic) and this has become one of the most common sources of the problem as the use of steroid therapy has increased.

Adrenal insufficiency may have its primary cause in the adrenal cortex itself or in the hypothalamic-pituitary axes (p. 103) in the brain. Table 2 gives a summary of these causes and shows how they may be simply classified. It follows that if the hypothalamic-pituitary complex is either absent (as in anencephaly) or, if the pituitary has been destroyed by disease (Simmonds' disease, Sheehan's syndrome), then the source of ACTH which is required to maintain and stimulate the adrenal cortex must also be absent and that the gland will atrophy. Similar circumstances must prevail if a patient is subjected to prolonged corticosteroid therapy which suppresses the release of ACTH and this therapy is then suddenly withdrawn. The symptoms of hypocorticalism are all due directly or indirectly to insufficient circulating levels of adrenocorticoids (natural or synthetic). There is a general picture of prostration, vomiting, excessive fatigue, muscle weakness and loss of weight. In chronic cases the skin and mucous membranes in the mouth become dry and pigmented and this may be accompanied by low sodium and high potassium levels in the plasma although these latter features are not necessarily present. In the acute form prostration and vomiting may be rapidly followed by death unless steroid therapy is instituted immediately.

Since hypocorticalism of adrenal origin may be considered as an inability of the adrenal cortex to respond to stress, convenient and rapid means of diagnosis have been evolved which include the i.m. administration of a small dose of the synthetic ACTH Synacthen, (0-25 mg) and the determination of plasma cortisol values before and 30 minutes after the injection. The normal range of values is 5-25 μg cortisol per 100 ml. plasma and, in the normal subject the resting value approximately doubles in the 30 min. following the Synacthen injection. The patient suffering from hypocorticalism of adrenal origin will show a poor or insignificant response to the stimulus. This test, however, should be regarded simply as a preliminary one and confirmation of the diagnosis requires more prolonged stimulation with ACTH over a period of at least three days and the determination of the urinary output of corticosteroid metabolites (17-hydroxycorticosteroids) during this time.

Pituitary function may also be assessed by means of a drug called Metopirone (Metyrapone) (Fig. 31) which selectively inhibits
adrenal 11β-hydroxylase. It thus interferes with the normal negative feedback system of the hypothalamic-pituitary-adrenal axis by reducing the quantity of cortisol synthesized and causing increased secretion of ACTH in the normal subject. This excess ACTH stimulates the adrenal gland to produce a large amount of 11-

![Chemical structure of cortisol and metopirone](image)

Fig. 31. (a) Dexamethasone, (b) Metopirone (metyrapone).

deoxycortisol (since 11β-hydroxylation is blocked) which is excreted as tetrahydro-11-deoxycortisol in the urine (cf. Fig. 28) and can be measured as a member of the 17-hydroxycorticosteroid group in the usual way. The test is normally carried out by observing the increase in urinary 17-hydroxycorticosteroid excretion following administration of 750 mg metopirone every 4 h for 24 hours. Tests have also been devised that involve the measurement of 11-deoxycortisol in the plasma following a similar dose regime. In the patient whose hypothalamic-pituitary system is unable to respond to low plasma cortisol concentration no stimulation will be observed.

Adrenal haemorrhage (Waterhouse-Friderichsen syndrome) is a relatively rare condition and although it is often classified as a hypocortical condition there is really little basis for this view. At post-mortem, various types of adrenal haemorrhage (adrenal apoplexy) may be seen leading to the supposition that acute adrenal insufficiency might be the cause of death. However, determination of plasma cortisol values on these patients during the acute phase of the condition or subsequent to death has revealed that the patient dies “in the midst of plenty”. A number of types of the syndrome have been recognized: (i) the classical Waterhouse-Friderichsen syndrome is seen in those with an apparently severe pulmonary infection, accompanied by a widespread purpuric rash, pyrexia and convulsions. This is followed by hypotension and death, the onset of which may be extremely rapid; (ii) A group presenting with severe abdominal pain, and vomiting resulting from peritoneal rupture of the adrenal; (iii) The third group is even more difficult to diagnose since the recognition of adrenal haemorrhage is only made at post-mortem after a history of severe infection or prolonged stress following accidental burning, or even surgery.
It would seem from the evidence available at present that these patients do not die of adrenal insufficiency but from acute endotoxic shock.

B. CONGENITAL ADRENAL HYPOPLASIA

This is an extremely rare condition in cases other than those associated with absence of the anterior pituitary or the hypothalamus such as anencephaly. The adrenal glands of these children are very small (their combined weight may be as low as 0.26 g) and have a characteristic histological appearance. The gland has a uniform pattern consisting of large compact cells, several times the size of normal adrenal cells and is said to be of the cytomegalic type of congenital adrenal hypoplasia. The lesion may be familial since the condition was found in three brothers of one family and in two children of another. There is a history of adrenal insufficiency dating from birth and of onset of vomiting after a few days of life. Dehydration and pigmentation commonly follow, and the condition rapidly terminates in death unless it is recognized and steroid therapy commenced.

G. CONGENITAL ADRENAL HYPERPLASIA

This defect is an inborn error of metabolism and is due to the partial (or even total in the most severely affected cases) deficiency of one or other of the enzymes required to synthesize cortisol or aldosterone in the adrenal cortex. The condition is relatively rare but of those recorded the commonest type is that of C$_{21}$-hydroxylase deficiency. This results in a relative inability to synthesize cortisol or aldosterone and in the mildly affected cases, the subject's feedback mechanism can compensate by secreting a higher than normal amount of ACTH. The higher concentration of ACTH in the plasma results in bilateral adrenal hyperplasia, and the larger more active glands can then produce adequate quantities of cortisol and aldosterone. However, as a "by-product" of this process, there is a consequent increase in the adrenal concentration of 17a-hydroxyprogesterone (Fig. 20). Its metabolite, pregnanetriol (5β-pregnane-3a,17α,20α-triol) (Fig. 12) is excreted in the urine in abnormally large amounts, making the condition comparatively easy to diagnose. Unfortunately, the abnormally high amounts of 21-deoxysteroid precursors in the adrenal can also be transformed to the C$_{19}$-steroids DHA, androstenedione and testosterone. Consequently virilism is a common finding in female infants with this defect. Characteristic changes may be observed which vary from an enlarged clitoris to a phallus complete with urethra. The labia may also be enlarged or even fused.
to form a scrotal sac with the vagina opening into the urethra. Hence the condition is also commonly known as the adrenogenital syndrome and it is sometimes impossible to determine the child's true sex without the results of chromosomal studies. In male children the defect may go unnoticed for a time but eventually precocious sexual development, premature puberty, appearance of hair and adult-type muscle development may be observed—the infant Hercules.

In the most severe cases, however, the metabolic block is so complete that the natural feedback system cannot compensate and shortly after birth the child may go into adrenal crisis with vomiting, dehydration and loss of salt with consequent hypertension being the early symptoms. Again, without correct treatment, death will inevitably ensue within a relatively short time.

As mentioned above, other types of deficiency have been observed and indeed lack of any enzyme involved in the transformation of cholesterol to the completed adrenal hormones may give rise to bilateral congenital adrenal hyperplasia. A 20,22-desmolase defect producing an inability to convert cholesterol to pregnenolone has been implicated in the condition known as congenital lipoid hyperplasia which results in typically cholesterol-rich adrenal glands. Deficiencies of 11β-, 17α- and 18-hydroxylases and of 3β-hydroxysteroid dehydrogenase/isomerase have also been described. Again, reviews by Symington (1969) and Visser (1966) could be referred to for a more detailed treatment of the subject.

D. HYPERCORTICALISM

The symptoms of overproduction of cortisol were described by Harvey Cushing in 1932. Typical features are plethoric moon-face, obesity with a curious distribution of fat on the shoulders known as "buffalo-hump", osteoporosis, low resistance to infection, ease of bruising, in certain cases pigmentation of skin and mucous membranes, virilism (rarely feminization), hypertension, cardiovascular disease and occasionally purple striae on the trunk of the body. The condition is commonly known as Cushing's Syndrome and is due to excess circulating levels of cortisol. It can be produced (iatrogenic) by overadministration of either corticosterone (natural or synthetic) or ACTH but the natural incidence of the disease is due to hyperactivity of the anterior pituitary resulting in a subsequent increased secretion of ACTH and causes bilateral adrenal hyperplasia. Cushing's Syndrome can also be due to the presence of an adrenal tumour, capable of secreting large amounts of cortisol. Measurement of urinary 17-hydroxycorticosteroids reveals values in excess of the appropriate normal range. Cortisol secretion rates (mg/24 h)
may also be determined by oral administration of \(^3\)H-labelled cortisol, collection of the urine throughout the following 24 hours and isolation of the specific cortisol metabolite, tetrahydrocortisone (Fig. 28). Plasma cortisol concentration normally shows a circadian rhythm with the low point occurring late at night and the peak in the early morning just before wakening (Fig. 32). In Cushing's Syndrome this pattern is usually absent, providing a useful means of distinguishing the disease from simple obesity in which raised plasma cortisol values are often observed, but in which the circadian rhythm is maintained. It is an obvious practical necessity to be able to distinguish between Cushing's Syndrome of pituitary and of adrenal origin and, if an ACTH stimulation test is carried out, those patients with bilateral adrenal hyperplasia due to hyper-pituitarism tend to show an exaggerated response, since the adrenal glands in such cases are hypersensitive to ACTH stimulation. On the other hand, most adrenal tumours (adenomas and carcinomas) do not respond greatly to ACTH stimulation since, in general, tumours tend to be functionally autonomous. Exceptions to this rule, however, have been recorded on a number of occasions and the evidence suggests that the type of cell present in the tumour dictates its responsiveness to stimulation. Adenomas comprising mainly "clear" type cells tend to respond whereas those with mainly "compact" type cells do not, which is in line with the behaviour of the normal cells of either type described earlier. The most important single test

![Fig. 32. Diurnal variations of plasma cortisol concentration.](image-url)
for the diagnosis of Cushing's Syndrome is the dexamethasone suppression test. Dexamethasone is a synthetic steroid (Fig. 31a) which has been developed by the pharmaceutical industry as a more active glucocorticoid than cortisol (it is 30 times more active than cortisol) but without the sodium-retaining properties of the natural hormone. It is also capable of influencing the hypothalamus in exactly the same fashion as the natural hormone. The test is normally carried out over a period of five days. Dexamethasone is administered orally at two dose levels and 24-hour urine specimens collected throughout. The urinary 17-hydroxycorticosteroid and 17-oxosteroid levels are then measured. Figure 33 shows the typical responses of various types of case, (a) normal (b) obesity (c) hyperpituitarism and (d) adrenal tumour. The normal 17-oxosteroid levels show a similar pattern but in the case of adrenal carcinoma extremely high 17-oxosteroid values (several hundred mg/24 h) can be observed. This is thought to be due to the inability of the dedifferentiated carcinoma cells to perform the more complex hydroxylations required to synthesize corticosteroids so that the relatively simple steroid DHA is secreted in large quantities. The excretion of these large quantities of DHA and its metabolites in the urine is thus often a useful indication in distinguishing between a large benign adenoma and a true carcinoma when interpreting X-ray evidence.
THE ADRENAL CORTEX

References


II. INVESTIGATIONS RELATING TO CANCER OF THE BREAST.

A. STEROID METABOLISM IN BREAST TUMOUR TISSUE.
Short Communications

Steroid Metabolism by Human Breast Tumours

By D. Jones, E. H. D. Cameron and K. Griffiths
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(Received 12 December 1969)

Extensive studies relating urinary 11-deoxy-17-oxo steroids and prognosis in patients with advanced breast carcinoma (Bulbrook, 1965) have directed attention to the major source of urinary C19 steroids, DHA5-sulphate, which is secreted by the adrenal and is present in plasma in high concentration (Yamaji & Ibayashi, 1969). Its metabolism by extra-adrenal tissue is therefore of particular interest. Adams & Wong (1968a, 1969) demonstrated in breast tumour tissue the presence of steroid-metabolizing enzymes capable of converting cholesterol into pregnenolone, 17α-hydroxyprogesterone into androstenedione (androst-4-ene-3,17-dione) and testosterone into oestradiol [oestr-1,3,5(10)-triene-3,16α,17β-triol]. This communication now presents results from studies similar to those of Adams & Wong (1968a), together with preliminary data from the perfusion of [7α-3H]DHA sulphate through human breast tumours in situ. Important differences, however, were found in the nature of some of the metabolites isolated, which bear fundamentally on the concept of hormone action in the breast.

Tumour tissue was maintained at 0°C after removal from the patient until prepared for incubation 30 min later. The tissue was thinly sliced, the slices were chopped into smaller segments with a razor blade and portions were incubated with radioactive steroids (Table 1) in Krebs–Ringer bicarbonate-glucose medium (Cohen, 1957) (12.5 ml/g of tissue), shaking at 37°C in an atmosphere of O2 + CO2 (95:5) for 2 h without addition of cofactors. Purity of incubated precursors was checked by isotope-dilution analysis before incubation. Reactions were stopped by addition of acetone, and known quantities in the range 300–500 μg each of the non-radioactive carrier steroids, shown in Table 1, added in ethanol. Mixtures were homogenized in a Silverson mixer and separated into neutral, phenolic and conjugated fractions as described by Fahmy, Griffiths, Turnbull & Symington (1968).

Perfusion studies were performed on tumours localized in the medial half of the breast, supplied predominantly by the internal mammary artery, which was exposed during exploration of the second or third intercostal space for lymph-node biopsy. A ligature was tied proximal to the point of injection, and [7α-3H]DHA sulphate (approx. 33 nmol, specific radioactivity 0.36 μCi/nmol) administered as a single injection within 30 s. After removal of the breast, the tissue was dissected from surrounding tissues, maintained at 0°C until transferred to the laboratory, and sliced into 20 ml of acetone, to which 300 μg of carrier non-radioactive steroids was then added (Table 1). The mixture was homogenized, and the steroids were fractionated as before and separated on thin layers of silica gel HF254/366 (E. Merck A.G., Darmstadt, Germany) (Fahmy et al. 1968). The following solvent systems were used, the proportions being by volume: I, chloroform-acetone (37:3); II, cyclohexane-ethyl acetate (7:3); III, benzene-ethyl acetate (9:1); IV, cyclohexane-ethyl acetate–ethanol (9:9:2); V, hexane–ethyl acetate (1:1); VI, cyclohexane–ethyl acetate (9:11); VII, ethyl acetate–2-methylpropan-2-ol–5x-NH3 (50:41:9); VIII, chloroform–methanol–water (187:12:1). Steroids were measured after elution and derivatives were prepared as described by Griffiths, Grant, Browning, Cunningham & Barr (1966). Radioactivity was measured by a Nuclear–Chicago liquid-scintillation spectrometer (model 6860). The mean of the specific radioactivities was used to calculate the percentage conversion from the precursor incubated or perfused.

16α-Hydroxy-DHA was analysed by g.l.c., after conversion into the bistrimethylsilyl ether, on a 150 cm column of 1.5% QF–1 on Supasorb AW–HMDS (100–120 mesh) (British Drug Houses Ltd., Poole, Dorset, U.K.) at 183°C. 5x-Dihydrotestosterone (17β-hydroxy-5x-androstan-3-one) and its derivatives were determined on a 150 cm column of 1% XE–60 on Gas–Chrom Q (Applied Science Laboratories, State College, Pa., U.S.A.) at 200°C.

Evidence for the identification of the metabolites formed in the incubation and the percentage conversions are given in Table 1. Extensive metabolism of DHA sulphate was shown to occur,

Abbreviation: DHA, dehydroepiandrosterone.
Table 1. Evidence for the identification of metabolites formed during the incubations

Experimental details are given in the text. Percentages of the total radioactivity incubated are given in parentheses. The perfusion studies were carried out (a) with 12.5 μCi of DHA sulphate and (b) with 11.8 μCi of DHA sulphate. Specific radioactivities are given as d.p.m./μmol. Values in parentheses indicate the percentages of the original precursor perfused found in the metabolite; values in square brackets refer to the percentages of radioactivity taken up by the tumour that was found in the metabolite.

<table>
<thead>
<tr>
<th>Compound isolated and derivatives formed</th>
<th>Solvent system used for purification</th>
<th>Radioactive steroids incubated and specific radioactivities of isolated carrier steroids (d.p.m./nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[7α-3H]DHA sulphate</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>VII</td>
<td>34008</td>
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<tr>
<td>3β-Acetoxyandrost-5-en-17-one</td>
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Perfusion studies

(a) 16.2 g of tumour tissue extracted, providing 260000 d.p.m. in acetone fraction

<table>
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<tr>
<th>Compound isolated and derivatives formed</th>
<th>Solvent system used for purification</th>
<th>Radioactive steroids incubated and specific radioactivities of isolated carrier steroids (d.p.m./nmol)</th>
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<tr>
<td>DHA</td>
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<td>Testosterone</td>
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<td>290</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>II</td>
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</table>

(b) 14.2 g of tumour tissue extracted, providing 49600 d.p.m. in acetone fraction
although androstenedione and testosterone were formed only in low yields, confirming the rather more tentative evidence of Adams & Wong (1968a), who demonstrated the release of labelled sulphate on incubation of DHA $[^{35}S]$sulphate with homogenates of breast tumours. No evidence was obtained for 16α-hydroxy-DHA sulphate or androst-5-ene-3β,17β-diol sulphate formation. DHA and androstenediones were converted into 16α-hydroxy-testosterone, although no evidence for oestriol synthesis was obtained even after incubation with testosterone. The isolated carrier oestradiol was found labelled after extensive chromatography, but the label disappeared on methyl ether and acetate formation. Some evidence for oestrone formation was found. This is contrary to the observation of Adams & Wong (1968a) using microsomal preparations of breast tumour, although they did not prepare derivatives of their oestradiol, and it was suggested by Dao (1969) that contamination of the carrier oestradiol with labelled androst-5-ene-3β,16α,17β-triol might account for their results. 16α-Hydroxy-DHA was added as carrier to the incubation of tumour tissue with DHA sulphate. Extensive chromatography in systems I, IV and VIII with radio-scanning indicated the carrier to be labelled. It was then acetylated, run in systems I and II, hydrolysed and re-run, and the specific radioactivity was determined and a conversion value of 0.02% was calculated from this. Testosterone was transformed into 5α-dihydrotestosterone in relatively large yields by the human breast tumour tissue, a conversion demonstrated in rat mammary fibroadenoma by King, Gordon & Helfenstein (1964). 5α-Dihydrotestosterone, currently of interest in relation to its androgenic action in prostatic tissue (Bruchovski & Wilson, 1968; Baulieu, Lasnitski & Robel, 1968), also inhibits the growth of the oestradiol-17β-dependent rat mammary fibroadenoma (Huggins & Mainzer, 1957).

The preliminary perfusion studies also showed a limited formation of DHA and androstenedione from DHA sulphate in vivo. The suggestion of Adams & Wong (1968b) that breast tumour metabolism of plasma steroids may account for the abnormal patterns of urinary steroid metabolites received little support from these studies, although such metabolism may provide a specific local steroid environment within the tumour cells on which growth of the tumour is dependent. It is of note that patients with advanced localized breast cancer have lower concentrations of urinary aetiocholanolone than have patients with primary or the advanced generalized type of disease (Gleave et al. 1970).

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The extensive studies of Bulbrook and his colleagues (1, 2, 3) relating 11-deoxy-17-oxosteroids and prognosis in patients with cancer of the breast directed attention to the major source of these urinary C\textsubscript{19} steroids, dehydroepiandrosterone sulfate (DHEA sulfate), which is secreted by the adrenal gland and is normally present in high concentrations in plasma (4). It seemed that abnormalities in the secretion or metabolism of DHEA sulfate could in some way be concerned with the etiology of breast cancer and so, concurrently with Dr. Adams and his colleagues, we in Cardiff commenced a series of experiments designed to assess the capacity of human breast tumor tissue to metabolize various C\textsubscript{19} steroids.

Tumor tissue, which was maintained at 0\textdegree C after removal from the patient until it was incubated, usually 30 min later, was thinly sliced with a razor blade and chopped into smaller segments. Tissue was then incubated with radioactive steroids in Krebs-Ringer bicarbonate glucose medium (12.5 ml/g tissue) shaking at 37\textdegree C, in an atmosphere of O\textsubscript{2}:CO\textsubscript{2} (95:5) for 2 hr. No cofactors were added to the incubation. The arbitrary nature of such in vitro studies makes it difficult to decide the nature and amount of cofactors which could be added, and in their absence we routinely incubate whole cell preparation of endocrine tissues. Purity of the incubated radioactive steroid precursors was checked by isotope-dilution analysis.

Figure 1 shows the metabolites formed and their yields from the incubation in vitro of labeled DHEA sulfate, DHEA, androstene-
Fig. 1. Incubation of labeled steroids with breast tumor tissue.
Transformation of Steroids by Mammary Cancer Tissue

dione, and testosterone with the breast tumor preparations. A preliminary account of these experiments has already been communicated (5).

An observation which Dr. Adams has described (see chap. 8), the deconjugation of DHEA sulfate and its transformation to other steroids including androstenedione and testosterone, was shown with these tissue preparations. Since we obtained no evidence for the synthesis of 16α-hydroxy-DHEA sulfate or androst-5-ene-3β,17β-diol sulfate, we must assume at present that 16α-hydroxy-DHEA is formed from DHEA.

Estrone was synthesized in small yields from both DHEA and androstenedione, although, contrary to the observation of Dr. Adams, no estriol could be detected despite repeated attempts to demonstrate its formation. A radioactive material with a chromatographic mobility identical to that of estriol was formed, but also separated completely from authentic carrier after methyl ether or acetate formation. Dr. Dao (6) has suggested that this "contaminant" may well be the androst-5-ene-3β,16α,17β-triol which is difficult to separate from estriol, and this possibility has now been verified (7). Noteworthy was the demonstrated 16α-hydroxylation of testosterone, also described by Adams and Wong (8).

Of particular interest however, was the formation of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) from testosterone, a compound which was shown by Huggins and Mainzer (9) to inhibit growth of 17β-estradiol-dependent rat mammary fibroadenoma and is currently considered to be the principal androgen within the cells of the prostate gland. The basis for this concept has been the finding that 5α-dihydrotestosterone was preferentially retained by rat prostatic nuclei after the tissue was incubated with labeled testosterone (10) or after testosterone was administered in vivo (11). Baulieu, Lasnitzki, and Robel (12, 13) have further shown that this compound will stimulate cell division and induce epithelial hyperplasia of the prostate tissue maintained in culture media. King (14) has also demonstrated the transformation of testosterone to 5α-dihydrotestosterone in rat mammary fibroadenoma and adenocarcinoma.

Results from investigations in which labeled steroids were perfused through human breast tumor tissue have tended to confirm these in vitro biosynthetic patterns. Perfusion studies were performed on tumors localized in the medial half of the breast, supplied predominantly by the internal mammary artery, which was exposed
during exploration of the second or third intercostal space for lymph-node biopsy. A ligature was tied proximal to the point of injection and either \((7\alpha^-3\text{H})\) DHEA sulfate (ca. 33 nmole, specific activity 0.36 \(\mu\text{Ci/nmole}\)) or \((7\alpha^-3\text{H})\) androstenedione (ca. 2 nmole, specific activity 5 \(\mu\text{Ci/nmole}\)) administered as a single injection within 30 sec (fig. 2).

After the breast was removed, the tumor was dissected from surrounding tissues and maintained at 0°C until it was transferred to the laboratory, and then the radioactive steroids were analyzed

![Fig. 2. Schematic diagram of the mammary gland illustrating the point of injection of \((7\alpha^-3\text{H})\) DHEA sulfate into the internal mammary artery.](image)

(5). In one experiment, venous blood was collected from the side of the tumor opposite the point of substrate infusion and the radioactive steroids were measured. Results are shown in tables 1 and 2. Total radioactivity extracted from each tumor is listed together with the identity of the metabolites, and the radioactivity associated with each is listed as a percentage of the total.

DHEA sulfate was transformed to DHEA and androstenedione, and carrier testosterone was also labeled, although there was insufficient radioactivity for good identification. Testosterone and \(5\alpha\)-dihydrotestosterone were formed from the \((7\alpha^-3\text{H})\) androstenedione in reasonable yields. In the one experiment—and it must be regarded as a preliminary finding—it was interesting that no \(5\alpha\)-dihydrotestosterone was isolated in the venous blood which was collected. This could mean that \(5\alpha\)-dihydrotestosterone is retained by the tumor cell and is subsequently metabolized to the various saturated \(C_{19}\) steroids.
The close relationship between the metabolism of testosterone by the breast tumor tissue and by the prostate is interesting. Some of the metabolites formed from testosterone by human breast tissue in vitro are shown in Table 3. Table 4 shows the metabolic products from the incubation of testosterone with canine prostatic tissue (16). Similar metabolic activity has been described for human prostatic

### Table 1: Perfusion of Human Breast Tumors with \(7\alpha-^3\text{H}\) DHEA Sulfate

<table>
<thead>
<tr>
<th>Total Radioactivity Isolated (dpm)</th>
<th>Steroid Isolated</th>
<th>Radioactivity Associated with Steroid (as % of total dpm isolated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 260,000 (tissue)</td>
<td>DHEA</td>
<td>3.27</td>
</tr>
<tr>
<td>2. 49,600 (tissue)</td>
<td>DHEA</td>
<td>16.25</td>
</tr>
<tr>
<td>3. 96,000 (tissue)</td>
<td>DHEA</td>
<td>8.88</td>
</tr>
<tr>
<td>4. 14,000 (tissue)</td>
<td>DHEA</td>
<td>1.89</td>
</tr>
</tbody>
</table>

### Table 2: Perfusion of Human Breast Tumors with \(7\alpha-^3\text{H}\) \(\Delta^4\)-Androstenedione

<table>
<thead>
<tr>
<th>Total Radioactivity Isolated (dpm)</th>
<th>Steroid Isolated</th>
<th>Radioactivity Associated with Steroid (as % of total dpm isolated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 29,600 (tissue)</td>
<td>(\Delta^4)-Androstenedione</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>5α-Dihydrotestosterone</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Epitestosterone</td>
<td>0</td>
</tr>
<tr>
<td>2. 47,700 (tissue)</td>
<td>(\Delta^4)-Androstenedione</td>
<td>63.0</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>5α-Dihydrotestosterone</td>
<td>0.43</td>
</tr>
<tr>
<td>3. 19,800 (blood)</td>
<td>(\Delta^4)-Androstenedione</td>
<td>50.4</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>5α-Dihydrotestosterone</td>
<td>0</td>
</tr>
</tbody>
</table>
### TABLE 3: INCUBATIONS OF MINCED HUMAN BREAST TISSUE WITH RADIOACTIVE STEROID SUBSTRATES

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Steroid Isolated</th>
<th>% Radioactivity Associated with Steroid Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4-14C) Testosterone</td>
<td>Testosterone</td>
<td>80.70</td>
</tr>
<tr>
<td></td>
<td>5α-Dihydrotestosterone</td>
<td>5.10</td>
</tr>
<tr>
<td></td>
<td>5α-Androstane-3β, 17β-diol</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>5α-Androstane-3α, 17β-diol</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5α-Androstane-3β, 17α-diol</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5α-Androstane-3α, 17β-diol Trace</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>Epitestosterone</td>
<td>Trace</td>
</tr>
<tr>
<td>(4-14C) Testosterone</td>
<td>Testosterone</td>
<td>86.60</td>
</tr>
<tr>
<td></td>
<td>5α-Dihydrotestosterone</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Androstenedione</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>5α-Androstanediols</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Epitestosterone</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Source: Jones et al. (5).

### TABLE 4: NORMAL DOG PROSTATE MINCE INCUBATED WITH 50 μCi (7α-3H) TESTOSTERONE FOR 1 HR

<table>
<thead>
<tr>
<th>Steroid Investigated</th>
<th>Whole Tissue</th>
<th>Nuclear Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>51.36</td>
<td>1.25</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0.62</td>
<td>0.59</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>19.96</td>
<td>1.30</td>
</tr>
<tr>
<td>5α-Androstane-3α, 17α-diol</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>5α-Androstane-3α, 17β-diol</td>
<td>0.86</td>
<td>0.03</td>
</tr>
<tr>
<td>5α-Androstane-3β, 17α-diol</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Androstane-3β-17β-diol</td>
<td>0.30</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Source: Harper et al. (15).
Transformation of Steroids by Mammary Cancer Tissue

Although 5α-androstanediols were formed by the prostatic tissue, no 5β-epimers were found in these studies; 5α-dihydrotestosterone, epitestosterone, and androstenedione were all synthesized from testosterone. Further, a similar pattern of metabolism was obtained (table 5) by incubating testosterone with nuclei isolated from human breast tumor tissue by procedures described by King, Cowan, and Inman (18). The presence of the 5α-reductase in the breast tumor nuclear preparation is of considerable interest in relation to the observations of Bruchovsky and Wilson (19) that this enzyme is normally found in the nuclei of androgen-responsive tissues. The possibility that 5α-dihydrotestosterone may be concerned in some regulatory mechanism within the breast tumor cell is thus worthy of serious consideration, and the identification of a 5α-dihydrotestosterone-protein receptor in human breast tumor tissue would open up new fields in the endocrinology of breast cancer. The 5α-androstanediols which have been shown to have biological activity in the prostate (12, 13) may also have some role in the breast tissue cell.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Steroid Isolated</th>
<th>% Radioactivity Associated with Steroid Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(7α-3H) Testosterone</td>
<td>Testosterone</td>
<td>88.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epitestosterone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Dihydrotestosterone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3α, 17β-diol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3β, 17β-diol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3α, 17α-diol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3β, 17α-diol</td>
</tr>
<tr>
<td>(7α-3H) Testosterone</td>
<td>Testosterone</td>
<td>95.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epitestosterone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Dihydrotestosterone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3α, 17β-diol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3β, 17β-diol</td>
</tr>
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<td></td>
<td></td>
<td>5α-Androstane-3α, 17α-diol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5α-Androstane-3β, 17α-diol</td>
</tr>
</tbody>
</table>
Two observations may therefore be made from the studies described. First, in vitro and in vivo studies have proved conclusively that breast tumor tissue can synthesize steroid hormones from C19 steroid precursors present in the plasma. That such hormones can be synthesized from endogenous cholesterol, as suggested by Dr. Adams, is also a possibility, but it may be that the pathway from cholesterol → DHEA, excluding pregnenolone and recently shown to be present in endocrine tissues (20), is of more importance. This biosynthetic capacity of the breast tumor tissue could provide a particular intracellular hormone environment on which the growth and metabolism of the tissue depended. The relatively low conversions shown to occur also suggest that the abnormal urinary steroid excretion pattern described by Bulbrook and his colleagues (2, 3) is not the result of extensive steroid metabolism by the tumor tissue. Some of our clinical studies on patients with breast disease in South Wales appear to add further support to this contention.

Estimation of urinary steroids have been made on 177 women with breast disease; 63 patients had benign disease of the breast, proved by excision biopsy of a palpable lump, 30 had primary cancer, and 63 had advanced cancer of the breast subsequently treated by hormone administration or ablation of endocrine glands. Twenty-one patients in the advanced group had disease limited to the breasts, chest wall, regional lymph nodes, or pleural cavity (localized disease), and 42 had osseous or visceral metastases (generalized disease), with or without local disease. Steroid analysis was also performed on 21 patients without known breast disease or malignant disease of any kind who had been admitted to the hospital for elective general surgery. Procedures involved in the analysis of 17-hydroxycorticosteroids and 11-deoxy-17-ketosteroids were described by Gleave (21) in the first Tenovus Workshop on Breast Cancer and have been described elsewhere (22). Comparison of the logarithmic values of etiocholanolone showed that, although there was no difference between the mean values of the normal, benign, and primary groups, there was a difference when the advanced group as a whole was compared with each of the other groups. Regression lines were drawn confirming that patients with advanced disease were different from others and had significantly lower logarithmic levels of etiocholanolone regardless of age (fig. 3). Further examination of this difference indicated that patients with advanced localized disease were distinct from all others and that these patients had significantly lower levels
Transformation of Steroids by Mammary Cancer Tissue

of etiocholanolone when adjusted for age (fig. 4) (22). Thus, it would seem that these abnormal levels of urinary steroids are confined to patients with advanced cancer, and primarily to those with localized disease. Of further interest are the results from the analysis of DHEA sulfate in the plasma from patients in these various groups. Again, the plasma DHEA sulfate was lower in the advanced group than in the others (fig. 5).

![Graph showing regression of log. etiocholanolone on age](image)

**Fig. 3.** All groups of patients over thirty-eight years of age; pooled regression of log. etiocholanolone on age.

![Graph showing regression of log. etiocholanolone on age with subgroups](image)

**Fig. 4.** All groups of patients over thirty-eight years of age; pooled regression of log. etiocholanolone on age (including subgroups).
Fig. 5. Log plasma DHEA-S

Acknowledgments

The authors wish to acknowledge the generous financial support of the Tenovus Organization and also wish to thank Mr. Ralph Marshal, Department of Medical Illustrations, Royal Infirmary, Cardiff, for his help in preparing the illustrations.

References


Measurement of endogenous steroid levels in human breast tumours by mass fragmentography. By D. S. Millington, T. Jones, D. Jenner and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff, CP4 4XX

Although it is now well established that steroid-specific protein-receptors for oestradiol-17β can be demonstrated in a proportion of human breast tumours, the tissue concentrations of this oestrogen in the tissue have not previously been determined. Furthermore, although Adams & Wong (1968) described the presence of various steroid metabolizing enzymes in human breast tumour tissue and this was to a certain degree supported by the studies of Jones, Cameron, Griffiths, Gleave & Forrest (1970), little is known concerning the C₁₉-steroid concentration within this tissue.

The studies now reported describe the use of the technique of combined mass spectrometry and gas chromatography for the analysis of endogenous steroid levels in breast tumours. The instrument, a Varian-MAT 731, was used in the highly specific and sensitive mode of single ion detection (mass fragmentography), a procedure now in use for the analysis of steroids in urine (Maume, Bournot, Lhuguenot, Baron, Barbier, Maume, Prost & Padieu, 1973) and plasma (Adlercreutz & Hunneman, 1973).

The breast tumour tissue was homogenized in acetone and water, centrifuged, the supernatant was evaporated, and the extract partitioned between methanol and light petroleum (b.p. 80-100 °C). The methanolic extract was dried and the residue partitioned between water and ether. The ether extracts were then evaporated, and the residue dissolved in ethanol for storage at −20 °C. A sample was prepared for analysis by removing an aliquot from the solution, corresponding to 500 mg tissue, evaporating the ethanol and treating the residue with bis-trimethylsilylacetamide (15 μl) and light petroleum (10 μl). After standing for 16 h at 20 °C, 1 μl of the solution was injected onto the gas chromatograph-mass spectrometer system.

Although mass fragmentography is normally carried out at low resolution (e.g. 1000, 10% valley) by monitoring simultaneously two or more ions that are characteristic of the compound being assayed, it was elected to monitor only the molecular ion peaks of the compounds of interest at high resolution (10000). While this simpler procedure leads to a tenfold drop in sensitivity on the varian-MAT 731 mass spectrometer, interference from overlapping gas chromatography components having ions with the same nominal mass was minimized. Moreover, sample detection could still be carried out in the concentration range of 0.1–10 ng/μl for both oestrogens and androgens.

Studies with five primary postmenopausal breast tumours gave the following steroid concentrations, the figures respectively, being given in ng/g wet weight tissue: oestradiol-17β, 500, 16, 15,000, 15, 10; oestradiol, —, —; oestrone, 8, 3, 20, 17, —; dehydroepiandrosterone, 35, 15, 30, 25, —; testosterone, 55, 25, 100, 25, 30; androsterone, 75, 10, 55, 50, 100; epiandrosterone, 38, 10, 60, —, 38. 5α-Dihydrotestosterone levels were below 5 ng/g in all cases. Dashes indicate levels below the limit of reliable detection corresponding to 1 ng/g for the oestrogens and 5 ng/g for the C₁₉ steroids.

The generous financial support of the Tenovus Organization is gratefully acknowledged.

REFERENCES


Endogenous Steroid Concentrations in Human Breast Tumours Determined by High-Resolution Mass Fragmentography

By D. Millington, Diane A. Jenner, T. Jones and K. Griffiths
Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XX, U.K.

(Received 4 February 1974)

A pilot study of the endogenous steroid concentrations in human breast tumours was performed. The technique of high-resolution molecular-ion monitoring during combined g.l.c.-mass spectrometry was used to determine oestrone, oestriol-17β and oestriol in concentrations above 1 ng/g wet wt. of tissue, and dehydroepiandrosterone, testosterone, androstenedione (3α-hydroxy-5α-androstan-17-one) and 3β-hydroxy-5α-androstan-17-one in concentrations exceeding 5 ng/g, in extracts of five primary breast tumours.

Several reports have recently appeared on a possible relationship between the presence or absence of oestradiol-17β 'receptor' proteins in human breast tumours and the response of the patient to subsequent endocrine therapy (Engelsman et al., 1973; Maass et al., 1972; Brecher et al., 1971). The techniques used to assay the binding of oestradiol-17β, however, reveal the presence only of unoccupied binding sites, and no attempt has yet been made to determine the number of binding sites occupied by the endogenous oestradiol-17β. At attempt was made by Maass et al. (1972) to relate plasma oestradiol-17β concentration to the receptor capacity of human breast-tumour tissue. Although no relationship was found to exist for plasma oestrogen concentrations below 200 pg/ml, no binding capacity could be detected in patients with plasma oestradiol-17β concentrations exceeding 300 pg/ml. Similar observations were made by Trams et al. (1973) for the binding of oestradiol-17β in human uterine tissue. High plasma oestrogen concentrations were found to relate to low binding capacity.

The relationship between endogenous steroid concentrations and oestradiol-17β receptor concentration in breast tumours has yet to be determined. As a preliminary to this investigation a technique for endogenous steroid analysis has been assessed and the results of the study are now reported. The study involved the technique of combined g.l.c.-mass spectrometry, using the highly specific and sensitive mode of single ion detection (mass fragmentography) at high resolution (10 000, 10% valley). Low-resolution mass fragmentography has previously been used for the analysis of oestrogens in urine (Maume et al., 1973) and in plasma (Adlercreutz & Hunneman, 1973).

Materials and methods

Samples of primary breast-tumour tissue were obtained at mastectomy and stored at −15°C until used. Chopped tissue was then homogenized in 50 ml of acetone, by using a Silverson tissue grinder, centrifuged (800 g, 10 min) and the acetone was decanted. The tissue was resuspended in acetone and the extraction procedure repeated twice more. The combined acetone extracts were dried under vacuum and the residue redissolved in acq. 80% (v/v) methanol (20 ml). This solution was extracted with an equal volume of light petroleum (b.p. 80–100°C) to remove lipids, and the light petroleum was then back-extracted with an equal volume of 80% methanol. The conjugated steroids were removed by redissolving the dried combined aqueous-methanol extract in ether and extracting with an equal volume of water. The water was then back-extracted twice with ether. The final ethereal solution was dried and the residue redissolved in ethanol. This solution and standard steroid solutions were stored at −20°C.

Samples were prepared for g.l.c. analysis by transferring measured volumes of the stock solution into a 1 ml screw-cap vial and treating the residue after evaporation with bis(trimethylsilyl)acetamide (30 μl) and light petroleum (b.p. 60–80°C) (20 μl). The tightly capped vials were left overnight at 20°C before analysis.

Combined g.l.c.-mass spectrometry was carried out by injecting 1 μl of the sample solution into a Varian 2700 gas chromatograph fitted with a Pyrex column (2 m x 2 mm internal diam.) packed with a homogeneous mixture of 3% OV-225 and 1% OV-210 on Supasorb (100–120 mesh) in the ratio 1:2. The injector and detector temperatures were 250°C and 275°C respectively, and the oven temperature was kept at 255°C. The gas chromatograph was operated with a helium flow rate of 25 ml/min, in tandem with a Varian–MAT 731 mass spectrometer, via a two-stage Watson–Biemann separator at a temperature of 250°C. A glass-lined probe at 240°C completed the all-glass introduction system to the ion source, which was operated at 150°C with an electron beam energy of 70 eV (11 A). The assay for
each steroid derivative was then conveniently set up by monitoring the intensity of the molecular-ion peak on a potentiometric recorder at a resolution of 10 000 (10% valley). Under these conditions, the recorder response varied linearly with sample concentration in the range 0.1–10 ng/µl and the detection limit was approx. 10 pg/µl for the oestrogens and 30 pg/µl for the C19 steroids. The two parameters of g.l.c. retention time and high-resolution molecular-ion detection were sufficient to detect and quantify each component regardless of the complexity of the mixture and the presence of overlapping g.l.c. peaks.

Results and discussion

The g.l.c. trace of a standard solution of the silylated oestrogens of particular interest in these investigations is shown in Fig. 1. In each of these derivatives, the molecular-ion peak was also the most intense ion in the mass spectrum under the conditions used, and therefore was the most convenient for single ion monitoring. Fig. 1 also shows the mass fragmentograms obtained by monitoring the signal at m/e 416.257, corresponding to the molecular weight of the bis(trimethylsilyl) ether of oestradiol-17β, in a blank solution (b) and in one of the breast tumours (c). Each of the tumour extracts was successively analysed in this way for oestradiol-17β; then the process repeated for the determination of oestrone, by high-resolution monitoring of the ions at m/e 504.291 and 342.201 respectively. The isomeric C19 steroids dehydroepiandrosterone and testosterone were well separated on the gas chromatograph after trimethylsilylation and were measured simultaneously by monitoring m/e 360.248, and androsterone (3α-hydroxy-5α-androstan-17-one, 3-epi-androsterone (3β-hydroxy-5α-androstan-17-one) and

![Fig. 1. G.l.c.–mass spectrometry of steroids](image)

(a) G.l.c. separation of oestradiol-17β [as bis(trimethylsilyl) ether] (i), oestrone [as tris(trimethylsilyl) ether] (ii) and oestrone (as trimethylsilyl ether and ketone) (iii). Experimental details are described under ‘Materials and methods’. (b) and (c) Mass fragmentograms of oestradiol-17β, monitoring m/e 416.257 at a resolution of 10 000 (10% valley) in a blank solution (b) and in the extract of sample 6 (c) respectively.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Dehydroepiandrosterone</th>
<th>Testosterone</th>
<th>3α-Hydroxy-5α-androstan-17-one</th>
<th>3β-Hydroxy-5α-androstan-17-one</th>
<th>Oestrone</th>
<th>Oestradiol-17β</th>
<th>Oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. M.B.</td>
<td>35</td>
<td>55</td>
<td>75</td>
<td>38</td>
<td>8</td>
<td>300</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2. R.L.</td>
<td>15</td>
<td>25</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>16</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3. M.H.</td>
<td>30</td>
<td>100</td>
<td>55</td>
<td>60</td>
<td>20</td>
<td>15×10³</td>
<td>22</td>
</tr>
<tr>
<td>4. E.D.</td>
<td>25</td>
<td>25</td>
<td>50</td>
<td>&lt;5</td>
<td>17</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>5. E.J.</td>
<td>&lt;5</td>
<td>30</td>
<td>38</td>
<td>38</td>
<td>&lt;1</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6. L.B.</td>
<td>70</td>
<td>&lt;5</td>
<td>100</td>
<td>20</td>
<td>6</td>
<td>45</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 1. Concentrations of endogenous steroids in breast tumours

Results are given in ng/g wet wt. of tissue. In all cases, the concentration of 5α-dihydrotestosterone was below 5 ng/g.
5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) were also assayed simultaneously by monitoring m/e 362.264. Peak heights were determined for all steroids isolated from tumour tissue and related to the peak height of steroid standards from similar fragmentograms.

Results are given in Table 1 for the analysis of the endogenous steroid content of five primary breast tumours removed from postmenopausal women. Sample 6 was a sample of normal breast tissue.

The results of the present observations do not take into account the errors introduced by the extraction procedures. It is clear, however, that the technique of high-resolution mass fragmentography is ideal for the determination, not only of the endogenous oestradiol-17β concentrations in breast tissue, but also of the other steroids that make up the hormonal milieu of the tumour.

We are grateful to the Tenovus Organization, Cardiff, for their very generous financial support. We also thank Mr. R. Blamey, Nottingham General Hospital, and Mr. M. Baum, University Hospital of Wales, Cardiff, for the samples of breast-tumour tissue.

Specificity of steroid binding by the oestrogen receptor of rat mammary tumours induced by 7,12-dimethylbenz(a)anthracene. By Wendy Powell-Jones, P. Davies, D. W. Wilson and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XX

Mammary tumours induced by 7,12-dimethylbenz(a)anthracene (DMBA) in rats share similar characteristics with human breast carcinoma. One of these is the dependence of tumour growth on oestrogenic hormones, involving the interaction of oestradiol with cytoplasmic macromolecules which transport the steroid to the nuclei and elicit a transcriptional response to the steroid hormone. The demonstration of receptor proteins allows investigations into the anti-oestrogenic potential and therapeutic value of synthetic compounds by their ability to decrease oestradiol binding to the receptor. Moreover, a varying affinity of the receptor protein for steroids other than oestradiol-17β may indicate a system controlling oestrogen action or mediating responses at different intracellular sites.

Mammary tumours induced in virgin female Sprague–Dawley rats reached a suitable size for experimentation (approx. 2 x 2 cm) 7–12 weeks after intubation of DMBA (20 mg in 1 ml sesame oil). Cytosol and nuclear fractions were prepared as described by Powell-Jones, Jenner, Blamey, Davies & Griffiths (1975). Specific binding of [2,4,6,7-3H]oestradiol (sp. act. 85 Ci/mmol) at final concentrations ranging from 0·01 to 10 nmol/l to proteins was studied by sucrose-density-gradient centrifugation or by charcoal adsorption techniques. Data from the latter were analysed according to Scatchard (1949) or by a computer program utilizing a law of mass action model governing protein–ligand interaction and incorporating an iterative curve fitting technique based on the method of least squares. Competitive binding studies were carried out using the following substances at concentrations up to 10,000 times that of [3H]oestradiol: diethylstilboestrol (DES), dibutylhydrolstilboestrol (DHBS), ICI 46,474 (the trans isomer of 1-[p-(β-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1-ene), ICI 79,792 (1-(4- [β-bis-(2-chloroethyl)amino]ethoxyphenyl)-trans-diphenylbut-1-ene hydrochloride), ICI 85,966 (3,4-bis-[p-[(N-bis-2-chloroethyl)carbamoyl]phenyl]hex-3-ene), oestrone, progesterone, and a number of androgens and androgen precursors.

The synthetic substances most effective at displacing [3H]oestradiol from receptor proteins, when added directly to cytosols were DES and ICI 46,474, while DHBS and ICI 79,792 produced similar results at higher concentrations, ICI 85,966 was effective only occasionally. After incubation of tissue minces with [3H]oestradiol, a 3H-labelled steroid–receptor complex was extracted from purified nuclei. This could be prevented by inclusion of DES and DHBS, but only by prolonged exposure to ICI 46,474 and ICI 79,792 before addition of 3H-labelled steroid. The cellular uptake of these compounds is apparently slow compared with oestradiol, DES and DHBS. Under these conditions, ICI 85,966 was consistently effective, possibly by metabolism to DES.

[3H]Oestradiol binding by receptor proteins was significantly reduced by androst-5-ene-3β,17β-diol, and several other androgenic steroids had varying inhibitory effects. Progesterone had no effect. The oestrogen receptor apparently has a range of affinities for a number of steroids, including potential androgens, possibly involved in control of oestrogen-mediated transcriptional processes in these mammary tumours.

REFERENCES
Analysis of Dehydroepiandrosterone and Androstenediol in Human Breast Tissue Using High Resolution Gas Chromatography–Mass Spectrometry

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Abstract—Dehydroepiandrosterone (DHA) and 5-androstenediol have been measured in human breast tissue by a method consisting of extraction and purification with final analysis by gas chromatography–mass spectrometry. The method has been shown to be specific and reproducible, measuring only free DHA and not its sulphate. Preliminary results indicate that levels of DHA are in the range 15–200 ng/g tissue whereas in fibroadenoma the amounts are much higher (200–1000 ng/g). Androstenediol is present in tumours and benign tissues in similar amounts (10–200 ng/g).

INTRODUCTION

The relationship of C19-steroids to the responsiveness of breast tumours to endocrine therapy has been the subject of many studies and a number of groups have reported that the estimation of 11-deoxy-17-oxosteroids in urine samples from their patients with breast cancer has been of value in discriminating between those who will and those who will not benefit from adrenalectomy or hypophysectomy [1–3]. Although similar investigations from our laboratories [4] supported the view that steroid excretion in many patients with breast cancer differed from the normal population, the results did not agree completely with those previous reports, abnormalities being found only in patients with advanced cancer of the breast.

Further studies from these laboratories [5, 6] and from others [7] were directed to the paraendocrine behaviour of breast tumour tissue and its capacity to metabolise plasma C19-steroids, particularly dehydroepiandrosterone sulphate (DHA sulphate) and DHA, to compounds such as testosterone, androst-5-en-3β,17β-diol (androstenediol), 17β-hydroxy-5α-androstan-3-one and oestrone. Obviously, the C19-steroid concentration in the breast tumour may well play a role in regulating the oestrogenic stimulation of this tissue and Poortman, Prenen, Schwarz and Thijssen [8] have reported that androstenediol will displace oestradiol-17β from the cytoplasmic receptor in breast tumours. Concurrent studies from this Institute indicate that a 50-fold excess of androstenediol will result in approximately 50% displacement of oestradiol-17β [Powell-Jones, submitted for publication].

As part of this investigation into the role of C19-steroids in breast cancer, the present communication deals with the assay of DHA and androstenediol in breast tissue. The procedure is based on a simple extraction and purification with quantitation by gas chromatography–mass spectrometry using high resolution selected ion detection and internal standards to monitor losses during extraction.

MATERIAL AND METHODS

Chemicals

DHA and androstenediol were obtained from Sigma Chemical Co., London; 3α-hydroxy-5-androsten-17-one and 5α-androstanediol (5α-androstan-3α,17β-diol) from Steraloids Inc., U.S.A. Bis (N,O) trimethylsilyl acetamide (BSA) was purchased from Jones Chromatography, Llanbradach, Mid-Glam., U.K. and polyfluorokerosene from Koch-Light Ltd., England. All solvents were of analar grade and were distilled before use.
Tissues

Breast tissue, received from a number of centres, was homogenised by powdering in a Thermovac automatic frozen tissue pulveriser (Telcolab Corp., N.Y.) and then suspended as a 15% (w/v) homogenate in 10 mM Tris-HCl buffer pH 7.4 containing EDTA (1 mM) and sodium azide (3 mM), using a glass–glass homogeniser. Portions of the homogenate were stored at −20°C until extracted.

Extraction of steroids

Internal standards, 3α-hydroxy-5-androsten-17-one (20 ng) and 5α-androstanediol (5 ng), in ethanol, were added to a stoppered glass tube followed by 0.5–2.0 ml of homogenate. After mixing vigorously the tubes were left at 4°C overnight.

Lipid material was extracted twice with ethanol/acetone (1:1, v/v) (5 ml) by agitation for 2 min on a vortex mixer, and the combined extracts evaporated to dryness under a stream of nitrogen in a water bath at 40°C. Most of the non-steroidal lipid was then removed using a modification of the method of Ismail, Love and McKinney [9]. Ethyl acetate (50 ml) was added to the residue followed by 1% aqueous CaCl₂ solution (5 ml) and the tubes placed in a water bath at 50°C for 10 min without shaking. The tubes were then carefully removed, placed in ice for a further 10 min and then slowly filtered through a scinttered glass funnel (porosity 1). The funnels were washed with ice-cold CaCl₂ solution (1 ml).

The filtrate was extracted twice with diethyl ether (5 ml) and the solvent evaporated to dryness under nitrogen. Residues were transferred in a small volume of ethanol to tubes for derivative formation.

Derivative formation

Trimethylsilyl ethers of the steroids were prepared by drying the tubes in vacuo over phosphorus pentoxide for 1 hr and adding BSA (20 μl) to the residue. The tubes were tightly stoppered, left at room-temperature overnight and the derivatised extracts analysed within 48 hr.

High resolution molecular ion monitoring

Details of the procedure by these laboratories for selected ion monitoring at high resolution have been described [10, 11]. A Varian 2700 gas chromatograph fitted with a 2 m x 2 mm i.d. helical glass column containing 3% OV-17 on Gaschorm Q (100–120 mesh) was interfaced to a Varian MAT 731 mass spectrometer by a two stage Watson–Biemann type separator followed by a glass lined probe. The ion source temperature was 150°C and the gas chromatograph injector, separator and probe temperatures were 275°C, 250°C and 245°C respectively. The electron beam energy was 70 eV with the resolving power at 8500–10,000 (10% valley) at the mass to be monitored, which was located with the aid of the reference compound polyfluorokerosene. In all cases the most intense peak was the molecular ion of the steroid derivative, and this was the ion monitored. The steroid extract (1–2 μl) was injected using BSA as solvent.

Determination of results

The output of the mass spectrometric detector was displayed on a potentiometric recorder and the respective peak heights of the steroid and internal standard were measured and compared with a standard curve for quantitation. DHA and androstenediol were assayed in the same extract in two separate injections.

RESULTS AND DISCUSSION

In order to show that there was little likelihood of any endogenous material interfering with the peak associated with the internal standards selected, a number of breast tissues (normal, benign and neoplastic) were extracted with no steroids added. In no case was there found a significant peak with the same ion mass nor a gas chromatograph retention time identical with the derivative of 3α-hydroxy-5-androsten-17-one or 5α-androstanediol. For the assay of DHA the ion mass monitored was 360.24845 and a typical trace of one standard is shown in Fig. 1(a), demonstrating an adequate gas chromatographic separation of the two compounds. In the majority of tissue extracts a peak of unknown material with this mass was found, but this had a very much shorter retention time on the column (Fig. 1b).

For the androstenediol assay it was necessary to monitor at an ion mass of 436.3192 and then at 434.30364 for, respectively, 5α-androstenediol and androstenediol derivatives. Figure 2 shows typical traces of a standard (Fig. 2a) and of a tissue extract (Fig. 2b).

Standard curves were set up with every group of tissues extracted and a linear relationship was found between the ratio of steroid to internal standard and the amount of steroid, both for DHA (Fig. 3) and androstenediol (Fig. 4). Although the between-assay variation in stan-
Analysis of Dehydroepiandrosterone and Androstenediol

Fig. 1. Separation and detection of dehydroepiandrosterone and 3α-hydroxy-5\(^\alpha\)-androsten-17-one as the trimethylsilyl derivatives by gas chromatography-mass spectrometry. (a) Typical trace of pure standards, (b) Typical trace of a tissue extract.

Fig. 2. Separation and detection of androstenediol and 3α,17α-androstane-3α,17α-diol as the trimethylsilyl derivatives, by gas chromatography-mass spectrometry. Ion mass 436.3192 monitored for internal standard and 434.30364 for androstenediol. (a) Typical trace of standard, (b) Typical trace of a tissue extract.

Fig. 3. Typical calibration curve for dehydroepiandrosterone assay.
standard curves was small, it was considered necessary to include a curve with each extraction batch.

A number of experiments were performed to test the efficiency of extraction of DHA and androstenediol from tissues. No differences were found between the steroid content of extracts of tissue prepared as described or when tissue was extensively homogenised with the acetone/ethanol mixture. Furthermore, the efficiency of the extraction procedure did not depend upon the amount of tissue present up to 300 mg (2 ml of homogenate) as shown in Table 1.

Table 1. Endogenous DHA and androstenediol in different quantities of 15% homogenate of a primary breast tumour

<table>
<thead>
<tr>
<th>Amount of homogenate (ml)</th>
<th>Steroid content (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHA</td>
</tr>
<tr>
<td></td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
</tr>
</tbody>
</table>

*Derivatised extract hydrolysed before analysis completed.

Because of the high levels of DHA sulphate in plasma, it is possible that some of the DHA detected could have come from contaminating plasma, following hydrolysis of the sulphate during processing. Table 2 shows the DHA measured in samples of tissue from two fibroadenomata, generally more vascular than malignant tissue, following the addition of 35 ng of either DHA or its sulphate before extraction. It can be seen that whereas recovery following the addition of the free steroid is good, the sulphate contributed nothing to the measurement. Therefore the method estimates only the free steroid content.

The reproducibility of the assay obviously will depend largely upon the homogeneity of the prepared sample, and breast tissue is well known to be difficult to homogenise. Table 3 shows the values obtained following the extraction of five 1 ml aliquots of a 15% homogenate of a fibroadenoma. The one sample with low levels of both steroids probably demonstrates the heterogeneity of the homogenised preparation before extraction.

Table 2. DHA measured in 1 ml portions of a 15% homogenate of two fibroadenomata either alone or following addition of free DHA (35 ng) or of its sulphate (35 ng)

<table>
<thead>
<tr>
<th>Additions</th>
<th>Dehydroepiandrosterone (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Fibroadenoma 1  Fibroadenoma 2</td>
</tr>
<tr>
<td>DHA (35 ng)</td>
<td>35.0  55.8</td>
</tr>
<tr>
<td>DHA sulphate (35 ng)</td>
<td>4.0  17.5</td>
</tr>
</tbody>
</table>

Table 3. DHA and androstenediol in replicate 1 ml portions of a 15% homogenate of a fibroadenoma

<table>
<thead>
<tr>
<th>DHA (ng)</th>
<th>Androstenediol (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.7</td>
<td>8.0</td>
</tr>
<tr>
<td>56.2</td>
<td>8.5</td>
</tr>
<tr>
<td>34.5</td>
<td>7.8</td>
</tr>
<tr>
<td>51.7</td>
<td>9.0</td>
</tr>
<tr>
<td>52.3</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Mean 49.5  8.3

Coefficient of variation 17 6
In this pilot study on the amount of DHA and androstenediol in breast tissues, a small number of malignant tumours and fibroadenomata have been assayed. As shown in Table 4, the concentration of these steroids is sufficiently high to be accurately determined in both types of tissue. Although the levels of DHA in the benign condition appear to be considerably higher than in the malignant tumours, it should be pointed out that these results are not corrected for cellularity, either by protein or by DNA estimation. Further work is necessary to determine whether such analyses can be related to subsequent development of the disease or to its clinical management.

Acknowledgements—The authors are grateful to the Tenovus Organisation in Cardiff and to the M.R.C. (Grant No. G974/125G) for financial support for this work.

### Table 4. DHA and androstenediol content in primary breast tumours and fibroadenomata

<table>
<thead>
<tr>
<th>Tumours</th>
<th>DHA</th>
<th>Androstenediol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ng/g)</td>
<td>(ng/g)</td>
</tr>
<tr>
<td>40-1</td>
<td>16.0</td>
<td>16.7</td>
</tr>
<tr>
<td>40-3</td>
<td>26.7</td>
<td></td>
</tr>
<tr>
<td>41-1</td>
<td>46.7</td>
<td>22.0</td>
</tr>
<tr>
<td>42-3</td>
<td>36.7</td>
<td>53.3</td>
</tr>
<tr>
<td>28-6</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>34-10</td>
<td>40.0</td>
<td>13.3</td>
</tr>
<tr>
<td>39-1</td>
<td>44.7</td>
<td>26.0</td>
</tr>
<tr>
<td>33-4</td>
<td>233.5</td>
<td>200.0</td>
</tr>
<tr>
<td>Fibroadenomata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36-1</td>
<td>~366.7</td>
<td>57.3</td>
</tr>
<tr>
<td>28-3</td>
<td>~783.3</td>
<td></td>
</tr>
<tr>
<td>36-2</td>
<td>~1000.0</td>
<td>42.0</td>
</tr>
<tr>
<td>43-6</td>
<td>245.3</td>
<td>134.7</td>
</tr>
<tr>
<td>28-4</td>
<td>330.0</td>
<td>55.3</td>
</tr>
</tbody>
</table>

REFERENCES

The Specificity of the Oestrogen Receptor of DMBA-induced Mammary Tumours of the Rat

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Abstract—The comparative abilities of several naturally-occurring steroids to compete for [3H]oestradiol-17β-binding sites in rat mammary tumour cytosol have been investigated. Apart from compounds containing a phenolic A-ring, the most effective compounds were 5-androstene-3β, 17β-diol, 5-androstene-3α, 17β-diol and 5α-androstane-3β, 17β-diol. 5-Androsten-3β, 17β-diol appears to compete by binding to the same site as oestradiol-17β. Those compounds most effective at diminishing cytoplasmic [3H]oestradiol-17β binding also depressed nuclear binding of [3H]oestradiol-17β. The presence of a nominal oestrogen receptor with a range of affinity for a number of steroids, including potential androgens, may indicate the presence of a regulatory mechanism superimposed on the basic oestrogen controlled system.

INTRODUCTION

Evidence which ascribes a role for androgens in the hitherto-designated oestrogen-target organs continues to accumulate. Certain of these tissues contain a discrete protein entity with definite selective affinity for androgenic steroids [1–3], while other reports [4, 5] suggest that the oestrogen receptor itself may sequester potential androgens to some extent. Previously [6], we have suggested that a macromolecule with varying ranges of affinity for steroids with different physiological roles may provide a regulatory system in the normal cell. Obviously, such a system achieves greater importance in the cancerous cell in view of its exploitation in therapy and, possibly, prognosis. This paper describes the study of the specificity of the cytoplasmic oestrogen receptors of mammary tumours induced in rats by 7,12-dimethylbenz[a]anthracene.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50 ± 2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of 4 and allowed food and water ad libitum. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size was recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration were not used in this study.

Preparation of subcellular fractions

Mammary tumours were homogenized [7] and cytoplasmic [8] and nuclear fractions [9] were prepared as previously described. The basic buffer system employed throughout comprised 50 mM-Tris-HCl buffer, pH 7.4, containing 5 mM-EDTA and 1 mM-dithiothreitol.

Detection of specific oestrogen-binding components in mammary tumour subcellular fractions

The labelling of subcellular fractions with [2, 4, 6, 7(α)-3H] oestradiol-17β (specific radioactivity 85–110 Ci/m mole; Radiochemical Centre, Amersham, Bucks., U.K.), and subsequent sedimentation analysis has been adequately described [8, 10]. The quantitative assessment of receptor proteins selectively retaining [3H]oestradiol-17β was performed by a charcoal adsorption technique [11] and analysis according to Scatchard [12]. The ability of certain other steroids to prevent the binding of...
[3H]-oestradiol-17β to specific cytoplasmic receptors was examined by the following two methods:

(a) Constant amounts of cytosol (100 μl) were incubated with a saturating concentration of [3H]oestradiol-17β (5 nmole/l) in the presence of varying concentrations of non-radioactive competitor (see 'Results'). After incubation (2 or 16 hr at 4°C) 200 μl of charcoal suspension (0.5% (w/v) Norit A; 0.05% (w/v) Dextran T-70; 0.1% (w/v) gelatin) was added. After removal of charcoal (800 g for 15 min), aliquots (100 μl) of the supernatant were assessed for bound radioactivity in 5 ml of scintillation fluid (5 g 2, 5-diphenyloxazole, 500 ml Triton X-100, 1 l. toluene) in a Nuclear Chicago (Mark 11) liquid scintillation spectrometer at a counting efficiency for tritium of 30–40%, as determined by external standardisation.

(b) Constant quantities of cytosol (100 μl) were incubated with three constant concentrations (0.18, 0.46 and 0.92 nmole/l) of [3H]oestradiol-17β in the presence of varying concentrations of nonradioactive competitor (as detailed in the 'Results' section). After incubation (2 hr at 4°C), charcoal suspension (200 μl) was added and removed (as described above) and aliquots (100 μl) of supernatant were assessed for bound radioactivity. Data were expressed graphically in the form of single and double reciprocal plots.

Chemical analyses

The protein concentration of cytosol fractions was estimated using the method of Lowry et al. [13].

RESULTS

Characteristics of [3H]oestradiol-17β-binding components in rat mammary tumour subcellular fractions

Sedimentation profiles of specific oestrogen receptor proteins from rat mammary tumour cytosol [9] and nuclei [10] have received adequate exposure. It is sufficient to reiterate that two species of [3H]oestradiol-17β-binding proteins are found in cytosol, one of sedimentation coefficient 8S which shows specificity by accepted criteria and one of sedimentation coefficient 4S which comprises predominantly nonspecific components. Saturation analysis indicated the presence of a single class of high-affinity binding sites with dissociation constants in the range 50–1000 pmole/l and with concentrations in the range 10–200 pmole/mg protein. Those cytosols used in the competition studies had receptor concentrations >80 pmole/mg protein. Salt-extraction [10] of nuclei labelled with [3H]oestradiol-17β yielded a single peak of protein-bound radioactivity on sucrose gradients corresponding to a sedimentation coefficient 4–5 S.

Competition for [3H]oestradiol-17β-binding sites in mammary tumour cytosol

The comparative abilities of a number of steroids to compete with [3H]oestradiol-17β for receptor sites is shown in Figs. 1 and 2.

Fig. 1. Competition for [3H]oestradiol-17β binding sites. Samples of cytosol were incubated with [3H]oestradiol-17β (5 nmole/l) alone or in the presence of increasing concentrations of other steroids. Free and nonspecifically bound steroid was removed by charcoal adsorption and retained radioactivity was assessed by liquid scintillation spectrometry. Competing steroids were (a) oestradiol-17β ( ), oestradiol-17β ( ), oestrone ( ) and oestriol ( ); (b) oestradiol-17β ( ), 5-androstene-3β, 17β-diol ( ), 5-androstene-3β, 17β-diol ( ) and 5-androstene-3β, 17β-diol ( ). Binding is expressed as a percentage of charcoal-resistant binding in the presence of [3H]oestradiol-17β alone (100 pmole/mg protein).

Fig. 2. Competition for oestradiol-17β binding sites. Details are essentially the same as for Fig. 1., but competing steroids were (a) oestradiol-17β ( ), 5α-androstane-3β, 17β-diol ( ), 5α-androstane-3α, 17α-diol ( ), 5α-androstane-3β, 17α-diol ( ), 5α-androstane-3α, 17β-diol ( ), and (b) oestradiol-17β ( ), 5α-dihydrotestosterone ( ), testosterone ( ), androstenedione or epitestosterone ( ), dehydroepiandrosterone ( ) and progesterone or corticosterone ( ).
Predictably, the most effective competitor was oestradiol-17β, together with oestradiol-17α (Fig. 1a). Oestriol and oestrone also competed very well (Fig. 1a). Of the three androstenediols studied, 5α-androstene-3β, 17β-diol was the most effective compound, with 5α-androstene-3α, 17β-diol able to reduce binding of [3H]oestradiol-17β by 50–60% at the highest concentration used (Fig. 1b). 4-Androstene-3β, 17β-diol was less effective, causing a maximum decrease in specific binding of 30–40% (Fig. 1b). The 5α-androstenediols also showed variable potency, 5α-androstane-3β, 17β-diol being most effective (~55% decrease), followed by 5α-androstane-3α, 17α-diol (40%), 5α-androstane-3β, 17α-diol (35%) and 5α-androstane-3α, 17β-diol (~30%) (Fig. 2a). It must be emphasised that these degrees of competition are those achieved by the highest concentrations used i.e. 2500 nmole/l, or a 500-fold higher concentration than that of [3H]oestradiol-17β. The compounds mentioned in Fig. 2b, therefore, can be regarded as relatively ineffective in decreasing the specific binding of [3H]oestradiol-17β.

In view of the substantial reduction in [3H]oestradiol-17β binding brought about by compounds such as 5α-androstene-3β, 17β-diol it was thought worthwhile to test the hypothesis that these steroids are able to bind to the same site as oestradiol-17β, since competition could also be induced if the second steroid bound to an alternative site to alter protein conformation. This was done by monitoring competition under conditions of varying [3H]oestradiol-17β concentration and varying concentrations of competing steroid (Figs. 3–6). Data obtained from competition studies using unlabelled oestradiol-

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Fig. 3. Competition by unlabelled oestradiol-17β for specific [3H]oestradiol-17β binding sites in mammary tumour cytosol. Aliquots (100 µl) of cytosol were incubated (2 hr at 4°C) with three constant concentrations of [3H]oestradiol-17β (0.18, 0.46 and 0.92 nmole/l), alone or together with varying concentrations of oestradiol-17β in a total vol of 200 µl. After incubation, charcoal suspension (200 µl) was added and after 1 hr at 4°C was sedimented (900 g for 15 min) and aliquots (200 µl) of bound supernatant assessed for bound radioactivity. The concentrations of unlabelled oestradiol-17β used were 0.367 nmole/l (△), 3.67 nmole/l (●) and 9.19 nmole/l (○). (a) when the reciprocal values of bound [3H]oestradiol-17β was plotted against the reciprocal value of total [3H]oestradiol-17β. Control systems containing [3H]oestradiol alone (□). (b) the reciprocal value obtained for bound [3H]oestradiol-17β were plotted against total unlabelled oestradiol-17β concentrations, at concentrations of [3H]oestradiol-17β of 0.18 nmole/l (○), 0.46 nmole/l (●) and 0.92 nmole/l (△).

Fig. 4. Competition by oestradiol for specific oestradiol-17β binding sites in mammary tumour cytosol. Details are essentially those in Fig. 3, except that oestriol was used as competitor. (a) [3H]oestradiol-17β alone (□) and with oestriol at concentrations of 0.367 nmole/l (△), 3.67 nmole/l (●) and 7.35 nmole/l (○). (b) [3H]oestradiol-17β at concentrations of 0.18 nmole/l (○), 0.46 nmole/l (●) and 0.92 nmole/l (△).
17β, oestradiol (a steroidal oestrogen), diethylstilboestrol (a nonsteroidal oestrogen) and 5-androstene-3β, 17β-diol [the most effective of potential androgens in the competition studies (Fig. 1b)] as the competing substances were expressed graphically in the form of double reciprocal plots (Figs. 3(a)–6(a), respectively). The reciprocal value of bound [3H]oestradiol-17β was plotted against the reciprocal value of total [3H]oestradiol-17β. The patterns obtained were characteristic of competitive inhibition in that the presence of competitor altered the slope but not the intercept on the ordinate. To eliminate the possibility of allosteric inhibition, a plot of the reciprocal of bound radioactivity against the concentration of competitor was also used [Figs. 3(b)–6(b)]. Whereas allosteric inhibition would have resulted in hyperbolic curves, the substances under investigation produced a linear relationship indicative of direct competition. These data suggest that these compounds were competing with [3H]oestradiol-17β for the same binding site.

**Compartment for oestrogen-binding sites in rat mammary tumour nuclei**

Using methods described previously [10], the ability of various compounds to prevent the nuclear uptake of [3H]oestradiol-17β was studied (see Fig. 7). A 1000-fold higher concentration of either oestradiol-17β or diethylstilboestrol completely depressed the 4-5S peak of radioactivity present in nuclear extracts after incubation with [3H]oestradiol-17β alone, while similar concentrations of testosterone or 5α-dihydrotestosterone only slightly decreased the binding peak (Fig. 7). The inclusion of a 1000-fold higher concentration of 5-androstene-3β, 17β-diol or 5α-androstane-3β, 17β-diol resulted in a significant decrease in the binding of [3H]oestradiol-17β (Fig. 7).

**DISCUSSION**

The ability of certain compounds to compete with [3H]oestradiol-17β for specific binding sites has proved of great value in the development of anti-oestrogens [8, 14, 15], which may retard growth processes by impeding the obligatory retention step in mechanism of action of steroids. However, the possibility that naturally-occurring steroids may also compete for oestrogen-binding sites provides an attractive scenario for regulation of oestrogen action at the physiological level.

The data presented in Figs. 1 and 2 show that, apart from those compounds with a phenolic A-ring, i.e. naturally-occurring steroids with vary-
Sedimentation analysis of the effect of various compounds on the binding of $[^3H]oestradiol-17\beta$ by mammary tumour nuclei. Samples (1 g) of tumour tissue were minced and incubated (15 min at 30°C) in Eagle's basal medium (10 ml) supplemented with $[^3H]oestradiol-17\beta$ (5 n mole/l) alone (O) or together with oestradiol-17\beta or diethylstilboestrol (●), 5-androstene-3β, 17β-diol (△), 5α-androstane-3β, 17β-diol (●), testosterone (— — — —) or 5α-dihydrotestosterone (■), (all at 5 n mole/l). After incubation, nuclei were purified, extracted with KCl (0.4 mole/l) and samples of extract analysed on sucrose gradients [10]. Sedimentation marker (arrow) was bovine serum albumin ($\beta_{2\mu}$, 4.68). Direction of centrifugation was from left to right.

In view of postulated regulatory roles of 5-androstene-3β, 17β-diol [4, 18], it is interesting to note that it behaves similarly to oestrogenic compounds by apparently binding to the same site as oestradiol-17β (Fig. 6). It is not unusual for 5α-dihydrotestosterone to be inactive as regards the oestradiol receptor (Fig. 2b) [20] since a spectrum of androgenic compounds have been shown to exert physiological effects [see ref. 21], among them 5α-androstane-3β, 17β-diol [22] and 5-androstene-3β, 17β-diol [23]. Together with the data presented in Figs. 1 and 2, these observations suggest that the variety of ‘active’ androgens might be reflected in diverse binding characteristics, ranging from nominal androgen receptors to nominal oestrogen receptors.

Those substances which diminish cytosol retention of $[^3H]oestradiol-17\beta$ also prevent nuclear accumulation of $[^3H]$steroid (Fig. 7). This is not unexpected and does not preclude androgen-induced translocation of receptor. We have previously shown [24] that pharmacological doses of androgen can translocate receptor to nuclei and that 5-androstene-3β, 17β-diol was most efficient in this respect. This data [24] agrees with concepts that not only those androgens which bind well to the oestrogen receptor promote its translocation, but also those which bind poorly may effect translocation if the intracellular concentration is sufficiently high [5, 25].

The role of androgens in oestrogen-target tissues remains obscure. Androgen-induced transfer of receptor to nuclei of immature rat uteri has been suggested to provide a pool of receptor inaccessible to oestrogen resulting in depletion of intracellular oestrogen-receptor complex and denigration of synthetic processes [25]. However, high doses of androgen can translocate oestrogen-receptor to nuclei of the same tissue and promote protein synthesis quantitatively and qualitatively similar to that produced by physiological quantities of oestrogen [26]. This would ascribe to androgens uterotrophic and antiuterotrophic responses induced by the same mechanism. However, the involvement of pharmacological doses of an-
hydrogens may result in aberrations. At physiological concentrations, androgens may deplete binding of oestradiol-17β but may not effect the necessary transformations for nuclear transfer. In this respect, a regulatory mechanism may operate which may be relevant to the study of human breast cancer, especially in view of observations on the competitive properties of 5-androstene-3β, 17β-diol [4, 5 and above].

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REFERENCES

Specificity of Oestrogen Receptor


Interaction of Androgens with Oestradiol-17\textbeta\ Receptor Proteins in DMBA-Induced Mammary Tumours—a Possible Oncolytic Mechanism

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Abstract—A series of androgens have been examined for their ability to (1) compete with \[^{3}\text{H}\]oestradiol-17\textbeta\ for the cytoplasmic oestradiol-17\textbeta\ receptor protein isolated from DMBA-induced mammary tumours and (2) facilitate the nuclear translocation of the oestrogen receptor. The most effective androgen examined was androst-5-ene-3\beta,17\beta-diol. Compounds that did not compete with \[^{3}\text{H}\]oestradiol-17\textbeta\ for cytoplasmic oestradiol-17\textbeta\ binding sites were unable to instigate the translocation of the oestrogen receptor. Androgen-transferred oestrogen receptors were found to have a relatively short nuclear retention time and readily bound \[^{3}\text{H}\]oestradiol-17\textbeta\ at 4°C.

INTRODUCTION
A number of reports have demonstrated an oncolytic action for androgens in both human [1, 2] and rat [3–6] mammary tumours. The mechanism by which such compounds inhibit tumour growth has for the most part remained unknown. Since we have previously demonstrated that the growth of the DMBA-induced rat mammary tumour requires the presence of cytoplasmic oestradiol-17\textbeta\ receptor proteins [7–9] the tumour represents a good model system by which the hypothesis that the oncolytic effects of androgens are mediated via the oestrogen receptor system can be tested. The present study therefore examines the direct action of testosterone and other C\textsubscript{19}-steroids on the binding of oestradiol-17\textbeta\ to its receptor protein in both cytoplasmic and nuclear compartments of DMBA-induced mammary tumours.

A preliminary report of this work has been presented [10].

MATERIAL AND METHODS
Animals
Mammary tumours were induced in virgin female Sprague–Dawley rats (50±2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of 4 and fed diet and water ad libitum. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23 week period following carcinogen administration were not used in this study.

Preparation of subcellular fractions
Soluble supernatant (cytosol) preparations were obtained as described by Nicholson, Golder, Davies and Griffiths [8] using a homogenisation procedure resulting in maximum breakdown of tumour cell membranes with minimum damage to cell organelles [11]. Nuclei were prepared by a method described by Davies and Griffiths [12].

Competitive binding studies on cytoplasmic fractions
Aliquots (200 \textmu l) of tumour cytosol preparations (approximately 20 mg protein/ml) from ovariectomised animals (operation performed one day previously) were incubated for 2 hr at 4°C with equal vol of 50 mM-Tris–HCl buffer, pH 7.4, containing 5 mM EDTA and 1 mM dithiothreitol (Tris–HCl buffer) and also \[^{3}\text{H}\]oestradiol-17\textbeta\ (5 nM; [2, 4, 6, 7 \textsuperscript{3}\text{H}]-

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oestradiol-17β, specific activity 85 Ci/mnmole, Radiochemical Centre, Amersham) in the presence and absence of various amounts of diethylstilboestrol or other potential ligands (5 nM–5 μM). After incubation, tubes were maintained at 4°C and an equal vol (400 μl) of charcoal suspension (0.5% (w/v) Norit-A, 0.05% (w/v) Dextran T-70, 0.1% (w/v) gelatin) in Tris-HCl buffer added. The resultant mixture was shaken for 60 min at 4°C and charcoal removed by centrifugation at 800 g for 10 min. Aliquots (200 μl) of the supernatant containing protein-bound [3H]oestradiol-17β were added to 6 ml scintillation fluid (1 litre toluene, 500 ml Triton X-100, 10 g PPO) and radioactivity measured in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation.

**Determination of nuclear sites by [3H]oestradiol-17β exchange—total sites**

The method for the determination of nuclear oestradiol-17β binding sites is a slight modification of that described in a previous communication [9]. Briefly, receptor protein present in the nuclear fraction (200 μl aliquots) was precipitated by protamine sulphate (1 mg/ml in 50 mM Tris-HCl buffer). [3H]Oestradiol-17β (20 nM) in Tris-HCl buffer (200 μl) containing 5 mM sodium bisulphite or [3H] oestradiol-17β plus a 1000-fold higher concentration of cold diethylstilboestrol was added to the precipitated receptor and the mixtures incubated for either 4 hr at 15°C or 16 hr at 4°C. After incubation, the tubes were cooled at 4°C and washed repeatedly in Tris-HCl buffer. The final pellet was extracted with ethanol, centrifuged and the ethanolic supernatant counted as before.

**Determination of oestrogen binding sites following the in vivo administration of either oestradiol-17β or various C19-steroids**

(a) Thirty ovariectomised animals bearing 50 mammary tumours were divided randomly into ten groups (5 tumours/group). Each group received either oestradiol-17β (0.5 or 5 μg), testosterone (1 mg), 5α-dihydrotestosterone (1 mg), epistosterone (1 mg), androstenedione (1 mg), 5α-androstane-3β, 17β-diol (100 μg or 1 mg), and androst-5-ene-3β, 17β-diol (androstenediol) (100 μg or 1 mg) in 1 ml vehicle (steroid solutions were prepared by addition of the requisite compound to propylene glycol and diluting to volume with 0.15M NaCl) by i.v. injection. Tumour biopsy samples (approx. 200 mg tissue) were removed aseptically from Nembutal*-anaesthetized animals at time 0 (prior to injection) and at 10, 25, 40 and 60 min after injection. Cytosol fractions were assayed for accessible oestradiol-17β binding sites while nuclear fractions were assayed for both total and accessible sites.

(b) Fifteen ovariectomised animals bearing 20 tumours were divided randomly into 4 groups (5 tumours/group). The experimental design was as described in (a) except that animals were treated with either oestradiol-17β (5 μg), 5α-dihydrotestosterone (1 mg), 5α-androstane-3β, 17β-diol (1 mg) or androstenediol (1 mg) and tumour tissue was biopsied at time 0, and at 0.5, 1, 2, 4, 8, 16 and 24 hr after injection. Nuclear fractions were assayed for both total and accessible oestradiol-17β binding sites.

**Chemical analyses**

The DNA content of nuclear fractions and the protein content of cytosol fractions were estimated using the methods of Burton [13] and Lowry, Rosebrough, Farr and Randall [14], respectively.

**RESULTS**

**Specificity of the accessible cytoplasmic oestradiol-17β binding assay**

The data presented in Fig. 1 shows the competition by various non-radioactive steroids with [3H]oestradiol-17β for accessible cytoplasmic binding sites. Addition of 25 nM unlabelled oestradiol-17β decreased the binding of [3H]oestradiol-17β to approximately 40% of the control value. The binding continued to decrease with increasing oestradiol-17β concentrations. There appears also to be competition for receptor binding between [3H]-oestradiol-17β and the dihydroxysteroids. Androstenediol and 5α-androstane-3β, 17β-diol in a 1000-fold excess decreased the binding of [3H]oestradiol-17β to 25 and 42% of the control value respectively. Of the other C19-steroids tested, testosterone and 5α-dihydrotestosterone were partially inhibitory but only at very high concentrations, while androstenedione, epitestosterone and dihydroepiandrosterone were without any substantial effect.

In **vivo effects of oestradiol-17β and C19-steroids on** [3H]oestradiol-17β binding

Within 40 min following the **in vivo** administration of 0.5 μg oestradiol-17β, an approximately 50% reduction in the binding of [3H]oestradiol-17β to specific cytoplasmic binding proteins was observed (Fig. 2). This corresponds to a concomitant progressive increase in specific nuclear oestradiol-17β binding sites. Elevation of the level of oestradiol-17β adminis-
Interaction of Androgens with Oestradiol-17β Receptor Proteins

Fig. 1. Specificity of [3H]oestradiol-17β binding assays. Cytosol fractions were incubated for 2 hr at 4°C with a saturating concentration of [3H]oestradiol-17β (5 nM) in the presence or absence of increasing concentrations (1, 5, 50, 500 and 1000-fold excess) of the potential oestrogen antagonists. After incubation free steroid was removed by charcoal adsorption.

Fig. 2. Effect of in vivo administration of oestradiol-17β on cytoplasmic and nuclear oestradiol-17β binding sites. Rats were injected with oestradiol-17β (0.5 µg, Δ or 5 µg, ○) at time 0 and tumour biopsy samples removed at varying time intervals thereafter. (a) Cytosol preparations from the biopsies were incubated at 4°C for 16 hr with a saturating concentration of [3H]oestradiol-17β (5 nM) or [3H]oestradiol-17β plus 1000-fold excess of stilboestrol. (b) Nuclear fractions were incubated for either 4 hr at 15°C (●, △) or 16 hr at 4°C (○, □) with a saturating concentration of [3H]oestradiol-17β (20 nM) or [3H]oestradiol-17β plus a 1000-fold excess of stilboestrol.

Androstenedione
Epitestosterone
Dehydroepiandrosterone
Testosterone
Dihydrotestosterone
5α-androstane-3β,17β-diol
Oestradiol-17β

Androstenedione
epitestosterone
dehydroepiandrosterone
testosterone
dihydrotestosterone
5α-androstane-3β,17β-diol
Oestradiol-17β

Fig. 3. Effect of in vivo to 5 µg resulted in a more pronounced translocation of cytoplasmic binding sites to the nuclear fraction. Like oestradiol-17β, single injections of either androstenediol (Fig. 3) or 5α-androstane-3β,17β-diol (Fig. 4) reduced accessible cytoplasmic oestradiol-17β binding sites in a dose-dependent manner. The nuclear binding sites, however, unlike the oestradiol-17β-translocated receptor, appear to be present in a form which is readily accessible to exogenous hormone at 4°C. In vivo administration of testosterone resulted in a slight decrease of cytoplasmic binding sites at the higher dose level and a corresponding low nuclear uptake of the receptor complex (Fig. 5). A considerable amount of nuclear binding occurred at 4°C. Administration of dihydrotestosterone was more effective than testosterone at reducing cytoplasmic oestradiol-17β binding sites in vivo, but less so than either oestradiol-17β, androstenediol or 5α-androstane-3β,17β-diol (Fig. 5). Again, considerable translocation of the
receptor complex to the nucleus occurred. The receptor bound [3H]oestradiol-17β with equal efficiency at 15 or 4°C. Androstenedione and epitestosterone were without effect on receptor translocation (not illustrated).

Extension of the experimental time period to 24 hr after oestradiol-17β administration (Fig. 6) showed that nuclear binding sites, as determined by exchange assay at 15°C, peak at 2 hr and remain elevated over the 24 hr period. Accessible nuclear binding sites were low throughout the experimental period. The pattern of nuclear binding following androstenediol and 5α-androstane-3β, 17β-diol was substantially different from that produced by oestradiol-17β. Maximum nuclear binding occurred at 2 hr. However, unlike the oestradiol-17β-mediated response, the levels were not maintained over the 24 hr period and returned to values indistinguishable from control levels by 4 hr. 5α-Dihydrotestosterone behaved in a manner similar to androstenediol and 5α-androstane-3β, 17β-diol.
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**DISCUSSION**

The data presented in Fig. 1 show that androstenediol and 5α-androstane-3β,17β-diol were the most effective compounds examined in reducing the specific binding of [3H]-oestradiol-17β to its cytoplasmic binding protein. Substantial inhibition of [3H]-oestradiol-17β binding was observed at concentrations of these dihydroxy steroids as low as 25 nmole/l, that is at a concentration only 5-fold higher than that of the radioligand. The relative affinity of androstenediol for the oestradiol-17β receptor protein based on comparative binding
studies is approximately 10–20 fold lower than that observed for oestradiol-17β. These observations are consistent with our earlier findings [15] and also with the suggested physiological [15, 16] and pharmacological [10] role of these substances in the growth of mammary tumours.

It is now quite clear that at relatively high dose levels certain androgens can bind to the cytoplasmic oestrogen receptor and induce its translocation to the tumour cell nuclei in a dose-dependent manner. The process appears to be related to the relative affinities of the compounds for the oestradiol-17β receptor protein. Androgens that do not compete with oestradiol-17β for its specific binding protein are unable to affect the translocation process. Similar observations have been made with 5α-dihydrotestosterone in rat uteri by Ruh, Wassilak and Ruh [17] and Rochefort and Garcia [18]. These data are not consistent with specificity previously attributed to the translocation phenomenon [19].

The androgen-transferred receptor is different from the oestrogen receptor complex in both its short nuclear retention time and also in its ability to extensively bind [3H]oestradiol-17β at 4°C. Such data may infer different conformational states for the respective receptors. An analogous finding has been observed with the receptor complex induced by the antioestrogen, tamoxifen, and that formed by oestradiol-17β [20]. Sucrose density gradient centrifugation analysis of rat mammary tumour cytosol fractions reveals that [3H]oestradiol-17β binds specifically with the sedimentation coefficient of 8S [20–22], while [3H]tamoxifen gives a value of 4–5S [20]. The exact role or significance of the 4–5 S moiety is unknown. Rochefort, Liong and Capony [23] demonstrated that the uterine nuclear oestrogen receptor translocated by 5α-dihydrotestosterone bound [3H]oestradiol-17β at 4°C and displayed the characteristic 5 S nuclear oestradiol-17β binding pattern. This latter characteristic does not in itself exclude differences between the respective complexes; rather it may emphasise the relative limitations of the techniques available.

The transitory nuclear retention observed for androgen-transferred oestrogen receptor is similar in character to that observed for oestriol. This steroid apparently acts as a weak oestrogen principally because of its short nuclear retention time [24, 25]. When oestriol is present continually, either by multiple injections [25] or by chemical modification of the compound [26], thus maintaining the nuclear concentration of the receptor complex, it acts as a potent oestrogen without antagonistic properties. In view of these data it may prove valuable to maintain the nuclear concentration of the androgen-transferred oestrogen receptor complex and determine whether it too acts as an oestrogen or has enhanced anti-tumour activity.

In summary, we have shown that certain androgens, most notably androstenediol, translocate the oestrogen receptor to tumour cell nuclei. We propose that this property may form the basis for the known anti-tumour activity of androgens.

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REFERENCES

Interaction of Androgens with Oestradiol-17β Receptor Proteins


Dehydroepiandrosterone and Androstenediol in Human Primary Breast Tumours*

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Abstract—Dehydroepiandrosterone (DHA) and 5-androstene-3β,17β-diol have been measured in a series of primary human breast tumours. All the tissues contained significant quantities of DHA and there was a negative correlation of these values with the age of the patient. Only just over one half of the tumours contained detectable levels of androstenediol but there was an over-all correlation of this steroid with the DHA, although concentrations of DHA were generally much greater. No relationship between the steroid content of the tumour and the oestradiol androgen receptor levels was established although receptor was not measurable in the relatively small number of tumours which contained high levels of either steroid. Quantities of endogenous C19-steroids would seem to be high enough to exert some influence on the activity of the oestrogen receptor in vivo.

INTRODUCTION

The relationship of C19-steroids to breast cancer is not clearly understood although the evidence from studies of urinary steroid excretion [1-4], plasma steroid assays [5-6] and data on the parapendocrine behaviour of some tumours [7-9] would indicate that involvement of dehydroepiandrosterone (DHA) or its sulphate is of major importance.

More recently [10-11] it has been shown that androgens can directly displace oestradiol from the oestrogen receptor protein present in a number of breast tumours and may exert an influence on the activity of the tumour cells. In order to determine whether C19-steroids are present in sufficient concentration within the tissue itself to affect tumour growth or activity DHA and 5-androstene-3β,17β-diol (androstenediol) levels in a series of human primary breast tumours have been determined using a gas chromatographic—mass spectrometric technique previously described [12] and are compared to the level of cytoplasmic oestrogen receptor in the tissues.

MATERIAL AND METHODS

Tissues

Primary breast tumours were collected by the Department of Surgery, University of Liverpool, stored dry at -20°C and transported to the Tevens Institute, Cardiff in dry ice. A 15% (w/v) homogenate of the tumours was made in 10mM Tris—HCl buffer for assay of oestrogen receptor and portions of the total homogenate were stored at -20°C until assayed for steroid content.

Assay of DHA and androstenediol

The details of the method of analysis have been published in full [12]. Briefly the procedure consisted of equilibration of homogenate with internal standards to monitor recovery of steroid followed by extraction of lipid materials with acetone/ethanol. Much of the non-steroidal lipid was removed by solution of steroid in hot aqueous CaCl2 [13], and the partially purified, dried residue derivatised by the addition of bis-trimethylsilylacetamide (BSA). After standing at room temperature overnight, aliquots (1.5 μl) of the BSA were injected into a g.l.c. column containing 3% OV-17 and the eluate passed into a Varian MAT 731 mass spectrometer set up for high resolution single ion monitoring. Standards were run with every set of extractions and results

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expressed as ng of steroid per g wet weight of tissue.

**Oestrogen receptor assay**

The oestrogen receptor content of the tissues was measured by methods routinely used in the Institute. The procedure involved the incubation of portions of a high speed supernatant with varying amounts of $[^3H]$oestradiol with separation of free and bound steroid by dextran coated charcoal. Binding site concentration was determined using equations derived from the Law of Mass Action and processed by computer using a Newton–Raphson iterative curve fitting technique [14]. The results were expressed as fmole of high affinity oestradiol binding per mg cytosol protein.

**RESULTS**

The assay procedures have been shown to measure specifically non-conjugated DHA and androstenediol [12] and in this series a large number of primary breast tumours have been analysed for both steroids. All the tissues examined were found to contain measurable quantities of DHA in levels ranging from 2.5 to 466.0 ng/gm tissue. There was a highly significant ($P<0.001$) correlation between the DHA content of the tumour and the age of the patient, younger women generally bearing tumours with considerably higher amounts of DHA than older women (Fig. 1). The negative correlation was greater when the values of DHA were expressed as logarithms ($R = -0.44$).

In contrast androstenediol was measurable in just over one half of the tumours, 42% of the tissues contained less than 1 ng steroid/gm. The range of values in those tumours where androstenediol was present was much lower than those of DHA, up to 40 ng/gm (Fig. 2). No significant correlation was found between androstenediol levels in the tumour and the age of the patient although the highest amounts were found in tissues taken from younger patients (Fig. 2). Despite this there was a very significant ($P<0.001$) positive correlation ($R = 0.47$) between DHA and androstenediol levels in tumours where both these steroids were measured in one extract (Fig. 3).

Since the correlation of DHA with age of patient could be due to the menopausal status of the women it is of interest that amounts of DHA, but not of androstenediol, were significantly ($P<0.05$, Mann–Whitney U test) higher in tumours from women who were pre-menopausal. The range of values were 5.4–300.0 ng/g in this group compared to 3.2–81.8 ng/g in tissues from women known to be at least two years past the menopause.

No significant correlations were seen between the levels of DHA or of androstenediol in the whole tumour and the amount of available oestradiol receptor sites in the cytoplasm. As can be seen from Figs. 1 and 2, tumours classified as receptor negative or positive (greater than

Fig. 1. DHA levels in human primary breast tumours. The lines are the regression lines, and the correlation between steroid levels and age ($R = 0.38$) is significant ($P<0.001$). $\square =$ oestradiol receptor negative and $\square =$ oestradiol receptor positive tumours.
DHA and Androstenediol in Human Primary Breast Tumours

Fig. 2. Androstenediol levels in human primary breast tumours. □ = oestradiol receptor negative and ■ = oestradiol receptor positive tumours.

Fig. 3. Correlation between DHA and androstenediol content of primary breast tumours, both steroids measured in one extract. The two lines are the regression lines and the correlation between DHA and androstenediol levels ($R = 0.47$) is significant ($P < 0.001$). □ = oestradiol receptor negative and ■ = oestradiol receptor positive tumours.
5 f.mole/mg cytosol protein) were found to contain high or low amounts of either steroid. Furthermore, tumours containing non-measurable amounts of androstenediol had the same distribution of receptor as those containing greater than the limit of the assay. However, tumours containing a high level of DHA (greater than 100 ng/g) were found to be mainly oestriadiol receptor negative (7 out of 10 tissues), and similarly tumours with high levels of androstenediol (greater than 20 ng/g) also lacked measurable cytoplasmic receptor (5 out of 6 tissues). In the three tumours containing high levels of both steroids (Fig. 3) no specific cytoplasmic oestriadiol binding could be detected.

**DISCUSSION**

All the tumours investigated contained measurable amounts of non-conjugated DHA at levels considerably greater than would be expected from contamination by plasma. Plasma DHA levels have been quoted [15] as being around 4.5 ng/ml in pre-menopausal women which drop to approximately 1.5 ng/ml after the menopause, whereas levels in these primary breast tumours were from 2.5 ng/ml to over 450.0 ng/gm. The correlation found between the level of this weak androgen in the tumour and the age of the patient might suggest that one possible source of the steroid may be the DHA sulphate found in high concentration in the plasma (over 2 µg/ml in pre-menopausal women and about 450 ng/ml post-menopause [15]). This would indicate that virtually all the breast tissues examined contained an active steroid sulphatase enzyme system, a concept which would agree with earlier work from these laboratories both in vitro [7] and in vivo, by perfusion [8], one of the principal metabolites of [3H]DHA sulphate was shown to be the free steroid.

Dao and Libby [16] have suggested, however, that the level of steroid sulphotransferase activity correlates with response to endocrine therapy and more recently Godefroi et al. [17] have tentatively shown that there is a correlation between sulphotransferase activity and oestrogen receptor levels. The amounts of DHA found in these tumours may thus reflect a balance between the activities of sulphatase and sulphotransferase enzyme systems.

In the present series no correlation has been found between DHA levels in the tumour and the quantity of oestrogen receptor in the cytoplasm, with the exception that when amounts of endogenous steroid are high, tumours seem more likely to contain less available receptor sites. It is possible that if all the DHA is in the cytosol fraction there may be a direct effect on the oestrogen receptor assay although it has recently been shown [11] that DHA itself does not displace oestriadiol from its receptor markedly even at very high concentrations.

If a relationship does exist between DHA in the tumour and physiological activity of the oestrogen receptor it would appear to be more likely that a metabolite of the C₁₉-steroid is responsible, possibly oestriadiol itself or probably a C₁₉-steroid containing an hydroxyl group at the 3 and 17 positions. Many such steroids do displace oestriadiol from its receptor [11], the most effective being androstenediol. Since this steroid is formed from DHA in both human [8] and rat [18] breast tumours, its concentration in the tissue may be very important, both in relation to the assay for the receptor, but more significantly, in its ability to modify the activity of the cell.

The correlation between DHA and androstenediol (Fig. 3) suggests that in many of the tumours there is an active 17β-hydroxysteroid dehydrogenase enzyme system. Although it has been reported that a similar conversion of oestrone to oestriadiol and of androstenedione to testosterone occurs more frequently in neoplastic breast tissue than in normal tissue [19], it was of interest to find that in many tumours there were non-detectable levels of androstenediol. In these tumours it may be that this enzyme system is absent or that any androstenediol formed is rapidly metabolised to other compounds and does not accumulate within the tissue. In those tumours where androstenediol was found, however, the concentrations were again considerably greater than could be explained by reported plasma levels of this steroid [15].

Although it remains to be seen whether the DHA and androstenediol being measured are present in the cytoplasm of the cell or bound to particulate material or indeed merely stored in the fat particles in the tissue, the levels are, in many tumours, very high and may well be capable of affecting the oestrogen receptor and thus the activity of the cell. In some cases, these C₁₉-steroids in the primary tumour may act as a store of steroid precursor material from which oestrogens could be synthesised. It will be of particular interest to see whether the steroid content bears any relationship to the course of the disease or to the response to therapy of subsequent metastatic deposits.

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REFERENCES

PURIFICATION OF THE CYTOPLASMIC OESTROGEN RECEPTOR FROM MAMMARY TUMOURS INDUCED IN RATS WITH DIMETHYLBENZANTHRACENE

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SUMMARY

The oestrogen receptor from mammary tumours induced in rats by dimethylbenz(a)-anthracene has been extensively purified by affinity chromatography and isoelectric focusing. The unpurified cytoplasmic 8 S oestradiol receptor had a molecular weight of 240 000, a Stokes radius of 5·4 nm and a frictional ratio of 1·32; the \( K_d \) (dissociation constant) at 4 °C for \(^{3}H\)oestradiol-17\( \beta \) was 0·184 nmol/l. At the end of affinity chromatography the molecular weight was still 240 000 but under the conditions of isoelectric focusing it was reduced to 110 000, with a Stokes radius of 4·0 nm, a frictional ratio of 1·26 and an isoelectric point of 6·4.

INTRODUCTION

The identification of binding proteins, which specifically retain oestrogens, in cytoplasmic extracts of target tissues has rapidly advanced the understanding of the biological response of the tissue to the steroid. The evolution of a mechanistic theory in which transcriptional processes and macromolecular syntheses are dependent on and follow the interaction of oestradiol and its receptor as well as the translocation of the resultant complex to nuclear sites has underlined the presence of the receptor as obligatory to the hormonal dependence of a tissue. The definition of cellular response by the absence or presence of the receptor has become important in the therapy of cancer of the breast. Anomalous results are being reduced gradually by a fuller comprehension of the individual processes involved in the mechanism of hormone action, and by improved techniques in the detection and identification of receptor proteins. However, available routine assays still rely on the capacity of the receptor to retain a tritiated ligand, whereas the ideal methodology requires direct measurement of the protein.

The development of a specific immunoassay would assist greatly in the estimation of low concentrations of oestrogen receptor. A highly purified protein preparation is necessary to achieve this. The achievements of DeSombre, Chabaud, Puca & Jensen (1971) and Sica, Parikh, Nola, Puca & Cuatrecasas (1973) using calf uterus, and of Ratajczak & Hahnel (1976) and Coffer, Milton, Pryse-Davies & King (1977) with human myometrium are encouraging. As the greatest interest is directed towards the detection of oestrogen receptors in human tumours, the preferable source of receptors would be human tissue, at least until any species or organ differences can be resolved. This may not be possible because of the lack of the necessary quantities of tissue. The task of purifying the receptor from mammary tumours induced in rats with 7,12-dimethylbenz(a)-anthracene has, therefore, been undertaken. These tumours are in plentiful supply and the oestrogen receptor which has been recovered has, at least in crude cytoplasmic preparations, similar characteristics to the receptor protein.

**MATERIALS AND METHODS**

**Chemicals**

[2,4,6,7-3H]Oestradiol-17β (specific activity 83–115 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks. [4-14C]Oestradiol-17β,17-hemisuccinate (specific activity 9-94 mCi/mol) was synthesized at the Tenovus Institute by Mr G. F. Read by the method of Abraham & Grover (1971) and its purity checked by thin-layer chromatography and field desorption mass spectrometry. Unlabelled oestradiol-17β,17-hemisuccinate was purchased from Steraloids Inc., Wilton, New Hampshire, U.S.A. Oestradiol-17β, 7,12-dimethylbenz(a)-anthracene (DMBA), Trizma base, dithiothreitol, charcoal (Norit A), thioglycerol and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were bought from Sigma (London) Chemical Co. Ltd, Kingston-upon-Thames, Surrey. Ammonium sulphate (especially low in heavy metals) and indanetione hydrate were obtained from British Drug Houses, Poole, Dorset. Cytochrome C, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase and ferritin were products of Boehringer Corporation (London) Ltd, London, W5. Diethylstilboestrol was from Koch-Light Laboratories, Colnbrook, Bucks. and the multichain copolymer, poly(L-lysyl-DL-alanine) (alanine : lysine, 8:2:1 (w/w); approx. mol. wt 78 800) was bought from Miles Laboratories, Slough. All types of Sephadex and dextran, Sepharose 4B and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia (U.K.) Ltd, London, W5, and Ampholine carrier ampholytes were purchased from LKB Instruments Ltd, Croydon, Surrey. All other chemicals were of the highest available commercial grade and were dissolved in water distilled from all-glass apparatus.

**Buffers**

The following buffer systems were used routinely: buffer A: Tris–HCl (10 mmol/l), pH 7-4, containing EDTA (1·5 mmol/l) and dithiothreitol (0·25 mmol/l); buffer B: Tris–HCl (10 mmol/l), pH 7-4, containing EDTA (1·5 mmol/l), thioglycerol (12 mmol/l), glycerol (10%, v/v) and KCl (0·2 mol/l); buffer C: essentially the same as buffer B but with KCl at 0·6 mol/l; buffer D: essentially the same as buffer B but with KCl at 1·0 mol/l. Solutions of ammonium sulphate were adjusted to the required pH value (see below) with aqueous NH₃ (2 mol/l).

**Dextran-coated charcoal**

Suspensions of dextran-coated charcoal comprised charcoal (0·5%, w/v), dextran T-70 (0·05%, w/v) and gelatin (0·1%, w/v) in either buffer A or buffer B.

**Animals and tissues**

The induction of mammary tumours with DMBA in virgin female Sprague–Dawley rats and their assessment for use in experiments have been described by Nicholson, Davies & Griffiths (1977). The tumours were dissected free from connective and necrotic tissues after removal from the animals and were either used immediately or were frozen at —70 °C and used within 4 weeks.

**Preparation of subcellular fractions**

All experimental procedures were carried out at 0–4 °C except where otherwise specified.

**Preparation of cytosol**

Pooled frozen tissue (100–120 g) was allowed to thaw, and was finely minced and then homogenized in 4 vol. buffer A in a Potter–Elvehjem ground-glass homogenizer using a
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Teflon pestle with a clearance of 0.15-0.23 mm. The pestle was driven by a motor (TriR Instruments, Jamaica, New York, U.S.A.) at 3000-3500 rev./min, four or five passes being sufficient to produce a satisfactory homogenate. Cytosol was obtained by centrifugation of the homogenate at 100 000 g for 1 h in a Beckman L2-65 ultracentrifuge using the SW.27.1 (6 x 17 ml) swinging-bucket rotor (rav, 11.7 cm). The SW.50.1 (6 x 5 ml; rav, 8.35 cm) or the SW.60 (6 x 4 ml) swinging-bucket rotor (rav, 9.17 cm) was used for pilot experiments or the processing of smaller quantities of tissue. All high-speed centrifugation procedures described here involved the use of the most suitable of these rotors.

Fractionation of cytosol proteins with a salt

Ammonium sulphate (0.5 vol. of a saturated solution in buffer A) was added to cytosol in a dropwise manner with continual stirring. The resulting precipitate was collected by centrifugation at 12 000 g for 20 min and dissolved initially in a volume of buffer B equivalent to one-tenth of the original cytosol volume by gentle homogenization by hand. In some cases, further dilution was necessary before further purification. Indissoluble material was sedimented at 100 000 g for 30 min.

Preparation of poly(L-lysyl-DL-alanine)-Sepharose

Poly(L-lysyl-DL-alanine)-Sepharose was prepared using the method of Sica et al. (1973). Cyanogen bromide-activated Sepharose 4B (15 g) was washed under suction in a glass funnel (coarse disk) with 1 litre HCl (1.0 mmol/l) followed by 1 litre ice-cold water. The agarose cake was added to a solution (60 ml) comprising the multichain copolymer (75 mg) in NaHCO3 buffer (0.2 mol/l), pH 9.0. The reaction proceeded during gentle agitation on a rolling end-over-end mechanical shaker for 24 h at 4 °C followed by further agitation for 4 h at room temperature. The reaction mixture was filtered and washed (over 30 min at 24 °C) in the funnel with 1 litre NaCl (1.0 mol/l). The gel was then incubated (2 h at 24 °C) with 200 ml glycine (1.0 mol/l), pH 9.0, to inactivate unreacted groupings on the Sepharose gel. Finally, the gel was washed, without suction, with 3-4 litres NaCl (1.0 mol/l) over 3 h, followed by 1 litre of water. The substitution of the copolymer as judged by the quantity of protein measured in the filtrate by the method of Moor & Stein (1948) was 1-3 mg/ml packed gel.

Coupling of the ligand

The ligand was coupled by adapting methods described by Sica et al. (1973). Poly(L-lysyl-DL-alanine)-agarose from the previous stage was suspended in a solution of [14C]oestradiol-17β,17-hemisuccinate [50 mg (133 μmol) in 25 ml aqueous dioxan (70% v/v)]. Two portions (125 mg) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were added, 4 h apart, to the mixture being gently agitated at room temperature. After 20 h at 24 °C, the suspension was filtered and washed at 24 °C with the following solvents (extent of washing time in parentheses): 1 litre dioxan (45 min), 6 litres 80% (v/v) methanol (3 days) and 1 litre water (30 min). The substitution of oestradiol-17β,17-hemisuccinate, as determined by incorporated 14C-labelled steroid after extensive washing, was 1-23 μmol/ml packed gel. The substituted gel was stored as a suspension in buffer B containing sodium azide (0.01%, w/v) at 4 °C until used.

Affinity chromatography of oestrogen receptor protein

The affinity matrix, prepared as described above, was diluted before use by mixing 3 ml (packed volume) oestradiol-17β,17-hemisuccinyl-poly(L-lysyl-DL-alanine)-Sepharose with 7 ml (packed volume) unsubstituted Sepharose 4B. The diluted matrix was washed with 1 litre methanol (80%, v/v) over 24 h at room temperature, followed by 1 litre water (30 min) and finally 1 litre buffer B (30 min) at 4 °C.

The redissolved precipitate (40 ml) obtained by fractionation of cytosol with ammonium sulphate was stirred very gently with the affinity matrix (previously suspended in 10 ml
buffer B) overnight at 4 °C. The mixture was transferred to a column fitted with a reservoir and after filtration the matrix was washed consecutively with 70 ml each of buffer C, buffer D and buffer B.

The adsorbent cake was removed from the column and suspended in buffer B (7 ml) containing ['H]oestradiol-17β (9·3 μmol/l; specific activity 5·4 Ci/mmol) and the receptor protein was eluted from the affinity matrix by batchwise incubation (15 min at 30 °C) with occasional stirring. The slurry was filtered and washed with a further 3 ml buffer B heated to 30 °C. The pooled eluate was incubated for 1 h at 4 °C and the bulk of the free ['H]oestradiol-17β was removed by treatment (10 min at 4 °C) with the pellet sedimented from 10 ml charcoal suspension. In some cases, the eluate was concentrated to two-thirds of its original volume by dialysis against Aquacide 11-A (Calbiochem Ltd, Herts.).

Assay of receptor binding capacity

Saturation analysis

Portions (200 μl) of receptor-containing samples were incubated (4 h at 4 °C) with an equal volume of ['H]oestradiol-17β (0·25–4 nmol/l), in the presence or absence of a 100-fold higher concentration of diethylstilboestrol. After incubation, charcoal suspension (400 μl) was added and the mixtures were retained at 4 °C for 30 min. Charcoal was sedimented (800 g for 15 min) and aliquots (200 μl) of supernatant fluid were assessed for retained radioactivity.

Sedimentation analysis

The sedimentation characteristics of ['H]oestradiol-17β binding entities were analysed by centrifugation through gradients of sucrose as previously described (Powell-Jones et al. 1975). Specific binding was calculated from differences in the profiles obtained in the absence and presence of a 100-fold excess of diethylstilboestrol.

Electrofocusing

Electrofocusing of ['H]oestradiol-receptor preparations was carried out in an LKB 440 ml or LKB 110 ml column, depending on the stage of the purification and the volumes and protein concentrations involved. A 0–50% (v/v) linear sucrose-density gradient containing 2% Ampholines (pH 5–8) was prepared. When the affinity matrix eluate was processed, the gradient also contained a uniform concentration of 5% (v/v) glycerol. The dense solution at the base of the column contained the anode electrolyte, comprising 0·2% (v/v) sulphuric acid, 55% (w/v) sucrose and 5% (v/v) glycerol. The density gradient was prepared and equilibrated for 16–20 h at 4 °C before electrofocusing of the sample. Samples (4 ml) of the affinity matrix eluate, previously desalted by dialysis against buffer B, were adjusted to the required concentrations of glycerol and Ampholine and were applied to the top of the column which was protected from contact with the upper cathode solution (2% ethanolamine) by a carefully applied layer of 2-5% (v/v) glycerol. Electrofocusing was continued for a further 24 h at a constant 500 V; the temperature of the cooling water being retained throughout at 4 °C. After focusing, fractions (3 ml from the 440 ml column; 1·8 ml from the 110 ml column) were collected at a flow rate of 30 ml/h, and the pH of the fractions was measured at 4 °C.

Counting of radioactivity

Radioactivity in the samples was counted in a Nuclear Chicago (Mark I or II) scintillation spectrometer in a phosphor comprising 5 g 2,5-diphenyloxazole in a mixture of 500 ml Triton X-100 and 1 litre toluene at an efficiency of 15–35% for [3H] and 40% for [14C] as determined by external standardization.
Purification of oestrogen receptors

**Gel-exclusion chromatography**

Columns (1.6 x 100 cm) were packed with Sephadex G-200 (pre-swollen in either buffer A or buffer B) to give a bed volume of 179 ml and a void volume, determined by the use of blue dextran, of 62 ml. Samples (3 ml) containing [3H]oestradiol receptor complexes were applied to the gel and were eluted at a flow rate of 13 ml/h. Portions (0.5 ml) of the fractions (1.9 ml) were assessed for radioactivity content and protein concentration.

**Determination of molecular weight**

The molecular weights of the oestradiol receptor proteins at various stages of purification were determined from a comparison of the elution volumes of the receptor proteins with those of a series of proteins of known molecular weights from columns of Sephadex G-200.

**Calculation of Stokes radius**

The distribution coefficients ($K_D$) of protein standards were determined after chromatography on Sephadex G-200 from the relationship $K_D = (V_e - V_o)/(V_t - V_e)$, where $V_o$ is the exclusion volume, $V_t$ the bed volume and $V_e$ the elution volume of the protein. The linear relationship obtained by a plot of $K_D$ vs. log Stokes radius was used to estimate the Stokes radius of the oestradiol receptor. Values of Stokes radius for the protein standards were obtained from Siegal & Monty (1966) and Page & Godin (1969).

**Calculation of frictional ratio**

The frictional ratio for oestradiol receptor preparations was calculated from the estimations of molecular weight and Stokes radius according to the equation:

\[
\text{frictional ratio} = a \left[ \frac{4\pi N}{3\delta M} \right]^\frac{1}{2},
\]

where $a$ is the Stokes radius, $N$ is Avogadro’s number, $\delta$ is the partial specific volume, assumed to be 0.725 cm$^3$/g (Martin & Ames, 1961) and $M$ is the molecular weight.

Axial ratios were derived according to Oncley (1941).

**Estimation of protein concentration**

When sufficient material was available, protein concentration was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Protein content of chromatography eluates was monitored at 280 nm by spectrophotometry.

**RESULTS**

Pertinent aspects of each stage of the purification procedure will be described, followed by details of the characterization of oestrogen–receptor complexes at the various stages, and by a critical analysis of the degree of purification achieved. Relevant data for the purification of receptor from three batches of cytosol are presented in Table 1.

**Tissue**

The maximum time for which DMBA-induced tumours were stored in liquid nitrogen was 4 weeks. Over this period no loss of oestrogen receptor occurred. Ratios of tissue homogenate : buffer of 1 : 4 were routinely used, this proportion being consistent with good yields of receptor and manageable volumes for processing. This latter consideration also militates against the processing of tissue weights of greater than 120 g.

**Fractionation of cytosol with salt**

Selective precipitation of the oestrogen receptor by 33% saturation of cytosol with ammonium sulphate reduced protein concentration and cytosol volume, and removed cytoplasmic
Table 1. Purification of oestrogen receptor from rat mammary tumours

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sample no.</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>(total mg)</th>
<th>(disintegrations min$^{-1}$ µg$^{-1}$)</th>
<th>$\times 10^3$ (total disintegrations/min)</th>
<th>Recovery of receptor (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>1</td>
<td>203</td>
<td>13·3</td>
<td>2699</td>
<td>1·7</td>
<td>45</td>
<td>100</td>
<td>$\times 1$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>240</td>
<td>16·1</td>
<td>3864</td>
<td>3·0</td>
<td>115</td>
<td>100</td>
<td>$\times 1$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>300</td>
<td>16·0</td>
<td>4800</td>
<td>1·5</td>
<td>72</td>
<td>100</td>
<td>$\times 1$</td>
</tr>
<tr>
<td>Redissolved pellet</td>
<td>1</td>
<td>30</td>
<td>9·7</td>
<td>291</td>
<td>9·5</td>
<td>28</td>
<td>62</td>
<td>$\times 5·5$</td>
</tr>
<tr>
<td>after fractionation</td>
<td>2</td>
<td>40</td>
<td>9·8</td>
<td>392</td>
<td>9·9</td>
<td>38</td>
<td>33</td>
<td>$\times 3·3$</td>
</tr>
<tr>
<td>with (NH$_4$)$_2$SO$_4$</td>
<td>3</td>
<td>110</td>
<td>8·3</td>
<td>935</td>
<td>5·3</td>
<td>49</td>
<td>68</td>
<td>$\times 3·3$</td>
</tr>
<tr>
<td>Eluate from</td>
<td>1</td>
<td>7</td>
<td>0·03</td>
<td>0·21</td>
<td>$13·4 \times 10^3$</td>
<td>23</td>
<td>62</td>
<td>$\times 7882$</td>
</tr>
<tr>
<td>affinity matrix</td>
<td>2</td>
<td>6</td>
<td>0·02</td>
<td>0·12</td>
<td>$20 \times 10^3$</td>
<td>24</td>
<td>21</td>
<td>$\times 6666$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30</td>
<td>0·01</td>
<td>0·3</td>
<td>$11·8 \times 10^3$</td>
<td>35</td>
<td>49</td>
<td>$\times 7860$</td>
</tr>
</tbody>
</table>

*Specific binding of [H]$\text{oestradiol-17\beta}$. 
components which cleave ligand from the matrix (Sica et al. 1973; Kuhn, Schrader, Smith & O’Malley, 1975). A recovery of greater than 60% and a purification of three- to sixfold were obtained consistently after this step (see Table 1).

**Affinity chromatography**

Coupling of the copolymer and the ligand to Sepharose was followed by extensive washing with organic solvents over long periods to ensure the removal of free or non-covalently bound ligand (Sica et al. 1973). If this process was eliminated or shortened, complexed receptor appeared in the column eluent after the application of samples and this resulted in poor recoveries of receptor bound to the matrix. Free steroid in the medium was measured as follows: samples of the washing material (buffer A) were extracted with ether, after evaporation of the ether, the residue was incubated with portions of cytosol and was then incubated with [³H]oestradiol overnight. After the washing procedure, the inhibition of binding of [³H]oestradiol was less than 2%.

The amount of ligand originally coupled to the gel was much greater than that which was available to interact with the receptor protein. Increasing dilutions of substituted or unsubstituted Sepharose were incubated (16 h at 4 °C) with freshly prepared salt-fractionated cytosol. The gel was sedimented and samples of the supernatant fluid were incubated with [³H]oestradiol in the absence or presence of a 100-fold higher concentration of diethylstilboestrol. The adsorbent was capable of binding 1-54 pmol receptor protein/ml packed gel. It was observed that gel diluted three to five times was capable of retaining >90% of total receptor protein in the sample. From this information, and from an estimation of oestogen receptor in cytosol samples, the necessary dilution of gel which would ensure an excess of available ligand over receptor could be calculated. The procedure was more beneficial and effective if fractionated cytosol was mixed with the affinity matrix and incubated overnight before packing the matrix as a column, rather than if the sample was applied to a pre-packed column of matrix.

The recovery of binding activity was determined by passage of the eluate from the affinity column through columns of Sephadex G-25, and was shown to be 71% of the total receptor in fractionated cytosol. At this stage, the recovery of receptor-bound radioactivity in the affinity eluate was 49% of that in cytosol, with an overall purification of 7860-fold.

**Electrofocusing**

Profiles of [³H]oestradiol-binding obtained by electrofocusing crude cytosol and affinity eluate are compared in Fig. 1. Labelled cytosol yielded two peaks localized in the pH range 6-4-6-8 (Fig. 1a). Both of these peaks were abolished by 100-fold excess of diethylstilboestrol. A radioactive peak found at pH < 5-0 comprised non-specific binding proteins (not shown). The affinity matrix eluate also yielded two peaks of radioactivity (Fig. 1b), a large peak at pH < 5-0 representing non-specific contaminants and a smaller peak at pH 6-4 which was the [³H]oestradiol–receptor complex. These peaks correspond to those shown by Puca, Nola, Sica & Bresciani (1971), Ratajczak & Hahnel (1976) and Molinari, Medici, Moncharmont & Puca (1977).

**Characterization of the oestrogen receptor**

Sedimentation analysis of unfractionated [³H]labelled cytosol showed two distinct [³H]oestradiol-binding components with characteristic sedimentation coefficients of 4 S and 8 S (Fig. 2a). Fractionation of cytosol with ammonium sulphate and redissolution of the pellet in buffer resulted in a single [³H]oestradiol-binding peak with a sedimentation coefficient of 4 S-5 S (Fig. 2b). Scatchard (1949) analysis of data obtained by saturation analyses showed a single specific component with a dissociation constant ($K_m$) of about 0-184 nmol/l (mean value of 21 determinations).
Fig. 1. Electrofocusing profiles of oestradiol–receptor complex. Left-hand ordinates indicate radioactivity and right-hand ordinates indicate pH. (a) Cytoplasmic fractions (10 ml), after incubation (4 h at 4 °C) with [3H]oestradiol (4 nmol/l) alone (○) or in the presence of 400 nmol non-radioactive diethylstilboestrol/l (●) were processed in an LKB 440 ml electrofocusing column. The sample was positioned in the upper third of the column. A gradient of pH 5–8 was used. The current was maintained for 43 h at 400–500 V before fractions of 3 ml were collected. Measurement of pH (broken line) was done at 4 °C. (b) Purified oestradiol receptor from the affinity eluate (4 ml) was processed in an LKB 110 ml electrofocusing column. The sample was positioned near the top of the pH gradient (pH 5–8), and focused for 36 h at 400 V, after which 35-drop fractions were collected and radioactivity (○) and pH (broken line) were measured.

Fig. 2. Sedimentation analysis of oestradiol receptor–complex. Samples were incubated (4 h at 4 °C) with [3H]oestradiol (4 nmol/l) alone (○) or in the presence of 400 nmol non-radioactive diethylstilboestrol/l (●), and analysed on sucrose gradients (see Materials and Methods). The sedimentation marker (arrows) was bovine serum albumin (s20, w; 4-6 S). The direction of centrifugation was from left to right. (a) Cytosol samples were analysed at low ionic strength. (b) Cytosol was fractionated by ammonium sulphate and the redissolved pellet was analysed at high ionic strength (0-4 mol KCl/l).

Analyses of 3H-labelled fractions during the purification procedure on Sephadex G-200 are shown in Fig. 3. Cytosol yielded two peaks of protein-bound radioactivity with elution volumes of 70 and 110 ml (Fig. 3a), whereas salt-fractionated cytosol (Fig. 3b) and affinity eluates (Fig. 3c) revealed a single [3H]oestradiol-binding component with an elution volume of 65 ml. The electrofocused oestradiol–receptor complex was eluted as a single peak with an elution volume of 93 ml (Fig. 3d).
Purification of oestrogen receptors

(a) Cytosol fraction after incubation (4 h at 4 °C) with [3H]oestradiol (4 nmol/l).

(b) Ammonium sulphate fractionated cytosol. The redissolved pellet was incubated (4 h at 4 °C) with [3H]oestradiol (4 nmol/l).

(c) Affinity chromatography eluate.

(d) Electrofocused fractions (of affinity eluate) were pooled and chromatographed. Insufficient protein was present to be recorded by monitoring at 280 nm.

Fraction number

Radioactivity × 10^-3 (counts/min)

Absorbance at 280 nm

Fig. 3. Gel exclusion chromatography of oestradiol receptors throughout the purification stages. A column of Sephadex G-200 (1-6 x 89 cm) was packed and equilibrated with buffer A (Tris-HCl (10 mmol/l), pH 7-4, containing EDTA (1-5 mmol/l) and dithiothreitol (0-25 mmol/l)) at a flow rate of 13 ml/h. Samples (3-4 ml) were applied to the column and eluted with the same medium, and 1-9 ml fractions were collected. Radioactivity (○) was counted and absorbance (broken line) was measured at 280 nm.

Table 2. Physical parameters of the oestrogen receptor from rat mammary tumours at different stages of purification

<table>
<thead>
<tr>
<th></th>
<th>Cytosol</th>
<th>Ammonium sulphate</th>
<th>Affinity eluate</th>
<th>Focused affinity eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B*</td>
<td>Fractionated cytosol</td>
<td></td>
</tr>
<tr>
<td>Molecular weight (x 10^-s)</td>
<td>240</td>
<td>60</td>
<td>240+</td>
<td>240</td>
</tr>
<tr>
<td>Stokes radius (nm)</td>
<td>5-4</td>
<td>3-0</td>
<td>6-0</td>
<td>6-0</td>
</tr>
<tr>
<td>Frictional ratio</td>
<td>1-32</td>
<td>1-17</td>
<td>1-46</td>
<td>1-46</td>
</tr>
<tr>
<td>Axial ratio:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prolate</td>
<td>2-6</td>
<td>2-6</td>
<td>2-5</td>
<td>2-5</td>
</tr>
<tr>
<td>oblate</td>
<td>0-38</td>
<td>0-37</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>8 S</td>
<td>4 S</td>
<td>4 S+</td>
<td>—</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>6-4-6-8</td>
<td>6-4-6-8</td>
<td>—</td>
<td>6-4</td>
</tr>
</tbody>
</table>

*A and B refer to the two protein species distinguishable on sedimentation analysis which specifically retain [3H]oestradiol by the criterion of competition by diethylstilboestrol.

†The sedimentation coefficient was determined by analysis on gradients of sucrose containing a salt, whereas molecular weight determinations were made after removal of a salt.
Fig. 4. Correlation of elution profiles and Stokes radius. Proteins (2-5 mg) of known Stokes radius (cytochrome C, 1.7 nm; chymotrypsinogen A, 2.2 nm; ovalbumin, 2.8 nm; bovine serum albumin, 3.5 nm; aldolase, 4.4 nm; catalase, 5.2 nm; ferritin, 7.9 nm) were eluted with buffer A (Tris-HCl (10 mmol/l), pH 7.4, containing EDTA (1.5 mmol/l) and dithiothreitol (0.25 mmol/l)) from a column of Sephadex G-200 with a bed volume \((V_t)\) of 179 ml, and a void volume \((V_0)\) of 62 ml. The elution volume of the proteins was determined by measuring the absorbance of aldolase at 220 nm; chymotrypsinogen A, ovalbumin and ferritin at 230 nm; catalase at 240 nm; bovine serum albumin at 280 nm and cytochrome C at 412 nm. Distribution coefficient \((K_D)\) of protein standards were calculated as described in Materials and Methods, and log Stokes radius was plotted v. \(K_D\). Stokes radii of oestrogen receptors were obtained from the curve by observing their elution profiles on the same Sephadex G-200 column. Vertical arrows indicate the distribution coefficients of the lower (1B) and higher (1A) molecular weight entities in cytosol, and receptors present after ammonium sulphate fractionation (2), affinity chromatography (3) and electrophoresis (4).

Fig. 5. Effect of pH on the sedimentation characteristics of oestrogen receptors. Mammary tumour cytosol, prepared in buffers of differing pH, was incubated (4 h at 4 °C) with \([3H]\)oestradiol (4 nmol/l) in the presence or absence of non-radioactive diethylstilboestrol (DES) (400 nmol/l). Samples (400 μl) were analysed on a 5-20% sucrose gradient, made in buffers at the corresponding pH value to that of the cytosol samples. The sedimentation marker (arrows) was bovine serum albumin \((s_{20,w}, 4.6 S)\). The direction of centrifugation was from left to right. (a) Profiles obtained with \(^{3}H\)-labelled cytosol prepared in imidazole–HCl buffer (50 mmol/l) at pH 7.4, with (△) and without (○) DES, and cytosol prepared in similar buffer, but at pH 6.4, with (△) and without (○) DES. (b) Profiles obtained with \(^{3}H\)-labelled cytosol prepared in Tris–HCl buffer pH 7.4 (10 mmol/l Tris–HCl, 1.5 mmol EDTA/l and 0.25 mmol dithiothreitol/l), with (△) and without (○) DES, and cytosol prepared in similar buffer, but at pH 8.0, without (○) DES. For a given cytosol, profiles were similar in either Tris–HCl buffers or imidazole–HCl buffer, at pH 7.4.
Purification of oestrogen receptors

Physical characteristics
The column of Sephadex G-200 used in these experiments was calibrated as described in Materials and Methods and the results are shown in Fig. 4. Analyses of the receptor protein at various stages of purification are shown in Table 2. The apparent reduction in molecular weight of the receptor protein after electrofocusing may be due to exposure to ampholytes in the region of pH 8 during this procedure (Fig. 5).

DISCUSSION
A consistent overall purification of the oestrogen receptor of approximately 7000-fold was achieved at the end of affinity chromatography, which compares well with the figures obtained for the receptor from human tissue (Ratajczak & Hahnel, 1976; Coffer et al. 1977). As protein content after isoelectric focusing could not be measured without using unacceptably large amounts of purified material, conservative estimates had to be made regarding the degree of purification and recovery after this stage. It was assumed that 50% of the purified oestrogen receptor was recovered after focusing, corresponding to 22.5% of cytosol receptor. Further purification of about fivefold resulted in an overall purification of approximately 40,000-fold. From the molecular weight of the receptor (240,000, see Table 2) and an average cytosol concentration of 48.75 fmol receptor/mg protein, then each gram original cytosol protein contained 11.7 µg receptor protein. Based on this, a purification of 85,000-fold would be required to achieve homogeneity and the affinity eluate would contain 2% receptor. The observed isoelectric point of the oestrogen receptor (6-4) correlates with those of 6-6 and 6-8 reported for the calf uterine receptor (Sica et al. 1973) and with those of 6-5 and 6-15 reported for the human myometrial receptor by Ratajczak & Hahnel (1976) and Coffer et al. (1977) respectively. The receptor protein was found as an 8 S entity until the focusing procedure was carried out. The reasons for the retention of this conformation are somewhat obscure. The cytosolic calcium-dependent factor (Puca et al. 1971) seems to be resistant to freezing and thawing, at least in calf uterus cytosol (Molinari et al. 1977), but its deactivation, or lower effectiveness, in mammary tumour cytosol cannot be excluded. Furthermore, the possibility of enzymic effects upon this factor cannot be eliminated. In the absence of such a factor, the formation of the 4-5 S receptor will be reversible, even in the presence of calcium ions and KCl, and the 8 S entity will reappear upon removal of KCl.

The purified receptor proved unstable if stored at 0-4 °C or at -20 °C, but could be retained for 2-3 weeks at -70 °C without appreciable loss of binding activity and other properties. This method of purification represents a practical method of obtaining oestrogen receptor protein of sufficient purity to be used for the production of antibodies. Whether an immunoassay based on antiserum to oestrogen receptor from rat mammary tumour will effectively quantitate the various forms of receptor in human tissue requires further study.

The authors wish to thank the Tenovus Organization and the Iraqi Government (Ministry of Higher Education and Scientific Research) for their generous financial support.

REFERENCES


Purification of Estrogen Receptor from DMBA-Induced Mammary Tumors of the Rat

N. Al-Nuaimi, P. Davies, and K. Griffiths

The study of the specific interaction between estradiol and its binding protein in cytosol of both human and experimental animal mammary tissue has been of great value in explaining the biological response to the steroid and the subsequent reactions and products resulting from its translocation to the nucleus. Measurement of estradiol receptors in breast cancer cells has achieved great importance in choosing the best therapy for breast cancer patients. Assessment of cellular concentrations of the receptor protein itself would, therefore, be more accurate than the available assays, which determine the extent of the binding of the steroid. The development of a specific immunoassay method would greatly assist in the estimation of small concentrations of estradiol receptor protein. A highly purified receptor protein is a prerequisite for this to be achieved. This paper describes the purification of estradiol receptor protein from mammary tumors induced in rats with 7, 12-dimethyl-benz (a) anthracene (DMBA) that has similar characteristics to the receptor protein found in human breast tumors. The methods used are salt fractionation, affinity chromatography, and isoelectric focusing.

The cytosol was initially made 30% with ammonium sulfate after being prepared by the centrifugation of tissue homogenate at 100,000 g. The precipitated protein contained 60% of the total estradiol binding protein, giving a purification of three-to-five fold. The affinity matrix was prepared by the coupling of cyanogen bromide activated sepharose 4B with a multichain copolymer, poly (L-lysyl-DL-alanine), followed by coupling with estradiol-17-hemisuccinate to obtain estradiol hemisuccinyl-poly (L-lysyl-DL-alanine)-agarose. The amount of copolymer coupled to the sepharose was 1.3 mg/ml packed gel. The substitution of estradiol hemisuccinate was measured either by the incorporation of "C-labeled estradiol hemisuccinate or by radioimmunoassay. This was 3.7 μmol/ml packed gel. To avoid any loss of receptor protein during affinity chromatography, the matrix was washed extensively in 80% aqueous methanol until no free steroid was detected in the washes, and then was equilibrated with the experi-
mental buffer (10 mM Tris HCl, 1.5 mM EDTA, 1 mM DTT, and 0.2 M KCl) before use. Salt-fractionated cytosol was incubated overnight with the affinity matrix with gentle stirring. All processes were carried out at 4°C unless otherwise specified. The incubation mixture was then packed in a column and washed with the experimental buffer containing 1 M KCl, and finally with the incubation buffer.

The receptor protein was eluted from the affinity matrix by incubation with buffer containing an excess of [3H]estradiol (9.3 µM, S.A. 5.4 Ci/mmol) for 30 min at 30°C. The eluate was dialyzed, then treated with dextran-coated charcoal to remove unbound [3H]estradiol. A sample was taken from each step of the purification method for assessing estradiol receptor concentration and measuring the purification progress by charcoal adsorption technique, sucrose density gradient analysis, Sephadex G-200 column chromatography, and isoelectric focusing where applicable. A recovery of receptor-bound radioactivity in the affinity matrix eluate (Table I) was 49% of that present in the original cytosol, with an overall purification at this stage of 7,860-fold.

Further purification of the receptor protein was achieved by isoelectric focusing using a standard Ampholine column LKB-8102 (440 ml) and a sucrose gradient (pH 3-10). The receptor protein has an isoelectric focusing point of 7.3 (Fig. 1). Accurate measurement of protein concentration of the purified fractions was not possible. On the assumption that 24% of receptor was present in the original cell supernatant, a purification of greater than 50,000-fold has been achieved. By gel exclusion on Sephadex G-200 (Fig. 2) the molecular characteristics of the receptor were determined. Purified receptor protein in the affinity matrix eluate had a molecular weight of about 240,000 daltons, a Stokes radius of 60 Å, and a frictional ratio (f/fo) of 1.46 (Table II). No significant differences in these parameters has been observed among receptor preparations at various stages of purification.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (dpm/mg)</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>300</td>
<td>4,800</td>
<td>1.5 x 10^5</td>
<td>7.2 x 10^7</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate pellet</td>
<td>110</td>
<td>935</td>
<td>5.3 x 10^5</td>
<td>4.9 x 10^7</td>
<td>68</td>
</tr>
<tr>
<td>Affinity column effluent</td>
<td>180</td>
<td>756</td>
<td>2.4 x 10^5</td>
<td>1.8 x 10^7</td>
<td>25</td>
</tr>
<tr>
<td>Affinity column eluate</td>
<td>30</td>
<td>0.3</td>
<td>11.8 x 10^5</td>
<td>3.5 x 10^7</td>
<td>49</td>
</tr>
</tbody>
</table>
Purification of Estrogen Receptor

Fig. 1. Isoelectric focusing of estradiol receptor proteins.

Fig. 2. Chromatography of the affinity eluate on Sephardex G-200.

Table II. Molecular Parameters of Estradiol Receptor Protein at Various Purification Stages

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Stokes radius ((\bar{\lambda}))</th>
<th>(K_{d})</th>
<th>(f/F_{0})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>240,000</td>
<td>54</td>
<td>0.07</td>
<td>1.32</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>60,000</td>
<td>30</td>
<td>0.41</td>
<td>1.17</td>
</tr>
<tr>
<td>Ammonium sulphate pellet</td>
<td>240,000</td>
<td>60</td>
<td>0.022</td>
<td>1.46</td>
</tr>
<tr>
<td>Affinity column eluate</td>
<td>240,000</td>
<td>60</td>
<td>0.022</td>
<td>1.46</td>
</tr>
</tbody>
</table>
II. INVESTIGATIONS RELATING TO CANCER OF THE BREAST.

B. HORMONE LEVELS IN BIOLOGICAL FLUIDS.
Benign and Malignant Breast Disease in South Wales: A Study of Urinary Steroids

H. CAMPBELL,∥ M.B., F.F.S.

Summary: The levels of aetiocholanolone, androsterone, and 17-hydroxy corticosteroids were measured in women without known disease of the breast, in women with benign breast disease, and in women with primary and advanced breast cancer. Statistical analysis showed there was no difference in the excretion of urinary 17-hydroxy corticosteroids in the various groups of patients. Detailed analysis of the aetiocholanolone and androsterone levels, however, indicated that patients with advanced localized disease excreted significantly less than those in the other groups.

Introduction

The initial report (Bulbrook, Greenwood and Hayward, 1960) showing a statistical link between the urinary excretion of 17-hydroxy corticosteroids (17-OHCS) and 11-deoxy-17-ketosteroids (11-DOKS) and the response of patients with breast cancer to endocrine ablation has been followed by several similar observations in other centres. The current position regarding steroid excretion in patients with breast cancer has been amply covered (Bulbrook, 1965; Hayward and Bulbrook, 1968; Bulbrook, 1969). In summary, the findings were that those women with advanced cancer of the breast who had a relatively high ratio of 11-DOKS to 17-OHCS in the urine (as expressed by the discriminant function 80-80 (17-OHCS in mg) + aetiocholanolone in μg) had a better response to endocrine ablation, particularly hypophysectomy.

The predictive value of this “discriminant function” is strengthened by inclusion of the free period and menopausal status (Atkins et al., 1964). The results from other centres have shown that the ability to “discriminate” between those who would respond and those who would not varies considerably, and unanimity in the field has yet to be achieved. The results of the Portsmouth group (Miller, Durant, Jacobs, and Allison, 1967; Miller and Durant, 1968), who have collaborated closely with Bulbrook and his colleagues on methodological problems (Thomas, Bulbrook, Durant, Miller, and Ross, 1969) agree closely. On the other hand, two other centres were unable to show satisfactory discrimination between the responders and non-responders (Ahlquist, Jackson, and Stewart, 1968; Wade, Davis, Tweedie, Clarke, and Haggart, 1969), and indeed Thomas, Bulbrook, and Hayward (1967) did not obtain quite such good discrimination in their second and somewhat larger series studied. Thus the value of these urinary steroid analyses has remained uncertain.

Discrimination apart, Bulbrook, Hayward, and their colleagues (Hayward and Bulbrook, 1968) have also shown that about half the patients with primary breast cancer have abnormally low excretion of aetiocholanolone and that the survival rates, up to eight years after mastectomy, are greater in those patients with normal values. This was the first observation that abnormal values of urinary steroids may be present at the stage of primary disease and that they may have an influence on prognosis.

The present study was undertaken to examine the patterns of steroid excretion in women with benign and malignant diseases of the breast in South Wales.

Patients and Methods

Estimations of urinary steroids were made in a total of 177 women. Of these 21 were patients without known breast disease or malignant disease of any kind who had been admitted to hospital for elective general surgical operations; 63 had benign disease of the breast, proved by excision biopsy of a palpable lump, 30 had primary breast cancer, subsequently treated by mastectomy, and 63 were patients with advanced breast cancer, subsequently treated by hormone administration or ablation of endocrine glands. Twenty-one patients in this advanced group had disease limited to the breasts, chest walls, regional lymph nodes or pleural cavity (localized disease), and 42 had osseous and/or visceral metastases (generalized disease) with or without localized disease.

Solvents and Reagents.—Ammonium sulphate, trimethylchlorosilane, hexamethyldisilazane, propane-1,2-diol, and sodium dichromate were of ordinary B.D.H. laboratory grade. All other chemicals were of analytical grade. Ethanol (A.R. grade) was obtained from Burroughs, London, and used without further purification. Ethyl acetate was purified by washing three times with a one-tenth-volume of distilled water, dried over anhydrous K₂CO₃, and distilled. Analytical grade diethyl ether was tested for impurities by shaking a 5-ml portion with 2 ml of 2% K₂CO₃. Only batches giving a completely colourless aqueous phase were used. Hexane and heptane were each purified by passage through a 1,300-cm. column (3 cm. internal diameter) of activated (grade 1) basic Wools alumina (Camlab, Cambridge). Both solvents were then distilled.

β-Glucuronidase (2 x 10⁵ units/g.) was purchased from Baylows Chemicals, Edinburgh.

Chromatography Apparatus and Reagents.—Paper chromatography was performed on strips of Whatman No. 4 chromatography paper (Reeves Angel, London) in the apparatus described by Thomas and Bulbrook (1964). Gas-liquid chromatography was carried out on a Pye (Cambridge) 104 Chromatograph (Model 24) with a flame ionization detector linked to a Leeds Northrup Speedomax W Recorder. Stationary phases XE60, SE30, and NPGS (neopentylglycol succinate) and GasChrom Q support were obtained from Applied Science Laboratories, Philadelphia.
Procedure in Detail

17-OHCS were measured according to the method of Few (1961). The technique used for the determination of the 11-DOKS and androsterone, aetiocholanolone, and dehydroepiandrosterone was essentially similar to that of Thomas and Buell (1964) as modified by Thomas (1965). Since, however, various modifications in this method were introduced, the complete experimental approach used is now given for the sake of clarity.

Urinary Collection.—Two consecutive 24-hour collections were made for each patient. Since the levels of urinary steroids vary considerably in response to stress, the conditions under which the collections were made were standardized as carefully as possible. All patients were in hospital (most were admitted to the same ward) and the collections made before treatment was carried out. The specimens were kept cool during collection and combined on completion of the 48-hour period. A portion was then stored in the frozen state without preservative until analysed.

Extraction.—Fifty millilitres of the combined specimen was brought to pH 6.5, using Na HSO_4, or NaOH, and placed in a 250-ml. separatory funnel. Known quantities (≈ 0.1 μg) each of [4,4-^14]C]DHAS sulphate (DHAS) and [7 α-^3H]DHA glucosiduronate (DHAG) (New England Nuclear Co., Boston) were added in a small volume of ethanol. Ammonium sulphate (25 g.) was then dissolved in the urine and the steroid conjugates were extracted with three 25-ml. portions of ethanol (3:1 v/v) mixture. Extracts were combined and the droplets of aqueous layer allowed to settle. On removal of this material the organic phase was shaken with excess ether to precipitate dissolved ammonium sulphate. The mixture was then filtered through Whatman No. 4 filter paper into a 250-ml. RB flask and taken almost to dryness in a rotary evaporator. The residue was dissolved in 10 ml of ethanol, centrifuged and 8 ml (equivalent to 40 ml of urine) of the supernatant was dried in a stream of filtered air at 40°C. in a B4 Test-tube.

Hydrolysis and Solvolysis.—8-Gluconuridase powder, assayed by the method of Talalay, Fishman, and Huggins (1946), was homogenized in acetate buffer (0.5 M, pH 4.0) and centrifuged to give a clear supernatant. This supernatant was diluted with acetate buffer to give a final concentration of about 2,000 units/ml. Then 5 ml of this solution was added to the dry steroid extract and the mixture incubated for 16 hours at 40°C. After the hydrolysis, 16-4 ml of a 27% (w/v) NaCl solution was added to the incubation mixture and the pH reduced to 1.0 with 4N H_2SO_4. Extraction of free steroids and conjugates was effected by shaking twice with 20-ml volumes of ethyl acetate. Pooled extracts were then evaporated to dryness at 50°C. (Burstein and Lieberman, 1958). On reduction of the volume to about 10 ml, 30 ml of n-heptane was added and the mixture shaken successively three times with 5 ml of 5% Na_2SO_4 in Na_2O (v/v) and twice with 2-5 ml of distilled water. As also found by Thomas and Buell (1964) this procedure effectively removed most of the urinary pigments and on evaporating the solution to dryness the residue was transferred to a B4 Test-tube with three more 4-ml volumes of ethanol. Finally, this solution was taken to dryness and the residue concentrated in the bottom of the tube.

Paper Chromatography.—Strips of Whatman No. 4 chromatography paper were prepared according to the design of Thomas and Bulbrook (1964). The extract was applied to the origin and the bottom half of the chromatogram dipped in a 30% v/v solution of propane-1,2-diol in methanol. This solution was then allowed to "run up" to the origin by capillary action, and when the process was complete the chromatogram was removed, shaken, and allowed to dry for 10 minutes. Chromatography was then carried out exactly as described by the above authors and the heptane eluate (fraction, 0.05-3.5 ml.) evaporated to dryness in a B4 Test-tube.

Radioactivity Measurement and Derivative Formation.—The dry residue was dissolved in 1 ml of n-heptane containing 10 μg of Sr-androstan-3α,17β-diol and 0.1 ml of this solution was removed and placed in a liquid scintillation phial. On evaporation of the solvent the residue in the phial was dissolved in 10 ml of toluene containing 3 g/l. PPO and 0.1 g/l. dimethyl POPOP (Thorn Electronics, London) and the aliquot of this solution (0.5 ml) was carried through a liquid scintillation spectrometer Mk. I. The remainder of the ethanoic solution was meanwhile taken to dryness in a stream of N_2, desiccated for 30 minutes, and redissolved in a mixture of n-hexane (200 μl), hexamethyldisilazane (250 μl.), and trimethylchlorosilane (100 μl). On standing overnight a portion of the solution was injected without further treatment on to the column of the gas chromatograph. This procedure was found to give quantitative trimethylsilyl ether formation.

Gas-Liquid Chromatography.—Initially of 275-cm. glass column (4 mm. internal diameter) was packed with GasChrom Q (100-120 mesh) supporting 0.35% SE-30 and 0.60% XE-60 prepared as a "hybrid" column by successively evaporating solutions of each phase down on to the support. This type of column, however, did not give adequate separation of androsterone and aetiocholanolone in our hands, and consequently the last 60 cm. (detector end) was replaced by GasChrom Q supporting a 1% NPGS phase. The column was conditioned at 210°C. for 48 hours and operated isothermally at 190°C. with a N_2 flow rate of 60 ml/min. This column was found to give excellent separation of all the steroids encountered, including the contaminant described by Thomas (1965), and was thence used throughout the study.

Results

Preliminary statistical analysis of the urinary steroid levels showed that the absolute values of each steroid were normally distributed around the mean, but had a definitely skew distribution. A unimodal and symmetrical distribution was, however, obtained by conversion of the values to logarithms to the base 10, and logarithmic values were therefore used in all subsequent analyses.

Comparison of the age distribution within each group (Table I) showed an obvious difference. The mean ages of the primary and advanced groups were similar. Those of the normal and benign groups, however, were lower, differing from the primary and advanced by seven and fifteen years, respectively.

C_19 steroid Analysis

Comparison of the logarithmic values of aetiocholanolone showed that though there was no difference between the mean values of the normal, benign, and primary groups (Table II) there was a difference when the advanced group as a whole was compared with each of the other groups. This difference reached statistical significance in the case of the benign and primary groups (P<0.01), and the fact that it did not reach significance when the advanced group was compared with the normal group (P<0.2) probably reflects the small size of the sample. Similarly, when the mean logarithmic values for androsterone in each group were compared there was no difference between the normal, benign, and

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Patients</th>
<th>Mean Age ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>21</td>
<td>48.6 ± 15.1</td>
</tr>
<tr>
<td>Benign</td>
<td>63</td>
<td>38.2 ± 9.9</td>
</tr>
<tr>
<td>Primary</td>
<td>30</td>
<td>53.6 ± 10.4</td>
</tr>
<tr>
<td>Advanced</td>
<td>63</td>
<td>53.4 ± 8.7</td>
</tr>
<tr>
<td>Local</td>
<td>42</td>
<td>53.3 ± 9.3</td>
</tr>
<tr>
<td>General</td>
<td>200</td>
<td>57.6 ± 7.4</td>
</tr>
</tbody>
</table>

**Table II.—Mean of Logarithmic Values of the 17-OHCS, Aetiocholanolone, and Androsterone with the Standard Deviation for the Normal, Benign, Primary, and Advanced Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean of Log 17-OHCS ± Standard Deviation</th>
<th>Mean of Log Aetiocholanolone ± Standard Deviation</th>
<th>Mean of Log Androsterone ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.912 ± 0.264</td>
<td>2.756 ± 0.346</td>
<td>2.362 ± 0.554</td>
</tr>
<tr>
<td>Benign</td>
<td>0.854 ± 0.205</td>
<td>2.376 ± 0.190</td>
<td>2.294 ± 0.376</td>
</tr>
<tr>
<td>Primary</td>
<td>0.986 ± 0.217</td>
<td>2.657 ± 0.415</td>
<td>2.576 ± 0.504</td>
</tr>
<tr>
<td>Advanced</td>
<td>0.819 ± 0.252</td>
<td>2.563 ± 0.407</td>
<td>2.526 ± 0.500</td>
</tr>
<tr>
<td>Local</td>
<td>0.822 ± 0.279</td>
<td>2.304 ± 0.416</td>
<td>2.602 ± 0.505</td>
</tr>
<tr>
<td>General</td>
<td>0.818 ± 0.242</td>
<td>2.304 ± 0.416</td>
<td>2.602 ± 0.505</td>
</tr>
</tbody>
</table>
arithmic values of androsterone and There was again fitting about 500 years of age. The groups was adjusted for between the primary and secondary groups. A significant difference was, however, found when the advanced group was compared with the benign and primary groups (P<0.01), though no difference was found between the advanced and normal groups (P>0.5).

More detailed statistical analysis of the urinary C17-steroid excretion values were then undertaken. Since there was an age difference between the groups studied, the logarithmic values of aetiocholanolone were related to age for each patient. The values for normal patients and those with benign disease were found to increase during early adult life and then decrease with advancing age. The best calculated curve was a parabola which gave a maximum aetiocholanolone excretion between 38 and 40 years of age in each of the two groups (Fig. 1). Since there were few patients with breast cancer below the age of 38, the further analysis was limited to patients over that age when a linear regression fitted the association between the logarithmic values of aetiocholanolone and age.

Analysis of covariance showed that the regression coefficients for the normal, benign, primary, and advanced groups were similar (Table III) and that a common regression coefficient could be used. Following from this, regression lines were drawn which showed that patients with advanced disease were different from the others and had significantly lower logarithmic levels of aetiocholanolone regardless of age (Fig. 2). To examine this difference further, the patients were considered according to the distribution of their disease, and regression lines were drawn separately for those with advanced local and those with advanced general disease (Fig. 3). They confirmed that the group with advanced localized disease was distinct from all others and that such patients have significantly lower levels of aetiocholanolone.

Calculations of the pooled standard deviation were determined for patients aged 50 and used to test the significance of difference between pairs of expected mean values. These studies confirmed that the group with advanced localized disease was distinct from all others and that such patients have significantly lower levels of aetiocholanolone when adjusted for age. When the difference in logarithmic values between the advanced localized disease and each of the other groups was converted back to a primary number, it was about 500 μg., or 45% of the mean value of each of the other groups.

The normal and primary groups behaved in a similar manner whereas the benign group had levels of aetiocholanolone little different from those of the patients in the advanced group. The differences between these four groups did not, however, reach statistical significance.

A similar analysis of the androsterone results was undertaken. There was a similar relationship between logarithmic values of androsterone and age, the results again fitting a parabolic curve reaching a peak at about 40 years of age. As with aetiocholanolone, however, the age of 38 was used for the further analysis. Analysis of covariance showed again that a common coefficient could be used, and the drawn regression lines (Fig. 4) indicated that the group

<table>
<thead>
<tr>
<th>Group of Patients</th>
<th>Degrees of Freedom</th>
<th>Elevation:</th>
<th>Slope:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>31</td>
<td>-0.018</td>
<td>0.032</td>
</tr>
<tr>
<td>Benign</td>
<td>31</td>
<td>-0.018</td>
<td>0.032</td>
</tr>
<tr>
<td>Primary</td>
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<td>0.032</td>
</tr>
<tr>
<td>Local</td>
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<td>-0.018</td>
<td>0.032</td>
</tr>
<tr>
<td>General</td>
<td>31</td>
<td>-0.018</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Slope: F = 0.454, D.F. 4,135, Not significant.
Elevation: F = 1.042, D.F. 4,139, Significant at 0.05 level.

![Fig. 1. Relationship of urinary excretion of aetiocholanolone (log μg./24 hr.) to age in 21 normal women and 61 patients with benign disease of the breast.](image1)

![Fig. 2. Relationship of urinary excretion of aetiocholanolone (log μg./24 hr.) to age in 16 normal women, 39 women with benign cancer, and 90 women with malignant breast disease over 38 years of age. Twenty-nine of these had primary and 61 advanced cancer.](image2)

![Fig. 3. Relationship of urinary excretion of aetiocholanolone (log μg./24 hr.) to age, including subgroups of advanced cancer of the breast. These consisted of 21 patients with localized and 40 with generalized disease.](image3)
with advanced localized breast cancer again, regardless of age, had the lowest levels of androsterone. The difference was not as great as shown with aetiocholanolone.

17-OHCS Analysis

The mean values of 17-OHCS excretion did not vary between the different groups (Table I). Furthermore, detailed analysis did not show the parabolic relationship between urinary excretion and age, and a regression analysis failed to reveal any differences (Fig. 5).

Discussion

The results we describe support the view of others (Bulbrook et al., 1960; Bulbrook, 1965; Juret, 1968; Wilson and Moore, 1968) that steroid excretion in many patients with breast cancer differs from that of the normal population. Equally clearly our conclusions regarding this difference throughout the spectrum of breast disease do not entirely coincide with some previous opinions. The figures show that in the population studied by the methodology used in our laboratory 11-DOKS excretion in normal women and in those with benign breast disease or primary breast cancer are individually indistinguishable. Abnormal levels of 11-DOKS were found only in patients with advanced cancer of the breast and, so far as our data are concerned, no abnormality of steroid excretion was present in patients with primary cancer of the breast. This was in contrast to earlier observations of Hayward and Bulbrook (1968).

It was also noted that patients with advanced localized disease predominantly show abnormal steroid excretion, an interesting observation in the light of experience that such patients tend to respond badly to endocrine ablation. Furthermore, in a controlled randomized trial to determine whether early endocrine ablation (steroid implant of the pituitary) carried any advantage over delaying it until other simpler forms of treatment have been tried, we found that patients with localized disease again behaved differently from those in whom widespread dissemination had occurred (Stewart et al., 1969).

Rationalization of these observations in physiological terms is somewhat difficult. Perhaps the discriminating power of the Bulbrook discriminant function is in some way related to the distribution of the disease. Note worthy are the observations of Bulbrook (1969) that the original results from the application of the discriminant function to patients with primary breast cancer (Hayward and Bulbrook, 1968) required some modification when larger numbers were studied, though they could still state that assays of 11-DOKS and 17-OHCS are of value in identifying patients with primary breast cancer at high risk of recurrence (Bulbrook, 1969). Our results would therefore indicate that an abnormal level of urinary steroids is confined to patients with advanced cancer of the breast, and would appear to be a result of rather than a precedent to spread of the disease. Interestingly, however, recent work on the extent of metabolism by breast tissue of steroids found in the plasma (Jones, Cameron, Griffiths, Gleave, and Forrest, 1970; Griffiths, Jones, Cameron, Gleave, and Forrest, 1970) would not support the view that such metabolism contributes significantly to the pattern of 11-DOKS excretion. Obviously a great deal still to be learnt about the endocrinology of breast cancer.

We wish to express our gratitude to the Tenovus Organization for generous financial support. We are also grateful to Dr. R. D. Bulbrook, and to Mr. B. S. Thomas, of the Imperial Cancer Research Fund, London, for their unstinted encouragement and practical assistance. Thanks are also due to Mrs. Ursula Jones and the staff of the department of surgery, Cardiff Royal Infirmary, for their assistance in the collection of urine specimens. Mr. D. Wilson, Miss R. Gardner, and Miss T. Gardner performed a major part of the urinary analyses, and Miss K. Davis kindly helped with some of the statistical analyses. We would also thank Mr. R. Marshall and his staff of the department of medical illustration for their assistance in the preparation of the diagrams.

REFERENCES


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Plasma Dehydroepiandrosterone Sulphate Levels in Patients with Benign and Malignant Breast Disease

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INTRODUCTION

In the last 10 years a number of groups [1-4] have shown that urinary 11-deoxy-17-oxosteroid excretion can be used to help discriminate between patients with breast cancer who will or will not benefit from adrenalectomy or hypophysectomy. Recently we have demonstrated that patients with advanced cancer of the breast, untreatable by local methods but requiring administration of hormones, endocrine surgery or cytotoxic drugs, excrete significant lower levels of aetiocholanolone and androsterone than patients with benign breast diseases or primary breast cancer. This difference was found to be due to patients with advanced disease localized to the breasts, chest wall, regional lymph nodes, pleura or lymphangitis carcinomatosa and such patients affected the mean of the whole advanced group which

Abstract—The plasma concentrations of dehydroepiandrosterone (DHA) sulphate and 11β-hydroxycorticosteroids (11-OHCS) were studied in groups of women with benign breast disease and those with primary and advanced breast cancer. Statistical analysis of the results obtained revealed that there was no difference in the levels of plasma 11-OHCS in the various groups of patients. However, patients with advanced breast cancer had lower plasma concentrations of DHA sulphate when compared to each of the other groups. The difference between the advanced and benign groups achieved significance (p < 0.05).

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group for comparison with those suffering from malignant disease.

**PATIENTS AND METHODS**

The levels of the plasma steroids were estimated in 83 women; 34 had benign breast disease; 23 had primary breast cancer; 26 had advanced localized or generalized cancer (21 advanced general, 5 advanced local).

**Plasma and urine specimens**

Plasma for the determination of steroid concentrations was separated from whole blood immediately after collection and this was stored in a frozen state until the estimations were done. All the patients included in the primary and advanced groups were in hospital and specimens were taken at a standard time (9.0 a.m.). Some of the patients in the benign group were in hospital at the time of collection but some were out-patients.

Urine collections were obtained as described previously [5].

**Adrenal stimulation test**

Stimulation of the adrenal cortex was provoked with β1-25-corticotrophin (Pentacosactride) kindly made available by Messrs. Sandoz Ltd. The dose given in each case was adjusted to the body weight of the patient (2-7 i.u./kg). This dose was calculated from a standard dose and average body weight. Basal samples of blood were taken at 9.00 a.m. and further samples at 3 and 4 hr, respectively, after administration of β1-25-corticotrophin, which was given as a single intravenous injection immediately after the first venepuncture. Patients remained in a resting state until the test was completed. The plasma was separated in the manner described above and again stored in a frozen state until the steroid levels were measured.

**Plasma 11-OHCS determination**

Plasma 11-OHCS levels were estimated by a modification of the Mattingly [10] method. Fluorescence was determined by means of an Aminco Bowman Spectrophotofluorometer using a Xenon lamp in order to achieve greater specificity (excitation wave length 475 nm; emission wave length 535 nm).

**Plasma DHA-sulphate determination**

To each 4 ml plasma specimen 0-025 μCi [7α-3H]DHA sulphate (Radiochemical Centre, Amersham, Bucks.; spec. act. 1000 mCi/mMol) was added in 500 μl ethanol to act as an internal standard for recovery estimation. Samples were then extracted twice with 4 volumes of ice-cold acetone and the pooled extracts reduced in volume to approximately 2 ml under vacuum (recovery 70-6% ± 3-25). Following the addition of 20 ml methanol, fatty material was removed by extraction with an equal volume of 80-100°C light petroleum. The aqueous methanolic layer was again reduced in volume to approximately 2 ml, diluted with 10 ml distilled water, and extracted twice with 30 ml diethyl ether to remove unconjugated steroids. To the remaining aqueous phase was added (NH4)2SO4 (6 g) and the conjugated steroid fraction was obtained by extraction with 3 × 30 ml diethyl ether: ethanol (3:1). Pooled extracts were taken to dryness on a rotary evaporator and the residue chromatographed on a thin layer of silica gel HF254/356 (Merck, Darmstadt) in the system tert-butanol: ethyl acetate: 5N NH4OH (82:100:40, v/v). The area of silica gel containing the DHA sulphate was located by means of 10 μg standards run on the same plate and made visible as fluorescent spots under u.v. light (366 nm). The DHA sulphate area from the extract lane was then eluted by shaking the silica gel with 5 ml ethyl acetate and 3 ml saturated NaCl solution. Following a further 2 extractions with 5 ml volumes of ethyl acetate, the pooled extracts were reduced in volume to 10 ml. After the addition of 10 ml distilled water, 50 μl 16N H2SO4 and 2 g NaCl, solvolysis [11] was carried out by shaking the mixture thoroughly and incubating at 50°C for 4 hr. After further shaking, the ethyl acetate layer was removed and the aqueous phase extracted once more with 10 ml ethyl acetate. The pooled extracts were then washed twice with 5 ml 5% NaHCO3 solution and twice with 5 ml distilled water before being re-chromatographed on a thin layer of silica gel in the system n-hexane : ethyl acetate (1:1, v/v). When the appropriate portion of silica gel had been scraped off the plate into a test-tube, the DHA was eluted by addition of 2 ml distilled water and extraction with 3 × 5 ml benzene (final recovery, 38-7% ± 11-30). The dried residue was re-dissolved in ethanol (1 ml) containing 1 μg 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) for subsequent use as an internal standard for gas chromatography. A portion of this solution (100 μl) was removed for determination of radioactivity (MK I Nuclear Chicago Liquid Scintillation Spectrometer). The remainder was again taken to dryness, transferred to a 300 μl capacity glass stoppered test-tube and dried in a vacuum desiccator for 60-90 min at
room temperature. The 3-heptafluorobutyrate derivative was prepared [12] by dissolving the residue in 20 μl benzene and 20 μl heptafluorobutyric anhydride (Koch-Light, Colnbrook) and incubating the mixture at 65° for 30 min in a sand bath. Three standards of 0.5 μg, 1 μg and 2 μg DHA, each containing 1 μg of 5α-dihydrotestosterone were also prepared. When the reaction was complete, the reagents were removed by placing the tubes in a heated vacuum desiccator for exactly 2 min. The residues were then re-dissolved in 500 μl benzene and portions subjected to gas chromatographic analysis using a Pye 104 Chromatograph (Model 74—electron capture detector) on a column of 1% XE60 on Gas Chrom Q (Applied Science Laboratories, Philadelphia) at 185°C.

Urinary eetiocholanolone and androsterone determination

These measurements were performed as described previously [5].

RESULTS

The initial statistical analysis of the levels of plasma steroids showed that the absolute values of each were not normally distributed around their mean but had a markedly skew distribution. This is seen in Fig. 1a which shows the values of DHA sulphate for the patients with benign diseases. However, logarithmic conversion to the base 10 gave a unimodal and symmetrical distribution (Fig. 1b) and logarithmic values were therefore used in all further analysis. This finding was similar to our experience in a previous study of urinary steroid levels in similar groups of patients [5].

Comparison of the mean age of patients within each group shows no difference between the benign and advanced groups or between the primary and advanced groups (Table 1). However, there was a difference (p < 0.01) in mean age between the benign and primary groups of 6 years.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Mean age ± standard deviation</th>
<th>Mean log value of cortisol ± standard deviation</th>
<th>Mean log value of DHA-S ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>34</td>
<td>46.59 ± 6.16</td>
<td>1.44 ± 0.11</td>
<td>2.04 ± 0.22</td>
</tr>
<tr>
<td>Primary</td>
<td>23</td>
<td>54.17 ± 5.67</td>
<td>1.39 ± 0.19</td>
<td>1.92 ± 0.28</td>
</tr>
<tr>
<td>Advanced</td>
<td>26</td>
<td>51.69 ± 6.34</td>
<td>1.43 ± 0.19</td>
<td>1.77 ± 0.41</td>
</tr>
</tbody>
</table>
DHA sulphate

Since some patients with benign disease were in hospital at the time of collection of the plasma samples and others were out-patients, comparison of the mean logarithmic values of DHA sulphate for those sub-groups was made (Table 2) but no difference was found \( (p < 0.2) \).

Comparison of the mean logarithmic values of DHA sulphate showed no difference between the benign and primary groups or between the primary and advanced groups (Table 1). There was however, a significant difference between the benign and advanced groups since patients with advanced breast cancer were found to have significantly lower levels of plasma DHA sulphate than the patients with benign breast diseases \( (p < 0.01) \).

No correlation was found between the logarithmic values of DHA sulphate and age for any of the groups (Table 3). This may reflect the lack of sufficiently large numbers of patients in the earlier and later decades of life since the majority in the groups studied were between the ages of 40 and 60 years.

Relation of urinary metabolites to plasma DHA sulphate

Twenty-two patients for whom plasma DHA sulphate was measured also had estimations of the 2 major urinary metabolites, aetiocholanolone and androsterone, performed. The coefficient of correlation between logarithmic values of the sum of aetiocholanolone and androsterone, and the logarithmic values of plasma DHA sulphate was calculated \( (r = 0.4344) \). This relationship reached significance \( (p < 0.05) \) (Table 4). These results are shown in Fig. 2 together with the calculated regression line.

<table>
<thead>
<tr>
<th>Group</th>
<th>Correlation coefficient ( (r) )</th>
<th>Significance ( (p) )</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>-0.1109</td>
<td>&gt;0.1</td>
<td>34</td>
</tr>
<tr>
<td>Primary</td>
<td>-0.1967</td>
<td>&gt;0.1</td>
<td>23</td>
</tr>
<tr>
<td>Advanced</td>
<td>-0.2812</td>
<td>&gt;0.1</td>
<td>26</td>
</tr>
</tbody>
</table>

11-hydroxycorticosteroids

No difference \( (p < 0.3) \) was found in the levels of plasma 11-OHCS between those patients with benign diseases who were in hospital at the time of collection or those who were out-patients (Table 2). Comparison of the mean logarithmic values of 11-OHCS for each group showed that there was no difference between them (Table 1).

![Fig. 2](image-url)
Table 4. Coefficient of correlation between the logarithm of the sum of aetiocholanolone and androsterone and logarithmic values of plasma DHA sulphate in the benign, primary and advanced groups and the degrees of significance

<table>
<thead>
<tr>
<th>Group</th>
<th>Correlation coefficient (r)</th>
<th>Significance (p)</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>0.8993</td>
<td>&lt;0.02</td>
<td>5</td>
</tr>
<tr>
<td>Primary</td>
<td>-0.2365</td>
<td>&gt;0.1</td>
<td>6</td>
</tr>
<tr>
<td>Advanced</td>
<td>0.3686</td>
<td>&gt;0.1</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>0.4344</td>
<td>&lt;0.05</td>
<td>22</td>
</tr>
</tbody>
</table>

Adrenal stimulation with β¹-²⁵-corticotrophin

The mean logarithmic values of plasma 11-OHCS and DHA sulphate for patients in all groups before and at 3 and 4 hr after the injection of β¹-²⁵-corticotrophin are shown in Table 5. No differences were found between the groups for the mean logarithmic values of either steroid. The mean values of 11-OHCS in each group were above the upper limit of the normal range (5-25 μg%), suggesting that all patients were subject to some degree of stress. There was considerable difference in the levels of DHA sulphate in the patients studied both before and after injection of β¹-²⁵-corticotrophin and the responses showed marked variability.

Table 5. Mean logarithmic values of cortisol and DHA-sulphate with standard deviations before and at 3 and 4 hr after β¹-²⁵-corticotrophin in benign, primary and advanced groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Mean log values of cortisol ± standard deviation</th>
<th>Mean log values of DHA-S ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
<td>3 hr</td>
</tr>
<tr>
<td>Benign</td>
<td>2</td>
<td>1.42 ± 0.07</td>
<td>1.75 ± 0.05</td>
</tr>
<tr>
<td>Primary</td>
<td>5</td>
<td>1.43 ± 0.07</td>
<td>1.81 ± 0.05</td>
</tr>
<tr>
<td>Advanced</td>
<td>8</td>
<td>1.47 ± 0.14</td>
<td>1.88 ± 0.10</td>
</tr>
</tbody>
</table>

The lower concentrations of DHA sulphate in patients with advanced breast cancer might suggest that previous findings of lower urinary levels of aetiocholanolone and androsterone in such women simply reflects the concentrations of their plasma precursors. However, as Wang [9] has pointed out, care has to be exercised in drawing too close a parallel between plasma, DHA sulphate and urinary 11-deoxy-17-oxosteroid levels, since these metabolites can have a number of origins other than DHA sulphate, some of which may be of substantial quantitative importance. For example, the pool sizes and plasma concentrations of free DHA may be small but its metabolic clearance rate is very much greater than DHA sulphate [13, 14].

Although Wang [9] was unable to demonstrate a significant correlation between plasma DHA sulphate concentration and urinary 11-deoxy-17-oxosteroid excretion (p = 0.1) he stated that total plasma androsterone sulphate and DHA sulphate of patients with advanced breast cancer did show correlation with the urinary steroids. In contrast, the present study established that, in the series examined, plasma DHA sulphate concentration was correlated with the sum of its main urinary metabolites aetiocholanolone and androsterone (p < 0.05) (Table 4).

Earlier studies [5] revealed no difference in urinary 17-hydroxycorticosteroid excretion between various groups of patients. In the present investigations, no difference in plasma 11-OHCS concentrations were observed, again suggesting that this aspect of adrenal function is of little value in investigations into the aetiology of the disease (c.f. Wilson and Moore [3]).

The β¹-²⁵-corticotrophin adrenal stimulation test also appeared to provide no useful information with regard to the natural history of the disease, but it was intriguing to note the apparent lack of consistent response to this ACTH preparation with respect to plasma DHA sulphate concentrations throughout the
population studied. No difference in plasma 11-OHCS and DHA sulphate concentrations between patients with benign breast disease and those with primary breast cancer could be detected. Similarly Wang [9] has reported that no differences could be observed in plasma DHA sulphate levels of normal women and women with early breast cancer. Indeed, he observed no differences between the latter groups and the group with advanced breast cancer. However, in view of the correlation found between plasma DHA sulphate concentration and urinary 11-deoxy-17-oxosteroid excretion, our observation of lower plasma DHA sulphate levels in patients with advanced breast cancer agrees with the significantly lower urinary 11-deoxy-17-oxosteroid levels detected in the advanced group [5] (particularly in those with advanced localized disease). Hence, this observation is compatible with the view that these levels also may be characteristic of the spread of the disease rather than its type. This picture does not entirely agree with that of Bulbrook et al. [1, 15] who showed that both advanced and early breast cancer groups had abnormally low urinary 11-deoxy-17-oxosteroid output. Sufficient data to demonstrate any statistically significant difference between the plasma DHA sulphate concentrations of those with localized and those with generalized spread of their disease are not yet available.

Thus, such differences as are detectable between groups of patients with breast disease remain small and overlap of results is extensive. In some respects, the days of accumulation of large series of hormone assay figures for statistical analysis may be numbered and valuable clues hitherto provided by such series should now be applied to more intensive investigations of much smaller numbers of individuals.

Acknowledgements—The authors wish to record their sincere appreciation of continuing generous financial support by Tenovus. We thank Miss T. Gardner and Miss R. Gardner for patient and skilled technical assistance. Dr. E. R. Evans of Messrs. Sandoz, London, kindly provided the $^13$-corticotrophin for the adrenal stimulation test, and our thanks are also due to Mr. Ralph Marshall and his Staff of the Department of Medical Illustration, Cardiff Royal Infirmary, for his assistance in reproducing our diagrams.

REFERENCES

    Steroids 10, 509 (1967).
13. E. Sandberg, E. Gurpide and S. Lieberman, Quantitative studies on the
    clearance rates of dehydroepiandrosterone testosterone and their sulphate
15. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, Abnormal
    excretion of urinary steroids by women with early breast cancer. Lancet II, 1238
    (1962).
Summary.—Urinary aetiocholanolone levels have been measured in 417 women aged between 20 and 70 years. The women were drawn from South East Scotland and South Wales and consisted of patients with either benign or malignant disease of the breast and control patients suffering from no detectable breast disorder. The pattern of aetiocholanolone excretion with respect to age and menopausal status has been defined in each group of patients.

No significant differences in urinary levels have been detected between patients with breast disease, whether benign or malignant, and control patients. More detailed examination of the 201 women with early cancer of the breast has also shown that there is no consistent correlation between pre-operative aetiocholanolone levels and factors of prognostic significance detectable at the time of primary treatment—tumour size, grade, round cell infiltration, histological involvement of nodes by tumour and the clinical palpability of lymph nodes.

It would seem, therefore, that the prognostic value of pre-operative aetiocholanolone measurements is somewhat limited in patients with early breast cancer. It is noted, however, that low levels of aetiocholanolone are associated with post-menopausal patients, a group in which the prognosis is generally poorer than that in pre-menopausal women.

Considerable interest in the relationship of urinary androgen metabolites with breast cancer has been provoked by the studies of Bulbrook and his colleagues (Bulbrook et al., 1962). These workers have indicated that (a) some women with advanced cancer have subnormal levels of urinary aetiocholanolone and are unlikely to respond to major endocrine ablation (Atkins et al., 1968a, b); (b) a proportion of women with early breast cancer also have abnormally low aetiocholanolone levels (Bulbrook et al., 1962) and that this is associated with poor prognosis (Hayward and Bulbrook, 1968); (c) “normal” women who subsequently develop breast cancer have lower mean excretion of aetiocholanolone than matched controls (Bulbrook, Hayward and Spicer, 1971).

Whilst it is now accepted that low aetiocholanolone levels are associated with a proportion of patients with advanced breast cancer who have a poor prognosis (Kumaoka et al., 1968; Cameron et al., 1970), the situation in patients with early disease remains equivocal, several studies indicating that aetiocholanolone levels are normal in these women (Alquist, Jackson and Stewart, 1968; Wade et al., 1969; Cameron et al., 1970). With the recent finding that C19 androgenic steroids may act as precursors for tumour growth promoting hormones (Miller and Forrest, 1974), it is even more important to resolve this conflict.
In an effort to do so, we have retrospectively examined the results of urinary aetiocholanolone measurements performed in two major centres in normal women and in patients with either early cancer or benign disease of the breast. These have been analysed with regard to age and menopausal status, in order to define the normal pattern of excretion in patients and controls, and then related to other factors of known prognostic significance.

**PATIENTS AND METHODS**

Urine was obtained from 417 hospital in-patients in Cardiff and Edinburgh. Patients were excluded from the study if they had known endocrine disease, had been receiving steroid preparations of any type or had a history of cancer at any site other than in the breast.

**Control patients.**—These were 23 women from Cardiff and 22 from Edinburgh who were admitted to hospital for elective surgery for conditions not involving the breast. None was suspected of having malignant disease of any kind. The majority were to undergo cholecystectomy, but others included patients awaiting surgery for hernias, duodenal ulcers and varicose veins. These patients were therefore subjected to similar pre-operative stress as subjects with breast disorders.

**Benign breast disease.**—The 163 women in this group had benign disease of the breast proved by excision biopsy. No further histological classification of the 76 women from Cardiff was made but the pathological records were checked to ensure that the diagnosis of benign disease was correct.

Sufficient numbers of pre-menopausal women from Edinburgh with benign breast disease were studied to subdivide this group further.

**Group 1**—fibrocystic disease of the breast (17 patients) (those patients in whom the predominant histological abnormalities were the presence of fibrosis and cyst formation);

**Group 2**—epitheliosis (17 patients) (lesions in which epitheliosis of a marked degree was present);

**Group 3**—fibroadenomata (25 patients).

**Cancer patients.**—There were 60 patients from Cardiff and 141 patients from Edinburgh with invasive carcinoma of the breast. All of these cases were deemed to have operable or early cancer of the breast and the majority were of international clinical Stages I and II. A few cases were in international clinical Stage III, solely on account of a primary tumour of a size greater than 5 cm diameter.

**Menstrual status.**—Each of the groups has been further divided according to their menstrual status, by the following criteria:

**Pre-menopausal**—patients with regular menstrual periods;

**Menopausal**—patients whose periods were diminishing in frequency or whose last period was within 2 years of primary treatment;

**Post-menopausal**—patients whose last period had occurred more than 2 years before treatment.

**Tumour grade.**—Histological grade of the primary tumour was assessed in 191 of the 201 patients, using the criteria of Patey and Scarff (1928, 1929). In this classification, increasing grade reflects an increase in tumour malignancy (Champion and Wallace, 1971).

**Round cell infiltration.**—At the time of grading, a semi-quantitative assessment of round cell infiltration was also carried out, as described by Champion, Wallace and Prescott (1972).

**Tumour size.**—The clinical size of the tumour was measured by 2 independent observers at the time of presentation. The tumour was measured in 2 planes at right-angles and the larger diameter was recorded in cm.

**Lymph node involvement.**—The clinical status of the axillary nodes was assessed by 2 observers before operation. Microscopical examination for the presence of tumour was carried out in all cases from which lymph nodes had been removed. In Cardiff, where the standard operations were either a simple mastectomy with pectoral node biopsy or a modified radical mastectomy, axillary node histology was available. In Edinburgh, where simple and radical mastectomies were performed, it was possible to make a histological assessment of the radical mastectomy group only (68 patients), no axillary nodes being avail-
able from those patients undergoing simple mastectomy.

Urine collection and analysis.—Urine was collected from all patients pre-operatively; in Cardiff for 48 h and in Edinburgh for 24 h before operation.

The techniques used for estimation of aetiocholanolone were based on that described by Thomas and Bulbrook (1964), and Thomas (1965). Different modifications of these basic methods were introduced separately in Cardiff (Cameron et al., 1970) and Edinburgh (Sneddon, 1969). These variations have led to minor differences between centres with respect to extraction, purification and measurement of aetiocholanolone, all of which are unlikely to affect quantitation. One major difference between the methods employed in the 2 centres, however, was the use of conjugated internal standards in Cardiff and free steroid standards in Edinburgh in order to monitor manipulative losses. This is likely to result in different absolute values for aetiocholanolone in any given specimen of urine. The results from the 2 centres are therefore presented separately. Nevertheless, any differences in aetiocholanolone values between the centres ought to be consistent, and the same relationship between aetiocholanolone and other parameters therefore would be expected in results from both Cardiff and Edinburgh.

RESULTS

The mean and standard deviation for the ages of the patient groups under study in Cardiff and Edinburgh is shown in Table I. The pre-menopausal, benign and control groups from Cardiff are considerably younger than the corresponding cancer group. To achieve comparability, only individuals aged 30 or more have been compared in the subsequent tests of significance (see below).

As statistical analysis of urinary steroids with age revealed a log normal distribution (Cameron et al., 1970), aetiocholanolone levels have been expressed in logarithms to the base 10 of the concentration (µg/24 h).

Age and menopausal status

Scattergrams relating aetiocholanolone levels to age and menopausal status in the patients with early breast cancer, from Cardiff are shown in Fig. 1 and from Edinburgh in Fig. 2. These figures also show the regression lines of aetiocholanolone with age for the pre- and post-menopausal patient groups. The regression coefficients for these lines do not differ significantly from zero. Age per se, therefore, has little effect on aetiocholanolone excretion in patients from either city. There is, however, a significant difference between the mean levels of aetiocholanolone in the pre- and post-menopausal patients, there being a substantial reduction in aetiocholanolone excretion after the menopause.

Analysis of the control and benign disease groups (restricted to 30 years and older) also revealed a similar pattern. Once subdivided into pre- and post-menopausal patients, age had no significant effect on the level of urinary aetiocholanolone. The pre-menopausal patients, however, showed significantly increased levels compared with post-menopausal patients (Fig. 3, 4).

Information was available from a number of patients under the age of 30 years—15 patients with benign disease and 5 control patients from Cardiff. These data are included in Fig. 5 and 6, which show the relationship between the aetiocholanolone excretion for all of

| Table I.—Mean Age and Standard Deviation of Patient Groups under Study |
|----------------|----------------|----------------|----------------|
|                | Pre-menopausal |                | Post-menopausal |
|                | Cardiff        | Edinburgh      | Cardiff        | Edinburgh      |
| Controls       | 33.0±12.3      | 39.1±7.3       | 63.1±7.2       | 60.7±5.0       |
| Benign disease | 35.0±8.7       | 40.3±5.4       | 50.0±8.2       | 57.0±6.3       |
| Cancer         | 43.1±6.78      | 44.0±4.5       | 60.7±5.8       | 59.7±6.5       |
the pre-menopausal control patients and for those with benign breast disease. In the benign group, there is a gradual increase in aetiocholanolone levels up to the age of 30 years, beyond which the mean level of excretion becomes relatively constant. This relationship is not apparent in the small number of women in the control group.

**Variation with breast disease**

This information is presented in Fig. 3 and 4. Analysis of variance showed no significant difference in mean aetio-
URINARY AETIOCHOLANOLONE IN PATIENTS WITH EARLY BREAST CANCER 623

Fig. 3.—Urinary aetiocholanolone levels by menopausal status in control subjects and patients with early breast cancer and benign breast disease from Cardiff (lines refer to mean ± standard error for the Groups).

Fig. 4.—Urinary aetiocholanolone levels by menopausal status in control subjects and patients with early breast cancer and benign breast disease from Edinburgh (lines refer mean ± standard error for the groups).
Fig. 5.—Scattergram of aetiocholanolone levels against age in 58 premenopausal patients with benign breast disease from Cardiff.

Fig. 6.—Scattergram of aetiocholanolone levels against age in 12 premenopausal control subjects from Cardiff.

Fig. 7.—Urinary aetiocholanolone level in premenopausal patients with either fibroadenomata, fibrocystic disease or epitheliosis of the breast (lines refer to mean ± standard error for the groups).
cholanolone levels between benign, cancer and control patients, whether pre- or post-menopausal, in women from either Cardiff or Edinburgh. The further subdivision of the Edinburgh patients with benign breast disease into those with fibroadenomata, fibroadenosis or epitheliosis, revealed no differences between these groups (Fig. 7).

Correlation with other clinical and tumour parameters

Size, malignancy grade and round cell infiltration of the primary tumour, together with clinical palpability of lymph nodes, were assessed in 191 patients from the 2 centres. Histological examination of lymph nodes was performed on 120 patients. The partial correlation coefficients of aetiocholanolone with each of the other variables are shown in Table II.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiff (52 patients)</th>
<th>Radical mastectomy (68 patients)</th>
<th>Total group (139 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.29</td>
<td>-0.02</td>
<td>-0.02</td>
</tr>
<tr>
<td>Grade</td>
<td>-0.30*</td>
<td>0.01</td>
<td>-0.19*</td>
</tr>
<tr>
<td>Round cell involvement</td>
<td>-0.30*</td>
<td>0.15</td>
<td>0.18*</td>
</tr>
<tr>
<td>Clinical involvement</td>
<td>0.11</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Histological involvement</td>
<td>0.12</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>0.22*</td>
<td>0.07</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* $P < 0.05$.

These coefficients reflect the association between aetiocholanolone levels and each of these variables, after mutual association with menopausal status and with each of the other variables, has been taken into account (Steel and Torrie, 1960). In the Edinburgh series, the full set of partial correlation coefficients could only be obtained for the 68 patients undergoing radical mastectomy, from whom histological examination of nodes was possible.

No consistent correlation was found between aetiocholanolone levels and any of these factors of prognostic significance. Significant but diverging relationships were, however, observed in Cardiff and in the full Edinburgh series with regard to both tumour grade and round cell infiltration. No obvious reasons are apparent for these paradoxical observations.

Discussion

Using data obtained separately from the 2 centres, it has been possible to define the excretion of aetiocholanolone in control women and patients with benign and malignant breast disease, with respect to their age and menstrual status. By examining the data from patients with early breast cancer, it has also been possible to relate these aetiocholanolone levels to other factors known to be of prognostic significance.

The relationship between urinary aetiocholanolone and age has previously been expressed in the form of a quadratic equation (Cameron et al., 1970), increasing age being associated with decreasing aetiocholanolone excretion. Whilst the present results are in accord with these findings, they also indicate that age per se is not associated with lowered aetiocholanolone levels. If the results are re-plotted after dividing the patients into pre- and post-menopausal groups, then age alone fails to influence urinary aetiocholanolone excretion significantly. It seems, therefore, that the reduction in aetiocholanolone observed with increasing age is a result of the increasing proportion of post-menopausal women. This relationship is also observed in control patients and in those with benign breast disease.

The present study, using a large number of patients, confirms the findings of Wade et al. (1969) and Cameron et al. (1970) that the mean urinary aetiocholanolone levels of patients with early
breast cancer are no different from those with either benign disease or in-patient controls. The observation that patients with epitheliosis (believed to be a pre-malignant condition) have normal aetiocholanolone levels does not support the suggestion that low aetiocholanolone levels may occur in women susceptible to breast cancer.

It has not been possible to demonstrate any consistent relationship between pre-operative urinary aetiocholanolone and any single one of a series of clinical and morphological factors of known prognostic significance in women with early breast cancer. These observations, therefore, are at variance with the suggestion that aetiocholanolone is of major prognostic significance in early breast cancer (Hayward and Bulbrook, 1968). In this respect, the time of the urine collection may be of importance. The data in the present study is based upon pre-operative specimens whereas those in Bulbrook's study related to urine collected 10 days after mastectomy. It is now known that mastectomy, possibly via the stress of surgical procedures, influences both urinary and plasma levels of androgenic steroids (Hayward and Bulbrook, 1968; Wang et al., 1974). Conversely, patients awaiting surgery are likely to suffer pre-operative stress and this may also contribute to differences in pre- and post-operative steroid excretion.

It should be noted, however, that this study shows one group of patients, i.e. the post-menopausal women, who are likely to have low urinary aetiocholanolone levels. It is therefore interesting that pre-menopausal women over 35 years with breast cancer have a better prognosis than those women who are post-menopausal (Hamilton et al., 1974). We therefore emphasize the importance of menopausal status in early breast cancer and suggest that it should be taken into account when assessing the possible predictive value of urinary aetiocholanolone.

This work was supported in Cardiff by Tenovus, and in Edinburgh by a grant to Sir John Bruce from the Cancer Research Campaign. The authors wish to thank N. Gleave, H. Stewart, M. M. Roberts and N. Campbell for their help in compiling data for the Cardiff patients. Similar thanks are offered to the surgeons and radiotherapists in Edinburgh who took part in the Edinburgh Breast Trial, and to Mr T. McNair who allowed us to study his patients as a control group. The authors are also grateful to the pathologists in both Cardiff and Edinburgh for their co-operation and generous provision of histological material.

REFERENCES


URINARY AETIOCHOLANOLONE IN PATIENTS WITH EARLY BREAST CANCER

Miller, W. R. & Forrest, A. P. M. (1974) Oestra-
diol Synthesis by a Human Breast Carcinoma. 
Lancet, ii, 866.


Sneddon, A. (1969) Estimation of Urinary 11-
deoxy-17oxosteroids using Isotopically Labelled Internal Standards. J. Endocr., 43, 487.


Thomas, B. S. & Bulbrook, R. D. (1964) A Rapid Method for the Estimation of Total 11-deoxy-17-


simple mastectomy followed by a watch policy, simple mastectomy followed by routine postoperative radiotherapy, and radical mastectomy. The chemotherapy regimen will be a two-drug combination. A smaller group will receive a four-drug regimen similar to that used in late cancer of the breast, but at a reduced dosage. Questions about the most effective form of primary treatment and chemotherapy can only be answered quickly as a result of cooperation involving many centres.

We thank Prof. J. G. Murray for his help and encouragement, our trial pathologists Prof. G. A. Gresham and Dr C. W. Elston for their invaluable contribution, and all the participants in the Cancer Research Campaign Breast Study, Miss Janet Humm and Mrs Joyce Quigley for typing the manuscript; Mrs Penny Besteridge for technical assistance; and the Cancer Research Campaign for financial support.

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REFERENCES


CIRCULATING HORMONE CONCENTRATIONS IN WOMEN WITH BREAST CANCER

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SUMMARY

Multiple plasma-hormone concentrations were measured in sequential plasma-samples from six women with breast cancer and were compared to concentrations in six control women matched for age, years since menopause, and parity. All hormone concentrations in all the women studied were within normal limits. However, within the normal range the plasma-testosterone concentrations in each cancer patient were significantly higher than in each matched control.

INTRODUCTION

It is well recognised that hormones are involved in the development and growth of breast cancer. However, studies of circulating hormone concentrations have not indicated that women with breast cancer differ in oestrogen, androgen, or prolactin concentrations from normal controls. In these studies single daily samples of plasma have generally been used for the estimates. In the study we now report, sequential samples of blood were taken at 15-minute intervals throughout the day from individual patients with breast cancer and from matched controls. Concentrations of cortisol, luteinising hormone (L.H.), and follicle-stimulating-hormone (F.S.H.), as well as oestradiol, testosterone, and prolactin, were estimated.

PATIENTS AND METHODS

Six patients with breast cancer were studied: two had early breast-cancer, two had advanced local disease, and two had disseminated disease. Four patients were tested when in hospital and two attended as outpatients. All were at least 5 years since their menopause and were otherwise fit, and all had offered voluntarily to take part in the study. Women of similar age, parity, and years since their last regular menstrual period were used. These also were volunteers whose cooperation was sought after identifying their compatibility with the patients from the records of the blood transfusion service. Details of patients and controls are shown in table 1.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Age (yr)</th>
<th>Years since menopause</th>
<th>Parity</th>
<th>Cancer patients</th>
<th>Age (yr)</th>
<th>Years since menopause</th>
<th>Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>21</td>
<td>**</td>
<td>1</td>
<td>75</td>
<td>22</td>
<td>**</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>15</td>
<td>**</td>
<td>1</td>
<td>65</td>
<td>17</td>
<td>**</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>12</td>
<td>**</td>
<td>1</td>
<td>75</td>
<td>22</td>
<td>**</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>12</td>
<td>**</td>
<td>1</td>
<td>65</td>
<td>17</td>
<td>**</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>21</td>
<td>**</td>
<td>1</td>
<td>75</td>
<td>22</td>
<td>**</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>20</td>
<td>**</td>
<td>1</td>
<td>65</td>
<td>17</td>
<td>**</td>
</tr>
</tbody>
</table>

Patients and their controls started their tests between 9.20 and 10.30 A.M. A "Teflon" cannula (18G) was inserted into an arm vein and 10 ml blood withdrawn at intervals of 15 min for 8 h and immediately placed in heparinised tubes. Between sampling, patients walked freely round the ward and took normal meals. The total duration of sampling varied in individuals and controls, and usually lasted between 6 and 8 h. The blood was immediately centrifuged and the plasma stored at −20°C. Once all the samples were collected they were sent in batches to the Teneusse Institute, Cardiff, for assay of prolactin, L.H., F.S.H., oestradiol, testosterone, and cortisol.

Hormone Assays

Prolactin was assayed by a double antibody radioimmunosay method described by Boyes and Cole. Results were expressed in terms of M.R.C. standard 71/222. L.H. and F.S.H. were assayed by methods described by Groom et al., L.H. was expressed in terms of M.R.C. standard 68/40 and F.S.H. in terms of second International Reference Preparation human menopausal gonadotrophin. Testosterone was assayed by the method of Fahmy et al. and cortisol by the method of Joyce et al. Plasma-oestradiol-17β was assayed by the radioimmunoassay method described by Goldner et al.

DATA ANALYSIS

The mean concentration of each hormone for the six patients during the 15 min period was calculated and compared graphically with the mean of the six controls. In these graphs, means were calculated for a period of time by the clock. Some points on the graph at either end therefore represent the means of five or even four patients. The mean values for each patient in the whole period of the
test were compared with those of their matched controls by Wilcoxon's matched-pairs signed-ranks test. For this calculation only samples of those 15 min periods by the clock for which there were both test and control samples were included. A statistical analysis of the sequential samples in both patients and controls was carried out to determine whether there was a detectable pattern in hormone concentration during the 8 h period. The test of significance was based on a comparison of the observed number of turning points to be expected if the sequence was random (E(T)) with the observed number T. These values were obtained for each woman separately.

Results

The mean concentrations of oestradiol-17β, L.H., F.S.H., and cortisol did not differ between the six patients with breast cancer and their matched controls. Prolactin and testosterone concentrations did differ (see accompanying figure). The difference in prolactin concentrations was sharply accentuated by the levels in one patient (no. 4) who was subsequently found to have been taking thyroxine 0.1 mg daily and spironolactone and hydroflumethiazide 25 mg three times daily before the test; accordingly the prolactin readings for this patient have been excluded from the figure.

The means of the samples for each patient are compared with those of their time-matched control samples in Table II. Significant differences at the 5% level were observed only in testosterone concentrations which were higher in every woman with breast cancer than in the matched control. Five patients also had higher serum-oestradiol-17β concentrations, but this only reached significance at the 10% level.

The pattern of sequential sampling in patients and controls and the significance of differences are shown in Table III. L.H. showed no significant pattern. However, oestradiol-17β and testosterone both showed significantly more turning-points than would be expected by random fluctuation. The results for prolactin, F.S.H., and cortisol showed significantly fewer turning-points than would be expected by random fluctuation, indicating changes in these variables during the observation period. Cortisol concentrations showed a

![Graph: Plasma prolactin and testosterone concentrations in patients with breast cancer and in controls.](https://example.com/graph.png)

| Table II: Comparisons of sample means for each patient compared with those of each time-matched control. |

<table>
<thead>
<tr>
<th>Patient and matched control no.</th>
<th>Cancer (mm. U./ml)</th>
<th>Cancer (mg/ml)</th>
<th>Cancer (pg/ml)</th>
<th>Cancer (mmU/ml)</th>
<th>Cancer (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.5</td>
<td>16.3</td>
<td>1.2</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>25.0</td>
<td>16.2</td>
<td>1.0</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>25.1</td>
<td>16.3</td>
<td>1.1</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>25.2</td>
<td>16.4</td>
<td>1.2</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>5</td>
<td>25.3</td>
<td>16.5</td>
<td>1.3</td>
<td>0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Mean difference: 0.17

N.S.: p < 0.05

(With Wilcoxon's test) N.S.

N.S.: N.S.

N.S.: not significant.

| Table III: Pattern of sequential sampling in patients and controls and significances of differences. |

<table>
<thead>
<tr>
<th>Cancer patients</th>
<th>Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients</td>
<td>Controls</td>
<td>Total</td>
</tr>
<tr>
<td>T</td>
<td>E(T)</td>
<td>var(T)</td>
</tr>
<tr>
<td>L.H. Prolactin</td>
<td>74</td>
<td>98.0</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>121</td>
<td>114.7</td>
</tr>
<tr>
<td>Testosterone</td>
<td>150</td>
<td>119.8</td>
</tr>
<tr>
<td>F.S.H. Cortisol</td>
<td>114</td>
<td>115.8</td>
</tr>
</tbody>
</table>

* p < 0.001

# \sqrt{var(T)}
pronounced fall 2 h or more after starting the test, usually followed by a later rise. The pattern for prolactin was not consistent for all women and generally showed only undulations from sample to sample. However, in six women prolactin concentration was reduced over the first 2 or 3 h. F.S.H. concentrations did not change much during the period of the test; departures from randomness were due to inconsistent undulations in level from sample to sample.

Discussion

All the observed concentrations of plasma hormones were within the limits of normal for postmenopausal women. We have no evidence to indicate that women with breast cancer have abnormal hormone concentrations.

Within this normal range, each patient with breast cancer had significantly higher testosterone values than her matched control at all times of the day. This difference was significant at the 5% level. Levels of oestradiol-17β in women with cancer were also higher than those in their matched controls, but significant differences were not proven. In making comparisons of six variables in two groups of six women, there is an appreciable chance that at least one variable would demonstrate a statistically significant difference at the 5% level, even if there are no differences in the concentrations in the populations from which these groups were derived. Furthermore, if the six variables were interdependent, there would be a probability of 17% that at least one variable would show a statistical difference at the 5% level, using Wilcoxon’s test on six matched pairs of subjects. The results must therefore be interpreted with some caution.

Apart from the obvious circadian rhythm of cortisol concentration, the sequential samples did not follow any consistent pattern of change. The absence of a significant difference from randomness of 17β concentrations is contrary to previous findings. Both oestradiol-17β and testosterone showed significantly more turning points than would be expected by random fluctuations—i.e., there was a zigzag pattern of change. The number of samples which could be included in a single run of a testosterone assay was limited to 20 and for this reason alternate samples from an individual were taken for each run. This explains the apparent zigzag serial pattern of testosterone but not of oestradiol-17β. It seems likely that this was chance variation.

We are aware of only two previous studies where plasma-testosterone concentrations have been measured in women with early or late breast cancer.16 17 In neither were differences observed from normal controls. However, sequential sampling was not carried out and radioimmunoassay was not used.

Although most previous work has indicated that low excretion of androgen metabolites may be associated with an increased risk of breast cancer,18 there is some evidence of a converse effect. Japanese women, who have a low risk of the disease, have been reported to have lower urinary excretion of androgen metabolites than their Western counterparts;19 conversely, women with breast cancer in Britain secrete sebum at a higher rate than normal.20 21 While the significance of the increased circulating testosterone levels which we have observed must remain in doubt until larger numbers have been studied, our report that testosterone can be metabolised by human breast cancer to form oestradiol-17β suggests that this may result in higher oestrogen concentrations within the tumour.22 23

We thank Dr R. J. D. Cash and Dr A. E. Robertson of the Blood Transfusion Service, Royal Infirmary, Edinburgh, and Mrs T. B. Anthony of the Blood Transfusion Service of Galashiels, and the patients and other women who took part in this study for their cooperation.

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REFERENCES

17. Horn, H., Gordon, G. Oncology, 1974, 30, 147.

PANCREATIC-POLYPEPTIDE RESPONSE TO FOOD IN DUODENAL-ULCER PATIENTS BEFORE AND AFTER VAGOTOMY

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Summary In 21 normal fasting subjects basal serum-pancreatic-polypeptide (H.P.P.), as measured by a specific radioimmunoassay, was 43±4 pmol/l (mean±s.e.m.). This basal concentration was of the same magnitude as that of other gastrointestinal peptide hormones. In normal subjects the H.P.P. response to food was biphasic. A rapid eightfold increase, reaching a maximum 20–30 min after beginning of the meal, was followed by a secondary, prolonged increase. H.P.P. did not return to basal concentration within five hours. 14 duodenal-ulcer (D.U.) patients were studied
The existence of two different autosomal loci, one controlling enzyme activity in leucocytes, the other in plasma, could help explain the disease fusosidosis. An individual who is homozygous for low-activity alleles at both loci would be expected to develop the disorder. However, on this basis, the expected incidence in the general population would be 0.0053 (i.e., $q_1^2 \times q_2^2$, roughly 5 per 1000. In fact the condition seems to be very rare, and it appears likely that the expression of the disease may depend on other genetic factors.

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J. R. PLAYFER

PLASMA-HORMONES DURING ANAESTHESIA FOR BREAST SURGERY

Sir,—In the search for a biochemical means of discriminating between hormone dependent and independent breast cancers, hormone-receptor levels and endogenous tissue hormone levels are being investigated. Since many measurements of receptors involve saturation of these, and since plasma levels influence endogenous tissue levels, we have examined the effects of anesthetic procedures on concentrations of prolactin, growth hormone, gonadotrophins, and oestradiol-17β in 30 consecutive operations on women undergoing general anaesthesia for breast-related conditions. Blood-samples were obtained before administration of anaesthetic and at the time that tissue was removed (4-3 h later). Hormone concentrations were measured by radioimmunoassay.

Prolactin concentration more than doubled peroperatively in 22 patients and was halved in only 2 patients (fig. 1). Growth hormone decreased in as many patients as it increased, greater than twofold changes occurring in 22 cases. In contrast, for oestradiol-17β, lutetiumising hormone, and follicle-stimulating hormone, only 1, 2, and 5 patients, respectively, experienced greater than twofold changes in hormone concentration (fig. 1).

Altogether 15 drugs were used to induce and maintain anaesthesia (fig. 2). When the patients were arranged in the order of increasing prolactin increment (as in fig. 1), it became apparent that halothane had been administered to those in whom the greatest peroperative increase ($p<0.005$ by the runs test) had been observed. Other agents to give significant effects on prolactin were pancuronium ($p<0.01$) and neostigmine ($p<0.05$).

When patients were ranked according to oestradiol increment, papaveretum was associated with a peroperative decrease in concentration ($p<0.05$ which became $p<0.01$ by including those patients who had been given morphine and 1. Nicholson, R. I., Goldner, M. P., Davies, F., Griffiths, K. Eur. J. Cancer 1976, 17, 711.

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JOHANNES GORMSEN

HANS MEINERTZ

5. Division of Heart and Vascular Diseases, National Heart, Lung, and Blood Institute, Bethesda, Maryland. Personal communication.

Fig. 1.—Cumulative frequency distribution of hormone concentrations expressed as ratios.

penicillin-induced haemolytic anaemia. The standard adult dose of penicillin-G administered to establish an established case of clostridial myonecrosis with normal renal function is 72 megauinitis/day, in 3 megauinitis hourly intravenous increments, for as long as 7 days.

The rationale for such a raised penicillin dose is the provision of a high diffusion gradient from viable perfused tissue to necrotic non-perfused tissue, the nidus in which the anaerobic, penicillin-sensitive clostridial organisms flourish. In the light of a possible bleeding tendency induced by penicillin, such gas-gangrene patients must be monitored very carefully, because increased vascular oozes within the devitalised limb may jeopardise further muscle vitality and encourage clostridial resurgence.

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IAN P. U侵蚀

QUANTITATIVE THROAT-SWAB CULTURE

Sir,—The criticism of our study of quantitative throat swab culture (July 10, p. 61) raised by Dr George and Mr Purdham (Sept. 11, p. 582) was apparently prompted by the difficulties they experienced in the collection of adequate specimens. We had recognised, before our study began, the existence of such problems in collection and also in transport of specimens. Accordingly the design of the study included prior training of the participants and batch testing of the materials used. The adequacy of collection and transport of specimens was confirmed by the isolation-rates of streptococci observed in the patients and symptom-free carriers. In both instances the rates were comparable with those reported in similar studies elsewhere.

The theme of the report of our study was restricted to the value of a standardised laboratory method of quantitative throat culture in the diagnosis of streptococcal pharyngitis. Considerable emphasis has been given to the collection and transport of specimens in the past, whereas quantitative laboratory examination received little attention. The report, therefore, was not enlarged to include a discussion of details of collection or transport which we assumed would be understood to be according to well-established practice.

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Australia

S. M. BELL
D. D. SMITH

EARLY USES OF POISON OAK

Sir,—The Indians of the Western Hemisphere have provided modern medicine with such powerful and useful naturally occurring botanical substances as curare and caffeine. Poison oak (Toxicodendron diversiformum) grows profusely throughout a large part of California; it has been estimated to be responsible for thousands of lost work days and is the leading cause of occupational dermatitis. Physicians may therefore also be interested in the early observations made by California Indians with regard to the medical applications of poison oak.

California Indians noted the efficacy of desensitisation therapy (i.e., that ingestion of young poison-oak leaves in the spring attenuated attacks of phytocontact dermatitis later in the year), but it is not generally appreciated that they also successfully employed immunotherapy for the treatment of necrosis. Balls4 writes: "The juice from stems, leaves, or roots was used as a cure for warts. The wart was either cut off close to the base or pricked or cut several times, and the fresh juice applied immediately. The application had to be repeated several times before the wart would disappear."

Circadian variation in urinary melatonin in clinically healthy women in Japan and the United States of America


Chronobiology Laboratories, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis (Minnesota 55455, USA); Department of Psychiatry, Karolinska Institute, St Görans Hospital, S-11281 Stockholm (Sweden); Department of Medicine, University of Florence, Florence (Italy); Tenovus Institute, Welsh National University, Cardiff (Wales, U.K.); 2nd Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka City (Japan), and St Paul-Ramsey Hospital, St Paul (Minnesota 55101, USA), 31 July 1978

Summary. Urinary melatonin excretion is lower in East-Asian (Japanese) than in North-American (whites of mixed ethnic origin) women. Moreover, a statistically significant circadian rhythm is demonstrated by population-mean cosinor in the data pool from both groups of women. Furthermore, statistical significance characterizes interactions of effects from geographic differences (between ethnic groups) with temporal factors. Such spatio-temporal interactions await further scrutiny with a view inter alia of carcinogenesis as it is influenced by a spectrum of intermodulating rhythms.

Circadian rhythms have been reported for urinary excretion and blood levels of melatonin in human beings, rats and calves, along with changes in serum melatonin during the menstrual cycle. Apart from basic physiologic and pharmacologic aspects beyond our scope, interest in this variable stems from the question whether melatonin excretion may vary in populations with different breast cancer risk. To study this possibility, adult female subjects, some menstrually cycling, others post-menopausal (table 1) were admitted to the Clinical Research Center at the University of Minnesota in Minneapolis between 08.00 h and 09.00 h, for a stay of about 28 h between mid-July and early September 1977. Each subject was instructed to note at home the time of her 1st morning urinary voiding on the day of admission. Thereafter urine specimens were collected in the hospital approximately every 2 h while the subject was awake and whenever a subject awoke from sleep, until the time of discharge (about 28 h later). The time and total volume of each voiding was noted by nurses for each urine specimen, and aliquots were distributed into plastic bottles and frozen at −20°C. Each subject collected 10-12 timed voidings during hospitalization, with times differing among subjects. As part of a larger study, blood samples were also drawn every 20 min during the span of hospitalization, for analyses of hormones, including prolactin. For each subject, an analysis for melatonin was performed on 6 of the timed voidings, usually including the one collected just before sleep, the one just after arising, and the others spaced as evenly as possible throughout the span of wakefulness. Similar sampling procedures were also carried out in Kyushu, Japan, and the samples were sent from Kyushu to Minnesota in dry ice. All samples arrived in Minnesota completely frozen and were immediately stored at −20°C. Both Minnesota and Japanese samples were then transported to Sweden, again in dry ice, and again stored at −20°C at St Görans Hospital until melatonin could be determined by radioimmunoassay. On the data thus obtained, several analyses were carried out to examine the possibility of a difference between the 2 populations, and also to search for a time effect. Unequal numbers of samples were available from Japan and Minnesota in given time intervals for consideration in an idealized day (table 2). For an analysis of variance (requiring equal numbers) samples were picked from among the totals available, using from the larger number of Minnesota samples primarily samples of subjects belonging to the high breast cancer risk group, since breast cancer risk was one of the points of interest. At the outset only the data between 06.00 h and 21.00 h (shown in table 2) were used. Thereafter, the analysis was repeated with the addition of data.
Chronogram at left shows urinary melatonin excretion by Japanese and Minnesotan women at several timepoints during a 1-day span; amplitude and acrophase estimates from all 19 subjects were combined in mean cosinor displayed at right. Each woman contributed 6-8 samples collected over 1 single 24-h span; data for each population averaged across all subjects for 3-h intervals (no Japanese samples between 21.00 and 24.00 h).

between 00.00 h and 06.00 h from each of 8 subjects. The mean value (±SE) for the latter span (not shown in table 2) from the samples collected in Minnesota was 176.60±53.32 as compared to 86.49±22.92 for samples collected in Kyushu.

Prior to carrying out an analysis of variance on the original data, the assumption of homogeneity of variances was tested by the Fmax statistic proposed by Hartley, which yielded Fmax = 86.26 with (12,7) degrees of freedom. Consequently, this assumption had to be rejected. When logarithmic transformation was applied to the original data, the desired homogeneity was achieved. The analysis of variance summary in table 3 showed statistically significant differences between groups. Moreover, the time-group interaction is statistically significant, thereby attesting to the fact that the intergroup difference depends upon time.

When all data, i.e., including those collected between 00.00 h and 06.00 h are considered, time also constitutes a statistically significant source of variation, as seen in the analysis of variance summary in table 4. Again the interaction effect nearly achieved statistical significance at the 5% level (0.05 < p < 0.06).

These results should be re-examined for the same effects by a graphic presentation of all data and, hence, the figure presents the means of the transformed data and shows that a very low value occurred in the timespan from 06.00 h to 09.00 h in the Japanese sample tested. Moreover, when lines are drawn to connect the values for each group, they barely intersect at one of the timepoints here considered, i.e., the Japanese have consistently lower values of melatonin excretion compared to the North Americans. Thus, statistically significant differences among timespans and between groups were attained, the latter difference demonstrable even with a simple non-parametric sign test.

An interesting separate question in the present study is whether there is a significant difference in mean melatonin values for the night span, from 00.00 h to 04.00 h, when compared to the combined mean of the other spans. A simple contrast test was applied to the (log-transformed) data and confirmed the above hypothesis, yielding a p-value < 0.05. Hence, these data on 19 subjects extend the scope of earlier studies showing high melatonin excretion during the time of darkness (and habitual rest).

The results summarized with ANOVA must be viewed with the realization that the process of choosing 96 observations from a larger total is somewhat arbitrary. The selection of data from the larger set was originally carried out to permit a balanced table of data for computational purposes. Nonetheless, the results here summarized have important potential for exploiting rhythms which may lead to an early detection or warning of impending breast cancer.

The data from both groups were subjected to a mean-cosinor analysis9,10, to yield the results shown in the figure. The question may be raised whether it is permissible to pool all data from Minnesota and Japan for such cosinor analysis after a significant population difference has been documented. As noted in connection with cosinor techniques, one can discuss populations, groups within populations and individuals in a given group and consider them concomitantly, provided that one makes some allowance for any repeated tests on the same data. With the realization that only the mean cosinor is done, it can be seen that the cosinor's 95% confidence region does not overlap the pole, a finding documenting a statistically significant rhythm. Table 5 shows an agreement between the current results and other studies on urine. It can be seen that the

<table>
<thead>
<tr>
<th>Geographic site</th>
<th>Number of Subjects</th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
<th>Subtotals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota (low)</td>
<td>1 (35%)</td>
<td>3 (55%-59%)</td>
<td>4 (55%-59%)</td>
<td>11</td>
</tr>
<tr>
<td>Minnesota (high)</td>
<td>3 (34%-35%)</td>
<td>4 (46%-57%)</td>
<td>8 (46%-57%)</td>
<td>11</td>
</tr>
<tr>
<td>Japanese (low)*</td>
<td>4 (30-35%)</td>
<td>4 (52-59%)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Subtotals</td>
<td>8</td>
<td>8</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* Japanese volunteers all were selected according to criteria indicating a low breast cancer risk.

Table 2. Urinary melatonin excretion by clinically healthy women in USA and Japan. Mean values for consecutive 3-h spans.

<table>
<thead>
<tr>
<th>Geographic site</th>
<th>Time span</th>
<th>6.00-9.00</th>
<th>9.00-12.00</th>
<th>12.00-15.00</th>
<th>15.00-18.00</th>
<th>18.00-21.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota</td>
<td>101.50</td>
<td>87.89</td>
<td>45.55</td>
<td>65.44</td>
<td>65.70</td>
<td></td>
</tr>
<tr>
<td>Kyushu</td>
<td>18.22</td>
<td>50.66</td>
<td>45.39</td>
<td>35.02</td>
<td>36.62</td>
<td></td>
</tr>
</tbody>
</table>

* Data from 8 subjects in each location covering only wakefulness span to balance both N of subjects and N of samples.
Table 3. Analysis of variance summary*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (G)</td>
<td>1</td>
<td>13.45</td>
<td>13.41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Time (T)</td>
<td>4</td>
<td>6.47</td>
<td>1.61</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Interaction (T × G)</td>
<td>4</td>
<td>10.77</td>
<td>2.68</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Error</td>
<td>70</td>
<td>70.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>100.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* On transformed data. Homogeneity of variances had to be rejected (Fmax = 31.46). Hence, natural logarithmic transformation was used before ANOVA. After transformation the homogeneity of variances was accepted (Fmax = 9.11).

Table 4. Analysis of variance summary*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (G)</td>
<td>1</td>
<td>15.05</td>
<td>10.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Time (T)</td>
<td>5</td>
<td>19.55</td>
<td>4.18</td>
<td>0.01</td>
</tr>
<tr>
<td>Interaction (G × T)</td>
<td>5</td>
<td>10.86</td>
<td>2.32</td>
<td>0.05</td>
</tr>
<tr>
<td>Error</td>
<td>94</td>
<td>78.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>113.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Logarithmically-transformed data.

difference between the acrophase found earlier in data for Lynch and that in this study is only 2°; i.e., 8 min, since 360° are equated to 24 h.

The question may also be raised how differences in melatonin excretion may be related to differences in risk of breast cancer between Minnesotan and Fukuoakan Japanese women. Since pinealectomy was reported with accelerated tumor growth1,2 and since pineal extracts reportedly counteracted malignancy3, pineal function may be involved in inhibition of carcinogenesis, acting conceivably via intermediary metabolism. However, if the pineal

equated spectrum the facts that a spectrum of rhythms with different frequencies - with 1 cycle in about 24 h (circadian), in about 7 days (circaseptan), in about 1 month (circatrigintan) and about 1 year (circannual) characterize the urinary excretion of 17-ketosteroids4. These frequencies also may be reflected in urinary melatonin excretion. Among other effects, these frequencies may influence cyclic processes.

When approaches based on homeostatic single samples fail to discern the mechanism underlying variability both in health and disease (in the latter case chronopathology quantifiable possibly as a rhythm alteration), a spectral analysis completed on a reference sample of test individuals from populations at different risk for a given cancer (such as that of the breast) may yield information of interest to both students of oncology and of chronobiology.

Table 5. Apparently similar timing of circadian melatonin rhythm in urine and plasma of several species

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Diurnally (D) or nocturnally (N) active</th>
<th>Circadian acrophase (95% confidence limits)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human beings</td>
<td>Urine</td>
<td>D</td>
<td>-6° (+4°, -9°)</td>
<td>This study</td>
</tr>
<tr>
<td>Human beings</td>
<td>Urine</td>
<td>D</td>
<td>-4° (+3°, -7°)</td>
<td>Lynch5</td>
</tr>
<tr>
<td>Human beings</td>
<td>Plasma</td>
<td>D</td>
<td>-27° (+11°, -43°)</td>
<td>Rollag4</td>
</tr>
<tr>
<td>Sheep</td>
<td>Serum</td>
<td>D</td>
<td>0° (+23°, -3°)</td>
<td>Hedlund5</td>
</tr>
<tr>
<td>Cattle</td>
<td>Plasma</td>
<td>D</td>
<td>-9° (+35°, -3°)</td>
<td>Lynch5</td>
</tr>
<tr>
<td>Rats</td>
<td>Urine</td>
<td>N</td>
<td>-25° (+5°, -3°)</td>
<td></td>
</tr>
</tbody>
</table>

Acrophase: Timing of high values in relation to middle of daily dark span, with 360°=24 h. 15°=1 h, based on fitting 24 h cosine curve in the case of human beings, sheep and cattle, in the case of rats a rough macroscopic approximation is listed, the excretion during a 12-h dark span being compared with that during a 12-h light span.

1 Supported by U.S. Public Health Service (5-K6-GM-13981-17), National Cancer Institute (1R01-CA-14445-05 and N01-CR-5-5702), National Institute of Occupational Safety and Health (OH-00631-01), National Institute of Aging (AG-00158), Environmental Protection Agency (R804573-01-0), Danish Medical Research Council grant 3371 and the St Paul Ramsey Medical Research and Education Foundation, R. B. Southern and Jung-Kean Lee, Scientists, Chronobiology Laboratories, University of Minnesota provided valuable help.


11 E. Katagiri, Osaka Igakki Zasshi. 43, 315 (1944).

12 T. K. Das Gupta and J. J. Lee, Cancer Res. 27, 1306 (1967).


The effect of chemotherapy on pituitary function in patients with advanced breast cancer

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(Accepted for publication 4 March 1978)

Fifteen women with advanced breast cancer were treated by 3 different regimes. Group 1 underwent yttrium implantation of the pituitary alone, group 2 was given triple chemotherapy alone and group 3 was treated with yttrium implant and chemotherapy. Pituitary function studies, which included stimulated levels of plasma LH, FSH, GH and prolactin were carried out before and one month after commencing treatment. The results showed that a 28 day cycle of chemotherapy did not produce an effect on pituitary function, and its addition to the yttrium implant procedure did not affect the response of the hormones to stimulants, compared to yttrium implant alone.

Introduction

The availability of specific stimulants of pituitary hormone release and of radioimmunoassays of plasma protein and peptide hormones has allowed the development of a range of tests of pituitary function which are precise and sensitive. The present study reports the results of tests of secretion of growth hormone, prolactin, thyroid stimulating hormone and gonadotropins in patients randomly allocated for treatment by pituitary ablation alone, by multiple drug chemotherapy alone and by their combination. The objective of the study was to determine the effect of multiple drug chemotherapy on pituitary function.
into 3 groups, one of which was treated by yttrium-90 implantation or the pituitary alone; one by chemotherapy alone and the third by yttrium-90 implantation plus chemotherapy.

In most patients a period of 4 weeks without systemic therapy preceded yttrium-90 implantation and/or chemotherapy. However, in 4 patients the progress of the disease was such that these treatments were preceded with before this rest period had elapsed. All 4 had been taking stilboestrol.

The yttrium-90 implantation of the pituitary was performed by the screw technique (Forrest, Blair & Valentine, 1958). Chemotherapy was by the CMF regime in which 2 doses of 5FU (700 mg/m²) and methotrexate (50 mg/m²) were given i.v. on days 1 and 8 of a 28-day cycle and cyclophosphamide (100 mg/m²/day) orally for days 1 to 14 inclusively. Days 14 to 28 were rest days.

Pituitary function studies were carried out during the week prior to the operation or the commencement of chemotherapy and were repeated exactly 1 month later. In the case of those patients treated by both, chemotherapy was started on the day following the operative procedure.

The tests were carried out with the patients in hospital and fasted overnight (from midnight) and kept in bed during the test. A 17 g Teflon catheter was placed into an antecubital vein and a basal blood samples taken at intervals of 15 min. A butterfly needle was inserted into the dorsum of the other hand and 100 μg LHRH plus 200 μg TRH given in a bolus injection followed by 10 ml saline. A constant infusion pump set to deliver 0.04 units insulin per kg body weight per h was connected by cannula to the needle, the appropriate solution of insulin being administered at a rate of 80 ml/h (Carter, Dozois & Kirkpatrick, 1972).

Blood samples were taken at 10 min intervals for 1 h and at 90 and 120 min. The blood was collected in heparinized tubes, centrifuged at 4°C and the plasma stored at -18°C.

The concentrations of growth hormone in basal and 30, 60, 90 and 120 min samples and those of LH, FSH and prolactin in basal and 10, 20, 30, 40 and 60 min samples were estimated by radioimmunoassay by standard methods as described previously (McFadyen et al., 1979). Blood glucose was estimated in those samples used for insulin assay by the Gulicium/glucose oxidase method.

The increments of hormones were calculated by subtracting the basal levels from the average of those at specified times after injection. The times used for estimating the average peak response were, as previously defined: 60, 90 and 120 min for growth hormone; 30 and 60 min for LH and FSH and 10, 20 and 30 min for prolactin (McFadyen et al., 1979).

Results

The basal levels and increments of the hormones for the tests before and after treatment are shown in Figures 1 and 2.

All patients treated by yttrium-90 implantation (either with or without chemotherapy) had significantly lowered increments for all hormones (P<0.5% Wilcoxon
Figure 1. Basal hormone levels pre- and post- yttrium, chemotherapy and yttrium and chemotherapy.

signed rank test). No such lowering of hormone increments could be detected for chemotherapy.

Basal levels of LH and FSH were also significantly lowered (P<5% Wilcoxon signed rank test) after yttrium with or without chemotherapy but although the average basal levels of prolactin and growth hormone levels were lower after these treatments, this could have been a chance result (P>5% Wilcoxon signed rank test).

In contradistinction these patients who received chemotherapy alone had no reduction in their basal or incremental hormone levels. The post-treatment values of all patients on chemotherapy were no different from the pre-treatment values for all patients. Three patients who were treated by chemotherapy alone, and also one who had a yttrium-90 implant, had abnormally low levels of gonadotrophins when first tested. The patients had taken stilboestrol within a month of starting chemotherapy.
Comment

Although the number of patients in these groups is small, and although the results in the control tests in the chemotherapy group were comprised by the administration of stilboestrol, the results of this investigation indicate that a 28-day cycle of triple chemotherapy does not suppress pituitary function. Nor did the addition of chemotherapy to the implantation of yttrium-90 into the pituitary affect the hormonal response to stimulation compared to the effect of yttrium-90 implantation alone.

Further studies are under way to determine the long-term effects of chemotherapy on endocrine function. Meantime our results suggest that it has no immediate effect on pituitary function as assessed by tests of stimulation of pituitary hormone release.
work, and to thank Hoechst U.K. for the generous supply of LITRET. Appreciation is expressed to Staff Nurse Watters for all her help.

References
International Geographic Studies of Oncological Interest on Chronobiological Variables

Franz Halberg, Germaine Cornélissen, Robert B. Sothern, Lee Anne Wallach, Erna Halberg, Andrew Ahlgren, Marilyn Kuzel, Alan Radke, Jose Barbosa, Frederick Goetz, Joseph Buckley, Jack Mandel, Leonard Schuman (Minneapolis, Minnesota, USA);

Erhard Haus, David Lakatua, Linda Sackett, Harriet Berg, Hans W. Wendt (St. Paul, Minnesota, USA);

Terukazu Kawasaki, Michio Ueno, Keiko Uezono, Midori Matsuoka, Teruo Omee (Fukuoka, Japan);

Brunetto Tarquini, Mario Cagnoni (Florence, Italy);

Mauricio García Sainz, Edmee Perez Vega (Mexico City, Mexico);

Douglas Wilson, Keith Griffiths, (Cardiff, Wales, Great Britain);

Luciano Donati, Patrizio Tatti, Mario Vasta, Jacopo Locatelli, A. Camagne (Urbino, Italy);

Renato Lauro (Rome, Italy);

George Tritsch (Buffalo, New York, USA);

Lennard Wetterberg (Stockholm, Sweden)

INTRODUCTION

For a given variable of oncologic interest displaying rhythmicity the more prominent and predictable is its variability with time, the greater is the urgency of procuring reference intervals ("normal values") that are time-qualified. Many oncologic variables show predictable changes recurring at similar intervals in similar sequences—in brief, rhythms. Actually, more often than not, statistically validated rhythms of several frequencies are found to characterize a series of data. Some of these rhythms are more prominent than others (18). Circadian rhythms in plasma cortisol, among the more prominent ones, have already been analyzed by special rhythmometric procedures on healthy human beings living on different continents. Good agreement of the circadian acrophase characterized the results from such studies (15), whether it was determined for an individual or for a group.

* Senior investigators italicized.

It seems important to extend studies on the individual assessability (i.e. inferential statistical quantification) of a given rhythm in conjunction with studies of the corresponding rhythm in peer groups. What is yet more important in dealing with rhythmic functions is to provide an optimal specification for serial sampling times and also for single samples (14). The latter qualification for single samples (that are currently almost exclusively used for oncologic interpretation) is the most valuable, when cost-effective and appropriate, but may not substitute in all cases for an eventual requirement of cost-effective repetitive sampling. The clinical interpretation of single samples or time series from rhythmic variables is best done, whenever possible, on an individualized basis, in addition to comparisons with corresponding distributions from a peer group. Whether one's interest is in the epidemiology of cancer, in its early diagnosis, or in its treatment, time-qualified reference intervals may avoid many pitfalls (55, 56). Stud-
ies along these several lines are under way with cooperation among investigators in Italy (8, 55), India (16, 19), United Kingdom (35), Sweden (62), Mexico (13), Japan, and the United States. The design of cooperative work in the latter two locations and in Italy concerning the epidemiology of breast cancer, and some early results, may be of methodologic interest more broadly to oncologists. In this cooperative endeavor, blood samples for hormonal evaluation along with other physiologic variables were taken around the clock several times a year in different geographic locations from subjects with different ethnic backgrounds. This strategically placed sampling assessed a set of bioperiodicities in variables of potential interest to research on cancer in the context of diseases with so-called competing risks. In so doing, a data base was secured so that eventually sampling requirements may be specified for a cost-effective time-qualified evaluation, clinical as well as epidemiologic, of individuals as well as of groups.

Methodology for sampling and analysis thus becomes available to implement research on a rational prevention and/or treatment of disease relying on an individualized, rather than a group, definition of health by the establishment of time-qualified reference intervals and characteristics. In several variables, large differences were detectable among groups being compared by rhythmometric procedures. By contrast, correlation matrices limited, for instance, to data from a single season led to confusing and, therefore, contradictory results.

Sampling of blood around the clock four times a year and the application of rhythmometric procedures revealed why previous results on but a single hormone, prolactin, could be so controversial (20, 25). Differences in characteristics of circadian and circannual rhythms in prolactin and other hormones were observed between Japanese and North American women, as well as between women with fibrocystic mastopathy and healthy controls in Italy.

Correlations were found in this study between the breast cancer risk represented (according to conventional criteria) by individuals and some of the hormonal end points gathered (with proper regard for rhythms or as rhythm characteristics at one or the other frequency). Some correlations will be presented without any causal implications primarily for the purpose of illustrating possible relationships that have to be investigated with much larger numbers of individuals, yet with fewer strategically placed samples. Eventually, this task, in view of the results presented, will be feasible on one or the other of the hormones investigated with far fewer time-specified single or multiple samples.

A risk scale was needed which, as opposed to the usual approach conventionally aimed at assessing the risk of a population, attempts to use population experience in an individualized fashion. The results from the use of such a scale will await much additional study with more rigorous data applicable to a given population. It cannot be overemphasized that it is premature at this time to extrapolate beyond the scope of these data, which are still awaiting intensive scrutiny, or to account for any geographic-ethnic, climatic, dietary, or other underlying factors. It is within our scope to emphasize, first, that differences demonstrated by the chronoepidemiologic approach are not only statistically significant but also are partly replicated and, second, that these differences, e.g. in certain circannual rhythm amplitudes, are not detectable by sampling at certain hours or seasons fixed by convenience rather than pertinence.

SUBJECTS AND METHODS

Three age groups totalling about 12 women volunteers were selected in Kyushu, Japan, and nearly twice that number in Minnesota. The group of Japanese women was considered to have a low risk of breast cancer, whereas in Minnesota two groups were formed according to the conventional epidemiologic criteria to distinguish low and high risk of breast cancer (using breast cancer in the immediate family, e.g. mother or sister = high risk; lack of any cancer in the immediate family = low risk). Each group consisted of three age groups: early post-pubertal, young adult, and postmenopausal women.

The comparison of hormonal function in Japanese and North American women brought together samples from two populations (a) differing greatly with respect to death rates from breast cancer and (b) documented as such by a reasonable number of autopsies. However, one has to be aware of regional differences in breast cancer risk that may characterize a given country, notably when limitations on the number of subjects sampled restrict the use of subjects to a single region. Takeshi Hirayama (27) points out that adjusted and crude death rates for breast cancer in Fukuoka (where the Japanese subjects were sampled) were 4.9 and 6.3/100,000, respectively, as compared to 4.6 and 5.8 for all...
made to take their contribution into consideration. Most subjects were sampled throughout a 24-hr span four times a year, once in each season, and in a different menstrual stage (early follicular, late follicular, early luteal, and late luteal) in each season, according to a Latin square design (47). Figures 37.2 and 37.3 display the design. During each 24-hour span 1 ml of blood was withdrawn through an i.v. catheter every 20 min and an additional 12.5 ml (for a total of 13.5 ml) of blood was taken every 100 min. Pituitary, adrenal, ovarian, thyroidal, and pancreatic functions were assessed by chemical analyses of these blood samples. Plasma prolactin and cortisol were determined every 20 min, while values for other hormones (thyroid-stimulating hormone (TSH), thyroxine (T4), triiodothyronine (T3), insulin, luteinizing hormone (LH), dehydroepiandrosterone sulfate (DHEA-S), estrone (E1), estradiol (E2), estranol (E3), aldosterone, and 17-OH-progesterone) were determined every 100 min, each by radioimmunoassay. Reagents for the determinations of human prolactin were obtained from Calbiochem, Calif., with human pituitary prolactin used as standard, 1 ng being equivalent to 0.6 ng WHO 75/504 human prolactin reference standard. Reagents for plasma cortisol were obtained from Clinical Assays, Cambridge, Mass. E1, E2, E3, and 17-OH-progesterone were determined following LH-20 column chromatography with antisera obtained from Steraloids. This method has been modified after Jaffe and Berson (29). Plasma insulin was determined with reagents obtained from Becton, Dickinson. Total DHEA-S was determined directly in plasma with reagents obtained from Radioassay Systems Laboratories using 7-3H-DHEA as standard. Plasma aldosterone determinations were modified by reducing sample and reagent volumes and by using a double antibody instead of a charcoal separation technique. Reagents were obtained from Diagnostics Products and 125I-labeled aldosterone standard. Reagents for the determination of human luteinizing hormone (hLH) in plasma were obtained from BIORIA, Montreal, Canada. The method is adapted from Midgley (41).

Plasma T4 and T3 were determined by radioimmunoassay with reagents obtained from Bio-Rad Laboratories, Richmond, Calif., and Panex Laboratories, Santa Monica, Calif., respectively. Human TSH in plasma was determined by solid-phase radioimmunoassay, reagents obtained from Beckman Instruments, Fullerton, Calif. Quality control lyophilized serum speci-
Figure 37.2. Individual sampling schemes (spans) for assessment of hemic, urinary, and other rhythms. Experimental design of Minnesota-Japan chronoeplidemiologic study: schedule of hospital admission and at-home self-measurements of subjects classified according to age and geographic location, with specification of menstrual cycle stage on day of admission. (See Figure 37.3 for sampling intervals during sampling spans shown.)

mens were obtained from the following manufacturers:

Ortho Diagnostics: Ortho-V and Ortho-VI for aldosterone, Ortho-I, II, III, and IV for cortisol, E1, E2, E3, and 17-OH-progesterone, insulin, TSH, T3, and LH.

Nuclear Medical Systems: NMS-IIa for human prolactin, E1, E2, and E3, 17-OH-progesterone.

Beckman: Beckman control for serum TSH, Monitrol-I and Monitrol-II for the determination of serum T4. References to the methods and coefficients of variation are summarized in Table 37.1.

In addition to blood hormones, adenosine deaminase activity in red blood cells was determined for some of the subjects (59). Blood was centrifuged at 500 x g for 10 min and plasma removed. The red cells were gently washed with saline, recentrifuged at 500 x g for 10 minutes; the supernatant saline was removed and the
packed red cells were frozen at $-20^\circ C$ until analysis for adenosine deaminase by an automated procedure developed by Tritsch and Mittelman (60). Ammonia produced during the conversion of adenosine into inosine by the enzyme was quantified by Nesslerization. Erythrocyte adenosine deaminase activity at $37^\circ C$ was expressed as $\mu$mol of $NH_3$/h per milliliter of packed erythrocytes and also per gram of hemoglobin.

Several physiologic variables also were monitored in the hope of elaborating relations among factors directly relevant to cancer, on the one hand, and of providing information on potential marker rhythms, on the other. The marker rhythm information is needed not only because it is more easily accessible but, what is critical, because it can be obtained with a density and over spans that may not be practicable for hormones—for economic or other reasons, such as an excessive requirement for blood or an undue interference with the daily routine of the individual investigated.

Blood pressure, breast surface temperature, activity, and EKG were automatically monitored and urine was collected every 2 hr throughout the 24-hr stay in a research center, in Minneapolis, Minn., and Fukuoka, Kyushu, Japan. Breast surface temperature was measured automatically with a so-called Medilog (Ambulatory Monitoring Inc., Ardsley, New York), and/or was self-measured with a telethermometer manufactured by the Yellow Springs Instrument Company (Yellow Springs, Ohio), and blood pressure was measured with an Arteriosonde (Roche Medical Electronics Division, Hoffmann-La Roche, Inc., Cranbury, New Jersey). Breast surface temperature data automatically recorded on tape in an analog form were digitized and edited. Data thus were available for a given subject for one or several spans of about 24 hr, with a sampling interval of 1 min. In order to improve the signal-to-noise ratio, these data were averaged over 15-min spans.

Wrist activity was measured for 24 hr at each
admission to hospital, using a piezo-electric transducer and Medilog recorder. Activity recordings were replayed in a computer system in order to digitize at 16 times a second the recorded voltages and to compute successive 5-min cumulations (33). The waking and rest and/or sleep portions of the recordings were analyzed separately by variance spectra (30).

In addition, each subject self-measured her blood pressure, pulse, and oral temperature and collected morning (and in a few cases, around-the-clock) urines for at least one menstrual cycle (or 1 month) bracketing the hospital admission.

To complement this main investigation, a study was also carried out during a second winter season on 20 Japanese and 16 Minnesotan young women, mostly low risk, between 18 and 24 years of age. In this study, blood was sampled every 4 hr during a 24-hr span and analyzed for plasma prolactin. In addition, urine was collected every 2 hr during wakefulness and whenever a subject awoke from sleep, for assay of melatonin.

Four-hourly blood sampling throughout a 24-hr span was also applied to 22 women with fibrocystic mastopathy and to 18 clinically healthy controls in a study in Florence, Italy (55).

With the same design as that of the Kyushu-Minnesota chronoepidemiologic study, blood also was sampled through an i.v. catheter every 20 min for 24 hr at the Center for Social Medicine of the Ospedale di Urbino, in Urbino, Italy, for plasma prolactin determination by radioimmunoassay, but this assay was carried out in the Department of Medicine at the University of Rome, Italy. Hence the data of this parallel study are not strictly comparable in terms of radioimmunoassay for prolactin determination.

The single cosinor procedure (21) was used to assess any rhythmic (e.g. circadian or circannual) variation. In this procedure, a cosine curve with a period selected on the basis of a priori knowledge is fitted to the data, using the method of least squares. An F-statistic is then used to test the zero-amplitude hypothesis for a circadian, circannual, and other rhythm. For statistically validated rhythms (P ≤ 0.05), the following parameters are estimated: the mesor (rhythm-adjusted mean), the amplitude (half the total extent of predictable change) and the acrophase (crest time of the best-fitting cosine function, in relation to a given reference time—such as local midnight or preferably midrest (midsleep) at the outset for circadian rhythms and midnight on December 22 of the year preceding data collection for circannual rhythms).

It is often desirable, with a small sample size available, to consider all rhythm characteristics from the time series on hand, whether or not the zero-amplitude assumption is rejected. As discussed earlier by Halberg et al. (24), the rhythm characteristics are then regarded as imputations, i.e. as intermediate calculations. In this case, additional methods along more classical lines are desired whenever possible. A mesor, for instance, could be replaced by the series mean; in the actual cases discussed (since the vast majority of samples, except for a few missed

Table 37.1

<table>
<thead>
<tr>
<th>Variable</th>
<th>CV</th>
<th>Reference</th>
</tr>
</thead>
</table>

* DHEA-S, dehydrospirostanol; E1, estrone; E2, estradiol; E3, estriol; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; T4, thyroxine; and T3, triiodothyronine.
time points, was obtained at equidistant intervals over integral multiples of at least the circadian period being analyzed) means and mesors will differ rarely and if so only slightly. In any event, in dealing with “imputed” amplitudes, one can gain insight into the statistical significance of a relationship between dynamic indices that would go unassessed in a conventional analysis of variance. It is pertinent, further, that the use of imputation in an operation bootstrap may lead to statistically significant conclusions for the group in cases when sampling is insufficient to establish the corresponding relation for the individual.

Wave-form may also be portrayed by plotting a plexogram (5) whenever data covering several cycles are available. In this procedure, the different cycles are folded onto one, thus providing confidence intervals for the mean at each time-point within that (folded) cycle. When only short but equidistant time series can be obtained, a harmonic interpolation serves to compute a paraphase, i.e. the lag from a reference time of the peak in the harmonically interpolated function (6). This method reconstructs a continuous function from the samples on the basis of a spectral decomposition, using all harmonics. Whenever information is available concerning the variance of the noise affecting the data, it is also possible to derive confidence limits around the reconstructed curve or wave-form.

Several authors have previously defined breast cancer risk factors and have suggested their use. Robert L. Egan (10) estimated risk for asymptomatic and minimally symptomatic breast cancer patients, demonstrating the feasibility of using simultaneously large numbers of risk factors in a systematic way to pinpoint patients with mammary cancer. Breast cancer patients have been found to marry later, have shorter lactational histories, and have fewer children when compared to controls (37). A study by Sartwell et al. (53) showed breast cancer to be associated with nulliparity, first pregnancy over 20 years of age, premenopausal status, and frequency of artificial menopause.

Hormonal status has also been repeatedly considered as a risk factor. Farewell et al. (11) included etiocholanolone excretion with three historical risk factors—age of menarche, family history, and age at first pregnancy. In studying menstrual patterns, Wallace et al. (61) suggested that late menopause may be a breast cancer risk factor resulting from relative estrogen excess and progesterone lack. Cole and Cramer (4) suggested the saturated fatty acid component of the diet as another risk factor. Miller (42) furthers this idea by suggesting that dietary factors mediated through a hormonal mechanism play a major role in breast cancer etiology.

The risk scale for this chronoepidemiologic breast cancer study was developed using risk factors as identified by MacMahon et al. (36) and Choi et al. (3). The risk factor scale is based upon the following items:

1. Geographic area of residence
   - North America and Northern Europe +5
   - South America and Southern Europe +2.5
2. Benign breast disease +4
3. Other primary cancers
   - Cancer of major salivary glands +4
   - Cancer of colon and/or uterus +2
4. Familial aggregation +3
5. Obesity
   - ≥ 60 kg in postmenopausal women ≥ 70 +3 years
6. Age
   - After 30 years of age (for every decade) +2
7. Late first pregnancy After 30 years of age +2
8. Early menarche Before 16 years of age +2
9. Late menopause After 55 years of age +2
   a. Early pregnancy Before 25 years of age −0.5
   b. Surgical menopause −0.4

The total risk for an individual was calculated by simply adding all the risk numbers relevant to that individual from the foregoing risk scale.

We illustrate below the procedure used to assign a risk number to an individual: if none of the above listed factors apply, the individual's total breast cancer risk is equated to unity. The risk number of 5 is assigned to a woman in North America, without any other pertinent characteristic listed above. If this woman has benign breast disease, her risk (+4) becomes 9, as long as she lives most of the time in North America.

A statistical correlation was computed between the individual's total risks and certain first order statistics, such as circannual prolactin or TSH amplitudes. Using a linear regression procedure, the total risk can thus be correlated with the circannual amplitude expressed in nanograms per milliliter and also as percentage of mesor.

To explore the potential clinical usefulness of thermorhythmometry, breast surface temperatures also were monitored with a 10-channel Yellow Springs telethermometer from the nipple and four quadrants of each breast during wakefulness, at 2-hr intervals, for 4 days from three groups each composed of eight women; 1,
sumably healthy; 2, with nonmalignant conditions; and 3, with breast cancer, at the Hospital de Oncologia, Mexico City, Mexico. A so-called chronobiologic window (22)—with 0.2-hr intervals between consecutive trial periods ranging from 20 to 28 hr—was fitted to the ten series from each woman.

RESULTS

Individualized Circadian Rhythm Assessment for Cortisol and Prolactin

For each hormone, in each season, the 24-hr series from each subject was analyzed by the single cosinor. Figure 37.4 portrays the observed hierarchy of statistical significance for individualized circadian variation in 13 of the hormones studied. For prolactin and cortisol, the characteristics of a 24-hr synchronized rhythm were confidently measured with their probable errors on an individual basis for almost all subjects, in Kyushu and Minnesota as well as in Italy.

![Figure 37.4](image)

Figure 37.4. Comparison of statistical significance of circadian rhythm, assessed by least-squares fit of a 24-hour cosine curve for 13 hormones in plasma of healthy women sampled for individualized rhythm assessment. P from F-test of zero-amplitude hypothesis. Since the hierarchical statistical significance represented by P-values is sampling-dependent, prolactin and cortisol, measured every 20 min, were also analyzed at 100-min intervals as for the other hormones. Total number (N) of determinations = 40765.
**Table 37.2**

Circadian Mean Cosinors of Several Hormones in Human Plasma from Two Populations (Japan and Minnesota) at Four Different Seasons

<table>
<thead>
<tr>
<th>Hormone (units)</th>
<th>Winter</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hormone (Units)</strong></td>
<td>P</td>
<td>M (±SE)</td>
<td>A</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>&lt;0.001</td>
<td>24.8 (+2.4)</td>
<td>16.6</td>
</tr>
<tr>
<td>Cortisol (μg/dl)</td>
<td>&lt;0.001</td>
<td>6.9 (+0.4)</td>
<td>4.2</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>0.08</td>
<td>23.7 (+3.6)</td>
<td>7.6</td>
</tr>
<tr>
<td>TSH (μIU/ml)</td>
<td>0.02</td>
<td>4.3 (+0.6)</td>
<td>0.3</td>
</tr>
<tr>
<td>DHEA-S (ng/ml)</td>
<td>&lt;0.001</td>
<td>1390.0 (+200.0)</td>
<td>250.0</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>0.76</td>
<td>104.0 (+7.1)</td>
<td>3.4</td>
</tr>
<tr>
<td>17-OH-Progesterone (pg/ml)</td>
<td>0.00</td>
<td>552.0 (+182.0)</td>
<td>42.0</td>
</tr>
<tr>
<td>T4 (μg/dl)</td>
<td>&lt;0.001</td>
<td>7.1 (+0.3)</td>
<td>0.4</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>0.002</td>
<td>5.2 (+0.4)</td>
<td>1.5</td>
</tr>
<tr>
<td>T3 (μg/ml)</td>
<td>0.15</td>
<td>81.0 (+15.5)</td>
<td>6.5</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>0.64</td>
<td>94.8 (+33.2)</td>
<td>7.3</td>
</tr>
<tr>
<td>T3 (pg/ml)</td>
<td>0.85</td>
<td>18.9 (+3.6)</td>
<td>1.1</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>0.03</td>
<td>34.4 (+7.3)</td>
<td>1.1</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>&lt;0.001</td>
<td>19.3 (+2.4)</td>
<td>14.2</td>
</tr>
<tr>
<td>Cortisol (μg/dl)</td>
<td>&lt;0.001</td>
<td>10.9 (+0.6)</td>
<td>5.3</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>0.007</td>
<td>23.3 (+2.7)</td>
<td>8.9</td>
</tr>
<tr>
<td>TSH (μIU/ml)</td>
<td>0.06</td>
<td>4.1 (+0.9)</td>
<td>0.5</td>
</tr>
<tr>
<td>DHEA-S (ng/ml)</td>
<td>0.05</td>
<td>1860.0 (+350.0)</td>
<td>280.0</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>0.02</td>
<td>105.0 (+7.3)</td>
<td>12.0</td>
</tr>
<tr>
<td>17-OH-Progesterone (pg/ml)</td>
<td>0.04</td>
<td>423.0 (+85.0)</td>
<td>38.0</td>
</tr>
<tr>
<td>T4 (μg/dl)</td>
<td>0.02</td>
<td>4.9 (+0.6)</td>
<td>1.7</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>0.03</td>
<td>91.4 (+12.6)</td>
<td>2.6</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>0.48</td>
<td>62.1 (+17.5)</td>
<td>4.2</td>
</tr>
<tr>
<td>T3 (pg/ml)</td>
<td>0.10</td>
<td>20.7 (+6.5)</td>
<td>11.1</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>0.17</td>
<td>30.5 (+6.9)</td>
<td>2.6</td>
</tr>
</tbody>
</table>

| **Hormone (Units)**             | | | |
| Prolactin (ng/ml)               | | | |
| Cortisol (μg/dl)                | | | |
| Insulin (μU/ml)                 | | | |
| TSH (μIU/ml)                    | | | |
| DHEA-S (ng/ml)                  | | | |
| T3 (ng/dl)                      | | | |
| 17-OH-Progesterone (pg/ml)      | | | |
| T4 (μg/dl)                      | | | |
| Aldosterone (ng/dl)             | | | |
| E2 (pg/ml)                      | | | |
| E3 (pg/ml)                      | | | |
| LH (mIU/ml)                     | | | |

**Notes:**

- N, number of subjects; P, P-value derived from the zero amplitude test; M, mean; SE, standard error; A, amplitude; φ, acrophase (360° = 24 h; 0° = 00:00); TSH, thyroid-stimulating hormone; DHEA-S, dehydroepiandrosterone sulfate; T3, triiodothyronine; T4, thyroxine; E1, estrone; E2, estradiol; E3, estriol; LH, luteinizing hormone; mIU, milli-international unit.
different behavior of the estrogens and the luteinizing hormone as compared to the other hormones, at least an unfavorable signal-to-noise ratio, wherein the uncertainties associated with the technique of hormone determination may play a sizable role and more refined techniques will have to scrutinize the noise term.

Circannual Hormonal Rhythms

For each hormone, circannual mean-cosinors (24) were obtained from the least squares fit of a 365.25-day cosine curve to the circadian mesors of those clinically healthy women who contributed data during four seasons. Table 37.3 shows that in Minnesota a circannual rhythm could be demonstrated at or near the 5% level for plasma insulin, TSH, E1, aldosterone and DHEA-S. In Japan, a statistically significant circannual rhythm was found for plasma prolactin and estriol while any circannual variation in plasma TSH and DHEA-S was associated with a P of only ~0.10.

Circatrigintan Hormonal Variation

Apart from circadian and circannual rhythms in human plasma hormones, other components may also contribute to the variability of the data. A circatrigintan (about 30-day) prolactin rhythm, difficult to assess in this investigation, was demonstrated by our analysis of longitudinal.

Table 37.3
Circannual Variation Investigated by Population-Mean Cosinor Procedure on 13 Hormones in Clinically Healthy Women

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>JAPAN (N = 113)</th>
<th>MINNESOTA (N = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PR M (±SE)</td>
<td>A/M</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>74 26.8±8.3</td>
<td>19.9</td>
</tr>
<tr>
<td>Cortisol (μg/dl)</td>
<td>41 8.6±0.5</td>
<td></td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>64 24.0±2.8</td>
<td></td>
</tr>
<tr>
<td>T4 (μg/dl)</td>
<td>70 7.4±0.3</td>
<td></td>
</tr>
<tr>
<td>TSH (μU/ml)</td>
<td>72 4.1±0.2</td>
<td></td>
</tr>
<tr>
<td>17-OH Progesterone (pg/ml)</td>
<td>70 474.0±111.0</td>
<td></td>
</tr>
<tr>
<td>E1 (pg/ml)</td>
<td>62 69.9±1.1</td>
<td></td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>73 70.4±17.2</td>
<td></td>
</tr>
<tr>
<td>DHEA-S (ng/ml)</td>
<td>74 34.1±2.8</td>
<td>72.0</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>61 4.9±0.4</td>
<td></td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>57 1519.0±253.0</td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>63 103.3±4.6</td>
<td></td>
</tr>
</tbody>
</table>

* Each contributing 72 plasma samples during four or more seasons.

A N, number of subjects; PR, percentage rhythm; M, mean (or mean); SE, standard error; A, amplitude; φ, acrophase (reference: December 22; 360° = 365.25 days); A/M and φ given only when P-value (from zero amplitude test) ≤ 0.05.
contribute a very large error term. Moreover, circatrigintan rhythms may differ in frequency among different individuals or at least may exhibit less frequency and/or acrophase synchronization than circadian rhythms. From the viewpoint of intra- as well as interindividual synchronization, they may compare unfavorably with circannual and circadian rhythms.

Ultradian Hormonal Variation

On prolactin and cortisol, sampled every 20 min, ultradian frequencies were investigated also. Using the same zero-amplitude test as that used in the cosinor procedure, each harmonic of the 24-hr period was tested for statistical "significance" over the possible spectral range. All statistically significant "candidate" harmonics were then ranked by increasing amplitude: the most prominent frequency was thus assigned rank 1, the second most prominent was assigned rank 2, and so on. These ultradian ranks were highly variable.

It is interesting to search for differences in the spectral representations of cortisol and prolactin. For both hormones, the circadian component accounted for the largest share of variability in the data (~46% for prolactin and ~53% for cortisol). The circadian rhythm was the most prominent in 66% of all individual profiles on prolactin, and in 97% of the cortisol profiles. Whatever its origin, nonsinusoidality or other, a statistically "significant" 12-hr harmonic was present in 78% of the series for prolactin, accounting for ~18% of the total variance, and in only 44% of the series for cortisol, accounting for only ~10% of the total variance. A statistically "significant" component with a period less than 6 hr could be demonstrated in 22% of the prolactin profiles and in 33% of the cortisol profiles. The mean period of all ultradians with a period less than 6 hr was between 3 and 4 hr for both prolactin and cortisol.

Circadian Hormonal Wave-Form

In order to obtain a better idea of the waveform for prolactin and cortisol rhythms, data from all volunteers sampled during the winter in Japan and in Minnesota were averaged over consecutive 3-hr spans to yield circadian "plexograms." Figures 37.5 and 37.6 portray the plexograms for prolactin and cortisol, respectively, in each of the two geographic locations, while Figures 37.7 and 37.8 give the corresponding curves reconstructed (from the 3-hr averages) by harmonic interpolation. Figures 37.5 and 37.6 reveal that, at night, prolactin concentrations are higher in Japanese than in Minnesotan women. With harmonic interpolation, a second peak in late afternoon (prolactin) or early afternoon (cortisol) also could be found for the Japanese subjects. This result is interesting in regard to a publication by Kwa et al. (34) reporting an evening elevation of plasma prolactin in postmenopausal women and to another publication by Tarquini et al. (54) on abnormalities in evening plasma prolactin concentrations in nulliparous women with benign or malignant breast disease. Table 37.4 compares acrophases (from the fit of a 24-hr cosine curve) and paraphases (from harmonic interpolation) for prolactin and cortisol in both geographic locations. Very good agreement of these two indices is achieved in the case of prolactin, while a difference of about 2 hr is observed in the case of cortisol. This may be explained by the fact that the cortisol peak is much sharper than the prolactin peak. Of inter-

![Figure 37.5. Plexogram of circadian prolactin rhythms obtained by pooling, over 3-hr spans, winter data for women from all Minnesotans (above) and all Japanese (below).]
occurred in the early afternoon. It can also be seen from Table 37.5 that the mesor and the amplitude of the circadian rhythm in systolic blood pressure underwent circannual changes.

Figures 37.12 and 37.13 summarize the blood pressure results for all Minnesotan subjects pooled over all seasons.

Table 37.6 gives an account of the number of individual series for which a statistically significant circadian rhythm could be demonstrated by rejecting the zero-amplitude hypothesis (single cosinor procedure).

A circadian rhythm of breast surface temperature was demonstrated. Frequency analysis, similar to a discrete Fourier transform, applied to the standard deviations of the 15-min averages yielded ultradians with a period of about 2 hr, at least in some of the series analyzed thus far. Further analyses will have to be carried out to investigate any possible rhythmicity in the noise term.

Daytime wrist activity was analyzed by computing an averaged spectrum. The percentage variance at 12-16 cycles/day was found to be more than double the random expectation. This

Potential Marker Rhythms: Blood Pressure, Breast Temperature, and Wrist Activity

In the case of a physiologic variable, it is interesting to note that with blood pressure data taken every 10 min, the circadian rhythm could be assessed in most subjects. The acrophases, however, were quite different from one individual to another. This was not observed for the circadian rhythm in prolactin or cortisol (see Figures 37.9-37.11) and may indicate the action of a completely different mechanism in the physiology of the individual.

A population-mean cosinor analysis was performed on blood pressure data in each season (considering all subjects sampled) to yield the results shown in Table 37.5. From the systolic and diastolic blood pressures, the pulse pressure and mean arterial pressure were computed and analyzed. The acrophases for all four variables

Figure 37.6. Plexogram of circadian cortisol rhythms obtained by pooling, over 3-hr spans, winter data for women from all Minnesotans (above) and all Japanese (below).

est is the good agreement of acrophases and paraphases between the two populations.

Figure 37.7. Wave-form of circadian prolactin rhythms reconstructed by harmonic interpolation from data for women from Minnesota (above) and Japan (below), summarized as plexograms in Figure 37.5.
result was also confirmed by a t-test and Wilcoxon signed rank test comparing the variance at this frequency for the group of subjects investigated with the random expectation, which was exceeded significantly \((P < 0.005)\). Plots of the wrist activity data analyzed thus far, however, revealed no regular or stable ultradian rhythms, and the spectral analyses demonstrated no variance peaks in the predicted 12–16 cycles/day frequency band. Thus, there was no well-defined ultradian rhythm, although most of the variance was concentrated below four cycles/day, in the circadian range and its harmonics.

**Time-Specified Reference Intervals**

Against the background of such a spectrum of rhythms, one can determine proper reference intervals. Time-unqualified tolerance intervals will not be appropriate since a large share of variability can be accounted for by circadian, circannual, or other variations. On a short-term basis, circadian time-specified tolerance intervals—so-called chronodesms (17, 23)—can be determined to provide reference intervals against which time-qualified single samples can be interpreted cost-effectively. Chronodesms present the advantage of being usually narrower than time-unqualified “normal ranges,” since part of the variance on the latter is due to the rhythmic behavior of the physiologic variable. This means that a given value can be too high at one time, too low at another, or “normal” at yet another time (Fig. 37.14).

The fact that 7 out of 13 hormones could be shown to exhibit marked circannual variation (Table 37.3), even with relatively few subjects, suggests the need for supplementary circannual chronodesms, in addition to circadian ones, as reference standards in cancer epidemiology and probably in the clinic quite broadly.

**Observed Differences**

**Prolactin in Kyushu and Minnesota—First Study**

Age-specific mortality and morbidity rates attributed to breast cancer (27, 28) are 6 times larger in the U.S.A. than they are in Japan. Among the hormones suspected of playing a role in the development of human breast cancer, prolactin has aroused much interest. Prolactin was indeed shown (1, 40, 48) to be essential for the promotion of both spontaneous and carcinogen-induced mammary tumors in rodents. The role of prolactin in the pathogenesis of human breast cancer, however, is uncertain (2) and controversial.

**Figure 37.8.** Wave-form of circadian cortisol rhythms reconstructed by harmonic interpolation, from data for women from Minnesota (above) and Japan (below), summarized as plexograms in Figure 37.6.

<table>
<thead>
<tr>
<th>Acrophase</th>
<th>Paraphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone</td>
<td>Japan</td>
</tr>
<tr>
<td>Prolactin</td>
<td>-48 ± 17</td>
</tr>
<tr>
<td>Cortisol</td>
<td>-135 ± 17</td>
</tr>
</tbody>
</table>

*Acrophases and parapases expressed in degrees; 360° = 24 hr; reference time, local midnight (P-values all ≤0.05). SE, standard error.
Figure 37.9. Circadian acrophases of systolic blood pressure in adolescent, young adult, and postmenopausal women from Japan and the two breast cancer risk groups from Minnesota in each of the four seasons show high variability despite individual statistical significance of circadian rhythm.

This chronoepidemiologic study has demonstrated differences between women from different ethnic-geographic backgrounds in plasma prolactin, with the magnitude and even the sign of some of these differences dependent upon circadian and circannual rhythm stage. The mesor and amplitude of the circadian rhythm in prolactin and the circannual variations in circadian prolactin mesor and amplitude are much more prominent in Japanese women than in Minnesotan women (Fig. 37.15 and 37.16). Moreover, the circannual prolactin mesors in Japanese are higher than the corresponding values in Minnesotan women.

These differences can also be seen from Figure 37.17, displaying mean prolactin circadian profiles for subjects in Minnesota and in Japan for all four seasons. Here again, one can see that Minnesotan women tend to have lower prolactin values than Japanese women when prolactin concentrations are high, i.e. during the night. This difference is more accentuated in winter and can hardly be discerned in summer.

A large but time-restricted difference in plasma prolactin concentration between Japanese and Minnesotan subjects could be observed during the winter season. The difference was less prominent during the other seasons and was statistically not significant during summer. This finding indicates the importance of sampling time in investigations of the role of certain hormones in oncogenesis. It also may explain previous discrepancies and controversies reported in the literature and provides a clue to possible differences in circadian physiological time structure of groups of women with different risks of developing breast cancer.

Kyushu-Minnesota Replication of Prolactin Study

This large but strictly time-dependent difference between Japanese and Minnesotan women
was confirmed in groups of 20 young and 15 adult subjects examined at one circannual stage (first part of March) in Fukuoka City, Kyushu, Japan and Minneapolis, Minnesota, respectively, the Japanese showing higher average circadian prolactin mesors and a higher circadian amplitude than did the Minnesotans, Figure 37.18. This observation emphasizes the critical importance of appropriate timing of sampling of endocrine functions of potential interest for oncogenesis by single or by a limited number of samples chosen on the basis of chronobiologic individual or group reference values.

Prolactin Study in Urbino, Italy

The circadian prolactin amplitude and mesor of a postmenopausal woman, 55 years of age, were lower than the mesors and amplitudes of three series from a 23-year-old woman, Table 37.8 and Figure 37.19. This result is in keeping with a statistically significant decrease in prolactin mesor found in groups of women studied in Minnesota before and after menopause.

Prolactin Study in Florence, Italy

A statistically significant mean circadian rhythm in serum prolactin was observed in groups of 22 women with fibrocystic mastopathy and of 18 clinically healthy women, sampled in Italy with circadian but not circannual idio-cyclicity (see below) (55). The circadian amplitude and acrophase were similar in the two groups but the mesor was statistically significantly higher in patients with fibrocystic mastopathy as compared to the controls. This result suggests that circadian mesor-hyperprolactinemia is a feature associated with fibrocystic mastopathy (Fig. 37.20).

A circannual rhythm in circadian mesors of
serum prolactin also was detected in healthy Italian women (55). With the same sampling schedule, a circannual rhythm in serum prolactin was not detected in women with fibrocystic mastopathy, although circadian rhythms could be found also in this group of subjects, which on the basis of a different risk factor show a higher likelihood of developing breast cancer. As in the comparison of women in Kyushu and Minnesota, the extent of predictable circannual prolactin variation in these Italian subjects decreased as the risk of breast cancer increased.

### Circannual Prolactin Amplitude and Breast Cancer Risk

In correlating the total breast cancer risk in any one subject in the Minnesota-Kyushu study with her circannual amplitude of prolactin, expressed as nanograms per milliliter, a negative correlation (−0.42; \(P = 0.025\)) was clearly apparent and is visualized in Figure 37.21A. When the circannual amplitude of prolactin is expressed as percentage of mesor (rather than nanograms per milliliter), its correlation coefficient with the

**Table 37.5**

<table>
<thead>
<tr>
<th>Season</th>
<th>P-value</th>
<th>Number of series</th>
<th>Mesor</th>
<th>Amplitude</th>
<th>Acrophase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>0.02</td>
<td>19</td>
<td>105.2</td>
<td>3.2</td>
<td>−225</td>
</tr>
<tr>
<td>Spring</td>
<td>0.07</td>
<td>20</td>
<td>101.3</td>
<td>2.4</td>
<td>−227</td>
</tr>
<tr>
<td>Summer</td>
<td>0.07</td>
<td>20</td>
<td>100.5</td>
<td>2.6</td>
<td>−252</td>
</tr>
<tr>
<td>Fall</td>
<td>&lt;0.01</td>
<td>18</td>
<td>105.3</td>
<td>7.8</td>
<td>−235</td>
</tr>
<tr>
<td>Diastolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>0.03</td>
<td>19</td>
<td>66.7</td>
<td>1.5</td>
<td>−197</td>
</tr>
<tr>
<td>Spring</td>
<td>0.11</td>
<td>20</td>
<td>66.8</td>
<td>1.5</td>
<td>−199</td>
</tr>
<tr>
<td>Summer</td>
<td>0.33</td>
<td>20</td>
<td>66.6</td>
<td>1.5</td>
<td>−250</td>
</tr>
<tr>
<td>Fall</td>
<td>&lt;0.01</td>
<td>18</td>
<td>66.2</td>
<td>4.2</td>
<td>−229</td>
</tr>
</tbody>
</table>

\*\(360° = 24\) hr; \(0° = \) local midnight.

**Figure 37.11.** As opposed to systolic (see Fig. 37.9) and diastolic (see Fig. 37.10) blood pressure, circadian acrophases of plasma prolactin, and cortisol of adolescent, young adult, and postmenopausal women from Japan and the breast cancer risk groups from Minnesota in each of the four seasons show a higher degree of synchronization.
risk of breast cancer is of borderline statistical significance ($P \approx 0.06$). It also can be seen from Figure 37.21A that, from the viewpoint of the total risk, some of the women who were grouped originally as high risk in Minnesota, on the basis of a relative with breast cancer, were actually at no higher risk than some of the women rated as low risk for breast cancer, when all factors are considered. When, however, a t-test was applied to compare total breast cancer risks between the two groups of Minnesotan subjects a statistically significant difference was found ($P < 0.05$). Referring to Figure 37.21A, a possible relationship between the circannual amplitude of prolactin and total breast cancer risk deserves scrutiny in larger samples of women. This can now be done with more cost-effective sampling aimed at assessing over a longer time-span serum prolactin rhythms with a better optimization schedule than was maintained in this investigation.

Although Figure 37.21A suggests a negative correlation for prolactin and a positive one for TSH as a function of total breast cancer risk load, one should be aware of statistical difficul-

### Table

<table>
<thead>
<tr>
<th>Key to Pressures</th>
<th>$P$</th>
<th>PR</th>
<th>NO. SER.</th>
<th>MESOR</th>
<th>Amplitude</th>
<th>Acrophase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A SYSTOLIC</td>
<td>&lt; 0.001</td>
<td>21</td>
<td>83</td>
<td>103.1</td>
<td>4.1 (2.6, 5.6)</td>
<td>-232° (-218, -244)</td>
</tr>
<tr>
<td>B DIASTOLIC</td>
<td>&lt; 0.001</td>
<td>20</td>
<td>83</td>
<td>66.6</td>
<td>2.2 (1.1, 3.4)</td>
<td>-221° (-195, -236)</td>
</tr>
</tbody>
</table>

*360° = 24 H; 0° = Local Midnight

**Figure 37.12.** When pooling over all subjects and all seasons, a circadian population-mean cosinor yields a statistically significant ($P < 0.05$) circadian rhythm of systolic and diastolic blood pressure.
that the period best characterizing the data set on any one day would almost certainly differ from precisely 24 hr. It was further emphasized that no implication was made as to any precise period length by the fit of, say, a 24-hr period; it was thereby solely assumed that an a priori 24-hr period would be close enough to the actual period to demonstrate substantially the same parameters and significance; the 24-hr period would thus serve only as a simple representative of the circadian region of the spectrum.

The fact that acrophases for different groups of subjects—H, B, and C—are close to each other when a 24-hr period is being fitted to the data on human breast temperatures is likely due to the fact that all subjects lived on a regular 24-hr routine. The conclusions to be promptly derived from the results of these fits are: (a) that there is a high likelihood that a circadian period characterized the data; (b) that this period was in the neighborhood of 24 hr in all three groups; (c) that, nonetheless, the circadian period differed among the several groups investigated, H, B, and C; and (d) that the technical limitations of analyses based upon only a few days’ data are not likely to account for this intergroup difference. (These limitations will be amplified later in this paper.) Study of more individuals for longer spans, including recordings under conditions of isolation from any 24-hr routine, will be desirable to clarify the question whether multiple periods may underlie the results.

**DISCUSSION**

In this first report of a large international cooperation, many problems arise that are not solved. They are here presented as an indication of the need to control seemingly trivial factors such as the inequalities of the samples of blood withdrawn at different consecutive times and the need to establish for each of the variables being examined that any differences in amount do not play a role. Such illustrative problems serve to indicate the tasks on hand in devising pertinent sampling procedures and thus may serve others who may build upon the results of this study.

Interaction of human plasma prolactin rhythms with different frequencies, including circadian mesor and amplitude both modulated by circatrigintan rhythms (except for postmenopausal group) and by circannual rhythms.
ties associated with the assignment of a $P$-value for these correlations. A test of lack of fit indicates the inappropriateness of a linear model of TSH ($F(11,15) = 2.67, P = 0.05$). Moreover, in addition to the hypothesis of “no error” in the independent variable (total breast cancer risk load), the usual assumptions are those of normality and homogeneity of variance of the error term. These assumptions have been tested by $\chi^2$ for goodness of fit and Bartlett’s test for homogeneity of variance. The normality hypothesis was rejected for the case of TSH ($\chi^2(3) = 13.6, P < 0.05$) but not for prolactin ($\chi^2(3) = 5.9, P > 0.05$). Homogeneity of variance along the independent variable was not satisfied for either prolactin ($F(3,1037) = 5.33, P < 0.05$) or TSH ($F(3,1037) = 5.39, P < 0.05$).

**Broader Circannual Hormonal Rhythmometry**

Quite apart from any negative correlation between the circadian amplitude of plasma prolactin on the one hand, and breast cancer risk on
Table 37.6
Rhythmic Summary of Blood Pressure in Women of Various Ages in Different Seasons and Different Geographic Locations

<table>
<thead>
<tr>
<th>Group</th>
<th>% Rhythm (±SE)</th>
<th>Mesor (Torr ±SE)</th>
<th>Double Amplitude (Torr ±SE)</th>
<th>% of series with P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U.S. Systolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad</td>
<td>14.0 (±2.8)</td>
<td>98.4 (±1.8)</td>
<td>9.7 (±1.2)</td>
<td>4 (18) 2 (9) 0 (0)</td>
</tr>
<tr>
<td>YA</td>
<td>23.5 (±2.8)</td>
<td>102.1 (±1.4)</td>
<td>13.1 (±1.3)</td>
<td>0 (0) 0 (0) 2 (6)</td>
</tr>
<tr>
<td>PM</td>
<td>22.1 (±3.3)</td>
<td>106.2 (±1.8)</td>
<td>15.2 (±1.7)</td>
<td>1 (4) 1 (4) 0 (6)</td>
</tr>
<tr>
<td>W</td>
<td>21.8 (±3.1)</td>
<td>106.2 (±2.0)</td>
<td>13.4 (±1.5)</td>
<td>0 (0) 1 (4) 1 (4)</td>
</tr>
<tr>
<td>Sp</td>
<td>15.2 (±3.1)</td>
<td>101.3 (±2.4)</td>
<td>9.4 (±1.4)</td>
<td>4 (20) 0 (0) 0 (0)</td>
</tr>
<tr>
<td>S</td>
<td>16.4 (±2.7)</td>
<td>100.5 (±1.7)</td>
<td>11.5 (±1.2)</td>
<td>1 (5) 2 (10) 0 (0)</td>
</tr>
<tr>
<td>F</td>
<td>29.3 (±5.1)</td>
<td>105.5 (±2.1)</td>
<td>18.1 (±2.4)</td>
<td>0 (0) 0 (0) 1 (6)</td>
</tr>
<tr>
<td>U.S. Diastolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad</td>
<td>10.0 (±2.5)</td>
<td>62.4 (±0.9)</td>
<td>5.8 (±0.9)</td>
<td>7 (32) 2 (9) 2 (6)</td>
</tr>
<tr>
<td>YA</td>
<td>21.7 (±3.0)</td>
<td>65.5 (±0.8)</td>
<td>9.4 (±1.0)</td>
<td>3 (9) 0 (0) 5 (16)</td>
</tr>
<tr>
<td>PM</td>
<td>24.1 (±3.8)</td>
<td>71.7 (±1.1)</td>
<td>10.8 (±1.4)</td>
<td>4 (15) 2 (7) 0 (0)</td>
</tr>
<tr>
<td>W</td>
<td>20.0 (±3.5)</td>
<td>67.1 (±1.2)</td>
<td>9.0 (±1.2)</td>
<td>4 (17) 1 (4) 2 (6)</td>
</tr>
<tr>
<td>Sp</td>
<td>18.6 (±3.5)</td>
<td>66.8 (±1.5)</td>
<td>7.8 (±1.3)</td>
<td>4 (20) 1 (5) 2 (10)</td>
</tr>
<tr>
<td>S</td>
<td>19.1 (±3.8)</td>
<td>66.6 (±1.3)</td>
<td>8.8 (±1.3)</td>
<td>3 (15) 1 (5) 2 (10)</td>
</tr>
<tr>
<td>F</td>
<td>21.9 (±5.1)</td>
<td>66.3 (±1.6)</td>
<td>9.9 (±1.6)</td>
<td>3 (18) 1 (6) 1 (6)</td>
</tr>
<tr>
<td>Japan Systolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad</td>
<td>20.3 (±4.1)</td>
<td>105.2 (±3.2)</td>
<td>9.1 (±1.5)</td>
<td>1 (13) 0 (0) 1 (13)</td>
</tr>
<tr>
<td>YA</td>
<td>4.3 (±4.6)</td>
<td>102.0 (±2.2)</td>
<td>4.3 (±1.6)</td>
<td>3 (50) 2 (33) 0 (0)</td>
</tr>
<tr>
<td>PM</td>
<td>22.6 (±4.5)</td>
<td>110.2 (±4.4)</td>
<td>14.9 (±1.7)</td>
<td>1 (13) 0 (0) 0 (0)</td>
</tr>
<tr>
<td>W</td>
<td>21.8 (±3.9)</td>
<td>105.7 (±2.3)</td>
<td>11.8 (±1.8)</td>
<td>1 (9) 1 (9) 0 (9)</td>
</tr>
<tr>
<td>Sp</td>
<td>11.7 (±3.5)</td>
<td>106.5 (±2.7)</td>
<td>7.9 (±1.7)</td>
<td>4 (30) 1 (9) 1 (9)</td>
</tr>
<tr>
<td>Japan Diastolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad</td>
<td>10.1 (±2.7)</td>
<td>63.8 (±2.7)</td>
<td>6.9 (±1.0)</td>
<td>2 (25) 0 (0) 0 (0)</td>
</tr>
<tr>
<td>YA</td>
<td>13.6 (±5.4)</td>
<td>66.1 (±0.8)</td>
<td>8.6 (±2.0)</td>
<td>1 (17) 0 (0) 2 (33)</td>
</tr>
<tr>
<td>PM</td>
<td>15.4 (±4.0)</td>
<td>75.3 (±2.6)</td>
<td>8.6 (±1.6)</td>
<td>1 (25) 0 (0) 1 (13)</td>
</tr>
<tr>
<td>W</td>
<td>10.4 (±1.7)</td>
<td>69.1 (±2.4)</td>
<td>7.7 (±0.9)</td>
<td>1 (9) 0 (0) 2 (18)</td>
</tr>
<tr>
<td>Sp</td>
<td>15.7 (±4.0)</td>
<td>68.1 (±2.7)</td>
<td>8.3 (±1.5)</td>
<td>3 (27) 0 (0) 1 (9)</td>
</tr>
</tbody>
</table>

Ad, adolescent; YA, young adult; PM, postmenopausal woman; W, winter; Sp, spring; S, summer, F, fall.

Ad, on the other hand, it seemed of interest to explore more broadly the imputations (17) of circannual mesor and amplitude in relation to breast cancer risk for the thirteen hormones examined systematically in the Kyushu-Minnesota study. Table 37.9 summarizes the statistically significant aspects of a correlation matrix computed between breast cancer risk and imputations of circannual rhythm characteristics from data on those volunteers who provided blood for four different seasons. This matrix revealed, in addition to the negative correlation with breast cancer risk of the prolactin amplitude, a negative correlation with the prolactin mesor and a positive correlation with the LH mesor and the TSH amplitude. Thyroidal function has been discussed in relation to breast cancer risk by Mittra (43) and Mittra and Hayward (44, 45). Hence, the circumstance that it is the circannual amplitude of TSH which seems to discriminate between the groups with high and low risk is of particular interest, notably since a relation of the TSH mesor to

Ad, adolescent; YA, young adult; PM, postmenopausal woman; W, winter; Sp, spring; S, summer, F, fall.

Ad, on the other hand, it seemed of interest to explore more broadly the imputations (17) of circannual mesor and amplitude in relation to breast cancer risk for the thirteen hormones examined systematically in the Kyushu-Minnesota study. Table 37.9 summarizes the statistically significant aspects of a correlation matrix computed between breast cancer risk and imputations of circannual rhythm characteristics from data on those volunteers who provided blood for four different seasons. This matrix revealed, in addition to the negative correlation with breast cancer risk of the prolactin amplitude, a negative correlation with the prolactin mesor and a positive correlation with the LH mesor and the TSH amplitude. Thyroidal function has been discussed in relation to breast cancer risk by Mittra (43) and Mittra and Hayward (44, 45). Hence, the circumstance that it is the circannual amplitude of TSH which seems to discriminate between the groups with high and low risk is of particular interest, notably since a relation of the TSH mesor to
breast cancer risk is only of borderline statistical significance, whereas relation to the circannual amplitude, indeed, seems to be established and should be checked in further work.

**Urinary Melatonin**

We here summarize results discussed elsewhere (62). Urine specimens, analyzed for melatonin, revealed statistically significant differences between groups ($P < 0.01$) as well as a statistically significant time-group interaction, thereby attesting to the fact that the intergroup difference depends upon time. It can be seen from Figure 37.22 that Japanese have consistently lower values of melatonin excretion than do North Americans. Analyses of values from the night span confirmed the fact, already observed in earlier studies (for review, see Refs. 19 and 51), that melatonin excretion is higher during the time of darkness (habitual rest). Figure 37.22 also summarizes the results from a population mean-cosinor analysis after pooling data from Minnesota and Japan, thus documenting a statistically significant circadian rhythm in urinary melatonin.

**Other Hormones**

Differences in other hormones were less obvious. In contrast to prolactin, plasma cortisol during the winter has a larger circadian mesor and amplitude in Minnesota than in Japan (Table 37.10). In the early morning hours (~09:00), a difference in cortisol of about 4 μg/dl may be observed between the two populations. During the other seasons, a statistically significant difference in all characteristics of the cortisol rhythm was also observed between Japanese and Minnesotan low-risk subjects, as shown by a Hotelling $T^2$ test.

North American women had a higher circadian aldosterone mesor in the winter than did Japanese subjects. In the summer, Minnesotans also had a higher circadian DHEA-S amplitude than did Japanese subjects. Differences between Japanese and Minnesotans (both risk categories) also were observed in the amplitude/mesor ratio for plasma insulin during the summer.

**Age-Related Differences**

Langlands et al. (35) report that deaths occurring in patients with breast cancer show a circannual pattern differing in relation to menopause. When analyses are carried out separately for women diagnosed before and after menopause, circannual rhythms were found to be in antiphase.

The investigation that is the subject of this report was planned to include four subjects from each of the three age categories and each of the three risk categories for a total of 36 subjects, so that sampling at different seasons and menstrual stages could proceed according to a balanced Latin-square design. For a variety of reasons, some subjects had to be replaced part-way through the study, and for some 24-hr sampling sessions, not all the planned blood samples could be obtained or analyzed. To avoid possible bias from unbalanced sampling of the various rhythms, the analyses for an age effect included only those subjects sampled in all four seasons (and menstrual stages) from whom most, if not all, of the planned blood samples were obtained.
Misleading single sampling would suggest:

Plasma Prolactin in Japanese women is higher than the same as that in women in USA.

Figure 37.16. Geographic difference in circannual rhythm of human plasma prolactin. Contradictory comparisons of circadian mesor could be obtained by sampling in different seasons. Moreover, controlling time of year (or time of day, Fig. 37.15) may not avoid misleading results due to circannual (or circadian, as the case may be) variations, whereas assessment of circannual (or circadian) rhythm characteristics may reveal important chronoepidemiologic or chronoprophylactic results. Conclusions from single samples obtained in different seasons (or times of day) can be resolved as differences of circannual (circadian) prolactin rhythm between Japanese and American women. Each subject provided 72 blood samples (8640 determinations, each in duplicate). W, Winter; Sp, Spring; S, Summer; F, Fall.

during each 24 hr span. An added consideration in the latter case was whether missing samples were more or less evenly distributed through the 24 hr so that their absence would not be expected to bias results.

Rather than simply analyzing the overall mean value for a given hormone on each subject, we chose to use estimates based on the fit of a 24-hr cosine (see above) so that the hormone's circadian amplitude as well as its mesor could be examined. It should be noted that the mesor estimate in this procedure is identical with the 24-hr arithmetic mean if the data are equally spaced throughout an entire cycle.

For each subject, the mean value of a circadian parameter (e.g. mesor) for a given hormone across all four seasons (menstrual stages) represented that subject in a two-way analysis of variance (ANOVA) for age and risk factors. In dealing with uneven numbers of values in different age versus risk cells, the conventional experimental approach (46) was used to partition the total sum of squares in testing the statistical significance of main effects and interaction.

Results concerning the effects of age on circadian mesors and circadian amplitudes of 12 hormones (including prolactin) are summarized in Tables 37.11A and 37.11B, respectively. Estriol (E3) was not included because of inadequate data satisfying the criteria indicated above. Inferences as to the statistical significance of main effects (age and risk) are not valid if there is interaction or if inhomogeneity of variance is
Figure 37.17. Comparison of plasma prolactin in clinically healthy Japanese and Minnesotan women in four seasons. Samples taken at 20-min intervals over a 24-hr span in Fukuoka City, Japan and Minneapolis, Minn.

indicated. In such cases, resort was made to a contrast test using separate variance estimates from each cell involved in the contrast to estimate the standard error. Such a test of an age effect for these hormones exhibiting inhomogeneity of variance or an interaction effect involved a contrast (46) between the mature-adult (age 29-36) and the postmenopausal (age 44-59) groups. A contrast involving the young-adult group as a whole could not be made because the U.S.A. low-risk, young-adult cell had only one value for all hormones.

Even allowing for an effect of multiple inference, an effect of age is clearly apparent for the circadian mesors of LH, prolactin, estrone (E1), estradiol (E2), and 17-OH-progesterone. In the case of DHEA-S, an interaction between age and risk effects plus inhomogeneity of variance among the nine age-versus-risk cells invalidate the indication of an apparently highly significant age effect in the ANOVA. A P-value of 0.09 in the contrast between the mature adult and postmenopausal age groups does not lend much support to the conclusion of an age effect on DHEA-S, especially if one considers multiple inferences. For these five hormones exhibiting a clear age effect, LH undergoes a marked increase while prolactin, the estrogens, and 17-OH-progesterone decrease between the age classes of 29-36 and 44-59 years.

LH, E2, and 17-OH-progesterone also exhibit a clear effect of age on the circadian amplitude, with an increase for LH and a decrease for the other two hormones. In addition, the results indicate a statistically significant decrease in amplitude of DHEA-S while an age effect on the amplitudes of circadian rhythms in prolactin, E1, and aldosterone may be considered of borderline statistical significance. It should be borne in mind that amplitude estimates were obtained by fitting a 24-hr cosine curve to the data from each 24-hr sampling span. For these hormones with 15 or fewer samples/24-hr span (i.e. all hormones except prolactin and cortisol), the cosine fit often was not statistically significant, as indicated by a zero-amplitude test; nevertheless,
the estimates were considered indicative of the extent of variation on the circadian time scale, for the purposes of investigating the age effect.

Results from testing of a possible breast cancer risk effect on circadian mesors and amplitudes are not tabulated, since there are only two cases (the aldosterone mesor and the prolactin amplitude) in which interpretation of a low P-value was not compromised by the presence of an interaction effect. The P-value for an effect of risk status on the aldosterone mesor was 0.01 while that for an effect of risk on the prolactin amplitude was 0.03.

A comparison of circannual amplitudes of the adolescent (15-21 year old) and the young adult (29-36 year old) groups consisting of 8 and 10 subjects, respectively, with those of the postmenopausal (44-59 year old) group (10 subjects) reveals a decrease in mean circannual amplitude as a function of age for aldosterone, 17-OH-progesterone, estrone, estradiol and prolactin and an increase for LH. These changes were statistically significant below the 5% level (Table 37.7). These finding are qualified by possible effects of unbalanced representation of menstrual stages, limited numbers of circadian mesors provided for the fit of a 1-year cosine curve and by any novelty effect biasing the data.

The circadian stage-dependent difference in chronogram.

![Figure 37.18](image)

**Figure 37.18.** Difference of plasma prolactin between clinically healthy Japanese and American women in March, 1978 when circadian rhythm is near its maximum, confirmed by complementary study. Fifteen whites of mixed ethnic background (18-24 years old) in Minnesota and 20 Japanese (~20 years old) in Kyushu, Japan.

Population-mean-cosinor analyses were also applied to blood pressure data from subjects of different ages (pooling over all seasons). Results are summarized in Table 37.12. The circadian blood pressure mesor increases with age. This statement is rhythm-qualified and hence seemingly secure since it is based on measurements made about every 10 min for 24 hr. This circumstance, however, does not solve the obvious problem associated with transverse observations involving sampling on different groups of individuals (defined as anidiocyclic below).

<table>
<thead>
<tr>
<th>Age Group*</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>2.84</td>
<td>4.85</td>
<td>1.68</td>
</tr>
<tr>
<td>17-OH-Progesterone (ng/ml)</td>
<td>491</td>
<td>615</td>
<td>73</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>66.97</td>
<td>71.73</td>
<td>12.08</td>
</tr>
<tr>
<td>Estrone (pg/ml)</td>
<td>47.19</td>
<td>45.41</td>
<td>21.19</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>7.11</td>
<td>8.82</td>
<td>2.38</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>11.48</td>
<td>7.14</td>
<td>16.74</td>
</tr>
</tbody>
</table>

* I, II and III indicate mean values for amplitude of subjects in age groups 15-21, 29-36, and 44-59 years.

**Table 37.7** Circannual Amplitude of Plasma Aldosterone, 17-OH-Progesterone, Estradiol, Estrone, Prolactin, and LH in Women after Menopause
Table 37.8
Circadian Prolactin Rhythm In a Premenopausal and A Post-menopausal Woman (Urbino, Italy)

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (yr)</th>
<th>Date (mo/day)</th>
<th>P</th>
<th>PR%</th>
<th>M (ng/ml) (± SE)</th>
<th>A (ng/ml) (± SE)</th>
<th>Acrophase° #</th>
<th>Deg (± SE) hr/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>07/26</td>
<td>0.021</td>
<td>14</td>
<td>4.24 (±0.12)</td>
<td>0.51 (±0.18)</td>
<td>-64 (±17)</td>
<td>04:19</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>10/17</td>
<td>0.125</td>
<td>6</td>
<td>16.09 (±1.00)</td>
<td>4.70 (±2.26)</td>
<td>-69 (±26)</td>
<td>04:39</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>01/23</td>
<td>0.001</td>
<td>28</td>
<td>12.16 (±0.72)</td>
<td>5.40 (±1.03)</td>
<td>-81 (±10)</td>
<td>05:28</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>04/08</td>
<td>0.001</td>
<td>36</td>
<td>6.74 (±0.41)</td>
<td>3.68 (±0.58)</td>
<td>-105 (±9)</td>
<td>07:00</td>
</tr>
</tbody>
</table>

* P-value in testing zero-amplitude hypothesis, PR, percentage rhythm; M, mesor; SE, standard error; A, amplitude
* From local 00:00; 360° = 24 hr

**Figure 37.19.** Lower circadian mesors and amplitudes in serum prolactin of a postmenopausal woman as compared to a 23-year old woman, both sampled in Urbino, Italy.

Sampling on the same individual (idiocyclicity) is required to explore the possibility that the mesor of some individuals may not change with age and that the age-associated increase seen in transverse studies may result from the circumstance that, with advancing age, some individuals develop mesor-hypertension.

Table 37.12 shows further that a circadian
amplitude increases with age in these clinically healthy subjects, in keeping with an early observation made on mesor-hypertensive patients by Menzel (personal communication). The circadian acrophase seems to remain unchanged.

Hormonal Models

From the Latin-square design of this study, it was possible to assess circannual or circatrigintan (menstrual) rhythms. Most subjects were

Figure 37.20. Circannual chronopathology. Obliteration of circannual but not of circadian rhythm in serum prolactin in fibrocystic mastopathy (FM) as compared to clinical health (H) for women sampled in Florence, Italy. While the circadian amplitude (expressed in ng/ml or as percentage of mesor) is not affected in presence of benign disease of the breast, the circannual amplitude is drastically reduced. The alteration of a circannual prolactin rhythm might thus be a harbinger of increased breast cancer risk.
A negative correlation between the total relative breast cancer risk evaluated from epidemiologic criteria and the circannual prolactin amplitude corroborates the finding that an elevation of breast cancer risk is associated with a decrease in circannual amplitude (based on least squares fit of 365.25-day cosine curve to circadian mesors assessed in each of the four seasons.) (see Fig. 37.16 and 37.20). Note further the positive correlation between epidemiologically assessed breast cancer risk and the circannual amplitude of thyroid-stimulating hormone (TSH). Clinical hypothyroidism has empirically been associated with breast cancer risk. If, then, this topic is still controversial, this may, perhaps, be accounted for by the circannual-stage-dependence of the correlation.

It is also noteworthy that in prostatic cancer (38) (a condition characterized by geographic differences in morbidity and mortality similar to those of breast cancer), the extent of circannual variation also changes as a function of risk and/or cancer. In blood sampled with serial independence in the morning at different times of the year, a prominent circannual rhythm in TSH of healthy subjects is lost in prostatic cancer (and perhaps even in men at high risk of prostatic cancer). For prolactin, a circannual rhythm becomes demonstrable in the case of prostatic cancer, while it is not demonstrable with serially independent sampling in healthy men of low or high prostatic cancer risk.

Thus, TSH and prolactin show opposite behavior along the 1-yr scale in cancers of both breast and prostate (rather than responding in the same fashion, as is the case along the scale of minutes to hours—following the application of stimuli such as Thyrotropin Releasing Hormone (26)). It is yet more interesting that the circannual relations of plasma TSH and prolactin to the risk of breast cancer are opposite to those with respect to prostatic cancer (and, as noted, opposite to each other in each cancer).

The two correlations shown in the figure are but part of a large correlation matrix. The circumstance is noted that correlations emerged as statistically significant for the very hormones which clinicians have long considered had some relation to breast cancer, yet thus far could not rigorously establish such a relation as biologically significant, perhaps because of all-too-limited sampling.

While these conclusions rest on large samples, they describe only a small number of subjects. Moreover, all conditions required to apply a linear regression between two variables are not satisfied. A test for lack of fit indicates that the model is not adequate for TSH; the error term is not normally distributed. In addition, the assumption of homogeneity of variance is not verified for prolactin as well as for TSH. Finally, in the case of prolactin, there seems to be an age effect on both this circannual amplitude (decreases with age) and the breast cancer risk (increases with age). This may account for the negative correlation illustrated in the figure. Hence the correlations in the figure are of ordering rather than documenting value. They are intended to emphasize that circannual rhythmicity deserves further study in relation to carcinogenesis. If such correlations can be confirmed and if the circannual rhythms involved should prove to be determinants of carcinogenesis in the human breast, these same correlations will point to the possibility of a chemoprevention of breast cancer.
Figure 37.21B  Negative correlation between the circannual aldosterone amplitude (based on least squares fit of 365.25-day cosine curve to circadian mesors assessed in each of the four seasons on each of the women investigated in Minnesota and Japan) and the circadian diastolic blood pressure mesor (in winter, the season when blood pressure profiles at about 10-min intervals were obtained in both locations) observed in women in Minnesota \( (N = 13, \text{---, left}) \), in Kyushu, Japan \( (N = 10, \text{---, center}) \), and both \( (N = 23, \text{---, right}) \).  † Results from heterogeneous sample, ostensibly not meeting conditions for significance testing.

Figure 37.21C  Negative correlation of the circannual aldosterone amplitude and both the circadian diastolic blood pressure mesor and the individual risk of diseases associated with high blood pressure. Results from 23 presumably healthy women, 13 in the USA (■) and 10 in Japan (●); estimate of circadian diastolic pressure mesor based on appropriate data obtained only in winter; circannual aldosterone amplitude determined by fit of 65.25-day cosine curve to circadian mesors assessed in each of the four seasons; factors determining risk value included familial morbidity or mortality, obesity, "high" heart rate and a history of blood pressure "spiking."  † Results from heterogeneous sample, ostensibly not meeting conditions for significance testing.
Figure 37.22. Circadian variation in urinary excretion of melatonin in 11 white women of mixed ethnic background in Minnesota and 8 Japanese women in Kyushu, Japan. Chronogram (left) shows urinary melatonin excretion by Japanese and Minnesotan women at several timepoints during 1-day span. Each woman contributed six to eight samples collected over a single 24-hr span; data for each population averaged across all subjects for 3-hr intervals (no Japanese samples between 21:00 and 24:00 hr). Amplitude and acrophase estimates from all subjects were combined in population-mean cosinor (right).

<table>
<thead>
<tr>
<th>Table 37.9</th>
<th>Correlations Between Relative Breast Cancer Risk and (Imputations of) Circannual Rhythm Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Variable</td>
</tr>
<tr>
<td>Amplitude</td>
<td>TSH</td>
</tr>
<tr>
<td></td>
<td>Prolactin</td>
</tr>
<tr>
<td>Mesor</td>
<td>TSH</td>
</tr>
<tr>
<td></td>
<td>Prolactin</td>
</tr>
<tr>
<td></td>
<td>LH</td>
</tr>
</tbody>
</table>

* \( r^* \), correlation coefficient; \( P \), probability; TSH, thyroid-stimulating hormone; LH, luteinizing hormone.

<table>
<thead>
<tr>
<th>Table 37.10</th>
<th>Circadian Mesor and Amplitude of Cortisol in Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td>Minnesota</td>
</tr>
<tr>
<td>Mesor (( \mu \text{g/d} ))</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>4.4 ± 0.3</td>
</tr>
</tbody>
</table>

The data on hand (four spans of 24 hr—about 3 months apart—during which the sampling rate was three times an hour) were not sufficient to allow us to fit concomitantly the different fre-
Results from Table 37.11b

Table 37.11A
Results from Two-Way ANOVA* for Effects of Age on Circadian Mesor (~24-Hr Mean) of 12 Plasma Hormones in Women Sampled in Each of Four Menstrual Stages and Four Seasons

<table>
<thead>
<tr>
<th>Hormone (units)</th>
<th>N°</th>
<th>Age*</th>
<th>ANOVA*</th>
<th>Contrast (II vs. III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (mIU/ml)</td>
<td>27</td>
<td>I</td>
<td>14.9</td>
<td>74.80 &lt;0.001</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>29</td>
<td>II</td>
<td>22.9</td>
<td>10.37 &lt;0.001</td>
</tr>
<tr>
<td>E1 (pg/ml)</td>
<td>27</td>
<td>III</td>
<td>75.0</td>
<td>14.66 &lt;0.001</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>26</td>
<td>I</td>
<td>81.3</td>
<td>14.52 &lt;0.001</td>
</tr>
<tr>
<td>17-OH Progesterone (pg/ml)</td>
<td>29</td>
<td>II</td>
<td>602.0</td>
<td>22.50 &lt;0.001*</td>
</tr>
<tr>
<td>Cortisol (pg/dl)</td>
<td>30</td>
<td>III</td>
<td>8.7</td>
<td>0.12</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>25</td>
<td>I</td>
<td>6.1</td>
<td>2.36</td>
</tr>
<tr>
<td>DHEA-S (ng/ml)</td>
<td>28</td>
<td>II</td>
<td>3090.0</td>
<td>20.65 0.001*</td>
</tr>
<tr>
<td>TSH (IU/ml)</td>
<td>29</td>
<td>III</td>
<td>3.3</td>
<td>1.51</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>28</td>
<td>I</td>
<td>94.7</td>
<td>0.35</td>
</tr>
<tr>
<td>T4 (ng/dl)</td>
<td>28</td>
<td>II</td>
<td>8.8</td>
<td>0.15</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>29</td>
<td>III</td>
<td>26.9</td>
<td>3.08</td>
</tr>
</tbody>
</table>

*ANOVA, analysis of variance. F = value taken by F-test in ANOVA, P = corresponding P-value. LH, luteinizing hormone; E1, estrone; E2, estradiol; DHEA-S, dehydroepiandrosterone sulfate; TSH, thyrotropic hormone; T3, triiodothyronine; T4, thyroxine.

Table 37.11b
Results from Two-way ANOVA* for Effects of Age on Circadian Amplitude of 12 Plasma Hormones in Women Sampled in Each of Four Menstrual Stages and Four Seasons

<table>
<thead>
<tr>
<th>Hormone (units)</th>
<th>N°</th>
<th>Age*</th>
<th>ANOVA*</th>
<th>Contrast (II vs. III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (mIU/ml)</td>
<td>27</td>
<td>I</td>
<td>4.0</td>
<td>38.99 &lt;0.001*</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>29</td>
<td>II</td>
<td>12.4</td>
<td>4.34 0.027</td>
</tr>
<tr>
<td>E1 (pg/ml)</td>
<td>27</td>
<td>III</td>
<td>17.6</td>
<td>4.30 0.03*</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>26</td>
<td>I</td>
<td>28.0</td>
<td>10.00 &lt;0.001*</td>
</tr>
<tr>
<td>17-OH Progesterone (pg/ml)</td>
<td>29</td>
<td>II</td>
<td>181.0</td>
<td>10.00 &lt;0.001</td>
</tr>
<tr>
<td>Cortisol (µU/dl)</td>
<td>30</td>
<td>III</td>
<td>7.1</td>
<td>1.23</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>25</td>
<td>I</td>
<td>4.1</td>
<td>11.49 &lt;0.001*</td>
</tr>
<tr>
<td>DHEA-S (ng/ml)</td>
<td>28</td>
<td>II</td>
<td>380.0</td>
<td>19.62 &lt;0.001</td>
</tr>
<tr>
<td>TSH (µU/ml)</td>
<td>29</td>
<td>III</td>
<td>6.73</td>
<td>2.48</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>28</td>
<td>I</td>
<td>12.9</td>
<td>0.17</td>
</tr>
<tr>
<td>T4 (µU/dl)</td>
<td>29</td>
<td>II</td>
<td>0.58</td>
<td>2.01</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>29</td>
<td>III</td>
<td>20.5</td>
<td>2.65</td>
</tr>
</tbody>
</table>

* See footnotes for Table 37.11A; substitute "circadian amplitude" for "circadian mesor." Adapted from W. Nelson et al., J. Gerontology, 35: 512–519, 1980.
circatrigintan and circannual rhythms modulating the circadian amplitude; subscripts 4 and 5 refer to the superimposed circatrigintan and circannual rhythms (modulating the circadian mesor). In other words, waves 2 and 3 were obtained by considering the circadian amplitudes (A) while waves 4 and 5 were obtained by considering the circadian mesors (M). The phases $\phi_i$ in equation (1) are related to the respective acrophases $\phi_i$ by the equation

$$\phi_i = \frac{2\pi}{\tau_i} \phi_i \text{ (where } \phi_i \text{ and } \phi_i \text{ are expressed in radians)} \text{ (2)}$$

For two hormones—prolactin and cortisol—six groups were considered: the three risk groups (pooled over age), i.e. Japan, Minnesota low-risk, and Minnesota high-risk; and the three age groups (pooled over risk and geographic location), i.e. adolescents, young adults, and postmenopausal women. The sequence considered in Figure 37.23 consists of: (a) the circadian rhythm over a 60-day span; (b) the circatrigintan rhythm (wave 4) superimposed on the circadian rhythm (modulating the circadian mesor) during a 60-day span; (c) the circatrigintan rhythms modulating both the circadian mesor and amplitude (waves 2 and 4) over a 60-day span; (d) the latter model extended over a 365-day span; (e) the same model as in (d) but with the circannual rhythm superimposed (wave 5: mesor modulation); and (f) the final model consisting of all 5 waves over a full year span.

It has to be noted that in order to visualize the full range of variation and to give a better idea of the envelope, the circadian phase ($\phi_i$) was fixed to $-0.5$. Because of the finite number of points used to construct the model, the amplitude may be clipped if $\phi_i$ is not fixed to this value. Tables 37.13 and 37.14 show the parameter values used for waves 1–5 in the theoretical models. Since the postmenopausal women were admitted irrespective of any circatrigintan rhythm, this component was not evaluated.

In the case of prolactin, the circadian mesor and amplitude tended to be higher in Japanese than in Minnesotan subjects. The amplitudes of circannual rhythms in the circadian mesor and amplitude were also larger in the Japanese.

Prolactin concentrations were higher in adolescents and young adults than in postmenopausal women. The circatrigintan rhythms modulating both the circadian mesor and amplitude were more pronounced in young adults than in adolescents, Figure 37.24.

In the case of cortisol, the mesors and relative amplitudes were about the same for the Japanese and the two Minnesota risk categories. As a result of phase relationships among the different components, however, the lower limit of variation of cortisol was almost constant in Minnesotans, whereas in the Japanese it was influenced by the circannual rhythm. Only slight differences were observed among age categories. For young adults, the lower limit of cortisol variation throughout the year remained almost constant, whereas the circannual variation was clearly visible at the two other age categories.

### Change in Circadian Period of Human Breast Surface Temperature

Breast surface temperatures, measured with a 10-channel Yellow Springs telethermometer in Mexico City (from the nipple and four quadrants of each breast during wakefulness, at 2-hr intervals, for 4 days from three groups each composed of eight women)—in health (H), benign breast disease (B), and breast cancer (C)—indicate a shortening of the circadian period in the presence of benign disease and further in the presence of cancer—Figures 37.25 and 37.26. One
1. Mean circadian rhythm (60-day span)
2. Circadian mesor modulated by circatrigintan rhythm (60-day span)
3. Circadian mesor and amplitude modulated by circatrigintan rhythms (60-day span)
4. Circadian mesor and amplitude modulated by circatrigintan rhythms (365-day span)
5. Circadian mesor and amplitude modulated by circatrigintan rhythms - mesor also modulated by circannual rhythm (365-day span)
6. Circadian mesor and amplitude both modulated by circatrigintan rhythms and circannual rhythms (365-day span)

Figure 37.23. Stepwise theoretical reconstruction of partial spectral structure of human plasma prolactin for group of Japanese women (based on parameter estimates obtained from separate least-squares fittings of cosine curves with periods of 24 hours (circadian), 28 days (circatrigintan) and 365 days (circannual) to data on plasma prolactin obtained every 20 min for 24 hours, ~4 times a year on a few women). Didactic example modeling the interaction of human plasma prolactin rhythms with different frequencies, including modulation of circadian mesor and amplitude by circatrigintan and circannual rhythms.
Table 37.13  Parameter Values Used in Theoretical Reconstruction of Cortisol Variability Due to Circadian, Circatrigintan, and Circannual Rhythms

<table>
<thead>
<tr>
<th>M*</th>
<th>A</th>
<th>τ</th>
<th>φ</th>
<th></th>
<th>M*</th>
<th>A</th>
<th>τ</th>
<th>φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 8.37</td>
<td>5.03</td>
<td>1</td>
<td>-0.35 (-0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 0.5</td>
<td>0.17</td>
<td>28</td>
<td>-5.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.5</td>
<td>0.15</td>
<td>365</td>
<td>-223.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 0.0</td>
<td>0.54</td>
<td>28</td>
<td>-1.40</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5. 5.0</td>
<td>1.27</td>
<td>365</td>
<td>-172.36</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Minnesota low risk</td>
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<td></td>
</tr>
<tr>
<td>1. 0.44</td>
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<td>1</td>
<td>-0.39 (-0.5)</td>
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</tr>
<tr>
<td>2. 0.5</td>
<td>0.06</td>
<td>28</td>
<td>-18.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.5</td>
<td>0.15</td>
<td>365</td>
<td>-215.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4. 0.0</td>
<td>0.18</td>
<td>28</td>
<td>-23.96</td>
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<td></td>
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</tr>
<tr>
<td>5. 0.5</td>
<td>0.95</td>
<td>365</td>
<td>-208.86</td>
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</tr>
<tr>
<td>Minnesota high risk</td>
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<td></td>
</tr>
<tr>
<td>1. 0.91</td>
<td>5.20</td>
<td>1</td>
<td>-0.37 (-0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 0.5</td>
<td>0.04</td>
<td>28</td>
<td>-17.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.5</td>
<td>0.07</td>
<td>365</td>
<td>-256.51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 0.0</td>
<td>0.04</td>
<td>28</td>
<td>-15.63</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5. 0.0</td>
<td>0.62</td>
<td>365</td>
<td>-200.75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M* mesor; A amplitude; τ period; φ phase, related to acrophase by equation φ = (2π/τ)φ, expressed in radians. Circadian φ adjusted to -0.5 for comparison of groups.

Table 37.14  Parameter Values Used in Theoretical Reconstruction of Prolactin Variability Due to Circadian, Circatrigintan and Circannual Rhythms

<table>
<thead>
<tr>
<th>M*</th>
<th>A</th>
<th>τ</th>
<th>φ</th>
<th></th>
<th>M*</th>
<th>A</th>
<th>τ</th>
<th>φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 21.35</td>
<td>11.93</td>
<td>1</td>
<td>-0.12 (-0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 0.5</td>
<td>0.26</td>
<td>28</td>
<td>-5.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.5</td>
<td>0.41</td>
<td>365</td>
<td>-86.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 0.0</td>
<td>1.52</td>
<td>28</td>
<td>-15.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 0.0</td>
<td>5.8</td>
<td>365</td>
<td>-15.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minnesota Low Risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 15.47</td>
<td>6.92</td>
<td>1</td>
<td>-0.15 (-0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 0.5</td>
<td>0.27</td>
<td>28</td>
<td>-10.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.5</td>
<td>0.11</td>
<td>365</td>
<td>-131.81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 0.0</td>
<td>1.69</td>
<td>28</td>
<td>-7.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 0.0</td>
<td>2.59</td>
<td>365</td>
<td>-140.93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Minnesota High Risk</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1. 17.13</td>
<td>8.89</td>
<td>1</td>
<td>-0.13 (-0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 0.5</td>
<td>0.11</td>
<td>28</td>
<td>-13.38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.5</td>
<td>0.13</td>
<td>365</td>
<td>-29.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 0.0</td>
<td>1.57</td>
<td>28</td>
<td>-17.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 0.0</td>
<td>2.58</td>
<td>365</td>
<td>-44.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M* mesor; A amplitude; τ period; φ phase, related to acrophase by equation φ = (2π/τ)φ, expressed in radians. Circadian φ adjusted to -0.5 for comparison of groups.

might question the validity of these results, since they were based on "imputations" rather than on statistically validated rhythm characteristics. Nevertheless, the same finding is observed when the mean "best-fitting" circadian period is computed after (a) disregarding period imputations that yielded best-fitting periods at one of the limits of the chronobiologic window investigated (20 or 28 hr) and (b) considering only the best-fitting periods that showed a rhythm significant at the 5% level.

The circadian acrophases were found to be similar for groups H, B, and C when a 24-hr cosine was fitted to the data. In this connection, it is important to note that when the population-mean cosinor was first proposed (24), it was emphasized that a given precise period such as the 24-hr period was fitted with the expectation
Figures 37.25. (left) and 37.26. (right): Average period of circadian temperature rhythm on human breast surface in health, benign disease, and cancer. Shortening of circadian period length of breast surface temperature rhythm in women with carcinoma as compared to healthy controls, with intermediate result obtained from women with benign disease of the breast. Ordinates on Figure 37.26 were scaled in an admittedly arbitrary fashion by assigning one for the healthy controls, two to women with benign disease, and three to women with carcinoma. Thereby, it was assumed, for the purpose of this computation, that there was an even change from health to benign disease to cancer. All period values in a given ordinate category were equally weighted for the computation of a slope-test. The linear trend thus formed constitutes a rough approximation. It suggests a shortening of circadian period with increasing severity of the disease. Based on least squares fitting of cosine curves with periods ranging from 20-28 hr. Best circadian period: that of cosine with smallest residual error. Recordings at five sites (four quadrants and nipple) from each breast. Ten series/subject, each series covering 96 hr, at 2-hr intervals daily from 08:00 to 22:00 hr. F, F-test; p, P-value (associated to F-test), S.E., standard error; t, t-[t-test] and p, P-value (associated to t-test).

Sampling

In turning to the effect of blood sample size, several differences relative to the amount of blood withdrawn were observed. According to the original protocol, small amounts of blood (1 ml) were withdrawn every 20 min in order to evaluate prolactin and cortisol concentration, whereas every 100 min a larger amount of blood (13.5 ml) was withdrawn so that eleven additional hormones could also be evaluated. Thus it was possible to investigate the effect of sampling by comparing results obtained from the large and small blood samples for prolactin and cortisol.

Circadian rhythm characteristics were derived from all samples ($\Delta t = 20$ min) and from the 100-min samples only, in each of the four seasons. The differences between the rhythm characteristics from the two series ($\Delta t = 100$ min; $\Delta t = 20$ min) were computed for each season. A highly statistically significant linear correlation between the rhythm characteristics determined from the 20-min and 100-min samples was demonstrated. Figure 37.27 illustrates the goodness of the linear regression in the case of prolactin mesors, determined in the four seasons, in the classical regression problem, and when the straight line is constrained to pass through the origin. Although very good correla-
tion coefficients were obtained, a t-test for paired "observations" demonstrates a consistent, statistically significant difference in mesor for all seasons except for cortisol in summer, Tables 37.15A and 37.16A. This finding is corroborated by two nonparametric inferential statistical tests, i.e., the sign test (Tables 37.15B and 37.16B) and Wilcoxon's signed ranked test (Ta-
Table 37.15
Detection of Differences Between Circadian Parameter Estimates for Prolactin Determined with Large Blood Samples (every 100 min) and Small Blood Samples (every 20 min)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Test (t, P) on differences in rhythm characteristics obtained with large and small samples (paired &quot;observations&quot;)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>(3.66, &lt;0.05)</td>
<td>(2.66, &lt;0.05)</td>
<td>(2.76, &lt;0.05)</td>
<td>(5.93, &lt;0.05)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>(0.66, &gt;0.05)</td>
<td>(1.71, &gt;0.05)</td>
<td>(2.09, &gt;0.05)</td>
<td>(3.84, &lt;0.05)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>(0.67, &gt;0.05)</td>
<td>(1.69, &gt;0.05)</td>
<td>(1.11, &gt;0.05)</td>
<td>(0.26, &gt;0.05)</td>
</tr>
<tr>
<td>Percentage Rhythm</td>
<td>(3.27, &lt;0.05)</td>
<td>(2.23, &lt;0.05)</td>
<td>(1.07, &gt;0.05)</td>
<td>(2.02, &gt;0.05)</td>
</tr>
</tbody>
</table>

B. P-values resulting from sign test (nonparametric test)

<table>
<thead>
<tr>
<th></th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesor</td>
<td>&lt;=0.01</td>
<td>&lt;=0.01</td>
<td>0.01</td>
<td>&gt;0.00</td>
</tr>
<tr>
<td>Amplitude</td>
<td>&gt;0.05</td>
<td>0.10</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Acrophase</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Percentage Rhythm</td>
<td>0.10</td>
<td>0.10</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

C. Wilcoxon's signed ranked test (T, P)

<table>
<thead>
<tr>
<th></th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesor</td>
<td>(435.0, &lt;0.05)</td>
<td>(402.0, &lt;0.05)</td>
<td>(403.0, &lt;0.05)</td>
<td>(507.0, &lt;0.05)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>(302.5, &gt;0.05)</td>
<td>(346.5, &lt;0.05)</td>
<td>(354.5, &lt;0.05)</td>
<td>(453.5, &lt;0.05)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>(163.0, &gt;0.05)</td>
<td>(144.5, &lt;0.05)</td>
<td>(180.5, &gt;0.05)</td>
<td>(163.5, &gt;0.05)</td>
</tr>
<tr>
<td>Percentage Rhythm</td>
<td>(400.5, &lt;0.05)</td>
<td>(220.0, &gt;0.05)</td>
<td>(231.5, &gt;0.05)</td>
<td>(329.5, &gt;0.05)</td>
</tr>
</tbody>
</table>

\* t = \( \bar{D}/S_0 \) where \( \bar{D} \) is the mean difference observed between the two series of data and \( S_0 \) is the standard error of \( \bar{D}. P \) is the P-value associated with t.

\* T = value taken by the test statistic in the Wilcoxon's signed ranked test; P = associated P-value.

The chemical analyses of the 20-min and 100-min samples were done at the same time in the same series of samples and with equal sample size in each assay. A relatively larger degree of evaporation from the tubes containing the smaller (0.5 ml) amount of plasma as compared with the three 2-ml samples of the 100-min specimen cannot be responsible for the differences, for the values obtained in the 100-min samples are slightly larger than those in the 20-min samples. A similar finding was obtained for adenosine deaminase activity (ADA), an enzyme evaluated in samples of packed erythrocytes from some of the subjects. A t-test for comparison of effect due to the amount of blood withdrawn was carried out. In the series investigated, there was a highly statistically significant difference (P <

bles 37.15C and 37.16C). It can thus be concluded that a slightly and statistically significantly larger mesor estimate is obtained with prolactin and cortisol data from larger volumes of blood withdrawn every 100 min, as compared to smaller volumes taken every 20 min.2
0.001) between mean values determined on samples representing different amounts of withdrawn blood: when a larger amount of blood was withdrawn, the values tended to be higher. This effect was observed only for ADA expressed in terms of μmole of NH₃ produced per milliliter of packed red cells and not for ADA expressed in terms of μmole of NH₃ produced per hr per gram of hemoglobin. Differences in values observed when expressed as enzyme activity per milliliter of packed cells as compared to enzyme activity per gram of hemoglobin are likely to be due to differences in the packing of red cells during centrifugation.

In discussing the apparently inverse relationship between circannual prolactin amplitude and the total risk of developing breast cancer, the shortcomings of the risk scale must be emphasized. First, the value for risk used may be a poor measure of risk. It is an estimate of increased risk linearly combined from weighted dichotomization of the characteristics investigated in separate epidemiological studies. Information has been lost in the dichotomizations, and the total risk ignores any interactions among the various risk factors. Furthermore, no consideration is given to relationships between breast cancer factors on the one hand, and possibly competing and interacting risks of other diseases on the other hand. Since epidemiological data for a risk interaction equation are not known to us, no attempt was made to correlate the risk factors with each other.

The advantage of the risk scale is that it uses the available data on risk factors as completely as possible, not only by ascertaining the presence or absence of a given risk factor but also by scaling it according to the information on increased risk determined by prior epidemiological studies. The risk scale is amenable to adjustment as epidemiological data on new risk factors are ascertained and corroborated. This method of individualized risk assessment and correlation deserves further testing and development with special endeavors to account for interactions (50).

With regard to an individual assessment of risk, it is interesting to note recent results obtained by Toti et al. (58). In an analysis of a screening program for the detection of breast cancer in Italy, 15 biological variables extracted from the subjects' histories were used to construct discriminant functions between women with breast cancer and control women without breast cancer. Four variables (age at first examination, number of pregnancies, number of sucklings, symptoms in breast at any time) were found to provide adequate separation of the two groups of women. Although there were problems with false positives and false negatives, Toti et al. state that "...individual values of a discriminant function may be associated with the risk of developing breast cancer in normal women." They suggest that it may be possible in screening programs to consider those women having a discriminant value below a given threshold as being at a higher risk than those above the threshold and therefore requiring follow-up.

Methodologic Considerations

Rhythms with many frequencies in each of several body functions create a dynamic hormonal environment. The complexity of this situation determines from a methodologic viewpoint a given study's minimum duration and sampling frequency in certain cases, such as in work on prolactin. In order to shed light on reproductive and other hormonal processes, as well as on causes of breast cancer and other major diseases, research projects must eventually account for the spectrum of rhythms, from ultradian over circadian to circannual.

Important effects are likely to be missed if sampling is inappropriate. Differences between Japanese and Minnesotans in overall prolactin circadian mesor or amplitude would not have been detected if sampling had been restricted to the summer. Even on the scale of a single day, differences between Japanese and Minnesotans might not have been detected if sampling were limited to a single clock-hour chosen by convenience rather than pertinence.

When dealing with a spectrum of rhythms with different frequencies, several intricate methodologic problems arise, mainly related to sampling rate and size. Straightforward testing of the zero-amplitude assumption with the cosinor procedure on autoregressive data will employ an overestimated number of degrees of freedom and thus lead to a too-optimistic P-value. Methods for arriving at the correct number of degrees of freedom are therefore needed. Moreover, if the time series on hand is not sinusoidal and short, the estimation of a best-fitting period may be the more misleading, the smaller the number of cycles covered by the data. In order to obtain a reasonable estimation of the period length, four to five complete cycles of data are needed (Fig. 37.28).

Whenever the period length is known, methods other than the cosinor procedure are avail-
Figure 37.28. Effect of sampling span and waveform upon period of best fitting cosine in chronobiological window. Cosine curve fitted by least-squares: at least five consecutive cycles should be considered in order to obtain a reasonable evaluation (within ~5%) of the true period length.

In such a hybrid approach, several individuals are followed-up longitudinally while new groups of subjects are added, thus providing simultaneously for longitudinal and transverse samples. Sampling is further complicated if the experiments are carried out in several locations, leading to an additional source of variation. In some cases, for instance in animal experiments synchronized with several different lighting regimens, it is possible to achieve serially independent "around-the-clock" data by experiments done only during working hours; this is achieved by using different rooms, each being set to a different lighting regimen, in which the animals have been stabilized with validated different timing.

Since specifications concerning the experimental set-up are usually lengthy, some terms

able to account for any asymmetry in the waveform. Such methods include harmonic interpolation (6), where the data on hand are used to reconstruct a continuous time function, thus allowing visualization of the shape of the bioperiodicity. Other methods are also available, where, for instance, more complex models (e.g. models including higher harmonics) are fitted by least squares to the data on hand (57).

When designing demanding and expensive experiments in biology or medicine, it is important to examine all possible benefits from different sampling schemes, whether from repeated measurements on the same individual in a longitudinal survey or from sampling transversely in groups of similar individuals. A practical solution may be achieved when a compromise between the two sampling schedules is followed:
are here proposed to facilitate precise and concise experimental descriptions. Clear, succinct composite terms seem efficient for this purpose. They can be built according to part or all of the following pattern. All the proposed new compounds would end with "-cyclic" to denote repetitive sampling:

<table>
<thead>
<tr>
<th>Prefixes</th>
<th>Required Stems</th>
<th>Uniform Suffix</th>
</tr>
</thead>
<tbody>
<tr>
<td>met-</td>
<td>-idio-</td>
<td>-cyclic</td>
</tr>
<tr>
<td>(if staggered)</td>
<td>(individual)</td>
<td></td>
</tr>
<tr>
<td>-an-</td>
<td>-eco-</td>
<td></td>
</tr>
<tr>
<td>(negation)</td>
<td>(related to location)</td>
<td></td>
</tr>
</tbody>
</table>

(1) The prefix "met-" allows the choice among several staggered schedules instead of a regular (not staggered) regimen.
(2) The prefix "an-" indicates the absence of the next part of the word.
(3) The main specification in relation to an individual is the "-idio-" and in relation to a location the "-eco-".
(4) The final "-cyclic" implies specified equidistant or unequidistant placement of repeated observations.

One could consider for future work subjects (e.g., permanent night workers) with a consistently changed temporal placement along the 24-hr scale of certain of their circadian rhythms. Thus, one could institute certain "metanidioanecycocyclic" schemes to compare different circadian stages of a given rhythm, measured simultaneously in different individuals in different locations—in an approach to an idealized circadian cycle. The "met-" in this case, would indicate the use of staggered work environments.

Correlation matrices were computed first without eliminating outliers because of the small total numbers of subjects in the groups being compared. Further investigations will have to clarify whether and if so to what extent outliers bias the results obtained thus far.

For some analyses beyond our scope, tests for outliers were performed. Thus, in a summary of results obtained by applying the population-mean cosinor to the risk or age categories, the Hotelling's $T^2$ statistic was utilized to test for any statistically significant differences between two populations with respect to parameters of circadian rhythms (mesors, amplitudes, and acrophases), jointly considered. A preliminary examination of circadian parameters, however, revealed that some subjects had an unusually high mesor compared to others in their respective categories. Assuming multivariate normality of parameters, a simple test for detecting outliers (7) was applied to the mesors in each category compared. To apply the test, the mesors in a given category were ordered from the smallest to the largest and denoted as $M_{(1)}, M_{(2)},$ and $M_{(n)}$, where $n$ was the total number of mesors. The test statistic, $r$, was then computed for the largest or smallest value, as follows:

$$r(n) = \frac{M_{(n)} - M_{(n-1)}}{M_{(n)} - M_{(1)}}$$

$$r(1) = \frac{M_{(1)} - M_{(n)}}{M_{(1)} - M_{(1)}}$$

The calculated $r(n)$ or $r(1)$ was then compared with tabulated critical values of $r$ (9). If an outlier was detected it was deleted and the procedure was repeated on remaining values.

Indeed, the inclusion of outliers in a first analysis does not lessen the need to eliminate those outliers which, in retrospect, indicate that some

---

**Table 37.17**

Schedule for Stepwise Seasonal Sampling for Estimation of Circannual and Circadian Parameters; Endocrine Rhythm Parameters Correlated with the Risks of Developing Breast Cancer (BC) and/or Diseases Associated with an Elevated Blood Pressure (BP)

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Fall</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>&lt;---</td>
<td>Circannual Amplitude</td>
<td>----&gt;</td>
<td></td>
</tr>
<tr>
<td>DHEA-S*</td>
<td>M*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>A*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td></td>
<td>M, A**</td>
<td>M, A*</td>
<td></td>
</tr>
<tr>
<td>Prolactin</td>
<td>M**</td>
<td>M, A**</td>
<td>M, A*</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td></td>
<td>M**</td>
<td>M*</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td></td>
<td>&lt;---</td>
<td>Circannual Amplitude</td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td></td>
<td>M**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td>M**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td></td>
<td>M**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td></td>
<td>&lt;---</td>
<td>Circannual Amplitude</td>
<td></td>
</tr>
</tbody>
</table>

* If not specified, frequency is circadian.

* DHEA-S, dehydroepiandrosterone sulfate; M, mesor; E2, estradiol; A, amplitude; E3, estranol; LH, luteinizing hormone; E1, estrone; T4, thyroxine; TSH, thyroid-stimulating hormone; *, correlation with blood-pressure-associated disease risk; **, correlation with breast cancer risk; =, correlation with both risks; ---, circannual urinary sampling; ----, circannual blood sampling.
subjects may lie remote from the mean in one or several respects.

Much has been written about biochemical individuality without taking rhythms into account. These authors do not wish to commit the reverse mistake in not considering biochemical individuality while considering rhythms. Rhythms have long been recognized as a feature of biochemical and other individuality. Individuality, as it may appear from consecutive comparisons of subjects, is more readily pursued when, for example, a circadian rhythm in cortisol can be documented by sampling on a single day for 132 out of 133 series. Many more individuals on many more days will have to be assessed to segregate at each pertinent frequency the biochemical individuality of a single subject, qualified as to the rhythms’ mesor, acrophase, amplitude, and wave-form. Finally, it may be noted that the comparison of healthy populations with no known disease also constitutes an attempt to define health positively and in an individualized fashion. Thus, this approach complements the continued definition of an index of health tied to causes of death (sic), an approach that continues to be used at this time.

This study was based on relatively few individuals. It had to be carried out (for economic reasons) without the originally planned inclusion of a high-risk group in Japan. Nonetheless, it has sufficed to yield suggestions for further work that can be supported by a set of statistically significant results (Table 37.17). The many samples from each of a very few individuals lead to conclusions that can now be properly tested on many more individuals with relatively few samples and even with single samples for one or the other variable, as the case may be. Various breast cancer risk factors that could not be separated because of the lack of a Japanese high-risk group and because dietary and other conditions, including climate, were not controlled, confound the results. For this reason the circumstance is noteworthy that all of these factors are controlled to some extent in the Italian study involving groups of subjects exposed to the same climate of Tuscany, presumably a comparable diet, and but a single risk factor. The agreement of certain results that are comparable in the Italian and Kyushuan-Minnesotan studies is the more noteworthy.

CONCLUSION

Apart from the slight differences observed in the amounts of blood withdrawn that still need further investigation, reliable determination of a spectrum of rhythms could be made for most of the hormones evaluated in these international studies. It can thus be concluded that rhythms with many frequencies in several body functions together create a dynamic hormonal environment. The complexity of this environment determines from a methodologic viewpoint the desirable minimum duration and sampling frequency of a certain study in certain circumstances, such as in work on cortisol and prolactin, in order to shed light on reproductive and other hormonal processes, as well as on causes of breast cancer and other major diseases.

An increasingly rational individualized prevention and/or treatment of disease requires an individualized and time-qualified definition of health by the establishment of reference intervals and rhythm characteristics of hormonal and other physiologic variability. With these reference intervals and rhythm characteristics available, one can better interpret with single samples or time series an increased risk of a certain disease or the inception of the disease. The practitioner needs rhythm-qualified peer-group and individualized reference intervals for the study of single samples, and estimates of rhythm characteristics for reference in the study of time series. Diagnostic nihilism and over-prescription of tests are equally probable consequences of sampling without proper qualifications as to individualized timing—whenever it is unambiguously demonstrated that without a rhythm-qualified interpretation, a single sample may be interpreted as too high at one time, too low at another, and “normal” at a third time.

From the viewpoint of benefit versus cost, time-specified single samples and time series interpreted against appropriately time-qualified tolerance intervals constitute for several variables a highly rational approach in medical research and practice (Table 37.18). This approach is desirable, in any event, in order to avoid misinterpretation. By contrast, without (individualized or at least peer-group) reference intervals that are qualified by a spectrum of rhythms, the interpretation of single samples is likely to be an undiscriminating endeavor, often wasteful because useless, and occasionally harmful. Figures 37.29 and 37.30 describe tolerance intervals along the scales of a day and Figures 37.30 and 37.31 with seasons. While circadian mesors of aldosterone estimated for the Minnesotan subjects should not get higher than ~12.5 ng/dl, in spring, values as high as ~22 ng/dl are still included in the (0.90, 0.90) winter tolerance interval. With rhythmometric precautions “sins of
omission" such as relying on time-unspecified single samples can be avoided and, as this chronoepidemiologic study has revealed, large differences may be found in rhythms among certain populations, also characterized by large differences in morbidity and mortality from breast cancer among other conditions.

SUMMARY

Several hormones as well as other physiologic variables of potential interest for breast cancer research have been shown to undergo rhythmic variation. The bioperiodicities involved concern

Table 37.18
Circadian Chronodesm for Plasma Prolactin Concentration Established on the Basis of Data from Adolescent Japanese Women in Winter (Group I) and Validated 1 Year Later on a Larger Group of 20 Women of Same Age and Ethnicity (Group II)

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Circadian Prolactin Concentration (Group I) (ng/ml)</th>
<th>Percent of Data from Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower limit</td>
<td>Higher limit</td>
<td>Chronicosis</td>
</tr>
<tr>
<td>00°00-02°00</td>
<td>10.7 158.5</td>
<td>15%</td>
</tr>
<tr>
<td>02°00-04°00</td>
<td>15.8 112.2</td>
<td>0%</td>
</tr>
<tr>
<td>04°00-06°00</td>
<td>10.7 134.9</td>
<td>17.5%</td>
</tr>
<tr>
<td>06°00-08°00</td>
<td>10.7 102.3</td>
<td>10%</td>
</tr>
<tr>
<td>08°00-10°00</td>
<td>2.6 65.1</td>
<td>10%</td>
</tr>
<tr>
<td>10°00-12°00</td>
<td>2.6 40.7</td>
<td>10%</td>
</tr>
<tr>
<td>12°00-14°00</td>
<td>5.6 28.8</td>
<td>10%</td>
</tr>
<tr>
<td>14°00-16°00</td>
<td>5.3 31.4</td>
<td>10%</td>
</tr>
<tr>
<td>16°00-18°00</td>
<td>5.0 33.9</td>
<td>5%</td>
</tr>
<tr>
<td>18°00-20°00</td>
<td>4.3 89.1</td>
<td>5%</td>
</tr>
<tr>
<td>20°00-22°00</td>
<td>5.5 50.1</td>
<td>10.7%</td>
</tr>
<tr>
<td>22°00-24°00</td>
<td>3.4 50.1</td>
<td>10.7%</td>
</tr>
</tbody>
</table>

* It seems pertinent that in a recent issue of the New England Journal of Medicine, Zervas and Martin (64) state that excessive prolactin secretion (by pituitary adenomas) can be readily detected by measuring basal (morning) plasma concentration. For these authors prolactin concentrations larger than 100 ng/ml are considered abnormal and indicative of a tumor, whereas normal values are considered to be less than 15 ng/ml. Time-specific tolerance intervals such as those reported in this table indicated that prolactin concentration in the morning is still decreasing from overnight high values. Prolactin assessment will be more discriminating, in the diagnosis of tumors and other disorders, if careful consideration is given to rhythms, i.e., if samples are obtained at appropriate times. The problem on hand should also be taken into account. One must consider with the expected change (e.g., elevation of prolactin in patients with certain pituitary tumors) also the physiologic timing of the sleep-wakefulness or activity-rest pattern. By taking rhythmic variations into account, chronodesms will usually yield narrower intervals than conventional "normal ranges". The set of time-specific tolerance intervals shown in this table is expected to contain at least about 90% of the distribution of data with 90% confidence. As expected, on the average, in a replication about 90% of the data fall within the chronodesm.

Figure 37.29. Circadian cortisol merodesm (bars) established from data (•) on young women (15-21 yr) in winter. As for an individual (Fig. 37.14), a value may be found for a pair of young women that is well within the reference interval at several times, yet below or above the tolerance interval at other times.

Figure 37.30. Extent of agreement of chronodesms constructed for two seasons.*

*When applied directly to the data collected, chronodesms may sometimes yield a zero lower limit when no zeros recur in the original data and may yield even negative lower limits. In these cases, it is recommended to compute the chronodesm after log-transformation of the data. Limits thus obtained are then retransformed into original units. These limits usually are different from those obtained without log-transformation.
Figure 37.31. Circannual merodesm for aldosterone in blood plasma. Circannual and circadian chronomesms may have to be derived, as suggested by large differences in upper tolerance limits established for plasma aldosterone of healthy Minnesotan women. (In each season circadian mesor of each subject estimated by fitting 24-hr cosine curve to data obtained throughout 24 hr. Tolerance interval (with 90% chance of including 90% of distribution) for circadian mesors was then computed. Only upper tolerance limits are pertinent (lower limits are computational artifacts: zero.) Statistically-significant effect of season on circadian mesor indicated by ANOVA (analysis of variance) (with repeated measures). F, 4.16; P, 0.02.  

several frequencies, indicating the need to investigate, when pertinent, not only single samples and not even solely a circadian rhythm, but multiple frequencies. Exploration of a spectrum of rhythms with several frequencies suggested differences between Japanese (mostly Kyushuans) and North American (mostly Minnesotans) women in plasma prolactin, aldosterone, insulin, estrone and estriol, and urinary melatonin. Rhythmometry on strategically placed and sequenced time series allowed the individualized quantification of circadian rhythms in plasma prolactin and cortisol for most (>90%) Japanese and Minnesotan subjects. Statistically significant differences in circadian parameters between the two populations were thus demonstrated. In addition, rhythm-qualified tolerance intervals could be derived for a cost-effective interpretation of subsequent time-qualified single samples. Circannual rhythms had to be taken into consideration as well, since circannual (about-yearly) variation in several plasma hormones could be described in one geographic location but not in another—insulin, estrone, aldosterone, and DHEA-S in Minnesota, prolactin and estriol in Japan. A reduction in extent of predictable circannual variation in plasma prolactin of Minnesotans, as compared to Kyushuans, women—differing in breast cancer risk—was paralleled by lesser circannual variation in patients with fibrocystic mastopathy, as compared to clinically healthy women, found in a separate study of serum prolactin in Italy. The geographic-ethnic and/or otherwise determined differences between Kyushuans and Minnesotans were shown to be statistically significant and were partly replicated, yet they would not have been detectable by sampling at certain hours or seasons fixed only by convenience rather than pertinence.

The paper emphasizes, in fact, the importance of sampling at a time chosen according to clinical pertinence rather than to convenience. Important hormonal differences between populations differing in risk of breast cancer are detectable only if bioperiodicities are properly assessed. Sampling requirements concerning density, duration of observation span, cost-effectiveness of spot-checks or adequate time-qualified single samples and other sampling problems related to clinical protocols are thoroughly examined in a few cases. In so doing, a feasibility check of the chronobiologic approach is provided. Added service by chronobiologists to oncologists must be extended 1) in depth and scope, 2) to additional frequencies, 3) to more hormonal and other variables, and 4) with (a) strategically placed but fewer samples and (b) for more individuals. The rhythmometric highways yet to be laid are ambitious and demanding endeavors. Once completed, they promise to facilitate rapid traffic to the goals, among others, of cancer prevention and treatment.

Addendum. Further work on the samples from the Japanese-American chronoepidemiologic study has revealed differences in plasma renin activity associated in part with differences in diet. Further evidence from the same samples also was obtained on geographic differences in plasma aldosterone and was complemented by findings of differences in urinary aldosterone and electrolytes (31, 32). These findings are of interest for students of diseases associated with high blood pressure as well as of breast cancer and allow considerations of competing risks—to be considered in any chronoepidemiologic endeavor. Such risks come to the fore in circannual rhythm parameters at a time when evidence from casual single samples or even from circadian rhythm parameters is noncontributory.

A recent clinical symposia issue published by CIBA (Volume 32, 2, 1980, 32 pp.) in discussing “breast lumps” reflects caution toward thermography. The latter procedure is not even mentioned—a decision apparently justified in view of the experience thus far with diagnostic uses of
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thermography for dealing with breast lumps. As yet that
publication does not refer to thermorhythmometry, a de¬
cision again justified since the promise of this procedure
discussed in the foregoing chapter and elsewhere notwith¬
standing, large-scale tests are essential before it may be
advocated for practical application.

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21.

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25.


carcinogenesis—prob¬

2.

Cappelaere, P. (1975): Prolactine et

3.

Choi, N. W., Howe, E. R„ Miller, A. B., Matthews, V., Morgan, R. W„
and analysis, in Biological Rhythms, Documenta Ceigy. pp. 6-8. Ciba-

cancers

28.

mammaires. Pathol. Biol., 23:

161.

4.

5.

Geigy Ltd., Basel, Switzerland.
Prins, J., Cornelissen, G„ Hillman, D., Halberg, F„ and Van Dyck, C.
(in press): Harmonic interpolation yields paraphases and orthophases
for biologic rhythms, in Proc. XIII Int. Con/. Int. Soc. Chronobiol., D. K.
Hayes, F. Halberg, L. E. Scheving (eds) II Ponte, Milan, Italy.
Donati, L., Lauro, R., Vasta, M., Locatelli, I., Camagna, A., Tatti, P., and
Halberg, F. (1979): Orcadian plasma prolactin hemopsy—variation as¬
sessed individually in a menstrually-cycling and a post-menopausal
Italian woman. Chronobiologia, 6.-93.
Egan, R. L. (1979): Estimated risk and occurrence of breast cancer in
assymptomatic and minimally symptomatic patients. Cancer, 43:871-

6. De

7.
8.

9.
10.

29.
30.

31.

32.

33.

34.

37. Malhotra, S. L (1977): A study of cancer of the breast with special
38.

Sainz, M., Tarquini, B., Cagnoni, M., Castellanos, J. M., Garcia
Pcna, J., and Halberg, F. (1979): Thermorrhythmometry in health (H),

benign breast disease (B) and breast cancer (C) and obliteration of
rhythm in serum prolactin infibrocystic
Halberg, F. (1965): Some aspects of biologic data analysis: longitudinal
and transverse profiles of rhythms, in Circadian Clocks, pp. 13-221
Halberg. F. (1977): Biological as well as physical parameters relate to

14.

15.
16.

Post Graduate Institute of Medical Education and Research. Chandi-

17.

garth, -India.
Glossary of chronobiology Chronobiologio. 4 Suppl. 1.

Different timing of circannual rhythm in mortality of women with
breast cancer diagnosed before and after menopause, in Proc. 8th Int.
Scientific Mtg. Int. Epidemiological Assoc., San Juan. Puerto Rico, Sep¬

36.

13. Garcia

circannual, but not of circadian

Kawasaki, T., Ueno, M„ Uezono, K., Matsuoka, M., Omae, T., Halberg,
circadian mesors for plasma renin activity in healthy young women in
Kawasaki, T., Ueno, M., Uezono, K., Omae, T., Haus, E., and Halberg, F.
(1979): Plasma and urinary aldosterone and urinary electrolytes in
healthy young women in Japan and U.S.A. Chronobiologia, 6:116.
Wrist actigraphic measures of sleep and rhythms. EJectroencephalogr.
Clin. Neurophysiol., 44.674-676.
Kwa, H. G., Bulbrook, R. D„ Cleton, F„ Verstraeten, A. A., Hayward, J.
prolactin in nulliparous and obese post-menopausal women. Int. J.

tember 17-23.

"11.

86:257-262.

Hirayama, T. (ed.) (1977): Comparative Epidemiology of Cancer in the
U.S. and Japan—Mortality. The U.S.-Japan Cooperative Cancer Re¬
search Program, Japan Society for the Promotion of Science.
Hirayama, T. (ed) (1978): Comparative Epidemiology of Cancer in the
U.S. and Japan—Morbidity. The U.S.-Japan Cooperative Cancer Re¬
search Program, Japan Society for the Promotion of Science.

Cancer, 22691-693.
351

877.

Farewell, V. T., Math, B., and Math, M. (1977): The combined effect of
cycle of daytime serum prolactin in man and monkey. Acta Endocrinol.,

Halberg, F., Johnson, E. A., Nelson, W„ Runge, W„ and Sothern, R.
(1972): Autorhythmometry—procedures for physiologic self-measure¬
Halberg, F., Katinas, G. S., Chiba, Y„ Garcia Sainz, M., Kovats, T. G.,
(1973): Chronobiologic glossary of the International Society for the
phase—an aspect of temporal morphology; procedures and illustrative
examples, in The Cellular Aspects of Biorhythms, pp. 20-48, edited by
Haus, E., Lakatua, D. J., Halberg, F., Halberg, E., Cornelissen, G., Sackett,
L. L., Berg, H. G., Kawasaki, T., Ueno, M., Uezono, K„ Matsuoka, M„
and Omae, T. (1980): Chronobiologic studies of plasma prolactin in
women in Kyushu, Japan and Minnesota, USA J. Clin. Endocrinol
Metab. 51:632-640, 1980.

26.
27.

References

resolution of low-frequency, small-amplitude rhythms in excreted 17ketosteroid; probable androgen-induced circaseptan desynchronization.
Acta Endocrinol., 50: Suppl. 103, 5-54.
Halberg. F., Gupta, B. D., Haus, E., Halberg. E., Deka, A. C., Nelson, W.,
Sothern, R. B„ Cornelissen, G., Lee, J. K., Lakatua, D. J„ Scheving, L E.,
and Burns, E. R. (1977): Steps toward a cancer chronopolytherapy. in
Proc. XlVth Int. Cong. Therapeutics, pp. 151-196, Montpellier, France,
Halberg, P., Haus, E., Tarquini, B„ Cagnoni, M., Cornelissen, C., Lakatua,
D„ Kawasaki, T., Wallach, L. A:, Halberg,- E:, and Omae,~T.f1979):
Circannual and circadian variations in some blood hormones, notably
prolactin. In: Internal Medicine, Proc. XlVth Int. Cong. Internal Medi¬
cine, ISM Rome, Italy, held in October, 1978, L Condorelli, U. Teodori,
M. Sangiorgie and R. Neri Semeri, Eds., Excerpta Medica, Amsterdam/

Circannual variation in serum TSH and prolactin of prostatic cancer
patients. Proc. 3rd Conf. Indian Soc. Chronobiolo., Varanasi, India,

39.

40.

Marquardt, D. W. (1963): An algorithm for least squares estimation of
Meites, J. (1972): Relation of prolactin to mammary tumorigcnesis and
growth in rats, in Prolactin and Carcinogenesis, 4th Tenovus Workshup.
Cardiff. Wales

41.

gonadotropin and human luteinizing hormone. Endocrinology. 79 19-18

42.


cancers.

Cancer

Res.. 38 3985-3990
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11974).

Manimotropic effect of prolactin enhanced by thyroid¬



STEROIDS IN SALIVA
FOR ASSESSING ENDOCRINE FUNCTION

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Assay of steroids in saliva rather than plasma is attractive because samples can be easily collected at frequent intervals by stress free non-invasive techniques. Salivary sampling regimens would also appear to have many of the advantages associated with the collection of 24 h urine samples and these samples unlike those of urine are not associated with problems of 'completeness of collection'. Data derived on assay of multiple saliva samples collected at 30 or 60 min intervals provides an integrated assessment of basal endocrine activity over prolonged time periods. Since 500 μl aliquots of saliva can be collected at 10 min intervals assays for salivary steroids may be more useful than those in plasma or urine in short term stimulation and suppression tests.

Plasma 'free' steroid concentrations are thought to provide more clinically useful information than that derived by determining 'total' steroid concentrations. Techniques for determining 'free' steroids in plasma are difficult and are unsuitable for routine use. In marked contrast salivary steroid assays are simple, high-throughput procedures which may well provide equally useful clinical data since steroid concentrations in saliva reflect the 'free' fraction in plasma (Smith et al., 1979; Baxendale et al., 1980).

Clinical studies featuring assays for salivary cortisol and progesterone (Walker et al., 1978; 1979) indicate that these procedures may well play an important role in assessing adrenal and ovarian activity. Concentrations of these steroids in saliva rarely exceed 5% of those in plasma; sensitive in-house immunoassays were therefore developed (Riad-Fahmy et al., 1981).

![Graph](image-url)

Fig. 1. (a) Cortisol levels in a volunteer and (b) two Cushingoid patients.
Cortisol concentrations in matched samples of plasma and saliva provided by a healthy volunteer at 15 min intervals throughout the day showed that the circadian rhythm in adrenal secretory activity was reflected equally well in plasma and saliva (Fig. 1a). In two Cushingoid patients, both having secondary adrenal hyperplasia this circadian rhythm was absent (Fig. 1b).

In two Cushingoid patients, both having secondary adrenal hyperplasia, this circadian rhythm was absent (Fig. 1b).

Subject with Adrenal Atrophy

Oestrogens are known to increase circulating transcortin concentrations so in a group of women taking oestrogen-containing contraceptive preparations plasma cortisol concentrations are slightly elevated and a greater than normal response to Synacthen occurs. In contrast salivary cortisol concentrations are within the normal range and a normal but slightly delayed response to Synacthen is observed. A patient having secondary adrenal atrophy failed to respond to Synacthen since plasma and salivary cortisol concentrations showed no change.

A group of healthy volunteers undergoing a typical short term Synacthen stimulation test had mean baseline plasma cortisol concentrations (450 nmol/L) (Fig. 2a) which were higher than those in saliva (Fig. 2b). Following stimulation cortisol concentrations in plasma showed a 3-fold increase whereas those in saliva rose 8-fold. This disproportionate increase in saliva probably arises because plasma cortisol concentrations of 500 nmol/L saturate all available binding sites on transcortin (Fig. 2c). Following Synacthen stimulation concentrations rise and the increased cortisol is redistributed in the albumin-bound and free fractions in plasma; the disproportionate increase in plasma free cortisol being reflected in saliva.

Oestrogens are known to increase circulating transcortin concentrations so in a group of women taking oestrogen-containing contraceptive preparations plasma cortisol concentrations are slightly elevated and a greater than normal response to Synacthen occurs. In contrast salivary cortisol concentrations are within the normal range and a normal but slightly delayed response to Synacthen is observed. A patient having secondary adrenal atrophy failed to respond to Synacthen since plasma and salivary cortisol concentrations showed no change.

![Fig. 2. (a & b) Cortisol levels following Synacthen stimulation, (c) distribution of cortisol in plasma.](image)

![Fig. 3. Cortisol levels following dexamethasone in Cushingoid patients.](image)
Dexamethasone suppression tests are widely used in investigations of Cushingoid patients. It was therefore interesting to find that following administration of dexamethasone the high cortisol concentrations in matched samples of plasma and saliva provided by a patient having a pituitary tumour were reduced (Fig. 3a) whereas those in a patient having an ectopic source of ACTH remained virtually unchanged (Fig. 3b).

Fig. 4. Cortisol levels in volunteers and patients during insulin stress tests.

Insulin tolerance tests are widely used for assessing patency of the hypothalano-pituitary-adrenal axis as a whole. In healthy volunteers, who all achieved adequate hypoglycaemia during the course of this test the mean percentage increase above base line levels in cortisol concentrations in plasma and saliva were in good agreement (Fig. 4a). The lack of response to this test in two patients is indicated by plasma and salivary concentrations which show no significant increase above basal values (Fig. 4b). These results taken as a whole indicate that cortisol concentrations in saliva are as useful as those in plasma for assessing basal adrenal function and the changes in activity throughout dynamic test procedures. Further studies have revealed that in normal women progesterone concentrations in saliva reflect those in plasma, suggesting that salivary levels of this steroid provide a reliable indication of ovulation and the functional capacity of the corpus luteum (Fig. 5a). The normal range for salivary progesterone (Fig. 5b) was determined by assaying the daily samples provided by a group of normal women (n=15); since these women also provided matched plasma samples dating of the cycle by conventional procedure was possible. Studies designed to assess the usefulness of salivary progesterone assays in infertile women featured a group of 20 patients having the defects listed in Table 1.

Table 1.

<table>
<thead>
<tr>
<th>Diagnosis (No. Patients)</th>
<th>Diagnostic criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteal phase abnormality (10)</td>
<td>Short luteal phase; Poor luteal phase rise in BBT; Plasma progesterone &lt;35 nmol/L (day 24)</td>
</tr>
<tr>
<td>Anovulatory cycles (8)</td>
<td>Monophasic BBT; Oligomenorrhea &gt;6 weeks duration</td>
</tr>
<tr>
<td>Hyperprolactinaemia (2)</td>
<td>Prolactin &gt;500 IU/L on at least two occasions</td>
</tr>
</tbody>
</table>
Fig. 5. Salivary progesterone levels: (a) correlation with plasma levels (b) normal range.

Some patients (6/10) having luteal phase abnormalities had salivary progesterone concentrations in the luteal phase like those in patient J.C., values being only marginally higher than those in the follicular phase of the cycle (Fig. 6a). Others (4/10) were like patient L.D., who had levels within the normal range but the luteal phase was of short duration (Fig. 6b).

Fig. 6. Salivary progesterone levels in patients with luteal phase defects.

Judged by salivary progesterone concentrations some of these patients responded to a 50 mg dose of clomiphene (Fig. 7a) whereas others failed to respond to doses of 150 mg (Fig. 7b).

Fig. 7. Salivary progesterone levels following clomiphene treatment.
Most patients (7/8) having anovulatory cycles had consistently low pretreatment salivary progesterone concentrations but one patient had a bizarre pattern, levels fluctuated widely achieving values exceeding 250 pmol/L on five occasions during the 28 days for which she was investigated. One of the hyperprolactinaemic patients (Fig. 8) had circulating prolactin concentrations 4 times greater than normal. This patient was amenorrhoeic and, as might be expected had low pretreatment salivary progesterone concentrations. Following treatment the improved ovarian function was indicated by rising progesterone values. In the luteal phase of the cycle which followed a short interval of bleeding salivary progesterone concentrations were within the normal range. Subsequent investigations showed that conception occurred in this cycle.

Salivary progesterone concentrations in the daily samples of saliva provided by infertile women would therefore appear to be of value in the initial assessment of ovarian activity and for monitoring the response to ovulation induction therapy. The possibility of long term control and manipulation of endocrine systems is well illustrated by the use of synthetic steroids in fertility control programmes. Assays for synthetic steroids may therefore have an important role to play in the design of new contraceptive formulations tailored to the needs of different ethnic groups. Such assays would be useful in initial pharmacokinetic studies and could well prove vital in large scale field investigations since in many developing countries various cultural and religious taboos make plasma sampling regimes almost impossible. Recent studies indicate that salivary sampling regimens may be used to monitor not only the bioavailability of norethisterone (Turkes et al., 1980) but also to assess the effect of a given norethisterone containing preparation on ovarian function. A group of healthy women who were known to be menstruating normally provided matched samples of plasma and saliva both before and after taking a norethisterone only formulation (norethisterone 500 μg). Samples collected throughout the pretreatment cycles were assayed for progesterone only but both norethisterone and progesterone were determined in the 'treatment' cycle. Pretreatment plasma and salivary progesterone concentrations in subjects C.R. and C.B. were consistent with ovulation and formation of a fully functional corpus luteum. In the 'pill taking' cycles however norethisterone markedly reduced ovarian activity in subject C.R. since plasma and salivary progesterone concentrations were consistently low. In the other subject (C.B.) norethisterone appeared to have little effect since plasma and salivary progesterone concentrations indicated normal luteal function (Figs. 9a & b).
Fig. 9. Salivary steroids in studies of fertility control.

These studies suggest that steroid concentrations in saliva may well have an important role to play in future investigations of endocrine function.

REFERENCES


Steroids in Saliva for Assessing Endocrine Function

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SOME difficulties in clinical studies of endocrine function based on plasma sampling regimens include time-consuming venipuncture and measurement of the "total" rather than the "free" biologically active fraction in plasma. Simple methods for determining plasma free steroids have not yet been developed, and most current procedures involve technically demanding ultrafiltration or equilibrium dialysis. In this context measurement of steroids in saliva is attractive.

Steroid concentrations in saliva are independent of flow rate and reflect those in the free fraction in plasma. Recent improvements in immunoassay techniques have allowed development of simple, high output assays for salivary steroids which are well suited for routine use. Since saliva samples can be collected at frequent intervals by both adults and children, they facilitate short term dynamic tests, pharmacokinetic analyses, and studies of chronobiological changes. Problems of viscosity, which restrict processing of freshly collected saliva, may be resolved by deep-freezing, and storage of samples at −20°C for prolonged periods is acceptable.

All steroids of diagnostic significance in the routine assessment of endocrine activity can now be measured in saliva. Established procedures include well validated, "in house" immunoassays and the use of suitably modified commercial kits. Data derived from measurement of steroids that provide an index of adrenal activity [cortisol, dehydroepiandrosterone-sulfate (DHA-SO₄), 17α-hydroxyprogesterone, and aldosterone] and those reflecting gonadal function (progesterone, estradiol, and testosterone) provide clinically useful information. Assays for salivary steroids may therefore have an important role to play in future investigations of endocrine function.

Introduction

The routine biochemical assessment of endocrine function is currently based on the determination of either urinary steroid levels or total circulating plasma hormone concentrations. Steroid concentrations in urine, collected over a 24-h period, which provide an integrated assessment of endocrine function, are complicated by difficulties of ensuring completeness of sample collection. Use of a factor, based on the relative constancy of urinary creatinine excretion, was thought to correct for "lost" samples, but more recent reports highlight the fallacy of this procedure (1, 2) and cast doubt on the effectiveness of collecting only early morning urine samples. Investigations featuring the determination of steroid concentrations in plasma have the disadvantage of requiring multiple-sampling regimens. The wide episodic fluctuations in circulating steroid levels make analysis of single samples useful only in screening procedures. Multiple-sampling regimens (3, 4) are required for accurate assessment of basal endocrine activity. Dynamic tests, designed to investigate patency of the hypothalamo-pituitary-adrenal/gonadal axes also rely on multiple plasma-sampling regimens. Collection of these samples is time consuming for clinicians and may be distressing for some patients. When the limitations associated with both plasma and urinary sampling are considered, it is not surprising to find that attention is now being directed towards the part that saliva may have to play in studies of endocrine activity.

Salivary sampling regimens have the advantage of frequent, easy collection by noninvasive, stress-free techniques. Patients find little difficulty in salivating directly into disposable tubes, providing adequate volumes (3 ml) for determining a steroid hormone profile in approximately 10 min. Assay of samples collected at 1- to 2-h intervals during waking hours provides an accurate assessment of baseline endocrine activity. Since smaller aliquots (500 µl) can be collected at 15- or even 10-min intervals, salivary sampling could well be more useful than that of either plasma or urine in short term dynamic tests.

Storage of saliva samples before assay poses no problems since steroid concentrations in saliva show no significant differences on storage at −20°C for periods ranging from 6-9 months or at 4°C for about 7 days. A limited study (Walker, R. F., unpublished observation) suggests that salivary testosterone and progesterone values show little if any change on standing at room temperature for
48 h. Further data indicate that this also holds true for salivary estriol concentrations (5). It may therefore be possible to mail samples to central laboratories for assay. Processing freshly collected saliva is frequently complicated by problems of viscosity. This difficulty can however be easily resolved by deep-freezing the samples either in a freezing mixture (acetone-CO₂) or overnight at -20 C. Proteins and/or mucopolysaccharides are precipitated, leaving a clear, easily dispensed supernatant. The hazard to health for technicians performing salivary steroid assays is, as far as we can ascertain, no greater than that involved in assaying plasma or urine samples. Noteworthy exceptions are samples contaminated by spum, since these may be associated with an increased risk of tubercular infection.

Influence of Flow Rate on Salivary Steroid Levels

Possibly the greatest deterrent to the introduction of assays for salivary steroids in routine practice has been the popular misconception that steroid concentrations in saliva are dependent upon flow rate. Studies designed to investigate the influence of flow rate on salivary concentrations of low molecular weight compounds, including drugs and steroids, usually feature samples collected at normal and maximally stimulated flow rates. Sucking solid objects and sweets or chewing gums all increase flow rates, but a convenient technique for achieving maximum stimulation is the dropwise addition of a citric acid syrup to the tongue. Problems of contamination may be avoided by collecting parotid fluid rather than mixed whole saliva, and a specially constructed device (6) fits easily over the gland, facilitating the collection of parotid saliva. The concentrations of 17α-hydroxyprogesterone, progesterone, and cortisol in matched samples of "normal" and "maximally stimulated" saliva showed excellent agreement (Fig. 1).

Further studies (7) indicate that the concentration of other small molecules, including diphenylhydantoin, remains constant despite a 6-fold increase in flow rate. Overall, there is good experimental evidence that the salivary concentration of many drugs (8) and steroid hormones is independent of flow rate.

Steroid Concentrations in Saliva vs. Plasma Free Values

Accumulating evidence indicates that the concentrations of steroids in saliva, like those in urine, reflect the free nonprotein-bound fraction in plasma. Studies by Smith et al. (9) provided the first data supporting this relationship. They determined testosterone in the free fraction of plasma and also in saliva in samples provided by a group of normal women and by patients with the polycystic ovarian syndrome; the good agreement between experimental and theoretical data is illustrated in Fig. 2. A similar relationship was found between plasma free (271 ± 63 pmol/l) and salivary testosterone (274 ± 73 pmol/l) concentrations in normal men (10), and an excellent correlation has now been established (11) between plasma free progesterone, determined by equilibrium dialysis, and salivary progesterone concentrations in women during the second and third trimester of pregnancy. Data derived from a review by Horning et al. (8) clearly show that for the drugs listed in Table 1, the relationship between plasma free and salivary drug concentrations is maintained, even though binding to plasma proteins may show considerable differences.

![Fig. 1. Comparison between steroid concentrations in matched samples of parotid fluid (O—O) collected with citric acid stimulation and of mixed whole saliva (●—●) collected with no stimulation, illustrating lack of dependence of steroid concentration on flow rate. a, Comparison of cortisol concentrations at times when the adrenal activity was maximal (0700 h) and minimal (0100 h). b, Comparison of 17α-hydroxyprogesterone (17-OHP) concentrations in a patient with CAH after administration of cortisol. c, Comparison of progesterone concentrations throughout the menstrual cycle in a normal subject.](image-url)
Transfer of Steroids from Plasma to Saliva

Factors controlling the dynamic interrelationship between steroid concentrations in plasma and saliva remain
to be determined. It is generally accepted that transfer is rapid and analogous to ultrafiltration. In the light of this
generally held belief, it is pertinent to consider recent reports (12, 13) calling attention to fundamental con¬
trdictions in accepted concepts (14, 15) governing hormone delivery to target tissues. Reevaluation of the kinetics of
hormone transfer from blood to target cells has led Ekins (12, 13) to propose the alternative model of hormone
delivery illustrated in Fig. 3. Based on this model the rate of delivery to some tissues is governed by the plasma
free hormone concentration as measured "in vitro," whereas delivery to other tissues having high clearance
rates is dependent in part on bound hormone concentrations. Therefore, specific binding proteins have a poten¬
tial, although as yet unproven, capacity for influencing the differential distribution of a hormone among various
target tissues.

Based on the model of Ekins, the rapid loss of steroids in saliva would make the salivary gland a tissue having a high clearance rate. Transfer of hormones to the gland would therefore depend on concentrations in plasma of both the free and bound hormone. This does not appear to hold true, since the studies of Wang and her colleagues (16) have shown that in a group of thyrotoxic patients, having sex hormone-binding globulin (SHBG) levels and plasma total testosterone values that were markedly elevated, salivary testosterone concentrations were very similar to those in normal men. This finding, together with other studies indicating good agreement in salivary and plasma free steroid concentrations, suggests that the model of Tait and Burstein (15) may provide a more realistic approach to factors influencing the transfer of steroid from plasma to salivary tissue, and justifies the efforts of biochemists to develop assays for monitoring free hormone concentrations.

![Graph showing correlation between plasma free testosterone concentrations and salivary testosterone concentrations](image)

**Table 1.** Relationship between plasma free and salivary drug concentrations

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ratio of drug concentrations</th>
<th>Binding by plasma proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free in plasma/total in plasma</td>
<td>Saliva/total in plasma</td>
</tr>
<tr>
<td>Antipyrene</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Aminopurine</td>
<td>0.85</td>
<td>0.8</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.77</td>
<td>0.78</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>0.09</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Data from Ref. 8.

![Diagram showing proposed model of hormone delivery](image)
Assays for Salivary Steroids

A simple method for measuring the free cortisol index in plasma has been described (17), but most procedures involve equilibrium dialysis or ultrafiltration. The well-recognized difficulties associated with both these techniques make them unsuitable for routine use. Assays for salivary steroids, on the other hand, are technically simple. This, when considered in conjunction with the relationship existing between salivary and plasma free values, suggests that assays for steroids will have a role to play in endocrinological investigations. Since hormonally active steroids circulating in plasma are largely bound by specific binding proteins, concentrations in saliva are low. The routine determination of steroid hormones in saliva is therefore critically dependent on the development of sensitive, specific immunoassays, having the output required for processing the large sample numbers generated by saliva-sample regimens. Assays have now been established for most, if not all, steroids of diagnostic value in the routine assessment of endocrine dysfunction. Although many questions remain unanswered, the prospect of frequent sampling coupled with relatively simple assay techniques suggests that measurement of salivary steroids will be a useful additional parameter for assessing endocrine activity.

Salivary Cortisol

The pioneering studies of Shannon, Katz, and their associates (18-20), which first focused attention on the role of salivary steroids in assessing adrenal activity, featured the determination of 17-hydroxycorticosteroids in parotid fluid. Since a device for collecting parotid fluid was essential, and the insensitivity of the assay technique required collection of large sample volumes (10 ml), it was not surprising to find that this monitoring procedure failed at that time to find general acceptance. More recent evidence suggested that, with the development of simple RIAs requiring only small aliquots of mixed whole saliva, reappraisal of the clinical value of salivary sampling regimens was justified (21).

Assays for salivary cortisol

The RIAs for cortisol used in our studies were simple, direct procedures (22, 23). Absence of binding proteins in saliva allowed assay of salivary cortisol at pH 7.4, but assay for total cortisol in plasma required a low pH to eliminate binding to plasma proteins. The determination of plasma free steroid featured equilibrium dialysis. Since cortisol concentrations in the dialysate were determined by the same procedure as that used for saliva, possible inaccuracies associated with the purity of the tritiated label were avoided. The specificity of these procedures was confirmed by the excellent agreement in results with those of a reference gas chromatography-mass spectrometry technique (Fig. 4). Other workers have also established immunoassays for measuring salivary cortisol. The group in Japan used an in house assay procedure (24) that included extraction with dichloromethane. Since this complex, insensitive assay had relatively high inter-assay coefficients of variation, the results need to be interpreted with caution. By contrast, Al-Ansari et al. (25) reported development of a simple direct assay by slight modification of a commercial kit originally designed for use with plasma or urine.

Salivary cortisol vs. plasma free cortisol

Reports in the literature (26) indicate that enzyme systems converting cortisol to cortisone are present in salivary glands and that the total cortisone/total cortisol ratio in human plasma differed from that in saliva. It is therefore a matter of some importance to establish whether the relationship between plasma free and salivary cortisol is maintained under these circumstances. In our studies (27) a healthy volunteer, with adrenal activity suppressed by prior administration of dexamethasone, received an infusion of cortisol hemisuccinate (rate of infusion, 2-5 mg/h). Matched samples of plasma and saliva were collected during infusion (3 h) and for a subsequent 2-h period. Data derived from determining cortisol concentrations in saliva, and the free and total values in plasma are presented in Fig. 5. The excellent agreement in plasma free and salivary cortisol levels over a wide range of concentrations is apparent. Recent more extensive studies (24) have confirmed that the relationship between plasma free and salivary cortisol holds true despite cortisol/cortisone interconversion by the salivary glands (Fig. 6). A possible explanation for this anomaly could be that cortisol transfer from blood to the gland is not rate limiting, but can be increased to compensate for that lost by further metabolism.

Fig. 4. Comparison of plasma and salivary cortisol concentrations, determined by direct RIA techniques, with those obtained by a reference gas chromatography-mass spectrometry (GC-MS) procedure. For plasma: $F_{RIA} = 0.967F_{GC-MS} + 2.37$, $r = 0.97$. For saliva: $F_{RIA} = 1.01F_{GC-MS} - 0.90$, $r = 0.99$. 
Circadian rhythm of salivary cortisol

Normal adrenal activity displays a marked circadian rhythm, and data presented in Fig. 7 provide clear cut, experimental evidence to indicate that these rhythms are reflected in the circadian variation in salivary cortisol concentrations in healthy volunteers. Although levels of cortisol were low in saliva, they clearly indicated the same rhythmic changes as those in plasma. It has proved possible to obtain saliva samples from children aged less than 5 yr and from newborn infants in the first 24 h of life. Children aged 3 yr or more found no difficulty in salivating directly into disposable, wide bore tubes, but in infants and babies samples were obtained by gently aspirating saliva from the floor of the mouth using a wide bore plastic tube attached to a syringe. In young children salivary cortisol levels showed a circadian rhythm and were in the adult normal range. In newborn infants, however, evidence for this rhythm was lacking and salivary cortisol values were high (Fig. 8). Basic factors
controlling adrenal secretory activity in the newborn remain to be defined. Zurbrugg (28) noted that adrenal activity, as reflected by changes in circulating cortisol, had an built in 12-h rhythm and that the variations in plasma cortisol values were not related to sleep/wakefulness. In marked contrast, other investigators (29) failed to provide evidence for rhythmic changes in adrenal activity. These workers found mean plasma cortisol levels during deep sleep, quiet wakefulness, and crying were 1.9, 4.7, and 13.0 μg/dl, respectively. This trend towards successively higher cortisol levels along the continuum of increasing psychophysiological arousal led to the conclusion that adrenal activity was related to behavioral status rather than to a built in synchronizer. Further work is required to resolve these differences, and salivary sampling, which avoids ethical restrictions imposed on studies involving normal newborn infants, may well help to delineate factors controlling adrenal activity in this younger age group.

Normal ranges for salivary cortisol

Reported normal ranges for salivary cortisol in early morning samples (Table 2) show that levels in men, women, and children were not significantly different. The range of values observed in our study using an in house assay (22) when compared with those obtained using a commercially available kit (25) were in good agreement. Therefore, assays for salivary cortisol need not be restricted to the relatively few centers having access to in house reagents. Commercial kits when suitably modified achieve adequate sensitivity for measuring salivary cortisol concentrations. The higher values reported by Umeda et al. (24) may in part be due to the use of a relatively complex, insensitive assay system. The volumes of saliva required were 100-200 times greater than those used in our studies, and this may well cause increased interference by nonsteroidal impurities.

Clinical studies featuring salivary cortisol

In collaborative investigations (27) designed to establish the usefulness of salivary sampling in clinical studies of adrenal dysfunction, total cortisol concentrations were determined in matched samples of plasma and saliva provided by healthy, nonobese volunteers and by inpatients having the various abnormalities listed in Table 3.

Absence of circadian rhythm. Loss of circadian variation in adrenal secretory activity is frequently associated with defects in the hypothalamo-pituitary-adrenal axis. The absence of rhythmic changes in salivary cortisol values in patients (n = 3) with subsequently proven hypercortisolism is clearly indicated in Fig. 7.

Dynamic tests. The functional integrity of the hypothalamo-pituitary-adrenal axis is usually assessed using various dynamic tests.

Insulin tolerance test: Insulin tolerance tests, designed to assess the patency of the hypothalamo-pituitary-adrenal axis as a whole, were performed on volunteers and patients. Insulin 0.1-0.2 μg/kg; Actrapid, Novo Labs., Wilton, CT) was given as an iv bolus after an overnight fast; all participants achieved adequate hypoglycemia (blood sugar less than 38 mg/dl) in this test. Data derived from the assay of these matched samples are presented in Table 4. The incremental response in plasma and salivary cortisol concentrations, expressed as a percentage of basal values, is illustrated for healthy volunteers in Fig. 9 and for patients having adequate and inadequate responses, judged by generally accepted criteria, in Fig. 10. Considering the responses in normal subjects, an arbitrarily selected value of 150% increase over basal values in saliva appeared to be a reasonable indication of normal response. Using this selected value the same clinical decision regarding the presence or absence of a response in patients could be made, on the basis of either plasma or salivary sampling regimens.

Standard "short" Synacthen test: The standard short Synacthen test (250 μg im; Ciba-Geigy Corp., Summit,
Table 4. Cortisol (F) responses to insulin-induced hypoglycemia

<table>
<thead>
<tr>
<th></th>
<th>% Incremental response</th>
<th>Maximum increase (nmol/l)</th>
<th>Maximum incremental increase (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma F</td>
<td>Saliva F</td>
<td>Plasma F</td>
</tr>
<tr>
<td>Normal subjects</td>
<td>(n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>159 ± 67</td>
<td>161 ± 41</td>
<td>507 ± 120</td>
</tr>
<tr>
<td>Patients, adequate response (n = 13)</td>
<td>(42.1)</td>
<td>(25.4)</td>
<td>(23.6)</td>
</tr>
<tr>
<td>Patients, inadequate response (n = 7)</td>
<td>(38.8)</td>
<td>(32.2)</td>
<td>(44.5)</td>
</tr>
</tbody>
</table>

Values shown are the mean ± SD; numbers in parentheses are the coefficient of variation.

NJ) is frequently used to assess adrenal reserve. Data presented in Fig. 11 show the response in healthy volunteers to Synacthen administration. Baseline cortisol concentrations in saliva (10 nmol/l) were low compared with those in plasma (450 nmol/l), but after adrenal stimulation mean peak values in saliva showed a 7-fold increase compared with a 3-fold increase in plasma. This disproportionate increase probably occurred because, at plasma concentrations of around 500 nmol/l (31), all available binding sites of transcortin are saturated. Therefore, after Synacthen stimulation the increased plasma cortisol would be redistributed between the albumin-bound and free fractions, with a disproportionate increase in plasma free moiety, which would be reflected in the salivary concentrations of the hormone. Others (25) have also observed this disproportionate rise in salivary cortisol values after administration of Synacthen to healthy volunteers; they agree with our explanation for this apparent anomaly.

Fig. 9. Salivary and plasma cortisol concentrations (mean percentage incremental change ± 1 SD) in normal subjects (n = 6) after insulin-induced hypoglycemia.

Estrogens are known to increase circulating transcortin concentrations, and women taking estrogen-containing oral contraceptive preparations have slightly elevated baseline levels of cortisol in plasma and a greater than normal response to Synacthen. Salivary cortisol concentrations in such women are, however, within the normal range and a normal although slightly delayed response to Synacthen is observed (22). Patients (n = 4) with secondary adrenal atrophy failed to respond to Synacthen, and plasma and salivary cortisol concentrations remained at or below the sensitivity of the assay procedure (saliva, 1 nmol/l; plasma, 28 nmol/l).

Dexamethasone suppression test: Tests featuring the suppression of adrenal activity with dexamethasone, a synthetic glucocorticosteroid, are widely used in investigations of patients suspected of having Cushing's syndrome. The potential value of salivary cortisol concentrations for indicating the response to such synthetic steroids is demonstrated by results derived from the assay of matched samples provided by a healthy volunteer after administration of dexamethasone. The suppressed adrenal activity with concomitant reduction in cortisol secretion was reflected equally well by decreased hormone concentrations in plasma and saliva (Fig. 12).
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Subject with Adrenal Atrophy

- Mean levels in females taking oestrogen oral contraceptive

Normal plasma Cortisol response

Mean ± SD n=7

Normal salivary Cortisol response

Mean ± SD n=7

Fig. 11. Plasma and salivary cortisol concentrations (mean ± 1 SD) after adrenal stimulation with Synacthen (250 µg im) in normal subjects (n = 7) and female subjects taking an estrogen contraceptive preparation, and the lack of adrenal response observed in a subject with secondary adrenal atrophy.

700-
600-
500-
400-
300-
200-
100-
0-

Fig. 12. Plasma and salivary cortisol concentrations in a normal subject after adrenal suppression with dexamethasone (0.5 mg) administered at the times indicated by the arrows.

The studies of Al-Ansari et al. (25) are in keeping with this observation since, in a group of healthy volunteers of both sexes, overnight dexamethasone suppression caused a marked reduction in early morning salivary cortisol levels.

The excellent correlation in results observed on assay of matched samples of plasma and saliva, provided by Cushingoid patients before and during high dose dexamethasone suppression tests, suggests that salivary sampling regimens have great potential for the differential diagnosis of Cushing's syndrome. In a patient (J.H.) with histological evidence of a pituitary tumor, dexamethasone administration markedly reduced cortisol levels in both plasma and saliva (Fig. 13); in another patient (W.B.) with an ectopic source of ACTH, dexamethasone caused only a limited suppression in adrenal activity.

Cortisol levels in saliva therefore provide as good an indication of adrenal activity as those in plasma, and the evidence clearly suggests that such assays have a role to play in screening for adrenal dysfunction and in the differential diagnosis of defects in the hypothalamo-pituitary-adrenal axis.

DHA-SO₄

Recent unpublished studies from this Institute have indicated that it is possible to determine salivary DHA-SO₄ concentrations by a simple, nonextraction technique. The antiserum used in the assay was raised to a DHA 3-hemisuccinate conjugate and had essentially complete cross-reactivity with the sulfate. Since preliminary studies (32, 33) had shown that unconjugated DHA comprised only approximately 2% of the total salivary DHA-SO₄, it was considered that its removal by solvent extraction, besides being time consuming, would add little to the accuracy of the assay and could be detrimental to precision. Results obtained by this simple immunoassay correlate well with those obtained using the gas chromatography-mass spectrometry procedure.

Factors influencing salivary DHA-SO₄ concentrations

Salivary levels of DHA-SO₄ showed a less clear circadian rhythm than that of cortisol, probably explicable by
the low metabolic clearance rate of approximately 15 l/h (34) and the formation of DHA-SO₄ by extraadrenal sulfation. No significant circatrigintan (monthly) rhythm was demonstrated in female subjects having apparently normal ovulatory menstrual cycles assessed by salivary progesterone analysis. As expected, no significant change in salivary DHA-SO₄ was seen after a simple overnight dexamethasone suppression test, although after prolonged, high dose administration, levels were markedly reduced (Fig. 14). The failure to completely suppress salivary DHA-SO₄ is possibly due to continuing androgen production by the testis/ovary.

Although there was a wide range of salivary DHA-SO₄ concentrations in adults (Fig. 15), there was evidence of a progressive fall with age in males, consistent with previous studies on plasma DHA-SO₄ levels (35) and with our own data for salivary testosterone (36).

**Clinical studies featuring salivary DHA-SO₄**

On the basis of the early work of Bulbrook (37), which indicated that low androgen excretion was a risk factor for carcinoma of the breast, a study was established in association with the Nottingham Screening Programme for Breast Cancer to determine cortisol and DHA-SO₄ concentrations in samples of saliva of women with primary breast cancer and in matched controls. Samples were taken regularly, approximately 10 throughout the day (0800–2300 h), for two consecutive days and cortisol and DHA-SO₄ concentrations were measured. The typi-

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**Fig. 13.** Plasma and salivary cortisol concentrations in two patients with hypercortisolism before and during oral administration of dexamethasone (2 mg) at the time indicated by the arrows.

**Fig. 14.** Plasma and salivary cortisol and DHA-SO₄ concentrations in a normal subject. Days 1 and 2 indicate the diurnal variation of cortisol and DHA-SO₄. Days 3 and 4 show the fall in cortisol and DHA-SO₄ after adrenal suppression with dexamethasone (2 mg) administered at the times indicated by the arrows.
Cal circadian rhythms of cortisol secretion were observed in both groups, with an overall indication that cortisol levels in the cancer patients were higher than the controls throughout the day. A less distinct rhythmic secretion of DHA-SO₄ was apparent, with a tendency for androgen levels to be lower in the patients with breast cancer. Clear group separation was however seen when the cortisol to DHA-SO₄ ratios were plotted (Fig. 16). Although these data in postmenopausal women are interesting, it is important to recognize that Bulbrook (37) and his colleague (38) tend to believe that low androgen secretion with regard to breast cancer incidence relates primarily to the younger premenopausal women, and the significance of this disappears as women approach the menopause. The potential value of salivary steroid analysis in studies relating to the etiology of breast cancer is, however, apparent.

**Salivary 17α-Hydroxyprogesterone**

In children having congenital adrenal hyperplasia (CAH), adrenal dysfunction is usually due to a C-21 hydroxylase enzyme deficiency (39). The adrenal gland in these patients secretes little cortisol, whereas the production of biosynthetic precursors, including 17α-hydroxyprogesterone, is high. Such patients are usually treated with orally active glucocorticosteroids that feed back at the hypothalamo-pituitary level to inhibit the secretion of ACTH. Adrenal activity is thereby reduced, and 17α-hydroxyprogesterone concentrations fall. Measurement of circulating 17α-hydroxyprogesterone concentrations is therefore of value in the initial diagnosis of CAH, but its usefulness in monitoring subsequent glucocorticosteroid therapy remains a matter of some debate. The results of such studies are difficult to interpret, being complicated by many factors including the intrinsic circadian rhythm in adrenal activity, and the timing of blood samples in relation to glucocorticosteroid administration (40-42). A further difficulty includes repeated venipuncture in young children, and the more frequent, stress-free sampling possible using salivary regimens is particularly attractive in these circumstances.

**Clinical studies featuring salivary 17α-hydroxyprogesterone**

The clinical studies of Walker et al. (43), designed to assess the role of salivary 17α-hydroxyprogesterone in monitoring treatment for CAH, included 14 patients, aged 1-16 yr, all having a C-21 hydroxylase deficiency. All patients received cortisol (15-20 mg/m²·day) divided into 3 doses throughout the day; the salt-losers (n = 8) also received fludrocortisone daily (0.1-0.5 mg; Florinef, E. R. Squibb, Hounslow, Middlesex, U.K.). Matched samples of plasma and saliva were collected between 0900 h and 1000 h from these patients and from agematched children with no obvious endocrinological abnormality. Concentrations of 17α-hydroxyprogesterone in these matched samples (n = 27), determined by standard “with extraction” RIA procedures (44), showed good correlation (r = 0.91) over a wide range of values. In a group of normal children (n = 32), age matched with the CAH patients, salivary 17α-hydroxyprogesterone concentrations had a median value of 390 pmol/l (range, 90-1520 pmol/l); in the CAH patients, concentrations of this steroid showed considerable variation (range, 67-26,000 pmol/l). More recent studies (45) have confirmed these observations (Fig. 17). Price and his colleagues (45) noted...
that many pediatricians aim to maintain plasma values of this steroid at approximately 25 nmol/l, which would correspond to a salivary level of around 1000 pmol/l. Although it has not yet been possible to determine salivary 17α-hydroxyprogesterone in an untreated patient, the high concentration of this steroid in poorly controlled patients (Fig. 18) suggests that this assay will be of value in the initial diagnosis of C-21 hydroxylase defects. All patients with salivary 17α-hydroxyprogesterone levels below the lower limit of normality had clinical signs of glucocorticosteroid excess. Assay of this steroid in saliva may therefore be particularly useful in detecting excessive glucocorticosteroid therapy before clinical signs of hypercortisolism appear.

A 15-yr-old, inadequately treated, female salt-loser (patient S.A.) provided matched samples of plasma and saliva at frequent intervals for 24 h. The concentrations of 17α-hydroxyprogesterone throughout the day in these matched samples showed a similar pattern (Fig. 19). Peak values in saliva exceed 18,000 pmol/l, levels nearly 50-fold greater than the median value 380 pmol/l observed in healthy children. Recent studies (41) suggest that in well controlled patients circulating 17α-hydroxyprogesterone concentrations remain within the normal range throughout the 24 h and that monitoring, based on the collection of single plasma samples, is possible. The
widely fluctuating levels of 17α-hydroxyprogesterone in this patient (Fig. 19) clearly emphasize the ineffectiveness of procedures based on single sampling regimens to monitor these poorly controlled patients. The routine, stress-free collection of small aliquots (500 μl) of saliva at 1- or 2-h intervals ensures more effective replacement therapy. Multiple-sampling regimens based on the collection of daily saliva samples just before administering replacement therapy may also have a role to play in patient compliance studies.

The short Synacthen test is sometimes used as an aid in the initial diagnosis of CAH. Data derived from the assay of matched samples provided by two CAH patients (J.H. and C.B.) after Synacthen administration show that the changing concentrations of 17α-hydroxyprogesterone in plasma are quickly and accurately reflected in the steroid levels in saliva (Fig. 20). The elevated salivary 17α-hydroxyprogesterone concentrations in these patients after stimulation closely approximated the stimulated values observed in healthy volunteers, probably because both patients were receiving replacement therapy. Both these patients showed suppressed baseline concentrations, with those in patient J.H. (45 pmol/l) being lower than those in C.B. (180 pmol/l), a finding consistent with the more marked clinical features of glucocorticosteroid excess observed in this patient.

Assays for salivary 17α-hydroxyprogesterone facilitate more detailed monitoring of therapy in CAH patients and may allow early detection of over- or undertreatment, thereby ensuring that each child is provided with the best possible opportunity of attaining optimum growth potential.

**Aldosterone**

Salivary aldosterone concentrations in children have been determined (46) using a RIA procedure based largely on that of Abraham et al. (47), for the determination of plasma concentrations of this hormone. In normal children (n = 12) aged 4–16 yr and having an unknown sodium intake, the mean salivary aldosterone concentration was 75 pmol/l (range, 30–210 pmol/l), a
value in good agreement with that observed in hospitalized children (mean, 93 pmol/l) having a regular sodium intake. These children had no known or suspected defects in the biosynthesis of glucocorticosteroids or of plasma proteins. During changes in sodium intake, aldosterone concentrations in plasma and saliva showed excellent correlation (Fig. 21). The ratio of aldosterone concentrations in plasma and saliva under these conditions was relatively constant for each individual, but in the group as a whole the ratio showed considerable variation, (0.16-0.45; mean, 0.27). The administration of Synacthen and dexamethasone caused consistent alterations in this ratio, Synacthen increasing (mean, 0.36) and dexamethasone decreasing (mean, 0.19) this ratio. It is suggested that these differences are due to competition between cortisol and aldosterone for available binding sites on transcortin.

**Salivary Testosterone**

Circulating testosterone concentrations show well defined circadian and circatrigintan variations that necessitate correctly timed, multiple plasma-sampling regimens for accurate assessment of basal endocrine activity (4, 48). Considered in relation to reports indicating that plasma free testosterone levels provide information of greater diagnostic significance than total plasma concentrations (49), this suggests that assays for salivary testosterone may be particularly useful in routine practice. Several groups have investigated possible factors influencing salivary levels of this hormone before attempting to assess the value of salivary testosterone assays in routine practice. The following are factors that have been investigated.

![Graph](attachment:image.png)

**Fig. 21.** Comparison of plasma and salivary aldosterone concentrations during periods of changes in dietary sodium (n = 30, r = 0.9044, P < 0.001). Data from Ref. 46.

![Graph](attachment:image.png)

**Fig. 22.** Circadian variation in salivary testosterone concentrations in a normal male, illustrating the effect of an altered activity cycle.

**Circadian rhythms**

In healthy men and women, salivary testosterone concentrations were found to show a well marked circadian rhythm (10, 50-53), with levels in early morning samples being almost double those in late evening samples. It has been noted (54) that in men this rhythm appears to be markedly dependent on the maintenance of a "normal" sleep/wake activity pattern. Men on a normal sleep/wake schedule, who rise at 0800 h and retire at 2300 h have high salivary testosterone concentrations in early morning samples. A phase shift of 8 h introduced into this activity pattern, with the subjects rising at 2400 h
and retiring at 1600 h, induced alterations in the periodicity of salivary testosterone concentrations. Levels were found to be high in samples collected just after rising (2400 h) and low at 0800 h (Fig. 22). In contrast, the rhythm in salivary cortisol concentrations showed little variation after altering the sleep/wake pattern, with cortisol levels remaining high in the 0800 h sample and low at 2400 h. The men involved in this study were members of an Arctic expedition who were exposed to continuous daylight and therefore relied on clock time for regulating their day. Later work (10) has confirmed that the changing levels of salivary testosterone in men are not dependent on time of day or posture but upon time of waking. Since the relationship between salivary testosterone and free and total values in plasma was maintained in early morning and late evening samples, changes in testosterone production rather than binding to plasma proteins were thought to account for the circadian rhythm in salivary testosterone levels.

**Exercise**

Participants in the study of Campbell *et al.* (54) were subjected to strenuous physical exertion throughout the course of an Arctic expedition, and it is interesting to note the influence of this factor on salivary testosterone values. Data derived from the assay of samples collected at 2-h intervals throughout normal waking hours before leaving the United Kingdom and after their return and at early and late stages of the expedition are presented in Table 5. In both the early and late study periods during the expedition, mean salivary testosterone levels were lower than those observed in the United Kingdom, whereas salivary cortisol concentrations showed no sig-

**Table 5.** Mean salivary concentrations of cortisol (nmol/l) and testosterone (pmol/l) on 2 consecutive days in normal male subjects (n = 4) before (UK pre), during (Spitz I, II, III), and after (UK post) an expedition to Spitzbergen.

<table>
<thead>
<tr>
<th>Time period</th>
<th>Spitz</th>
<th>UK post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>4.47</td>
<td>5.29</td>
</tr>
<tr>
<td>SD</td>
<td>1.36</td>
<td>2.79</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>220</td>
<td>146</td>
</tr>
<tr>
<td>SD</td>
<td>43</td>
<td>30</td>
</tr>
<tr>
<td>P value*</td>
<td>0.0013</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

* vs. UK pre period (Student’s *t* test).

**Fig. 23.** Salivary testosterone concentrations in normal male subjects as a function of age. □, Mean ± SD.
significant differences. It has been noted previously (55) that decreased plasma testosterone values were associated with strenuous physical exercise.

**Age**

The protein binding studies of Vermeulen and co-workers (49, 56) indicate that plasma free testosterone concentrations decrease with age in men. In keeping with this observation, our own studies (36) have shown that the mean salivary testosterone concentration in healthy old men in the eighth decade (100 pmol/l) is lower (Fig. 23) than that in young adult males (236 pmol/l).

Tames and Swift (53) have recently obtained saliva samples between 0900 and 1000 h from almost 1000 male school children. These volunteers were from mixed socioeconomic groups and ethnic backgrounds; their ages ranged from 9-17 yr. Measurement of testosterone in these samples showed uniformly low values in the 9-yr-old group, but with progressing age, levels increased (Table 6). A marked increase occurred between the 12- and 13-yr-old groups, and mean values for the 13- and 14-yr-old groups were significantly different (P < 0.05). Values closely approximating the male adult range were observed in the 17-yr-old group. This clear-cut, age dependence of salivary testosterone concentrations, also observed by others (57), emphasizes the need to establish normal ranges, based on decades of age, for the correct interpretation of clinical data.

**Circatrigintan variation**

In healthy women in the reproductive age, studies by Turkes et al. (52) indicate a circatrigintan variation in salivary testosterone concentrations. The range of values expected throughout the normal menstrual cycle was determined by assaying saliva samples, collected daily at 0900 h, by a group of women who were menstruating regularly. These women also provided daily, matched samples of plasma around the time of ovulation so that dating of the cycle by conventional plasma hormone analysis was possible. In the follicular and luteal phase of the cycle, salivary testosterone concentrations showed no significant difference. Just before ovulation concentrations increased, and maximum values were reached around the time of ovulation (Fig. 24). These cyclical changes in salivary testosterone concentrations parallel those reported in plasma (48).

**Role of salivary testosterone in clinical studies**

**Male infertility.** The value of assays for salivary testosterone in studies of male infertility remains to be determined. Preliminary investigations suggest they may be useful, since in a group of normal men undergoing a typical 3-day stimulation test with human CG, the consequent increase in circulating testosterone concentrations was accurately reflected by a corresponding increase in salivary testosterone (Fig. 25). Others (57) have shown that in men with hypogonadotrophic hypogonadism, salivary testosterone concentrations are less than 10% of those in age-matched, healthy volunteers. Wang and her colleagues (16) have noted that after administration of testosterone enanthate to a patient with hypogonadotrophic hypogonadism and to healthy males, plasma

---

**Table 6. The mean (±sd) salivary testosterone concentrations of adolescent school boys**

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Conc. (pmol/l)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>76 ± 42</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>82 ± 29</td>
<td>74</td>
</tr>
<tr>
<td>11</td>
<td>122 ± 56</td>
<td>63</td>
</tr>
<tr>
<td>12</td>
<td>165 ± 86</td>
<td>90</td>
</tr>
<tr>
<td>13</td>
<td>235 ± 230</td>
<td>226</td>
</tr>
<tr>
<td>14</td>
<td>347 ± 122</td>
<td>108</td>
</tr>
<tr>
<td>15</td>
<td>368 ± 172</td>
<td>161</td>
</tr>
<tr>
<td>16</td>
<td>369 ± 156</td>
<td>184</td>
</tr>
<tr>
<td>17</td>
<td>380 ± 98</td>
<td>68</td>
</tr>
</tbody>
</table>

[Adapted with permission from F. J. Tames and A. D. Swift: Immunoeassays for Clinical Chemistry (edited by W. M. Hunter and J. E. T. Corrie), Churchill Livingstone, Edinburgh, in press (53).]

---

**Fig. 24. Salivary testosterone concentrations (mean ± SEM; □) in a group of normal females (n = 10). Comparative data from an infertile hirsute female (——) are also shown.**

**Fig. 25. Plasma and salivary testosterone concentrations determined in male volunteers after human CG stimulation.**
and salivary testosterone concentrations showed parallel increases.

**Conditions in which binding to plasma proteins varies.**

**Increased sex hormone-binding protein:** In the study of Wang et al. (16) plasma total and salivary testosterone levels together with circulating SHBG values were measured in normal men \((n=23)\) and male patients \((n=8)\) with thyrotoxicosis. The mean value for the plasma total testosterone concentrations in patients \((13.48 \pm 3.74 \text{ ng/ml})\) was significantly higher than that in normal men \((6.09 \pm 1.8 \text{ ng/ml})\). Levels of SHBG in the hyperthyroid men were also markedly elevated \((6.5 \pm 2.2 \text{ vs. } 0.8 \pm 0.3 \mu g \text{ dihydrotestosterone bound/100 \mu l plasma})\). In marked contrast, the mean value for salivary testosterone in the hyperthyroid patients \((84.4 \pm 26 \text{ pg/ml})\) was strictly comparable with that of normal men \((84.0 \pm 35 \text{ pg/ml})\). These results provide a useful indication of the value of salivary testosterone measurements in conditions associated with increased SHBG levels. These workers, like Smith et al. (9), found no evidence indicating the existence in saliva of any appreciable amounts of SHBG.

**Decreased sex hormone-binding protein:** According to Vermeulen (34) true idiopathic hirsutism is associated with plasma testosterone concentrations in the normal range, and in these women hirsutism may be due either to a target tissue defect or to a decreased concentration of SHBG in plasma. Decreased circulating concentrations of SHBG would lead to an increase in the plasma free moiety, and this in turn would be reflected in saliva.

Preliminary studies (52) indicate that measurement of salivary testosterone may be used to differentiate those patients with reduced circulating levels of SHBG from those with end-organ defects or increased concentrations of androgenic steroids other than testosterone. A hirsute patient who was menstruating regularly and was found to have plasma testosterone concentrations within the normal range on at least three occasions provided daily saliva samples for about 30 days. In nearly all samples, salivary testosterone levels were markedly elevated (Fig. 24), suggesting that hirsutism in this patient may be due to increased plasma free steroid caused by decreased circulating SHBG concentrations. Other women \((n=4)\) presenting with true idiopathic hirsutism and infertility had salivary testosterone levels closely approximating to those seen in normal women.

More extensive studies (9, 58) also indicate that measurement of salivary testosterone provides useful information regarding biologically available androgen without recourse to the complicated techniques for determining plasma free values. In a group of patients \((n=17)\) diagnosed as having polycystic ovaries (PCO) by laparoscopy, Smith et al. (9) found a single salivary value to be of greater diagnostic significance than any of the currently used plasma androgen assays including testosterone, dihydrotestosterone, androstenedione, and DHA-SO₄. Most but not all \((14 \text{ of } 17)\) patients in this group had elevated salivary testosterone concentrations. Baxendale and James (58) have also assayed samples provided by normal women \((n=34)\) and patients having PCO \((n=14)\) or idiopathic hirsutism \((n=30)\). The mean salivary testosterone values observed in women with PCO \((20.6 \pm 8.5 \text{ pg/ml})\) and idiopathic hirsutism \((13.9 \pm 5.6 \text{ pg/ml})\) were significantly higher than those in normal women \((7.7 \pm 2.6 \text{ pg/ml})\). Both groups of workers have noted that, although there was a highly significant correlation between plasma free and salivary testosterone in normal women and in patients, the slope of the curve differed significantly from unity. This difference in the slope from the predicted value in women, but not in men, may in part be due to possible inaccuracies associated with the determination of the low concentration of testosterone present in female samples (58). It could however arise if some form of active transport were involved. It is known that the low concentrations of albumin in saliva (thought to be around 1% of plasma values) originate from serum (59). It is therefore possible that transfer of small amounts of albumin-bound testosterone may also account in part for the higher levels in saliva compared with the unbound moiety in plasma. This anomaly is not necessarily important since the data of Smith et al. (9) indicate that salivary values are directly proportional to the calculated value for the plasma free moiety.

**Normal range of salivary testosterone concentrations in healthy volunteers**

The data presented in Table 7 allow comparison of reported values for testosterone in the early morning saliva samples of healthy volunteers. RIAs for determining salivary testosterone all featured antisera raised against testosterone-3 conjugates; the two enzyme immunoassays (52, 60) both utilized antitestosterone-11 isera. All antisera cross-reacted to a greater \([59.7\% (53)]\) or lesser \([14\% (16)]\) extent with a major reduced metabolite of testosterone commonly known as 5α-dihydrotestosterone (5αT). RIA of male samples with and without preassay chromatographic purification led the groups associated with Wang (16), Baxendale (10), and Luisi (57) to conclude that the antisera used in their studies was specific and that preassay purification was not required in routine use. It is therefore surprising to find that the values reported by Luisi et al. (57) are far higher than those of all other workers except Landman et al. (50), who provided no details of cross-reactivity. With these two exceptions there is remarkably good agreement in the normal range for salivary testosterone in the adult male; RIAs and enzyme immunoassays both provided results in keeping with those obtained by gas chromatography-mass spectrometry techniques.
Fall, 1982

STEROIDS IN SALIVA

Table 7. Salivary testosterone concentrations

<table>
<thead>
<tr>
<th>Assay procedure</th>
<th>No. of volunteers</th>
<th>Age (yr)</th>
<th>Salivary testosterone (pmol/l)</th>
<th>Anti-</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Males</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>12</td>
<td>25-55</td>
<td>1025 ± 125</td>
<td>T-3</td>
<td>50</td>
</tr>
<tr>
<td>RIA</td>
<td>12</td>
<td>25-35</td>
<td>1025 ± 125</td>
<td>T-3</td>
<td>57</td>
</tr>
<tr>
<td>RIA</td>
<td>26</td>
<td>19-39</td>
<td>875-1205</td>
<td>T-3</td>
<td>58</td>
</tr>
<tr>
<td>RIA</td>
<td>25</td>
<td>40-59</td>
<td>705-1094</td>
<td>T-3</td>
<td>53</td>
</tr>
<tr>
<td>RIA</td>
<td>15</td>
<td>60-78</td>
<td>362-573</td>
<td>T-3</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>12</td>
<td>18-24</td>
<td>118-288 (FP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>12</td>
<td>18-24</td>
<td>210-375 (LP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>24</td>
<td>NS</td>
<td>274 ± 73</td>
<td>T-3</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>34</td>
<td>NS</td>
<td>327 ± 73</td>
<td>T-3</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>47</td>
<td>19-58</td>
<td>412 ± 154</td>
<td>T-3</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
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<td>20-30</td>
<td>186 ± 112</td>
<td>T-3</td>
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</tr>
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<td>236</td>
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<tr>
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<tr>
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<tr>
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<td>70-100</td>
<td>106</td>
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<td></td>
</tr>
<tr>
<td>RIA</td>
<td>36</td>
<td>18-36</td>
<td>368 ± 167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
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<td>NS</td>
<td>321 ± 122</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>36</td>
<td>NS</td>
<td>112-312</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>23</td>
<td>NS</td>
<td>148-538 (FP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>19-32</td>
<td>NS</td>
<td>14-83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>19-32</td>
<td>NS</td>
<td>14-83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>7</td>
<td>21-29</td>
<td>50-218 (FP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-MS</td>
<td>4</td>
<td>NS</td>
<td>243-486</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EIA, Enzymeimmunoassay; GC-MS, gas chromatography-mass spectrometry; T-3, testosterone-3 conjugates; T-11, testosterone-11 conjugates; FP, follicular phase; LP, luteal phase; NS, not stated; NA, not applicable.

Reported values for salivary testosterone in women (Table 7) show a wide variation. Since the values of Turkas et al. (52) (using an assay in which interference by 5αT was negligible) and those of Tames and Swift (53) (using an antiseraum having around 60% cross-reactivity with 5αT) were very similar, it is possible that compounds other than 5αT cause interference in immunoassays for testosterone in female saliva. The lower salivary testosterone levels reported by Smith et al. (9) and by Baxendale and James (58) are consistent with the generally accepted plasma free values for this hormone. Data presented by the latter group have shown that RIAs of female saliva samples after thin layer preassay purification were roughly half those obtained when this step was excluded. These workers conclude that chromatography is required to remove “polar artifacts,” but the nature of these artifacts remains to be determined. The need to improve the specificity of the nonchromatographic immunoassays used for measuring salivary testosterone in female saliva, before attempting to assess the role that these assays may have to play in infertility studies, is apparent.

Progesterone

In studies of female infertility, measurement of progesterone is thought to be a useful index of ovulation and the biosynthetic capacity of the corpus luteum since little steroid is stored by the luteal tissue and the half-life of the hormone in plasma is short (61). Although data derived from daily plasma sampling regimens provide an accurate assessment of ovarian function (62), the expensive clinical support required for such sampling procedures severely restricts their usefulness in the routine investigation of infertile patients. Therefore, most clinicians, at least in the United Kingdom, rely on measurement of progesterone in plasma samples collected at fortnightly intervals, together with basal body temperature charts and, where possible, an endometrial biopsy, for monitoring hormonal status. Regimens like this provide data that may be difficult to interpret. Results illustrated in Fig. 26 show that in the matched samples of plasma and saliva provided by a normal woman, progesterone concentrations in both saliva and plasma related well to the time of ovulation and the formation of a functional corpus luteum. The corresponding monophasic basal body temperature chart suggested ovulatory failure. Since saliva samples can be collected at daily intervals in the patients' own home, it is possible that measurement of progesterone in these samples would provide more useful information than current procedures for monitoring ovulation and luteal function in infertile women.
Assays for salivary progesterone

We (63), like Luisi et al. (64), used a specific antiserum raised against a progesterone-11-hemisuccinate conjugate in establishing RIAs for salivary progesterone. Differences in methodology arose due to the use of the Italian group of an extraction procedure featuring diethyl ether rather than petroleum ether and the separation of bound from free steroid by a 20% polyethylene glycol solution rather than by dextran-coated charcoal.

Normal range for salivary progesterone throughout the menstrual cycle

The normal pattern of salivary progesterone concentrations throughout the menstrual cycle was established by assaying daily samples provided by a group of normal women (n = 15). These women, aged 18-34 yr, had taken no contraceptive steroid preparations for at least 6 months and had a history of regular menstruation. The provision of matched samples of plasma allowed dating of the cycle by the conventional procedure of determining plasma LH and FSH levels in relation to the plasma concentration of estradiol and progesterone (Fig. 27). In the follicular phase of the cycle, salivary progesterone levels did not exceed 150 pmol/l, but in the early luteal phase, concentrations increased with peak values ranging from 300-750 pmol/l around day 21 of the cycle. Progesterone levels declined just before menses and at menstruation were usually less than 150 pmol/l. This cyclic variation in salivary progesterone values throughout the normal menstrual cycle has recently been confirmed by other workers (64). These findings are in marked contrast to those of Gombe (65), who failed to demonstrate a correlation in plasma and salivary progesterone concentrations in women of reproductive age.

Maximum values for salivary progesterone, observed around days 6-8 after ovulation, were in our experience 4 times lower than those of the Italian group. The latter reported peak levels of around 3500 pmol/l and claimed that their values were more in keeping with reported concentrations for the plasma free moiety (66). A recent review (61) indicated a lack of consensus opinion regarding plasma free progesterone concentrations. Reported values vary from 2-10% of total plasma concentrations and are therefore of little help in resolving this anomaly. In the absence of a reference gas chromatography-mass spectrometry technique for salivary progesterone, data made available by Swift (personal communication) are of interest. He found that in normal women (n = 40) in the luteal phase of the cycle, salivary progesterone values determined by RIA varied from 600-1400 pmol/l, a result suggesting possible inaccuracy in the methodology of Luisi et al. (64) or intrinsic racial differences in factors controlling the relationship between plasma and salivary progesterone concentrations.

Fig. 26. a, Plasma and salivary progesterone concentrations throughout the menstrual cycle of a normal female; b, corresponding basal body temperature chart (BBT). × × ×, pyrexia due to laryngitis.

Fig. 27. Salivary progesterone concentrations (mean ± 1 sd) in normal women (n = 15) throughout the menstrual cycle. Day 0 represents the day of ovulation, dated by determination of the midcycle LH surge in plasma in the normal subjects.
Table 8. Type of ovulatory failure in infertile women (n = 20)

<table>
<thead>
<tr>
<th>Type of failure</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteal phase insufficiency</td>
<td>10</td>
</tr>
<tr>
<td>Anovulatory cycles</td>
<td>8</td>
</tr>
<tr>
<td>Hyperprolactinaemia</td>
<td>2</td>
</tr>
</tbody>
</table>

A further point of difference arises when the plasma total progesterone to salivary progesterone ratio throughout the cycle is considered. In our experience this ratio remained virtually unchanged, a finding in keeping with reports indicating that the percentage of unbound progesterone in plasma remains constant throughout the normal menstrual cycle. Others (64), however, have noted a 10-fold variation in this ratio due to the disproportionate increase in salivary progesterone during the luteal phase of the cycle. The reason for this difference remains to be determined.

Clinical studies featuring salivary progesterone

As yet only the preliminary data provided by our group (67) are available for assessing the role of assays for salivary progesterone in clinical studies of female infertility. Women attending infertility clinics were screened and only those known to have no tubular defects or "partner" problems were included in the study. The twenty patients selected for further investigation were divided into groups based on the generally accepted criteria listed in Table 8. All patients collected samples of saliva daily for about 1 month before and during ovulation-induction therapy. Women having luteal phase abnormalities and those with anovulatory cycles received clomiphene citrate treatment, whereas the 2 patients with hyperprolactinemia were treated with bromocriptine.

Luteal phase abnormalities. In some of the patients having luteal phase abnormalities (6 of 10), salivary progesterone levels were only marginally increased in the second half of the cycle, the concentration invariably remaining below the normal range (Fig. 28). The remaining 4 patients with luteal phase abnormalities had pretreatment salivary progesterone concentrations that closely approximated to those of healthy volunteers, except the luteal phase was of a short duration (9.2 ± 1.3 days) compared to that in normal women (13.4 ± 1.7 days). Representative data from 1 of these women are shown in Fig. 29. Some patients with luteal phase abnormalities appeared to respond to a low dose of clomiphene citrate (50 mg), since they achieved levels of salivary progesterone that were within the normal range (Fig. 30). However, other patients failed to respond to even high dosage regimens (150 mg). Progesterone concentrations in saliva samples provided by one patient (S.J.) throughout two consecutive, high dose treatment cycles (Fig. 31) indicate the patterns typical of women failing to respond to clomiphene citrate treatment. One patient in the group, when treated with 100 mg doses of clomiphene citrate, achieved salivary progesterone concentrations...
that were about 3 times higher than those in normal women, a result in keeping with the early classic studies of Ross and co-workers (68), who reported an exaggerated response to clomiphene citrate in some patients.

Anovulatory cycles. Nearly all patients with anovulatory cycles (7 of 8) had low levels of progesterone in saliva; in many instances these values closely approximated to the lower limit of sensitivity of the assay (56 pmol/l). One notable exception, patient A.R., had pretreatment salivary progesterone levels showing a bizarre pattern: levels fluctuated widely, occasionally reaching values similar to those seen in the luteal phase of normal women (Fig. 32).

Hyperprolactinemia. The two patients with hyperprolactinemia were amenorrheic and, as might be expected, had low pretreatment salivary progesterone levels. After low dose (1.25 mg) bromocriptine treatment, the improved ovarian function in both patients was reflected in a corresponding rise in salivary progesterone levels. Patient D.W. responded well to high dose (2.5 mg) bromocriptine, and in the luteal phase of the cycle that followed a short interval of bleeding, salivary progesterone levels were within the normal range and subsequent investigations revealed that the patient conceived during this cycle (Fig. 33). The response of the other hyperprolactinemic patient to high dose bromocriptine was not so marked; salivary progesterone concentrations were originally lower than those observed in normal women and the luteal phase was of short (10 days) duration.

Role of salivary progesterone assays in research programs

Basic studies in reproductive endocrinology.

Current concepts of reproductive endocrinology are very largely based on the hormonal profiles observed in low parity, regularly menstruating women in developed countries. In many developing countries, however, multiparous women of low socioeconomic status have only brief periods of recurring menstruation between pregnancies, due to a prolonged, postpartum lactational amenorrhea. Although in evolutionary terms such patterns might be considered more normal than those of women in developed countries, little is known of the endocrino-

Fig. 30. Salivary progesterone concentrations in an infertile woman (JC), compared to the range (mean ± SD; n = 15) in normal women, before (a) and during (b) attempted induction of ovulation with clomiphene citrate (50 mg/day for days 5–9; Clomid, Richardson-Merrell, Cincinnati, OH).

Fig. 31. Salivary progesterone concentrations compared to the range in normal women (mean ± SD; n = 15) and basal body temperature (BBT) chart of patient S.J. during two consecutive attempts to induce ovulation with clomiphene citrate (Clomid, 150 mg/day on days 5–9).
logical changes in such women. Salivary sampling regimens are ideal for these studies since such procedures are not hindered by the many and various fears associated with the collection of blood samples. Preliminary investigations (69) have indicated that women of low socioeconomic status in Bangladesh have endocrinological patterns very different from those seen in the United Kingdom. Low salivary progesterone concentrations are frequently observed throughout the cycle, suggesting a high incidence of anovulatory cycles or of luteal phase defects. There is also an impression that early spontaneous abortions are common, but these may not be easily distinguished from normal menstruation due to the irregularity of the cycles. A clear understanding of the endocrinological changes occurring in such populations and of the extent to which ill health and malnutrition influences hormonal status clearly requires further extensive study.

Etiology of breast cancer. Salivary sampling regimens may well facilitate investigations into the relationship between menstrual irregularities and the incidence of both benign and neoplastic lesions of the breast. The familial aspect of the incidence of breast cancer may relate to the hereditary nature of certain menstrual abnormalities; fewer premenopausal women with breast cancer are, for example, reported to show a normal secretory endometrium than a corresponding normal group of women (70). Certain “risk factors,” generally considered to be associated with the development of breast cancer, could be related to luteal phase defects occurring throughout reproductive life (71). The risk of breast cancer is reduced by early full term pregnancy (72) and is increased in nulliparous women (73). Early pregnancy indicates the establishment of effective ovulation and luteal function, whereas late pregnancy might be associated with subnormal fertility. Investigations of normal women, utilizing assays for salivary progesterone and salivary estradiol (see below), may provide direct evidence for a link between infertility and an increased risk of breast cancer.

The suitability of salivary assays in extended studies is particularly marked since they allow, for example, comparison of luteal function throughout consecutive menstrual cycles (Fig. 34). One adult volunteer (subject A.R.) was found to have an exceptionally regular pattern, but other healthy women were more like subjects A.C. and M.A.I. in having a somewhat greater variation in timing of ovulation and in the activity of the corpus luteum.

Hormonal changes throughout adolescence. Abnormal patterns in ovarian activity are thought to occur for some years after menarche, and anovulatory cycles are relatively frequent. Only limited data are available on hormonal patterns at this phase of reproductive life, due to difficulties in collecting either daily plasma or urine samples. Our experience suggests that measurement of salivary steroids is of value in monitoring changes in hormonal status in young girls over long time intervals. Study of a premenarcheal girl, aged 11½ yr at the start of the study, who collected saliva daily at 0800 h for 9 months, indicated that salivary progesterone concentrations occasionally reached values associated with the luteal phase of normal postmenarcheal women (Fig. 35), but with no apparent circatrigintan rhythm. Studies of normal post-menarcheal girls have suggested a similar elevated, nonrhythmic profile immediately after menarche (Fig. 36), with levels of salivary progesterone falling with increasing postmenarcheal age. The data at 2 yr post menarche are compatible with those of an anovulatory cycle in an adult.

Salivary progesterone concentrations have also been determined in girls with CAH due to a defect in the C-21 hydroxylase enzyme system. One of these girls (patient C.B.), although having regular intervals of bleeding, had salivary progesterone levels that were well below the normal range (Fig. 37). These values closely approximated to those seen in some infertile women known to have luteal phase abnormalities. This patient was well
controlled clinically and salivary 17α-hydroxyprogesterone concentrations in the luteal phase of the cycle approximated to those observed in normal women. Another, poorly controlled CAH patient (C.H.), who when well controlled reported regular intervals of bleeding, had, as might be expected, no regular pattern in salivary progesterone concentrations (Fig. 38).

In the decade before the menopause, as at menarche, abnormal endocrine patterns are frequently observed. It is considered possible that abnormality in hormonal patterns during this phase of reproductive life may also relate to the etiology of breast cancer. Salivary sampling regimens, which offer a simple, economical method of monitoring endocrine status, may therefore be useful in determining whether patient selection for intensive screening of breast cancer is feasible.

**Estrogens**

Assays for determining salivary concentrations of the three classic estrogens estrone, estradiol and estriol, have been developed, and preliminary studies indicate the possible usefulness of these procedures.

**Estrone**

Estrone concentrations have been determined in saliva samples of normal men (74). In the matched plasma and saliva samples provided by a group of 14 normal men aged 18–33 yr, concentrations of estrone ranged from 28.2–83.7 pmol/l in saliva vs. 92–250 pmol/l in plasma; plasma and salivary levels showed excellent correlation ($r = 0.95$, $y = 0.331$, $x = 1.244$). However, the clinical relevance of this procedure is not immediately obvious.
More recent work by this group (75) indicated that women of proven fertility and infertile women had salivary patterns of estrone that showed no significant differences; normal values ranged from 33.0–86.3 pmol/l vs. 31.5–100 pmol/l in infertile women.

Studies by Elattar (76) relate to the metabolism of steroids including estrone by normal human saliva and saliva collected from patients with chronic gingival inflammation. No estrone metabolites were detected in the saliva of subjects with normal gingiva, whereas in the inflamed saliva of one patient limited conversion (1.82%) to estradiol was observed.

Estradiol

Current initial investigations of ovarian dysfunction frequently include measurement of estradiol concentrations in either plasma or urine, although recent reports suggest that salivary levels of this hormone may be of value (75). Salivary estradiol levels throughout the normal menstrual cycle have, like those in plasma or urine, a biphasic pattern (Fig. 39), with the peak values, observed in the follicular and the luteal phase of the cycle, being around 20 pmol/l (77, 78). Measurement of these low levels of estradiol in saliva by conventional RIAs is
technically difficult and offsets the advantage of easy sampling. This limitation may only be temporary since immunoassays with chemiluminescent end points may have improved sensitivity (79).

Time taken for improving the methodology for salivary estradiol seems justified because these assays may well make a significant contribution to future breast cancer research programs. Although the determination of total plasma estrogen levels has not given any indication of how they may be related to the etiology of breast cancer (80), high plasma free estradiol concentrations (81, 82) have recently been reported in patients having this disease. Since salivary steroid concentrations reflect the free

![Graph 36](image1)

**Fig. 36.** Salivary progesterone concentrations over a 30-day time span in young normal females of varying postmenarcheal ages. The shaded area represents intervals of menses. Y, Year.

![Graph 37](image2)

**Fig. 37.** Variation throughout a 30-day study, between menses, of salivary progesterone (—) and 17α-hydroxyprogesterone (17-OHP; ••••) in a well controlled patient (C.B.) with CAH. EZ, Normal range for salivary progesterone concentrations (n = 10).

![Graph 38](image3)

**Fig. 38.** Variation throughout a 30-day study of salivary progesterone (—) and 17α-hydroxyprogesterone (17-OHP; ••••) in a poorly controlled subject (C.H.) with CAH. EZ. Normal range of salivary progesterone concentrations (n = 10).
fraction in plasma, the determination of salivary levels of
this hormonally active steroid, considered in conjunction
with the frequent easy sampling of saliva, offers a new
approach to studies relating to the growth and develop-
ment of hormone-dependent tumors of the breast.

**Estriol**

The clinical usefulness of assays for estriol in assessing
fetal well being is still a matter of some debate. Well
conducted clinical trials featuring daily plasma or urinary
sample collections provide encouraging results, whereas
those derived from routine studies that rely on samples
collected once or at most twice per week are disappoint-
ing. According to Goebelsmann (83), the infrequent sam-
ping regimens used in routine practice may be largely
responsible for these conflicting results. Since saliva sam-
ple collections are easily collected by the patients them-
sevels, measurement of salivary estriol may be clinically useful.

**Assays for salivary estriol/salivary estrogens in preg-
nancy.** Early assays for salivary estrogens in pregnancy
used nonspecific antisera raised against 17β-estradiol-
hemisuccinate conjugates (84, 85). This is in marked
contrast to more recent procedures that use specific
antiestriol sera (86, 87) or a commercial kit (5). Most
workers have determined the free steroid in saliva, but
Fischer-Rasmussen and his colleagues (87) have included
a β-glucuronidase hydrolysis step in their methodology.

**Salivary estriol values in normal pregnancy.** The ranges
of salivary estriol concentrations observed during the
third trimester of normal pregnancy are remarkably
consistent and are about 1% of total plasma values, i.e. 10% of
unconjugated plasma estriol. Although Fischer-Ras-
mussen et al. (87) measured total salivary estriol, their
results (2.8–7.2 nmol/l) are not appreciably higher than
those of others who measured only the free steroid in

---

**Fig. 39.** The concentration of estradiol (mean ± SEM) in saliva at each
day of the menstrual cycle relative to the day of the plasma LH peak
in 10 subjects in whom the LH peak was detected (77).

**Fig. 40.** Provisional normal range (□□□) for salivary free estriol, constructed retrospectively from results from women who had given birth to a
normal infant. Mean concentrations from thrice-daily samples are plotted for two other such women (●, ○) through the third trimester of pregnancy.
saliva [2.0–6.8 nmol/l (86); 1.44–8.33 nmol/l (5)]. This could be because concentrations of estriol conjugates in saliva are low, and samples assayed with and without hydrolysis show no significant differences (Truran, P. L., personal communication). Heap and Broad (84), using a nonspecific antiserum, report values for total estrogen in late pregnancy of 0.11–1.55 nmol/l. These lower values appear anomalous since they are obtained with the use of a nonspecific antiserum.

Estriol concentrations in samples of saliva collected at intervals (0900, 1400, 2100 h) throughout the day showed considerable variation (20%) even in normal pregnancy. This finding suggests that measurement of estriol in an aliquot from three pooled samples would facilitate clinical studies without imposing undue strain on laboratory facilities. The smoothly rising mean salivary free unconjugated estriol concentrations in the daily samples, collected from 30 weeks to term in two normal pregnancies (Fig. 40), illustrate the value of this approach. Other reports on the possible utility of salivary estriol assays have shown a parallel rise between salivary and plasma estriol as gestation progresses (5) and a significant correlation between salivary and plasma estriol concentrations (87). It may therefore be assumed that low maternal plasma estriol concentrations due to a poorly functioning feto-placental unit would be reflected in low salivary levels of this steroid. This assumption awaits confirmation.

**Long Term Control of Endocrine Systems**

The possibility of long term control and manipulation of endocrine systems is well illustrated by the use of synthetic steroids in fertility control programs. Assays for synthetic steroids in saliva may have an important role to play in the design of new contraceptive formulations tailored to the needs of different ethnic groups. Such assays would be useful in initial pharmacokinetic studies and could prove vital in large scale field investigations, since in many developing countries, various cultural and religious taboos make plasma-sampling regimens almost impossible.

Recent studies (88) have shown that after oral administration of a norethisterone-containing contraceptive preparation (norethisterone, 500 µg; ethynyl estradiol, 35 µg), the changing concentrations of norethisterone in plasma are quickly and accurately reflected in saliva (Fig. 41). Peak values were observed in both plasma and saliva within 2 h of administration; in plasma these values were around 30 nmol/l, but those in saliva were far lower, being only about 700 pmol/l. Medroxyprogesterone acetate, like norethisterone, is also widely used in contraceptive preparations. After oral administration of medroxyprogesterone acetate (5 mg; Provera, Upjohn Co., Kalama zoo, MI) the changing concentrations of this progestagen in plasma (Fig. 42) were quickly paralleled by corresponding changes in salivary values (89). The low concentrations of medroxyprogesterone acetate and norethisterone in saliva are consistent with studies indicating that synthetic steroids, like the naturally occurring hormones, are extensively bound by plasma proteins (90). They are also consistent with more recent work (91), indicating an excellent correlation in plasma free and salivary norethisterone values. Measurable amounts of norethisterone were present in small aliquots of saliva (100 µl) for 6–14 h after dosage with an appropriate preparation (norethisterone, 500 µg; ethynyl estradiol, 35 µg). Since determination of norethisterone in larger volumes of saliva could facilitate distinction from zero for longer time intervals, salivary sampling may be useful in checks on patient compliance.

Recent studies (89) indicate that salivary sampling regimens may also be used to assess the effect of a given norethisterone-containing preparation on ovarian function. A group of healthy women who were known to be menstruating normally provided matched samples of plasma and saliva both before and after taking a norethisterone-only formulation (norethisterone, 500 µg). Sam-
Fig. 43. Concentrations of progesterone in plasma and saliva throughout a control cycle and of progesterone and norethisterone (NET) in plasma and saliva throughout a cycle when NET (500 µg/day) was taken in two female subjects.

Figures show the concentration of progesterone and NET in plasma and saliva over the course of the cycle. The graphs illustrate the differences in concentrations between the control cycle and the cycle with NET treatment.

In women in developing countries the incidence of general intestinal tract infections, tuberculosis, and other disease states is high. These conditions themselves, or medications given as treatment, may alter the bioavailable concentrations of contraceptive steroids. There is, therefore, a need to reevaluate the efficacy of contraceptive formulations under local conditions. In such projects salivary sampling could be invaluable.

Discussion

The limitations associated with plasma sampling and the measurement of total circulating steroid concentrations have become increasingly apparent over the last decade. Techniques similar to those used for assessing free T4 in plasma (12, 13) have not yet been developed for steroid hormones. This could be due to the relatively low specific activity of suitable marker steroids. In this context, the resurgence of interest in the measurement of urinary and salivary steroids is understandable. The replacement of old, nonspecific colorimetric group assays for urinary steroids by relatively specific immunoassays has done much to make urinary sampling more accepta-
ble. The high concentrations of steroid conjugates present in urine facilitate development of simple, direct immunonassays (92). However, the possibility that urinary steroid conjugates may be derived from compounds other than free hormones circulating in plasma, when considered in relation to the limitations associated with single, early morning sampling regimens, could restrict the usefulness of urinary sampling procedures. The alternative approach to noninvasive sampling featuring saliva is therefore attractive, but is as yet not fully established. The increasing number of research groups currently working in this area suggests that, with the development of sensitive immunonassays, this technique is likely to play a permanent and important role both in routine practice and in endocrinological research.

References

14. Robbins J, Rall JE 1957 The interaction of thyroid hormones and protein in biological fluids. Recent Prog Horm Res 13:161
18. Shannon LL, Prigmore JR, Brooks RA, Feller RP 1959 The 17-

hydroxycorticosteroids of parotid fluid, serum and urine following intramuscular administration of repository corticotropin. J Clin Endocrinol Metab 19:1477
41. Hughes IA, Winter JSD 1976 The application of a serum 17a-hydroxyprogesterone radioimmunoassay to the diagnosis and management of congenital adrenal hyperplasia. J Pediatr 88:766
42. Bacon GE, Spenser ML, Kelch RP 1977 Effect of cortisol treatment on hormonal relationships in congenital adrenal hyperplasia. Clin Endocrinol (Oxf) 6:113
43. Walker RF, Hughes IA, Riad-Fahmy D 1979 Salivary 17a-hydroxyprogesterone in congenital adrenal hyperplasia. Clin Endocrinol (Oxf) 11:831


52. Turkes AO, Turkes A, Joyce BG, Riad-Fahmy D 1980 A sensitive enzymeimmunoassay with a fluorometric end-point for the determination of testosterone in female plasma and saliva. Steroids 35:69


60. Turkes A, Turkes AO, Joyce BG, Read GF, Riad-Fahmy D 1979 A sensitive enzymeimmunoassay for testosterone in plasma and saliva. Steroids 33:347


70. Gratton R 1964 The premenstrual endometrial pattern of women with breast cancer. Cancer 17:1119


73. MacMahon B 1986 Cohort fertility and increasing breast cancer incidence. Cancer 57:1350


78. Chersiuk SL, Rincón-Rodríguez I, Solt SB, Donaldson, A, Jeffcoat SI, Simple, direct assays for the measurement of estradiol and progesterone in saliva. In: Radioimmunoassay and Related Procedures in Medicine, IAEA, Vienna, in press


91. Odlin V, Johansson EDB 1981 Free norethisterone as reflected by saliva concentrations of norethisterone during oral contraceptive use. Acta Endocrinol (Copenh) 98:470


CIRCADIAN RHYTHMS OF TESTOSTERONE AND CORTISOL IN SALIVA: EFFECTS OF ACTIVITY-PHASE SHIFTS AND CONTINUOUS DAYLIGHT

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The circadian variation of cortisol secretion by the human adrenal is well documented (Hellman et al., Krieger et al.). This inherent circadian variation, although not directly related to sleep, can be modified by alterations in the sleep-wake span (Migeon et al., Martel et al., Krieger and Rizzo). It is now generally accepted that testosterone concentrations in plasma exhibit a circadian variation, concentrations of testosterone being highest in the morning and lowest in the evening. The mechanism(s) responsible for the 'nocturnal' increase in testosterone, are, however, not understood, although it does appear that the variation in testosterone concentrations throughout the day may be a reflection of changes in the rate of secretion of testosterone by the testis. Various factors have been implicated including the episodic secretion of luteinizing hormone (Murray and Corker, Naftolin et al.), the nocturnal increase in prolactin (Rubin et al.) or changes in the blood flow through the gonads related to the activity of the sympathetic nervous system and adrenal medulla at different times of the day (Lincoln et al.). Perturbation of normal sleep or alteration of the daily sleep-wake schedule has been shown to affect the characteristics of the circadian variation of both cortisol and testosterone (Rowe et al., Weitzman et al.).

Recent investigations at the Tenovus Institute, Cardiff, have demonstrated a circadian variation in salivary testosterone concentrations similar to that reported for plasma testosterone (Walker et al.). A well-defined circadian rhythm has also been shown for salivary cortisol concentrations (Walker et al.).

Key-words: Circadian rhythms; Cortisol; Men; Phase shift; Resynchronization; Saliva; Sleep-wake schedule; Testosterone.

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The effect of continuous daylight and a phase shift in the sleep-wake schedule on circadian secretions of cortisol and testosterone, as judged from the levels of these steroids in saliva, were studied to gain further insight into the mechanism(s) concerned with nocturnal changes in their secretion.

The study reported here describes rhythms of testosterone and cortisol concentrations in saliva samples collected from healthy male subjects: 1. while on a normal sleep-wake schedule in Britain, 2. during a similar schedule in continuous daylight, 3. during an 8-h phase shift in sleep-wake schedule under these conditions, and 4. finally, after a return to the normal sleep-wake schedule.

MATERIALS AND METHODS

Subjects

The subjects were 4 healthy men (3 aged 19 years and one aged 33 years) who were members of a mountaineering and scientific expedition to the West coast of Spitzbergen (15°E, 78°N) during the summer of 1979.

The setting

Spitzbergen is an island situated some 600 miles north of the North Cape of Norway and is about 700 miles from the North Pole. Although so far north, the climate on the West coast is influenced by the Gulf Stream so that the temperatures, at the time at which this study was performed (late July and early August) were about 5°-10° C. Daylight was continuous and there was no significant circadian variation either in temperature or in light intensity. Subjects slept in tents under conditions of diffused light and were generally more active than in Britain. They were socially isolated from other communities.

Sampling procedure

Saliva samples were collected in plain glass tubes. They were taken during normal waking hours, every 2 h for 2 days, 1 week before leaving Britain (phase A). This sampling procedure was repeated 1 week and 2 weeks after arrival in Spitzbergen (phase B and C respectively) and 1 month after return to Britain (phase E). For their first 3 weeks in Spitzbergen the subjects followed a normal sleep-wake schedule of rising at about 0800 and going to bed at about 2300-0000. After 3 weeks, this schedule was suddenly shifted 8 h out of phase when the subjects retired to bed at 1600 and arose at 0000. This schedule (phase D) continued for 4 days and saliva samples were collected over this period at intervals of 2 h or 4 h.

Storage of samples

Samples collected in Britain were deep-frozen immediately and remained so until analysis. In Spitzbergen this procedure was impracticable. Samples were therefore buried in snow banks or immersed in glacial streams until they could be taken into the main settlement where they were deep frozen. This usually occurred at intervals of about a week. The samples were then transferred deep-frozen to Britain for analysis.
Fig. 1 - Concentration (mean ± SD) of testosterone (■—) and cortisol (□—) in saliva collected from healthy male subjects (n = 4) during spans A-E; for comparative purposes only the first 48 h span is illustrated for D. Concentrations are expressed as a percentage of their daily mean values (100%). The shaded area represents sleep spans.
The effects of storage and shipping on the saliva samples were investigated by comparing levels of testosterone and cortisol in saliva samples collected and analyzed in Britain before departure with duplicates taken to Spitzbergen by the subjects, placed with the samples collected there, and shipped back to Britain where they were analyzed. There was no statistical difference between levels of testosterone or cortisol in the samples analyzed before going to Spitzbergen and again on return to Britain.

Radioimmunoassay of cortisol and testosterone in saliva

These have been previously described (Walker et al., Walker et al.) and the radioimmunoassay data processing carried out using the procedure of Rodbard and Hutt.

Statistical analysis of the salivary hormone data

Data have been analyzed graphically and the group mean cosinor technique has been used to elicit estimates for rhythm parameters for testosterone and cortisol data obtained for each phase of sample collection. Tests of significance for the presence of circadian rhythms and their comparisons have been in concordance with those published elsewhere (Halberg et al.). Serial section analysis was also performed on the data for phase D of the study to test the stationarity of the hormone rhythms.

Testosterone rhythms: group cosinor analysis

Group cosinor analyses were used to analyze testosterone concentrations in each of the study phases, A-E, investigated and a statistical summary is shown in Tab. 1. Analysis of the data for all study phases indicated a highly significant circadian rhythm (p < 0.001). Mesors were equivocal for phases in the U.K. before (A) and after (E) the expedition and there may be a negative trend in relation to the duration of subjects' stay in Spitzbergen. Amplitudes were equivocal for all phases except B and in this case amplitude and acrophase were significantly different as compared to those obtained for the U.K.

<table>
<thead>
<tr>
<th>phase of study</th>
<th>mesor (SEM) pmol/L</th>
<th>amplitude (95% CL) pmol/L</th>
<th>acrophase (95% CL) reference to midnight</th>
<th>p-value for 24-h period</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>224 (8.1)</td>
<td>41 (18-68)</td>
<td>-99 (-149 - 66)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>B</td>
<td>228 (17.0)</td>
<td>109 (47-172)</td>
<td>-51 (-74 - 25)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>C</td>
<td>182 (6.6)</td>
<td>53 (33-75)</td>
<td>-110 (-137 - 90)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>D</td>
<td>141 (5.1)</td>
<td>40 (24-57)</td>
<td>-19 (-41 - 346)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>E</td>
<td>225 (6.8)</td>
<td>42 (23-63)</td>
<td>-119 (-158 - 88)</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

Tab. 1 - Group cosinor analysis of data from 4 subjects. Circadian rhythm parameters and associated uncertainties for concentrations of salivary testosterone estimated from samples collected over 48 h and 96 h spans for phases A, B, C, E and D of the study, respectively. Phases A and E refer to a 48 h span prior to and 1 month following the Spitzbergen expedition. Phases B and C refer to the same span 1 and 2 weeks after domicile in Spitzbergen respectively, and phase D is the 96-h span with the 8-h phase shift 3 weeks after domicile in Spitzbergen.
DISCUSSION

Statistical evaluation of a circadian rhythm, employing a longitudinal study, of necessity requires a frequent, serial-sampling regimen. Previous investigations have demonstrated the value of salivary steroid determinations in the assessment of adrenal function (Walker et al. 16, 18) and of gonadal function (Walker et al. 17, 19). Collection of saliva samples for the characterization of biological rhythms is eminently suitable for studies like those reported here, since biological samples may be collected by the participants themselves by a non-invasive, stress-free procedure which does not cause undue perturbation in their activity patterns.

<table>
<thead>
<tr>
<th>phase of study</th>
<th>mesor (SEM) nmol/L</th>
<th>amplitude (95% CL) nmol/L</th>
<th>acrophase (95% CL) reference to midnight</th>
<th>p-value for 24 h period</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.28 (0.456)</td>
<td>2.49 (1.23-3.86)</td>
<td>-114° (-158° -80°)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>6.12 (0.662)</td>
<td>3.49 (1.64-5.53)</td>
<td>-123° (-171° -88°)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>5.36 (0.553)</td>
<td>2.84 (1.10-4.75)</td>
<td>-114° (-164° -81°)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>D</td>
<td>5.45 (0.375)</td>
<td>3.35 (2.02-4.69)</td>
<td>-73° (-94° -50°)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>E</td>
<td>4.78 (0.549)</td>
<td>2.90 (1.43-4.48)</td>
<td>-122° (-166° -86°)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Tab. 2. - Group cosinor analysis of data from 4 subjects. Circadian rhythm parameters and associated uncertainties for concentrations of salivary cortisol estimated from samples collected over 48 h and 96 h spans for phases A, B, C, E and D of the study, respectively. Phases A and E refer to a 48-h span prior to and 1 month following the Spitzbergen expedition. Phases B and C refer to the same span 1 and 2 weeks after domicile in Spitzbergen respectively, and phase D is the 96-h span with the 8-h phase shift 3 weeks after domicile in Spitzbergen.

phases A and E (p<0.05). When group acrophases were compared, a highly significant difference was observed for phases C and D (p<0.01). The 8-h shift in sleep-wake schedule resulted in a mean 6.4 h shift in testosterone acrophase. Serial section analysis of window length and increment of 24 h failed to demonstrate any statistical difference in rhythm parameters for phase D data.

Cortisol rhythms: group cosinor analysis

Group cosinor analyses were performed as described for testosterone and results are displayed in Tab. 2. Data reveal highly significant circadian rhythms (p<0.001) for all the study phases investigated. Meters, amplitudes and acrophases were considered to be statistically equivocal (p>0.05) except for acrophase values concerning phases C and D where a difference of 2.7 h was significant (p<0.05). It was also interesting to note that the mesor and amplitude appeared to be elevated, indicative perhaps of stress in a hostile environment. Serial section analysis, as described for testosterone, again failed to reveal any systematic changes in rhythm parameters that might indicate non-stationarity of the rhythm.
For the group of subjects studied a statistically significant circadian rhythm was demonstrated for both salivary cortisol and testosterone by cosinor analysis of the data. Previous studies (Weitzman et al.\textsuperscript{39}) have demonstrated a persistence of the circadian rhythm of plasma cortisol concentrations under conditions of continuous daylight within the Arctic Circle except for the fact that subjects slept in darkened rooms. Manipulation of the day-length under these conditions has also confirmed that there is a regular circadian rhythm of cortisol in plasma (Simpson\textsuperscript{19}). The 24-h cycle of cortisol has, however, been shown to undergo a 180° phase inversion with reversal of sleep-wake rhythms and to be partially entrained to the non-24 h sleep-wake rhythms (Migeon et al.\textsuperscript{3}, Martel et al.\textsuperscript{8}, Krieger and Rizzo\textsuperscript{4}, Crafts et al.\textsuperscript{1}, Hale et al.\textsuperscript{9}). These studies of phase shift and change in day-length periodicity have clearly shown that dissociation and desynchrony in the rate of adaptation of multiple physiological variables do occur, and that the greater the departure from the 'normal' 24 h day, the greater the resistance to adaption.

The subjects studied in this report collected saliva samples immediately after an 8-h phase shift in their normal sleep-wake span. As in the investigations of plasma cortisol, a persistence was observed in the circadian rhythm of salivary cortisol with no significant alteration in the rhythm parameters except for an approximate 8-h shift of the acrophase.

The phase shift in the salivary testosterone acrophase was, however, more evident than that observed for cortisol and was similar to the actual change in the phase of the sleep-wake schedule. This observation is in agreement with the studies of Rowe et al.\textsuperscript{13} who observed a 5-h phase shift in the circadian rhythm of plasma testosterone in a healthy male subject living on a remote island. When the subject underwent an alteration in his sleep-wake schedule of 5 h, the circadian rhythm of plasma testosterone was observed to have re-aligned itself with the new sleep-wake schedule within 10 days. In the study reported here, all the subjects demonstrated an immediate alteration in the circadian variation in salivary testosterone when subjected to the 8-h phase shift.

It was also interesting to note that mesor values of testosterone in saliva samples collected in Spitzbergen phases C and D were significantly lower than those observed in samples collected in the U.K. The lower values of salivary testosterone concentrations cannot be attributed to the effect of storage on the samples, since no significant degradation in either testosterone or cortisol concentrations could be observed in the control salivas which underwent the same rigours as the samples collected in the 'field'. The volunteers investigated in this study were subjected to both strenuous physical and mental activity during their stay in Spitzbergen. Such fatigue has been reported (Lincoln et al.\textsuperscript{7}) to cause the most conspicuous circadian changes in plasma testosterone concentrations with an evening trough being especially evident. Thus, the observed depression of salivary testosterone mesor values may well be due to the greater activity of these subjects whilst in Spitzbergen.

In conclusion, the results of this investigation would suggest that the early morning rise in salivary testosterone concentrations is related, almost directly, to activity while cortisol rhythms would appear less related to activity and more associated with the resynchronization of the endogenous rhythm of cortisol secretion.
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SUMMARY

The variations in concentrations of testosterone and cortisol in saliva samples collected by 4 healthy young men whilst on a scientific expedition from Britain to Spitzbergen are described. The rhythms observed in steroid concentrations in saliva were characterized mathematically by cosinor analysis of the levels of testosterone and cortisol determined in saliva samples collected before, during and after the expedition. Whilst in Spitzbergen, the subjects were maintained initially on the British sleep-wake schedule which was later subjected to an 8-h phase change. Variations in testosterone concentrations in saliva appeared to be largely related to subject activity rather than any photoperiodic effect with rapid resynchronization of the circadian acrophase in response to the phase shift of the sleep-wake schedule. In contrast, variation in salivary cortisol levels appeared to be less related to activity and more dependent on the slower resynchronization of an endogenous rhythm of cortisol secretion.

REFERENCES


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CIRCADIAN RHYTHMS OF STEROIDS IN SALIVA


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Salivary Cortisol and Dehydroepiandrosterone Sulphate Levels in Postmenopausal Women with Primary Breast Cancer*

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Abstract—Cortisol and dehydroepiandrosterone sulphate (DHAS) were measured in saliva from postmenopausal women with primary breast cancer and a control group of comparable age. Specimens were collected at 2-hr intervals during wake-sleep for two consecutive days, and circadian rhythmicity was demonstrated for each of the hormones in both populations. The marginally elevated levels of cortisol and lower levels of DHAS associated with the cancer group, and the large inter-subject variation, make it unlikely that these hormone measurements would be of value in identifying women at risk of developing breast cancer.

INTRODUCTION

CLASSICAL epidemiological studies in the field of breast cancer clearly imply that endocrine status must be considered a determinant of risk. Early age at menarche is associated with increased risk, whereas early first-term pregnancy is protective [1]. Family history must also be considered a risk factor [2], but equally of interest, however, developing from the earlier 'discriminant function' studies of Hayward, Bulbrook and their colleagues [3, 4], has been the indication that low excretion of 'urinary androgens', in particular aetiocholanolone (<1 mg/24 hr), also constituted a risk factor [5]. A prospective study of women in Guernsey [6] suggested that the precancer subject could be identified from controls on the basis of urinary levels of 17-hydroxycorticosteroid and aetiocholanolone up to a decade before diagnosis [7, 8], and the concept of abnormal adrenal steroid metabolism in the breast cancer subject has been supported by the studies of Deshpande et al. [9].

Furthermore, low plasma concentrations of DHA sulphate, DHA and androster-5ene-3β,17β-diol have been found in women from Guernsey with a high probability of developing breast cancer [10], and these results correlated well with urinary aetiocholanolone excretion. Although Wang originally reported [11] normal plasma levels of DHA sulphate in patients with breast cancer, he was subsequently able to confirm the report from this laboratory [12] that the concentrations of DHA sulphate in plasma from women with advanced disease were subnormal. Rose et al. [13] and Zumoff et al. [14] have since reported that certain breast cancer patients have low levels of this adrenal androgen. The latter study was particularly effective, the authors recognising the unrepresentative nature and difficulties of single-sample analysis and the marked circadian fluctuation of plasma DHA sulphate, reported on a '24-hr mean plasma level' derived from the analysis of a pooled sample from aliquots of 72 specimens collected every 20 min throughout a day. Unfortunately patient numbers in the study were small.

Recent studies from this institute [15] have drawn attention to the value of salivary steroid analyses for the assessment of endocrine status and to their potential in the investigation of breast cancer aetiology. Steroid changes in saliva correlate well with those in plasma, but of particular interest is the close approximation between concentrations of the free 'biologically active' levels of steroid in plasma and the salivary hormone concentration. Such an approach provides a new opportunity to develop further endocrine studies into breast cancer using...
multiple-sampling procedures which are stress-free. Data from single-sample analysis, even collected at a fixed time during the day, can be misleading if rhythmic changes are present [16].

In this study cortisol and DHA sulphate have been measured in saliva collected throughout two days from postmenopausal women with primary breast cancer and from normal controls. The possible identification of a salivary 'discriminant function' could offer the means of recognising the individual high-risk subject, the extensive rhythm-sampling maximising the probability of separating the two groups.

**MATERIALS AND METHODS**

**Subjects**

The subjects were 50 of the volunteer postmenopausal women who had been referred by G.P.s to the Nottingham City Hospital as part of a national breast cancer screening programme. After clinical diagnosis of breast cancer had been made, each patient returned to their domiciliary environment and completed the salivary sampling laid down in the study protocol. Histological confirmation of primary breast cancer was subsequently made from a biopsy specimen prior to surgery, which was 4-6 days after the initial clinical diagnosis. Control subjects were 82 volunteer women who had been visiting a well-woman breast screening clinic for the sole purpose of attending a lecture. These controls had no familial history of breast cancer and no personal history of breast disease or endocrine cancer. Although the controls were not exactly age-matched with the patients, they did, however, span the period from 45 to 66 years of age, covering the major span of the cancer group, which was aged 49-78 years.

**Samples**

Subjects were supplied with an instruction/record card and a compact plastic box containing an insert for holding 20 5-ml glass sample tubes. Samples of whole saliva were taken at 2-hr intervals during wake-span (07.00-23.00 hr) for 2 consecutive days with an additional sample taken at 07.00 hr on the third day. Samples were immediately placed in the plastic box and transferred to the freezing compartment of the subject's domestic refrigerator. Samples were transported to the Tenovus Institute by special courier, where they were stored at -20°C. Immediately prior to analysis samples were thawed and centrifuged at 700 g for 10 min to remove any particulate matter. Experiments designed to simulate the transit and storage of samples revealed no noticeable changes in either cortisol or dehydroepiandrosterone sulphate levels under these conditions.

**Steroid analysis in saliva**

The procedure for cortisol determination has been previously described [17]. The estimated intra- and inter-assay variations over the working range of the standard curve, expressed as % coefficient of variation (% CV), were approximately 3-8 and 10-17 respectively. An assay to determine DHA sulphate concentrations in saliva was developed using sheep antiserum raised against a DHA-3-hemisuccinate ovalbumin conjugate and a [1, 2, 6, 7-3H]-DHA radioligand. This was considered to be a valid assay for DHAS since the DHA/DHAS ratio in saliva is only about 3%. Duplicate aliquots of saliva (50 μl) were added to 100 μl of label (25,000 counts/min; sp. act. 60-90 Ci/mmol) dissolved in phosphate-buffered saline (0.01 M phosphate, 9 g/1 saline, pH 7.4) and the solution incubated for 60 min. Dextran-coated charcoal (1.0 ml, 0.025 g/1 dextran, 2.6 g/1 charcoal) was added, tubes shaken and left at 4°C for 12 min, followed by centrifugation (700 g; 10 min) and the supernatant decanted into scintillant vials for counting. The estimated intra- and inter-assay variations (% CV) for the working range of the standard curve were 3-12 and 7-22 respectively, and the specificity of the antiserum as indicated using the method of Abraham [18] is given in Table 1. A standard curve showing the precision of the response metemeter is shown in Fig. 1. The assay method was compared with a high-resolution gas chromatography-mass spectrometry procedure established in this institute and a satisfactory correlation (r = 0.8; n = 12) was observed.

**Processing of RIA data and Quality Control**

Procedures for the processing of RIA data have been described elsewhere [19]. Each analytical batch contained 19 samples, taken in order of collection, from one particular subject selected at random from either the cancer or the control group of women. The sequential arrangement of

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydroepiandrosterone (DHA)</td>
<td>100</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>92</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Aetiocholanolone</td>
<td>&lt;1</td>
</tr>
<tr>
<td>17a-Hydroxyprogesterone</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Androsten-5-ene-3β,17β-diol</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>
selected hormone concentrations coefficient assay. The intra-assay Fig. 1. Representative standard curve for DHA sulphate assay. The intra-assay precision of the response meter at selected hormone concentrations are expressed as a % coefficient of variation. B/T represents the ratio of bound counts to total counts in the assay.

standards, subject and quality control samples was as follows: (i) duplicate standard hormone concentrations; (ii) duplicate aliquots of low, medium and high titre quality control samples; (iii) duplicate aliquots of subject samples; and (iv) a repeat arrangement of quality control samples to monitor potential intra-assay drift. Cumulative sum techniques were used to monitor changes in: (i) mean hormone concentrations; (ii) imprecision; and (iii) drift, as described previously [20]. The control parameters and target and control statistics used to monitor analytical performance are described elsewhere for use with a computer program specially developed for radioimmunoassay [21].

RESULTS AND DISCUSSION

Concentrations of cortisol and DHA sulphate in saliva exhibited considerable between-subject variability in both cancer and control patients. Values for the arithmetic and geometric means for cortisol and DHA sulphate in saliva, together with their respective standard errors of the mean, are given in Table 2. These means and standard errors have been calculated from the average of each population computed for each time period.

Graphs of the arithmetic and geometric means, together with the standard error of the mean for each time period, are shown for cortisol and DHA sulphate in Figs 2 and 3 respectively. Chronograms [22], tolerance levels containing 90% of the population with 90% confidence, are shown for cortisol and DHA sulphate in Figs 4 and 5, respectively.

Discriminant function and canonical variate analyses were performed at each time period on the control and cancer populations to determine whether or not a significant separation between the two groups could be achieved based solely on cortisol and DHA sulphate measurements. The analysis was carried out using the General Statistics Program, GENSTAT [23], available on the South West Universities Computer Network in the United Kingdom. Canonical variate analysis, the principles of which have been described elsewhere [19], seeks to determine those linear orthogonal combinations of variates, such as the concentrations of cortisol and DHA sulphate, which maximise the ratio of the between-group to the within-group variances. In the analysis of two groups the first canonical variate is exactly equivalent to Fisher's linear discriminant. Using raw data, little discrimination could be achieved between the two groups at each of the time periods, with the possible exception of 21.00 hr.

Cortisol levels in saliva in both groups of subjects (Fig. 2) exhibited circadian rhythmicity; levels are highest during waking hours and lowest towards 21.00 hr, with signs of elevation at 23.00 hr. It is apparent that the geometric mean, as would be expected a priori, gives lower values than the arithmetic mean. The results indicate that cortisol levels in patients are higher than their controls. Statistical significance, however, remains difficult to assess due to the 'error' structure between individuals.

![Graph](image)

**Table 2. Mean salivary cortisol and DHA sulphate concentrations and standard errors of the mean (SEM) for normal and log-normal distributions for postmenopausal women with primary breast cancer and their controls**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Group</th>
<th>Arithmetic mean</th>
<th>SEM</th>
<th>Geometric mean</th>
<th>± SEM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>control</td>
<td>6.84</td>
<td>0.981</td>
<td>5.89</td>
<td>6.68-5.19</td>
</tr>
<tr>
<td>(nmol/l)</td>
<td>cancer</td>
<td>8.33</td>
<td>0.821</td>
<td>7.71</td>
<td>8.43-7.05</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>control</td>
<td>3.78</td>
<td>0.307</td>
<td>3.68</td>
<td>3.88-3.40</td>
</tr>
<tr>
<td>(nmol/l)</td>
<td>cancer</td>
<td>3.70</td>
<td>0.229</td>
<td>3.55</td>
<td>3.80-3.40</td>
</tr>
</tbody>
</table>

*SEM transposed to arithmetic scale.*
DHA sulphate similarly exhibits circadian rhythmicity although not so pronounced, values again being highest during waking hours. When arithmetic and geometric means are compared it is interesting to note that in the latter case the results indicate that lower levels of DHA sulphate are associated with breast cancer patients, adding support to previous evidence for low androgen production in relation to breast cancer.

It is noticeable that the concentration of DHA sulphate in saliva is only 10% of that calculated for plasma-free DHA sulphate, obtained from literature values for the equilibrium association constant and plasma DHA sulphate and albumin concentrations. Despite the acceptable level of accuracy of the DHA sulphate methodology, failure to demonstrate a high correlation between matched plasma and saliva samples in other studies may indicate that the mechanism of DHA sulphate secretion from the salivary glands is different from that of unconjugated steroids. Until reliable methods for determining free DHA sulphate in plasma become available these discrepancies will remain unresolved.

In conclusion, these studies have demonstrated and quantified circadian variations of cortisol and dehydroepiandrosterone sulphate in postmenopausal women with primary breast cancer and their controls. Furthermore, the advantages of salivary hormone determinations in assessing the endocrine status of individuals, particularly where large numbers of samples are required to investigate chronobiological variations, have been demonstrated. Whilst an increase in salivary cortisol and a decrease in DHAS would be consistent with previous findings, the wide variation in individual concentrations compared to the differences between groups makes it unlikely that the determination of salivary concentrations of cortisol or DHAS would be valuable in identifying a population of women at risk of developing breast cancer.
Cortisol and DHA Sulphate in Breast Cancer

Fig. 3. Graphical representation of (a) arithmetic and (b) geometric mean salivary DHA sulphate concentrations for each population measured at various time intervals during wake-span for 2 consecutive days. Data are for postmenopausal women with primary breast cancer (O--O) and their controls (●--●). Error bars represent standard errors of the mean.

Fig. 4. Salivary cortisol chronodesms for postmenopausal women with primary breast cancer (open columns) and their controls (hatched columns). Chronodesms contain 90% of the respective populations with 90% confidence.

Fig. 5. Salivary DHA sulphate chronodesms for postmenopausal women with primary breast cancer (open columns) and their controls (hatched columns). Chronodesms contain 90% of the respective populations with 90% confidence.
Acknowledgements—We thank our colleague Dr. S. J. Gaskell for the gas chromatography-mass spectrometric analyses, Ms. J. Sanderson and J. Wyatt for recruitment of volunteers and sample collection, and Mrs. J. Nott and Mrs. R. Jones for expert technical assistance.

REFERENCES


II. INVESTIGATIONS RELATING TO CANCER OF THE BREAST.

C. CHRONOBIOLOGY: BREAST SKIN TEMPERATURE RHYTHMS AND BREAST DISEASE.
Breast skin temperature rhythms throughout the menstrual cycle. By D. W. Wilson, G. V. Groom, C. G. Pierrrepoint, E. Phillips, D. R. Fahmy, H. Simpson*, F. Halberg† and K. Griffiths. Tenovus Institute for Cancer Research, The Welsh National School of Medicine, Cardiff, CF4 4XX, *NIH, Bethesda, Maryland, U.S.A. and †Chronobiology Laboratories, University of Minnesota, U.S.A.

Circadian and circatrigintan (around 30 ± 5 days) breast skin temperature rhythms have been examined in a woman throughout an ovulatory cycle by monitoring breast skin temperatures at 30 min intervals daily over a period of 35 days. The subject, aged 37, had had four full-term pregnancies and had otherwise experienced regular 28–30 day ovulatory cycles, monitored by oral temperature, for the past 20 years. She had received no hormone medication during the 12 months before data collection. Ovulation was confirmed by hormone analysis during the time span of temperature measurement.

Since regions of the breast have different sources of blood supply and varying degrees of vascularization (Vorherr, 1974), it was important to attach a temperature sensor to the surface and centre of each of the four breast quadrants. Control sensors were attached over the sternum just above the breasts and also at a point level with the nipple on the side of the left chest wall. All these areas were washed daily to remove dead skin and sensors were attached using hypoallergenic tape. Electronic thermometers were obtained from Light Laboratories, Brighton.

Preliminary data established that identical temperature changes were apparent for the same locations on the contralateral breast and contralateral control areas. The subject, habitually living on a diurnal activity, nocturnal rest schedule, carried out her normal daily life and no attempt was made to control her environment.

Temperature data were subjected to rhythmometric techniques (Halberg, Carandente, Cornélissen & Katinas, 1977), which enabled values for the mesor, amplitude and acrophase of circadian rhythms in skin temperature to be calculated.

Breast sensors in the lower inner, lower outer, upper outer and upper inner quadrants and those on the sternum and chest wall were designated as sensors 1 to 6 respectively. Each area exhibited statistically significant circadian rhythms (P<0.001) in 24 h periods. Serial section (interval of 120 h) analysis also showed that both the circadian mesor and amplitude exhibited a circatrigintan rhythm. Both parameters appeared to peak around mid-cycle, but the amplitude changes were more pronounced. Minimum and maximum mesor values (°C) for sensors 1 to 6 during the cycle were, respectively, 34.01 and 35.29; 34.18 and 35.59; 34.01 and 34.14; 35.0 and 35.4; 33.90 and 34.96 and 34.20 and 34.73. The corresponding values for the amplitudes were 0.49 and 1.42; 0.51 and 1.32; 0.63 and 1.18; 0.34 and 0.64; 0.58 and 1.02 and 0.53 and 0.88.

The circadian rhythms at mid-cycle had a periodicity of 24 h with acrophase values occurring approximately at −323° (where midnight is 00° and 360° = 24 h) with 95% confidence intervals between −316° and −329°. The lower quadrants of the breast exhibited the maximum range of values for the mesors and amplitudes during the menstrual cycle. Except for the upper inner quadrant, these temperature characteristics were more pronounced than the control areas.

Analysis of breast and control skin temperature obtained from a menstrually cyclic woman with regular ovulatory cycles indicates that quantification of rhythms is possible and the time of ovulation can be predicted.

REFERENCES

Prolactin and breast skin temperature rhythms in relation to breast cancer. By M. J. Phillips, D. W. Wilson, D. Riad-Fahmy, G. V. Groom, M. E. A. Phillips, C. G. Pierrepont, H. W. Simpson*, H. W. Holliiday†, R. W. Blamey†, F. Halberg‡ and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX, *Department of Pathology, Royal Infirmary, Glasgow, G4 0SF, †Department of Surgery, City Hospital, Nottingham, NG5 1PB and ‡Chronobiology Laboratories, University of Minnesota, U.S.A.

Circadian rhythms of plasma prolactin and breast skin temperature were studied in postmenopausal women with primary breast cancer (n = 7; aged 65–82 years) together with a volunteer group of premenopausal, postmastectomy women (n = 4; aged 31–43 years; >18 months after surgery). All subjects were in a ward environment (nocturnal rest schedule, 22.00–06.00 h; ambient temperature 21–23 °C) and generally confined to bed for the duration of the study. Blood samples were collected at intervals of 2 h for approximately 2 days through an indwelling catheter in their left antecubital vein. Skin temperatures were monitored on all breasts at intervals of 30 min for 96 h. Equipment and location of the temperature sensors have been described elsewhere (Wilson, Groom, Pierrepont, Phillips, Fahmy, Simpson, Halberg & Griffiths, 1979). Ambient and oral temperatures were monitored every h and the dietary intake schedule controlled. Plasma prolactin and cortisol were measured by radioimmunoassay. Circadian rhythms of prolactin (units (MRC 75/504)/litre) and breast skin temperature (°C) were mathematically characterized, inter alia, by fitting a cosine function to the data (Cosinor analysis).

In the postmastectomy group, all prolactin rhythms were significant (P<0-002), values for the fitted mean, and the amplitude were 0-302 (0-067), 0-214 (0-103) units/l respectively and the time of the crest of the fitted rhythm from midnight was at 02.09 h (1-54 h). Standard deviations are given in parentheses, the acrophase is given in clock time and its s.d. as ‘decimal’ h. Corresponding values for the mesor, amplitude and timing of acrophase for the primary breast cancer group were 0-171 (0-065), 0-121 (0-096) units/l and 23.42 h (3-23 h) respectively, but only two subjects had significant rhythms (P<0-02). Maximum prolactin levels were observed in all subjects between midnight and 06.00 h, but higher mesor values were associated with the premenopausal, postmastectomy group. Circadian rhythms of plasma cortisol indicated that subjects were well synchronized. It is clear that a single blood sample is inadequate in characterizing the prolactin status of an individual.

In the primary cancer group, six out of seven subjects had significant circadian breast skin temperature rhythms (P<0-05) when compared with the contralateral site, the tumour area had an increased mesor value (change = 0-91; s.d. = 0-39), decreased amplitude (change = −0-17; s.d. = 0-12) and a tendency towards an earlier acrophase. These abnormal breast temperature characteristics may be exploited for the early detection of breast cancer. In the postmastectomy women, all subjects had a significant circadian rhythm (P<0-001). Group Cosinor analysis showed that the acrophase of breast temperature occurred at 18.56 h which is much nearer that of the tumour (20.00 h) than the contralateral breast (21.20 h) in women with breast cancer. In conclusion, prolactin and breast skin temperature rhythms have been investigated for potential indices of breast cancer risk.

REFERENCE

Characterisation of breast skin temperature rhythms of women in relation to menstrual status


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Abstract. Circadian breast skin temperature rhythms were characterised throughout the menstrual cycle, for various locations on the left breast of ambulatory women. All subjects exhibited highly significant circadian rhythms ($P < 0.001$). Changes in rhythm parameters, such as the mesor, amplitude and acrophase, were observed during the menstrual cycle. No consistent trend in these rhythm parameters was observed between subjects in relation to menstrual cycle stage. Experimental and statistical techniques used to characterise circadian rhythms in pre-menopausal women were applied to a post-menopausal woman with primary breast cancer. Comparison of rhythm parameters associated with the tumour area and corresponding site on the contralateral breast showed abnormal thermal characteristics such as elevated mesor values, decreased amplitude as well as changes in the timing of the acrophase. These properties may be exploited for the early detection of breast cancer. The project also involved the design and testing of an ambulatory device, known as the 'chronobra', for the measurement of breast skin temperature. The performance of the chronobra was in close agreement with reliable, conventional equipment. The chronobra now allows studies of breast skin temperature rhythms associated with breast disease to be extended.

Experience of human breast cancer has clearly indicated that malignant cells may disseminate at any stage of the disease and not merely when the tumour has reached a certain critical or detectable size (Duncan & Kerr 1976). Early detection of a small tumour may well improve prognosis, but research must be directed to the recognition of 'pre-neoplastic discriminants' of breast biology. Familial relationships (Macklin 1959; Anderson 1976), the different incidences of breast cancer in Western and Asian populations and the risk factors associated with age at first term-birth (MacMahon et al. 1970) or early menarche suggest that high risk groups might be identified. Furthermore, Jensen and her colleagues (Jensen et al. 1976; Brem et al. 1978) have described recognisable pre-neoplastic lesions in the human breast.

There would appear to be a possibility that early detection of breast cancer may be achieved with the aid of an ambulatory monitoring device for the measurement of breast skin temperature (Simpson 1977) provided the appropriate statistical techniques can be used to interpret the critical aspects of the data.

This investigation has the ultimate aim of determining whether specific temporal pathologies precede tumour development and if so, whether this can be exploited for diagnosis. The present report concerns the characterisation of circadian and cir-
catrigintan breast skin temperature rhythms of pre-menopausal women exhibiting normal ovulatory cycles and their relationship to hormonal profiles. The study was directed towards the accumulation of breast temperature data, from groups of subjects, making use of conventional equipment previously described (Wilson et al. 1979). These ‘normal’ data can be presented in the form of cosinor diagrams (Halberg et al. 1967, 1972) and time-qualified reference ranges termed chrono-desms (Halberg et al. 1978) against which results from other groups of women, possibly with early breast cancer, benign breast disease or ‘normal’ subjects from high or low risk groups, can be compared.

The eventual correlation of the breast temperature circadian and circatrigintan rhythms with rhythms of hormone concentrations in plasma and saliva should provide valuable data on the hormonal control of breast physiology. The practical limitations to collecting this type of chronobiological information necessitated the development of an ambulatory device which can automatically measure breast skin temperature and store the data over a substantial time-span. A device known as the ‘chronobra’ has been developed and basic information on this garment and associated electronics is described in this report.

Materials and Methods

Subjects
Nine pre-menopausal, clinically healthy subjects, aged 18–37, who were research staff volunteers from the Tenovus Institute, were studied either throughout the whole (4 subjects), or a large proportion of (5 subjects) a normal menstrual cycle. The latter group was studied during the late follicular-early luteal phase (days 8–23 of the cycle). Breast skin temperature measurements were made on these subjects, as they carried out their normal daily routines. Nocturnal-rest schedules were generally between 23.00 and 07.00 h. Blood and saliva samples, for hormone assay, were collected at 09.00 h on selected days. Oral temperatures were taken daily upon wakening but before rising. On one post-menopausal patient with primary breast cancer, temperature measurements were made at 30 min intervals over a 96 h time-span, prior to surgery. Temperatures were recorded using the manual conventional procedure, as described below, with the patient in a hospital bed.

Measurement of breast skin temperature

(a) Manual procedure. Each day, miniature semi-conductor, temperature sensors (4 mm dia. with the reverse side insulated) connected through fine electrical wire to small plugs, were attached by the subjects to each of the 4 quadrants of the left breast. The axes were defined vertically by the cephalo-caudal line and horizontally by the parasternal to mid-axillary line, the nipple being located at the origin. One sensor was attached to the sternum just above the sinus mammarianum, and another at a point approximately level with the nipple on the mid-axillary line of the left lateral chest wall. Areas of skin against which the sensors were placed, were carefully washed to remove dead tissue. Sensors were attached using hypoallergenic surgical tape (Micropore 3M Co. Ltd., St. Paul, Minnesota, USA). Temperature readings from the 6 sensors were recorded by plugging each in turn into an electronic thermometer (range 32–38°C in 0.05°C divisions: Light Laboratories, Brighton, England) as shown in Fig. 1A. Readings were generally taken at 30 min intervals throughout the course of the study and various activities, such as dietary intake, were recorded. All the sensors were interchangeable within 0.1°C.

(b) Automatic procedure. An ambulatory device, the chronobra, has been designed to automatically monitor and store breast skin temperature. The prototype, shown in Fig. 1B–D, currently provides for 16 channels of data, has a memory capacity of up to 8K bit words and a wide range of sampling times. The device is initialised and functionally checked by an interface unit, (Fig. 1E) which also effects extraction and storage of data from the memory system of the chronobra for subsequent analysis by a main frame- or mini-computer.

Hormone analyses

Blood samples were centrifuged for 5 min at approximately 1000 g within 10 min of collection and the plasma stored at -20°C. Saliva was also stored at -20°C after collection. Plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) were assayed by double antibody radioimmunoassays shown to be specific for these hormones (Groom et al. 1971). Concentrations of progesterone and 17α-hydroxyprogesterone in saliva were determined by procedures recently developed in the Institute (Walker et al. 1978, 1979a,b). Plasma oestradiol-17β concentration was measured by the routine Suprarregional Assay Service Laboratory in the Institute.

Numerical analysis

Breast skin temperature data were subjected to rhythmometry (Halberg et al. 1967, 1972, 1977) in which a cosine function of the form

\[ y(t) = M + A \cos(2\pi t + \phi) + \epsilon(t) \]

was used, with the patient in a hospital bed.
was fitted to the data using the method of least squares. The statistical analysis provided values and fiducial limits for the mesor, M, (mean of the fitted rhythm), amplitude, A, (this is a measure of one half the extent of the rhythmic change in the cycle estimated by the cosine function) and acrophase, φ, (the lag from local midnight to the crest time of the fitted cosine function), respectively. The breast skin temperature at a time t is given by \( y(t) \), the period of the rhythm is denoted by \( T \) and \( y_0 \) is the uncertain or uncontrollable or unobservable errors assumed to be independent normal variables with a mean of zero and similar variance. The percentage variability accounted for by the cosine fit together with the \( P \)-value for the statistical significance of the rhythm were also calculated.

The possibility that circadian rhythm characteristics may change during the menstrual cycle had also to be considered (Simpson & Halberg 1974), and such rhythm parameters were assessed using serial section analysis. A 'window' of fixed dimension was selected and data contained therein, were fitted by a cosine function using a fixed 24-hour period. Values of the rhythm parameters were then calculated. The window, located initially at the beginning of the data train, was then moved in fixed increments along the train and the whole process continued until all data had been scanned. The process is analogous to the more generally used 'moving' average technique (Chatfield 1975).

Circadian rhythms of breast skin temperature were displayed graphically in the form of a cosinor diagram with an attendant table summarising the statistical analysis.

Results

This report describes interim progress in the development and use of the chronobra, which automatically monitors changes in breast skin temperature over relatively long time-spans. At the same time, the potential value of circadian and circatrigintan rhythms of breast skin temperature is clearly illustrated by the preliminary results.

It was established in early experiments using the manual procedure, that the breast skin temperatures for corresponding sites on the left and right breast were similar and consequently, all subsequent measurements were made on the left breast.

Breast skin temperature data were also collected by the manual procedure from subjects studied throughout one menstrual cycle and measurements were taken every 30 min during wake-span. The effect of a gap in the sampling of breast skin temperatures from 23.00 to 07.00 h does produce rhythm parameters which are somewhat different from those collected over a full 24 h. Nevertheless the results obtained are comparable to each other. Fig. 2 shows the analysis of this type of data in the form of a single cosinor diagram for each sensor from one of the subjects studied. Representation of circadian breast skin temperature rhythms is shown in the diagram. The acrophase is displayed by the position of the pointer on the clock and its value is given in both clock time and degrees; the fiducial limits are also indicated. The length of the pointer is scaled according to the amplitude of the rhythm. The table provides a statistical summary of the cosine fit.

All subjects (n = 4) studied for the complete menstrual cycle exhibited a statistically significant circadian rhythm \( P < 0.001 \) with a period of approximately 24 h. The overall mean values for the breast mesor, amplitude and acrophase for these women exhibiting ovulatory cycles, assessed by plasma and saliva hormone concentrations, were 34.13°C, 0.63°C and -316° respectively. All circadian rhythms of breast skin temperature had acrophases approximating to 21.00 h. The acrophase associated with the mid-axillary line occurred approximately one hour earlier and the rhythm had an amplitude that was usually lower than that of the breast. Subjects studied during the late follicular-early luteal phase of the cycle displayed similar rhythm parameters as those previously described.

Changes in circadian rhythm parameters through the menstrual cycle are illustrated in Fig.

---

**Fig. 1.**

A manual and automated procedure for measurement and recording of breast skin temperature. A) Conventional thermometer with sensors taped to the skin and the manual recording of data. B – D) Essential design features of the prototype chronobra with 4 of the 16 channels in use. B) Diagrammatic presentation of sensors located inside the cups; for clarity only 2 sensors are visible and cotton channels, through which sensors are threaded, are omitted. C) The prototype chronobra recording breast skin temperature; sensors, channels and micro-computer are discernible. D) Micro-computer and sensors used to record and store temperature on left (SL1-SL2) and right (SR1-SR2) breast; special function switches, status light and interface lead are evident. E) Interface unit used to extract data from the chronobra at, for example, weekly or monthly intervals.
Cosinor diagram of circadian breast skin temperature rhythms 9 days before and 7 days after the onset of menses. Temperatures were recorded at 30 min intervals during wake-span on a subject with sensors located on the lower inner, lower outer, upper outer and upper inner breast quadrants, the left lateral chest wall and the sternum denoted by the key as A-F respectively. Definitions of the rhythm parameters and descriptions of the cosinor diagram are found in the text and references.

3. Spans of 48, 72, 96 and 120 h (windows) were incremented by 24 h intervals for the serial section analysis and values for the circadian rhythm parameters were calculated for each window throughout the data train. An example of a computer print-out of results from a 120 h serial section analysis of temperature measurements obtained from the lower inner quadrant of the left breast of one subject investigated for 34 days is shown in Fig. 3.

This subject was studied from day 19 of one cycle to day 24 of the next. Hormone analyses indicated that ovulation occurred on day 14. Circadian variation in breast skin temperature can be clearly seen...
in the chronogram (Fig. 3a) which is a plot of the raw temperature data as a function of time. The temperature varied daily from 32°C at 07.00 h to 36°C at 21.00 h. The chronogram indicated a circatrigintan rhythm peaking around the time of menstruation, with a nadir around day 8. Values for the significance of the rhythm ($P < 0.001$), the mesor (33–36°C), the mesor plus amplitude and acrophase, together with standard error, are also displayed in Fig. 3b–d. In an individual, and for each quadrant, these values can be seen to change through the menstrual cycle. Sampling frequency (Fig. 3e) is also displayed in the computer output for each section taken through the menstrual cycle.

Fig. 4 illustrates data on one subject showing hormone concentrations in both saliva and plasma.
oral temperatures and mean mesor temperatures for the breast. The characteristic LH and FSH peaks follow the usual oestradiol-17β surge prior to ovulation. Post-ovulatory rises of progesterone and 17α-hydroxyprogesterone concentrations in saliva were observed in all these subjects, again characteristic of normal ovulatory cycles. Although in this example, a rise in oral temperature after ovulation was clearly demonstrated, this was not the case in all subjects and it would seem that oral temperature is not a reliable indicator of ovulation, particularly in untrained subjects. Detailed salivary hormone analysis will be related to those temperature measurements. The application of rhythmometry of the data obtained from this preliminary study of subjects for one menstrual cycle indicates that while circatrigintan rhythms are evident, the relatively wide range of possible values for the rhythm parameters suggests that interpretation would be more meaningful from data accumulated from at least three consecutive menstrual cycles.

The performance of the chronobra was assessed
against temperature data obtained by the manual procedure in one subject for five weeks. The chronobra was arranged such that 4 sensors were in contact with the left breast. Sensors for the manual procedure were attached immediately adjacent to those of the chronobra and the performance of both systems recorded. Cosinor analysis of the full sets of data were comparable indicating that values for the circadian rhythm parameters were similar (unpublished data). Investigations are currently underway in the Institute to accurately calibrate the chronobra using thermometers certified by the British Standards Institution.

A preliminary study of breast rhythm parameters of a post-menopausal woman with primary breast cancer was encouraging. Sensors were attached to the skin over the tumour and to corresponding sites on the contralateral breast. Temperatures were recorded by the manual procedure and the rhythm characteristics are shown in Fig. 5. The acrophase of the circadian rhythm associated with the tumour is significantly different to that of the contralateral breast as shown in Fig. 6 and occurs approximately 5 h earlier. More detailed studies are in progress to attempt to exploit such differences as a means of detecting early breast cancer.

**Discussion**

The data reported clearly demonstrate the presence of circadian breast skin temperature rhythms in pre-menopausal subjects, studied during wake-span, who were on a normal daily routine. In

![Fig. 5.](image-url) Comparison of circadian breast skin temperature rhythms associated with the area of skin over the tumour and a corresponding site on the contralateral breast of a post-menopausal woman with primary breast cancer. An elevated mesor value, reduced amplitude and earlier acrophase for the tumour area are important findings. Temperatures were collected at 30 min intervals for 96 h throughout wake- and sleep-spans in a ward environment, and represents a cosine fit of the data averaged for duplicate areas on each breast.
Cosinor diagram of circadian breast skin temperature rhythm parameters for duplicate sensors over the tumour and similar sites on the contralateral breast. The mean temperature data for the cancerous and contralateral breast are shown in Fig. 5.

Fig. 6.

<table>
<thead>
<tr>
<th>VARIABLE AND ELLIPSE IDENT.</th>
<th>n OF DATA</th>
<th>MESOR</th>
<th>SEM</th>
<th>AMPLITUDE AND (95% LIMITS)</th>
<th>ACROPHASE AND (95% LIMITS)</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. CANCEROUS 1</td>
<td>191</td>
<td>36.11</td>
<td>0.02</td>
<td>0.23 (0.16, 0.30)</td>
<td>-283 (-265, -301)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B. CANCEROUS 2</td>
<td>191</td>
<td>35.24</td>
<td>0.02</td>
<td>0.28 (0.22, 0.34)</td>
<td>-272 (-258, -286)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C. CONTRALATERAL 1</td>
<td>191</td>
<td>34.73</td>
<td>0.05</td>
<td>0.63 (0.47, 0.79)</td>
<td>-342 (-327, -357)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D. CONTRALATERAL 2</td>
<td>128</td>
<td>34.92</td>
<td>0.06</td>
<td>0.90 (0.68, 1.12)</td>
<td>-360 (-346, -1.13)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Contrast, evidence for a well characterised circatrigintan rhythm is equivocal on the data collected over a time-span of only one menstrual cycle.

The cosinor diagrams described, provide a valuable means by which potential differences between a 'normal' and diseased breast might become evident. Just as a biopsy might be used to assess early breast pathology, so might a thermopsy be used to detect neoplasia before the disease becomes clinically manifest. This preliminary study, now described, provides encouraging evidence that the chronobra may detect early cancer, since in the patient investigated, a definite phase shift of nearly five hours was present in the temperature rhythm associated with the tumour. A major study of patients with breast cancer is now in progress in the hope of establishing the validity of these preliminary data and initial analyses suggest that an 'abnormal' rhythm, relating to the presence of cancer, can be detected.
Such studies support the early work of Mansfield and his colleagues (Mansfield et al. 1973) who studied surface temperatures over the normal and cancerous breast with a view to monitoring hormone therapy. They described certain changes in the rhythm parameters when the control and affected side were compared. Similar support for this concept was provided by surface tumour temperature data obtained by radiotelethermometry (Gautherie & Gros 1971). It is obvious that detailed, carefully controlled studies are required to establish the biological characteristics of the developing tumour, which might allow for early detection, or ultimately identify women with a high risk of developing breast cancer.

The detailed study, now described, set out to define some of the rhythm criteria which should be considered in the establishment of screening programmes. When circadian mesors for selected spans of the data were plotted through the menstrual cycle, there was often a nadir around midcycle as previously reported (Wilson et al. 1979), and circatrigintan rhythms, based solely on the P-value for a cosine fit, were demonstrated (P < 0.001).

To determine whether or not changes in breast skin temperature occur after ovulation, certain improvements to the protocol have been implemented in a study of a group of Cardiff women practising 'natural' family planning methods. The span of the study has been extended to a minimum of three months and the temperature data are being collected using the prototype chronobra which has produced data similar to that employing the manual procedure. The value of salivary steroid analysis to monitor ovulation and the endocrine changes in relation to menstruation is obvious. The assay for oestradiol-17B in saliva, recently developed at the Institute, will also be used. Circadian temperature rhythms in relation to hormone changes as reflected in the concentration of steroid in saliva, and prolactin in plasma, will form a part of the next phase of the study. The fact that the concentration of steroid in saliva is a measure of the free, non-protein bound level in plasma further improves the physiological nature of the study. Special attention is being directed to the design of the chronobra and its insulation in order to substantially reduce environmental noise and this may enable any biorhythms present to be elicited more readily.

This report describes preliminary data which have been obtained from early studies of breast temperature rhythms and it forms the guide-line for the development and use of chronodesms in breast cancer screening.

Acknowledgments
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References


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I. CERTAIN ASPECTS OF PRECANCER BREAST BIOLOGY

A. Precancer Biology: A Key Issue

The average duration of life from the onset of symptoms to death in untreated breast cancer is about three years (see discussion by Bloom et al [1962]). Mastectomy has been the primary treatment of this
disease for over a hundred years but, as an overall figure, only about half of the patients so treated will be alive ten years postoperatively (C.R.C. Trial, 1976). When the mastectomy is complemented by radiotherapy, the chance of local recurrence undoubtedly diminishes but long-term survival is little, if at all, affected (C.R.C. Trial, 1976).

Endocrine strategies of therapy programed by age of the patient or presence of receptors in the cancer [McGuire et al, 1978] are a beneficial therapeutic delaying tactic but again are unlikely to bring about a substantial reduction to the ultimate rate of mortality in those not cured by mastectomy. Whereas treatment is increasingly scientific, the age-adjusted death rate for breast cancer has not noticeably declined in recent decades [Logan, 1975; Thier, 1977]. Screening techniques (including self-examination) may lower mortality but the effect will probably be small. The reason is that as many as 27% of women treated for breast cancers as small as 1 cm in diameter developed disseminated tumor in a 20-year follow-up study [Duncan and Kerr, 1976]; the diagnosis of a tumor smaller than this size is technically difficult, yet the tumor already contains many billions of neoplastic cells which may disseminate at any time if metastasis happens to be a property of the particular malignant-tumor clone. Gullino [1977] has concluded from growth-rate extrapolations that a breast tumor is usually "subclinical" by current diagnostic standards for two-thirds of its total time course.

These remarks focus attention on the importance of studying the precancer biology of the breast in an attempt to reduce mortality. Future breast cancer research should place increasing emphasis on this aspect rather than on early detection or strategies for the eradication of an already established neoplasm. More prospectively oriented studies are needed (see eg. the Guernsey study of Bulbrook and Hayward [1967]).

The pessimist might point out that the incidence of breast cancer is age-linked and that the risk of neoplasm could be irrevocably associated with the aging process, ie, if the tendency for the formation of mutagenic cells were inevitable, there might be no precancer biology, and the only treatment would be preemptive removal of the mammary tissue. Some literature about breast cancer indicates that many think of the disease in this way, but an alternative to this gloomy perspective is offered by the hypothesis that breast cancer may be a focal manifestation of a prolonged mammary disease process, that precancer risk can be detected and that preemptive drug control should be possible. To
accept the former view is to ignore the epidemiological evidence and the experience from studies of cancer induction in an endocrine-sensitive gland as described by classical pathologists. These two lines of evidence will now be discussed and interwoven.

B. The Epidemiologist’s Perspective: The Important Role of the “Active” Ovary in Mammary Carcinogenesis

The current weight of epidemiological evidence indicates that a large proportion of human breast cancers are initiated in the early years of a woman’s life even though promotion and diagnosis of a tumor mass may not occur until many decades later when the woman is near the end of her natural life-span. Early menarche, frequent abortions, infertility, late menopause and late first pregnancy are all associated with increased risk of breast cancer [MacMahon et al, 1973]. Early menarche appears to be associated with a predisposition to develop breast cancer after the age of 45 years [Staszewski, 1971]. However, parity is associated with lower risk (although, paradoxically, in some cases a tumor already present may be rapidly promoted during the pregnancy [Haagensen, 1971]. Ovariectomy is also protective and risk of breast cancer is reduced by 75% in women who have had the operation before the age of 40 years [Feinleib, 1968].

These facts focus sharply on ovarian function as an important influence in breast carcinogenesis; consistent is the low incidence and later age of appearance of breast cancer in males [Haagensen, 1971]. Consistent too, is the fact that males taking exogenous estrogens to effect transexuality may develop breast cancer [Symmers, 1968]. Abundant evidence of the importance of ovarian factors also comes from rodent studies. Thus the classical work of Lathrop and Loeb [1961] demonstrated that the mammary tumor incidence in strains of mice with a high cancer risk is dependent on the age at ovariectomy. It is interesting, too, that mammary cancer may even be induced in male mice that are the recipients of ovarian grafts [Murray, 1928]. It has also been shown that the production of mammary tumors by estrogens is a dose-dependent phenomenon [Shimkin and Wyman, 1945].

Further implication of ovarian function comes from the greater-than-expected tendency of human endometrial and breast cancer to coexist. A 30% increase in carcinoma of the breast over the expected number in women 60 or more years old at the time of diagnosis of endometrial cancer has been reported by MacMahon and Austin [1969]. Both tu-
mors may contain estrogen receptors and the ovary or estrogens from a nonovarian source must be considered as the common link in carcinogenesis.

It is important that risk factors for cancer have similarity with risk factors for hyperplastic cystic disease of the human breast. Both diseases are common in the final reproductive years and both are commoner in unmarried women or in women who have not borne children [Haagensen, 1971]. Also, as mentioned, parity is associated with lowered risk for breast cancer and it is consistent that (clinically) cystic disease tends to disappear in the first half of pregnancy or, if not, during lactation [Lewis and Geschickter, 1934].

We also note Beatson's [1896] classical observations in Glasgow nearly a hundred years ago that the growth of disseminated breast cancer—a tissue derived from breast epithelium—may be (temporarily) checked or reversed by ovariectomy; and lesions such as cranial-nerve palsies due to metastasis may be relieved. In further considering the role of the ovary, one also has to remember that the amount of prolactin is to some extent dependent on the presence of estrogen [Furth, 1972]. This may be due to a suppression of prolactin-inhibiting factor by estrogen and/or a direct stimulating effect on the pituitary prolactin-producing cells [Bloodworth, 1968]. Removal of the ovary then presumably means diminution of estrogen, progesterone, and prolactin. After the menopause, ovarian estrogen production virtually stops and circulating estradiol and estrone are derived from the adrenal; the postmenopausal ovary, however, is responsible for 50% of plasma testosterone and 30% of androstenedione [Vermeulen, 1976], and it is worth noting that testosterone may be a pro-hormone for estradiol in postmenopausal women since it can undergo conversion in peripheral tissues [Lipsett, 1973]. An interesting chronobiological point is that trophic stimuli shift after the menopause from a (primary) menstrual periodicity to a circadian periodicity—the main cycle of the adrenal [Vermeulen, 1976]. The relevance of these postmenopausal factors is not as yet clear in terms of cancer biology.

C. The Pathologist's Perspective: Hyperplasia, a Risk State for Neoplasia

The foregoing epidemiological facts serve to implicate ovarian function as having a key role in mammary carcinogenesis or cocarcinogenesis. A classical pathologist considering breast cancer in this light would certainly expect changes in the target mammary tissue as a prelude to malignant change and, indeed, many have found them. Thus Nicholson [1921] said, "I have insisted for years that hyperplasia passes insensi-
bly into carcinoma and that this gradual change can nowhere be better studied than the breast and that tumour formation is multicentric rather than omnicentric.' Cheatle [1926] stated that he could (sometimes) trace a definite series of epithelial changes in the breast ending in carcinoma. Muir [1941] emphasized the importance of intraductal and intra-acinar epithelial hyperplasia as a possible precursor of breast neoplasia. He also noted that the proliferative changes could be a focal response to the presumptive hormonal stimulus. Willis [1960] emphasizes the dilemma of the pathologist in this regard. "Any competent pathologist who has examined a considerable number of surgically removed breasts will find it difficult to remain patient with those surgeons who deny the precancerous potential of cystic hyperplasia."

Endocrine-oncology abounds with examples of inappropriate hormonal stimulation effecting an adenomatous and sometimes malignant change in the target tissue. The outstanding example is the hyperplasia-adenomatous hyperplasia-adenocarcinoma sequence seen in the endometrium when there is prolonged estrogen stimulation [Novak and Woodruff, 1974; King, 1978] and especially when the estrogen is un-complemented by progesterone. Excessive ACTH in Cushing's syndrome, for example, may result in an "adenomatous" hyperplasia of the adrenal [Symington, 1969]. Adrenal carcinoma has been reported arising in a hyperplastic gland [Anderson et al., 1978]. Pituitary adenomata occur in many of the cases of "pituitary driven" Cushing's syndrome, and they may be due to continued abnormal stimulation of the pituitary by hypothalamic factors. This hypothalamic abnormality could account for the development of (large) ACTH secreting pituitary tumors in patients with Cushing's syndrome who have undergone adrenalectomy—Nelson's syndrome [Christy, 1965]. Cancer of the prostate frequently arises in the organ already the seat of benign nodular enlargement [Willis, 1948], though benign hyperplasia is classically periurethral and cancer peripheral. According to Bloodworth [1968], carcinoma occurs in 5-11% of nodular goiters, which is two to five times the expected occurrence. However, it again should be noted that when cancers do occur in this situation, they are between lobules rather than within lobules of thyroid tissue. Adenocarcinomata of the parathyroids may arise in a primary chief cell hyperplasia [Bloodworth, 1968]. Recently a link has been shown between the use of contraceptive pills and focal nodular hyperplasia of the liver. The hepatocytes, of course, contain specific steroid receptors (as does the breast epithelium). Further, there are case reports of malignant transformation in the nodules or coexistence of benign and malignant lesions [Vana et al., 1977]. This iatrogenic model could have a parallel in breast cancer biology. Anoth-
er relevant point is that the hyperplasia-neoplasia transition is also seen in the mammae of mice bearing the pituitary (prolactin) so-called "mammotrophic tumor" [Furth and Clifton, 1958].

In the context of the hyperplasia-neoplasia pathway, a case worked up by Foote and Stewart [1945] is of interest. A woman of 41 years was put on stilbestrol for one year for menopausal symptoms (total 131 mg). At the end of this time she noticed a left breast nodule which proved to be a 2-cm infiltrating cancer of "not unusual type." The remaining breast tissue showed severe hyperplasia to an extent that mimicked the lactating gland—a change that had never been seen in that pathologist's lab among thousands of mammae examined. Three months later a 2.5-cm infiltrating cancer was removed from the other breast which, being an apocrine-type cancer, was a presumptive new primary. The stilbestrol had been stopped after the first mastectomy and now there was a "considerable reduction in tissue resembling lactating breast." However, all the lesions seen in the other breast were still present to some extent. Foote and Stewart conclude that certain individuals may be hyper-responsive to estrogens. They quote the experiment of Reece [1943], who was able to initiate lactation in sterile heifers by diethylstilbestrol alone. They think that their case lies at the high end of an estrogen sensitivity spectrum and that the occurrence of two histologically dissimilar tumors in a very hyperplastic mass of tissue points strongly to a hyperplasia-neoplasia risk.

In the same context Barnes et al [1979] report a case of bilateral duct carcinomata, one of which was positive for both estrogen and progesterone receptors while the other was negative for both receptors. Such cases serve to hint that breast cancer is a focal manifestation of diseased mammary tissue in which hormones play an important role. The early literature on the risk of bilateral breast cancer has been well reviewed, and "general" figures for all types of breast cancer have been assessed at 9%, 6%, 5%, 3%, and 6%, whereas whole-organ sections showed that as many as 50% contained multiple independent foci [Reviewed by Sandison, 1962].

The fact is, though, that a transition from hyperplasia can be traced in only some breast cancer cases, and this aspect has led some writers to suggest that hyperplasia is sometimes a collateral manifestation of a neoplastic stimulus rather than lying on the direct path [Foulds, 1958]. Study of 300 radical mastectomies for carcinoma, 200 mastectomies for nonmalignant conditions, and 27 autopsy breasts led Stewart to conclude that "the female breast is a precancerous organ" (!) [Stewart, 1950; Foote and Stewart, 1945]. This remark (of despair?) perhaps exemplifies the mistake of trying to reconstruct events from one moment of time rather than postulating a dynamic series of changes in
which the antecedent event may have long since disappeared. With the hindsight of epidemiological facts on the time scale of breast cancer, and the importance of the ovary, one would now expect only the premenopausal cases to show the putative hyperplastic lesions; in the postmenopausal cases the withdrawal of ovarian hormone support would likely leave the neoplastic clone on its own. Why should the postmenopausal female breast have a higher cancer risk than the male breast if the antecedent reproductive span was not important? This hypothesis does not seem to be specifically discussed in previous literature; it is with the investigation of this concept that the current chapter is concerned.

It might be argued from the foregoing that if hyperplasia was always a risk state, then the physiological hyperplasia of pregnancy might be followed by greater risk of cancer. But as has already been pointed out, pregnancy is associated with lowered risk for the subsequent development of breast cancer. The key issue seems to be whether the hormone is provided in the right amount at the right time and whether the mammary lobules move forward in unison through successive menstrual cycles to anticipate the next phase. An attractive hypothesis is that the growing buds of mammary lobules exhibit menstrual cycles of proliferation and "atrophy" (eg, apoptosis) from the onset of puberty. As the reproductive life-span extends, there is a tendency for individual units to become out of phase. Menstrual cycles of hormones continue, however, and consequently individual mammary growth units are stimulated whether or not they are "out of phase." This imbalance is manifest clinically as the various forms of hyperplastic cystic disease and an increased risk of mutation in "out-of-phase" units. Analogy might be drawn with the adenomatous prostate or thyroid; both of these are uncommon in the young gland. In the case of pregnancy, lobules are brought into phase by the exceptional and sustained hormone levels and hence cancer risk is reduced because of the phase reset in all units—that is, provided the cancer is not already initiated. Alternatively the hyperplasia could represent a focally increased sensitivity following alteration in steroid receptor synthesis due to an oncogenic virus or other mutagenic agent [Furth, 1972].

D. A Census of Hyperplasia in Pericancerous Breast Tissue:
Glasgow Royal Infirmary 1976–77

This search was prompted by the issues discussed above together with clinical observations made in the course of breast cancer diagnosis. Specifically we noted that in some breast cancer cases there was a gradual spectrum of transition from hyperplasia to neoplasia exactly as
reported by earlier pathologists. We realized in addition that such cases were strongly clustered in the paramenopausal years. Figures 1–7 exemplify the type of change. All breasts considered contained an infiltrating cancer (except Figure 6, which is equivocal for carcinoma), but this is not necessarily shown in the particular section. It can be seen that the pericancerous breast tissue is involved in a hyperplastic re-

Figs. 1–8. Mastectomy histopathology illustrating the borderlands of hyperplasia and neoplasia. In all cases illustrated an infiltrative cancer was present somewhere in the breast (except Figure 6, where this diagnosis was difficult). These selected fields emphasize that there can be continuous histological spectrum from hyperplasia to neoplasia and that a distinction may be impossible using classical morphology. This fact is underscored by the independent pathological review of 506 breast cancers detected by the N.C.I. and A.C.S. in 1973 screening programs. Sixty-six of these cancers were "reinterpreted" as "benign" and 22 as "borderline." In fact who knows? (See discussion by Thier [1977].) In the case of endometrial hyperplasias pathologists have always recognized a "not cancer but better out" category—a situation which carries implications for the current discussions. The histopathology of cases 1 to 7 was classified as positive for WHO Al and AII lesions.

Fig. 1. Woman aged 43 years. Mastectomy performed for infiltrating carcinoma (WHO CII). Contralateral mastectomy two years previously for infiltrating carcinoma. (Married with two adopted children; no pregnancies; menarche at 14 years.) Field (× 93) selected away from carcinoma to illustrate borderlands of hyperplasia and neoplasia. (GRI No. 4784/77.)
sponse with a constellation of changes: cysts, papillary ingrowths, apocrine metaplasia, calcification, and, in some cases in the same field, foci of cancer. These cases are selected, and many specimens with infiltrative breast cancer do not show these changes (Fig. 8). Accordingly, we decided to assess all Glasgow Royal Infirmary 1976–77 infiltrative cancer mastectomy specimens for cysts and proliferative epithelial lesions as a function of age. The census focused on only two aspects of the pericancerous tissue so that the search could be carried out expeditiously, i.e., on simple cysts (W.H.O. A Ia lesions) and/or “regular typical epithelial proliferations in ducts or lobules” (W.H.O. A III lesions [Scarff and Torloni, 1968]). The histopathology census was done without knowledge of the patients’ age at mastectomy, reference to this being made only when the data were collated. Scoring of the mastectomies was on a simple all-or-none basis. The work was speeded up by a previous departmental decision to classify all specimens according to the Systematized Nomenclature of Pathology [College of American Pathologists, 1969]. In practice we were able to

Fig. 2. Woman aged 45 years. Mastectomy performed for infiltrating carcinoma (WHO CII). (Married with one child born when she was 27 years. No delay in conception, no abortions, menarche at 13 years.) Field selected × 93 to show a focus of equivocal hyperplasia/neoplasia and cysts. (GRI No. 5911/76.)
Fig. 3. Woman aged 33 years. Mastectomy performed for infiltrating carcinoma (WHO CII). (Married with five children, no miscarriages or delay in conception; aged 17 years at first pregnancy. Menarche at 12 years.) Fields show (a) scirrhous carcinoma (× 93) and (b) focus of apocrine hyperplasia/neoplasia (× 223) from same area as lower right hand corner of 3(a). (GRI No. 7279/77.)
assess the presence of cysts and hyperplasia in 164 mastectomies from the Glasgow Royal Infirmary material of 1976–77; in others the amount of paraffin-blocked, noncancerous mammary tissue was deemed an inadequate sample. The decision about acceptance or rejection was made without knowledge of the patient's age. The results of the census are summarized in Figure 9. Cysts, the commonest component of the hyperplasia constellation, were present in the pericancreous tissue of nearly all patients in the years leading up to the menopause, but in the elderly women the frequency of cysts fell to only a third; proliferative epithelial lesions were present in nearly two-thirds of the premenopausal and less than one-third of the postmenopausal cases.
Fig. 5. Woman aged 47 years. Para 2 + 0; 27 years at first pregnancy; 1 year delay before conception. (a) × 72. Last menstrual period just before mastectomy. Example of desquamative epithelial hyperplasia [Cheatle and Cutler 1932]. Note also epitheliosis. (b) The infiltrative carcinoma was 2 cm away from these lesions. (GRI No. 6674/77.)
Fig. 6. Woman aged 42 years. Severe cribriform epitheliosis or intraduct carcinoma? × 90. (GRI No. 6033/77.)

Fig. 7. Woman aged 41 years. Severe epitheliosis × 225. Infiltrative cancer elsewhere in breast. (GRI No. 9165/77.)
Fig. 8. Postmenopausal breast cancer (× 180). No focal hyperplasias or cysts. (GRI No. 9888/78.)

E. Discussion of the Census

The premenopausal figures seem high to us as practicing pathologists. For comparison we have the similar age groups in Sandison’s [1962] autopsy breast series (n = 677) from Glasgow’s Western Infirmary. This has an adjacent referral territory. (There is, however, some excess of social class 4-5 lowest in the Royal Infirmary’s population). Sandison studied at least one block of tissue from each breast of every patient coming to postmortem. The cases included 34 patients with a primary diagnosis of breast carcinoma and 6 with “occult” carcinoma [Sandison, 1962]. In breast tissue blocks from his specimens obtained from pre- or paramenopausal women aged 36–55, epitheliosis was present in 16% of cases, whereas in our cancer series it was present in 63%, i.e., nearly four times as many (Table 1). In contrast, epitheliosis was present in 26% of the postmenopausal breasts, obtained at postmortem a figure virtually identical with that for (our) pericancerous tissue (25%). Verification of the Sandison values comes from an autop-
Fig. 9. Retrospective census of focal hyperplasia of pericanerous tissue in all cases of infiltrative breast carcinoma treated by mastectomy in Glasgow Royal Infirmary 1976–77. Cysts were defined as WHO A1 lesions and proliferative epithelial lesions as WHO A3 lesions [Scarff and Torloni 1968]. It is routine practice to take one paraffin block from the breast tissue away from the neoplasm. When this was not available the case was rejected. The two pathologists assessing the sections for A1 and A3 lesions were unaware of any clinical details (eg, age) when the slides were assessed. Note the high incidence of cysts and proliferative lesions in premenopausal patients and the decreasing trend with age.* Cf, Figure 10 for “control” data, also Table 1.

*Postscript added in proof: The census has now been extended (N-500) essentially confirming the above findings except that a rising trend is seen in the very elderly 75y–90y.
TABLE I.

Control (Autopsies n = 677)  Test (Mastectomies n = 164)

<table>
<thead>
<tr>
<th>Age group</th>
<th>P.M.s n=</th>
<th>Epitheliosis n=</th>
<th>%</th>
<th>Mastectomies n=</th>
<th>Epitheliosis n=</th>
<th>%</th>
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<td></td>
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</tr>
<tr>
<td>36-40</td>
<td>33</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>7</td>
<td>59</td>
</tr>
<tr>
<td>41-45</td>
<td>45</td>
<td>12</td>
<td>27</td>
<td>25</td>
<td>18</td>
<td>72</td>
</tr>
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<td>46-50</td>
<td>58</td>
<td>8</td>
<td>14</td>
<td>18</td>
<td>11</td>
<td>61</td>
</tr>
<tr>
<td>51-55</td>
<td>98</td>
<td>17</td>
<td>17</td>
<td>28</td>
<td>16</td>
<td>58</td>
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<tr>
<td>Totals</td>
<td>234</td>
<td>39</td>
<td>x = 16%</td>
<td>83</td>
<td>52</td>
<td>x = 63%</td>
</tr>
<tr>
<td>Post menopausal</td>
<td></td>
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<td>56-60</td>
<td>94</td>
<td>22</td>
<td>23</td>
<td>22</td>
<td>5</td>
<td>23</td>
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<tr>
<td>61-65</td>
<td>138</td>
<td>37</td>
<td>27</td>
<td>26</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>66-70</td>
<td>114</td>
<td>31</td>
<td>27</td>
<td>17</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>71-75</td>
<td>97</td>
<td>26</td>
<td>27</td>
<td>16</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Totals</td>
<td>443</td>
<td>116</td>
<td>x = 26%</td>
<td>81</td>
<td>20</td>
<td>x = 25%</td>
</tr>
</tbody>
</table>

*Sandison [1962].

*In pericancerous tissue only.

sy series by Frantz [Frantz, et al, 1954; quoted by Sandison, 1962] (n = 225). The different incidence of epitheliosis in pericancerous tissue and autopsy controls (all diagnoses) is summarized in Figure 10.

Sandison’s data indicate that a minimum of 1:4 to 1:6 of Glasgow women from middle reproductive life onward have foci of epitheliosis in the mammary tissue. When the breast contains, in addition, an infiltrative cancer, there appears to be dramatic increase in the incidence of this phenomenon in premenopausal women so that nearly two-thirds will have it. In contrast the incidence of epitheliosis in the pericancerous tissue in the postmenopausal woman is similar to that in the “controls.”

The finding of focal hyperplasias in the majority of premenopausal patients suggests that, indeed, the critical tumor initiation—as expected—occurred in such an area. More of the cases could have been positive for focal hyperplasia but were missed because of inadequate sampling. This was a retrospective study and in some instances the pathologist did not observe the ordinary protocol for mastectomy ex-
amination of sampling block(s) of tissue away from the cancer but merely took enough for diagnosis. On this basis some of our cases were rejected because of an inadequate volume of tissue for assessment; most of these were postmenopausal. Furthermore, it could well be that the proportion of patients with hyperplasia was slightly overestimated, since in some instances it was difficult to distinguish morphologically between hyperplasia and neoplasia. In other words, cases recorded as "focal hyperplasia" could have been neoplasia. Gullino [1977], who uses angiogenesis in rabbit iris xenografts as a test for neoplastic transformation in hyperplastic tissue, finds that morphologically similar tissue may be positive or negative. However, all the observations in the present census were subject to the same error; since the age at mastectomy was not known by the pathologists during the histological assessment the results summarized in Figure 9 should not contain any bias altering the important finding of a slope.

![Chart](image)

Fig. 10. Frequency of proliferative breast lesions in pericancerous tissue (Fig. 9) versus consecutive autopsy findings of an adjacent hospital [Sandison, 1962]. Whereas in postmenopausal women the pericancerous tissue contained no more epitheliosis than expected, the premenopausal pericancerous tissue contained a large excess of epitheliosis above that which could be expected.
The findings are consistent with the idea that postmenopausal cancers are initiated premenopaually but that the putative hyperplastic clone where they initiated has long since regressed. The estimated time scale of tumor growth is not seriously in conflict with this suggestion (see discussion by Gullino [1977]). In Sandison’s [1962] study of the breast tissue at 677 consecutive autopsies (referred to earlier) he found six “occult” carcinomas. This finding suggests a long preclinical phase of the disease (cf, prostatic cancer). Consistent is the general agreement that the proportion of tumors with available cytosol estradiol-17β receptors is lower in premenopausal (32%) than in postmenopausal specimens (62%) [Barnes et al, 1979] and that degree of tissue differentiation and receptor presence and prognosis often go together. If the foregoing hypothesis is correct and if it is correct that postmenopausal cancers usually initiate in the premenopause, then one would expect tumors clinically manifest in the postmenopause to contain receptors and be better differentiated.

Another point is that the menopausal “hook” in breast cancer incidence in some data [de Ward, 1964] could be due to withdrawal of ovarian hormone promotion from tumors already initiated.

Haagensen [1971], quoting the age distribution of 2,017 cases with gross cystic disease, had virtually no postmenopausal cases; in contrast, the age distribution of his 70 cases of cystic disease specifically associated with the later development of cancer had a distinct shift toward an older age group. In our series, in which an infiltrative cancer was present in addition, microscopic evidence of cystic disease was present in a proportion of cases until the 70+ age group. These observations fit with the presence of a more prolonged (aggressive ?) form of cystic disease in cancer patients. Haagensen [1971] also reviewed the 300 cancerous breasts originally described by Foote and Stewart [1945]. Of the breasts exhibiting changes due to gross cystic disease, 90% were from patients under 55 years. As Haagensen points out, cysts are a premenopausal phenomenon, but both cystic disease and cancer can have “a common etiology going far back into the patient’s life history.”

If this construction is correct, then the majority of breast cancers are initiated and promoted during the functional life of the ovary. One old hypothesis that fits a remarkable number of epidemiological facts is that breast cancers result from a prolonged mammary stimulation from a subnormal secretion of progesterone (eg, anovulatory) cycles; in other words the breast is stimulated by the estrogen (and high prolactin ?) of the anovulatory cycle, but the sequence is not followed by adequate amounts of progesterone. Anovulatory cycles are a feature of
early and late reproductive life and of infertility; all these features are known to be associated with high risk. Consistent too, as mentioned, is the fact that cystic mastopathy may be “cured” by pregnancy, ie, the mammary epithelium is allowed to complete its cycle in the high progesterone state and, as also already mentioned, parity is protective against cancer risk [Bonser and Jull, 1960; Sherman and Korenman, 1974].

The current investigation indicates only a focal rather than diffuse hyperplasia of the breast tissue (cf, endometrial hyperplasia after iatrogenic estrogen), and this requires an explanation.

Estradiol receptor studies suggest that the neoplastic transform of the breast cell is accompanied by a strong activation (mutation?) of the appropriate structural gene [Leake et al, 1979]. Could the focal hyperplasia derive in fact from such a mechanism, and could the full neoplastic transformation occur out of the derived hyperplastic risk clone?

Other reasons for the focal nature of the hyperplasia might be 1) a reaction to material from an ectatic duct (discussed by Sandison [1962], ie, endogenous cocarcinogens. We have noticed histological appearances suggesting this possibility but only very infrequently. 2) There might be a phase delay in selected duct systems. In contrast to lactational changes, the menstrual cycle events appear to involve only a proportion of duct acinar systems. Particularly after anovulatory cycles, it may be that some duct systems become out of phase and that focal hyperplasia is the manifestation of this phenomenon. Anovulatory cycles could fit in with the epidemiological risk factors of early menarche, late menopause, infertility, nulliparity, etc.

F. Implications of the Census

In a previous section it was pointed out that even with highly successful breast cancer screening, breast cancer mortality would be unlikely to fall below 20-30%; in other words, the real future for breast cancer might lie in its prevention rather than cure. Epidemiologists have accumulated an extremely telling body of evidence implicating the cycling ovary (or rather the disordered cycling ovary) in the genesis of the disease. Classical pathology of endocrine organs indicates that cancer is likely to occur in the hyperplastic gland, and a search for focal hyperplasias revealed that nearly all premenopausal cases had some part of the “constellation” of hyperplastic changes; postmenopausal cases had much less. In other words the histopathology findings are consistent with the epidemiological facts: that the disease is induced during the life-span of the cycling ovary. The relative lack of
hyperplastic features in the postmenopausal cases suggests that in many cases the cancer was initiated premenopausally, then promoted slowly because of estrogen withdrawal and was not, for example, due to another initiation or promotional stimulus such as peripherally formed estrogen from ovarian androgen.

If this construction is allowed as a working hypothesis, then the screening of the mammary tissue for high risk should occur during the reproductive life-span. Soft tissue x-rays may be used for assessment of hyperplastic disease but may carry the attendant risk of inducing cancer per se. Clinical observations indicate that the cysts can change in size (cyclically), and Haagensen [1971] prefers for this reason to examine women with breast symptoms in the postmenstrual phase. But x-rays at clinical examination are unlikely to be sufficiently quantitative. Breasts do change some 20% in size during the menstrual cycle [Doring, 1953], presumably as the result of cyclical hormonal activity, but this cyclicity would be difficult to measure. Solid-state memory recordings of bra-strap tension might be effective if the signals were averaged over time to damp out respiration and other associated cycles of movement.

At this time heat signals from the surface are an excellent possibility for monitoring the menstrual and circadian cyclical changes. The idea of monitoring breast “activity” from surface heat is not new, for it has been known since the advent of the clinical thermometer that the surface of the lactating breast was hotter than the axilla and that the surface temperature of the pregnant breast was similar to the axilla, while the normal breast was colder than the axilla [Cheatle and Cutler, 1931].

Of course there is a contemporary consensus of opinion questioning the cost-benefit of some screening programs. Preclinical diagnosis of lung cancer by mass miniature radiology not infrequently changes a fit working man, after pneumonectomy, into an invalid for the long-odds gamble of cure. The situation with breast cancer would seem to be much more hopeful because the gland is superficial and is under relatively well-understood normal growth control. If precancer hyperplasias can be confirmed as a risk factor then presumably they can be reversed by strategic hormonal therapy, eg, by oophorectomy, by tamoxifen (an estrogen blocker), or by a progesterone pill (to complement the putative anovulatory cycles). Haagensen [1971] notes that pregnancy is beneficial to hyperplastic cystic disease. Is that because of the high progesterone levels? Symmers [1966] discusses the condition of “pubertal breast hyperplasias.” This is a reversible hyperplasia not to be
confused with so-called "virginal hypertrophy," and one wonders if it is due to the cumulation of anovulatory cycles at the start of the reproductive life-span and is cured by normal cycles of progesterone.

G. Definitions

**Hyperplasia** is a reversible proliferation of cells of a particular kind, eg, as a result of disturbed hormonal control of the tissue.

**Neoplasia** is an abnormal mode of growth of tissues which exceeds and is uncoordinated with that of normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change [Willis, 1948].

**Epitheliosis** is the multiplication of epithelial cells within existing gland structures without formation of new gland elements . . . a cellular filling of a lobule, not a branching, which indicates a more or less pathological response, though it may be only temporary activity and be followed by cell degeneration and cyst formation [Dawson, 1948].

**W.H.O. AI.a. lesions** are simple cysts. This means a cyst that has a lining of flattened epithelium with no overgrowth [Scarff and Torloni, 1968].

**W.H.O. AIII lesions** are regular typical epithelial proliferations in ducts or lobules. The epithelium may be solid, adenomatous, or papillary in type, but in all cases it is regular, and the nuclei are even in size and staining and do not show mitoses or hyperchromatism [Scarff and Torloni, 1968]. For practical purposes this is (arguably) the same as epitheliosis.

II. EARLY ATTEMPTS AT A METHOD TO MEASURE THE RISK STATE BY MAMMARY THERMAL CHRONOBIOLOGY

A. Introduction

Cheatle and Cutler [1931] reviewed the earlier German literature on the surface temperature of the breast in relation to its metabolic activity. Thus the lactating breast had a higher temperature than the axilla, and if milk secretion stopped the breast temperature fell. In pregnancy the breast temperature was as high as or higher than that in the axilla, and in normal nonpregnant women the breast temperature was lower than in the axilla.

The current study is particularly concerned with the finer temperature variations of the breast along the course of the menstrual cycle. Earlier views of thermographers that the infrared image was "warmer"
at the menses [Draper and Jones, 1969] were modified when a Cleveland group using liquid-crystal thermography discovered that there was, in addition, a transient peak at midcycle provisionally interpreted as a correlate of the ovulation process [Ewing et al, 1973]. These findings have been confirmed by Smith and colleagues (1975; 1977) using thermistors and also by the Strasbourg group using infrared thermography [Gautherie and Gros, 1976].

All in all, one concludes that there is a good body of indirect evidence to suggest that the surface temperature fluctuations of the breast may to some extent represent an internal bioassay for mammotrophic hormones (such as estrogen). It is also interesting and consistent that the breast undergoes systematic changes in volume during the menstrual cycle (about 20%; see Doring [1953]).

The breast also exhibits a circadian variation of surface temperature [Mansfield et al, 1974; Gautherie and Gros, 1976; discussion by Simpson, 1977a,b]. On a normal routine the temperature rises throughout the active day and falls when the subject is recumbent. The relative amount of postural vs endogenous effect has not yet been worked out. One possibility is that the temperature rise is prolactin-mediated since prolactin has a normal nocturnal peak and this fits the observed rise toward evening. Interestingly enough the first subject studied had persistent high diurnal and nocturnal prolactin levels, and no circadian rhythm was detected. This is very unusual. Against prolactin as a mediator is the fact that women in the third trimester of pregnancy have a well-defined circadian rhythm of breast temperature but do not have a circadian rhythm of prolactin concentration in the plasma [Meis et al, 1977].

The foregoing indicates that breast surface temperature is subject to cyclical variations, along the menstrual and circadian time scales, and that some of these variations are related to corresponding cycles in hormone variation. If this is true, and the data look promising, then by analogy with the electrocardiograph, we have a dynamic method of assessing the physiological and pathological function of the breast. The first part of this chapter provided evidence that focal epithelial hyperplasia is a (frequent) intermediate step in carcinogenesis and the critical question yet to be answered is whether the metabolic and/or vasodilative “heat” of this abnormality is measurable. One might, for example, expect increased average temperature or menstrual amplitude; with altered receptors status there could be circadian or menstrual phase changes; a third possibility is increased between-breast-site variance. Studies to test these possibilities are underway in America and Japan (Halberg et al, personal communication) and also in Great Britain (the authors together with Mr. Roger Blaney and Mr. Howard Holliday).
Moreover a single case study has been documented in which breast thermorhythmometry is correlated with postmastectomy morbid anatomy [Halberg et al, 1979].

The phenomenon of the "false positive" thermogram is an encouraging fact for correlation of surface heat abnormality and underlying epitheliosis. Various thermographers have documented [Stark and Way, 1974; Gautherie and Gross, 1976] a type of thermogram with increased surface heat and a vascular pattern interpreted as "suspicious." Very recently Gautherie has reviewed the experience of the Strasbourg group in this respect [Gautherie and Gros, 1980]. Of 58,000 patients seen at that clinic, some 1,245 had "questionable" or stage Th III thermograms. By conventional means of examination (including mammography and ultrasonography) there was no evidence of malignant disease. Yet within five years of the initial examination, over one-third of this group had histologically proven cancers. The question remaining to be answered is whether the thermic abnormality derives from focal epitheliosis.

B. Breast Temperature Data Obtained in Various Physiological States

Thus far our research has not reached the critical stage of testing different risk groups, but we have been confirming that a valid signal can be indeed obtained by recording temperatures thermistors taped under a bra. The form of this and its relative movement to so-called "basal body temperature" during the menstrual cycle indicates that it could be an internal bioassay for mammotrophic hormones.

Figures 11 and 12 illustrate the first subject systematically studied in terms of estrogens, breast temperature, and basal body temperature. It can be seen that the breast is "cold" at midcycle but, relative to deep body temperature, warms up toward the menses. This might be interpreted as a "pseudo-pregnancy" effect (cf the pseudo-decidua of the endometrium). As can be seen, this particular cycle was anovulatory since the total urinary estrogen was monophasic; furthermore, no pregnanediol was detected. The subject was infertile and this was one reason why she was willing to collect the very large amount of data. She was later found to have prolactin levels in day and night samples of blood above the peer-group chronodesm.

Figure 13 averages data from three consecutive cycles in one fertile woman. Note that the rise in breast temperature (relative to deep body temperature) is relatively early in the cycle. The data are incomplete and "synchronization" of the three cycles was effected by aligning the primary estrogen surge. Figure 14 summarizes the circadian variations
Fig. 11. Breast surface temperature was measured by a thermistor taped onto the breast surface just lateral to the nipple (36-year-old female) the wire from the sensor was led down the sleeve to a jack plug at the wrist. Readings were measured by plugging the lead into a Yellow Springs model 43 TI telethermometer and recording the value on a tabulation sheet. Readings were made at various times in the day as suited the subject's convenience. To account for circadian bias, readings were put into two hourly ranks (09:00-11:00; 11:00-13:00, etc) and the rank sum average was taken as the day reading. Plotted readings are three-day smoothed means. This was necessary since there were odd days of missing data, especially at weekends.

of the same data. To obtain equal circadian weighting—the numbers of observations were not always the same for different times of day—the circadian two-hour ranks were averaged and the average of the nine ranks taken as the “reading” for that day. Figure 15 summarizes circadian variations in a) a subject who was lactating (as predicated,
the breast temperatures are higher at corresponding times on the average); b) a clinically healthy medical student who provided similar data when the study was redone a year later; and c) in the hyperprolactinemic woman mentioned earlier. No regularity of circadian variation form is evident. The other circadian data have similar phasing to that described in the literature [Simpson, 1977].

The collection of data along the time scale of a menstrual cycle needs a special instrumentation, and for this a chronobra [Simpson, 1974, 1977a,b] is now constructed and is in the prototype (Fig. 16) testing stage.
FOLLICULAR PHASE (PRESUMED 16-22 DAYS BEFORE OVULATION)

LUTEAL PHASE (PRESUMED 14 DAYS AFTER OVULATION)

BREAST SURFACE TEMPERATURE CHANGES AVERAGED OVER THREE MENSTRUAL CYCLES IN ONE WOMAN AGED 27 YEARS

URINE TOTAL OESTROGENS (N=64)

URINARY TOTAL OESTROGENS (µmol/mol creatinine)

BREAST TEMP (N=760) %

ORAL TEMP (N=84)

ESTIMATED OVULATION (60 hr AFTER OESTROGEN SURGE)

FOLLICULAR PHASE (PRESUMED 16-22 DAYS BEFORE OVULATION)

LUTEAL PHASE (PRESUMED 14 DAYS AFTER OVULATION)

START OF NEXT CYCLE

DAYS BEFORE AND AFTER OVULATION
THE CIRCADIAN RHYTHM OF BREAST SURFACE TEMPERATURE
AVERAGED FOR SPANS BEFORE & AFTER THE OESTROGEN SURGE
OF A NORMAL MENSTRUAL CYCLE

Fig. 14. Circadian rhythm of breast temperature recorded via a thermistor taped to the same position of the breast each day and held in place by the bra. A wire was led down the sleeve to the wrist where a jack plug could be released and inserted into a Yellow Springs model 43 TI telethermometer. Most studies obtain similar data. Note that whereas deep-body temperature usually peaks before the end of the active span, breast temperature usually rises throughout the diurnal span. These data were obtained while the subject worked in the laboratory.

Fig. 13. Data and ranking obtained as in Figure 12. Data must be regarded as preliminary in terms of the ovulation timing since the three cycles of data, pooled in the figure, were incomplete, and precise alignment was uncertain. However, note the relative movement of breast vs basal body temperature, as before, with maximum breast temperature around the menses.
Fig. 15. Similar methods as to those used in Figure 14.

'PLAYTEX' 18 Hour Bandeau Bra or Similar

Note: Units built into existing fibrefill lining.
Units electrical interconnections via Flexible Film Wiring System.

Fig. 16. The chronobra. Breast temperatures are recorded by day automatically on the solid-state memory system, which is integral. Read-out is on a computer interphase via a jack plug.
III. THE FINAL PERSPECTIVE

If the mortality from breast cancer is to be improved or eliminated, then the precancer biology must be studied and a signal must be found on which preemptive therapeutic decisions can be taken. Evidence has been presented that there is a focal increase in proliferative lesions in the pericancerous tissue of the premenopausal breast. This finding will need confirmation and, if confirmed, then the next question will revolve around whether the proliferative lesions produce a heat signal that can be quantitated. Is this, in fact, the same heat signal as the “false positive” thermogram? In any event, the addition of the time dimension to any heat signal, and the correlation with menstrual and circadian phasing, could add greatly to the sophisticated interpretation of temperature signals. How much information is obtained from a tiny segment of the ECG, compared with the whole trace? Thermographers always point out the fact that their signals do not give etiological intelligence and that a fibroadenoma can be as hot as a cancer. But will there be a difference in rhythm? Since hyperplastic cystic disease may be associated with hyperprolactinemia [Cole et al. 1977; Tarquini et al., 1979], what happens to the parameters of the circadian rhythm of mammary heat in this condition? The answers to these questions should soon be at hand with studies on different risk groups (as defined by epidemiologists), with the help of radioimmunoassay techniques of hormone analysis, computer programs for time series analysis, and modern electronics integrated in the chronobra.

IV. REFERENCES


A Fresh Approach to Breast Cancer


Simpson HW (1977b): An outline of mammary chronopharmacology and pathology; a new
Simpson et al


NOTE ADDED IN PROOF

Gautherie (1980, personal communication) now advises that the age-frequency distribution of these cases is very similar to the peak of (pericancerous) hyperplasia described herein.
TOWARD A CHRONOPSY: PART III. AUTOMATIC MONITORING OF RECTAL, AXILLARY AND BREAST SURFACE TEMPERATURE AND OF WRIST ACTIVITY; EFFECTS OF AGE AND OF AMBULATORY SURGERY FOLLOWED BY NOSOCOMIAL INFECTION

Erna Halberg * Richard Fanning * Franz Halberg *
Germaine Cornélissen * Douglas Wilson ** Keith Griffiths **
Hugh Simpson *

At the University of Minnesota Hospitals and presumably at other centers, those leaving the outpatient clinic after ambulatory surgery, such as a dilatation and curettage (D and C), are given printed (Appendix A) as well as verbal instructions concerning precautionary measures at home and work. Should these precautionary measures include the use of automatic ambulatory devices or at least self-measurements for monitoring the patient's rhythms as indices of health? Could the post-operative ambulatory patient be seen after a time of a length depending on the evaluation of certain rhythms, compared before and after surgery rather than, as is current practice, after a somewhat arbitrarily fixed span? Symptoms which may occur at home, beyond nausea and vomiting and even cramps or extreme weakness and slight fever, may be ignored initially, with the usual statement that they are likely to pass. At least marked fever, however, should be recognized early and acted upon promptly.

Key-words: Automatic ambulatory monitoring; Chronopsy; Marker rhythm; Motor activity; Nosocomial infection; Rectal and surface temperature; Surgery.

Support: National Institute of General Medical Sciences (GM-13981), National Cancer Institute (CA 14445), National Institute of Aging (AG 00158), National Institute of Occupational Safety and Health (OH 00651).

* Dedicated to Howard Levine on the occasion of his retirement as Chief of Medicine at the New Britain General Hospital, New Britain, Connecticut, USA.

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Received November 11, 1980.

Chronobiologia 8, 253, 1981.
Conceivably, an alteration of the amplitude or timing of circadian temperature rhythm may precede the elevation of overall rhythm-adjusted temperature mean as a warning signal or it may be the sole sign of low toxicity (Halberg and Spink'). Any post-operative weakness may be reflected by a change in overall rhythm-adjusted mean. Characteristics of other circadian and ultradian rhythms, e.g., in motor activity of the non-dominant hand, may also provide important signals for the state of health. The latter variable is particularly easy to monitor, whereas body core temperature measurement orally or rectally involves interruption of one’s activities. Thus for ambulatory monitoring, a hand motion recorder would be the simplest instrument. Socially still acceptable, however, would be an instrument recording both surface temperature (e.g., axillary) and motion. One must first approximate by axillary temperature the information provided by the rectal measurements. It might be argued that such monitoring procedures are dispensable, since large-scale experience, if not statistics, support the current practice and justify the procedure of unmonitored ambulatory surgery at this time as a cost-effective treatment. If, in turn, by automatic monitoring instrumentation, additional information could be gained to reduce the danger to those few cases which may develop complications (notably infections), the practice of ambulatory surgery, supported by many justified reasons, could be rendered even safer. Accumulating experience from automatic measurements indicates that a small number of self-measurements may perhaps be specified and advocated for post-operative assessment in geographical locations where automatic instrumentation is as yet unavailable. The analyses of the data thus collected will be rendered easier and more practical with the implementation and general use of home computers now entering the market. By the same token, automatic instrumentation, in turn, could be extremely helpful in the hospital itself, since it could reduce staff duties, yet provide a more reliable and denser record amenable to routine analyses.

We refrain from the usual choices for hospitalization (expensive and conventionally involving but few measurements by nursing staff) interpreted solely for the presence or absence of fever or discharge to home usually with no measurement whatsoever unless there is a subjective impression of fever (see Appendix A). A third possibility is to adopt a procedure for obtaining a record of at least one or preferably several pertinent marker rhythms. Variables that can easily be monitored before as well as after surgery are preferred, provided they exhibit a stable and pertinent rhythm. The alteration of such marker rhythms may serve at least as a harbinger of some problem after surgery. If there is sufficient turn-around time, an appropriate useful record could be provided. The purpose of this report is to describe monitoring for marker rhythms in one of us (EH) whose medical history has been alluded to elsewhere (E. Halberg et al.').

BRIEF CASE HISTORY

The subject (a 60-year-old male) had 5 pregnancies and 2 full-term deliveries. She had no history of hormonal medications. She had undergone biopsies of the left breast in 1951 and in 1970; both revealed fibrocystic disease. A screening xero mammogram done on October 28, 1976, revealed a small cluster of calcifications in the upper outer quadrant of the left breast immediately posterior to the nipple and approximately 1 cm from the chest wall. The subject's mother had died from endometrial carcinoma and her sister was diagnosed as having breast cancer at age 49.
The subject was over 80 years of age at the time of her first pregnancy which aborted. Because of her high risk for breast cancer, the likelihood of repeated biopsies in the future, anxiety associated with the subject's pronounced concern about cancer, and her marked preference to have prophylactic mastectomy, and in view of the maturity of her character, it was elected to perform a bilateral subcutaneous mastectomy instead of a more limited breast biopsy. Cancer was not diagnosed but findings discussed elsewhere (E. Halberg et al.)

After being amenorrheic for about 4 years, a few months prior to mastectomy, the subject experienced vaginal bleeding associated with uterine cramping, preceded by a few days of mild mastalgia. After another span of about 42 months after mastectomy, the spontaneous vaginal bleeding recurred. When she was seen for this reason on April 4, 1980, she bled for several days. The decision to perform an ambulatory dilatation and curettage was made and executed on April 7, 1980, at 09°. The surgery was carried out under general anesthesia. The subject started to awake before the surgery was even completed. It yielded a very small amount of tissue without any recognizable pathology. Sections of the endocervical curettings showed fragments of histologically unremarkable squamous and columnar epithelium. Endometrial curettage showed fragments of inactive endometrium with no evidence of malignancy. Sections of the cervical biopsy showed chronic inflammation with no evidence of malignancy. The bleeding terminated within one week.

For several months prior to hospitalization for the D and C, the subject had carried instruments for ambulatory monitoring, first of rectal temperature only (solidorder, manufactured by Ambulatory Monitoring, 731 Saw Mill River Road, Ardsley, New York 10502, USA) and thereafter 2 TherMolog instruments (manufactured by Vitalog Corporation, 1058 California Avenue, Palo Alto, California 94306, USA), one for the continued monitoring of rectal temperature, the other for the monitoring of both axillary temperature and motor activity. Monitoring was continued during D and C and upon return to home after the D and C around 11° on April 7, 1980. On April 12, 1980, the subject was readmitted to the hospital, placed on intravenous feeding and antibiotic and monitoring was continued. From the outset, prior to D and C and thereafter, the activity of the non-dominant left wrist was monitored. Since the left arm was used for intravenous treatments during hospitalization, the right wrist was monitored at the time instead.

Instrumentation used by subject for automatic monitoring of marker rhythms

Technological advances made in electronic data collection allow rapid manual or automatic recording of data from ambulatory subjects pursuing their daily activities. Several devices for measurement of rhythmic functions can be used:

1. the Yellow Springs Instrument (YSI) tele-thermometer (TROLANDER) manufactured by the YSI Company of Ohio, can be used to monitor temperature. A Wheatstone bridge serves as the basic measurement device and temperature is sensed by thermistor probes, each containing a semiconductor in which a slight temperature change causes a large change in electrical resistance. Single or multiple channel instruments used in our laboratory have a temperature range from 20 °C to 42 °C and a reported accuracy of 0.2 °C. Their batteries have an approximate 1,000-h life. Instructions for using the Yellow Springs Instrument are given in Appendix B.
2. the Medilog, procured from Ambulatory Monitoring, Inc., Ardsley, New York, is a cassette tape device capable of monitoring on 4 channels. With instruments used in our laboratory, a 4th channel must be reserved for time. Thus, one may automatically record temperature from 4 sites for 1 day, but the 4 mercury RMIR batteries and the Memorex CI20 cassette tape must then be replaced. The Medilog also serves for monitoring EEG, ECG, respiration and motor activity for a 24-h span. Data are recorded in an analog form and need to be digitized before analysis. Pre-processing of the data for analysis also requires a computing system for the formation of card images containing the time-coded data. This can be done by using the playback unit supplied by Ambulatory Monitoring plus a processor such as a Varian 620 computer. Recording speed stability is better than 2% overall and the signal-to-noise ratio is better than 30 dB. It is also recommended that minimal maintenance be performed after each day of recording and that the instrument be calibrated (see Appendix C for instructions).

3. the Solicorder, manufactured by the Solicorder Company, Ardsley, New York, is capable of monitoring temperature in the range from 34°C to 40.3°C from one site for spans dependent upon measurement frequency (up to 8 days with a 6-min sampling interval or 2 weeks at 12-min intervals). With respect to data handling, the Solicorder has 2 advantages over the Medilog: it does not require digitization of collected data and can be interfaced with a minicomputer for data analysis. The Medilog, however, is capable of recording higher frequency data such as EEG and ECG.

4. the TherMolog, manufactured by the Vitalog Company, Palo Alto, California, is capable of concomitantly monitoring temperature and motor activity; it also does not require digitization of collected data and is sold with an Apple II microcomputer and a Sanyo TV monitor for data display and minimal analysis.

Instructions for using the Solicorder and TherMolog instruments are given in Appendix D. The probes used with the Medilog, Solicorder and Vitalog are those produced by VSI and have the same characteristics. Usually, accuracy of recording does not seem to constitute the major problem; problems may arise with defective probes, their calibration, contact with the skin when so applied, and batteries.

RESULTS

Figure 1 constitutes a so-called chronobiologic serial section (Halberg et al.), illustrating the evolution with time of the circadian rhythm characteristics for wrist activity. The first vertical dashed line (event line) corresponds to the time of ambulatory surgery and the second event line to the subsequent hospitalization and start of antibiotic treatment. The abscissa represents time in days (calendar date as well as day in relation to surgery). The graph is divided into 5 rows or sections, displaying different aspects of the data. In the top row of the figure, the data themselves are presented as they were monitored at 2-min intervals averaged every 12 min and stored in a solid-state memory for eventual analysis by a PDP 11/34 computer. The data on activity seem much more irregular after surgery than before. By the naked eye, however, one can only distinguish that there are many more points at the bottom of the ordinate scale (corresponding to no movement) than there were before surgery.

The 2 bottom rows, i.e. the fourth and fifth sections, show 1. the p-values obtained in testing zero-amplitude of the 24-h cosine curve fitted by least squares
HALBERG, R. BANNING, F. HALBERG, C. CORNELIUSSEN, D. WILSON, E. GRIFFITHS, H. SIMPSON

Fig. 1 - Chronobiologic serial section of automatically recorded wrist activity before, during and after hospitalization for nosocomial infection following ambulatory surgery (D+C, 60 year-old woman).

(Halberg et al.) to the data in each interval; and 2. the number of data per interval. It can be seen that as an end effect, the parameter estimation at the start and end of the observation span is biased by the smaller number of data available. The p-values should be regarded with the added consideration that with a sampling rate as high as 5 values/hour, the residuals are correlated.

The 2 rows in the middle (second and third sections) depict the changes with time of the circadian rhythm parameters: 1. the mesor or rhythm-adjusted mean plotted as the lower curve in the second row; 2. the amplitude or half the total extent of predictable change, shown as the difference between the 2 curves plotted in the second row; 3. the acrophase or timing of fitted peak values, illustrated in the third row.

One can see that the circadian rhythm of wrist activity (Fig. 1) is statistically significant (p<0.05; i.e. below the horizontal dotted line) throughout the whole observation span. The decrease of the mesor after surgery reflects a lowering of activity. The fact that the 2 curves in the second row come closer together after surgery indicates a clear decrease in amplitude of the circadian rhythm in wrist activity. The acrophase remains remarkably stable prior to surgery, whereas a consistent drift in acrophase characterizes wrist activity for the first 2 days after surgery. The appearance of a decrease in mesor and amplitude before surgery, i.e. before the vertical dashed event line, is an artifact due to the diluting effect of data after surgery included in the analysis of an interval centered before surgery.

Figure 2 represents the results obtained for the circadian rhythm in rectal temperature and should be interpreted in keeping with the explanation in Fig. 1. Again, the rhythm can be statistically validated (p<0.01) for most of the observation span. After surgery, a steady rise in mesor is observed while the subject is at home fol-
Fig. 2. Chronobiologic serial section of automatically recorded axillary temperature before, during, and after hospitalization for nosocomial infection following ambulatory surgery (D+C). 60-year-old woman.

Fig. 3. Chronobiologic serial section of automatically recorded axillary temperature before, during, and after hospitalization for nosocomial infection following ambulatory surgery (D+C). 60-year-old woman.
The amplitude of the circadian rhythm is decreased during the post-operative span and shows larger fluctuations than before surgery, where it is quite stable. The acrophase is also very stable prior to surgery and, as in the case of wrist activity, shows a drift corresponding to an acrophase delay after surgery. Of particular interest is the fact that this delay in the temperature acrophase is apparent ~18 h earlier than that of the wrist activity rhythm.

The effects of surgery on the circadian rhythm in axillary temperature are shown in Fig. 5. The rhythm is statistically validated (p < 0.05) throughout the observation span, except for a few short spans. A rise in mesor during the post-operative stay at home is in keeping with the rise observed for the rectal temperature mesor. The increased variability of the amplitude during this time is particularly noteworthy. Although the axillary temperature acrophase is more variable than that of rectal temperature, a fixed alteration is still apparent immediately following surgery. The diluting effect of data after surgery included in an interval centered before surgery is again seen.

Additional analyses by double demodulation (De Prins and Cornelissen) also indicate the stability of the acrophase and, to a lesser extent, the amplitude before ambulatory surgery and after recovery from nosocomial infection, for all 3 variables. Between the D and C and the subsequent hospitalization, however, the macroscopic pattern changes dramatically. These changes may be explained in part by the trend in the data caused by fever and lowering of activity. Similar changes that may be related to the nosocomial infection have not been seen in unpublished studies of uncomplicated surgery carried out in our laboratory on rats.

DISCUSSION

It will be very important to compare not only the immediate pre-operative and post-operative data but also any subsequent and the earlier long-term recording which one of us (EH) has kept up for a considerable span. Fig. 4 summarizes only part of the earlier studies, including measurements made in relation to a transequatorial flight. The contributions of fatigue and of any changes in circannual rhythm characteristics as a function of the environmental shift between winter and summer are confounded. This confounding also applies to earlier studies on transmeridian flights of similar duration (Levine et al.).

Also of interest are prominent changes with age in circadian rhythm characteristics of breast surface temperature obtained in a large chronoeidemiologic study (Halberg et al.), Tabs 1 and 2. The increase in amplitude with age is particularly noteworthy. It deserves follow-up studies on a larger scale with more miniaturized instrumentation. When developed, the chronobra (Simpson et al.; Wilson et al.) may meet this requirement of recording from multiple sites on the breast. With thermopiles from such instrumentation (E. Halberg et al.; Wilson et al.) sampling for hemopises, notably of prolactin (Haas et al.; Halberg et al.; Phillips et al.) may eventually be rendered cost-effective.

One of the purposes of this manuscript is to assess any merits of axillary and rectal temperature and motor activity monitoring in providing information on marker rhythms which may serve as reference functions in the clinic. If all 3 showed one or several rhythms with acceptable signal-to-noise ratios, the second purpose was to compare the relative merits and to ask whether monitoring may eventually be restricted to 2, if not a single variable, once sufficient information is
TOWARD A CHRONOPSY: PART III
available. Clearly, if the optimal variable should be activity rather than temperature, the use of temperature monitoring, apart from the collection of information on marker rhythms for the detection of classical fever, will have to be reconsidered.

We have seen that the first question, of the presence of a circadian rhythm, could be answered in the affirmative for all 3 variables. The second question, whether a single variable will suffice, awaits many more measurements before it can be answered.

In regard to the rectal temperature data before and after surgery, one finds at the time of surgery a drop almost certainly due to the general anesthesia and thereafter a rapid rise. Temperatures as high as the highest one on the preceding day are reached during the first post-surgical day, with an ensuing peak far beyond the highest values encountered before surgery. Had one monitored and prepared a gra-

<table>
<thead>
<tr>
<th>group</th>
<th>no. of series</th>
<th>no. of subjects</th>
<th>P</th>
<th>percent rhythm ± SE</th>
<th>mesor ± SE</th>
<th>amplitude ± SE</th>
<th>acrophase ± SE (95% CL)</th>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>15</td>
<td>8</td>
<td>&lt;0.01</td>
<td>57 ± 7</td>
<td>36.03 ± 0.19</td>
<td>0.19 (0.06, 0.36)</td>
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<td>0.02</td>
<td>45 ± 6</td>
<td>36.11 ± 0.18</td>
<td>0.17 (0.02, 0.41)</td>
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<td>post-menopausal</td>
<td>14</td>
<td>7</td>
<td>&lt;0.01</td>
<td>62 ± 5</td>
<td>55.73 ± 0.27</td>
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<td>13</td>
<td>8</td>
<td>0.06</td>
<td>35 ± 7</td>
<td>55.86 ± 0.26</td>
<td>0.20 ( - )</td>
<td>-517°( - )</td>
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<tr>
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<td>16</td>
<td>9</td>
<td>0.67</td>
<td>56 ± 7</td>
<td>55.98 ± 0.18</td>
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* In degrees, with 360° = 24 h, 15° = 1 h; reference = 00°.

Tab. 1 - Circadian variation of breast surface and axillary temperature of healthy Minnesotan women, summarised by population-mean cosinor (Halberg et al.)

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TOWARD A CHRONOPHY: PART III

a. circadian parameter estimates* from single cosinor:

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<td></td>
<td></td>
<td>menstrually cycling (no. = 16)</td>
<td>post-menopausal (no. = 14)</td>
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<tr>
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<td>C</td>
<td>55.98</td>
<td>55.77</td>
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<td></td>
<td>SE</td>
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<tr>
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<td>C</td>
<td>0.10</td>
<td>0.35</td>
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<tr>
<td></td>
<td>SE**</td>
<td>0.11</td>
<td>0.09</td>
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<tr>
<td>acrophase (Ø)</td>
<td>angular degrees</td>
<td>-62</td>
<td>-14</td>
</tr>
<tr>
<td></td>
<td>SE**</td>
<td>45</td>
<td>10</td>
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Sample mean of:

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<th>right breast</th>
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<td>0.46</td>
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<tr>
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<td>0.08</td>
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<tr>
<td>A % of M (A/M)</td>
<td>(%)</td>
<td>1.21</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.23</td>
<td>0.22</td>
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b. testing differences in parameters between pre- and post-menopausal subjects

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<th>kind of test</th>
<th>left breast</th>
<th>right breast</th>
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<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>(M, A, Ø jointly</td>
<td>T²</td>
<td>(2.26)</td>
<td>10.40</td>
</tr>
<tr>
<td>(A, Ø) jointly</td>
<td>T²</td>
<td>(2.37)</td>
<td>5.35</td>
</tr>
<tr>
<td>mesor (M)</td>
<td>t</td>
<td>(1.28)</td>
<td>7.55</td>
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<tr>
<td>amplitude (A)</td>
<td>t</td>
<td>(1.38)</td>
<td>0.04</td>
</tr>
<tr>
<td>A % of M (A/M)</td>
<td>t</td>
<td>(1.38)</td>
<td>0.06</td>
</tr>
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</table>

* Estimates summarizing imputations of individual parameters obtained by least squares fit of 24-h cosine curve to each subject's data; mesor = rhythm's mean value, amplitude = half of difference between rhythm's highest and lowest values; acrophase = timing of rhythm's highest value in relation to 000, with 360º = 24 h, 360º/24 h. ** The standard errors for amplitude and acrophase are approximations. ² Hotelling's T² statistic.

Tab. 2 - Age effect on circadian surface temperature rhythm of left but not of right human adult female breast.

...phic display of the data in a classical fashion, monitored as densely as in this case, it would have been clear that there was a response to ambulatory surgery. If, in turn, self-measurements would have been taken at much less dense intervals, it still seems likely that by curve-fitting, one could have detected what is apparent immediately, from the fit of a 24-h cosine curve, if one considers the average; the average rises promptly after surgery and remains high. This, however, is not the usual average; instead, it is a rhythm-adjusted mean (mesor), indicating that one deals with a mean for a rhythm characterizing the data.

The question whether there is also a change in frequency following surgery was tentatively explored by considering the acrophase. It is clear that the acrophases occur later and later, but an increasing trend remains unevaluated in the serial section, as it was carried out. A possible change in frequency suggested indirectly by a
change in acrophase is supported by the macroscopic inspection of the 'spikes' in the original chronogram.

To resolve the question whether the frequency did indeed change or not constitutes a two-fold problem for further research. It involves not only the need for trend identification and removal prior to frequency analysis, but also some endeavor toward separating the frequency and trend contributions from a host-microbial system in which the defenses of the former may respond in their known circadian cycling fashion to attacks by the latter (Halberg and Spink). Moreover, at least in vitro, certain microorganisms, as populations if not as individuals, exhibit spontaneous circadian rhythmicity (Rogers and Greenbank; Halberg and Conner) and, perhaps, rhythms also in responses to antibiotics.

To return to the data, the dramatic change of mesor after the D and C contributed at least in part to the apparent acrophase drift. Moreover, the acrophase change itself seems to be less pronounced in the axillary temperature than in the rectal one, although some delay is again obvious. With the change revealed by only a few days' data, one should not speak strictly of any transiently free-running periods, longer than 24 h, but rather a spectral solution contributed perhaps by several mechanisms in a host-microbial interaction.

In turning to body activity, an inspection of the chronogram shows the increase in irregularity after surgery as compared to before. Particularly interesting with respect to the changes in acrophase is the fact that after 3 post-operative days during which the acrophase was drifting, it returns to a position similar to that before surgery, when it seems to stabilize.

CONCLUSION

Much remains to be done concerning the microscopic interpretation of subtle and long-term changes, including those as a function of aging, discussed elsewhere (Halberg et al.). Rhythms can be viewed macroscopically, simply by inspection of original data or averages in displays. Depending on the extent and kind of noise in the data, such viewing can lead to impressions, abstractions or intuitive inferences. This approach does not require any inferential statistical point-and-interval estimates of rhythm characteristics. Any macroscopic approach to rhythms, however, has to be distinguished from a (complementary) 'microscopic' one. The latter relies on the objective quantification of temporal characteristics in biologic data based on the fitting of appropriate mathematical models, among other methods. Thereby, one may not only estimate temporal parameters on the basis of the best-fitting model, for instance, but one may also obtain time-specified reference standards ('chronodesms') for interpretation of single sample values (Halberg et al.).

Microscopic analyses reveal fever more reliably than spot-checks and in the case of continuous monitoring rather early, as in Figs 2 and 3. Such monitoring for fever may perhaps be advocated routinely for those subjected to ambulatory surgery, in order to detect an infection as soon as possible. What is yet more interesting is to systematically follow the relative effects of anesthesia, surgery and any infection, to better assess complications and/or to detect them earlier. Such an effort could complement the work started in the SENIC project.

It remains the subject of future research and is warranted on the basis of this study, which demonstrates that tools for both the automatic physiologic monitoring and the required numerical analysis are available, at least in some prototype form.
APPENDIX A

AMBULATORY SURGERY CENTER HOME CARE INSTRUCTIONS ADULTS

1. To ensure a safe recovery we ask that the following instructions be followed:
   A. When leaving the Ambulatory Surgery Center you must be accompanied by a responsible adult
      who can assist you.
   B. During the next 24 h after your anesthesia and surgery, follow these instructions:
      1. Do not drive or operate any machinery.
      2. Do not drink alcoholic beverages.
      3. Avoid climbing stairs.

2. HOME CARE AFTER AMBULATORY SURGERY

   Diet: Eat lightly today. Drink plenty of fluids. Tomorrow, resume your regular diet.
   Nausea: If you experience some nausea, try lying down and drinking fluids such as tea, soup or 7-UP. It is
   important to drink sufficient fluids.
   Drainage or dizziness: Some may be present 24 to 48 h. Stay home and rest the day of surgery. Do not
   drive or operate any machinery while dizziness, light-headedness or drowsiness persist.
   Fever: A low-grade fever is usual after even simple procedures. If it is elevated above 37.8 °C or lasts longer
   than 24 h, or is questionable in any way, do not hesitate to call your doctor or us.
   Discomfort: The amount of discomfort you may expect is unpredictable. Your doctor will advise you
   regarding pain medications.
   Activities: Rest on the day of surgery. Usually, you may resume normal activities over the next day or so.
   Your doctor may order specific limitations, however.
   Instruction: Generally, you will have a Band-Aid or a dressing over this. You may have a small amount of
   bleeding or drainage from this incision. The incision should be kept clean and dry at all times. If excessive
   pain or bleeding develops, consult your doctor or call us.
   Miscellaneous: You may experience a dry mouth, sore throat or nightmares from the anesthesia or medications
   used during surgery. These usually disappear in 12 to 24 hours.
   To call a doctor: Call and ask for the resident on call or call Ambulatory Surgery Center or the Emergency
   Department.

HOME CARE FOLLOWING A, B AND C

General: Follow the general instructions as well as the following:

Drainage: You will have a small to moderate amount of vaginal bleeding for several days: you may pass
some clots. This is normal. If an excessive amount of bleeding occurs, call your doctor.

Activities: Bed rest the day of surgery, then you may resume your normal activities as you feel like it, except:

1. No douching
2. No tampons for 72 h, use a pad
3. No heavy lifting for 1 week
4. No intercourse until bleeding has stopped
5. Bathe or shower, as you prefer

Discomfort: Most people have little or no discomfort. You could have some abdominal cramping or low
backache.

Call the doctor if the following occurs:
1. If there is fever
2. Excessive bleeding
3. Severe pain

Telephone numbers to call:

-------- for the resident on call for Gynecology
-------- for Ambulatory Surgery Center
-------- for the Emergency Department

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APPENDIX B
INSTRUCTIONS FOR TAKING BREAST TEMPERATURES WITH THE YELLOW SPRINGS INSTRUMENT

1. Scrub skin surface with a rough washcloth (in order to remove dead skin cells).
2. As shown in Fig. A, place 2 sensors on each breast, with the shiny metal surface against the skin. One sensor should rest halfway between the nipple and the upper edge of the breast tissue, vertically above the nipple and the second sensor halfway between the nipple and the lower edge of the breast tissue, vertically below the nipple. *
3. Secure each sensor with a non-allergenic tape (as on drawing). To prevent sudden movements from loosening this tape, the wire from the sensor is looped at a short distance from the sensor and also taped to the chest (Fig. B).

Fig. A - Positioning of sensor on breast.
Fig. B - Scheme to show how to secure and insulate sensors on breast.

4. Place a 5 cm x 7 cm x 1 cm insulating foam pad (Reson, 3M or equivalent) squarely over each of the 4 sensors.
5. In order to keep the wires of the sensors from tangling, it is best to tape them at intervals, including a site close to the plugs. The plugs should be numbered in sequence, corresponding to the number to be scratched on the black side of the sensor. The convention we use is as follows: left upper breast (LUB) = no. 1, left lower breast (LLB) = no. 2, right upper breast (RUB) = no. 3, right lower breast (RLB) = no. 4.
6. In order to take temperatures, each plug is inserted in sequence into the Yellow Springs Instrument. Before recording temperatures, turn the power switch of the telethermometer to 'on'. The red needle has to be superimposed on the red line on the left side of the scale on the telethermometer. If the needle and line are not superimposed, calibrate the thermometer by using the black adjustment button in the lower right corner of the meter's front panel. The red needle on the instrument and its image in the mirror have to be superimposed for accurate readings of each temperature. The temperature is then read in °F, for which the scale allows a more accurate reading. Results of analyses may then be converted to °C. The exact time is recorded with each set of readings. For the first time point we repeat the readings in succession, i.e. 1, 2, 3, 4 and again 1, 2, 3, 4 to assure that each sensor is recording the temperature accurately. If there is more than 0.2 °C discrepancy for one lead at the same time point, we repeat the sequence a third time.

If the difference is consistently ±0.2 °C, off for consecutive readings i.e. if the temperature seems to drift, the sensor should be replaced with a new sensor.

* Studies by liquid crystals or other means for thermography on the merits of identifying the sensor's best location with respect to vascular areas are recommended when means for this purpose are available.
Fig. C - Several sampling schemes for assessing breast surface rhythms of several frequencies.
7. When ultradian (menstrual) and circadian monitoring are both involved, temperatures are taken by the subject as often as possible while awake, but at least on getting up, before lunch, before supper and before retiring. For ultradian monitoring, breast temperature measurements are taken from day no. 1 of one menstruation to day no. 1 of the next menstruation (or for a span of at least 30 days for subjects before menarche or in menopause).

8. For ultradian (and circadian) monitoring, measurements every 20 min are desirable for a single wakefulness span (preferably at 3-day intervals for combined ultradian-to-circadian monitoring).

9. The simplest monitoring (but the one with most restricted promise of picking up prooepathology) is (exclusive) circadian monitoring. This may be done at 1. or 2-h intervals for one day (without interruption of sleep by night) or preferably for 4 days (4 consecutive wakefulness spans). Additional measurements at any convenient time are recommended. Several schemes are illustrated in Fig. C.

If for some reason the sensors have to be taken off, the position of each sensor location should be carefully marked with an indelible pen so that the sensors are replaced at the identical place each time. If the subject takes breast temperatures for one week or one menstrual cycle, sensors are taken off for each bath or shower and at night to avoid rash. The development of any rash should lead to discontinuance of monitoring for an appropriate span.

Since the tape has a tendency to stretch, tapes should be replaced once every 24 h to assure close contact between sensors and skin and also to inspect for rash. The times of retiring for sleep and of getting up, of meals, and any results of auscultationmetry (not only of breast temperature but also of other variables, such as blood pressure, Halberg et al.) should be recorded.

APPENDIX C

MEDILOG PREPARATION

1. Open Medilog, turn off and remove cassette. Then turn back on.

2. Dampen swab with ethyl alcohol and gently swab the tape head and capstan in that order. Avoid leaving any fibers from the swab on tape head or capstan.

3. Turn Medilog off.

4. Slowly but steadily pull white tab on battery carriage until one battery is free. Then the remaining batteries can be removed. Do not use any implements other than fingers in this operation.

5. Re-moisten swab and clean contacts at the ends of the battery carriage.

6. Install new batteries as shown next to battery carriage. Make certain fiberglass tab is under batteries so that batteries can be easily removed the next time.

7. Open new cassette and wind so that tape and not leader will be over the tape head. Usually a pencil or ball point pen can be used to wind the tape. Make sure that the tape moves freely. If the tape cannot be made to move freely, do not use the cassette.

8. Insert cassette into Medilog with full reel on appropriate side.

9. Turn Medilog on.

10. Set calibrator to lowest setting (29) and plug into the socket on the side of the Medilog which has 10 holes. Allow the Medilog to record with calibrator in this position for 5 min; then switch to second lowest setting (52) and record for 5 min. Check to make sure that the tape is moving, although very slowly. Advance the calibrator one position every 5 min until all positions (29, 52, 55, 58, 80) have been recorded for 5 min.

11. Unplug calibrator, record the exact time on the cassette along with the date, plug in the temperature probe and event marker harness. Close up Medilog and place in carrying case.
APPENDIX D
INSTRUCTIONS FOR USING SOLICORDER AND THERMOLOG DATA RECORDERS

1. DESCRIPTION OF THE RECORDING SYSTEMS
   a. Solicorder
      This temperature recorder is a rectangular plastic box weighing 270 g. 15.5 cm long, 7 cm wide and 3.5 cm thick. At one end of the box the probe can be plugged into a receptacle. A special button near the receptacle for the probe serves to check on the instrument's continued function. If the button is depressed a red light turned on automatically will indicate proper function and the failure of the red light to come on malfunction.

   b. TherMolog
      The TherMolog recorder is a plastic box of similar size as the Solicorder with 2 receptacles at one end. This instrument records both temperature and minor activity, the latter e.g. from wrist or foot. A nylon belt and pouch are provided for carrying this recorder. As compared to devices of similar cost, the multiple functional monitoring capability of the TherMolog and the versatility of the Apple II microcomputer have to be noted. The Apple II can be programmed to carry out numerical analyses such as the co-

2. PREPARATION OF CERTAIN RECORDERS FOR DATA COLLECTION
   a. First, a log sheet must be filled out for each recording span. Pay particular attention to the start times for both subject and machine. The subject start time begins when the probe is physically attached to the subject. The machine start time is the time when the power to the recorder is switched on.

   b. Solicorder; in keeping with details in the Solicorder pamphlet of instructions, connect the recorder to the D/R/CDU interface, erase the memory and load in a data 'preamble' which will identify and initialize the recording.

      TherMolog: turn the power on. To do this, use a screwdriver to unscrew the 2 small metal screws at the bottom of the recorder. Do not unscrew them completely, the plastic end-cap will come off after a few turns of each screw. Remove this end-cap. Be very careful to avoid contamination of the recorder when this cap is removed. Avoid touching the internal components, except the switches. There are 2 small black slide switches inside the recorder, located at the right side of the open end as the recorder is held with the "TemL 2" sticker upward. Just below these switches are explanations written on the blue circuit boards. The bottom of the 2 switches has labels 'OFF' and 'ON'. This is the power switch. When switched, this should be in the left or 'OFF' position. The switch above the 'ON' or 'OFF' switch should be in the right, or 'RUN' position. This switch should never be touched while recording data. If by mistake this switch is to the left, or 'DUMP' position, switch it to the right. Assuming that the upper switch points the right, the recorder can be switched 'ON' to start collecting data. Please record the complete time, including year, when the power switch is turned 'ON'. After starting the recorder, replace the end-cap. The end-cap need never be removed after the recording has begun. In particular, the power is not turned off after the recording has been completed. To do so would cause a loss of all data.

   c. Place the recorder in the carrying pouch, end-cap down, and so that the elastic loop will not interfere with the jacks at the top.

   d. Attach the probes to be used to the recorder. The TherMolog recorder has 2 jacks at the top end. The probes, one for temperature and one for activity, plug into these jacks. The jacks are slightly different to prevent mixup of the probe types. Slip the probe wires through the small slot in the carrying pouch and connect the probe connectors to the proper jacks. Ensure that the probes are plugged in completely.

   e. Connect the probes to the subject. The activity probe is usually worn on the non-dominant wrist. The cable should be worn under the clothing to prevent snagging. Rectal temperature probes are used with plastic sheaths for hygienic reasons. A sheath is slipped over the probe and lubricated with vaseline before the probe is inserted. This procedure is repeated with each reinsertion of the probe. Insert the probe to the depth of the sheath. The cable should be attached at the upright with 'Microgore' tape (IBM Company, St. Paul, Minnesota). An additional attachment should be made at the hip so that there are at least 2 attachments for the probe cable. Enough slack should be left between the attachments to prevent usual movements from pulling out the probe. The activity probe cable should also be secured with 'Microgore' tape at convenient places so as to allow for usual movements, while preventing undue slack (which would be bothersome on the body and could even result in the wearer's getting caught, e.g. on doorknobs and furniture). The cables are best passed to the recorder at waist level. Excess cable may be pulled through into the carrying pouch and looped loosely. Record the subject start time on the log sheet as soon as the probes are properly placed.
3. WHILE RECORDING IS IN PROGRESS:
   Record these events on the log sheet:
   a. Any times of probe removal or adjustment.
   b. Sleep-wakefulness spans.
   c. Meal times.
   d. Spans of strenuous or unusual activity.
   e. Any other circumstances which may affect the data.

   Attachment tape should be changed every day or whenever necessary. Monitor the rectal probe occasionally to be sure it has not slipped out.

4. INSTRUMENT REMOVAL WHEN RECORDING IS COMPLETED:
   a. Record the disconnection time on the log sheet.
   b. Unravel and unfasten the probes and remove any tape residue from the cables. Unplug the cables from the recorder. The rectal probes may be cleaned by application of a mild detergent followed by alcohol sterilization. Never expose the probes to high temperatures (e.g. never boil or autoclave them).
   c. Remove stain from rectal probe by soaking in hydrogen peroxide.

5. PROCEDURE FOR DUMPING THERMOLOG AND SOLCORDER DATA
   SPECIFICALLY FOR "BASIC DO" AND ONE DISK DRIVE

I. Setting up the Apple II computer
   a. Turn on the power switch located on the left side of the back panel of the Apple II computer.
   b. Turn on the power switch to the video monitor. The monitor will take about 15 seconds to warm up.
   c. Press the RESET button located at the upper right corner of the Apple console.

II. Loading of the data retrieval program
   a. Place the disk containing the system programs in the slot of the disk drive with the label upward. Push the disk gently as far as it will easily go, then close the latch which swings down from the top.
   b. To load the program:
      i. Key in C600G on the Apple console and hit the RETURN key to activate the disk drive.
      ii. The name of the disk, the volume number, and a listing of the files on the disk will appear on the screen. On each line appears a letter, a number and the name of the file. If this information does not appear, type in CATALOG and the information will be printed. A prompt character (either > or >) and a flashing square will appear at the bottom of the screen. If there is only a flashing square, there are more files on the disk. To list them, hit RETURN.
      iii. The data retrieval program is called RETRIEVE. Start it running by keying in RUN RETRIEVE.

III. Using the RETRIEVE program
   a. Tell the program which kind of recorder you are working with. The program will ask you to select i. TherMolog or ii. Solcorder recorder type. A list of options is then provided.
   b. To get instructions for the retrieval procedure, key in 1 followed by RETURN. The read-in procedure normally takes a couple of minutes. If there is an addressing error, it means there is something wrong with the recorder. Check the switch positions again and try again if in doubt. After the data have been read, slide the switch on the recorder back to RUN (if you are using a TherMolog recorder) and remove the recorder from the interface cable. If using a Solcorder machine, remove it from the DR/CIU interface.
   c. Assuming the data are correctly read, the parameter editing can start by keying in 2 and hitting RETURN. Fill in all parameters if in doubt about these parameters, a question mark may be keyed in to provide an explanation. Mistakes can be corrected after all the information has been entered. By entering the line number, the line may be changed. This portion of the program is terminated by hitting a RETURN instead of a new line number.
   d. Writing out information to a disk for storing data
      i. Take out the old disk.
      ii. Take the current disk on which data are being stored and place it into the drive.
      iii. Key in 3 to perform disk operations and respond with W (Writing) to the question.
      iv. The file name should be entered. To activate the disk drive, hit RETURN. When the write process is done, the program will return to the selection list again.
A Feasibility Study for Early Detection of Breast Cancer Using Breast Skin Temperature Rhythms

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Breast skin temperatures were measured at 30 min intervals for four days in 10 post-menopausal women, seven with primary breast cancer, one with benign breast disease and two with clinically healthy breasts. The rhythm characteristics of breast skin temperature for the area of skin over the tumour, between 3 and 5 cm in diameter, were compared with a similar site on the contralateral breast using methods of time series analysis. Results suggest that differences exist between the thermal characteristics of left and right breasts in healthy subjects and that these differences tend to be reinforced by a primary cancer in the left breast but reduced, or even reversed, by one in the right breast. It may be possible to exploit these effects to detect breast cancer at an early stage particularly when the device known as the "chronobra" is available for the automatic collection of breast skin temperature data.

Introduction
Considerable differences of opinion exist in the literature (Hitchcock et al., 1968; Davey et al., 1970; Furnival et al., 1970; Nathan et al., 1970; Price, 1970; Stark and Way, 1970; Davey et al., 1972; Nathan et al., 1972; Gautherie and Gros, 1980) concerning the value of thermography in detecting breast tumours and, consequently, its potential usefulness for breast cancer screening in "Well-Women" clinics (Davey et al., 1970). The large number of false positives and the unacceptable false negatives have meant that, generally, thermography has only a supporting role in the clinical management of women with carcinoma of the breast. Studies in the Tenovus Institute and elsewhere (Phillips et al., 1980; Mansfield et al., 1973;
Gautherie and Gros, 1977) of breast skin temperatures in pre- and post-menopausal women have clearly demonstrated the existence of statistically significant circadian rhythms which are affected by such factors as activity, dietary intake and the environment. It is not surprising, therefore, that a single snapshot of thermal radiation from the breasts cannot be truly representative of the underlying breast biology. Furthermore it has been suggested (Phillips et al., 1980; Mansfield et al., 1973; Gautherie and Gros, 1977) that these rhythms are affected by the presence of a tumour.

This report is concerned with the mathematical characterization of breast skin temperature series in order to examine the possibility that a tumour site can be distinguished from a similar site on the contralateral breast. This characterization has been achieved using the methods of time series analysis and it is hoped that these techniques will make possible the early detection of breast cancer. The finding of abnormalities in the temperature series could initiate a thorough clinical examination of the subject and instigate early treatment of any breast disease found.

Essential to any such programme of research, however, are the appropriate statistical methods that can be used to properly characterize these series of breast skin temperatures and a section of this report is devoted to these methods and their use in relation to the breast study.

Materials and methods

Patients

These were post-menopausal women between 58 and 82 years of age, seven with primary breast cancer, one with benign breast disease and two with clinically healthy breasts. Patients were studied in a ward environment where ambient temperature (21–23°C), timing of dietary intake, nocturnal rest schedule (2200–0600 hours) and activity were well controlled. A detailed questionnaire entitled “Questionnaire for volunteers in a chronoepidemiologic study on rhythms, reproduction and risk of breast cancer as well as cardiovascular disease” designed by F. Halberg, University of Minnesota, was completed for all patients studied. The pertinent features included general information, employment and travel history, gynaecological and obstetrical history, and general medical and familial history. A histopathological examination of all excised tumours was conducted.

Measurement of Temperature

Miniature thermistor sensors (4 mm diameter with reverse side insulated) connected through fine electrical wire to small plugs were attached to each quadrant of both breasts. The areas were defined vertically by the cephalo-caudal line and horizontally by the parasternal to mid-axillary line, the nipple located at the origin. A sensor was attached to the centre
of the skin overlying the tumour and a corresponding sensor located on a similar site on the contralateral breast. Sensors were also placed on the centres of the remaining breast quadrants. These were attached to areas of skin with hypoallergenic Micropore surgical tape purchased from Medical Products Division, 3M Co. Ltd., St Paul, Minnesota, USA; sensors were obtained from Light Laboratories, Brighton, England.

Breast temperatures were generally measured at 30 min intervals throughout a time span of up to 96 h prior to surgery. Temperatures were taken with patients wearing ordinary night-clothing with bed-clothing up to their midriff and were in a recumbent position, except for excursions at 2-hourly intervals to the bathroom, during the study.

**Statistical Analysis**

The objective of the statistical analysis is to describe these series of breast temperatures by a model which is simple, realistic and provides a satisfactory fit for all series of data collected. It is important that estimates of the parameter values of the model, derived from the data, are accurate and their standard errors reliable. This depends not only on the method of estimation but also on the actual model used since the calculated parameter values and their standard errors are model-dependent. Consequently a number of models were considered and are described below.

*The cosinor and extended models.* The simple cosinor model has been described (Halberg *et al.*, 1967) and applied to the analysis of breast skin temperature (Phillips *et al.*, 1980; Mansfield *et al.*, 1973; Gautherie and Gros, 1977). This model can be represented in the form

\[ y(t) = \beta_1 + \beta_2 \cos \omega t + \beta_3 \sin \omega t + \epsilon(t) \]

where \( y(t) \) is the temperature at time \( t \), \( \omega = 2\pi/24 \) radians/h, \( \beta_1, \beta_2, \beta_3 \) are regression parameters and \( \epsilon(t) \) denotes a random error term at time \( t \). This model can also be expressed in a form more familiar to the chronobiologist by

\[ y(t) = M + A \cos (\omega t + \phi) + \epsilon(t) \]

where \( M \) is the mean of the fitted rhythm (mesor), \( A \) is the amplitude of the fitted cosine function and \( \phi \) indicates the peak of the fitted rhythm relative to some reference point, such as midnight, and is known as the acrophase. This model can be viewed as a cosine wave with superimposed random errors.

The standard methods of analysis using this model assume that the errors have zero mean, constant variance and are uncorrelated and that a sinusoidal wave form is present. Spectral analysis (Fuller, 1976) of the data revealed a periodic effect of 12 h as well as one of 24 h. Consequently a
more realistic model is required to describe the data and one such model is

\[ y(t) = \beta_1 + \beta_2 \cos \omega t + \beta_3 \sin \omega t + \beta_4 \cos 2\omega t + \beta_5 \sin 2\omega t + \epsilon(t) \]

or, equivalently,

\[ y(t) = M + A_1 \cos (\omega t + \phi_1) + A_2 \cos (2\omega t + \phi_2) + \epsilon(t) \]

where \( A_1 \) and \( A_2 \) are the amplitudes of each rhythm component with corresponding phases \( \phi_1 \) and \( \phi_2 \). This model includes the fundamental (cosinor model) and the first harmonic of period 12 h. Additional harmonics were included in the investigation initially but produced only marginal improvements in the fit and for simplicity were subsequently omitted. Some patients showed variations at other frequencies but these did not appear consistently in all series and in the interests of generality were not included in the model. There will also, no doubt, be periodic variations at lower frequencies but the data were collected over too short a period for monthly or annual effects to be estimated and so these were ignored. It was found that the error terms \( \epsilon(t) \) are highly correlated and so it is inappropriate to use the ordinary least squares procedure to estimate the regression parameters since the calculated standard errors will seriously underestimate the true error (Fuller, 1976). This necessitated the use of a time series model.

**Time series model.** The type of correlation present in the errors was modelled using various statistical techniques (Fuller, 1976; Harvey and Phillips, 1979) and can be adequately described by

\[ \epsilon(t) = \alpha \epsilon(t-1) + z(t), \]

where \( \epsilon(t) \), the error at time \( t \), is a multiple \( \alpha \) of \( \epsilon(t-1) \), the error at time \( t-1 \), plus a random component \( z(t) \) which has zero mean and constant variance for all values of \( t \). Such a model is referred to as an autoregressive model of order 1 (AR(1)) (Box and Jenkins, 1970). This model can now be combined with the extended cosinor model so that the correlated errors can be taken into consideration when estimating regression parameters \( \beta_1 \) to \( \beta_5 \). Thus the full model is

\[ y(t) = \beta_1 + \beta_2 \cos \omega t + \beta_3 \sin \omega t + \beta_4 \cos 2\omega t + \beta_5 \sin 2\omega t + \epsilon(t), \]

where \( \epsilon(t) = \alpha \epsilon(t-1) + z(t). \)

**Results**

It is clearly impossible in the space allowed to present the analyses of 80 series of breast skin temperatures obtained in this study. The results presented mainly concern the comparison of a series of temperatures obtained
from a sensor over the tumour with a series from a similar site on the contralateral breast, for seven patients with primary breast cancer and for one patient with benign breast disease. For patients with clinically healthy breasts, results for the upper inner quadrant of both breasts were selected for presentation since this is the most common site of cancer observed in this study. For the purpose of distinguishing features of the results that are of essentially statistical or biological importance the results section of this report has been separated accordingly.

Statistical Results

Table 1 shows the results for three models for two patients with primary breast cancer located in the upper outer and upper inner quadrant respectively. Models I—III refer to the cosinor, extended cosinor (harmonic) and full model respectively. The percentage variation explained (PVE) was calculated for each series as follows:

\[
PVE = \frac{(\text{Variance of series} - \text{Variance of residuals})}{\text{Variance of series}} \times 100,
\]

due to a useful relative measure of the degree of fit for the various models. These two examples have been singled out since they represent the extremes of the data collected in terms of their rhythmic appearance as shown in Figure 1(a–d). In patient 1 the PVE is significantly increased when the harmonic is added and is further increased when the time series model is used. Patient 2 illustrates that models I and II account for hardly any of the variation, whereas model III produces a sharp rise in the PVE. The fact that the PVE, when model II is fitted, is less in patient 2 than patient 1 need cause little concern; it seems that the breast temperature of patient 2 merely has a much larger random component than patient 1, so that the resulting PVE underestimates the quality of fit. Consequently the full model was used to analyse the data from all patients and these results are shown in Table 2.

Table 2 contains the estimates of the regression parameters, \( \beta_1 \) to \( \beta_8 \), with their standard errors in parentheses. The variance of each time series, the residual variance associated with the fit of the model, the PVE and the value of \( \alpha \) in the AR(1) model are also presented. A broad cross-section of examples which show the fit of models II and III are displayed in Figure 2(i–iv). It can be seen that model III provides a good description of the data and is superior to models I and II.

In earlier work (Phillips et al., 1980; Mansfield et al., 1973; Gautherie and Gros, 1977) on breast skin temperature rhythms, emphasis was placed on the mesor, amplitude and acrophase. With respect to the extended cosinor model the fitted wave can assume a variety of shapes; sometimes
Table 1

Estimates $\hat{\beta}_1$ to $\hat{\beta}_5$ of the regression parameters $\beta_1$ to $\beta_5$, together with the initial variance, residual variance and PVE for each patient are shown, when models I–III are fitted. The standard errors of the estimates are given in parentheses. Also shown is the estimated $\alpha$ value for model III. In patients 1 and 2 the tumours were located in the upper outer (UO) and upper inner (UI) quadrants respectively, cancerous and contralateral site being indicated by (C) and (N). (a)–(d) refer to the plots in Figure 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Model</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\beta_3$</th>
<th>$\beta_4$</th>
<th>$\beta_5$</th>
<th>Initial variance</th>
<th>Residual variance</th>
<th>Percentage variation explained (PVE)</th>
<th>Value of $\alpha$ when AR(1) model fitted</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>I</td>
<td>35.06</td>
<td>0.790</td>
<td>-0.059</td>
<td></td>
<td></td>
<td>0.901</td>
<td>0.594</td>
<td>34.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>35.06</td>
<td>0.790</td>
<td>-0.058</td>
<td>0.459</td>
<td>0.483</td>
<td>(0.045)</td>
<td>(0.064)</td>
<td>(0.051)</td>
<td>(0.051)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>35.06</td>
<td>0.819</td>
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<td>0.482</td>
<td>(0.064)</td>
<td>(0.115)</td>
<td>(0.116)</td>
<td>(0.110)</td>
</tr>
<tr>
<td>(b)</td>
<td>I</td>
<td>34.64</td>
<td>1.04</td>
<td>0.259</td>
<td></td>
<td></td>
<td>1.284</td>
<td>0.720</td>
<td>43.9</td>
<td>-</td>
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<tr>
<td></td>
<td>II</td>
<td>34.64</td>
<td>1.04</td>
<td>-0.259</td>
<td>0.466</td>
<td>0.649</td>
<td>(0.050)</td>
<td>(0.071)</td>
<td>(0.071)</td>
<td>(0.071)</td>
</tr>
<tr>
<td></td>
<td>III</td>
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<td>0.251</td>
<td>0.466</td>
<td>0.653</td>
<td>(0.085)</td>
<td>(0.118)</td>
<td>(0.119)</td>
<td>(0.113)</td>
</tr>
<tr>
<td>(c)</td>
<td>I</td>
<td>35.58</td>
<td>-0.016</td>
<td>-0.186</td>
<td></td>
<td></td>
<td>0.371</td>
<td>0.364</td>
<td>2.7</td>
<td>-</td>
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<tr>
<td></td>
<td>II</td>
<td>35.58</td>
<td>-0.015</td>
<td>-0.186</td>
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<td>(0.070)</td>
<td>(0.071)</td>
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<tr>
<td></td>
<td>III</td>
<td>35.58</td>
<td>-0.023</td>
<td>-0.181</td>
<td>0.087</td>
<td>0.106</td>
<td>(0.060)</td>
<td>(0.130)</td>
<td>(0.134)</td>
<td>(0.121)</td>
</tr>
<tr>
<td>(d)</td>
<td>I</td>
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<td>-0.108</td>
<td>-0.240</td>
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<td>0.403</td>
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<td>-0.240</td>
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<td>0.105</td>
<td>(0.051)</td>
<td>(0.071)</td>
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<tr>
<td></td>
<td>III</td>
<td>35.33</td>
<td>-0.136</td>
<td>-0.243</td>
<td>0.001</td>
<td>0.110</td>
<td>(0.127)</td>
<td>(0.164)</td>
<td>(0.170)</td>
<td>(0.133)</td>
</tr>
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</table>

(a)–(d) refer to the plots in Figure 1.
The plots show the raw data (heavy line) and demonstrate the fit of the extended cosinor model (smooth curve), and the full model (III). Plots (a)–(d) refer to the sensors indicated in Table 1.
Table 2

Estimates $\hat{\beta}_1$ to $\hat{\beta}_5$ of the regression parameters $\beta_1$ to $\beta_5$, together with the initial variance, residual variance, PVE and estimated $\alpha$ value are shown for each patient when model III is fitted. The standard errors of the estimates are given in parentheses. Patients 1–4 and 5–7 had primary breast cancer of the right and left breast respectively. Patient 8 has benign breast disease in the left breast and patients 9 and 10 have clinically healthy breasts. The breast quadrants are denoted by UI (upper inner), UO (upper outer), LI (lower inner), LO (lower outer). Histopathology features for each tumour are shown in the legend of Table 3 (i)–(iv) refer to the plots in Figure 2.

<table>
<thead>
<tr>
<th>Patient code</th>
<th>$\hat{\beta}_1$</th>
<th>$\hat{\beta}_2$</th>
<th>$\hat{\beta}_3$</th>
<th>$\hat{\beta}_4$</th>
<th>$\hat{\beta}_5$</th>
<th>Initial variance</th>
<th>Residual variance</th>
<th>Percentage variation explained (PVE)</th>
<th>Value of $\alpha$, when AR(1) model fitted</th>
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</thead>
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<tr>
<td>1</td>
<td>UO(C)</td>
<td>35.56</td>
<td>0.0419</td>
<td>-0.038</td>
<td>0.0428</td>
<td>0.482</td>
<td>0.901</td>
<td>0.182</td>
<td>79.8</td>
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<td></td>
<td>UO(N)</td>
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<td>1.036</td>
<td>-0.261</td>
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<td>0.653</td>
<td>1.284</td>
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<td>83.4</td>
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<td>2</td>
<td>UI(C)</td>
<td>35.58</td>
<td>-0.023</td>
<td>-0.183</td>
<td>0.087</td>
<td>0.106</td>
<td>0.371</td>
<td>0.225</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>UI(N)</td>
<td>35.33</td>
<td>-0.136</td>
<td>-0.243</td>
<td>-0.001</td>
<td>0.110</td>
<td>0.403</td>
<td>0.172</td>
<td>57.3</td>
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<tr>
<td>3</td>
<td>UO(C)</td>
<td>34.78</td>
<td>0.235</td>
<td>-0.042</td>
<td>-0.012</td>
<td>0.031</td>
<td>0.593</td>
<td>0.345</td>
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<td>(i)UO(N)</td>
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<td>0.072</td>
<td>0.020</td>
<td>1.325</td>
<td>0.512</td>
<td>61.4</td>
<td>0.77</td>
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<tr>
<td></td>
<td>(ii) UI(C)</td>
<td>UI(N)</td>
<td></td>
<td>(iii) LO(C)</td>
<td>LO(N)</td>
<td></td>
<td>(iv) UI(R)</td>
<td>UI(L)</td>
<td></td>
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<td>-------</td>
<td>---</td>
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<td>59.4</td>
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<td>5</td>
<td>35.11</td>
<td>(0.077)</td>
<td>67.1</td>
<td>45.2</td>
<td>59.4</td>
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<td>0.46</td>
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<td>6</td>
<td>35.14</td>
<td>(0.079)</td>
<td>67.1</td>
<td>45.2</td>
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<td>7</td>
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<td>(0.077)</td>
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<td>45.2</td>
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<td>8</td>
<td>34.01</td>
<td>(0.076)</td>
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<td>45.2</td>
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<td>34.04</td>
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<td>43.2</td>
<td>49.1</td>
<td>0.46</td>
<td>0.59</td>
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</table>

The table above contains a series of values, likely representing various measurements or data points, organized in a grid format.
The plots show the raw data (heavy line) and demonstrate the fit of the extended cosinor model (smooth curve), and the full model (III). Plots (i)-(iv) refer to the sensors indicated in Table 2.
there will be one peak in every 24 h and sometimes two. The amplitudes
and phases of the two component waves are shown in Table 3, together
with standard errors in parentheses. Standard errors for amplitude and
phase are not easily calculated, but approximate values were obtained
using asymptotic results (Kendall and Stuart, 1967). These are also pre-
sent in Table 3. A study of Figures 1 and 2, together with the standard
errors listed in Table 3, indicates that, while the standard errors are gen-
erally large, they do seem to be realistic since it can be seen that the wave
forms could be translated by the amount (±2 standard errors) without
seriously affecting the fit.

The question of whether significant differences exist between the two
series, as judged by parameter estimates, has already been raised. The
differences in parameter values are presented in Table 3 together with
their standard errors. The calculation of these standard errors must take
into account the generally high positive correlation between the two series.
It is incorrect to simply take the square root of the sum of the variances in
this case. For example, in patient 10 the standard errors for individual
estimates of φ1 are 0.747 and 0.929 for the upper inner quadrants of the
right and left breast respectively; the standard error of the difference is
0.707 whereas the square root of the sum of the variances is 1.192.

**Biological Results**

A close study of the results from each sensor on every subject suggested
two sets of hypotheses for the rhythm characteristics of normal and caner-
ous subjects. For normal subjects these are:

(i) The mean temperature of the left breast is greater than that of the
right breast (supported by Evans and Gravelle, 1973).

(ii) The amplitude A1 and initial variance are smaller for the left
breast than for the right breast.

(iii) The fitted rhythm peaks earlier in the left breast than in the right.

The results suggested, furthermore, that in the neighbourhood of a
tumour the rhythm characteristics are affected in the following way:

(a) The mean temperature of the cancerous site is raised.

(b) The amplitude of the rhythm and the initial variance are reduced.

(c) The peak of the rhythm brought forward, i.e. the phase is increased.

For patients with a tumour in the left breast, this could result in the
differences for the mean temperature, amplitude and phase being accentu-
ated, as exemplified by patient 5 shown in Table 3. For patients with a
tumour in the right breast these differences may tend to be nullified and the
differential effects of the left breast may be observed to a lesser degree, if
Estimates of the rhythm parameters $\beta_1$, $A_1$, $\phi_1$, $A_2$, $\phi_2$, together with their estimated differences, and their associated standard errors in parentheses are shown. Histopathological description of tumours is given with patient identity in parentheses; invasive carcinoma with marked scirrhus reaction (1), moderately differentiated lobular carcinoma (2), moderately differentiated carcinoma (3 and 4), moderately differentiated invasive carcinoma (5), poorly differentiated invasive carcinoma (6) and well-differentiated invasive carcinoma (7). Patient 8 had benign breast disease with fibro-fatty tissue bearing a haemorrhagic cavity. (R) and (L) refer to right and left breast respectively.

<table>
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<th>Patient code</th>
<th>$\beta_1$</th>
<th>Mean</th>
<th>$A_1$</th>
<th>$A_1$ diff.</th>
<th>$\phi_1$</th>
<th>$\phi_1$ diff.</th>
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<th>$A_2$ diff.</th>
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<th>$\phi_2$ diff.</th>
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<td>(0.237)</td>
<td>(0.286)</td>
<td>(4.078)</td>
<td>(0.266)</td>
<td>(0.334)</td>
<td>(0.250)</td>
<td>(13.21)</td>
<td>(24.41)</td>
</tr>
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</table>
at all, as illustrated by patient 2 shown in Table 3. To illustrate this further, the results from all sensors (data not shown here) relating to cancer patients 1–7 were compared. The mean temperature level was raised in 24 cases out of 28 when compared with the contralateral breast and out of the four discrepancies three were in patients with a tumour in the right breast. With respect to amplitude, $A_1$, this was reduced in 19 cases in the breast bearing the tumour and of the nine discrepancies, six were in patients with a tumour in the right breast. In patients with cancer in the left breast, the temperature peaked earlier 11 times out of 12. For patients with cancer in the right breast the unaffected left breast peaked earlier 11 times out of 16 suggesting that the assumed difference in phase existing in the normal state is perhaps larger than the change produced by the presence of the cancerous tumour. Patient 8 with a benign growth conformed to neither the cancerous nor normal pattern and since there was only one patient of this type no general hypothesis has been formulated.

Correlation of histopathological features of the tumours with rhythm parameters of breast skin temperature was examined but no definite conclusions have yet been drawn from the limited amount of information available.

Conclusions and Discussion
A statistical model has been developed that adequately describes series of breast skin temperatures measured at 30 min intervals for up to 96 h for post-menopausal women, with either primary breast cancer, benign breast disease, or clinically healthy breasts, studied in a hospital environment. The model, which was the extended cosinor model with a time-series component, provided a good fit to the data for all patients studied.

Important implications for the detection of breast cancer could emerge if further work supports the hypotheses drawn as a result of these investigations. Since statistically significant differences in rhythm parameters may be present in a healthy subject between left and right breasts and no difference may indicate the presence of a tumour, effective screening could require monitoring the subject at suitable intervals to detect any changes in their thermal characteristics due to a developing tumour.

The feasibility of measuring breast skin temperatures for the early detection of breast cancer has not been fully proven but there is sufficient evidence from these patients with tumours of between 3 and 5 cm in diameter to suggest that there are differences in the thermal characteristics of circadian rhythms of breast skin temperatures that may be exploited for such a purpose. These results form the basis of a larger pilot study to be undertaken when the final version of the “chronobra”, a device that
automatically monitors and stores breast skin temperatures, becomes available later this year.

The results of this feasibility study therefore indicate that reliable screening of women for breast cancer may soon, with suitable equipment and a proper statistical approach, be a realistic possibility, this being of particular importance when mammography may be contra-indicated (Hartman, 1977). Of particular importance are those women who can be classified as belonging to a high risk category for breast cancer. This category would include, for example, women with a familial history of breast cancer, those who have been found to be false positives on mammographic examination (Gautherie and Gros, 1980) and those who have risk factors associated with age at first-term birth and menarche. It may even be possible to relate rhythm characteristics with pre-neoplasia of the breast (Jensen et al., 1976).

Acknowledgements

The authors are grateful to the Tenovus Organization for their generous financial support and to the Nuffield Foundation in providing a grant towards computer programming and data processing. The contributions of Mr Roger Blarney and Mr H. W. Holliday, Department of Surgery, University of Nottingham, and Dr H. W. Simpson, Department of Pathology, Royal Infirmary, Glasgow, are gratefully acknowledged. The authors from the Tenovus Institute are indebted to Professor F. Halberg, University of Minnesota, Minneapolis, for his collaboration in this project.

REFERENCES


Bimodal Age-Frequency Distribution of Epitheliosis in Cancer Mastectomies

Relevance to Preneoplasia

H. W. SIMPSON, MD, PhD, FRC PATH,* F. MUTCH, MB CHB,* F. HALBERG, MD,† K. GRIFFITHS, BSc, PhD,‡ AND D. WILSON, BSc, PhD‡

A census of epitheliosis in 500 consecutive cancer mastectomies has been carried out. The probability of concurrence of this phenomenon with cancer has a bimodal age-frequency distribution. It is high in cancer mastectomies from women in their early 40s, low in the late 50s and high again in the elderly. Epitheliosis during the reproductive life span is regarded as a reversible ovary-dependent abnormality. It is greatly increased in the premenopausal cancerous breast and it is thought that it carries increased risk for cancer initiation. In contrast the probability of epitheliosis in the breast in the elderly cancer patient is only slightly greater than in "noncancerous" post mortem breasts of similar age. Much of this "epitheliosis" may represent, in fact, indolent autonomous cancer though a small proportion could be epitheliosis supported by extraneous estrogen.


PATHOLOGISTS, both classical1,2 and modern,3,4 have noted transitions from mammary epithelial 'hyperplasia' (epitheliosis) to neoplasia in some cases of breast cancer. Jensen and her colleagues4,5,6,8 (see also complementary discussion by Cardiff et al.) have made exceptionally detailed dissections of cancer and autopsy breast tissue to reach important conclusions on the anatomical aspects of this association. Of particular interest is her study of the frequency and distribution of so-called "atypical lobules." These nodules of excess epithelium seen by the dissecting microscope correspond to epitheliosis in routine histopathologic studies. She found these structures more common in mammary tissue ipsilateral and contralateral to cancer. This suggests that cancer does not initiate in a normal mammary epithelium, but rather after a series of epithelial proliferations.

As the above concept stands in 1981, there is difficulty in the credible application of the (focal) epitheliosis–neoplasia concept to the generality of human breast cancer.

The problem is summarized in conclusion 5 of Sir Robert Muir's article on the genesis of mammary cancer written in 1941.2 He wrote "in a large proportion of cases of carcinoma of the breast, the stages of evolution of malignancy within ducts and acini cannot be followed and in some of these there is evidence that malignancy arises de novo without the occurrence of preliminary hyperplastic changes." Many pathologists would agree with this. It is easy to verify, certainly at ordinary levels of sampling, that many breast cancers reside in atrophic rather than "epitheliotic" breast tissue. In 1979, Gray,6 concluded from the examination of 178 cancer mastectomies that epithelial hypoplasia was present in 75 and "hyperplasia" in (only) 62.

We regard age as an important variable in all mammary indices and, thus, we decided to define the age-frequency distribution of epitheliosis in 500 consecutive Glasgow Royal Infirmary cancer mastectomies in the hope that it would give information on cancer pathogenesis.

Methods

A name and age list was prepared of 500 consecutive cases of breast cancer treated at Glasgow Royal Infirmary between 1974 and 1979. The filed histopathologic sections relating to these cases were assembled for review. The two pathologists (H.W.S. and F.M.) reassessed the slides covering about 20 cases at a session using a twin-headed microscope. During this review they were...
FIGS. 1-6. Selected sections from cancer mastectomies performed at Glasgow Royal Infirmary from 1975-1979. The diagnosis of invasive cancer was made from other sections of the breast tissue. The six sections illustrated exemplify lesions that were assessed as "epitheliosis" and not carcinoma in situ. The cases were unselected for age but fell as expected into two groups: premenopausal and the elderly.

Fig. 1. Section from a 43-year-old woman at mastectomy. Para 4 +0. Expansion of lobular alveolar units by epitheliosis with atypia from mild to severe.

Fig. 2. Section from a 44-year-old woman at mastectomy, considered to represent cribriform epitheliosis. While the spaces represent shrinkage artefact in some parts, in others the appearances are of a semilaminar construction.

Fig. 3. Section from a 46-year-old woman at mastectomy considered to represent "solid type" epitheliosis with only mild atypia.

Fig. 4. Section from a 55-year-old nulliparous woman at mastectomy. Cribriform epitheliosis with apocrine metaplasia and microcalcifications. In our experience the juxtaposition of epitheliosis, metaplasia, and cystic change was a definite entity suggesting a common origin for each or a time-dependent transition.

Fig. 5. Section from a 71-year-old woman at mastectomy who currently has a contralateral breast lump. Cribriform epitheliosis with mild atypia suggesting either a neoplastic pathogenesis or an extraovarian source of estrogen, e.g., adrenal.

Fig. 6. Section from a 74-year-old woman at mastectomy. Section represents cribriform epitheliosis.
unaware of the age of the particular patient. All sections relating to each case were examined. Normally they contained diagnostic “cancer areas,” uninvolved breast (including a block taken specifically away from the tumor) and a section through the nipple. All of the noncancerous breast tissue was assessed for epitheliosis except the tissue from the region of the nipple and the lactiferous sinuses. With these exclusions an average of two tissue sections of material from breast parenchyma proper was available for scoring. This was on a simple all-or-none basis. The pathologists sought to determine if the breast tissue showed unequivocal epitheliosis or not, i.e., “regular typical epithelial proliferations in ducts or lobules” (WHO AIII lesions). In the literature, the term epitheliosis has been used synonymously with this definition. Dawson, who originated the term, described it as a “multiplication of epithelial cells within existing ducts and ductules without the formation of new glandular elements.” As she said, this proliferation may be of “solid or papillary character or may form a multilayered cell lining.” During the census it was an absolute rule that all decisions on the presence or absence of epitheliosis were made at the time of looking at the slides and no changes were made thereafter. Doubtful cases were scored as negative for epitheliosis. Typical examples of cases scored positively for epitheliosis are illustrated in Figures 1–6. It should be noted that all epithelia (including apocrine or other metaplastic types) were scored positive for epitheliosis if they fulfilled Dawson’s criteria (we have not found a specific reference as to whether metaplastic epithelia are included in her definition).

**Results**

Results are presented as a graph (Fig. 7). They confirm and extend our earlier conclusions of a peak frequency of epitheliosis in cancer mastectomies from patients in the 41–45-year age rank and a trough in the 56–60-year rank. The increased number of specimens now studied has enabled us to define a second peak in the elderly (80–90 age group). This is significant at the 2.5% level by a Spearman test for a monotonic increase with age. The evidence for a sharp fall of cancer associated epitheliosis in mastectomies from patients aged
in their middle 40s to late 50s is discussed here *in extenso*, as we think it is a new and important finding.

The authors examined an estimated average of two paraffin blocks of noncancerous mammary tissue per case, and 69% of the 78 mastectomies falling into the 41-45-year age group contain such foci of epitheliosis. An estimate of the reliability of this percentage *vis-a-vis* a universal population, can be obtained from estimating this figure from each year's (1974 through 1979) cases separately. New means and their standard errors can then be calculated from the six estimates (Fig. 8). The individual yearly estimates are 69%, 58%, 67%, 71%, 73%, and 75%, respectively. In other words, the overall average (69%) is a reasonably reliable indicator of the "universal" population. This high frequency of epitheliosis is in sharp contrast to the low frequency found in the 65 mastectomies falling into the 56-60-year age group. Here the average incidence of epitheliosis was found to be only 17% with the individual estimates being scattered as follows: 8%, 21%, 29%, 30%, 0%, and 14%.

It is reaffirmed that these estimates were made by two pathologists with no knowledge of the patient's age at mastectomy. We conclude that a sharp fall in the epitheliosis incidence in cancer mastectomies from the 41-45-year age group to the 56-60-year age group does characterize the data and that there is a bimodality.

**Discussion**

The question arises as to whether the bimodal distribution of the frequency of epitheliosis is an age-related trend of normal mammary tissue or whether cancerous breasts exhibit an altered frequency. For comparison, we have a substantial census of the "normal" age-frequency distribution of epitheliosis estimated from 800 consecutive autopsies (unselected for diagnosis) at an adjacent Glasgow hospital by our colleague Dr A. T. Sandison. The sample size of tissue examined in each case of Sandison's study was of a similar order to that of our own and therefore it forms a valid comparison especially in regard to age trends. His tabulated data have been plotted so that a comparison may be made between the observed and expected frequency (Table 1;
compare Figs. 7 and 9). The graph also summarizes a similar "control" census of epitheliosis frequency derived from a New York autopsy population published by Frantz and colleagues.\textsuperscript{15} Perusal of these three studies show that, premenopausal cancer mastectomies contain a substantial increase of epitheliosis above the expected. It can be seen from Table 1 that cancerous breasts from patients aged 26–55 years contain 2.6–9.5 times the expected frequency. In contrast, postmenopausal cancerous breasts evidenced an observed frequency of epitheliosis above but yet remarkably close to that expected from the autopsy studies.

Jensen and colleagues showed by subgross dissection of 52 breasts that there was, on average, an increase in "atypical lobules" in cancer-associated tissue (that is above autopsy controls; 65 breasts dissected); and, further, that there was an age-related increase in this phenomenon until the seventh decade.\textsuperscript{4}

Our data confirm that there is an overall increase in epitheliosis in cancer-associated breast tissue (X2.8; table 1) and that over selected age ranges there are systematic increases with age, (i.e., age 26–40 years and 61 years upward).

However, our finding of a bimodal age-frequency distribution of epitheliosis in cancer-associated breast tissue is in partial conflict with her conclusions and a discussion is pertinent. First it is important in this context that there are between-country differences in the presence or absence of the so-called menopausal "hook" in the incidence of the disease.\textsuperscript{15–19} In high-incidence countries this fall or plateau seen, in medium-incidence countries, is obscured by steadily increasing rates; high-incidence countries are predominantly characterised by an increase in postmenopausal cases; thus, although the Scottish incidence of breast cancer is relatively high in world terms,\textsuperscript{19} in the current instance it is substantially less than in California where Jensen's study was carried out.\textsuperscript{19} Second, Jensen's sample size was relatively small being less than a quarter of our own and it is clearly an important principle in assessing population trends that the sample size is reasonably large. Third, in our own study a relatively constant amount of tissue was examined, and discrepancies could occur with conclusions from subgross dissection because in the latter the whole breast is examined. In the former, a block of a "fatty" breast would contain proportionally less epithelial tissue. Estimates for the age-frequency distribution of epitheliosis in postmortem material do exhibit modest falls (13%\textsuperscript{4} to 8%\textsuperscript{15}) at the time of menopause (Fig. 9), but these are small\textsuperscript{11} compared with the peak–trough difference in cancer-associated epitheliosis. The possibility that the bimodal distribution is due to a dilution of epithelial tissue by fat in the early post menopause is not a major explanation of our findings.

We are convinced that a bimodal age-distribution of epitheliosis does characterize the Glasgow population of cancer mastectomies.

Mammary epithelium is a hormone supported tissue and on first principles a pathologist would expect involution to occur at the menopause. Our data are consistent with this expectation. In contrast to the epitheliosis-laden breast tissue of the premenopausal cancer mastectomy, the same tissue from early postmenopausal women exhibits no significant increase of epitheliosis above autopsy controls. It can, therefore, be reasonably assumed that the autonomous cancer in this early postmenopausal group, having initiated in a focus of epitheliosis some years earlier, is now left without evidence of its origin.

With regard to the elderly (aged 80–90 years), our data suggest that at the very minimum half of the mastectomies contain lesions recognised as epitheliosis by the histopathologist (Table 1). However, Sandison\textsuperscript{14} finds only a slightly lower probability in his breast autopsy series. If this difference is real it must be small; it could represent an effect of nonovarian estrogen. But the primary conclusion is that "epitheliosis" in the elderly would seem to have little or no predictive value for cancer risk. A secondary conclusion might be that breast cancer in the elderly is a closely age-linked phenomenon, c.f., skin cancer, and that ordinary histopathology cannot discriminate between benign and malignant disease, i.e., that many of the lesions in this group diagnosed as epitheliosis represent indolent malignancy.

In summary, we postulate two primary populations of breast cancer differing in pathogenesis: (1) a "prime of life group" age 25–60+ at diagnosis initiated in epitheliosis supported by ovarian estrogen; and (2) an elderly group with some difference in etiologic causes of disease.

We believe it may be possible to identify the "prime of life" group by surface heat changes. Gautherie\textsuperscript{30} has described a thermographic signal associated with increased risk for the subsequent development of clinical breast cancer. It is interesting that patients exhibiting this Stage III thermogram fall into the age group of the first peak of the bimodal distribution of epitheliosis described herein. Vascular changes associated with epitheliosis could be a morphologic explanation of his signal. In this context we have developed the 16-channel thermochronobra which takes into account the ultradian, circadian, and menstrual rhythms of breast temperature in an attempt to obtain a better description of this signal in breasts considered to be at increased risk.\textsuperscript{3}
REFERENCES

Age Distribution of Epitheliosis in Cancer Mastectomies: Possible Relevance to “False-Positive” Thermograms
F. Mutch, H.W. Simpson, D. Wilson, K. Griffiths, and F. Halberg

We undertook a review of all cancer mastectomies performed in the Glasgow Royal Infirmary between 1974 and 1979. The histopathologic material from these cases was assessed by two pathologists who used a double-headed microscope. Normally, this material consisted of sections of the tumor, sections of uninvolved breast tissue remote from the tumor, and a section including the nipple. All the slides relating to each case were examined and cases with inadequate sampling of uninvolved breast tissue were excluded. The two examining pathologists were unaware of the ages of the patients they were reviewing.

All breast tissue not involved by tumor was assessed for the presence of epitheliosis, excluding material from the region of the nipple, lactiferous sinuses, and large ducts. Scoring of epitheliosis was on a simple “all or none” basis and no form of grading was attempted. The term epitheliosis was introduced by Dawson [1], who described it as the “multiplication of epithelial cells within existing ducts and ductules without the formation of new glandular elements.” We prefer this term to “epithelial hyperplasia,” as hyperplasia implies an increase in cell turnover, whereas thymidine labeling studies by Meyer [2] indicate a decrease rather than an increase in cell kinetics in such a situation. Some examples of cases scored positively for epitheliosis are shown in Figures 1–3. Doubtful cases were scored as negative. In total, 500 cases were studied.

RESULTS

Our results are presented in Table I. They show that the percentage of cancer mastectomies with coexistent epitheliosis is maximal in the age group 41–45
Figs. 1–3 Selected sections of the uninvolved breast tissue from cancer mastectomies to illustrate lesions scored positively for "epitheliosis." Although the cases illustrated were not specifically selected for age, all patients are in their forties.
years, where 69% of the cases have epitheliosis by our examination. A marked drop in cases with epitheliosis in the 56- to 60-year rank is also present. A second peak, less well defined, occurs in the elderly, (80+). This is significant by a Spearman test for a monotonic increase with age (at the 2.5% level).

We compared our results with the age-frequency distribution of epitheliosis in a "normal" population from a study by Sandison [3] at a neighboring hospital in Glasgow of 800 consecutive female autopsies that were completely unselected for diagnosis. Frantz et al [4] showed a pattern of epitheliosis frequency similar to Sandison's in a postmortem population in New York (Fig. 4).

Comparison of the observed frequency of epitheliosis in the nonneoplastic tissue of cancer mastectomies and that expected from Sandison's postmortem study showed that in patients less than 55 years old there was 2.6–9.5 times the expected frequency of epitheliosis. In patients over age 60 the observed-versus-expected ratio is close to unity.

DISCUSSION

Our data show that epitheliosis in cancer-associated mastectomies exhibits a bimodal age distribution. We feel that these two peaks represent different populations of breast cancer in terms of pathogenesis. This has been fully discussed in a previous paper [5]. In this paper, we would like to concentrate on the premenopausal peak of epitheliosis.
TABLE I. Census of Glasgow Royal Infirmary (GRI) Cancer Mastectomies: Percentage Containing Epitheliosis in Nonneoplastic Tissue

<table>
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<th>Age at mastectomy</th>
<th>GRI 1974–1979 No. cases</th>
<th>% Cases epitheliosis</th>
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<td>26–30</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>31–35</td>
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<td>36–40</td>
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Numerous investigators have attempted to define the relationship of benign breast disease to carcinoma. Those who find no relationship between the two have generally studied clinical benign breast disorders, such as gross cysts, rather than microscopic lesions [6]. In contrast, the microscopic lesion of “atypical epithelial hyperplasia” is regarded by many as carrying the greatest risk of subsequent breast cancer [7–9]. Few however, have considered this as a function of age. Ashikari and colleagues [10], are among the few to do so and they found atypical lesions most commonly in the age group 40–49 years (Fig. 5). In their study, the cumulative risk of breast cancer following diagnosis of atypical epithelial hyperplasia was approximately 4–5% at 30 months and 9% at 48 months. Similarly, Donnelly et al [11] showed that women with a previous histologically proven diagnosis of “chronic cystic mastitis” developed breast cancer 2.9 times more frequently than expected. However, breast cancer developed 10 times as often as expected in those patients aged 40–49 years at the time of diagnosis of malignancy. Using a subgross dissection technique, Wellings et al [12] showed an increase in “atypical lobules” in cancer-associated mastectomies compared to postmortem controls. They considered that these lesions were the human equivalent of the murine “hyperplastic alveolar nodules.” These epithelial aggregations have been shown by both Gullino [13] and Cardiff et al [14] to exhibit increased sensitivity for neoplastic transformation by classical chemical carcinogens.
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The sharp fall in epitheliosis in the age rank 56–60 years seen in our study might be explained if the epitheliosis is hormonally supported. Gullino [15] has concluded from growth rate extrapolations of breast cancer that one would expect an average elapse of 10 years between the initiation of one cancer cell and the presence of a palpable 1-cm diameter lump. Thus, a tumor initiated in a focus of estrogen-supported epitheliosis in a woman in her mid-40s would be seen in the mastectomy specimen taken in her mid-50s without the putative epitheliosis.
Fig. 5. Age-frequency distribution of “atypical lesions” (atypical lobular hyperplasia and atypical duct papillomatosis) found in a clinicopathologic study of atypical breast lesions by Ashikari and colleagues [10]. Redrawn from Ashikari et al [10].

An important practical question is whether focal epitheliosis can be detected thermographically. In a study, by Draper and Jones [16], of the surface thermal pattern types of the breast there were two particularly large categories. Category I consisted of “cold” breasts and category II of “warm” breasts, these temperatures being relative to the infraclavicular fossa. In cases of carcinoma they found a substantial reduction in the number of patients with ‘cold’ breasts and a similar reduction was found in cases diagnosed histologically as “cystic hyperplasia.” In addition, there was also a high rate of “false-positive” carcinoma diagnosed within the cystic hyperplasia group. While they conclude that “vascularity” or “inflammation” must account for the “warm” breast, the effect could be due to epitheliosis, particularly since they found that simple fibroadenosis does not tend to produce a “warm” breast. Of particular interest is the work of Gautherie and Gros [17] showing that 38% of women with “false-positive” (stage Th III) thermograms but normal physical, mammographic, or echographic examination results develop breast cancer within 4 years. Of equal interest is the finding that 44% of a similar group, considered to have benign disease at the initial exam-
Epitheliosis in Cancer Mastectomies

Epitheliosis, development of breast cancer within the same time period. This suggests that focal epitheliosis may be identifiable, especially since the age-frequency distribution of this "false-positive" group fits the premenopausal frequency peak seen in our study [18].

We have developed a thermometry device, the "chronobra" [19] that uses 16 heat sensors and a solid-state memory that adds ultradian, circadian, and menstrual time domains to breast thermography. Focal epitheliosis might be detected as an alteration of the amplitude, phase, or frequency of these rhythms [20] and so provide a new dimension to the treatment of breast cancer.

REFERENCES

Thermorhythmometry of the Breast: A Review to 1981
H. W. Simpson, D. Wilson, K. Griffiths, F. Mutch, F. Halberg, and M. Gautherie

Observations on the changes in breast temperature in relation to function were first reviewed by Cheatle and Cutler [1] in their classical monograph on mammary carcinoma published in 1931. Referring to work of the German scientists Heusler and Moll, they discuss the observed temperatures of the inframammary fold versus those of the axilla. Normally the breast is cooler than the axilla, but if the subject is pregnant then the breast temperature is the same or greater; alternatively if the breast is lactating it is greater still; and if only one breast is lactating then there is unilateral hyperthermia.

BREAST THERMAL PERIODICITIES

Circatrigintan (menstrual) Periods ($\tau = 30 \pm 5$ days)

It is remarkable that the large amount of breast thermography performed in the 1960s and motivated mainly by the goal of cancer detection did not generate more interest in the effect of the menstrual cycle on thermograms. Vascularity of the breast would seem likely to vary along the course of menstrual cycles, since from time immemorial some women have noted "engorgement" of the breast in the premenstrual or menstrual stage. As a resident surgeon, one of us was instructed not to admit patients for mastectomy during the pre- and intramenstrual stages to minimize operative bleeding. It is remarkable, therefore, that thermographers have commented on the "constant and unchanging" thermal pattern [2]. When one considers this attitude closely, one realizes that they were referring to the pattern of vascularity rather than absolute temperature. However, Draper and Jones [3] in a 1969 thermography review paper concluded that, relative to the infraclavicular temperature, breast temperature was maximal for a few days at the beginning of menstruation.

In 1973 Ewing et al [4] published a small study on the effect of activity, alcohol, smoking, and the menstrual cycle on liquid crystal breast thermography. They found that alcohol consumption and cigarette smoking could change the
vascular pattern substantially: But they also found in half their subjects, a maximum breast temperature, not at the menses, but at the expected time of ovulation; this phenomenon they considered sometimes lasted only for hours rather than days.

By classical thermometry Nassar and El Smith confirmed the existence of this phenomenon in 8 out of 13 subjects studied in 1975 [5] and in a 1977 study [6] they were able to demonstrate it in 7 out of 10 subjects. At least at first sight such a biphasic menstrual cycle would seem logical, since the corresponding estradiol cycle is normally biphasic even though individual hormonal cycles may show a surprising degree of variation [7].

In 1975 [8] and formally in 1977, Gautherie published [9] an analysis of menstrual cycle breast temperature changes in a 37-year-old woman with breast cancer. Over the noncancerous breast there was a rhythm with a period of 29.2 days and an amplitude of 0.6°C; in contrast, over the cancerous breast, no similar rhythm was detected by the analysis. There is some qualification of these data since the time series only extended to about half a menstrual cycle. Nevertheless, this was an important indication that the time structure can be deleted in disease states.

In 1976, [10,11] H.W. Simpson made breast temperature measurements and steroid measurements in a 36-year-old nulliparous woman who was experiencing fertility problems. In view of El Smith's work he thought he could obtain information on ovulation and so help the patient's problem. Her data are seen in Figures 1 and 2. A pronounced menstrual breast temperature rhythm is seen with a range of 1.15°C which corresponds to the Strasbourg findings of an amplitude of 0.6°C. The menstrual rhythm is monophasic; this is possibly explained by the patients' infertility and the absence of pregnanediol in the urine during the luteal phase of the cycle. These data may provide some explanation of the variability in the bimodality that was found by El Smith in his subjects. The data are also interesting in that they show a different breast and oral temperature phasing.

Gautherie at this time was also studying the menstrual changes by thermography. He measured the temperature difference between the breast and the surface temperature of the xiphisternal area [12]. After averaging the data over 23 young adults with "regular" menstrual cycles, he found a maximal difference around midcycle (compare breast surface and oral temperature in Fig. 1 at midcycle). This difference was accentuated when the breast temperature was measured over a mammary cutaneous blood vessel.

Marks [13] working with Smolensky, prepared a thesis on breast temperature in 1977. Forty-eight hour spans of data were obtained weekly for one menstrual cycle from 11 women aged 22-23 years. They experienced considerable trouble with the Medilog instrumentation. There did appear to be a menstrual variation, but the phase variability precluded any meaningful average description.
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Fig. 1. Breast surface and oral temperature changes and total urine estrogens along the course of the menstrual cycle (Simpson 1976 study) of a 37-year-old subject complaining of infertility. No midcycle estrogen surge is seen and pregnanediol was undetectable in the urine so that the results have to be viewed with caution in terms of normal breast surface temperature readings.

In 1978 [14] Simpson carried out a further longitudinal study of breast surface temperature and urinary steroids in a laboratory worker for three menstrual cycles (Fig. 3). These data provide some confirmatory information about the biphasic temperature changes as described by El Smith.

In 1980 Wilson made ambulatory studies along the menstrual cycle scale using a prototype version of the automated Chronobra instrumentation. The electronic system is described in general in References [10] and [11] and photos of the definitive model are seen in the instrument section of this paper (Figs. 9
and 10). Parallel studies were made of plasma FSH, LH, and salivary 17-hydroxyprogesterone and progesterone. An example of the computer output of analysis of one subject’s data is given in Figure 4. The 34 daytime series was analyzed by the least-squares fit of 24-hr cosine function to successive 120-hr sections of data by 24-hr increments. Ovulation according to the hormone data occurred on Day 14. In the graph, notice the nadir was on Days 8–12 (cf Fig. 3) and the maximum in the premenstruum. However, his other three subjects exhibited considerable variability in the character of the menstrual component (cf the experience of Marks) and he concluded that a more prolonged protocol was needed covering three cycles to get a good description of the menstrual cycle changes in breast surface temperature. It may be that environmental factors will have to be controlled to obtain homogenous data; alternatively, environ-

Fig. 2. Data as Figure 1, redrawn to show the relative movement of breast versus oral temperature.
Fig. 3. Breast surface temperatures measured with the Yellow Springs instrument in one woman aged 27 years (Simpson, 1977 data). Data are averaged three menstrual cycles which were synchronized in terms of the primary estrogen surge. Note again that the extent of change of the breast temperature is notably greater than that of basal body temperature. In this instance the nadir of breast temperature seems earlier than in basal body temperature but the data are not conclusive in this respect.

Menstrual temperature might also be monitored continually and data corrected for its perturbations.

In view of the difficulty of obtaining homogenous rhythm parameters for the menstrual changes of breast surface temperature, it would seem important to
Onset of menses for 28 day cycle
Sensor 1 - Left inner lower
Tenovus Breast Skin Temp Programme

Fig. 4. Computer printout summarizing 120-hr serial section analysis of circadian breast skin temperature data (°C) of Wilson. The data were analyzed in Halberg's laboratory at The University of Minnesota. Temperatures were measured at 30-min intervals during the wake span and obtained from the lower inner quadrant of the left breast of premenopausal women studied for 34 days. In the top part of the figure there is a plot of breast skin temperature against time (a chronogram). Immediately below this is a plot of the significance of the fit of the 24-hr cosine function. Next is a plot of the amplitude of the fit and below that of the circadian mesors with their respective standard errors. In the lower rectangle is a plot of the circadian acrophase with 95% confidence limits and at the very bottom of the figure is an indication of the number of samples collected during each interval analyzed [29]. (Permission received from Acta Endocrinologica to use this figure).
classify subjects according to the different thermography patterns, for example, those described by Draper and Jones [3] for premenopausal subjects. They found Group I “cold” breasts approximately 29%; Group II some vascularity 16% (also 28% nonspecific); Group III “warm” 20%; and Group IV “patchy vascularity” about 7%.

**Circaseptan (about weekly) Periods ($\tau = 7 \pm 3$ days)**

In 1975 & 1977 [8,9] Gautherie and Gros reported a weekly component* in the least-squares spectrum of breast temperatures on the noncancerous breast of a 37-year-old woman. This had more than half the amplitude of the 24-hr component which was 1.37°C. The data span was about two cycles. Credibility of this component is suggested by the following: (1) It is almost exactly 168 hr (= 7 days) on the least-squares spectrum and such a component is unlikely to be due to chance; (2) Gautherie had also previously demonstrated a 168-hr component in the least-squares spectrum of skin temperature oscillations in the finger pad [15]; and (3) These components are being increasingly validated in human chronobiology (eg, the risk of renal graft rejection, a phenomenon that might well be accelerated by vascular changes [16–18]).

This weekly component was, however, not present in a time series collected from the sensor sited over the cancer. This is of provocative scientific interest. One has to be reminded that both breasts are in the same external environment, on the same individual, and colonized by the same parent vascular system. The absence of both the menstrual rhythm component (previously described) and the absence of the circaseptan component over the cancerous breast would seem to be a clear example of time series deletion associated with neoplasia. An obvious suggestion is that the vascular system is outgrowing its nerve supply eg, from the sympathetic system.

**Circadian (about Daily) Periods ($\tau = 24 \pm 4$ hr)**

In 1969 Mansfield and his colleagues [19] documented the first surface temperature studies of the breast along the circadian scale. They observed that the temperature of the normal breast was characterized by a “cyclic pattern” reproducing itself at approximately 24-hr intervals. They provisionally concluded: (1) cancers (N = 2) exhibit a higher average temperature and smaller amplitude of their circadian variation; and (2) fibroadenomata (N = 2) are similar in temperature to the other breast.

This work was followed up in 1973 [20] with a cosinor analysis of breast surface temperature data obtained from 14 cases of advanced or recurrent cancer.

*See also circadian rhythm alteration of skin temperature in breast cancer by M. Gautherie, A. Wygodza, and P. Gueblez in this volume (B23) for documentation of other subjects with circaseptan components.
in patients on a routine of diurnal wakefulness and nocturnal rest. Though there was a problem of achieving the conventional 95% confidence levels the authors were able to conclude:

(1) the noncancerous breasts (the control side) exhibited a phase and frequency synchronized rhythm with a period at or close to 24 hr and an acrophase in the late evening or early morning hours; and

(2) the cancerous breast also exhibited a phase and frequency synchronized rhythm (at or close to 24 hr), but the acrophase was earlier (ie, in the afternoon). Furthermore, as noted previously, the circadian amplitudes were diminished, on average, over the carcinomata when these were compared with those from a corresponding anatomical site on the other breast.

An important motive in Mansfield’s work was the search for new tests of tumor responsiveness to therapeutic agents: In one case in which there was a good clinical response to stilbestrol, a reduction in circadian amplitude was noted over the tumor. Smolensky and colleagues [21], a little later than Mansfield, also investigated the potential of breast thermorhythmometry for assessment of treatment. They studied 12 cases. On average the mesors measured over the cancers were 1.2°C warmer and this Δ decreased to 0.94°C after therapy.

After preliminary communications in 1975 and 1976, Gautherie and Gros published a definitive study of thermorhythmometry in 26 cases of breast cancer in 1977 [8,9]. In addition to at least 9 days of breast temperature recordings, the physical, radiological, histological, and doubling time estimates were also recorded to characterize the tumor in each case. In 11 cases the cosinor (τ = 24-hr) parameter alterations were essentially similar to those discovered by Mansfield and colleagues [20], ie, the cancer circadian acrophase was earlier and the amplitude was less (see Fig.5). Cases exhibiting these characteristics fell naturally into a group. In this group the tumor exhibited relatively slow growth, low heat production, and better than average histological differentiation. The balance of cases (ie, those with a short doubling time, high heat production, and histological anaplasia) did not exhibit a phase and frequency synchronized thermal rhythm at or near 24 hr, but rather, in terms of the least-squares spectral analysis, a scatter of circadian periodicities between 20 and 24 hr.

The amount of this extension toward the ultradian band was positively correlated to the tumor’s assessed metabolic heat production and rate of growth. It is important in the interpretation of these findings to remember that in each instance the matched contralateral breast exhibited a periodicity close to (or at) 24 hr. This implies that the finding is not an environmental artifact. The field of mammary thermorhythmometry now awaits an independent confirmation of these findings with great interest.

In this context the contemporary publication of analyses of Garcia-Sainz data by Halberg [22] is of possible relevance. Four-day time series of breast temperatures were available from 24 women considered to have “healthy,” “benign,”
or "cancerous" breasts. Taking these three states in order on the supposition (not unreasonable) that there might be a gradation from normal through benign to malignant, a zero slope was rejected at the 5% level for no change of periodicity, i.e., $\tau = 24\,\text{hr} > \text{benign} > \text{malignant}$.

In June 1977 Marks [13] supervised by Smolensky, presented a thesis to Texas University concerned with the circadian (and menstrual) periodicities of breast surface temperature in 11 premenopausal women under ambulatory conditions. Their data reconfirm a circadian variation (peak 22:00–03:40) about a mesor of 35.3°C, but there were serious difficulties with the Medilog instrumentation and with the conventional cosinor analysis, since it only accounted for some 20% of the variability.

In September 1977 Simpson [10] presented some analyses of breast temperature at the International Congress of Therapeutics at Montpellier and later at a conference on Tumour Markers at the Tenovus Institute in Cardiff [11]. The 19-day time series analyses of oral and breast temperatures in a female medical student were presented. They exhibited a breast mesor of 35.2°C (cf, Mark's figure 35.3°C) and indicated a statistically significant phase difference of oral (15:36) and breast (18:28) acrophase (sleep 23:00–07:00). At the same meeting he presented circadian cosinor rhythmometry findings obtained on the surface over a mucus-secreting carcinoma in an elderly female. The circadian parameters
were apparently in conflict with the findings of Mansfield et al [20] and Gautherie and Gros [9]. In this instance the tumor exhibited a greater circadian amplitude and similar phase to that found on the contralateral side. It should be noted that the tumor was on the right breast (vide infra) and that the sensors were not sited by thermography.

Later that month Meis and colleagues [23] submitted data on pregnancy breast temperatures for the chronopharmacology conference at Tallahassee. The two subjects investigated were in the third trimester and temperatures were recorded continuously for 24 hr. As expected from the pregnancy status, the mesors were substantially higher (36.6 and 36.7°C) than normal (cf, 35.3 and 35.2°C above). It is relevant in this connection that we have documented some breast surface temperature data for a 26-year-old primapara [14] who was phasing out the breast feeding of her first child. Temperatures varied from 35.4 to 35.9°C along the circadian scale, and these values fall, as expected, between those of the normal breast and those for subjects in advanced pregnancy. In the data of Meis and colleagues the acrophases in the two subjects were 15:24 and 17:40 which is earlier than in normal breast. Before this can be attributed to the effect of pregnancy, one has to consider that it may be an effect of the hospital schedule. Lights are often switched on early in the morning.

In extensive review publications which appeared in 1978 [24] and in 1979 [25] Halberg and colleagues set the scene for breast thermometry as a “chronopsy” (cf, biopsy) for neoplasia. In one case thermorhythmetrical parameters were correlated in a temporospatial way with the spectrum of histopathological features of underlying hyperplastic cystic disease. The study was possible since temperature observations could precede planned bilateral prophylactic subcutaneous mastectomies. The more seriously affected left breast exhibited higher mesor and lower percentage of predictable circadian variation than the less seriously affected right breast. However, the suggestion of any cause and effect relationship is tentative. The authors are well aware of the shortcomings of this study, namely, that the sensors were not specifically sited in terms of blood vessels and/or local variations in mammary metabolism. More recently another possible qualification has occurred since Wilson, Griffiths, Simpson and others have suggested [26] that the left breast intrinsically exhibits a higher mesor and a lower circadian amplitude than the right breast. More work is required. Nevertheless, the “chronopsy” papers are conceptually important in chronopathological terms, particularly since reversible focal epithelial abnormalities appear to precede cancer [27] in, at least, premenopausal cases of the disease [14,28].

In 1979 Taggett-Anderson and colleagues [25] completed an interesting “baseline” type study (N = 7) of the circadian rhythm of breast temperature in college students. The observations (T = 4 days; \( \Delta t = 3 \) hr) were repeated 1 year later. Five out of the seven of the paired acrophases were within 2 hr of each other and averaged 18:01 overall. In other words they can be described as reasonably
steady over the span of 1 year.

In the same year Wilson and Griffiths and others carried out studies [29] on nine research staff volunteers whose breast surface temperature was measured during their diurnal activities for the whole (N = 4) or part (N = 5) of a menstrual cycle (Δt = 30 min). The overall mesor value of these measured over the whole cycle was 34.3°C, with an amplitude of 0.63°C and an acrophase of 316° (24 hr = 360°). The circadian acrophase over the midaxillary line (control) occurred about 1 hr earlier and the amplitude was less. Serial section (Fig. 4) analysis of one (ovulatory) menstrual cycle time series by Halberg indicated a mesor peak in the premenstruum and a nadir on Days 8–10. This is a little earlier than the midcycle low found in our earlier preliminary study [10,11]. Ovulation in this subject in terms of salivary and plasma estimations of progesterone, 17-hydroxyprogesterone, LH, and FSH occurred several days later on Day 15. On the other hand when all four subjects were considered there was considerable inconsistency of the circadian parameters, especially the mesor, along the menstrual cycle scale.

Parallel with this study we Griffiths, Simpson, Wilson and others also carried out circadian rhythm observations on seven postmenopausal women with breast cancer under the care of Mr. R.W. Blamey at the University of Nottingham [26] (T = 4 days; Δt = 15 min). In all instances the tumor exhibited an increased mesor (Δ = +0.91°C) and diminished amplitude (Δ = −0.17°C) as compared with that from a matched anatomical site on the other breast (eg, Fig. 6). This of course, is in agreement with the bulk of earlier work, but an additional finding was the normal asymmetry between left and right breasts, namely, a higher mesor and lower amplitude on the left. If this can be confirmed, and it is already hinted at in Halberg et al’s papers [24,25], qualification and caution are necessary in the interpretation of breast temperature data. A possible reason for left asymmetry would be the heating effect of the underlying heart.

**Ultradian Periods (τ = < 20 hr)**

In one subject studied via 14 breast temperature sensors (see Fig. 7A) at 64-sec intervals for 4 hr there was an invariable component in the least-squares spectrum between 20 and 24 min (X = 22.3 min). (Trial periods had been fitted to the data at 1-min intervals, and the spectrum ran from 5 min to 2 hr.) In 8/14 time series this was the primary period in the spectral range. Other common components were τ = 28 to 29 min, 40 to 42 min, 60 to 63 min, and 88 to 90 min (see Fig. 7B). Apart from the 28- to 29-min periodicities, the remainder seemed to form a group: τ = 22.2 × 1 min, 22.2 × 2 min, 22.2 × 3 min, and 22 × 4 min, and/or τ = 28.5 × 1, × 2, × 3. Phase analysis has been carried out on the 22.3- and 28.5-min periodic components in the 14 time series. For τ = 22.3 the left breast leads the right in 6/7 instances. The mean phase
Fig. 6. Least-square fits of cosine functions to time series obtained over the breast cancer in an elderly woman versus that obtained on a matched anatomical site on the other breast. Note the elevated mesor value reduced amplitude and earlier acrophase for the tumor. Temperatures were collected at 30-min intervals for a 96-hr span throughout wakefulness and sleep in a ward environment [29]. (Permission requested from Acta Endocrinologica to use this figure.)

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<th>ACROPHASE AND (95% LIMITS)</th>
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<td>&lt;0.001</td>
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Fig. 7. (A) A 4-hr study with 64-sec sampling on 14 chronобра channels were set up on the subject seen in Figure 9. During measurement, in order to minimize environmental effects, the subject sat up in bed with a shirt and fine wool jersey over the bra. Room temperature was 18–20°C. Inspection of the data reveals a strong circadian trend, but in addition there are seven to eight subsidiary cycles grossly evident. Time series analysis of these components to confirm the number of different ultradian frequencies is described in the text and in Figure 7B. (B) Histogram illustrating the number of breast temperature channels exhibiting periodicities in the range $\tau = 19$ to 93 min.
Chronobra studies of breast surface temperature synchronous circadian and ultradian rhythms.

7A

ULTRADIAN PERIODS IN BREAST SURFACE TEMPERATURE

7B

PERIODICITIES IN LEAST SQUARES SPECTRUM (MINS)
difference is 1.9 min and this is highly significant by a t test (P < 0.001). For 
\( \tau = 28.5 \) min, in \( \frac{5}{7} \) instances the right leads the left (ie, the reverse). The average difference is 2.7 min (P < 0.01). (Of course in cyclic phenomena the interpretation of "lead" and "lag" is relative. But it seems more reasonable from point of view of biology to accept the smaller difference.) The largest periods are provocatively close to the reported R.E.M. and "basic rest activity" periods in man [31]. Caution is required in the rhythmometric interpretation of these analyses since the ultradians contributed a very small proportion of the total variance. The results await confirmation.

It may also be relevant that Romano and Gizdulich have described an ultradian period in human forearm plethysmography studies which is almost precisely half the (presumed) fundamental described previously of 22.3 min [33].

Pathological Periods

In the breast cancer case described in extenso by Gautherie and Gros [8,9] the weekly, menstrual, and daily components were absent in the least-square spectral analysis for the cancerous breast time series. Instead, a new circadian component appeared at \( \tau = 21.1 \) hr (amplitude 0.62°C) and multiples \( \tau = 42.3 \) hr (amplitude 0.31°C) and \( \tau = 84.4 \) (amplitude also 0.31°C). It is important to point out that the circadian period is defined as a statistically significant rhythm with a period of 20 ± 4 hr so that the period here of 21.1 hr is still technically "circadian" even if it is approaching the ultradian band.

Gautherie and Gros also document the fact that the other 14 cancer cases characterized by high malignancy and heat production exhibited a fundamental period which was between 20 and 24 hr. Infradian multiples of this primary period (small in amplitude) were present in each case. These findings have not been investigated properly by other laboratories, but they are provocative for cancer biology in general and tissue kinetics in particular.

NOTES ON STATISTICAL ANALYSES

The characterization of rhythmic data and, specifically, the estimation of rhythm parameters require time series analysis. A typical first stage in the time series analysis of breast temperature data is the least-squares spectral analysis of Halberg. An example of this analysis is seen in Figure 8. When the main periodicities have been defined, it is normal to proceed to a version of the cosine vector analysis as follows:

(1) The single cosinor analysis [34] has proven a valuable rhythmometrical procedure. The model is of the form:

\[
y(t) = M + A \cos \left( \frac{2\pi t}{\tau} + \phi \right) + e(t)
\]
Fig. 8. An example of the least-squares spectrum analysis carried out by F. H. in this case on the data obtained of breast temperatures in a 19-year-old healthy medical student in 1976. Readings were obtained with the Yellow Springs Instrument, and the thermistor was placed just lateral to the nipple under 1 cm of insulating material. Readings were made during the daytime only, the subject carrying out diurnal activities in this laboratory. Note that there is a clear resolution of a significant circadian rhythm. The same subject also carried out oral temperatures in parallel with breast temperatures. The oral temperature also exhibited a significant circadian rhythm, the mesor was 2.1°C higher, the amplitude 0.5°C less, and the acrophase 2.8 hr earlier. These three parameter differences were all statistically different.
Fig. 9. The 16-channel chronobra in operation seen here during the interrogation of the 4-k semiconductor memory store. The data are being led into the Rockwell interactive computer on the right seen conveniently packed inside a briefcase. In turn the Rockwell has been programmed by the small magnetic tape recorder seen to its left.

where \( y(t) \) is the value of the physiological variable at time \( t \), \( M \) is the mean of the fitted function (mesor), \( A \) is the amplitude, and \( \phi \) is the timing of the crest of the rhythm from some reference point, usually local midnight, \( T \) is the period of the rhythm which for circadian rhythms are usually fixed at 24 hr, and \( e(t) \) is a random error term at time \( t \). In some data this model is certainly a good approximation of the biological rhythm form. For example, in the breast temperature studies of Halberg and colleagues [24] the percentage of rhythm accounted for in successive time series was 65, 31, 59, 59, 49, 48, 50, 66, and 66. These are considered to be a satisfactory approximation of the data, and one can have confidence in rhythm parameters under these circumstances, but Marks [13] found that the model accounted for only approximately 20% of variation in his average group data sets. Wilson’s experience is closer to that of Marks than that of Halberg and consequently he investigated methods of improving the model.
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Fig. 10. The 16-channel chronobra seen with the collar study sensors in situ and also showing the instrument pouch sited anteriorly.

(2) The extended cosinor analysis with a time series component. This model is of the form:

$$y(t) = M + A_1 \cos\left(\frac{2\pi t}{T} + \phi_1\right) + A_2 \cos\left(\frac{4\pi t}{T} + \phi_2\right) + e(t)$$

where $A_1$ and $A_2$ are the amplitude of 24 hr and 12-hr rhythmic components present in breast skin temperature data, and $\phi_1$ and $\phi_2$ are the corresponding phases, respectively. The time series component, $e(t)$, which models the correlation in the error term is given by:

$$e(t) = \alpha e(t-1) + z(t)$$

where $\alpha$ is the multiple of $e(t - 1)$ and $z(t)$ has zero mean and constant variance. This model is more fully described elsewhere [35]. Reliable values of the rhythm parameters and uncertainties in their estimation were obtained using a weighted
least-squares analysis of the harmonic model. In most situations studied, the usual cosinor model was found in reasonable agreement with the more sophisticated fully extended model; nevertheless, there was still an advantage from the use of the full model, with increased reliability of the estimates of the rhythm parameters, together with smaller standard errors.

NOTES ON INSTRUMENTATION

There is no question that the development of mammary thermorhythmometry has been seriously delayed by inadequate instrumentation. The early clinical studies of Gautherie and Gros [9] and Mansfield et al [20] were made by FM telemetry. Temperature-sensitive resistors were used with voltage-controlled oscillators to produce FM signals whose frequencies were proportional to skin temperature. The signals were telemetered to a local receiver which in turn sent them through a program quality-leased telephone line to a remote station where they were sampled at fixed intervals (eg, 5 min) and recorded digitally. This sophisticated method is prone to interference, eg, by other hospital equipment and it imposes limitations on the subject’s mobility. It is also a sound electronic principle to have the signal digitized proximal to the point of acquisition. Not many workers are currently using these FM telemetry methods for human studies because of the difficulties involved.

A very robust “manual” method is to use the Yellow Springs instrument (Ohio 45387) Model TI (range 30–41°C; accuracy 0.1°C; readability 0.05°C). Our own early data were collected with this instrument involving much labor but yielding certain results.

The Medilog instrument, made by Oxford Medical Systems Limited, Nuffield Way, Abingdon, OXON, OX14 1BZ England is a mini-tape recorder with a great storage capacity but only four channels; it is relatively bulky and, in the hands of Marks [13], had problems concerning the resistance calibration, variable tape speed, sporadic output, inadequate recording range, and problems with the digitization.

Instruments with semiconductor memories and no moving parts would seem to offer a better solution. Thus the Solicorder (Solicorder Co. Ardsley, New York) measures temperatures from 34 to 40.3°C from one site up to 8 days with intervals of 6 min; also the Thermalog Model TML-2 (by Vitalog, 1058 California Avenue, Palo Alto, California 94306). This instrument can record temperatures on one channel for nearly 23 days at 2-min intervals, but the range is too high for breast temperature (35.5 to 42.9°C) without modification.

Conceptually the idea of a custom-built brassière for the noninvasive detection of breast pathology through thermorhythmometry came in 1974 [36]. The integration of these ideas and the semiconductor electronic solutions came in 1975 [37] and 1977 [38]; fairly detailed electronic descriptions were published the
same year [10,11]. This instrument, the chronobra, is now in use in Scotland. The current model is a 16-channel device which synoptically measures breast skin and environmental temperature and logs the result in a 4-K miniaturized, digital semiconductor store. Sampling rates can be varied from every 64 sec through intermediate values, to a maximum of 68 min giving endurances from 4.5 hr to 12 days, respectively. The switches controlling the sampling interval are seen in the larger recessed vertical slot in the bra hardware package (Fig. 9); the smaller recessed slot contains switches concerned with the operation mode. The range of the 14 breast sensors is 26.8 to 39.6°C while that of the two ambient sensors seen in the upper part of the cups (Fig. 9.) is double this range with a center point adjusted to suit the environmental conditions.

Independent water bath tests of the 16-channel instrument have been made against a British Standards Institution grade mercury glass thermometer (30-50°C in 0.2°C divisions at the Tenovus Institute. Sensors are linear in response to temperature and the 82% of sensors have a gradient magnitude within the range 0.98-1.02. In the current instruments a small sensor offset (maximum less than 2°C) has to be accounted for when the data are analyzed. Clinical trials on ambulatory subjects are now in progress with 10 such instruments.

CONCLUSIONS

Basic rhythmometry of the breast has shown that alterations of function (lactation) and hormonal status (pregnancy) are associated with appropriate changes of overlying skin temperature.

Sophisticated rhythmometry (eg, by FM telemetry or by the chronobra with semiconductor memory) has revealed that there are circatrigintan (menstrual), circaseptan (weekly), circadian (about daily), and ultradian rhythms of breast surface temperature. The menstrual rhythm may exhibit differences from that of deep body temperature in terms of phasing and/or the presence or absence of a peak around ovulation in addition to the menstrual peak. A circaseptan rhythm has also been demonstrated in the noncancerous breast in subjects with this disease. The same rhythm was absent in data obtained from the skin surface of the cancer.

Circadian rhythms of breast temperatures have been confirmed by many different laboratories. The amplitude is greater and the phase later than that of deep body temperature under controlled conditions; this difference is more marked over the right breast than over the left possibly because it is further away from the warming effect of the heart. Generally when temperatures are measured over a cancer the average temperature is warmer, the amplitude less, and the phase earlier than expected. Part of this change may be an approximation of deep body temperature conditions due to greater vascularity; however, the latter does not apparently explain the circadian period shortening noted over relatively warm
fast-growing cancers or the absence of "normal" periodicities in these cases (eg, 24 hr, 7 day, or menstrual).

Grossly synchronized ultradian rhythms have been demonstrated in one subject with tentative periodicities near 22, 29, 41, 61, and 89 min when the time series for 14 channels were subjected to least-squares spectral analysis.

REFERENCES


29. Phillips MJ, Wilson DW, Simpson HW, Fahmy DR, Groom GV, Phillips MEA,


PROLACTIN AND BREAST SKIN TEMPERATURE RHYTHMS IN POSTMENOPAUSAL WOMEN WITH PRIMARY BREAST CANCER

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Chronobiology Laboratories, University of Minnesota, Minneapolis, Minnesota, USA °

Malignant cells may disseminate at any stage of tumour development, yet the early recognition of breast cancer, if it prompts treatment, may increase the 'chances' of cure. This study explored the feasibility of measuring breast skin temperatures for the early detection of breast cancer, together with any role circadian rhythms of prolactin may have in breast cancer.

Circadian temperature rhythms reportedly differ on the average for areas of the breast skin overlying a tumour when compared to a similar site on the contralateral breast (Mansfield et al. 13, Gautherie and Gros 7, Phillips et al. 8).

This report follows our earlier concern with the measurement and characterization of circadian breast skin temperatures (Wilson et al. 9, Phillips et al. 8) for improving upon the diagnosis of small tumours (Evans and Gravelle 5) with a safe and non-invasive procedure. Breast skin temperature rhythms were studied on postmenopausal women with breast tumours in

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order to gain information that would guide developments towards the use of an ambulatory device for measuring and storing breast temperature, known as the 'chronobra', for the purposes of detecting 1. breast cancer at an early stage and possibly 2. precancerous chronopathology (Halberg et al.8).

MATERIALS AND METHODS

Subjects
Postmenopausal women, 65-82 years of age, 7 with primary breast cancer and 1 with benign breast disease, lived in a hospital ward (nocturnal-rest schedule 2200–0600, ambient temperature 21°–23°C) and were confined to bed, except for a walk to the bathroom every 2 h, throughout the study. The timing of dietary intake and activity was controlled. The juxtaposition of bed-clothing with midriff, the choice of night-clothes, posture, particularly during sleep, were arranged or controlled in an attempt to minimize any bias that may have arisen for intra- and inter-mammary breast temperature measurements as a result of such external factors.

Histopathology
Tumour histology was routinely recorded.

Measurement of breast, oral and ambient temperature
The manual method of measuring breast surface temperature has been described in detail elsewhere (Wilson et al.19, Phillips et al.17). A sensor was placed on the area of skin overlying the tumour and on a similar site on the contralateral breast, and at the surface and centre of each remaining breast quadrant. Sensors were attached using hypoallergenic tape. Breast skin temperature measurements were made from each sensor at 30-min intervals, generally for 96 h. The readings were taken in a manner attempting to avoid any technical bias in the temperature measurements.

Oral and ambient temperature were monitored every 2 h for the duration of the study, with some exceptions for oral temperature during sleep.

Calibration of sensors
Each sensor was independently calibrated at the Tenovus Institute using a specially-made mercury-in-glass thermometer (H. Stout & Co. Ltd., Redhill, England) and calibration apparatus obtained from Light Laboratories, Brighton, England. The calibration apparatus consisted of an enclosed double-walled water tank fitted with a stirrer and heating coil; access for the thermometer and breast sensors was made via a cork insert in the roof of the tank. Each breast sensor lead was attached to the stem of the mercury-in-glass thermometer such that the sensors themselves were in close proximity to the thermometer bulb. Readings from the mercury-in-glass and 'Light' thermometer (at 0.05 °C intervals over the range of 32° to 38 °C) allowed one to assess the performance of the breast sensors.

All sensors had a linear response to temperature changes over the physiological range of the study. The inter-sensor variability showed that they were interchangeable within 0.05 °C.
Blood collection

Blood samples (10 ml) were collected in lithium heparin tubes at 2-h intervals for approximately 1-2 days via an indwelling catheter in the left antecubital vein. Samples were centrifuged for 5 min at 100 g and the plasma stored at -20 °C.

Measurement of plasma hormones

a. Cortisol was directly radioimmunoassayed with a system that utilized a $^{125}$I-radioligand in low pH buffer and a micro-cellulose solid-phase antiserum for separating bound from free hormone, as described in detail elsewhere (Fahmy et al.).

b. Prolactin was measured by a homologous double antibody radioimmunoassay which had a negligible cross-reaction with follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone and chorionic somatomammotrophin (Cole and Boyns).

Statistical methods of analysis

The characterization of data exhibiting rhythmic changes is best achieved using time series analysis. Two models were employed in data analysis and are described below.

a. The single-cosinor analysis. This is described in detail elsewhere.

b. The multiple component model with correlated noise. This model is intended for dense and hence often correlated data for which several approaches are now available.

The model is of the form

$$y(t) = M + \sum_{j=1}^{p} A_j \cos \left(\frac{2\pi j t}{\tau} + \phi_j \right) + z(t)$$

where $A_j$ are the amplitudes of rhythmic components with period $\tau/j$ and $\phi_j$ are the corresponding phases. The error term, $z(t)$, is considered to result from an autoregressive process (AR) to take into account the correlation between consecutive measurements:

$$z(t) = \sum_{k=1}^{q} \alpha_k z(t-k) + e(t)$$

where $\alpha_k$ are the coefficients of the AR process of order $q$ and $e(t)$ is assumed to be normally distributed with zero mean and unknown variance.

For the breast skin temperature data discussed in this paper, a two-component model (24-h and 12-h periods) with a first-order ($q = 1$) AR process was considered:

$$y(t) = M + A_1 \cos \left(\frac{2\pi t}{24} + \phi_1 \right) + A_2 \cos \left(\frac{2\pi t}{12} + \phi_2 \right) + z(t)$$

$$z(t) = \alpha z(t-1) + e(t).$$

This model is described elsewhere (Dunstan et al.). Reliable values of the rhythm parameters and uncertainties in their estimation were obtained using a weighted least-squares analysis of the above model.
RESULTS

Histology

The patients' biopsies show a heterogeneous population. Moreover, a classification of these histological results, if not as a timed biopsy (chronobiopsy), then as a spatially more rigorous biopsy (Halberg et al.), will be recommended for future studies, notably if there is a subcutaneous mastectomy that would allow the rigorous scrutiny of the entire breast tissue involved.

Patient 1: invasive carcinoma with marked scirrhous reaction; patient 2: moderately-differentiated lobular carcinoma; patients 3 and 4: moderately-differentiated carcinoma; patient 5: moderately-differentiated invasive carcinoma; patients 6 and 7: poorly-differentiated and well-differentiated invasive carcinoma, respectively, and patient 8: benign breast disease consisting of fibrofatty tissue bearing a haemorrhagic cavity.

Cortisol

Patients were reasonably synchronized as judged from their circadian rhythms of plasma Cortisol. The original data generally revealed maximal and minimal values at 0600 and 0000, respectively.

Circadian rhythms of plasma Cortisol were demonstrated in all subjects by rejection of the zero amplitude assumption in a single cosinor.

Prolactin

Statistically significant circadian rhythms (as defined by the above models) of plasma prolactin were observed (p<0.05) in only two primary breast cancer patients and in the patient with benign breast disease, as shown in Tab. 1. All subjects, however, showed a peak concentration of prolactin be-

<table>
<thead>
<tr>
<th>subject I.D.</th>
<th>no. of samples</th>
<th>prolactin units in U/l (MRC 75/504)</th>
<th>clock time (hours)</th>
<th>p-value for significance of a rhythm</th>
<th>'raw' prolactin data U/l (MRC 75/504)</th>
<th>max. value</th>
<th>min. value</th>
<th>max. clock hours</th>
<th>min. clock hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>right breast</td>
<td>14</td>
<td>0.124 0.011 0.020 (0.020–0.065)</td>
<td>2312</td>
<td>ns</td>
<td>0.18 0.06 14000</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>26</td>
<td>0.170 0.020 0.124 (0.033–0.195)</td>
<td>0440 (0014–0348)</td>
<td>&lt;0.001</td>
<td>0.53 0.04 12000</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>18</td>
<td>0.072 0.014 0.029 (0.005–0.093)</td>
<td>1818</td>
<td>ns</td>
<td>0.25 0.12 12000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.166 0.064 0.196 (0.032–0.426)</td>
<td>2208</td>
<td>ns</td>
<td>1.10 0.12 12000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>left breast</td>
<td>14</td>
<td>0.174 0.024 0.128 (0.036–0.221)</td>
<td>0116 (2212–0436)</td>
<td>0.008</td>
<td>0.45 0.05 12000</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>14</td>
<td>0.218 0.022 0.063 (0.021–0.147)</td>
<td>0424</td>
<td>ns</td>
<td>0.44 0.12 14000</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>14</td>
<td>0.275 0.121 0.286 (0.220–0.379)</td>
<td>0136</td>
<td>ns</td>
<td>0.25 0.07 12000</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>26</td>
<td>0.205 0.013 0.054 (0.008–0.160)</td>
<td>0308 (0100–0900)</td>
<td>0.020</td>
<td>0.39 0.11 20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tab. 1 - Circadian rhythm parameters for plasma prolactin, maximum and minimum values of raw data with corresponding times measured in clock-hours. Two subjects had non-significant rhythms (NS), probably because prolactin concentrations were less than the minimum detectable value. Standard deviations are given in parentheses, those for acrophase in 'decimal' hours.
tween 00° to 04°. Apart from the peaks within 4 h after 00°, no other characteristic was detected in the prolactin profile by the naked eye in time plots.

**Ambient temperature**

This was maintained between 21–23 °C for the duration of the study for all patients studied.

**Oral temperature**

Statistically significant circadian rhythms of oral temperature (p<0.001) were observed in all but two patients, 3 and 4. The 6 subjects displaying significant rhythms had a mean value (with standard deviation in parentheses) for the mesor, amplitude and acrophase of 36.75 (0.19) °C, 0.40 (0.12) °C and 17° (0.88) h, respectively. Maximal excursion of oral temperature occurred during late afternoon to early evening, whereas the nadir of the rhythm occurred around 0400 to 0600 just prior to full awakening.

No statistically significant differences were found either among individual patients or between groups of patients with primary breast cancer having tumours in either left or right breast, when considering either rhythm parameters of oral temperature or the original data. The failure to describe a statistically significant circadian oral temperature rhythm of two patients remains unexplained.

**Breast skin temperature**

Circadian rhythms of breast skin temperature were characterized using both formula [3] (corresponding to the model with 2 components and a first order AR noise model) (Dunstan et al.) and the more widely used single cosinor method (Halberg et al.). These results are conveniently viewed with respect to:

a. **Comparison of temperature rhythm parameters on the tumorous and contralateral sites.** Figure 1 illustrates, for patient 6, breast skin temperature data fitted by using formula [3] with α ≠ 0 and α = 0 for both the tumorous site (Fig. 1a) and the corresponding site on the contralateral breast (Fig. 1b).

Values for circadian rhythm parameters of breast temperature are shown in Tab. 2. The zero amplitude test of the cosinor method revealed statistical significance (p<0.05) in all but two cases, when correlations were ignored.

In all patients studied, including the patient with benign breast disease, using either model, the mesor was higher over the tumour than on the corresponding contralateral site. The mean mesor difference and its standard deviation for patients with breast carcinoma were 0.91° ± 0.40 °C. Fitting according to formula [3], as well as by single cosinor showed a concomitant reduction in amplitude with the mesor elevation over the tumour for all individual patients. The three acrophases associated with a cancer of the left breast were phase-advanced as compared to the contralateral breast. The 4 acrophases were barely or somewhat retarded relative to the contralateral site when the cancer was in the right breast, however.

b. **Comparison of rhythm parameters for sites on the left breast with those on the right.** For the 7 patients with breast cancer, 86% of the sensors (24/28) produced series with an elevated mesor over the tumour and also adjacent sites
on the breast, when compared to the corresponding contralateral area. Of the 4 ‘discrepancies’, 3 were found in patients with a tumour of the right breast. Secondly, 68% of sensors (19/28) produced series that exhibited a reduced amplitude in the tumour-bearing breast. Of the 9 ‘discrepancies’, 6 were in patients bearing a tumour in the right breast. Thirdly, the rhythm was phase-advanced in 92% of the time series collected (11/12) over a tumour in the left breast. It is interesting, however, that the proportion of sensors on a left breast showing phase-advancement was only 69% (11/16) when the tumour was present in the right breast, suggesting that the left breast is naturally phase-advanced, as compared to the right, and that a tumour in the right tends to nullify this difference.

The implication that differences between mesors and amplitudes for cancer and contralateral site may be nullified sometime during tumour growth in the right breast is somewhat insubstantial when based on these ‘discrepant’ values alone. However, unpublished data from the Tenovus Institute from similar studies of 6 clinically healthy postmenopausal women of similar age revealed that 42% (10) of the quadrants of the left breast had higher mesors than their corresponding counterparts on the right; 33%

Fig. 1 - These computer plots for patient 6 show raw data (solid squares) and demonstrate fit according to formula (3) with $a = 0$ (smooth curve) and $a \neq 0$ (open squares). Plot a. refers to data obtained from skin overlying tumour and b. represents data from a similar site on the contralateral breast. Identification of raw data and models may be achieved by the fact that both models begin at the same datum.
One rhythmic of ultradian and infradian data covering cannot be assumed and there may be a greater probability that a tumour in the right breast would nullify differences in these rhythm parameters.

- **Comparison of the two models.** The point estimates from the single cosinor model were found to be in reasonable agreement with that described by formula [3], but the use of formula [3] may increase the reliability of the rhythm parameter estimates and associated standard errors, as previously reported (DUNSTAN et al.4).

**DISCUSSION**

The models used here are still incomplete, although more complex models cannot be discussed in the case of breast temperature on the basis of data covering 4 days. It is already known that in health, there are circadian, ultradian and infradian components, the latter including circaseptan, circatripgintan and circannual rhythms. In health as well, there are trends with aging. With dense data covering long observation spans, the broad spectrum of rhythmic components with different frequencies and age trends is revealed. One also finds a number of types of noise, some correlated, some ap-
Apparently Gaussian. The spectral solutions that may be sought with such a full model of rhythms, trends and noises have been intimated for thermorhythmometry elsewhere (Halberg et al.), but remain beyond the scope of this report.

Circadian rhythms of cortisol and prolactin concentrations in plasma were investigated in patients with primary breast cancer 4 days prior to surgery. Cortisol was chosen as an internal marker rhythm to assess the comparative degree of synchrony of patients with their environment.

Investigations in this Institute have generally failed to show differences of prolactin concentration in single samples of plasma taken at 0900 between normal women and those with breast cancer (Boyns et al., Wilson et al.), although serum prolactin concentrations were higher in women with benign breast disease (Cole et al.). Others have reported differences (Murray et al., Rolandi et al.) although the current situation on the role of prolactin in human breast cancer remains confused (Nagasawa). Recent chronoe-pidemiological studies of Halberg and his colleagues (Halberg et al.), however, indicated that for such studies samples of plasma for prolactin analysis must be time-qualified with respect to circadian, circatrigintan and circannual stage. In the present study, plasma prolactin concentrations were monitored in the postmenopausal women with primary breast cancer, in order to provide additional information on their circadian rhythms. The data demonstrate that circadian rhythms of prolactin are present in some subjects and that the acrophase occurs during the early hours of the morning, as in the normal women (Halberg et al.); this has also been demonstrated for pre-menopausal women who have had a unilateral mastectomy (Phillips et al.).

Statistically significant circadian rhythms of oral temperature (p < 0.001) were demonstrated for all but two primary breast cancer subjects. The acrophases were at ~1600-1800, and since oral temperature is a reliable index of body core temperature, even among subjects with different genetic backgrounds (Halberg et al.), these patients were considered to be reasonably well synchronized for thermal behavior. Consequently, observations of breast skin temperature were considered to be representative of the breast itself.

Thermal asymmetry in human breasts has previously been demonstrated from independent studies of breast skin temperature rhythms (Halberg et al.). The studies now reported have shown that the left breast appears to have a higher mesor, a lower amplitude and an advanced acrophase when compared with the right breast. Examination of the differences in rhythm parameters between a breast cancer and a site on the contralateral breast indicates that a tumour in the left breast reinforces the positive difference in the mesor, further reduces the differences in amplitude and increases the degree of phase advancement. On the other hand, the presence of a tumour in the right breast tends to nullify any inter-mammary difference in rhythm characteristics.

It would seem, therefore, that if these thermorhythmometric properties of breast temperature are to be used for screening for cancer, then subjects would require regular monitoring so that persistent change in the inter-mammary difference of thermal rhythm characteristics, produced as a result of tumour development or a preneoplastic condition (Jensen et al.), might be detected.
It is now clear that a systematic research program is necessary for studying women with clinically healthy breasts and those with either small primary tumours or benign breast disease. It now seems reasonable to assume that changes in breast skin temperature rhythms, characterized by statistical techniques, may allow the early diagnosis of breast disease and possibly the identification of the women 'at risk'.

SUMMARY

Plasma prolactin and cortisol were determined and breast skin temperatures measured in 8 postmenopausal diurnally active-nocturnally resting women, 7 with primary breast cancer and 1 with benign breast disease. In all subjects, prolactin peaked during the early morning hours. Cortisol and oral temperature served as internal physiological markers of anticipated rhythmicity in adrenocortical function and metabolism. In all patients studied, the tumour site had an increased mesor (midline-estimating statistic of rhythm) and reduced amplitude of the circadian breast skin temperature rhythm when compared to a similar site on the contralateral breast. Thermal asymmetry was also observed between left and right breasts. A cancer, if in the left breast, may reinforce lower amplitude and perhaps a higher mesor (associated with the healthy left breast in these particular patients), whereas if it is located in the right breast, it tends to nullify inter-mammary differences in mesor and amplitude. Screening for breast cancer appears to require subjects being monitored on at least two occasions before thermal abnormalities due to tumour or preneoplasia become discernible.

REFERENCES


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II. INVESTIGATIONS RELATING TO CANCER OF THE BREAST.

D. PROLACTIN AND BREAST CANCER.
RESPONSE OF PLASMA PROLACTIN AND GROWTH HORMONE TO INSULIN HYPOGLYCAEMIA

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Summary The responses of plasma prolactin and growth hormone to insulin hypoglycaemia, induced by either a single injection or continuous intravenous infusion, were estimated by radioimmunoassay in post-vagotomy patients. Release of both hormones was stimulated by the standard single-injection test but only growth hormone release was stimulated by the insulin infusion. This test may have advantages in the assessment of pituitary function, especially in post-hypophysectomy patients, but in such an event an alternative stimulus, such as chlorpromazine, will be required to assess the prolactin response.

Introduction The response of circulating growth hormone to insulin hypoglycaemia is a well-established test of pituitary function. In our experience it has proved a valuable estimate of residual pituitary function after attempted ablation by yttrium-90 implantation. Insulin hypoglycaemia is also used as a stimulus of gastric-acid secretion in the standard Hollander test to assess the completeness of vagotomy.

The standard method of administering insulin in these tests is by a single intravenous injection varying from 0.1 to 0.2 units per kg. body-weight. A new test in which insulin is given by constant intravenous infusion has been described. This test has been shown to produce the same degree of gastric-acid secretion.
but much fewer side-effects than the standard test.\textsuperscript{3} It is now the method of choice in our gastric-secretion laboratory. The present investigations were undertaken to compare the growth-hormone and prolactin responses to these two types of test.

**Methods**

Nine patients, seven male and two female, with duodenal ulcer (eight post-vagotomy) were studied during 10 insulin tests of gastric secretion. 5 tests were of the standard Hollander type using a single intravenous injection of 0.2 units insulin per kg.; 5 were insulin-infusion tests in which the insulin was administered in a dose of 0.04 units per kg. per hour by constant intravenous infusion.\textsuperscript{3}

Samples of venous blood were withdrawn into lithium-heparin tubes by one of us (R. W.) before and at three intervals of 45 minutes after administration of the insulin. A specimen was taken for the estimation of blood-glucose by the glucose-oxidase method of Marks\textsuperscript{4} and the remainder of the blood-samples was centrifuged at 4°C and stored at \(-20°C\). 5–8 weeks later, the frozen plasma was sent to the Tenovus Institute for assay of prolactin and growth hormone.

Both hormones were estimated by radioimmunoassay. The system used for prolactin is a homologous assay \textsuperscript{6} which uses labelled human prolactin and rabbit antibodies to human amniotic prolactin.\textsuperscript{3} The assay for growth
hormone was the established double-antibody technique. Plasma concentrations were expressed in terms of milli-ampoules of prolactin (M.R.C. 71/222) per ml. and μu. growth hormone (1st International Reference Preparation) per ml.

Throughout the tests the output of gastric acid was also being monitored, but since eight of the patients had been treated by vagotomy, their gastric-acid results will not be discussed further.

Results

The growth-hormone response to the two methods of administering insulin is shown in fig. 1. In all patients there was a pronounced rise in circulating growth hormone within 90 minutes of insulin administration, which was usually sustained in the sample collected at 135 minutes. In contrast there was a pronounced difference between the effect of the two types of test on prolactin secretion (fig. 2). All patients given a single injection of insulin had a clear increase at 90 minutes, falling off at 135 minutes; but only one patient in the infusion group had any rise of prolactin level and that was slight.

The results are typified by patient 3 who had both tests performed. Similar growth-hormone responses occurred with both the injection and infusion techniques, but only with the former did a prolactin response occur.

The mean levels of blood-glucose are shown in fig. 3. Significantly lower levels of hypoglycaemia were
achieved by the standard Hollander test. The difference between the mean blood-glucose at 45 minutes in those patients receiving insulin by infusion (23.6 ± S.D. 6.84 mg. per 100 µlitre) and those receiving insulin in the Hollander test (9.20 ± S.D. 2.28 mg. per 100 µlitre) was highly significant (p < 0.005).

**Discussion**

These results indicate that the thresholds for stimulation of prolactin and growth hormone by insulin hypoglycaemia are different. Surprisingly, only blood-glucose levels of around 10 mg. per 100 ml. caused prolactin release, which would explain the differing results in the published reports of the effect of insulin-induced hypoglycaemia on this hormone. The mechanism for stimulation of the release of these hormones by insulin hypoglycaemia is likely to differ. Release of growth hormone is by a hypothalamic-releasing factor; that of prolactin predominantly by an inhibiting factor. Clearly both mechanisms are not equally sensitive to hypoglycaemia.

These results have great practical significance. That there are comparatively fewer side-effects in the insulin-infusion test has proved of advantage in testing gastric-acid secretion. Similar advantages should accompany the use of an infusion test to assess pituitary function, especially in the hypophysectomised patient.

The insulin-infusion test cannot be used to monitor prolactin secretion. An alternative, under study in our department, is a test in which the stimulus is chlorpromazine.
We thank Mrs. J. Dale for her help and cooperation with the gastric-acid studies; Mr. I. B. Macleod and Mr. J. R. Kirkpatrick who allowed us to collect blood-samples from their patients; and Prof. L. G. Whitby for his cooperation. Dr. V. J. Lewis kindly supplied the purified human prolactin. Prolactin standards were obtained from the Medical Research Council, Division of Biological Standards. Generous financial assistance was provided by the Tenovus organisation.

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REFERENCES
Abstract—The incidence of mammary tumours induced in three strains of rats is different. Plasma prolactin levels have been measured in these strains of rat during the dioestrous phase of the oestrous cycle. The strain showing the highest incidence of induced mammary tumours also showed the highest circulating prolactin levels.

INTRODUCTION

The incidence of rat mammary tumours induced by 7,12-dimethylbenz(a)anthracene (DMBA) is variable. As tumour growth is dependent upon serum prolactin levels [1, 2] we have tried to decide if a relationship exists between circulating prolactin levels and tumour incidence. In this study we have measured plasma prolactin in strains of rats with differing incidence of tumour induction.

METHOD

Animals—three strains of rats were studied. (i). A strain of Sprague-Dawley originally obtained from Oxfordshire Laboratories, but bred for the past 6 years in the small animal quarters of the Department of Clinical Surgery, University of Edinburgh. (ii). A strain of Sprague-Dawley obtained from the Animal Diseases Research Association (ADRA), Edinburgh. (iii). A strain of Long-Evans, a gift from Dr. C. B. Huggins of the Ben May Research Laboratory, Chicago.

Animals were housed at a temperature of 68°F ± 2°F with light provided from 0600 to 2000 hr daily.

Vaginal smears were performed at 50 days and 33 animals in the dioestrous phase of the oestrous cycle were selected. Blood was obtained between 0900 and 1100 hr by cardiac puncture after the animal had been anaesthetized with ether. The blood was centrifuged, the serum separated and stored at -20°C until assayed, the assay being performed within three weeks.

Measurement of plasma prolactin

Immunoreactive prolactin was measured in serum samples by double antibody radioimmunoassay using a kit distributed by the National Institute for Arthritis and Metabolic Diseases (NIAMD), Bethesda, Md., U.S.A. NIAMD-Rat prolactin 1-1 was labelled with (131I) iodine (Radiochemical Centre, Amersham) by the method of Greenwood, Hunter and Glover [3] and separated from iodide on columns of Biogel P-60 (Bio-Rad Laboratories). The labelled hormone was diluted in assay buffer (50 mM-phosphate buffer, pH 7.3, containing 0.01% merthiolate and 0.5% serum albumin) to give a concentration of approximately 20,000 counts/min/100 μl (5 ng/ml). Antiserum to rat prolactin (NIAMD-anti-rat prolactin S-1) was diluted (1/100,000) in 50 mM-phosphate buffer containing 50 mM-EDTA and 1/400-non-immune rabbit serum. Standard rat prolactin (NIAMD-rat prolactin RP-1) was diluted in assay buffer to give a series of stan-
standard solutions in the range 1–125 ng/ml. Anti-
serum to rabbit serum globulins was raised in sheep by the intramuscular injection of the antigen emulsified in Freund's complete adju-
vant and was diluted (1/10) in assay buffer. Standard curves were prepared as follows: 100 µl of prolactin standard was mixed with 200 µl of antibody to prolactin, 100 µl plasma from a hypophysectomized male rat and 300 µl assay buffer. After incubation for 1 day at 4°C, 100 µl 131-I-labelled prolactin was added and the solution incubated for 1 day at 4°C. Finally, 200 µl sheep-anti-rabbit globulin serum was added and the solution incubated overnight at 4°C. The tubes were centrifuged at 1500 g for 30 min at 4°C and the supernatants decanted. After draining, the tubes were counted in a Nuclear Chicago gamma counter. When serum samples were assayed, 100 µl samples replaced the standard prolactin solution and 100 µl assay buffer replaced plasma from a hypophysectomized male rat. Assays were carried out in duplicate, the radioactivity in each tube being expressed as a percentage of the radioactivity present in the tubes containing the zero standard. Standard curves were linearized by plotting on probability-log paper.

**Tumour induction**

A special 15% fat emulsion with 7,12-di-
methylbenz(a)-anthracene, 5 mg/G (supplied by Dr. P. Schurr, Upjohn Company, Kala-
mazoo), was used to induce mammary tumours. Three groups of rats aged 50 days ± 1 day were given 5 mg of DMBA in this fat emulsion by injection into a dorsal tail vein. Thereafter, the rats were examined regularly and tumour incidence noted. At 250 days of age, a post-mortem examination was performed and a portion of each mammary tumour taken for histological examination.

**RESULTS**

The incidence of mammary tumour is shown in Table 1. There was a much higher incidence of tumours in the Clinical Surgery Sprague-

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of rats</th>
<th>No. of rats with mammary carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Surgery</td>
<td>104</td>
<td>39</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADRA</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-Evans</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

Dawley strain than in the Long-Evans and ADRA Sprague-Dawley strains. Histological confirmation was obtained that every tumour was an adenocarcinoma.

The values of circulating prolactin are shown in Fig. 1. The concentration of prolactin in the serum of Clinical Surgery Sprague-Dawley rats were significantly higher than those of the other groups (p < 0.05, Kruskal-Wallis one-

way analysis of variance).

![Fig. 1. Plasma prolactin levels in different rat strains in the dioestrous phase of the oestrous cycle.](image)

**DISCUSSION**

Serum immunoreactive prolactin in the rat undergoes considerable fluctuations during the oestrous cycle, but the lowest concentrations are reached during dioestrous [4]. It is likely, therefore, that the rats in our study were bled at a time when serum prolactin levels were relatively steady. Stress produced by ether anaesthesia may induce release of prolactin from the pituitary [5]. In view of this, the serum prolactin concentrations that are reported here, cannot be regarded as representative of the lowest levels of the circulating hormone reached during dioestrous.

However, the strain of rats which showed the highest serum prolactin concentration after ether stress also showed the highest incidence of mammary tumours. The data suggest that the high incidence of tumours in Clinical Surgery Sprague-Dawley rats and the low incidence of tumours in Long-Evans and ADRA rats may be related to different concentrations of circulating pituitary prolactin in these strains.
REFERENCES


Plasma Prolactin in Breast Cancer

A. R. BOYNS, E. N. COLE, K. GRIFFITHS, M. M. ROBERTS, R. BUCHAN, R. G. WILSON and A. P. M. FORREST

Tenovus Institute and Department of Chemical Pathology, Welsh National School of Medicine, Department of Clinical Surgery, University of Edinburgh, Great Britain

Abstract—Plasma prolactin levels have been measured in normal women and in women with benign and malignant breast disease. No difference in circulating prolactin levels has been demonstrated between any of these groups of patients.

INTRODUCTION

It has been recognized for a number of years that pituitary prolactin has a powerful stimulating effect on the breast and on some types of breast cancer in experimental animals [1–4]. The demonstration of prolactin in cultured human and monkey pituitary glands by both Pasteels [5] and also by Friesen and his colleagues [6] allowed the development of specific radioimmunoassays for the hormone in human plasma [7, 8].

This communication describes the measurement of human prolactin in the plasma of patients with breast disease by means of a heterologous radioimmunoassay system.

MATERIAL AND METHODS

1. The assay

Human prolactin was determined by a heterologous double antibody radioimmunoassay. Standard human pituitary prolactin (71/222) and a human reference plasma (71/167) containing a high concentration of prolactin were obtained from the WHO International Laboratory for Biological Standards. Rabbit antiserum to human prolactin (AHPs-S-R) and ovine pituitary prolactin (OPr) (NIH-P-S8) were gifts from Dr. H. G. Friesen (Montreal) and Dr. V. C. Stevens (Columbus, Ohio) respectively. Antiserum to rabbit globulins (ARGS-S) was raised in sheep.

A series of standard solutions were prepared by diluting human prolactin (71/222) in 0.05 M-phosphate buffer pH 7.5, containing 5 mg/ml human albumin and 0.01% merthiolate (HSA buffer). AHPs-S-R was diluted (1/400) in 0.05 M-phosphate buffer pH 7.5, containing 0.05 M-EDTA. $^{131}$I-labelled OPr was prepared by a modification of the method of Greenwood et al. [9], and diluted in HSA buffer to a concentration of 5 ng/ml. ARGS-S was diluted (1/9) in HSA buffer.

The assay protocol was as follows. One hundred $\mu$l of each solution of standard human prolactin (71/222) was treated with 200 $\mu$l AHPs-S-R (1/400) and 100 $\mu$l HSA buffer. The solutions were mixed and incubated at 4°C for 2 days. Then 100 $\mu$l $^{131}$I-labelled OPr was added, mixed and incubated at 4°C for a further two days. Finally, 200 $\mu$l ARGS-S (1/9) was added. After mixing, the solutions were left overnight at 4°C. They were then centrifuged at 1500 g for 30 min at 4°C, the supernatants were decanted and the tubes counted in an auto-$\gamma$-counter. Radioactivity precipitated by antibody was expressed as a percentage of that bound in the absence of added standard prolactin. The results were plotted on linear-linear or on probability-log scales. The latter procedure usually gave a straight line relationship. When plasma samples were assayed, standard solutions of prolactin were replaced by 100 $\mu$l of the unknown plasma and plasma (100 $\mu$l)
from a hypophysectomized patient was included in the standard curve. Concentrations of prolactin were expressed in terms of milliampoules of standard.

The mean standard curve for ten assays is shown in Fig. 1. Human growth hormone, human follicle stimulating hormone, human lutetinising hormone, human thyroid stimulating hormone and human chorionic somatomammotrophin did not interfere with the assay system. The addition of plasma from an hypophysectomized patient reduced binding of $^{131}$I-labelled OPr from 35 to 28% of the total counts added in the absence of standard prolactin. Reference plasma (71/167), which contained a high concentration of prolactin, inhibited binding of $^{131}$I-labelled OPr to antibody. Serial dilutions of this plasma in hypox. plasma gave a line parallel to that of the standard (Fig. 2).

Human amniotic fluid contains high concentrations of prolactin [7]. Human mid-term amniotic fluid was extracted for prolactin by a modification of the method of Simkin and Goodhart [10]. Serial dilutions in buffer of this extract and a prolactin-rich fraction of human pituitary glands gave a curve parallel to that of the standard (Fig. 3).

2. Clinical study

Plasma prolactin was estimated in 90 women of whom 38 were in Cardiff and 52 in Edinburgh. Ten patients had been admitted to hospital for the treatment of conditions other than breast disease; 12 had benign disease of the breast; 27 had primary breast cancer and 41 had metastatic breast cancer, of whom 29 had localised disease and 12 generalised disease as defined previously by us [11].Twenty-four were pre-menopausal (having regular periods or up to 2 years from their last menstrual period); 17 were menopausal (2–5 years since their last menstrual period) and 48 were post-menopausal (more than 5 years from their last menstrual period).

In most cases, blood was collected by venepuncture at random times after admission to hospital but in a few patients samples were collected as outpatients. In patients admitted for breast biopsy or mastectomy collections of blood were made before operation. In patients with advanced disease, blood samples were collected before any form of treatment was initiated. As far as could be ascertained, no patient was receiving phenothiazines, L-dopa, monoamine oxidase inhibitors or other drugs known to affect the secretion of prolactin.

Following collection, venous blood was placed in heparinized containers which were immediately refrigerated, centrifuged and the plasma stored at $-30^\circ$C. All samples were stored under identical conditions but the time of storage varied, the maximum period being 20 months.

RESULTS

As the circulating levels appeared to have a log normal distribution, the logarithms of the measured values in milliamperes/ml of WHO 71/222 reference standard were used in the analysis. No differences were noted between the hospital controls, the patients with benign breast disease or the patients with primary or advanced breast cancer (Fig. 4, Table 1).

There was no significant difference in the ages of the patients studied, except for those with benign disease who were younger than the other groups.

The patients with breast cancer were divided into pre-menopausal, menopausal and post-menopausal groups. The mean levels of prolactin were significantly lower in the post-menopausal group, compared with the combined pre-menopausal and menopausal groups ($P < 0.05$, Table 3).

DISCUSSION

These preliminary results indicate that women with breast cancer do not show any striking differences in circulating prolactin levels compared with a small group of hospital control women. They also show that women with advanced breast cancer of either localized or generalized type do not have different levels from those with primary disease. Nor was any difference noted in the circulating levels of prolactin in women with benign disease compared with any of the other groups. Women with benign disease were significantly younger than either the hospital controls or breast cancer patients. Yet, when they were compared with women of similar menstrual status with breast cancer, no difference was seen.

There is a need to define more clearly the relationship between age, menstrual status and plasma prolactin in normal women, before accepting unequivocally that women with breast disease have normal levels for their age. This we are currently doing.

Acknowledgements—We are indebted to Tenovus (Cardiff) for their financial support and those Surgeons and Radiotherapists of the Cardiff Royal Infirmary, Velindre Hospital, Cardiff and the Royal Infirmary, Edinburgh, for their help and co-operation.
**Plasma Prolactin in Breast Cancer**

Fig. 1. Standard curve for Human Prolactin (71/222) diluted in HSA buffer. Mean + SEM (10 assays) HPL—Human Chorionic Somatomammotrophin (WHO 70/194).

HGH—Human Growth Hormone (1st IRP HGH).
FSH—Human Follicle Stimulating Hormone (CPD/8 But).
TSH—Human Thyroid Stimulating Hormone (DE 32.2 Hartree 28.6.69)).

Fig. 2. Standard curve for Human Prolactin (71/222) diluted in plasma from a hypophysectomized patient. Serum Standard Plasma 71/167 and post-menopausal plasma also diluted in hypox. plasma.

Fig. 3. Dilution curves in buffer of Human Prolactin (71/222), pituitary extract and amniotic fluid extract.

Fig. 4. Circulating prolactin levels (log milliampoules/ml) in patients with breast cancer, benign breast disease and hospital controls.

Fig. 5. Circulating prolactin levels (log milliampoules/ml) in patients with breast cancer, benign breast disease and hospital controls.

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>No. in Group</th>
<th>Mean age ± S.D.</th>
<th>Mean log Prolactin ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10</td>
<td>56.0 ± 14.3</td>
<td>1.411 ± 0.44</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td>12</td>
<td>38.8 ± 8.6</td>
<td>1.313 ± 0.48</td>
</tr>
<tr>
<td>Primary breast cancer</td>
<td>27</td>
<td>58.8 ± 12.2</td>
<td>1.214 ± 0.42</td>
</tr>
<tr>
<td>Localised advanced breast cancer</td>
<td>29</td>
<td>60.8 ± 12.9</td>
<td>1.279 ± 0.32</td>
</tr>
<tr>
<td>Generalised advanced breast cancer</td>
<td>12</td>
<td>53.8 ± 10.1</td>
<td>1.245 ± 0.50</td>
</tr>
</tbody>
</table>

Mean age and circulating prolactin levels (log Milliampoules/ml) in groups of patients studied.

Table 2.

<table>
<thead>
<tr>
<th></th>
<th>No. in group</th>
<th>Mean log prolactin ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign breast disease</td>
<td>11</td>
<td>1.316 ± 0.51</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>11</td>
<td>1.259 ± 0.48</td>
</tr>
</tbody>
</table>

Circulating prolactin levels (log Milliampoules/ml) in pre-menopausal patients with benign breast disease and breast cancer.

Table 3.

<table>
<thead>
<tr>
<th></th>
<th>No. in group</th>
<th>Mean log prolactin ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-menopausal</td>
<td>42</td>
<td>1.171 ± 0.37</td>
</tr>
<tr>
<td>Pre+ menopausal</td>
<td>25</td>
<td>1.384 ± 0.39</td>
</tr>
</tbody>
</table>

Circulating prolactin levels (log Milliampoules/ml) according to menstrual status in patients with breast cancer.

REFERENCES

PLASMA PROLACTIN AND BREAST CANCER

R. G. Wilson, MBChB, R. Buchan, MD, M. M. Roberts, MBChB.
A. P. M. Forrest, MD, A. R. Boyns, MBChB, PhD,
E. N. Cole, BSc, and K. Griffiths, PhD

Using a homologous radioimmunoassay we have compared plasma prolactin concentrations in 49 patients with breast cancer and 39 hospital controls. When a drug history was taken into account no difference was found. In addition we have studied the effect of stilbestrol in 6 females with advanced breast cancer. Remission of disease has been noted in the face of elevated prolactin levels.


In a previous study using a heterologous assay we reported that the concentration of prolactin in the plasma in women with benign and malignant disease of the breast did not differ from that found in normal women. In contrast, Murray using a similar assay, found that postmenopausal females with metastatic breast cancer had higher basal plasma prolactin levels than normal. We now report our experience in a further group of patients with breast cancer using a homologous radioimmunoassay.

**Materials and Methods**

Eighty-eight women were studied. Forty-nine had breast cancer; 14 primary, 18 locally advanced, and 17 disseminated disease. Thirty-nine women, who were admitted to hospital for surgical treatment of non-malignant disease, other than that of the breast, were used as controls.

A blood sample was taken from patients in the late afternoon of the day before their operation or at an equivalent time in non-operative cases. In six patients with advanced disease, samples were repeated after 1 month of treatment of their advanced breast cancer with oral stilbestrol 15 mg daily, and in four of these a further sample was taken after 3 months of therapy. At this time the disease response to this therapy was assessed by two of us (MMR and RB) using the criteria previously described.

Following collection the blood was placed on ice, centrifuged at 4°C, and the plasma stored at −20°C for a maximum time of 27 months. The assay of the concentration of prolactin was by a homologous radioimmunoassay using an antiserum raised in the rabbit to human amniotic prolactin, and iodinated human prolactin. The results are expressed in milliampules/ml of the Medical Research Council human prolactin standard (71/222). Two mamp is approximately equivalent to 1 ng of purified human prolactin.

**Results**

The distribution of plasma prolactin concentrations in the 88 patients is shown in Fig. 1. With the exception of 4 patients with advanced breast cancer whose prolactin levels ranged from 50 to > 100 mamp/ml, all other patients with malignant disease lay within the normal range. On further questioning it was found that these 4 patients were receiving drugs known to stimulate prolactin secretion, 2 each chlorpromazine and methyldopa. They were therefore excluded from the statistical analysis which indicated identical mean prolactin levels in control patients and those with primary or advanced disease (Table 1). A separate analysis on postmenopausal patients was also carried out and again showed no difference in mean plasma prolactin levels.

In all 6 patients receiving stilbestrol, the plasma prolactin rose within 1 month (Fig.
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Table 1. Mean Prolactin Levels in Breast Cancer Patients and Controls

<table>
<thead>
<tr>
<th>Patients</th>
<th>No.</th>
<th>Mean age in years</th>
<th>Mean prolactin level ± S.E. of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>39</td>
<td>48 ± 3.1</td>
<td>8.31 ± 1.16</td>
</tr>
<tr>
<td>Primary breast cancer</td>
<td>14</td>
<td>59.46</td>
<td>10.86 ± 2.20</td>
</tr>
<tr>
<td>Localized advanced</td>
<td>18</td>
<td>58.50</td>
<td>8.44 ± 0.99</td>
</tr>
<tr>
<td>Metastatic breast</td>
<td>13</td>
<td>53.62</td>
<td>7.69 ± 1.64</td>
</tr>
</tbody>
</table>

Fig. 1. Plasma prolactin levels in 49 patients with breast cancer and 39 controls.

2. One of the two patients with initially elevated levels was receiving a methyldopa and the other had been treated previously with norethisterone acetate (this patient was not included in the original 49 studied). Three patients had a persistent hyperprolactinemia after 3 months of treatment; two of these were considered to have had regression of their advanced disease as a result of Stilbestrol therapy.

Comment

Using a homologous radioimmunoassay we have confirmed our previous finding with a heterologous assay that patients with breast cancer have circulating plasma prolactin levels which do not differ from normal. Four patients who had elevated levels were found to be taking a prolactin-stimulating drug.

This stresses the importance of obtaining an accurate drug history from patients in whom plasma levels of prolactin are to be estimated. Care was taken to collect samples at the same time each day to minimize possible errors due to the diurnal rhythm.

Stilbestrol, in normal therapeutic doses, was found to elevate plasma prolactin even when regression of tumor was being observed. Meites observed this effect of estrogen in the rat.

Despite unequivocal evidence that hyperprolactinemia stimulates the induction and growth of experimental mammary tumors in the rat, there is little clear evidence for a relationship between circulating prolactin levels and human breast cancer. In fact it rests on 1) the observations of McCalister and Welbourn that the administration of ovine prolactin to hypophysectomised patients with breast cancer and bone metastases increased the output of urinary calcium, contrary to the findings of Lipsett and Bergensdal; 2) the report by Murray of elevated plasma prolactin levels in postmenopausal women with cancer of the breast, and 3) possible benefit of advanced cancer following the administration of L-dopa.

Fig. 2. Effect of Stilbestrol on plasma prolactin in six patients with breast cancer.
Our studies do not support the concept that this hormone plays a significant role in human disease. Not only were levels of prolactin in the plasma normal in women with breast cancer, but regression of advanced disease following stilbestrol therapy occurred in the face of elevated levels. A similar observation after pituitary stalk section was made by Turkington.13

However, before accepting unequivocally that circulating prolactin levels are normal in women with cancer of the breast, detailed sequential studies during periods of diurnal rhythm are required. This we are now doing.

REFERENCES


The Effect of Long Term Phenothiazine Therapy on Plasma Prolactin


Summary. Plasma prolactin has been estimated in 69 psychiatric in-patients receiving either thioridazine (20–300 mg./day) or chlorpromazine (50–200 mg./day) or other phenothiazines. A homologous radioimmuno-assay was used which depends on an antiserum to human amniotic prolactin. Prolactin levels were significantly increased in these patients compared with control patients, and were dose related. Simultaneous estimation of growth hormone revealed normal levels.

INTRODUCTION

Hyperprolactinaemia has been demonstrated by radioimmuno-assay in patients receiving drugs of the phenothiazine group, irrespective of their route of administration (Bryant and Greenwood, 1972; Frantz et al., 1972; Beumont et al., 1974). Galactorrhoea also may occur as a side-effect of the drug (Sulman and Winnik, 1956). Evidence that elevated plasma prolactin levels promote the induction and growth of experimental rat mammary tumours (Pearson et al., 1972) has led some to advocate caution in the use of these drugs in patients with breast cancer (Palmer and Maurer, 1972). Therefore we believed it important to define the effect of long-term phenothiazine therapy on the circulating levels of plasma prolactin, and this has been done in a group of psychiatric in-patients.

MATERIALS AND METHODS

Ninety-four female psychiatric in-patients from eight wards of the Royal Edinburgh Hospital were selected for inclusion in the study. Of these 69 had been on long-term therapy with drugs of the phenothiazine group for up to eight years; 49 were receiving thioridazine in doses ranging from 20 to 300 mg. daily, and 11 were taking chlorpromazine in doses of from 50 to 200 mg. daily, while the remaining 9 patients were receiving a variety of other phenothiazine drugs singly or in combination. Twenty-five patients not receiving any drug therapy were used as controls. The ward nursing staff were asked to examine the breasts of these patients during a routine bath for lumps or galactorrhoea. The examination included 'milking' of the breast.

A single blood sample was taken by venepuncture from each patient. All blood samples were taken between 1000 hours and 1700 hours on the same day, this being the period of time when there is a plateau of low plasma prolactin levels as measured during studies of the circadian rhythm of the hormone (Nokin et al., 1972; Sassin et al., 1972). The blood was received into lithium-heparin anticoagulant tubes and immediately placed on ice. The plasma was separated by centrifugation at 4 °C. and stored at −20 °C. for four weeks.

The prolactin concentration was assayed by the Tenuvus Institute in Cardiff. A homologous radioimmuno-assay system was used which depends on rabbit antiserum to human amniotic prolactin and iodinated human prolactin (Lewis) (Cole and Boyns, 1973). Growth hormone was assayed in 55 of the samples by the method similar to that of Schalch and Parker (1964). Prolactin results are expressed in mAmp./ml. of the MRC standard (71/222) (2 mAmp. = 0.02 μg.) and expressed in μU/ml. of the MRC standard (66/217).

RESULTS

Circulating prolactin was detected in all 94
plasma samples. The mean plasma prolactin (±S.E.) of the group of 25 control patients was 17.9±2.7 mAmp./ml. That of the 69 patients under treatment was significantly higher (55.9±5.3 mAmp./ml., p < 0.001).

The mean plasma prolactin values of the groups receiving thioridazine and chlorpromazine were not different (Table I). When the patients receiving thioridazine were subdivided into three groups according to dose, a significant dose-related effect emerged (Fig. 1).

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
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</thead>
<tbody>
<tr>
<td>Controls</td>
<td>25</td>
<td>79.3±1.3</td>
<td>17.9±2.7</td>
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<tr>
<td>Thioridazine</td>
<td>49</td>
<td>74.9±1.3</td>
<td>50.8±4.7</td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>11</td>
<td>71.5±4.2</td>
<td>60.5±18.9</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1.—Mean, plus or minus one standard error of mean, and range of plasma prolactin in control women and those receiving various doses of thioridazine. The difference between control and low (20–30 mg.) and medium (40–80 mg.), and between medium and high (>80 mg.) dose groups were significant at the level of p = 0.01–0.02.
Growth hormone was detected in all but one of the samples assayed. The mean value (±S.E.) of the control group (12) was 19·6±8·6 µU/ml, while that of the phenothiazine group (42) was 8·8±2·1 µU/ml. The high level of the control group is due to one patient having a value of 100 µU/ml and two 40 µU/ml. These findings confirm that phenothiazines do not stimulate growth hormone secretion.

Three patients in the group were found by the nursing staff to have breast abnormalities. One with suspected galactorrhoea (not confirmed by us on subsequent examination) was 83 years old. She was receiving chlorpromazine 100 mg. daily and had a grossly elevated plasma prolactin (115 mAmp./ml). One patient had had bilateral mastectomies (1942 and 1958) for carcinoma; this was before she began phenothiazine treatment. A third had a retracted nipple but no evidence of underlying breast disease.

**DISCUSSION**

Patients with psychiatric disorders are often treated for long periods with phenothiazine drugs. In this study we have confirmed, using a homologous radioimmuno-assay, that such treatment results in persistently elevated plasma prolactin levels. Individual patients, however, may show variations from day to day. This may be due to failure to take the drug, for example by hoarding, a practice well recognized amongst these patients, or to the need for a particular dose being unnecessary. The level of elevation of the plasma prolactin level in these patients is directly dose-related.

Although the group was small, there was no evidence of breast cancer amongst those studied. The low incidence of galactorrhoea in those rather elderly patients (mean age 73·2±1·22 years) confirms the findings of other workers. Both Hooper (1961) and Apostolakis (1972) found that the incidence of galactorrhoea during administration of phenothiazines was high during the reproductive period of life and fell in the post-menopausal period. Presumably oestrogens also are required for the initiation of lactation in these patients.

There is no doubt that hyperprolactinaemia has an important promoting effect on DMBA-induced mammary tumours in rats (DMBA is 7,12-dimethylbenzanthracene). Recently we have reported that their incidence in three strains of rat is related to the basal prolactin levels, those rats with the highest incidence of tumours also having the highest prolactin concentration in the plasma (Boyns et al., 1973a). No such relationship has yet been uncovered in humans. In two separate studies we have found that women with breast cancer have normal circulating prolactin levels (Boyns et al., 1973b; Wilson et al., 1973).

The incidence of breast cancer in females with psychiatric disorder has been reported as normal (Katz et al., 1967); we have no evidence to suggest that it is increased in those receiving phenothiazine therapy.

**ACKNOWLEDGEMENTS**

We are indebted to the sisters and ward staff from the wards of the Royal Edinburgh Hospital for their energetic help and co-operation; and to the consultant psychiatrists for their permission to carry out this study. Generous financial assistance was received from the Tenovus Organization.

**REFERENCES**


THE EFFECT OF LONG TERM PHENOThIAZINE THERAPY ON PLASMA PROLACTIN


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Prolactin in human breast tumours. By E. N. Cole, Meriel P. Golder and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX

Human primary breast tumours have been routinely homogenized and analysed for DNA, protein, and available oestradiol-17β receptor sites as part of a detailed investigation into endocrinological aspects of breast cancer. This programme has been extended to include the measurement of tissue prolactin.

With experience gained from studies of prolactin in rat tissues (Cole & Baker, 1975), 82 tumour homogenates have been analysed for immunoreactive human prolactin using the homologous radioimmunoassay of Cole & Boyns (1973). Each homogenate was centrifuged at 1000g for 20 min at 4°C and the supernatant removed. The loosely packed pellet was resuspended in 50 mM-bicarbonate buffer, pH 9.5, by vortex mixing and sonication in the presence of a glass bead (Simon, 1974). After an overnight extraction period at 4°C followed by centrifugation at 1000g for 20 min, a supernatant fraction was removed and the pellet resuspended in bicarbonate buffer. Two further supernatant fractions were obtained from 3 h washes of the pellet. Aliquots were taken from the four supernatants per tumour and were included in the prolactin radioimmunoassay at two dilutions. Prolactin concentration in plasma taken during mastectomy was determined from the appropriate standard curve.

When exogenous human prolactin was added to a pool of breast tumour homogenates, over 85% was recovered in the first supernatant fraction; there was an insignificant excess of prolactin in the overnight extract or subsequent washings. Therefore, the endogenous immunoreactive prolactin that is found in the first supernatant is likely to be loosely held in the extracellular fluid space, and not tightly bound.

Tumours were considered to contain prolactin if the overnight extract was more immunoreactive than the initial supernatant fraction and showed parallelism to authentic prolactin. In the series of 82 tissue homogenates, 42 tumours contained prolactin by these criteria. There was no correlation between prolactin and oestradiol-17β-receptor concentrations. In a series of 30 primary breast tumours for which endogenous oestradiol-17β concentrations were determined, 17 were receptor-negative (i.e., less than 5 fmol/mg protein) and 5 of these tissue extracts contained prolactin (median 0.95, range 0.50-1.91 μg/μg DNA: the prolactin standard is MRC 71/222). Of the 13 receptor-positive tumours, prolactin was present in 5, but at significantly lower concentrations (median 0.42, range 0.20-0.46 μg/μg DNA: 2P < 0.05 by Mann-Whitney U test). It remains to be determined whether prolactin content of primary tumours is a factor which relates to the course of breast cancer.

The authors thank the Tenovus Organization and the Medical Research Council for financial support. Various centres, especially Nottingham, Bristol and Bath, are gratefully acknowledged for providing breast tumours.

REFERENCES

Serum Prolactin Concentrations in Benign Breast Disease Throughout the Menstrual Cycle

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Abstract—Prolactin concentration has been estimated by homologous radioimmunoassay in morning serum samples taken daily throughout the menstrual cycle of normal women and women with benign breast disease. Whereas the median prolactin concentration in 550 sera from 24 normal cycles was 0-10 mU/ml, in 19 women with “fibroadenosis” the median of 500 values was 0-15, and was 0-27 mU/ml for 357 sera from 12 cycles of women who had had breast cysts aspirated. Abnormal daily or weekly prolactin profiles over the monthly cycle were found in benign breast disease, especially in women over 30 years old, and most marked in those with cystic disease. A significant positive correlation was present between age and prolactin in cystic disease, but not in the fibroadenosis or normal groups of women. The two benign breast diseases studied here would seem to be clinical conditions susceptible to treatment by prolactin-suppressive agents.

INTRODUCTION

It would now appear to be well accepted that prolactin has many varied actions [1] some of which may be concerned in controlling the physiological activity of mammary tissue. Thus growth, interaction with steroid hormones and electrolyte movement are all aspects of prolactin action which justify the study of this hormone in relation to the breast and in particular to breast diseases. The role of prolactin throughout the various stages of the menstrual cycle is however poorly understood, and even the monthly pattern of the hormone’s concentration in the blood has given rise to controversy [2]. Although the human breasts undergo cyclical changes in normally menstruating women, it is impossible at present to assess whether the cyclical nature of many benign breast complaints could be related to abnormal prolactin secretion. Furthermore, despite reports of normal concentrations in various benign breast diseases [3–5], these were based on single samples and do not exclude the possibility of abnormal prolactin profiles during some phases of the menstrual cycle.

The investigation now reported was undertaken to establish serum prolactin profiles over the complete menstrual cycle in two clinically different benign breast diseases in women from a wide range of age. Some patients were categorized as having “benign cystic disease of the breast” when breast discomfort or pain led to clinical examination that revealed a palpable lump containing fluid which could be aspirated. The term “fibroadenosis” was used here to denote a diffuse condition of painful, lumpy breasts [6]. These definitions were broad, and diseases of the mammary ducts, including nipple discharge, were excluded, as were solid lumps, whether benign adenomata or malignant cancer. Normal women were studied for comparison.

MATERIAL AND METHODS

Subjects and samples

The subjects were pre-menopausal women with benign disease of the breast. Eleven of them had had one or more cysts aspirated from one or both breasts, and a further 19 had painful lumpy breasts diagnosed clinically as

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having generalised fibroadenosis [6]. The existence of discrete fibroadenomata or microcysts cannot be excluded from the latter group. The range of age for women with cystic disease was 24–49 and for those with fibroadenosis, 24–49 years. These groups were sub-divided to include women in their 20s, 30s and 40s. Normal women aged 22–49 were also studied, but they are considered as one group because no age-related trends in serum prolactin were found in a detailed analysis of these 24 control cycles [7].

None of the subjects had a history of gynaecological disorders nor was taking drugs, hormone preparations or other agents known to affect prolactin, ovarian steroid, or gonadotrophin levels.

Samples of peripheral venous blood (10 ml) were obtained daily, or as often as possible, between 09.00 and 12.00 hr for at least one menstrual cycle. The blood was left to clot, centrifuged, and serum removed for storage at −20°C.

Measurement of prolactin, follicle stimulating hormone (FSH), oestradiol-17β and progesterone

Prolactin was measured by the radioimmunoassay established in these laboratories by Cole and Boyns [8]. Results are given as milliunits/ml M.R.C. Res. Std. A 71/222 where 1 mg = 50 ng prolactin by this assay system. Displacement of 5% of bound iodinated prolactin was achieved by 0.04 mg/ml. Serum samples were assayed in duplicate, all those for an individual subject being together in one of the 8 assays that were required. So far as was possible, each assay contained samples from normal, cystic and fibroadenosis groups, where age and the length of menstrual cycle were comparable for women of each group.

Measurements of FSH were limited to women over 40 yr old. The radioimmunoassay of Groom et al. [9] was used and the results served as a check on whether the women had normal cycles with a marked mid-cycle peak.

The results of oestradiol-17β and progesterone radioimmunoassays have been published for these subjects [1, 10].

Analysis of results

Prolactin concentrations were calculated from standard curves using the preferred equation of Taljedal and Wold [11]. Values of less than 0.01 were entered into subsequent calculations as 0.01 mu/ml.

Menstrual cycles varied greatly in length and so two reference points were used: the day of the mid-cycle peak of oestradiol-17β was designated Day 0, taking gonadotrophin and progesterone profiles into consideration; the day of appearance of menstrual bleeding was labelled M.

Serum prolactin concentrations approximated to a log-normal distribution and so Student's t-test was applied to mean logarithms for making planned comparisons [12]. Back transformation of mean logarithms yielded the geometric means which have been plotted for simplicity.

The experimental design supported planned comparisons between normal and each of the other groups, and also between benign disease groups within the same age range. Such comparisons would utilize all or similar portions of the prolactin profile over the menstrual cycle. For examining changes occurring within a cycle, the Student–Newman–Keuls procedure was applied as these were essentially unplanned comparisons. Significant effects were not found for any experimental group by this procedure because of inter-subject variation.

Correlation analysis was used for testing the presence of age-related trends of prolactin concentration.

RESULTS

When patients were grouped by diagnosis and age (Table 1), the expected age-distribution was apparent, with cystic disease tending to occur in later years than fibroadenosis. Cycle lengths tended to be more variable than normal for these breast disease patients although the median values were similar. However, the number of days from the mid-cycle oestradiol-17β peak to onset of menstruation was 16.4 ± 2.25 (S.D., 10 cycles) for patients with cystic disease, whereas the fibroadenosis and normal groups had shorter luteal phases (14.4 ± 2.48 and 14.9 ± 1.69 for 16 and 21 cycles respectively; 2P < 0.05 vs cystic group).

In Table 2, prolactin concentrations for each experimental group are given, using three statistics of location with their associated statistics of dispersion. The geometric mean lies closer to the median value than does the arithmetic mean in every case. Furthermore, the range which should contain 95% of the prolactin concentrations around the geometric mean corresponds quite well with the 25–97.5 percentiles of the raw data in every group, whereas the standard deviation of the arithmetic mean implies many negative values. Hence a logarithmic transformation was applied to prolactin concentrations, and back-transformation to give geometric means has been
Serum Prolactin Concentrations in Benign Breast Disease

Table 1. Allocation of subjects to groups, their age and the duration of their menstrual cycle

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Group</th>
<th>Age (yr)</th>
<th>Cycle length (days)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>—</td>
<td>35</td>
<td>22-49</td>
<td>24</td>
</tr>
<tr>
<td>Cystic disease</td>
<td>—</td>
<td>43</td>
<td>34-46</td>
<td>12</td>
</tr>
<tr>
<td>Fibroadenosis</td>
<td>—</td>
<td>32</td>
<td>24-49</td>
<td>19</td>
</tr>
<tr>
<td>Cystic disease</td>
<td>30s</td>
<td>35</td>
<td>34-37</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>40s</td>
<td>45</td>
<td>40-48</td>
<td>27</td>
</tr>
<tr>
<td>Fibroadenosis</td>
<td>20s</td>
<td>27</td>
<td>24-29</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>30s</td>
<td>33</td>
<td>31-38</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>40s</td>
<td>46</td>
<td>40-49</td>
<td>24</td>
</tr>
</tbody>
</table>

Menstrual cycles have been grouped by diagnosis and age. Note that 2 cycles were studied in one 22 yr old normal subject and also in a 33 yr old who had cystic disease.

Table 2. Comparison of serum prolactin concentrations between groups of subjects using statistics of location and dispersion that are derived from raw data (median with 2.5-97.5 percentiles) and by arithmetic (mean±S.D.) or logarithmic (geometric mean±1.96 S.D. range) computation

<table>
<thead>
<tr>
<th>Diagnosis and age group</th>
<th>Number of sera</th>
<th>Median</th>
<th>2.5–97.5 percentiles</th>
<th>Arithmetic</th>
<th>Geometric Mean</th>
<th>± 1.96 S.D. range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>—</td>
<td>550</td>
<td>0.10</td>
<td>0.01–0.38</td>
<td>0.13</td>
<td>0.091</td>
</tr>
<tr>
<td>Cystic disease</td>
<td>—</td>
<td>337</td>
<td>0.27</td>
<td>0.05–1.16</td>
<td>0.34</td>
<td>0.273</td>
</tr>
<tr>
<td>Fibroadenosis</td>
<td>—</td>
<td>500</td>
<td>0.15</td>
<td>0.02–0.88</td>
<td>0.22</td>
<td>0.212</td>
</tr>
<tr>
<td>Cystic disease</td>
<td>30s</td>
<td>125</td>
<td>0.17</td>
<td>0.04–1.02</td>
<td>0.24</td>
<td>0.243</td>
</tr>
<tr>
<td></td>
<td>40s</td>
<td>212</td>
<td>0.34</td>
<td>0.07–1.38</td>
<td>0.40</td>
<td>0.273</td>
</tr>
<tr>
<td>Fibroadenosis</td>
<td>20s</td>
<td>165</td>
<td>0.13</td>
<td>0.01–0.67</td>
<td>0.17</td>
<td>0.166</td>
</tr>
<tr>
<td></td>
<td>30s</td>
<td>217</td>
<td>0.15</td>
<td>0.04–0.91</td>
<td>0.26</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td>40s</td>
<td>118</td>
<td>0.17</td>
<td>0.04–0.91</td>
<td>0.23</td>
<td>0.205</td>
</tr>
</tbody>
</table>

Serum prolactin concentrations in µ/ml MRC Res. Std. A 71/222 are given for menstrual cycles from subjects grouped as in Table 1. The median and 2.5–97.5 percentiles were found by inspection; arithmetic mean and standard deviation (S.D.) were calculated in the usual way; geometric mean ± 1.96 S.D. range are back transformations of the logarithmic mean ± 1.96 S.D. where 1.96 = t (approximately) at the 5% probability level for the appropriate degrees of freedom.

Table 3. Significance levels by Student’s t-test for planned comparisons of logarithmically transformed prolactin concentrations sampled over complete menstrual cycles of subjects as in Tables 1 and 2

<table>
<thead>
<tr>
<th>Diagnosis and age group</th>
<th>Cystic disease</th>
<th>Fibroadenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30s</td>
<td>40s</td>
</tr>
<tr>
<td>Normal</td>
<td>20–50</td>
<td>***</td>
</tr>
<tr>
<td>Cystic disease</td>
<td>30s</td>
<td>—</td>
</tr>
<tr>
<td>Fibroadenosis</td>
<td>30s</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fibroadenosis</td>
<td>40s</td>
<td>—</td>
</tr>
</tbody>
</table>

Two-tailed probabilities: ***<0.0005; **<0.005; *<0.02; N.S. >0.16; — not tested.
Fig. 1. Relationship between serum prolactin concentration and age in patients with cystic breast disease. The equal frequency ellipse is calculated to enclose 95% of observations of age and the geometric mean prolactin level over the menstrual cycle for this group of women.

Fig. 2. Serum prolactin profiles of normal women, and patients with fibroadenosis or benign cystic disease of the breast. Geometric means are shown for days of the cycle aligned by reference to onset of menstrual bleeding (day M) and mid-cycle peak of oestradiol-17β (day 0).

adopted for clarity of presentation. Mean logarithms were used for significance testing by Student's t-test. As detailed in Table 3, though apparent from Table 2, prolactin concentrations were higher than normal in benign breast disease, especially in women who had had cysts aspirated, and predominantly so in the 40s age group. This group of older cystic patients had higher prolactin levels than either the younger group or the comparable age of fibroadenosis patients. An age-related trend was established for all the cystic patients (Fig. 1) by correlation analysis, which showed that geometric mean prolactin concentration over the complete menstrual cycle was positively correlated with age (Spearman rank correlation coefficient = 0.57, linear correlation coefficient r = 0.749, 10 degrees of freedom). No such correlations were significant for the fibroadenosis group, whether as a whole or as subgroups, nor for the normal subjects.

The daily patterns of geometric mean prolactin concentration are shown in Fig. 2. A striking feature of the benign breast cases is the
wild fluctuation of serum prolactin level occurring over a few days. The small mid-cycle peak of the normal profile appears pronounced in the cystic breast disease groups and in all but the youngest fibroadenosis group. An abnormal follicular phase peak was similarly present. In the luteal phase, the gradual increase from about day +4 after the mid-cycle oestradiol-17beta peak has become a sharp rise of prolactin concentration to a peak at about day +8. This is most clear for the group of women in their 30s in the cystic disease group because all five of them had similar luteal phases which differed by only 2 days.

Weekly patterns for serum prolactin are shown in Fig. 3. Day to day variations and errors due to misalignment of cycle days have been reduced by calculating mean levels over defined 5-day intervals in the menstrual, follicular, periovulatory and luteal phases. A clear pattern is found for normal subjects for whom luteal prolactin concentrations tend to be higher than in the follicular phase, and highest levels occur in the periovulatory phase (Fig. 3). In the benign breast disease patients, however, luteal phase prolactin concentrations were greater than in the periovulatory period, and follicular phase levels were higher than the normal pattern would predict in all but the youngest fibroadenosis group.

Significance levels are given in Table 4 for the planned comparisons that the data of Fig. 3 support. Prolactin concentrations of women with cystic disease were greater than in the normal women in the follicular and luteal phases, and for the remaining weeks in the older group. The cystic 30s group was significantly different from the cystic 40s, except in

Table 4. Significance levels for planned comparisons of prolactin concentrations sampled over 5-day intervals as in Fig. 3

<table>
<thead>
<tr>
<th>Diagnosis and age group</th>
<th>Phase</th>
<th>Cystic disease 30s</th>
<th>Fibroadenosis 20s</th>
<th>Cystic disease 40s</th>
<th>Fibroadenosis 30s</th>
<th>Fibroadenosis 40s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 20-50</td>
<td>M</td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>**</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cystic 30s</td>
<td>F</td>
<td>**</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>**</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fibroadenosis 30s</td>
<td>M</td>
<td>*</td>
<td>N.S.</td>
<td>*</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>**</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>N.S.</td>
<td>N.S.</td>
<td>*</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fibroadenosis 40s</td>
<td>M</td>
<td>**</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>**</td>
<td>N.S.</td>
<td>**</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>*</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Phases: M, menstrual, days M−2 to M+2; F, Follicular, days −8 to −4; O, periovulatory, days −1 to +3; L, luteal, days +6 to +10. Two-tailed probabilities: *** < 0.0005; ** < 0.005; * < 0.05; N.S. > 0.05; — not tested.
the luteal phase, but no significant concentration differences were found when compared to the 30s group of fibroadenosis patients. Yet the older cystic group differed from the same age of fibroadenosis patients at all stages of the cycle. Both the 30s and the 40s group of fibroadenosis patients had significantly raised prolactin concentrations throughout the menstrual cycle, but the youngest group had prolactin levels which were above normal when considered overall and yet non-significantly so in each phase. Table 4 and Fig. 3 suggest that the 30s and 40s groups of fibroadenosis patients have similar prolactin profiles. This contrasts with the gross differences of prolactin concentrations related to age in the women with cystic disease.

**DISCUSSION**

Earlier investigations from these laboratories [3] indicated plasma prolactin concentrations were similar in benign breast disease, breast cancer at various stages and in control patients admitted to hospital for non-breast conditions. At least 2 other studies [4, 5] have also reported that prolactin levels in benign breast disease were within the normal range. These studies were based however upon single samples taken at random times in the menstrual cycle, which in association with rather imprecise terms like "benign breast disease" or "(poly)cystic mastitis", lessens the apparent discrepancy of previous results with the data now described. Furthermore, only one serum in every four had a prolactin concentration beyond the range found in normal menstrual cycles, even in the cystic 40s group (56/212 sera with more than 0.47 mu/ml). This illustrates the advantage of repeated sampling throughout the menstrual cycle.

The two benign breast diseases which were chosen for study were diagnosed by routine criteria, obtaining histological confirmation if practical. If a patient had a gross cyst from which fluid could be aspirated, then she was placed in the cystic disease group. If breasts had painful lumps, then such a condition was broadly termed fibroadenosis on exclusion of cancer, fibroadenoma, duct ectasia, inflammatory reactions, nipple discharge and many other distinctly defined breast complaints. It is obvious that the fibroadenosis group was much more heterogeneous than the cystic disease group and could obviously include microcysts and sub-clinical forms of other breast conditions.

Both cystic disease and fibroadenosis tend to become more severe in the few days before menstruation, and the symptoms disappear after the menopause [13] thus relating benign breast disease to ovarian function. As illustrated in Fig. 2, serum prolactin does not appear to follow oestrogens in a well defined monthly cycle [5, 7, 14-17], although it would seem that oestrogens play an important role in controlling secretion of human prolactin, and Vekemans and Robyn [17] have found a significant decline of prolactin concentration at the menopause in normal women blood donors. In a case of premenstrual syndrome associated with thirst and water retention, prolactin-suppressive therapy with bromocriptine has successfully relieved all symptoms for over a year [18]. Therefore, a cyclical variation of clinical symptoms may be linked to changes in serum prolactin concentration.

In the cystic disease group, there was a clear correlation between prolactin and age that was not present in the other groups. This correlation was in the opposite direction to that found by Vekemans and Robyn in their 86 normal women over their wider age range of 18-65 yr. Although cystic disease and raised prolactin may be associated, this does not prove that one causes the other nor does it exclude a common causative factor. However, the well established role of prolactin in water and electrolyte balance [1] has counterparts in humans as shown by the lowering of serum prolactin by water loading [19] or the relief of water retention by suppression of prolactin secretion [18]. Furthermore, a correlation has been demonstrated between the mean plasma prolactin concentration of male subjects and urinary Na/K excretion [20, 21]. The sign of this correlation became negative when diuretics were administered, which suggested that the diuretic response of an individual was a function of his prolactin set-point. As a working hypothesis then, serum prolactin concentration could control the movement of water and electrolytes into microcysts. A gross cyst would be formed as a consequence of localized fluid influx, and a more generalized swelling of microcysts may account for the cyclical pain in fibroadenosis. Normal breasts, which may also have microcysts [13], are in a prolactin environment that does not favour cyst enlargement.

The incidence of breast cancer in patients with gross cystic disease has been found to be 4 times higher than expected [13], but the raised prolactin levels in cystic disease cannot be given a role in carcinogenesis on the present
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evidence since extrapolation is not possible from the 11 women of this study to the 1693 cases of cystic disease in the other study which included 72 that developed cancer [13]. Indeed, patients that have had mastectomy for breast cancer were found to have only minor abnormalities in their serum prolactin profiles throughout the menstrual cycle [7].

If the generally raised serum prolactin concentrations of these two benign breast conditions is a disease factor, then prolactin suppressive therapy would be effective. Such treatment is presently undergoing clinical trials which will be reported at a later date.

Acknowledgements—We thank the Tenovus Organisation, the Medical Research Council and the Cancer Research Campaign for generous financial support.

REFERENCES

Serum Prolactin Concentrations Throughout the Menstrual Cycle of Normal Women and Patients with Recent Breast Cancer*

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Abstract—Prolactin concentration has been estimated by radioimmunoassay of serum samples taken daily throughout the menstrual cycle of 11 patients who had undergone mastectomy for primary breast cancer and 32 normal women. Although there were no marked cyclical changes in prolactin level, concentrations were lowest in the follicular phase. Hence, comparison of normal and cancer subjects required detailed statistical analysis of results from comparable stages of the monthly cycle. Mid-cycle peaks of oestradiol and follicle stimulating hormone and the onset of menstrual bleeding were used as reference points. Prolactin concentrations were very similar in samples from normal and cancer groups, although at certain stages of the cycle some significant differences were found: on the fifth day preceding the mid-cycle oestradiol peak; during the follicular and periovulatory phases; and among the highest mid-cycle levels. At these stages of the cycle, samples from the breast cancer patients had a greater prolactin concentration than normal controls, although levels were within the normal range. The physiological relevance of these higher prolactin concentrations is uncertain and, in general, this detailed study clearly indicates little difference between serum levels of prolactin in normal women and in patients with breast cancer whose primary tumour had been removed more than three months previously.

INTRODUCTION

Although the mammotrophic-lactogenic and luteolytic-luteotrophic effects of prolactin are well established in experimental animals [1, 2], the physiological role of prolactin during the human menstrual cycle is not so well understood. Apart from generally higher plasma levels in the luteal phase, and a possible increase near mid cycle, no consistent patterns of prolactin concentration have been found from daily blood sampling [3-8]. Indeed, such studies have emphasized the marked variations in prolactin concentration which occur not only between individuals but also from day to day within subjects. Furthermore, because cycles of differing lengths are usually aligned for periovulatory events, prolactin concentrations at the time of menstruation have been given much less attention [9].

The effects of prolactin on experimental mammary tumours are well documented, and their relevance to human breast cancer has been reviewed recently [10]. Whereas basal plasma prolactin concentrations were correlated with differing susceptibility to chemical induction of mammary tumours in 3 strains of female rats [11], a finding which also appears true for male rats [12], our studies of human breast cancer patients have shown similar basal prolactin levels at various stages of the disease and in control subjects [13, 14]. These and other studies [15-17] indicate that if there are abnormal prolactin concentrations in breast cancer patients then the differences will be small. However, elevated prolactin concentrations in breast cancer patients have been found...
using a heterologous radioimmunoassay [18] and also using a bioassay [19].

In this detailed study, basal prolactin concentrations throughout the menstrual cycle have been compared in normal women and in breast cancer patients, to examine the possibility that there may be differences at some stages of the cycle only. As it would be unethical to delay mastectomy for more than a month while collecting the daily blood samples, patients were studied at least three months after removal of the primary tumour, when they had resumed regular cycles. Such patients may still be considered to have breast cancer, though occult, since breast carcinoma is regarded by many as a systemic disease [20, 21].

**MATERIAL AND METHODS**

**Subjects and samples**

Samples of peripheral venous blood (10 ml) were obtained daily, or as often as possible, between 09.00 and 12.00 hr for at least one menstrual cycle. The blood was allowed to clot, centrifuged and serum removed to be stored at −20°C. The subjects were 32 normal women, and 11 women who had undergone mastectomy for primary carcinoma of the breast but had since resumed regular menstrual cycles. None of the subjects had a history of gynaecological disorders nor was taking drugs, hormone preparations or other agents known to affect prolactin, ovarian steroid or gonadotrophin levels.

Of the normal women, 11 were selected to be matched controls for the group of cancer patients on the basis of cycle length, age and completeness of sampling. The normal women, including matched controls, were placed into 3 subgroups by age for data analysis.

**Measurement of prolactin, follicle stimulating hormone (FSH), oestriol-17β and progesterone**

Prolactin was measured by the radioimmunoassay established in these laboratories by Cole and Boyns [22]. Results are given as milliunits/mI M.R.C. Res. St. A 71/222 where 1 mu = 50 ng prolactin by this assay system. Serum samples were assayed in duplicate, all those for an individual subject being together in one of the 8 assays that were required. Each cancer patient was paired with her matched control and their samples also included within the same assays. Displacement of 5% of bound iodinated prolactin was achieved by 0.04 mu/ml prolactin.

Measurements of FSH were limited to women over 40 years old. The radioimmunoassay of Groom et al. [23] was used and the results served to check whether subjects had normal cycles with a marked mid-cycle peak.

Oestradiol-17β and progesterone were determined by radioimmunoassays and the results for these subjects have been reported [24, 25].

**Analysis of results**

Prolactin concentrations were calculated from radioimmunoassay standard curves by the preferred equation of Taljedal and Wold [26]. Values of less than 0.01 were entered into subsequent calculations as 0.01 mu/ml.

Menstrual cycles varied greatly in length and so two preference points were used: the day of the mid-cycle peak of oestradiol-17β was designated Day 0, taking gonadotrophin and progesterone profiles into consideration; the day of appearance of menstrual bleeding was labelled M.

Prolactin concentrations were first examined to assess whether they met the assumptions inherent in parametric tests of statistical significance [27]. A logarithmic transformation was found necessary. Back-transformation yielded geometric means, but the logarithmic values were tested for significant differences.

As Student's t-test should be reserved for planned comparisons [27], for which the experimental design allowed relatively few, the Student–Newman–Keuls (SNK) procedure was applied to transformed daily prolactin values from the cancer, matched control, and normal 40s groups. The range of values in the cancer group was not wide enough to hold significant differences, but prolactin levels on days −5 and +1 in the other two groups lay beyond the maximum non-significant ranges at the 5% level. Planned comparisons were therefore made in the remaining groups using data from days −5 and +1.

The experimental design could support the use of Student's t-test for the following planned comparisons:

(a) test for homogeneity of normal subgroups and whether the cancer group is different.

(b) test highest periovulatory prolactin levels in cancer vs matched control groups.

Additionally, planned comparisons were made using samples taken during defined 5-day intervals which were evenly spaced over 4 consecutive weeks: days −1, 0, +1, 2, 3 (periovulatory phase); days −8 to −4 (fol-
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RESULTS

Mid-cycle peaks of oestradiol-17β or FSH could be located in 25 normal cycles and in all the 11 cycles from the patients with breast cancer. Two of the normal subjects (aged 53 and 46) were menopausal, having generally elevated FSH levels; critical samples were unavailable for a further 6 subjects and these were therefore omitted from detailed data analysis. One other cycle was rejected because of low FSH levels in an abnormally long luteal phase.

The remaining 24 normal cycles were placed in 3 age groups (Table 1). Distribution of ages within each group was essentially random, and cycle lengths were variable with no age-related trend. Table 1 also shows median value with range of both age and cycle length for the breast cancer group, matched control group and the combined normal group. The 30-year-old in the matched control group was chosen for her 22 day cycle to pair with the 23 day cycle of a 45-year-old cancer patient.

Prolactin concentrations did not follow a Gaussian distribution but approximated to a log-normal distribution. This is evident from Table 2 where for each group the median value from raw data is compared with the calculated arithmetic and geometric means. In a normal distribution, median and mean values coincide: in Table 2, the geometric mean is the closer to the median. Furthermore,

Table 1. Allocation of subjects to groups, their age and the duration of their menstrual cycle

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Cycle length (days)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Normal 20s</td>
<td>25</td>
<td>22-29</td>
<td>30</td>
</tr>
<tr>
<td>Normal 30s</td>
<td>34</td>
<td>30-39</td>
<td>26</td>
</tr>
<tr>
<td>Normal 40s</td>
<td>44</td>
<td>40-49</td>
<td>27</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>44</td>
<td>38-48</td>
<td>27</td>
</tr>
<tr>
<td>Matched control</td>
<td>41</td>
<td>30-49</td>
<td>27</td>
</tr>
<tr>
<td>Combined normal</td>
<td>35</td>
<td>22-49</td>
<td>28</td>
</tr>
</tbody>
</table>

Menstrual cycles of 23 normal women have been grouped by age and additionally by matching to the 11 cancer patients on the basis of cycle length and age. Note that 2 cycles were studied from one 22 year old subject.

Table 2. Comparison of serum prolactin concentrations between groups of subjects using statistics of location and dispersion that are derived from raw data (median with 2.5-97.5 percentiles) and by arithmetic (mean, S.D.) or logarithmic (geometric mean ± 1.96 S.D. range) computation

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of samples</th>
<th>Median value</th>
<th>2.5-97.5 percentiles</th>
<th>Arithmetic mean</th>
<th>Geometric mean</th>
<th>± 1.96 S.D. range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 20s</td>
<td>187</td>
<td>0.10</td>
<td>0.01-0.45</td>
<td>0.15</td>
<td>0.133</td>
<td>0.085</td>
</tr>
<tr>
<td>Normal 30s</td>
<td>147</td>
<td>0.09</td>
<td>0.01-0.33</td>
<td>0.12</td>
<td>0.090</td>
<td>0.083</td>
</tr>
<tr>
<td>Normal 40s</td>
<td>226</td>
<td>0.10</td>
<td>0.03-0.28</td>
<td>0.12</td>
<td>0.101</td>
<td>0.074</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>292</td>
<td>0.11</td>
<td>0.02-0.44</td>
<td>0.15</td>
<td>0.112</td>
<td>0.114</td>
</tr>
<tr>
<td>Matched control</td>
<td>262</td>
<td>0.10</td>
<td>0.01-0.29</td>
<td>0.12</td>
<td>0.069</td>
<td>0.097</td>
</tr>
<tr>
<td>Combined normal</td>
<td>550</td>
<td>0.10</td>
<td>0.01-0.38</td>
<td>0.13</td>
<td>0.111</td>
<td>0.091</td>
</tr>
</tbody>
</table>

Serum prolactin concentrations in μg/ml MRC Res. Std. A 71/222 are given for menstrual cycles from subjects grouped as in Table 1. The median and 2.5-97.5 percentiles were found by inspection; arithmetic mean and standard deviation (S.D.) were calculated in the usual way; geometric mean ± 1.96 S.D. range are back transformations of the logarithmic mean ± 1.96 S.D., where 1.96 = t (approximately) at the 5% probability level for the appropriate degrees of freedom.
95% of raw data lie within the 2.5–97.5 percentiles by definition, and within ±1.96 S.D. of the mean of a normal distribution. These limits can be compared in Table 2 for each group of women, and it is obvious that a logarithmic transformation of prolactin values is reasonable.

Prolactin concentrations were similar in all groups of normal subjects and in the group of cancer patients (Table 2). The range of geometric mean prolactin for those individuals who were rejected from the normal group was 0–02–0·19, and this is similar to the ranges for normal subjects in their 20s, 30s and 40s or the range for breast cancer patients: 0·02–0·25; 0·02–0·21; 0·06–0·14; and 0·05–0·23 μu/ml respectively. There was no evidence for any trend in serum prolactin concentration with age (Fig. 1), and it can be seen that the geometric means for the cancer patients are within the range for the normal women.

Daily prolactin concentrations for the follicular, periovulatory and luteal phases and the period of menstruation showed fluctuations of the geometric mean which were small compared with the range of the values for each day. Figure 2 illustrates this for the combined normal group. Nevertheless, prolactin levels were generally higher in the luteal phase than in the follicular phase, and an elevation at midcycle was present (2P < 0·025 for day +1 vs −5 relative to the oestradiol-17β peak). A decline in serum prolactin occurred during the 2 days before onset of menstrual bleeding in 21 women out of 27 who were sampled on these days (significant at 1% level by a sign test).

When daily prolactin concentrations in breast cancer patients and matched controls were compared, a significant difference was found on day −5 in the follicular phase (2P < 0·01) but not for day +1. A mid-cycle elevation of serum prolactin in the cancer group could not be shown (2P > 0·10) whereas the significance level was 0·001 for day −5 vs +1 in the matched control group (Fig. 3). However, by using a paired comparison of the mean values shown in Fig. 3, Wilcoxon’s matched-pairs signed-rank test indicated an overall difference between the 2 groups at the 2% probability level. This statistical method takes no account of the considerable range of prolactin concentrations associated with each mean (Fig. 2).

As the differences in mean prolactin levels within 5-day intervals were generally non-
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Fig. 3. Comparison of daily prolactin concentrations in breast cancer patients and matched controls. Values are geometric means for 11 cancer patients (●) and 11 controls (○).

Fig. 4. Weekly patterns of serum prolactin in normal subjects, grouped according to age or selected as matched controls, and in breast cancer patients. Geometric mean with 95% confidence limits are shown for prolactin concentrations over 5-day intervals in the weeks of menstruation (M) and ovulation (O) or within the follicular (F) and luteal (L) phases.

significant, pooled values were calculated to represent the pattern of prolactin concentration week by week (Fig. 4). In each group, serum prolactin was lowest in the follicular phase and was significantly higher in the periovulatory phase \( (2P < 0.05, 20s; < 0.05, 30s; < 0.05, 40s; < 0.01, \text{combined normal age groups}; < 0.01, \text{cancer group}; < 0.01, \text{matched controls}). In all the groups of normal women, prolactin was higher in the week of menstruation than in the following week, significant at \( 2P < 0.01 \) for the combined group.

The weekly pattern of prolactin in serum from the cancer patients differs from normal (Fig. 4). Prolactin remained at the level of the menstrual phase during the follicular phase and was higher for the next 2 weeks. Consequently, there was a difference \( (2P < 0.05) \) in the follicular phase serum prolactin concentration between 55 samples from 11 breast cancer patients and either 100 samples from 23 normal women or 49 from the matched controls. Similarly, these cancer patients have higher prolactin concentrations than normal in the periovulatory phase. In addition, by taking the highest values in the periovulatory phase, the geometric mean prolactin for the cancer patients \( (0.29) \) is greater than for the matched controls \( (0.20; 2P < 0.025) \).

DISCUSSION

Using samples taken throughout the menstrual cycle, only small differences could be found in serum prolactin concentrations between normal women and a group of patients that had undergone mastectomy for primary breast cancer. Indeed, to show any significant differences in prolactin levels, both the design
of the radioimmunoassays and the statistical treatment of results required very careful consideration. The physiological relevance of the differences is obviously uncertain.

The a priori selection of control subjects was intended to minimize possible sources of error due to interassay variation, age-related trends and bias arising from, for example, a prolonged follicular phase which would tend to lower an individual’s mean prolactin level. In practice, however, the major variance components arose from within and between subjects. Hence, geometric mean prolactin values over the four 5-day intervals were within 2% of those given in Table 2 for which sets of results over the whole cycle were taken into consideration.

Several statistical treatments of the data were possible. Thus, prolactin concentrations could be normalized to reduce inter-subject variation and then tested for consistent cyclical changes. Alternatively, areas under the prolactin-time curve could be taken for the comparison of cancer and normal groups. Instead, the familiar Student’s t-test has been applied to data which have undergone transformation in order that the assumptions of parametric significance testing were better met.

Prolactin concentrations approximated quite closely to a log-normal distribution, and a slight skewness may be accounted for by less than 0.01 mu/ml prolactin in the “prolactin-free” plasma that was used in constructing the radioimmunoassay standard curve. This skewness is a likely cause of the over-estimation of standard deviation terms which is indicated by the ±1.96 S.D. limits being wider than the 2.5-97.5 percentiles (Table 2). The significance levels for differences between means are likely to be conservative estimates in consequence.

Daily serum prolactin concentrations were variable and there were no consistent patterns from one person to another during the menstrual cycle. This is in agreement with similar studies from other centres [3–7]. However, by pooling the results from the defined 5-day periods, a clear pattern of serum prolactin emerged. This pattern throughout the menstrual cycle was consistent insofar as follicular phase prolactin was lower than in the periovulatory or menstrual phases for the combined group of 24 normal cycles and in the subgroups of 8, 7 or 9 cycles by age, or the matched control cycles. However, the pattern for the breast cancer group differed because prolactin in the follicular phase remained at the level of the preceding week before increasing in the periovulatory and luteal phases (Fig. 4).

Comparison of patterns of serum prolactin week-by-week has advantages over the simple comparison of mean concentrations from one group of subjects with another. In addition to smoothing out daily fluctuations, assay and alignment errors should be minimal, and the errors due to the selection of patients and matched controls reduced. Thus, neither worry related to the mastectomy nor residual post-operative stress are likely to affect the pattern of prolactin levels over the month, whereas it could be argued that a single sample might well be affected.

The breast cancer patients for this study had undergone mastectomy for primary breast cancer and had since resumed normal cyclical activity. It is, of course, virtually impossible to obtain daily blood samples for a month from a woman with known or suspected breast cancer; it would be unethical to withhold treatment for this time, and hormone concentrations would be of doubtful value in a woman giving informed consent under such distressing circumstances.

The abnormal prolactin pattern in our patients may indicate some relationship between prolactin and carcinogenesis. The occurrence of significantly greater prolactin concentrations in the cancer patients during the periovulatory phase and the possible prolonged elevation at this stage (Fig. 3) certainly provide further evidence that the endocrinology of these women still differs if only minimally from the 23 normal subjects. Just as the disease persists in occult metastatic foci even after mastectomy [20, 21], so there may remain an abnormal prolactin balance in the breast cancer patients. However loss of breast tissue may itself affect prolactin secretion or utilization, although such a feedback mechanism from prolactin target tissue remains to be clarified. Mastectomy could result in neural stimulation of prolactin release, which might explain the generally elevated levels, but is unlikely to account for the observed difference in the weekly pattern.

Although differences in serum prolactin concentration were found, their interpretation requires care as it is probably unwise to generalize from a sample of only 11 premenopausal breast cancer patients. The differences between cancer and control levels are very small and their relationship to the disease is still uncertain.
REFERENCES


II. INVESTIGATIONS RELATING TO CANCER OF THE BREAST.

E. TAMOXIFEN: MODE OF ACTION.
Influence of Anti-Oestrogens on the Specific Binding in vitro of $[^3H]$Oestradiol by Cytosol of Rat Mammary Tumours and Human Breast Carcinomata

By WENDY POWELL-JONES,* DIANE A. JENNER,* ROGER W. BLAMEY,† PETER DAVIES* and KEITH GRIFFITHS*

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The anti-oestrogenic potential of two nitrogen-mustard-containing compounds, I.C.I. 79792 and I.C.I. 85966, was studied. I.C.I. 85966 usually did not decrease specific binding of $[^3H]$oestradiol by breast-tumour cytoplasmic proteins. I.C.I. 79792 decreased specific $[^3H]$oestradiol binding, but not to the same extent as similar concentrations of I.C.I. 46474, diethylstilboestrol or dibutyldihydrostilboestrol.

Certain compounds are able to decrease the specific uptake of oestradiol in vivo and in vitro by various target tissues of the rat (Terenius & Ljunckvist, 1972; Jensen et al., 1972; Geynet et al., 1972; Skidmore et al., 1972) and man (Jensen et al., 1972; Hahnel et al., 1973; Lunan & Green, 1974). These substances, some steroids and synthetic anti-oestrogens, are able to inhibit the selective transfer of oestrogens by specific receptor macromolecules to the chromatin of target tissues (Jensen et al., 1971; Jensen & DeSombre, 1973, 1972), and have been used in distinguishing between the interactions of oestradiol with specific and non-specific binding proteins (Jensen et al., 1972). Such anti-oestrogens have an important therapeutic role in the treatment of women with advanced carcinoma of the breast (Terenius, 1971). This present communication evaluates the anti-oestrogenic potential of certain substances, in particular two compounds containing nitrogen-mustard groupings, by their comparative ability to inhibit specific binding of $[^3H]$oestradiol by cytoplasmic proteins of human breast tumours and rat mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene.

Materials and Methods

Animals and tissue. Mammary tumours were induced in virgin female Sprague–Dawley rats by intubation of 7,12-dimethylbenz[a]anthracene (20 mg in 1 ml of sesame oil). Tumours reached a suitable size for experimentation (approx. 2 cm × 2 cm) 7–12 weeks after intubation. Rats bearing such tumours were killed by cervical dislocation and tumours were removed, placed in vessels surrounded with crushed ice, and used immediately.

Samples of human primary breast-tumour tissue were provided by the Department of Surgery, University of Nottingham, where they were stored at −20°C until collection. Frozen tissues were transported to the Tenovus Institute where they were retained at −20°C until used for analysis.

Chemicals. $[2,4,6,7-^3H]$Oestradiol (specific radioactivity 85 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive oestradiol, diethylstilboestrol $[3,4$-bis-$p$-(hydroxyphenyl)hex-3-ene] and dithiothreitol were provided by Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. meso-Dibutyldihydrostilboestrol [5,6-bis-$p$-(hydroxyphenyl)decane], I.C.I. 46474 [the $trans$ isomer of 1-$[p$-(β-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1-ene], I.C.I. 79792 (1-$[4$-$[p$-(2-chloroethyl)aminoethoxy]phenyl]-trans-diphenylbut-1-ene hydrochloride) and I.C.I. 85966 (3,4-bis-$p$-[N-(bis-2-chloroethyl)carbamoyl]phenyl)hex-3-ene) were gifts from Dr. A. L. Walpole and Dr. D. N. Richardson of I.C.I. Pharmaceuticals, Alderley Park, Cheshire, U.K. 7,12-Dimethylbenz[a]anthracene was supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other compounds were of AnalaR grade and were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

Preparation of soluble supernatants. N₂-frozen human breast-tumour tissue was pulverized in a spring-loaded plunger gun and mortar previously cooled in liquid N₂. The pulverized tissue was quickly transferred to a Potter–Elvehjem homogenizer and homogenized in 10 mm-Tris–HCl buffer, pH 7.4, containing 1 mm-EDTA and 0.25 mm-dithiothreitol (medium A: 5 ml/2 g of original tissue) at 4°C.

Rat mammary tumours were dissected free from adhering connective tissue and obvious necrotic material, and washed in medium A. Tissue was then minced with scissors and homogenized in medium A (5 ml/2 g of tissue) in a Potter–Elvehjem ground-glass homogenizer with a Teflon pestle driven by a motor (TriR Instruments, Jamaica, N.Y., U.S.A.) operating at 3000–3500 rev./min at 4°C.
Fig. 1. Effects of various substances on the specific binding of $[^{3}H]$oestradiol in cytosol of rat 7,12-dimethylbenz[a]anthracene-induced mammary tumours

Samples of cytosol prepared from rat mammary tumours were labelled at 0°C with $[^{3}H]$oestradiol (0.5 nm) in the absence or presence of various other compounds, and analysed by sucrose-density-gradient centrifugation (from left to right). The sedimentation marker (arrows) was bovine serum albumin ($s_{20, w} = 4.6 S$) in each case. (a) Cytosol labelled with $[^{3}H]$oestradiol alone (○) or in the presence of 50 nm-oestradiol (●) or I.C.I. 85966 (■); (b) cytosol labelled with $[^{3}H]$oestradiol alone (○) [curve from (a) for comparison] or in the presence of 50 nm-diethylstilboestrol (●) or dibutyldihydrostilboestrol (■); (c) cytosol labelled with $[^{3}H]$oestradiol alone (○) [curve from (a) as comparison] or in the presence of 50 nm-I.C.I. 46474 (●) or -I.C.I. 79792 (■); (d) cytosol labelled with $[^{3}H]$oestradiol alone (○) or in the presence of 50 nm-I.C.I. 79792 (●) or -I.C.I. 85966 (■).
Fig. 2. Effects of various substances on the specific binding of $[^3H]$oestradiol in cytosol of rat 7,12-dimethylbenz[a]anthracene-induced mammary tumours and human primary breast carcinomata

Samples of cytosol prepared from rat mammary tumours and breast carcinomata were labelled at 0°C with $[^3H]$oestradiol (0.5 nM) in the absence or presence of various other compounds. Free and non-specifically bound steroid were removed by treatment with dextran-coated charcoal and portions (400 µl) were layered on sucrose density gradients and centrifuged for 18 h at 100000g, at 3-4°C. Direction of centrifugation was from left to right. Sedimentation marker (arrows) was bovine serum albumin ($s_{20,w}$ 4.6S) in each case. (a) Rat mammary-tumour cytosol labelled with $[^3H]$oestradiol alone or in the presence of 5 nm-dibutylidihydrostilboestrol (O) or in the presence of 5 nm-diethylstilboestrol (O); (b) cytosol as in (a) labelled with $[^3H]$oestradiol alone or in the presence of 5 nm-I.C.I. 79792 (O), or in the presence of 5 nm-I.C.I. 46474 (O); (c) human breast-tumour cytosol labelled with $[^3H]$oestradiol alone (O) or in the presence of 50 nm-diethylstilboestrol (O), dibutylidihydrostilboestrol (O) or -I.C.I. 46474 (O); (d) human breast-tumour cytosol labelled with $[^3H]$oestradiol alone (O) or in the presence of 5 nm-dibutylidihydrostilboestrol (O), I.C.I. 46474 (O) or -dibutylidihydrostilboestrol or -I.C.I. 79792 (O).
Soluble supernatants (cytosol) were prepared by centrifugation of crude homogenates at 100,000g for 1 h at 3°C in the SW50.1 (6 x 5 ml) swinging-bucket rotor (rsv, 8.35 cm) in a Beckman L2-65B preparative ultracentrifuge.

**Labelling of cytosol receptor proteins with [3H]-oestradiol.** Portions of cytosol (0.5–1.0 ml) were incubated for 1 h at 0°C with [3H]-oestradiol at a final concentration of 500 pM in the absence and presence of an excess of various anti-oestrogens (as detailed in the text). In some experiments, free and non-specifically bound steroid were removed from the cytosol by treatment with dextran-coated charcoal (0.5% Norit-A, 0.05% dextran T-70, sedimented from 1 ml of medium A).

Linear 5 ml (5–20%, w/v) sucrose density gradients in a uniform concentration of medium A were prepared as described by Martin & Ames (1961). Samples of [3H]-labelled cytosol (400 µl) were layered over the gradients and centrifuged at 100,000g, for 18 h at 3–4°C. One gradient in each set was layered with bovine serum albumin (s20w 4.6S; 4 mg in 400 µl) as a sedimentation marker. Gradients were fractionated by upward displacement by sucrose (>40%, w/v) and three-drop fractions collected and analysed as described by Davies & Griffiths (1973).

**Results and Discussion**

Analysis of [3H]-labelled cytosol preparations from rat mammary tumours showed two peaks of protein-bound radioactivity corresponding to [3H]-oestradiol-receptor complexes of approximate sedimentation coefficients 4S and 8S (Fig. 1a). The low capacity of the 8S peak was demonstrated by the displacement of radioactivity to the high-capacity 4S peak by the presence of a 100-fold excess of unlabelled oestradiol (Fig. 1a). Whereas a similar concentration (50 nM) of either diethylstilboestrol or dibutyldihydrostilboestrol produced an identical displacement (Fig. 1b), diethylstilboestrol, but not dibutyldihydrostilboestrol, caused some depression of the 8S peak at a concentration of 5 nM (Fig. 2). I.C.I. 46474 abolished the 8S peak at a concentration of 50 nM (Fig. 1c) and caused a significant depression at 5 nM (Fig. 2b). I.C.I. 79792 (50 nM) was not as effective as I.C.I. 46474 at the same concentration (Fig. 1c) and had no effect at 5 nM (Fig. 2b). The other nitrogen-mustard-containing compound, I.C.I. 85966, generally failed to diminish [3H]-oestradiol binding to the 8S receptor (Fig. 1a), although it should be noted that in two of the rat mammary-tumour cytosols studied (Fig. 1d) this compound was more effective than I.C.I. 79792 and decreased specific binding of [3H]-oestradiol almost as efficiently as diethylstilboestrol, dibutyldihydrostilboestrol and I.C.I. 46474. This effect may be due to differing affinities of receptors for oestrogen and anti-oestrogens in different tumour cytosols, or to some entirely independent factor.

Cytosol prepared from samples of human breast carcinoma and labelled with [3H]oestradiol (0.5 nM) also showed two peaks of radioactivity when analysed by sucrose-density-gradient centrifugation. Removal with charcoal of free and loosely bound steroid indicated, however, high-affinity binding in both the 4S and 8S peaks (Fig. 2c), as previously reported (McGuire & DeLaGarza, 1973). Preliminary studies showed that this specific binding could be decreased by a 50 nM concentration of either diethylstilboestrol, dibutyldihydrostilboestrol and I.C.I. 46474 (Fig. 2c), but only dibutyldihydrostilboestrol was successful at 5 nM (Fig. 2d).

Diethylstilboestrol and I.C.I. 46474, therefore, were the two most active anti-oestrogens studied, whereas dibutyldihydrostilboestrol and I.C.I. 79792 were quite effective at higher concentrations. Dibutyldihydrostilboestrol may, however, prove useful in the clinical management of breast cancer, since it displays only 1% of the oestrogenicity of diethylstilboestrol (Grundy, 1957). This latter compound was more efficient than I.C.I. 46474 in displacing [3H]-oestradiol from human breast-carcinoma receptor proteins, whereas the converse was true for rat mammary-carcinoma receptor proteins; this may be due to a species variation in the protein. However, I.C.I. 46474 has been used clinically in the management of breast cancer (Cole et al., 1971; Ward, 1973).

The binding of oestradiol to receptor proteins has been postulated to occur by the initial attachment of the C-3 phenolic hydroxyl group to a highly specific site facilitating the attraction of the C-17 β-hydroxyl function to a less specific binding site (Hahnel & Twaddle, 1974). It is obvious that diethylstilboestrol and dibutyldihydrostilboestrol could both bind through the two phenolic hydroxyl groupings. The nitrogen-mustard derivative of diethylstilboestrol, I.C.I. 85966, may lose its ability to compete with [3H]-oestradiol because of the dichloroethanebromomou grouping. It is possible that its occasional effect (Fig. 1d) may be due to some metabolic process resulting in the loss of these groups. It has been suggested, however, that the inhibition of specific [3H]-oestradiol binding of I.C.I. 46474 is due to allosteric competition (Hahnel et al., 1973). This would be brought about by the association of the aromatic N-ethyl ether grouping to a site on the receptor protein other than that specific for oestradiol. Whether the inhibition is of a simple competitive or allosteric type, replacement of the NN-dimethyl group by the NN-(2-chloroethy)l group to produce I.C.I. 79792 may decrease the affinity at this site and may account for the lower anti-oestrogenic properties of this compound.

We are extremely grateful to the Tenovus Organisation for their generous financial support.
References
Jensen, E. V. & DeSombre, E. R. (1973) Science 182, 126-134
INFLUENCE OF ANTIOESTROGENS ON SPECIFIC BINDING OF
\( [^3H] \) OESTRADIOL IN VITRO BY NUCLEI FROM RAT MAMMARY TUMOURS

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Compounds having antioestrogenic properties are potentially useful for the treatment of certain patients with metastatic cancer of the breast. This report describes studies on the ability of various potential antioestrogens, in particular two nitrogen-mustard-containing substances, ICI 79792 (1-[4-\( \beta \)-bis(2-chloroethyl)amino]ethoxyphenyl]-trans diphenylbut-1-ene hydrochloride) and ICI 85966 (3,4-bis(\( \beta \)-[N-bis(2-chloroethyl)carbamoyl]phenyl)hex-3-ene), to prevent the uptake of \( [^3H] \) oestradiol into rat mammary tumour nuclei in vitro.

Mammary tumours were induced in virgin female Sprague-Dawley rats by intubation of 7,12-dimethylbenz(a)anthracene (20 mg in 1 ml sesame oil). Tumour tissue for experimentation (2 x 2 cm) developed 7–12 weeks after intubation. Tumours were dissected free of connective tissue and obvious necrotic areas and minced with scissors. Equal weights of mince (1 g) were incubated (15 min at 30°C) in Eagle’s basal medium (10 ml/g tissue) supplemented with \( [2,4,6,7-^3H] \) oestradiol (0-5 nmol/l, sp. act. 85 Ci/mmol) either alone or with non-radioactive competitor at either 50 or 500 nmol/l. In some instances, the tissue was pre-incubated (30 min at 30°C) in medium containing non-radioactive competitor before incubation with \( [^3H] \) oestradiol. Mincases were chilled on ice, drained, rinsed several times in cold \( H \)-free Eagle’s medium and homogenized in 0-25 M-sucrose–1 mM-MgCl\(_2\). Nuclei (800 g pellet) were washed twice in 0-25 M-sucrose–0-1% (v/v) Triton X-100, in 0-25 M-sucrose–1 mM-MgCl\(_2\) and extracted with 0-6 ml KCl (0-4 mol/l), at 4°C for 30 min. Aliquots of extract (0-4 ml) were analysed by sucrose-density-gradient centrifugation (Davies & Griffiths, 1973).

Incubation of minces with \( [^3H] \) oestradiol alone allowed the extraction from the nucleus of a \( ^3H \)-labelled steroid–receptor complex of sedimentation coefficient 4–5 S (Fig. 1 a). Inclusion of non-radioactive oestradiol, diethylstilboestrol [3,4-bis(\( \beta \)-hydroxyphenyl)hex-3-ene] or dibutyldihydrostilboestrol [5,6-bis(\( \beta \)-hydroxyphenyl)decane] at 50 nmol/l prevented this characteristic specific binding of \( [^3H] \) oestradiol (Fig. 1 a). However, inclusion of ICI 79792, ICI 85966 or ICI 46474 (the trans isomer of 1-[\( \beta \)-dimethylaminoethoxy]phenyl]1,2-diphenylbut-1-ene) at 50 or 500 nmol/l (Figs 1 a and b) failed to decrease the specific binding of \( [^3H] \) oestradiol. ICI 46474 (Lunan & Green, 1974; Powell-Jones, Jenner, Blamey, Davies & Griffiths, 1975) and ICI 79792 (Powell-Jones et al. 1975) have, however, some direct antioestrogenic effects on rodent and human tissues. Pre-incubation of the mammary tumour minces with antioestrogens before addition of \( [^3H] \) oestradiol (Fig. 1 c) showed that ICI 46474 and ICI 79792 depressed the uptake of \( [^3H] \) oestradiol, ICI 85966 was however more effective in this respect. Since this latter compound did not decrease specific \( [^3H] \) oestradiol binding when added directly to cytoplasmic extracts of rat mammary tumours (Powell-Jones et al. 1975) this effect on nuclear binding in whole-cell incubations may be due to its metabolism to diethylstilboestrol. In contrast, the ineffectiveness of ICI 46474 and ICI 79792 under these conditions, in relation to their effects on cytoplasmic binding of \( [^3H] \) oestradiol (Powell-Jones et al. 1975), may be due to their slower uptake compared with oestradiol, diethylstilboestrol and dibutyldihydrostilboestrol, since administration of ICI 46474 in vivo results in a diminution of tissue binding of \( [^3H] \) oestradiol (Nicholson & Golder, 1975) by rat mammary tumour tissue.
Fig. 1. (a) Mince incubated with $[^3]$H oestradiol alone (solid circles) or with 50 nmol/l concentrations of unlabelled oestradiol (open triangles), diethylstilboestrol or dibutyldihydrostilboestrol (solid triangles), or ICI 46474, ICI 79792 or ICI 85966 (open circles). (b) Mince incubated with $[^3]$H oestradiol alone (solid circles), or with 50 nmol/l concentrations of unlabelled oestradiol (open triangles), or 500 nmol/l concentrations of ICI 46474, ICI 79792 or ICI 85966 (open circles). (c) Mince pre-incubated (30 min at 30°C) with no additional substance (solid circles), unlabelled oestradiol (50 nmol/l) (open triangles), ICI 46474 or ICI 79792 (500 nmol/l) (open circles) or ICI 85966 (500 nmol/l) (solid triangles) before incubation with $[^3]$H oestradiol. Sedimentation marker (arrows) was in all cases bovine serum albumin ($s_{20,w}$ 4.6 S).

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REFERENCES

Effects of Oestradiol-17β and Tamoxifen on Total and Accessible Cytoplasmic Oestradiol-17β Receptors in DMBA-Induced Rat Mammary Tumours

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Abstract—An exchange assay for the measurement of total cytoplasmic oestradiol-17β binding sites in DMBA-induced rat mammary tumours has been established. The assay was used to examine the in vivo interactions of tamoxifen and oestradiol-17β with cytoplasmic receptor proteins for oestradiol-17β. Although certain quantitative differences were observed between the actions of tamoxifen and oestradiol-17β, the compounds show an initial similarity of response, each ligand promoting an early elevation in receptor levels. Tamoxifen, however, was unable to sustain the replenishment of the cytoplasmic receptor.

INTRODUCTION

The association of the oestradiol-17β molecule with a specific cytoplasmic binding protein and the subsequent translocation of the complex to the nucleus are thought to be fundamental in the action of oestradiol-17β [1]. Compounds which interfere with these events prevent oestrogens from exerting their full influence on target tissues. Such compounds are of particular interest in the management of metastatic breast cancer since approximately 30% of patients shown an objective remission of tumour growth following ablative or additive endocrine therapy.

This paper describes the influence of oestradiol-17β and the anti-oestrogen, tamoxifen (I.C.I. 46474: trans 1-(p-/f-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene) on the levels of total and accessible cytoplasmic oestradiol-17β binding sites in rat mammary tumours induced by 7,12-dimethylbenz(a)-anthracene (DMBA). Such studies can provide information on the mechanisms by which anti-oestrogens influence tumour growth. A preliminary report of this work has been presented [2].

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague-Dawley rats (50±2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of 4 and fed diet and water ad libitum. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration were not used in the study.

Preparation of cytosol fractions and assay for specific oestradiol-17β binding sites

(a) Accessible sites. Animals were killed by cervical dislocation, the tumours removed and dissected free of fat, washed with 0·25 M sucrose (adjusted to pH 7-4 with NaHCO₃),
cut into small pieces and passed through a stainless steel press (pore diameter, 1·5 mm). The brei was homogenized in 0·25 M sucrose using a Potter–Elvehjem glass-on-teflon homogeniser with a clearance of 14–16 μm (6 strokes, speed 2500 rev/min). This procedure results in maximum breakdown of tumour cell membranes with minimum damage to cell organelles [3]. A high-speed supernatant (cytosol) preparation was obtained by centrifugation of the homogenate in a Beckman L2-65B ultracentrifuge (T50 rotor, rav. 5·9 cm) for 45 min at 4°C. Aliquots (200 μl) of cytosol (approx. 20 mg protein/ml) were incubated (16 hr at 4°C) with equal volumes of 50 mM-Tris–HCl buffer, pH 7·4, containing 5 mM-EDTA and 1 mM-dithiothreitol (Tris–HCl buffer) and also containing various concentrations of [3H]oestradiol-17β (0·1–10 nmol/l) in the presence and absence of a 1000-fold higher concentration of unlabelled oestradiol-17β. After incubation, tubes were maintained at 4°C and an equal volume (400 μl) of charcoal suspension (0·5% w/v Norit-A, 0·05% w/v Dextran T-70, 0·1% w/v gelatin) in Tris–HCl buffer added. The resultant mixture was shaken for 60 min. at 4°C and charcoal removed by centrifugation at 800 g for 10 min. Aliquots (200 μl) of the supernatant containing protein-bound [3H]oestradiol-17β were added to 6 ml scintillation fluid (Aquasol, N.E.N. Chemicals Gmbh) and radioactivity measured in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation.

The protein content of cytosol fractions was estimated using the method of Lowry, Rosebrough, Farr and Randall [4].

(b) Total. Cytosol fractions were preincubated at 4°C with varying concentrations of either oestradiol-17β or tamoxifen (0–5 nmol/l). After incubation, tubes were maintained at 4°C and excess ligand removed by charcoal adsorption. Aliquots (200 μl) of the charcoal free supernatant were incubated at either 4, 10, 15, 25 or 37°C for periods up to 24 hr with an equal volume of Tris–HCl buffer containing a saturating concentration of [3H]oestradiol-17β (5 nmol/l) in the presence or absence of a 1000-fold higher concentration of unlabelled oestradiol-17β. The remaining experimental procedure was identical to that described earlier for the determination of accessible sites.

In other experiments cytosol fractions were preincubated with a saturating concentration of [3H]oestradiol-17β (5 nmol/l) and reincubated with unlabelled oestradiol-17β or tamoxifen (5 nmol/l).

**Determination of total and accessible cytoplasmic oestradiol-17β binding sites following in vivo administration of either oestradiol-17β or tamoxifen.**

Two experiments were undertaken:

(a) Ten rats bearing 15 tumours were divided randomly into three groups (5 tumours per group). Each group received either oestradiol-17β (5 μg) or tamoxifen (free base) (100 μg) in 0·1 ml vehicle (10% (v/v) ethanol in 0·15 M NaCl) by i.v. injection. Control animals received vehicle alone. Tumour biopv samples (approx. 200 mg of tissue) were removed aseptically from animals (anaesthetized with nembutal) at time 0 (prior to injection) and at 10, 20, 30 and 60 min. after injection. Aliquots of cytosol preparations were incubated at either 4°C for 16 hr (accessible sites) or 15°C for 2 hr (total sites) with a saturating concentration of [3H]oestradiol-17β or [3H]oestradiol-17β plus a 1000-fold excess of oestradiol-17β.

(b) Fifteen animals were divided randomly into three groups (5 tumours per group). The experimental design was as described in (a) except that tumour tissue was biopsied at time 0 and at 0·5, 1, 2, 4, 24 and 48 hr after injection.

**RESULTS**

**Measurement of total and accessible cytoplasmic oestradiol-17β binding sites.**

The establishment of conditions for the exchange of [3H]oestradiol-17β with oestradiol-17β or tamoxifen bound to a specific cytosol receptor protein involved (a) reduction of the interference of non-specific binding components and (b) determination of the optimum temperature to ensure the greatest rate of exchange with the minimum rate of degradation of specific binding sites.

(a) Allowance for non-specific oestradiol-17β binding sites. The binding of oestradiol-17β by non-specific components was a linear function of the [3H]oestradiol-17β concentration (Fig. 1a). Subtraction of this non-specific binding from the value obtained in the presence of [3H]oestradiol-17β alone, produces a curve demonstrating saturability of binding sites at approx. 2 nmol/l. These were termed specific sites. Analysis of the corrected values by the method of Scatchard [5] gives an apparent dissociation constant, Kd, of 230 pmol/l (Fig. 1b).

(b) Temperature optimisation of incubation conditions. Figure 2 illustrates (a) the rate of uptake of labelled oestradiol-17β by specific receptor proteins and (b) the thermostability of the
ligand–receptor complex formed. Rapid uptake of \( [\text{H}] \text{oestradiol-17\beta} \) was observed at all experimental temperatures. Determination of the number of binding sites at 10, 15 or 25°C produced levels of binding which were increased relative to the value obtained at 4°C. At these elevated temperatures, however, thermal degradation of the ligand receptor complex occurred. This was especially evident at 23°C and 37°C.

In order to establish whether incubations at temperatures above 4°C released oestradiol-17β previously bound to receptor, cytosols were preincubated at 4°C with a saturating concentration of cold oestradiol-17β and then reassayed in the presence of \( [\text{H}] \text{oestradiol-17\beta} \). Incubations performed at temperatures above 4°C resulted in the binding of \( [\text{H}] \text{oestradiol-17\beta} \) to otherwise occupied sites (Fig. 3a). The exchange was maximal at 15°C reaching 75% after a 2 hr incubation period. Similar results were obtained when cytosols were preincubated with tamoxifen (Fig. 3b). The exchange process is partially dependent upon the concentration of bound ligand present within the incubate (Fig. 4). Exchange values approached 100% in those cytosol fractions
previously incubated with non-saturating concentrations of either tamoxifen or oestradiol-17β. Incubations carried out at 37°C gave lower levels of [3H]oestradiol-17β binding than those observed at 10, 15 and 25°C.

Preincubation of cytosol preparations with a saturating concentration of [3H]oestradiol-17β (Fig. 5) at 4°C and reincubation of the [3H]-ligand-receptor complex with cold oestradiol-17β or tamoxifen at 15°C shows the events to be a true exchange process. Under the conditions employed, approximately 60% loss of bound [3H]oestradiol-17β was noted. In view of the results it seems likely that an incubation performed for 2 hr at 15°C provides a good estimation of the total number of binding sites for oestradiol-17β, while a similar incubation, performed at 4°C for 16 hr, measures only those sites which are accessible at the time of assay. These conditions of assay were used in the determination of total and accessible cytoplasmic oestradiol-17β binding sites in biopsy samples of DMBA-induced mammary tumour exposed, in vivo, to oestradiol-17β or tamoxifen.

Within 30 min following in vivo administration of oestradiol-17β (5 μg), very little binding, total or accessible, of [3H]oestradiol-17β to specific binding proteins was observed (Fig. 6a). Tamoxifen, like oestradiol-17β, produced an initial decrease in receptor levels, although the effect was less pronounced (Fig. 6b). Injection of saline vehicle had no appreciable effect on either total or accessible binding sites (Fig. 6c). A secondary period of elevated oestradiol-17β binding was observed after administration of either tamoxifen or oestradiol-17β (Fig. 7). The results indicate that accessible receptor levels are considerably increased during the 2–24 hr period following injection of oestradiol-17β and during the 2–4 hr period following treatment with tamoxifen. Total and accessible sites behave similarly, demonstrating the presence of specifically bound oestradiol-17β or tamoxifen within the cytosol. Tamoxifen, unlike oestradiol-17β, was unable to maintain elevated receptor levels, which decreased considerably in concentration during the 24–48 hr experimental period.

Fig. 5. Rate of exchange of bound [3H]oestradiol-17β.

CytoSol preparations were preincubated for 90 min at 4°C with a saturating concentration of [3H]oestradiol-17β (5-0 nmol/l). Excess [3H]oestradiol-17β was removed by charcoal adsorption and the ligand receptor complex reincubated for periods up to 360 min at 4°C (●—●) or 15°C (○—○) with either oestradiol-17β (5-0 nmol/l) or tamoxifen (5-0 nmol/l). Each result is expressed as a percentage of the total number of accessible oestradiol-17β binding sites determined in the absence of the preincubation step.

Fig. 6. Effect of in vivo administration of oestradiol-17β and tamoxifen on the cytoplasmic levels of total and accessible oestradiol-17β binding sites.

Rats were injected with either (a) 5 μg oestradiol-17β (b) 100 μg tamoxifen or (c) vehicle alone. Tumour biopsy samples were removed at time 0 and after 10, 20, 30 and 60 min. Cytosol preparations from the biopsies were incubated at either 4°C (accessible sites) (●) or 15°C (total sites) (○) for 120 min with a saturating concentration of [3H]oestradiol-17β (5-0 nmol/l) or [3H]oestradiol-17β plus a 1000-fold excess of unlabelled oestradiol-17β. Each result is expressed as a percentage of the value obtained by incubation of cytosol with labelled steroid at 4°C (accessible binding sites at time 0) and is the mean ± SEM of 5 separate tumours.

DISCUSSION

This report describes the development and use of a method to determine the total number of cytoplasmic oestradiol-17β binding sites present in DMBA-induced rat mammary tumours, regardless of whether the sites are occupied by oestradiol-17β or tamoxifen. The
criteria of minimum loss of binding sites with relatively high rates of exchange (approximately 75% exchange of specifically bound ligand with \[^{3}H\]oestradiol-17\(\beta\)) were met using an assay temperature of 15°C and an incubation period of 2 hr. Incubations at temperatures above 15°C resulted in considerable degradation of the ligand-receptor complex. It is evident, therefore, that although elevated temperatures increase the rate of dissociation of the ligand from the cytoplasmic oestradiol-17\(\beta\) binding protein [6, 7] they have limited application to a mammary tumour assay system. Accessible binding sites for oestradiol-17\(\beta\) or tamoxifen were determined at 4°C. At this temperature little dissociation of ligand from the cytoplasmic receptor occurs, a situation similar to that shown in rat uterine fractions [6-8].

Previously, it has been reported that the administration of tamoxifen and oestradiol-17\(\beta\) for 6 days to intact animals reduced the capacity of the DMBA-induced rat mammary tumour to bind \[^{3}H\]oestradiol-17\(\beta\) in vitro [9]. It was then not possible to decide whether this was due to a loss of binding protein or to a saturation of the available binding sites by the ligands. The present study extends the initial observations to include the early actions of oestradiol-17\(\beta\) and tamoxifen on receptor protein concentrations. Tamoxifen, like oestradiol-17\(\beta\), caused an initial decrease in total and accessible cytoplasmic binding sites for \[^{3}H\]oestradiol-17\(\beta\). The effect was more pronounced with oestradiol-17\(\beta\) and may reflect the relative depleting cytoplasmic receptor concentrations. A further association of the complex with acceptor sites in chromatin could increase transcription of the modified genome, resulting in the production of messenger RNA species coding for specific proteins, including the receptor for oestradiol-17\(\beta\). The administration in vivo of either substance brings about increased activity of RNA polymerase enzyme B in mammary tumour nuclei [12], correlating with the nuclear uptake of the ligand-receptor complex. Any quantitative changes in response to oestradiol-17\(\beta\) and tamoxifen may be a reflection of the relative ability of the substances to bring about transformation of the receptor to an active form, translocation to nuclei and association with specific nuclear sites.
The present studies, utilizing the total oestradiol-17β binding site assay, demonstrates the presence of considerable quantities of specifically bound ligand in cytosol fractions following the administration of either tamoxifen or oestradiol-17β. Noteboom and Gorski [14] and King and Gordon [15] have described a dual intracellular incorporation of labelled oestrogens in rat uteri following oestrogen treatment, observing that although most of the hormone was found in the nuclear fraction, a considerable portion (20-30%) was present in the high-speed supernatant or cytosol fraction. No further characterization into specific or non-specifically bound oestradiol-17β was undertaken by these authors. The biological significance of the specifically bound ligand present in cytosol fractions of DMBA-induced rat mammary tumours is not clear. It may represent a pool of ligand capable of eliciting gene expression if translocation of the ligand-receptor complex to the nucleus occurs. Alternatively, it may be defective ligand bound receptor present in either a pre- or post-nuclear form. Sucrose density gradient centrifugation of the bound material together with an examination of the characteristics of the ligand-receptor complex in a reconstituted cell-free system, as that described by Davies and Griffiths [16] for rat ventral prostate, may resolve the significance of this material to the cell.

Our observations suggest that although certain quantitative differences are noted between the early actions of tamoxifen and oestradiol-17β, the compounds show an initial similarity of response. This similarity is not prolonged. Tamoxifen, unlike oestradiol-17β, is unable to maintain elevated receptor concentrations during the remaining experimental period, as reported also by Clark, Anderson and Peck, [17] and Rocheft and Capony [18] for naf oxide and for CI628 by Katzenellenbogen and Furguson [8] in rat uteri. In addition the latter authors observed that CI628 induced oestrogen-like actions which included the synthesis of a specific uterine protein and elevated uterine weights. No elevation in accessible receptor concentrations was, however, noted. It is apparent that the anti-oestrogen, tamoxifen, like naf oxide and CI628 in rat uteri, is unable to sustain the replenishment of the cytoplasmic receptor in DMBA-induced rat mammary tumours. Although the reasons for this are not readily obvious, such actions would clearly induce tumour regression and is therefore consistent with our previous findings [9].

Research is now under way to investigate the action of tamoxifen in both nuclear and cytoplasmic compartments. Such data may elucidate the oestrogen-like and oestrogen antagonistic actions of tamoxifen.

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REFERENCES


Plasma Hormones in Patients with Advanced Breast Cancer Treated with Tamoxifen*

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†Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX, Wales

Abstract—Plasma concentrations of prolactin, FSH, LH and oestradiol-17β were measured in patients with advanced breast cancer being treated with tamoxifen. Plasma FSH levels dropped during the first month of treatment in patients who subsequently responded to therapy and in those who did not. After prolonged therapy plasma FSH in the responders returned to pre-treatment levels whereas those in the non-responders remained lower than the pre-treatment value. LH levels were lowered during tamoxifen therapy but there was no significant difference between the two groups of patients. Plasma prolactin and oestradiol-17β concentrations were unaffected by treatment.

INTRODUCTION

ALTHOUGH it is well recognized that a large proportion of patients with recurrent breast cancer require palliative treatment, only approximately one third of these respond to hormone therapy, whether additive or ablative. The selection of the type of treatment given is largely empirical and a more scientific basis is required.

Little is known regarding the biochemical effects of the hormonal therapy regimes which are used although it is accepted that these may involve a suppression of pituitary hormone secretion, a direct action on the tumour cells or an enhancement of the immune competence of the patient. Although a number of studies have been undertaken in an attempt to provide a scientific basis for the selection of therapy [1, 2], little is known about the endocrine changes that occur during the treatment of patients with various hormone regimes or of the plasma hormone concentrations of those who respond to therapy compared to those who do not.

In this study, plasma concentrations of prolactin, follicle stimulating hormone (FSH), luteinizing hormone (LH) and oestradiol-17β have been studied in patients with advanced breast cancer treated with the anti-oestrogen tamoxifen, (I.C.I. 46474). This compound has been used extensively in the treatment of the disease in recent years by other groups [3, 4]. There are however no reports of studies of plasma hormone changes in treated patients.

MATERIAL AND METHODS

Patients

Studies were carried out on patients attending the Combined Breast Clinic of the Departments of Surgery and Radiotherapy. All patients included in the study had either local or general carcinoma of the breast. They were either post-menopausal (in all cases more than two years since the last menstrual period) or had undergone oophorectomy. Thirty patients were treated with tamoxifen and of these 24 had sufficient blood samples taken for analysis. None had previously received any hormone treatment nor had undergone ablative endocrine surgery which would require maintenance therapy. None had received cytotoxic drugs.

Blood samples were taken from the patient on the morning before the commencement of treatment. Subsequent samples were taken at 7 and 14 days and at one, two and three months later.

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Patients were given tamoxifen (trans- (p-β-dimethylamino-ethoxyphenyl) - 1,2-diphenyl-but-1-ene, NovoGex, I.C.I. Ltd., Macclesfield, Cheshire) 20 mg twice a day for a minimum of one month.

Hormone assays

Blood was collected into lithium sequestrane tubes, centrifuged at 4°C, separated, and the plasma stored at -20°C until assayed.

Protein hormones

Prolactin was assayed by a double antibody radioimmunoassay using the method described by Cole & Boyns [5]. Results are expressed in terms of MRC Standard 71/222 and 1 mIU is equal to 50 ng in this assay system. LH and FSH were assayed using methods described previously [6], LH is expressed in terms of MRC Standard 68/40 and FSH in terms of 2nd IRP-HMG where 1 mIU is equivalent to 230 ng.

Plasma oestradiol-17β

Oestradiol-17β was determined by a RIA procedure, using an antiserum raised against an oestradiol-6-[O-carboxymethyl]-oxime bovine serum albumin conjugate. Steroids were extracted with diethyl ether (the recoveries ranged from 95-105%) and no purification procedures were incorporated in the assay since results, with and without chromatography, showed no significant difference. Free and bound steroid were separated using dextrancoated charcoal and the free steroid in a 500 µl aliquot dissolved in 5 ml Aquasol (NEN Chemicals) and counted in a liquid scintillation counter.

Data analysis

The clinical response to tamoxifen was assessed three months after beginning therapy according to the criteria described by Forrest [7], and patients were grouped as responders or non-responders. Their pretreatment plasma hormone values were compared and are presented as median and range because the distributions for prolactin and oestradiol-17β were markedly skewed. Within subjects, however, hormone concentrations approximated to a Gaussian distribution so that transformation of the data was not warranted. In order to minimize between subject variation hormone concentrations were first normalized by expressing each concentration as a fraction of the average for an individual. For both groups of patients at each time interval mean ± S.E.M. was calculated and the figures then scaled to make the pretreatment value 100.

RESULTS

Three months after the start of treatment all patients were assessed clinically and classified as responders, non-responders or those with an equivocal response. For the purpose of analysis the two patients assessed as equivocal responders were grouped with the non-responders. The distribution of sites of the disease with which the patient presented was similar between the two groups of patients (Table 1). There were no differences between the ages, years post-menopausal and cancer free intervals in all the patients studied (Table 2).

<table>
<thead>
<tr>
<th>Disease type</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responders</td>
</tr>
<tr>
<td>Local</td>
<td>5</td>
</tr>
<tr>
<td>General</td>
<td>1</td>
</tr>
<tr>
<td>Local and General</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10 (42%)</td>
</tr>
</tbody>
</table>

Patients were assessed 3 months after start of treatment and classed as responders or non-responders.

Table 3 shows the pre-treatment plasma hormone concentrations of the patients treated with tamoxifen. The results indicate that the nature of the response of the patients to the anti-oestrogen treatment is not determined by differences in the hormonal status of the patient before commencement of hormone therapy.

Plasma prolactin levels in the patients were unaffected by tamoxifen treatment (Fig. 1a). Although there was a wide range of concentrations before the start of therapy in both responders and non-responders, some patients having high prolactin levels and others very low levels, the values in the individual patients did not alter significantly during the treatment time and there was no difference between the two groups of patients. Plasma oestradiol-17β concentrations were also unaffected by tamoxifen treatment in either group of patients (Fig. 1b).

Gonadotrophin levels during treatment are shown in Fig. 2. Plasma FSH decreased in the
Plasma Hormones in Patients with Advanced Breast Cancer Treated with Tamoxifen

Table 2. Distribution of patients treated with tamoxifen

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Time since L.M.P. (yr)</th>
<th>Cancer free Interval (yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders</td>
<td>10</td>
<td>61-5 (56-80)</td>
<td>2-0 (0-12)</td>
</tr>
<tr>
<td>Non-responders</td>
<td>14</td>
<td>61-5 (54-82)</td>
<td>1-5 (0-6)</td>
</tr>
</tbody>
</table>

Patients were assessed 3 months after starting treatment and classed as responders or non-responders.

Values are expressed as the median and range.

There are no differences between responders or non-responders.

LMP = Last Menstrual period.

0 = Patients who presented with advanced disease.

Table 3. Plasma Hormone concentrations before starting treatment with tamoxifen

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Prolactin (mU/ml)</th>
<th>Oestradiol-17ß (pg/ml)</th>
<th>LH (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders</td>
<td>10</td>
<td>0-13 (0-04-0-98)</td>
<td>27 (16-52)</td>
<td>25 (15-48)</td>
<td>72 (33-112)</td>
</tr>
<tr>
<td>Non-responders</td>
<td>14</td>
<td>0-21 (0-04-0-41)</td>
<td>25 (16-52)</td>
<td>36 (17-50)</td>
<td>80 (28-124)</td>
</tr>
</tbody>
</table>

Results are expressed as Median and range.

There are no differences between responders and non-responders.

---

Fig. 1. Response of plasma prolactin and oestradiol-17ß to tamoxifen therapy in responding and non-responding breast cancer patients.

Values are expressed as mean ± S.E.M. of normalised data.

* = P < 0.05
† = Non-responders v. responders

Fig. 2. Response of plasma FSH and LH to tamoxifen therapy in responding and non-responding breast cancer patients.

Values are expressed as mean ± S.E.M. of normalised data.

* = P < 0.05
† = Non-responders v. responders
first week of tamoxifen therapy and at one month was significantly lower \((P < 0.05)\) than the pretreatment level in both responders and non-responders. However, within 2 and 3 months of the start of treatment, plasma FSH concentration in patients responding to tamoxifen had returned to the value found before tamoxifen treatment started, whereas in the non-responders, hormone concentrations were significantly lower \((P < 0.05)\) than the basal level throughout the course of therapy. There was a significant difference \((P < 0.05)\) between the FSH levels in the responders and non-responders in the second and third month of treatment.

By contrast, there was no difference between LH concentrations in the two groups of patients (Fig. 2b). After 1 week of treatment, plasma LH had decreased in both groups and remained significantly lower than the pretreatment value over the three-month period.

**DISCUSSION**

The remission rate of approximately 40\% which was induced by tamoxifen treatment in this series of patients is similar to that obtained by other groups who have also used this agent in the treatment of advanced breast cancer [3, 4]. This proportion of patients showing an objective response is comparable with the results of other studies from this group using other single hormone agents [8]. Using clomiphene, a compound closely related chemically to tamoxifen, a response rate of approximately 40\% has also been observed [9].

Evidence from animal studies suggests that the rat mammary tumour, induced by the administration of dimethylbenz-anthracene is dependent on the pituitary hormone prolactin [10, 11]. Although Murray [12] demonstrated elevated plasma prolactin levels in post-menopausal women with advanced breast cancer, other studies from the Tenovus Institute have, however, failed to demonstrate such a difference or any relationship between plasma prolactin concentration and breast disease [13, 14].

In this study, neither prolactin concentrations nor oestriadiol-17\(\beta\) levels altered during the course of tamoxifen therapy. The plasma prolactin concentration before treatment ranged from low normal through to high normal values but in each patient levels were not affected by treatment. This result appears to be in contrast to the action of tamoxifen in the pre-menopausal woman, when tamoxifen administration caused a decrease in plasma prolactin with a subsequent increase in oestriadiol-17\(\beta\) concentration [15].

Plasma FSH concentrations were lowered during the first week of treatment with tamoxifen in patients who subsequently responded to therapy and in those who did not. Significantly reduced levels were maintained during the first month of treatment but after prolonged therapy, plasma FSH in the responders returned to pre-treatment levels whereas those in the non-responders remained lower than the pretreatment value. At two and three months after the start of tamoxifen administration, FSH concentrations, in the group of non-responders remained significantly lower than in the responders.

Plasma FSH levels are also affected in a similar way when patients with advanced breast cancer were treated with clomiphene [9]. Both these compounds are generally regarded to be anti-oestrogens although the mechanism of action of neither is fully understood. Although tamoxifen is known to affect the binding of oestriadiol-17\(\beta\) to the cytoplasmic receptor protein in both the rat DMBA-tumour [16] and in human breast carcinoma [17], it would also seem to have some action on the hypothalamic-pituitary axis. The response of the rat to antioestrogen treatment appears to be dependent on dose since a large amount of tamoxifen administered over a long period of time can be antioestrogenic and cause an elevation of prolactin levels (R. Nicholson & M. P. Golder, unpublished observations) whereas lower doses are antioestrogenic [18, 19].

In view of the use of tamoxifen in pituitary-gonadal function tests in the pre-menopausal woman it will be interesting to study in greater depth pituitary function in post-menopausal women.

**Acknowledgements**—We thank Miss K. Baker for expert technical assistance.

We are grateful to Professor L. E. Hughes, Mr. M. Baum, and Dr. T. Friedman for allowing us to study patients under their care.

**REFERENCES**


EFFECT OF THE ANTI-OESTROGEN TAMOXIFEN ON PLASMA LEVELS OF LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE, PROLACTIN, OESTRADIOL AND PROGESTERONE IN NORMAL PRE-MENOPAUSAL WOMEN

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(Received 10 November 1975)

SUMMARY

Plasma levels of LH, FSH, prolactin, oestradiol and progesterone were determined daily during two consecutive menstrual cycles in six women volunteers. During the first (control) cycle no treatment was given and normal secretion of these hormones was observed. Oral administration of tamoxifen (20 mg/day), for either 5 or 10 days of the follicular phase of the second cycle, caused no change in either the overall length of the cycle or the time of occurrence of the mid-cycle gonadotrophin surge. There was little difference in the secretion of LH, FSH and progesterone during the control and test cycles.

A two- to eight-fold increase in oestradiol levels was observed during the test cycle which was most pronounced at the times of mid-cycle and mid-luteal hormone peaks. There was a significant decrease in plasma prolactin levels at mid-cycle but no real difference could be seen during the remainder of the cycle.

The data suggest that tamoxifen may act directly on the ovary to stimulate oestradiol release without intermediary gonadotrophin stimulation. As the drug apparently inhibited prolactin secretion even in the presence of high oestradiol levels, an alternative explanation may be that the reduced prolactin concentration permits augmented ovarian stimulation by normal concentrations of gonadotrophins.

INTRODUCTION

It is well recognized that a large proportion of patients treated for primary carcinoma of the breast, eventually require palliative therapy for recurrent disease. Approximately 30% of these patients respond well to various forms of endocrine therapy, suggesting that this metastatic tissue is hormone-dependent and responds to changes in the hormone status of the patient resulting from adrenalectomy or hypophysectomy. In recent years endocrine therapy has involved the administration of anti-oestrogens and one such compound, tamoxifen, has been found to be of particular value (Cole, Jones & Todd, 1971; Ward, 1973; Golder, Phillips, Preece, Jones, Henk & Griffiths, 1976) and at least as effective as diethylstilboestrol in postmenopausal women.

Little information is, however, available on the mechanism of action of tamoxifen or other similar compounds or hormones which are used for the treatment of advanced breast cancer. Although it would seem reasonable to suppose that tamoxifen has a direct action on breast tumour tissue, part of its effect in the patient may be due to its influence on either the hypothalamo-pituitary-ovarian or hypothalamo-pituitary-adrenal axes. It has been
demonstrated that tamoxifen will displace $[^3H]$oestradiol-17$\beta$ from high-affinity cytosol receptor proteins isolated from human breast tumours (Powell-Jones, Jenner, Blamey, Davies & Griffiths, 1975). At the same time, treatment with tamoxifen of post-menopausal patients with advanced carcinoma of the breast decreased plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the end of the first week but had no significant effect on plasma prolactin or oestradiol-17$\beta$ (Golder et al. 1976).

It was, therefore, considered necessary to study, as part of an investigation into the action of tamoxifen, the effects of the anti-oestrogen on plasma hormones of the normal pre-menopausal woman. This paper describes such an investigation.

**MATERIALS AND METHODS**

Six regularly menstruating women volunteers aged between 19 and 35 years, having given advised consent to the study, were bled daily over two consecutive menstrual cycles. None of the women had received steroid therapy. No treatment was given during the first cycle. During the next cycle, however, one group of three women received tamoxifen (20 mg/day; Nolvadex, I.C.I. 46474, I.C.I. Ltd, Cheshire) orally for 5 days and a further three women were given the drug for 10 days. In each case, treatment started on day 5 following commencement of menstrual bleeding. Blood was centrifuged and plasma stored at $-20^\circ$C before assay.

The plasma concentrations of LH, FSH, prolactin, oestradiol and progesterone were determined by radioimmunoassay procedures using methods similar to those already described (Groom, Groom, Cooke & Boyns, 1971; Cameron & Jones, 1972; Cole & Boyns, 1973; Scarisbrick & Cameron, 1975). Results were expressed in terms of MRC Standard A 63/15 for LH, 2nd IRP–HMG for FSH, MRC Standard 71/222 for prolactin and weight of pure steroid.

**Statistical analysis of results**

For each woman, the day of the LH peak was termed day 0 and the days of the cycle were determined from this. The mean and standard deviation of each hormone level on each day was then calculated.

For more detailed analysis in order to overcome differences in cycle length, day of LH peak and minor day-to-day hormone fluctuations, the moving average of the results from 4 consecutive days was calculated for each hormone throughout the cycle. For example, the mean hormone values for days 3, 4, 5 and 6 would be plotted for ‘day 4-5’, those for days 4, 5, 6 and 7 for ‘day 5-5’, etc. Using this technique, small, inconsequential differences in day-to-day hormone values are smoothed out so that only major changes and trends are considered in the statistics. The data for each hormone in control and treated cycles of each woman were then compared using the Wilcoxon Matched-Pairs Test for the following three periods: days $-10$ to $-1$ (follicular phase); days $-5$ to 4 (mid-cycle); and days 1–10 (luteal phase).

**RESULTS**

**Control cycles**

Figure 1a shows the mean (±s.d.) plasma hormone levels in the six women during the control menstrual cycles. Mean cycle length was $27±3$ days with a mid-cycle LH peak $14±2$ days following commencement of menstrual bleeding.

Levels of LH were about $6±4$ mu./ml during both follicular and luteal phases with a peak of $29±12$ mu./ml at mid-cycle. Plasma FSH levels peaked coincidentally with LH at
Fig. 1. Mean plasma levels of LH, FSH, prolactin, oestradiol and progesterone during the menstrual cycle. (a) Normal control cycle \((n = 6)\); (b) tamoxifen (20 mg/day) given for 5 days during the follicular phase \((n = 3)\); (c) tamoxifen (20 mg/day) given for 10 days during the follicular phase \((n = 3)\). Stippled area indicates ±s.d.
17 ± 9 µg/ml, but tended to be higher during the follicular phase (5 ± 3 µg/ml) than in the luteal phase (2 ± 1 µg/ml).

Prolactin levels fluctuated considerably throughout the cycle with a mean level of 0.20 µg/ml. Two major peaks in prolactin secretion of almost equal magnitude (mean 0.50 µg/ml) were however apparent, one about day 0 and a second at day 10. Of the individual women, four showed this marked double peak while two showed a more random pattern of prolactin secretion (Figs 2 and 3).

Early follicular phase oestradiol concentrations were approximately 70 pg/ml rising to a peak of about 300 pg/ml on day -1. A secondary oestradiol peak of about 180 pg/ml was observed in the luteal phase (days 7-11). Progesterone secretion was very low before day 1 rising to a peak (23 ng/ml) about day 9 and falling again before menstruation occurred.

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**Fig. 2.** Plasma levels of LH, FSH, prolactin, oestradiol and progesterone during two successive menstrual cycles. Tamoxifen (20 mg/day) was given for 5 days of the follicular phase of the second cycle. Hormone levels are expressed as moving means of 4 days results. Control cycle, closed symbols; test cycle, open symbols. *P* values are shown for comparisons of control and test cycle values within the brackets (see Methods for details).
Fig. 3. Plasma levels of LH, FSH, prolactin, oestradiol and progesterone during two successive menstrual cycles. Tamoxifen (20 mg/day) was given for 10 days of the follicular phase of the second cycle. Hormone levels are expressed as moving means of 4 days results. Control cycle, closed symbols; test cycle, open symbols. P values are shown for comparisons of control and test cycle values within the brackets (see Methods for details).

Treatment cycles

Figures 1b and 1c show the mean (± s.d.) concentrations of plasma hormones throughout the treatment cycles of women given tamoxifen for 5 and 10 days respectively. The mean length of these treatment cycles was 28 ± 1 days with an LH peak 14 ± 1 days following commencement of menstrual bleeding. No apparent change in the length of subsequent cycles was observed.

Levels of LH were approximately 4 ± 3 and 6 ± 3 mu./ml respectively during both follicular and luteal phases of the 5-day and 10-day treated groups of women. Mid-cycle peaks of LH reached 22 ± 13 and 25 ± 6 mu./ml in these groups. Plasma FSH levels peaked co-
incidentally with LH at about 20 mu./ml in both test groups. However, although there was no difference from the controls in the follicular and luteal phase levels of FSH in the 5-day treated group (5 ± 3 and 2 ± 1 mu./ml respectively), there was evidence of increased luteal phase FSH release in the 10-day treated group so that plasma concentrations in follicular and luteal phases were similar (6 ± 3 and 6 ± 2 mu./ml respectively).

Prolactin levels again varied considerably throughout the cycle of both treated groups with a mean level of about 0-20 mu./ml. In the 5-day treated group, a peak was still apparent at about mid-cycle (Fig. 1 b) but of reduced magnitude (0-30 mu./ml). A further reduction in mid-cycle prolactin levels was seen in the 10-day treated group (0-08 mu./ml). No marked change in the luteal phase prolactin concentration was observed in either treatment group (0-60 mu./ml) compared with the control cycles.

Plasma concentration of oestradiol increased from about 60 pg/ml during the early follicular phase to a mid-cycle peak of about 550 pg/ml in both groups of treated women. In one of the 10-day treated women a mid-cycle peak oestradiol level of 1400 pg/ml was observed (Fig. 3). Mean levels of 300 and 560 pg/ml were obtained for the luteal phase oestradiol peaks in the 5-day and 10-day treated women respectively (Figs 1 b and 1 c).

There was no real difference in progesterone secretion in either treatment group compared with the control cycles – levels were very low before day 1, rising to a peak (25 ng/ml) during the luteal phase.

Figures 2 and 3 show a direct comparison of the hormone levels expressed as moving means for each woman in her control and test cycle. No real change had occurred in the LH levels of any woman but FSH levels were slightly raised in two out of the three women taking the drug for 10 days. This increase was not consistent with any particular phase of the cycle. Prolactin levels were significantly reduced in every woman during the period around mid-cycle immediately after administration of the drug.

Plasma oestradiol concentrations were significantly raised in every woman following administration of the drug and this increase was observed during follicular, mid-cycle and luteal phases, i.e. during and after taking tamoxifen. A significant increase in plasma progesterone concentration was observed in the luteal phase of some of the women taking tamoxifen but this increase was not great.

**DISCUSSION**

The plasma concentrations of LH, FSH, oestradiol and progesterone in the control cycles of the women under investigation were similar to those previously reported for normal ovulatory women (Stevens & Vorss, 1967; Speroff & van de Wiele, 1971). Furthermore the levels of plasma prolactin observed during the control cycles of these women also showed the irregular variation previously observed (McNeilly & Chard, 1974). In some of the women, two distinct peaks of prolactin were observed, one at mid-cycle and the other during the luteal phase. In other subjects no significant peaks could be detected amongst the random fluctuations observed in the complete cycle. Similar prolactin profiles have been previously reported (Friesen, Hwang, Guyda, Tolis, Tyson & Myers, 1972; L'Hermite, Delvoye, Nokin, Vekemans & Robyn, 1972; McNeilly & Chard, 1974).

Tamoxifen had no effect on plasma LH levels in these premenopausal women, while only a slight increase in the concentration of plasma FSH was observed. There have been no other reports on tamoxifen action in normal women. Labhsetwar (1970 a, b, c) and McDonald (1973) concluded that in the rat, administration of tamoxifen inhibited ovulation by blocking the ovulatory gonadotrophin surge caused by positive feedback of oestrogen or progesterone on the hypothalamus. There is no evidence from the data presented here that tamoxifen has any direct action on the release of LH and FSH in premenopausal women.
Large increases in plasma oestradiol concentration occurred both during and after administration of tamoxifen, suggesting stimulation of ovarian activity. Successful use has been made of similar doses of tamoxifen to induce ovulation in anovulatory infertility (Klopper & Hall, 1971; Williamson & Ellis, 1973), but unfortunately no plasma hormone measurements were made in those studies. It is possible that tamoxifen acts directly on the ovary. The structurally similar synthetic oestrogens chlorotrianisene and clomiphene have been reported to stimulate gonadal function directly, without a requirement for gonadotrophin participation (Smith, 1965; Mayfield & Ward, 1966; Baker, Burger, de Kretser, Hudson & Straffon, 1973).

Relatively small changes were seen in plasma progesterone concentrations following tamoxifen treatment. There is no information on the effect of other anti-oestrogens on progesterone secretion in normal women, although rises have been reported in previously anovulatory women treated with such drugs (Loraine & Bell, 1970).

There was a significant decrease in plasma prolactin levels during the period of administration of the drug. Jordan, Koerner & Robison (1975) reported that tamoxifen inhibits oestrogen-stimulated prolactin release in rats, although at higher dose levels, the oestrogenic properties of the compound mask any inhibitory effect it has on prolactin release in the rat (Nicholson & Golder, 1975). In normal menstruating women, therefore, the association of increased plasma oestradiol with reduced plasma prolactin levels, indicates an antagonistic role of tamoxifen in the feedback mechanism of this steroid on pituitary prolactin release.

The reduction in plasma prolactin concentration by tamoxifen may be an important factor in the use of the drug to induce ovulation since hyperprolactinaemia has been associated with several forms of amenorrhoea (Rolland, Schellekens & Lequin, 1974; Thorner, McNeilly, Hagen & Besser, 1974). Furthermore, it seems reasonable to suggest that the lowered prolactin levels following tamoxifen treatment may have permitted an augmented action of gonadotrophins on the ovary in the normally menstruating women in this study, in a manner similar to that considered for amenorrhoea-galactorrhoea patients (Rolland et al. 1974). Such an explanation could account for the success of anti-oestrogenic drugs for the induction of ovulation.

Since prolactin is one of the factors controlling the development of the breast, one may also postulate that at least part of the action of tamoxifen as a treatment for breast cancer may involve its anti-prolactin property. No detectable changes in plasma prolactin concentrations were observed by Golder et al. (1976) in postmenopausal women with advanced breast cancer treated with tamoxifen. However, daily plasma samples were not available in this study and stress or some other random effect may have masked any changes in plasma prolactin levels, since Willis, London & Butt (1976) found a reduced basal as well as reduced thyrotrophin releasing hormone-stimulated prolactin level in their series of tamoxifen-treated advanced breast cancer patients.

We are grateful to Dr A. L. Walpole for supplying the tamoxifen. We thank Miss Janet Link for technical assistance with some of the assays and Mr F. Morgan for advice on the statistical treatment of the data. This work was made possible through the generous support of the Tenovus Organization.

REFERENCES


Effects of Oestradiol-17β and Tamoxifen on Nuclear Oestradiol-17β Receptors in DMBA-Induced Rat Mammary Tumours

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Abstract—An exchange assay has been established for the measurement of nuclear oestradiol-17β binding sites in DMBA-induced mammary tumours of the rat. The assay was used to examine the nuclear concentration of binding sites following the administration in vivo of tamoxifen and oestradiol-17β. Both compounds translocate receptor protein to the nucleus and cause an early elevation in cytoplasmic receptor levels. By 24 and 48 hr after tamoxifen administration, the intranuclear concentration of oestrogen receptor complex has returned to levels indistinguishable from pretreatment values. At this time, replenishment of the cytoplasmic receptor had not occurred and tumours were non-responsive to oestradiol-17β.

INTRODUCTION

The initial event in the antioestrogenic action of tamoxifen (ICI 46474; trans 1-(p,p'-dimethylaminooxyphenyl)-1,2-diphenylbut-1-ene) on mammary tumours induced in rats by 7,12-dimethylbenz(a)anthracene (DMBA) is the reduction of both total [1] and accessible [1–3] cytoplasmic binding sites for oestradiol-17β in vitro. Although the final result of tamoxifen administration i.e. tumour regression [2,4] is obvious, the precise sequence of events leading to this end-point remains unclear. This study examines the nuclear uptake of oestradiol-17β and tamoxifen as determined by an exchange assay, with the aim of providing further information as to the means by which antioestrogens can influence tumour growth.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague-Dawley rats (50 ± 2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of four and allowed diet and water ad libitum. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration were not used in the study.

Preparation of nuclear fractions and determination of oestradiol-17β/tamoxifen binding sites by the [3H]-oestradiol-17β exchange assay

Animals were given oestradiol-17β (5 µg) or tamoxifen (100 µg) in 100 µl vehicle (10% v/v ethanol in 0.15 M NaCl) by intravenous injection 1 hr before sacrifice. The tumours were removed and dissected free of fat, washed with 0.25 M sucrose (adjusted to pH 7.4 with NaHCO3), cut into small pieces and passed through a stainless steel press (pore diameter, 1.5 mm). The brei was collected in a beaker containing 0.25 M sucrose and homogenised with a Potter–Elvehjem glass-on-teflon homogeniser of clearance 14–16 µm (6 strokes, speed 2500 rev/min). Such a procedure has been demonstrated to give maximum breakdown of tumour cell membranes with minimal damage to cell organelles [5]. A nuclear preparation was

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obtained by centrifugation of the homogenate at 800 g for 10 min at 4°C. Nuclei were washed twice by resuspension and re-sedimentation from 0.25 M sucrose-3 mM MgCl₂ containing 0.1% (w/v) triton X-100, then washed and resuspended in 0.25 M sucrose-3 mM MgCl₂. Aliquots of the nuclear fraction (200 µl) were mixed with an equal volume of protamine sulphate [1 mg/ml in medium A (50 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA and 1 mM dithiothreitol)] and re-sedimented at 800 g for 10 min. Precipitated receptor was incubated for various times at various temperatures (see Results) with 200 µl [2,4,6,7-³H]oestradiol-17β (specific radioactivity 85 Ci/m-mole; Radiochemical Centre, Amersham, Bucks., U.K.) in medium A (final steroid concentration 20 nmole/l), in the absence or presence of unlabelled diethylstilboestrol (20 µmole/l). After incubation precipitates were cooled to 4°C and washed with cold medium A (three washes, each followed by centrifugation at 800 g for 10 min). The final pellet was extracted overnight with ethanol (0.5 ml), centrifuged and the ethanol-soluble supernatant decanted into vials. The extracts were dried and counted in 6 ml scintillation fluid (4 g, 2,5-diphenyloxazole, 0.05 g of 1,4-di [2-(5-phenyloxazolyl)]-benzene, 1,1-toluene) in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation.

In other experiments, animals were injected with 20 µCi [³H]oestradiol-17β 1 hr prior to removal of tumours. Under these conditions approximately 70% of the tumour-associated radioactivity was found in the nuclear fraction. Aliquots of the nuclear fraction (200 µl) were treated with protamine sulphate as previously described, centrifuged and the precipitated receptor incubated with a saturating concentration of either oestradiol-17β (20 nmole/l) or tamoxifen (20 nmole/l) at 4, 10, 15, 25 or 37°C for periods up to 4 hr.

Saturation analysis of the binding of [³H]oestradiol-17β to otherwise occupied nuclear sites was estimated by incubating aliquots of the protamine sulphate precipitated receptor in medium A containing various concentrations of [³H]oestradiol-17β (1-25 nmole/l) in the presence and absence of a 1000-fold higher concentration of unlabelled diethylstilboestrol for 2 hr at 15°C. After incubation tubes were treated as previously described.

The method for the determination of accessible cytoplasmic oestradiol-17β binding sites has been described in a previous communication [1]. Briefly, the supernatant from the 800 g centrifugation stage was subjected to further centrifugation at 105,000 g for 45 min at 4°C. Aliquots of cytosol were incubated for 16 hr at 4°C with equal volumes of medium A containing a saturating concentration of [³H]oestradiol-17β (5 nmole/l) in the presence and absence of a 100-fold higher concentration of unlabelled diethylstilboestrol. Excess steroid was removed by charcoal adsorption and aliquots of the charcoal-free supernatant counted for radioactivity.

For competitive binding studies various amounts of non-radioactive oestradiol-17β or other potential ligands (25-10,000 nmole/l) were added with [³H]oestradiol-17β (either 5 nmole/l for accessible cytoplasmic binding sites or 20 nmole/l for nuclear binding sites) to the reaction mixture and incubated as described previously. All values were corrected by subtraction of the binding value obtained in the presence of a 1000-fold excess of unlabelled oestradiol-17β.

The DNA content of nuclear fractions and the protein content of cytosol fractions were estimated using the methods of Burton [6] and Lowry et al. [7] respectively.

Determination of nuclear and cytoplasmic binding sites by the [³H]oestradiol-17β-exchange assay following the in vivo administration of either oestradiol-17β or tamoxifen.

Three experiments were undertaken.

(i) Eighteen rats bearing 25 tumours were divided randomly into five groups (5 tumours per group). Each group received either oestradiol-17β (0.5 µg or 5 µg) or tamoxifen (10 µg or 100 µg) in 0.1 ml vehicle [10% (v/v) ethanol in 0.15 M NaCl] by intravenous injection. Control animals received vehicle alone. Tumour biopsy samples (approximately 200 mg tissue) were removed aseptically from Nembutal® anaesthetized animals at time 0 (prior to injection) and at 18, 24, 36 and 72 hr after injection. Cytosol and nuclear preparations were obtained as previously described and portions incubated for either 2 hr at 15°C (total nuclear sites) or 16 hr at 4°C (accessible cytoplasmic sites) with a saturating concentration of [³H]oestradiol-17β (20 nmole/l) with and without unlabelled stilboestrol (20 µmole/l).

(ii) Fifteen animals were divided randomly into three groups (5 tumours per group). The experimental design was as described in (i) except that animals were treated with either oestradiol-17β (5 µg), tamoxifen (100 µg) or vehicle alone and tumour tissue was biopsied at time 0, and at 0, 1, 2, 4, 8, 16, 24 and 68 hr after injection.
(iii) Animals were treated as follows: Group 1, 4 animals bearing six tumours received injections of tamoxifen (100 μg) on two consecutive days. After a further 24 hr, animals were given oestradiol-17β (5 μg). Group 2 animals (four animals bearing five tumours) received saline injections for the first two days followed by oestradiol-17β (5 μg) on the third. Group 3 animals (three animals bearing five tumours) received saline injections alone. Tumour biopsy samples were removed at time 0, 24, 48, 52, 56 and 60 hr and assayed for cytoplasmic and nuclear oestradiol-17β binding sites.

RESULTS

Measurement of nuclear oestradiol-17β binding sites

The establishment of conditions for the exchange of [3H]oestradiol-17β with endogenous oestradiol-17β or tamoxifen specifically bound to nuclear oestradiol-17β receptors involved (a) determination of the optimum temperature to ensure the greatest rate of exchange with the minimum rate of degradation of binding sites and (b) reduction of the interference of non-specific binding components.

(a) Temperature optimisation of incubation conditions. Nuclear fractions prepared from mammary tumours pretreated in vivo with oestradiol-17β contain elements that bind [3H]oestradiol-17β (Fig. 1a). Rapid uptake of [3H]oestradiol-17β was observed at 15, 25 and 37°C. Continued incubation of the receptor complex resulted in thermal inactivation. This phenomenon was most evident at 25 and 37°C. Incubations of nuclear fractions at 4°C caused little [3H]oestradiol-17β binding. The maximum [3H]oestradiol-17β binding value was observed after a 2 hr incubation period at 15°C. Similar results were obtained when animals were pretreated with tamoxifen (Fig. 1b).

Pretreatment of animals with [3H]oestradiol-17β caused the rapid incorporation of radioactivity into mammary tumour nuclear fractions (Fig. 2). The uptake was maximal at 2 hr. Thin layer chromatography of acetone extracts of the nuclear fractions showed the radioactivity...
to be associated with oestradiol-17β. Further incubation of the nuclear preparation at 15°C for periods up to 4 hr resulted in a considerable loss of bound radioactivity (Fig. 3). No loss of [3H]oestradiol-17β was observed at 4°C. Incubation of the nuclear preparation at 15°C for periods up to 4 hr resulted in a considerable loss of bound radioactivity (Fig. 3). No loss of [3H]oestradiol-17β was observed at 4°C. In view of these results it was considered reasonable that an incubation performed for 2 hr at 15°C would provide an estimate of the oestradiol-17β binding sites present in nuclear fractions of DMBA-induced mammary tumours. Extension of the incubation period or an elevation in the incubation temperature resulted in a nett loss of bound material.

Fig. 3. Rate of exchange of bound [3H]oestradiol-17β. Animals were given 20 μCi [3H]oestradiol-17β 1 hr before sacrifice. Aliquots of the nuclear fraction were treated with protamine sulphate, centrifuged and the precipitated receptor incubated with either Tris buffer, , oestradiol-17β (20 nmole/l), □, or tamoxifen (20 nmole/l), at 4°C or 15°C for periods up to 4 hr.

Fig. 5. Specificity of [3H]oestradiol-17β binding assays. (a) Cytosol fractions were incubated for 16 hr at 4°C with a saturating concentration of [3H]oestradiol-17β (5 nmole/l) in the presence and absence of increasing concentrations (5, 50 and 500-fold excess) of the potential oestrogen antagonists. (b) Nuclear fractions were incubated for 2 hr at 15°C with a saturating concentration of [3H]oestradiol-17β (20 nmole/l) under similar conditions to those described in (a). , oestradiol-17β, Δ, diethylstilboestrol, □, tamoxifen, △, C1628, ▲, meso-DHBS, ×, testosterone and ■, corticosterone.

(b) Allowance for non-specific oestradiol-17β binding sites. The binding of oestradiol-17β by non-specific components was a linear function of the [3H]oestradiol-17β concentration (Fig.

![Graph A](https://via.placeholder.com/150)
![Graph B](https://via.placeholder.com/150)

Fig. 4. Determination of the number of specific nuclear binding sites for oestradiol-17β in rat mammary tumour tissue. (a) Nuclear fractions were incubated for 2 hr at 15°C with various concentrations of (1) [3H]oestradiol-17β () or (2) [3H]oestradiol-17β plus a 1000-fold excess of unlabelled diethylstilboestrol (△—△). Specific [3H]oestradiol-17β binding was obtained by subtraction of (2) from (1) (). Scatchard analysis of the corrected values.
4a). Subtraction of this non-specific binding from the value obtained in the presence of $[^3H]$oestradiol-17β alone, produced a curve demonstrating saturability of binding sites at approximately 15 n mole/l. Analysis of the corrected values by the method of Scatchard [8] gives an apparent dissociation constant, $K_d$, of 180 pmole/l (Fig. 4b).

Specificity of (a) the accessible cytoplasmic oestradiol-17β binding assay and (b) nuclear oestradiol-17β exchange assay

(a) The affinity of various steroid and non-steroid anti-oestrogens for the cytoplasmic oestradiol-17β receptor was examined by a competitive binding assay. This involved the incubation of cytosol with a fixed concentration of $[^3H]$oestradiol-17β (5 n mole/l) in the presence and absence of increasing concentrations of the potential oestrogen antagonists (Fig. 5a). Addition of 25 n mole/l oestradiol-17β decreased the binding of $[^3H]$oestradiol-17β to 41% of the control value. The binding continued to decrease with increasing oestradiol-17β concentrations. Testosterone slightly decreased the uptake of $[^3H]$oestradiol-17β whereas corticosterone was without effect. Of the non-steroidal compounds examined diethylstilboestrol, tamoxifen and CI628 were the most effective antagonists.

(b) Competitive binding assays performed on nuclear exchangeable material gave a similar gradation of results (Fig. 5b).

In vivo effects of oestradiol-17β and tamoxifen

Within 30 min following in vivo administration of 0.5 µg oestradiol-17β a 50% reduction in the binding of $[^3H]$oestradiol-17β to specific cytoplasmic binding proteins was observed (Fig. 6a). The effect was associated with a concomitant progressive increase in specific nuclear oestradiol-17β binding sites. The number of nuclear oestradiol-17β binding sites had increased from a control value of 133 fmole/mg DNA to 369 fmole/mg DNA by 60 min after oestradiol-17β treatment. Elevation of the level of oestradiol-17β (5 µg) administered in vivo caused a more pronounced depletion of cytoplasmic binding sites. Nuclear oestradiol-17β binding sites increased to 478 fmole/mg DNA after 60 min. Tamoxifen, like oestradiol-17β, showed similar properties although the response was not as great (Fig. 6b). Injection of saline vehicle had no appreciable effect on either cytoplasmic or nuclear binding sites (Fig. 6c).

A secondary period of elevated cytoplasmic

![Fig. 6. Effect of in vivo administration of oestradiol-17β and tamoxifen on cytoplasmic and nuclear oestradiol-17β binding sites. Rats were injected with either (a) oestradiol-17β (0.5 µg, ○ or 5 µg, □), (b) tamoxifen (10 µg, ○, or 100 µg, □) or (c) vehicle alone. Tumour biopsy samples were removed at time 0 and after 10, 25, 40 and 60 min. Cytoplasmic preparations from the biopsies were incubated at 4°C for 16 hr with a saturating concentration of $[^3H]$oestradiol-17β (3.0 n mole/l) or $[^3H]$oestradiol-17β plus 1000-fold excess of stilboestrol. Nuclear preparations were incubated for 2 hr at 15°C with a saturating concentration of $[^3H]$oestradiol-17β (20 n mole/l) or $[^3H]$oestradiol-17β plus a 1000-fold excess of stilboestrol. The results are the mean ± S.E.M. of five tumours per group.](image-url)
oestradiol-17β binding was observed after either tamoxifen or oestradiol-17β administration (Fig. 7). The results indicate that accessible receptor levels are considerably increased during the 4–24 hr period following oestradiol-17β injection and during the 4–16 hr period following tamoxifen treatment. Tamoxifen, unlike oestradiol-17β, was unable to maintain elevated receptor levels, which showed a considerable decrease in concentration during the 24–48 hr experimental period. Saline injections had no appreciable effect on accessible receptor levels. The concentration of nuclear oestradiol-17β binding sites increased to a maximum (979 fmole/mg DNA) 2 hr after oestradiol-17β administration (Fig. 7) and were still elevated over control values after 24 hr. Administration of tamoxifen produced a less rapid accumulation of nuclear oestradiol-17β binding sites which reached maximum value at 4 hr and returned to pre-stimulation levels at 24 hr.

The administration of tamoxifen (100 μg/day) to tumour-bearing animals on two consecutive days reduced the capacity of the mammary tumour cytosol to bind [3H]-oestradiol-17β (Fig. 8). A further injection of oestradiol-17β (5 μg) at this time did not result in any appreciable nuclear uptake of exchangeable oestradiol-17β or in a stimulation of accessible cytoplasmic oestradiol-17β binding sites. Mammary tumours present in saline treated animals retained their capacity to respond to oestradiol-17β administered in vivo.

**DISCUSSION**

This report describes the development and use of an exchange assay for the determination of nuclear oestradiol-17β binding sites present in DMBA-induced mammary adenocarcinomata of rats, irrespective of whether the sites are occupied by oestradiol-17β or tamoxifen. The criteria of minimum loss of binding sites with relatively high rates of exchange were met using an assay temperature of 15°C and an incubation period of 2 hr. The conditions of assay are therefore identical to those described for the estimation of total oestradiol-17β binding sites present in cytoplasmic fractions of DMBA-induced rat mammary tumours [1]. Specificity studies carried out on the binding of
Effects of Oestradiol-17β and Tamoxifen

[3H]oestradiol-17β to filled (nuclear) or unfilled (accessible cytoplasmic) binding sites demonstrates that the receptor protein has similar affinity characteristics for non-steroidal oestrogen antagonists in each state. These data suggest a common origin.

The relative ability of the non-steroidal oestrogen antagonists in competing for oestradiol-17β binding sites correlates closely with (a) their relative effectiveness in reducing the in vitro capacity of the DMBA-induced mammary tumour to bind [3H]oestradiol-17β following their in vivo administration and (b) their ability to cause a regression in tumour mass [2]. It was not possible in that earlier study to determine whether the loss of receptor-binding was due to a decrease of receptor protein or to a strong association of the ligand to the receptor complex, which through steric or transport effects resulted in a biologically inactive complex. The present study demonstrates that tamoxifen can compete with oestradiol-17β for its cytoplasmic receptor protein, although this may be allosteric rather than a direct competition [9]. Other investigations have also demonstrated that tamoxifen can effectively block the association of the oestradiol-17β molecule with its specific receptor protein in DMBA-induced mammary adenocarcinomata [3, 10]. Indeed, we have ascribed an affinity constant of 0.213 × 10^{-1} nmol (cmf 0.643 × 10^{-1} nmol for oestradiol-17β) to the interaction between tamoxifen and the oestradiol-17β receptor protein [11]. Sedimentation analysis of tamoxifen treated cytosol fractions [3, 10] demonstrates that the anti-oestrogen competes for 8S-oestrogen binding components.

Although the initial association of tamoxifen with its specific cytoplasmic binding site is undoubtedly important, this present study indicates that it forms only part of the complex mechanism by which tamoxifen influences tumour growth. After the in vivo injection of tamoxifen or oestradiol-17β, a rapid dose-dependent accumulation of nuclear [3H]-oestradiol-17β exchangeable material occurs. Maximum [3H]oestradiol-17β binding values were observed 2 hr following the administration of oestradiol-17β and 4 hr after tamoxifen treatment. Oestradiol-17β more effectively promoted the translocation process than did tamoxifen, which may reflect either their relative rates of entry into the tumour cells [1, 10, 12], or their different affinities for the oestradiol-17β receptor protein.

The translocation of the receptor complex to the nucleus results in an initial depletion of both total [1] and accessible cytoplasmic oestradiol-17β binding sites. Tamoxifen is able to elicit further oestrogen-like actions which result in elevated cytoplasmic binding site levels. The above data are not compatible with the hypothesis that the sole action of tamoxifen lies in its ability to compete with oestradiol-17β for its cytoplasmic binding sites. It would appear that tamoxifen, bound to the cytoplasmic binding protein, enters the nucleus and associates with chromatin acceptor sites, thus affecting the rates of transcription of the DNA template. Such a phenomenon has been demonstrated in other studies from this group [13] which show that the administration in vivo of either oestradiol-17β or tamoxifen brings about an early elevation in nuclear RNA polymerase B activity. It would seem reasonable that the secondary effects observed in the cytoplasmic oestradiol-17β receptor levels following tamoxifen treatment are a direct consequence of an interaction of the tamoxifen-receptor complex with chromatin, resulting in the production of mRNA species coding for specific proteins including the receptor for oestradiol-17β.

Tamoxifen, unlike oestradiol-17β, is unable to maintain elevated cytoplasmic receptor levels, which diminish during the 24–48 hr experimental period. The effect was also reported by Clark, Anderson and Peck [14] and Rochefort and Capony [15] for nafoxidine and for CI628 by Katzenellenbogen and Furguson [16] using rat uteri. The results of Clark et al. [14] clearly indicate that nafoxidine acts as an atypical oestrogen in that it can stimulate uterine growth over extended periods of time (up to 19 days), but when administered either simultaneously with oestradiol-17β or when injected 24 hr before oestradiol-17β, acts as an oestrogen-antagonist. Furthermore, the nafoxidine-receptor complex present in nuclear fractions remains elevated, although there is no apparent replenishment of the cytoplasmic oestradiol-17β binding protein. The elevated retention of the ligand-receptor complex was not noted in DMBA-tumour nuclear fractions following the administration of tamoxifen. This may in part account for its anti-tumour activity since continued high levels of ligand-receptor complex seem to be equatable with oestrogen-like responses. However, the small amounts of [3H]oestradiol-17β exchangeable material present in rat mammary tumour nuclei 24 hr and 48 hr after tamoxifen treatment should not be disregarded. It may represent a limited number of tamoxifen-receptor complexes in chromatin resulting from...
either a relatively strong association of the receptor with specific acceptor sites or it may be a consequence of a low clearance rate for tamoxifen and its metabolites from the animal [17].

Tamoxifen treatment therefore results in a depletion of tumour cytoplasmic oestradiol-17β binding sites, a condition which appears to be refractory to further oestrogen action, since in vivo administration of oestradiol-17β at this time fails to stimulate receptor uptake into tumour cells nuclei or replenish cytoplasmic binding sites. The tumour is therefore in an oestradiol-17β non-responsive state, a condition which must ultimately result in a regression of tumour mass.

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REFERENCES


11. W. Powell-Jones, Personal communication.


EARLY INCREASES IN RIBONUCLEIC ACID POLYMERASE ACTIVITIES OF DIMETHYLBENZANTHRACENE-INDUCED MAMMARY TUMOUR NUCLEI IN RESPONSE TO OESTRADIOL-17β AND TAMOXIFEN

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SUMMARY

Studies on the mode of action of tamoxifen have shown that this compound ultimately causes regression of mammary tumours induced in female rats by 7,12-dimethylbenz(a)anthracene, but induces preliminary effects similar to those produced by oestradiol-17β. Following a single intravenous injection of either substance, a sequence of events was observed which included depletion of cytoplasmic receptor, a concomitant increase in nuclear receptor and a subsequent replenishment of cytoplasmic receptor. Tamoxifen and oestradiol-17β induced a transient increase in RNA polymerase B activity, followed by increases in RNA polymerase A and, again, RNA polymerase B activity. Tamoxifen, unlike oestradiol-17β, could not maintain replenishment of cytoplasmic receptor, the increase in RNA polymerase A activity or the secondary rise in RNA polymerase B activity. The basic anti-oestrogenic properties of tamoxifen may be implicit in its inability to maintain oestrogenic stimulation, and may be linked to its retention time within the nuclei.

INTRODUCTION

The action of oestradiol-17β in a target tissue is apparently dependent upon the obligatory association of the steroid with a cytoplasmic receptor protein of selective high affinity (Jensen, Mohla, Gorell & DeSombre, 1974; O'Malley & Means, 1974). Subsequent events leading to growth response of the tissue are prevented if the ability of tissue components to bind oestrogen is decreased (Jensen, Jacobson, Smith, Jungblut & DeSombre, 1972). Therefore, the development of compounds exhibiting anti-oestrogenic properties is of importance, in view of their potential value in the management of metastatic breast cancer.

Previous studies (Nicholson & Golder, 1975; Jordan & Dowse, 1976; Nicholson, Golder, Davies & Griffiths, 1976) have shown that tamoxifen [I.C.I. 46474: 1-([p-β-dimethylaminoethoxyphenyl]-1,2-diphenylbut-1-ene] reduces the oestrogen-binding capacity in vitro of cytosol fractions derived from mammary tumours induced in rats by 7,12-dimethylbenz(a)anthracene (DMBA). However, there are indications (Nicholson et al. 1976; Nicholson, Davies & Griffiths, 1977) that tamoxifen can produce similar qualitative effects to oestradiol-17β in terms of translocation of receptor from the cytoplasm to the nucleus. This might possibly influence gene expression.

In the uterus, oestradiol-17β increases RNA synthesis (Hamilton, Teng, Means & Luck, 1971; Knowler & Smellie, 1971). This increase appears to be dependent upon an early rise in synthesis of DNA-like RNA without which the stimulation of total RNA synthesis cannot
occur (Knowler & Smellie, 1971; Luck & Hamilton, 1972; Borthwick & Smellie, 1975). Supporting evidence has been provided by an early stimulation of RNA polymerase B activity, followed by an increase in RNA polymerase A activity and a second rise in RNA polymerase B activity, after administration of oestradiol-17β (Glasser, Chytíl & Spelsberg, 1972; Borthwick & Smellie, 1975). The purpose of this investigation was to compare the early effects of oestradiol-17β and tamoxifen on similar systems in DMBA-induced mammary tumours, to clarify the means by which tamoxifen induces early oestrogen-like responses in these tissues but ultimately causes regression.

**MATERIALS AND METHODS**

**Animals**

Mammary tumours were induced in virgin female Sprague–Dawley rats (50 ± 2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of four and allowed food and water ad libitum. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals. Tumour size was recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23-week period after carcinogen administration were not used in the study. Experimental animals were given a single dose of either oestradiol-17β (5 μg) or tamoxifen (100 μg) in 100 μl vehicle [10% (v/v) ethanol in NaCl (0.15 mol/l)] by intravenous injection. Control animals received vehicle alone. Biopsy samples were taken at various times after injection (as detailed in the text).

**Chemicals**

[2,4,6,7-3H]Oestradiol (specific activity 85 Ci/mmol) and [5-3H]UTP (specific activity 10-1 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks. Non-radioactive oestradiol-17β, diethylstilboestrol [3,4-bis-(p-hydroxyphenyl)hex-3-ene] and dithiothreitol were provided by Koch–Light Laboratories Ltd, Colnbrook, Bucks. α-Amanitin, the disodium salt of ATP and the trisodium salts of CTP, GTP and UTP were bought from the Boehringer Corporation (London) Ltd, Ealing, London. Crystalline bovine serum albumin, calf-thymus DNA and Tris-base (99% pure) were obtained from the British Drug Houses Ltd, Poole, Dorset. The DMBA was a product of Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey. Tamoxifen was a gift from Drs A. L. Walpole and D. N. Richardson of I.C.I. Pharmaceuticals, Alderley Park, Cheshire. All other compounds were of Analar grade.

**Preparation of subcellular fractions**

Soluble supernatant (cytosol) preparations were obtained as described by Nicholson et al. (1976) using an homogenization procedure resulting in maximum breakdown of tumour cell membranes with minimum damage to cell organelles (Nicholson & Davies, 1974). Nuclei were prepared by a method described by Davies & Griffiths (1975).

**Measurement of specific binding sites for oestradiol-17β**

Accessible cytoplasmic oestradiol-17β-binding sites were estimated according to Nicholson et al. (1976). Nuclear oestradiol-17β-binding sites were measured by the method of Nicholson et al. (1977). Briefly, aliquots of nuclear fractions (200 μl, 50–100 μg DNA) were mixed with equal volumes of protamine sulphate solution (1 mg/ml) and the resulting precipitates were sedimented, washed, and incubated (2 h at 15 °C) with [3H]oestradiol-17β (200 μl; 20 nmol/l)], either alone or with non-radioactive diethylstilboestrol (20 pmol/l)]. After incubation, tubes were chilled and precipitates were sedimented and washed copiously before
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extraction (16 h) with ethanol (500 µl). Ethanolic supernatants were decanted into scintillation vials, dried and counted in a scintillation fluid (6 ml: 4 g PPO, 50 mg PPO per litre toluene) in a Nuclear Chicago Mark II Liquid Scintillation Spectrometer.

The above conditions of assay have been shown to be optimal for minimal degradation of receptor with maximum replacement of endogenously bound ligand with exogenous \[^3H\]oestradiol-17β.

Estimation of RNA polymerase activity

For the purposes of this study, it was necessary to measure activities of RNA polymerases A and B separately in intact nuclei, but in the presence of each other. This was achieved by using two assay mixtures, based on described procedures (Glasser et al. 1972; Borthwick & Smellie, 1975), which exploit the differential response of the two RNA polymerase species to salt, and to the fungal toxin, α-amanitin.

Reaction mixtures (500 µl) routinely contained Tris–HCl buffer (60 µmol), pH 7-9, KCl (15 µmol), dithiothreitol (200 nmol), NaF (300 nmol), ATP, GTP and CTP (all 300 nmol), \[^3H\]UTP (0-5 µCi), UTP (20 nmol) and 10% (v/v) glycerol. Assays for RNA polymerase A alone also contained MgCl₂ (2-5 µmol) and α-amanitin (40 ng). Assays for RNA polymerase B alone also contained MnCl₂ (1-5 µmol) and (NH₄)₂SO₄ (125 µmol). Solutions of (NH₄)₂SO₄ were adjusted to pH 7-9 with aqueous NH₃ (2 mol/l) before mixing with the other components of the assay mixture. Preliminary experiments confirmed that, under the latter conditions of assay, all RNA polymerase was sensitive to α-amanitin. Nuclear suspensions (50–100 µg DNA) were incubated in the medium for 15 min at 37 °C. Processing of acid-insoluble material for estimation of incorporation of \[^3H\]UMP into polyribonucleotide linkage, as well as descriptions of adequate control systems, has been described previously (Davies & Griffiths, 1973, 1974). Under these conditions of experimentation, 55 d.p.m. were equivalent to 1 pmol of incorporated \[^3H\]UMP.

Chemical analyses

Protein concentration of fractions were assessed by the method of Lowry, Rosebrough, Farr & Randall (1951), using crystalline bovine serum albumin as standard. DNA content of fractions was estimated by the procedure of Burton (1956), using calf-thymus DNA as standard.

RESULTS

The major purpose of these experiments was to observe any correlations between concentrations of cytoplasmic binding sites, nuclear binding sites, and the activities of RNA polymerases A and B, in mammary tumour cells after the administration of either oestradiol-17β or tamoxifen. As shown in Fig. 1a, within 1 h of injection, both compounds caused a decrease in cytoplasmic receptor sites below the levels seen in control cytosols, although the depletion brought about by oestradiol-17β was greater than that caused by tamoxifen. Concomitantly, a corresponding increase in nuclear receptor concentration was observed (Fig. 1b).

These studies were extended to include phenomena occurring up to 48 h after oestrogen or anti-oestrogen administration (Fig. 2). The depletion in cytoplasmic binding sites caused by either substance was maximal after 1 h, and was followed by a considerable rise or replenishment of cytoplasmic receptor (Fig. 2a). Replenishment induced by oestradiol-17β attained its highest level 16 h after injection, and had fallen to control levels 48 h after injection. The increase caused by tamoxifen, however, was maximal after 8 h, and fell to levels considerably below the control level by 48 h. Increases in nuclear receptor were greatest 2-4 h after administration of either substance (Fig. 2b) and gradually decreased to control levels over the 48 h period following injection.
Fig. 1. Intracellular receptor concentrations after treatment of rats with oestradiol-17β or tamoxifen. Mammary-tumour-bearing rats were given a single dose of 5 μg oestradiol-17β (○), 100 μg tamoxifen (●) or vehicle alone (△) by intravenous injection, and biopsy samples were removed at various times up to 60 min after treatment. Cytoplasmic (a) and nuclear (b) binding sites were measured as described in 'Materials and methods'. Values represent the mean ± S.E.M. of estimates carried out on subcellular fractions derived from mammary tumours from four to five animals.

Fig. 2. Receptor concentrations in subcellular fractions after treatment of rats with oestradiol-17β or tamoxifen. Mammary tumour-bearing rats were given a single dose of 5 μg oestradiol-17β (○), 100 μg tamoxifen (●) or vehicle alone (△) by intravenous injection, and biopsy samples were removed at various times up to 48 h after treatment. Cytoplasmic (a) and nuclear (b) binding sites were measured as described in 'Materials and methods'. Values are the means ± S.E.M. of results from mammary tumours from four to five animals.

Associated with the apparent transference of ligand-bound receptor into nuclei, a rapid increase of activity of RNA polymerase B was noted (Fig. 3b). The effect was more rapid after administration of oestradiol-17β, reaching a maximum 10–30 min after injection. Tamoxifen induced a more gradual increase in activity of RNA polymerase B, attaining highest levels after 40 min. Activity of RNA polymerase A was at control levels 60 min after the administration of either substance. At this time, a rise in RNA polymerase A activity in
Fig. 3. Alterations in activity of RNA polymerases resulting from administration of oestradiol-17\beta or tamoxifen. Mammary tumour-bearing rats were given a single dose of 5 µg oestradiol-17\beta (○), 100 µg tamoxifen (●) or vehicle alone (△) by intravenous injection, and biopsy samples were removed at various times up to 60 min after treatment. RNA polymerase A (a) and RNA polymerase B (b) were each measured under conditions which precluded activity of the other species. Control (100%) activities, in terms of [\text{H}]UMP incorporation, were 5.4 ± 0.6 pmol/100 µg DNA for RNA polymerase A and 18.3 ± 1.4 pmol/100 µg DNA for RNA polymerase B. Values are the means ± S.E.M. of four or five animals.

Fig. 4. Effects of oestradiol-17\beta or tamoxifen on activities of RNA polymerases. Mammary tumour-bearing rats were given a single dose of 5 µg oestradiol-17\beta (○), 100 µg tamoxifen (●) or vehicle alone (△) by intravenous injection and biopsy samples were removed at various times up to 24 h after treatment. The activities of RNA polymerase A (a) and RNA polymerase B (b) were measured in intact nuclei from mammary tumours. Values are the means ± S.E.M. of results from four or five animals.
mammary tumour nuclei of oestradiol-17β-treated rats was observed, but in mammary
tumour nuclei of tamoxifen-treated rats the activity of RNA polymerase A was not signifi-
cantly higher than in controls (Fig. 3a).

These effects were again studied over longer periods of time (Fig. 4). Oestradiol-17β and
tamoxifen caused increases in activity of RNA polymerase A in mammary tumour nuclei
(Fig. 4a). Oestradiol-17β-induced rises were greatest 2 h after injection and were maintained
at these high levels over the 24 h after injection. Increases caused by tamoxifen were lower
than those brought about by oestradiol-17β, reaching a maximum 4 h after injection and
soon returning to control levels. Both compounds, however, produced large secondary
increases in RNA polymerase B activity (Fig. 4b), although oestradiol-17β was able to
maintain increased activity whereas tamoxifen could not.

**DISCUSSION**

The results described in this paper indicate that tamoxifen and oestradiol share similar
properties in their ability to elicit three phenomena: (1) early depletion and later replenish-
ment of cytoplasmic receptor; (2) translocation of receptor into the nucleus; (3) stimulation
of the activities of RNA polymerases A and B. Obviously, based on the accepted hypothesis
of the mechanism of steroid hormone action (Jensen et al. 1974; O'Malley & Means, 1974)
these effects are interrelated.

Quantitative and temporal differences between the responses in RNA polymerase activity
to the two compounds may be traced to the comparative abilities of the substances to transfer
receptor efficiently from the cytoplasm to the nucleus and, possibly, to specific acceptor sites
within the chromatin. Similarly, the inability of tamoxifen to maintain high levels of RNA
polymerase activities and replenishment of cytoplasmic receptor may show the dependence
of the latter on the former and of both on the retention time of the compound within the
nuclei. This relative inefficacy of tamoxifen as an oestrogen may be also responsible for its
ability to cause tumour regression (Nicholson & Golder, 1975).

In order to stimulate or maintain true growth of a target tissue, the ligand–receptor com-
plex may need to occupy nuclear sites for a minimum finite period of time (Anderson, Peck
Katzenellenbogen (1976) examined extensively the temporal relationships between hormone–
receptor binding and early and late biological responses in the uterus. Chemical modifica-
tions of the oestriol molecule which result in prolonged stimulation of uterine growth and
metabolism also result in prolonged retention of the hormone–receptor complex in the
nucleus. This strongly supports the stated concept. Tamoxifen may not fulfill this criterion
as well as oestradiol-17β, and inefficient translocation of receptor by tamoxifen may result
in insufficient pools of receptor necessary for the continuation of various events. In this
case, the transient rise of RNA polymerase B over the first hour after tamoxifen administra-
tion, suggesting putative mRNA synthesis, may be sufficient to trigger possible protein
synthesis leading to greater production of total RNA, and maybe replenishment of receptor,
but this initial effect requires also the continued presence of the ligand–receptor complex.
Tissue regression following the entry of the tamoxifen–receptor complex into the nucleus
may be explained by changes in early protein synthesis and subsequent modification of the
function of the genome.

Tamoxifen, an efficient anti-oestrogen, exhibits several oestrogen-like characteristics.
Similar properties have been reported for other anti-oestrogenic substances (Katzenellen-
bogen & Ferguson, 1975). However, not all superficially oestrogenic compounds can stimu-
late true growth (Hardin, Clark, Glasser & Peck, 1976). A series of events is necessary for a
proliferative response, of which receptor translocation and stimulation of RNA polymerase
are among the very first. Failure to induce efficiently and maintain these responses may be the basis of the anti-oestrogenic properties of tamoxifen.

We have shown previously that the ultimate failure of tamoxifen to replenish the oestradiol-17β receptor protein appears to be refractory to further oestrogen action (Nicholson et al. 1977). These data, with our current findings, are consistent with the hypothesis (Clark, Peck & Anderson, 1974) that non-steroidal anti-oestrogens are antagonists because they cannot maintain receptor replenishment. Tissues are therefore non-responsive to oestradiol-17β, a condition that finally results in tissue atrophy or regression.

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REFERENCES


TAMOXIFEN BINDING IN MAMMARY TUMOURS IN RELATION TO RESPONSE

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The selective high-affinity binding of oestradiol-17β to its specific cytoplasmic receptor protein and the subsequent transfer to and retention of the receptor complex within the nucleus are thought to be obligatory features of the action of oestradiol-17β (1). Compounds which interfere with these events may prevent oestrogens from exerting their full influence on target tissues. A number of studies (2,3) have now shown that a series of compounds based on triphenylethylene inhibit the activity of oestradiol-17β, possibly by competing for receptor sites, thereby decreasing the number of oestradiol-17β-receptor complexes formed and transferred to the nucleus. Such anti-estrogenic compounds are of potential value in the management of metastatic breast cancer since approximately 30% of patients show an objective remission of tumour growth following ablative or endocrine therapy.

Tamoxifen, the trans isomer of 1-(p-(3-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene, has potent anti-estrogenic activity in the rat (4,5). In man, tamoxifen has been used to induce ovulation (6) and to regulate dysfunctional uterine bleeding (7). Its anti-estrogenic properties have led to its clinical use in advanced breast cancer in postmenopausal women (8,9,10). Tamoxifen produced objective breast tumour remissions in 22% (8), 36% (9) and 42% (10) of patients and partial remissions in a further 36% (9) and 37% (8) of tumours. Clearly a test pre-selecting those patients with tamoxifen-responsive tumours would be valuable. Jensen, Block and Smith (11) suggested that the presence of the oestradiol-17β receptor
in human breast cancer indicated the oestrogen dependence of the tumour. More recently, several investigators have reported that the receptor content of tumours was related to the response of the tumour to tamoxifen administration (12,13). Patients with oestradiol-17β-receptor-positive tumours show a more favourable response to the drug. Exceptions were, however, evident and in some cases the presence or absence of oestrogen receptors did not relate to the subsequent response. More detailed information concerning the mechanism of action of tamoxifen in breast tumour tissue was obviously required.

In the dimethylbenzanthracene (DMBA)-induced mammary tumour of the rat, a model-system for hormone-dependent human breast cancer, it is thought that tamoxifen bound to cytoplasmic oestradiol-17β receptor proteins (14,15,16) is translocated as a complex to the nucleus (17) and associates with chromatin (18). Any one, or all, of these steps may be critical to the mechanism by which tamoxifen influences tumour growth. Previously, the principal method that we have applied to the determination of the antioestrogenic nature of tamoxifen has involved the use of a technique, whereby $[^3H]$oestradiol-17β is specifically exchanged for receptor-bound antioestrogen (16,17). However, the current availability of tamoxifen in a form with high specific radioactivity (Fig. 1) permits a direct study of the binding properties of this compound to cytoplasmic proteins isolated from the DMBA-induced mammary tumour. It was hoped that such studies would be of prognostic value to determine tumour response to tamoxifen treatment and would form the basis for similar studies in man.

Fig. 1. Chemical structure of tamoxifen.
ASSOCIATION OF TAMOXIFEN WITH INTRACELLULAR BINDING COMPONENTS

Determination of the Number and Affinity of Cytoplasmic Binding Sites for \(^{3}\)H Oestradiol-17\(\beta\) and \(^{3}\)H Tamoxifen.

Our first studies compared the binding properties of \(^{3}\)H oestradiol-17\(\beta\) [6,7(n)-\(^{3}\)H\(\_\_\_\_\_\_\_\_\_\) oestradiol-17\(\beta\), specific activity, 42 Ci/mmol, Radiochemical Centre, Amersham, U.K.] and \(^{3}\)H tamoxifen (specific activity, 19.2 Ci/mmol, ICI Pharmaceuticals Limited, Alderly Edge, Cheshire, U.K.). This involved saturation analysis in which free radioactive steroid was adsorbed by dextran-coated charcoal (16). The binding obtained from incubating increasing

\[\text{Fig. 2. Determination of the number of specific oestradiol-17\(\beta\) and tamoxifen binding sites in rat mammary tumour tissue. (a) Cytosol fractions were incubated for 16h at 40\(^\circ\)C with various concentrations of either (i) \(^{3}\)H oestradiol-17\(\beta\) or (ii) \(^{3}\)H tamoxifen (Q) in the presence or absence of a 1000 fold excess of unlabelled diethylstilboestrol (O). Specific binding was obtained by subtraction of the counts obtained in the presence of diethylstilboestrol from those obtained in its absence. (b) Scatchard analysis of the corrected values.}\]
concentrations of radioligands with cytosol preparations was analysed by the method of Scatchard (19) and the dissociation constant, $K_D$, and the receptor site concentration, $P_Q$, determined (Fig. 2). Comparative binding studies were carried out on the same cytosol preparations to establish any relationship between the binding characteristics of the two compounds.

The binding of oestradiol-17β by non-specific components was a linear function of the $[^{3}H]$oestradiol-17β concentration (Fig. 2). Subtraction of this non-specific binding from the value obtained in the presence of $[^{3}H]$-oestradiol-17β alone, showed saturability of binding sites at approximately 3-5 nmol/l. Scatchard analysis of the corrected values yielded a $K_D$ of 1.4 nmol/l and a receptor site concentration of 153 fmol/mg protein. Similar incubations using $[^{3}H]$tamoxifen demonstrated a high non-specific binding component. Nevertheless, subtraction of this value from the value determined in the presence of $[^{3}H]$tamoxifen alone produced saturability of binding sites between 20 and 30 nmol/l. A $K_D$ of 10 nmol/l was obtained for $[^{3}H]$tamoxifen binding and a binding site concentration of 166 fmol/mg protein.

Eight tumours have been examined to date by this method. The dissociation constants obtained for $[^{3}H]$-tamoxifen binding were 5 to 10 fold higher than those for oestradiol-17β (not illustrated). The majority of tumours contained approximately the same number of binding sites.

Specificity of $[^{3}H]$Oestradiol-17β and $[^{3}H]$Tamoxifen Binding

The affinities of various steroids and tamoxifen for the cytoplasmic oestradiol-17β receptor were examined by a competitive binding assay. Cytosol was incubated with a fixed concentration of $[^{3}H]$oestradiol-17β (5 nmol/l) alone or with increasing concentrations of potential antagonists (Fig. 3). A 5 fold excess of either oestradiol-17β or diethylstilboestrol decreased the binding of $[^{3}H]$oestradiol-17β to approximately 45% of the control value. Binding decreased further with high concentrations of these competitors. Tamoxifen and 5-androstene-3β,17β-diol in a 1000 fold excess decreased the binding of $[^{3}H]$oestradiol-17β to 30 and 35% of the control value respectively, while 5α-androstane-3β,17β-diol reduced the binding to 54%. Of the other competitors
Fig. 3. Specificity of \(^{3}H\)oestradiol-17\(\beta\) and \(^{3}H\)tamoxifen binding assays. Cytosol fractions were incubated for 2h at 4°C with a saturating concentration of either \(^{3}H\)oestradiol-17\(\beta\) (5 nmol/l) or \(^{3}H\)tamoxifen (20 nmol/l) in the presence and absence of increasing concentrations (5 to 1000- fold excess of the potential antagonists. \(A\), oestradiol-17\(\beta\); \(B\), diethylstilboestrol; \(C\), tamoxifen; \(D\), 5-androstene-3\(\beta\), 17\(\beta\)-diol; \(E\), 5a-androstane-3\(\beta\), 17\(\beta\)-diol; \(F\), testosterone; \(G\), dihydrotestosterone; \(H\), progesterone, and \(J\), cortisol. Free and non-specifically bound radioactivity was removed by charcoal adsorption.

examined, only testosterone and dihydrotestosterone reduced \(^{3}H\)oestradiol-17\(\beta\) binding.

Competitive binding assays performed using \(^{3}H\)tamoxifen and the same competitors gave a similar gradation of results.

Sedimentation Analysis of \(^{3}H\)Oestradiol-17\(\beta\) and \(^{3}H\)Tamoxifen Binding Components

The sedimentation characteristics of cytosolic components binding \(^{3}H\)oestradiol-17\(\beta\) and \(^{3}H\)tamoxifen were examined on linear 5-20\% (w/v) sucrose density gradients under low salt conditions. Two procedures were used: (i) incubation of the cytosol fractions with the radioligands and competitors prior to their centrifugation through the sucrose gradients and (ii) centrifugation of the cytosol fractions through the gradients prior to their incubations with the radioligands (detailed in Fig. 4).
Fig. 4. Sucrose density gradient profiles of cytosol fractions. Method 1: Samples of cytosol prepared from a rat mammary tumour were labelled at 0°C with either (³H) oestradiol-17B (5nmol/l) or (³H) tamoxifen (20 nmol/l) in the presence or absence of various other compounds. Free and non-specifically bound steroid were removed by treatment with dextran-coated charcoal and portions (400 ul) were layered on linear 5-20% (w/v) sucrose density gradients under low salt conditions (sucrose solutions contained 10 mM Tris HCl buffer pH 7.4, which also included 5mM EDTA and 5mM dithiothreitol) and centrifuged for 18 h at 100,000 gav at 3-4°C. Direction of centrifugation was left to right. Sedimentation marker(arrow) was bovine serum albumin (S20, w1.6S) in each case. (a) Rat mammary tumour cytosol labelled with (³H)-oestradiol-17B alone (o) or in the presence of either 0.5 umol/l cold oestradiol-17B (●) or 5 umol/l cold tamoxifen (▲); (b) cytosol as in (a) labelled with (³H) tamoxifen alone (o) or in the presence of 20 umol/l oestradiol-17B (●).

Method 2: The experimental procedure was as described in Method (1) except the cytosol fractions were centrifuged through the gradients prior to their incubation with either (a) (³H) oestradiol-17B in the presence or absence of competitors or (b) (³H) tamoxifen with or without cold oestradiol-17B.
Analysis of the labelled cytosol preparations from the mammary tumour using method (i) showed the characteristic 8S binding peak for $[^3H]$oestradiol-17$\beta$ (Fig. 4a). The low capacity of this peak is demonstrated by the displacement of the radioactivity by a 100-fold higher concentration of oestradiol-17$\beta$ or a 1000-fold excess of tamoxifen. Similar incubations performed with $[^3H]$tamoxifen reveal only 4-5S binding components (Fig. 4b). There was no displaceable $[^3H]$tamoxifen binding in the 8S region of the gradient.

Centrifugation of the cytosol preparations prior to their incubation with the radioligands (method (ii)) demonstrated displaceable $[^3H]$oestradiol-17$\beta$ and $[^3H]$tamoxifen binding in the 7-8S region of the gradient (Fig. 4). A cytosol fraction prepared from a mammary tumour with no 8S oestradiol-17$\beta$ binding peak showed no displaceable $[^3H]$tamoxifen binding in either the 4 or 8S regions (not illustrated).

PROGNOSTIC DETERMINATION OF TUMOUR RESPONSE TO TAMOXIFEN TREATMENT

As with human mammary tumours there exists a fairly heterogeneous response of DMBA-induced rat mammary tumours to tamoxifen treatment. We therefore examined the binding of $[^3H]$oestradiol-17$\beta$ and $[^3H]$tamoxifen to mammary tumour cytosol fractions in an attempt to rationalise this phenomenon.

Mammary tumours used in this experiment were approximately 2 cm mean diameter and were biopsied before and after treatment. The biopsies were then examined for their capacity to bind $[^3H]$oestradiol-17$\beta$ and $[^3H]$tamoxifen in a specific manner. The values obtained were plotted against the response of the tumour to a four week treatment regime (100 $\mu$g/day for 5 days/week). Tumour size was recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain a size of 2 cm mean diameter in the 15 week period following carcinogen administration were excluded from the study.
Tumour Growth Characteristics on Tamoxifen Treatment

Analysis of the growth characteristics of mammary tumours following tamoxifen treatment showed three distinct patterns: continued growth, no growth and regression (not illustrated). The majority of tumours (approximately 80%) fell into this latter category.

\[^{3}\text{H}]\text{Oestradiol-17\beta}\) binding (displaceable by tamoxifen) and tumour growth

The data presented in Fig. 5 demonstrates that at low levels of oestradiol-17\beta binding (\(<8 \text{ fmol/mg protein}\)) the tumours remain either static or continue to grow. This group represents approximately 20% of the tumour population and was autonomous, since ovariectomy did not affect tumour growth. The cut off point of 8 \text{ fmol/mg protein} was determined previously by the response of the tumour population to ovariectomy (Nicholson, unpublished observation). As the concentration of oestrogen receptors increased there was an apparent increased probability of a more extensive tumour regression. Analysis of the log, transformed oestradiol-17\beta binding data (Fig. 6), demonstrated a correlation between receptor content and tumour response to tamoxifen treatment. The correlation was maintained when non-responsive tumours were removed from the calculation.

![Graphs showing relationship between oestradiol-17\beta binding and tamoxifen response](image)

**Fig. 5.** Relationship of \[^{3}\text{H}]\text{Oestradiol-17\beta}\) and \[^{3}\text{H}]\text{Tamoxifen}\) binding to tumour response to Tamoxifen treatment. Cytosol fractions obtained from biopsy samples were incubated for 2h at 4\(^\circ\)C with a saturating concentration of either \(^{3}\text{H}\) oestradiol-17\beta (5.0 \text{ nmol/l}) or \(^{3}\text{H}\) tamoxifen (20 \text{ nmol/l}) in the presence and absence of a 1000 fold higher concentration of tamoxifen on oestradiol-17\beta respectively. Free and non-specifically bound radioactivity was removed by charcoal adsorption. Tumour size was measured at 4 weeks as the mean of two diameters.
[\[^{3}H\] Tamoxifen Binding (Displaceable by Oestradiol-17β) and Tumour Growth

Figs. 5 and 6 show the data obtained from a direct study of displaceable [\[^{3}H\] tamoxifen binding to the cytoplasmic oestradiol-17β receptor. Although an overall correlation between tumour response to tamoxifen treatment and receptor content was observed, no predictive discriminant of receptor content and the degree of regression response to tamoxifen treatment was seen.

DISCUSSION

The aim of the present study was to examine the binding of [\[^{3}H\] tamoxifen to cytoplasmic binding components from DMBA-induced rat mammary adenocarcinomata in relation to two specific phenomena: (i) the mechanism by which this antioestrogen influences tumour growth and (ii) the prognostic determination of tumour response to tamoxifen treatment. These phenomena are obviously related.
Tamoxifen binds to the cytoplasmic oestradiol-17β receptor protein from the mammary tumour with approximately one tenth the affinity of that shown by oestradiol-17β. Scatchard analysis of the corrected data indicated a single class of binding sites with high affinity for these compounds. These data are in agreement with those obtained in rat uterine cytosol fractions (20,21). The apparently lower affinity of tamoxifen for the oestradiol-17β receptor based on comparative binding studies in relation to those obtained in direct binding studies (i.e. 40 and 5-10 fold respectively) is interesting, although no explanation is readily obvious. It is possible however that in the comparative binding studies tamoxifen may compete with and displace [3H]oestradiol-17β from a number of its non-specific binding sites thereby increasing the free [3H]oestradiol-17β to tamoxifen ratio and lowering the fold excess of tamoxifen. Alternatively since [3H]tamoxifen binds avidly to most surfaces, such a phenomenon could artefactually lower the unlabelled tamoxifen concentration in competitive binding studies. The relative contributions of these possibilities awaits clarification.

Specificity studies carried out on the binding of receptor saturating concentrations of [3H]oestradiol-17β and [3H]tamoxifen demonstrates that the binding protein or proteins have similar affinity characteristics for steroidal oestrogen antagonists. These data suggest a common binding site for these two compounds. However, the exact mechanism of interaction may differ. A consideration of the structures of tamoxifen (Fig. 1) and oestradiol-17β does not reveal many similarities. Hahnel, Twaddle and Ratajczak (22) have postulated that the attachment of oestradiol-17β to the receptor probably occurs via two groups, the phenolic hydroxyl group on C-3 to a highly specific site facilitating the attraction of the C-17β-hydroxyl function to a less specific binding site. The mechanism by which the antioestrogen, tamoxifen, binds to the receptor is less easy to define. It has been suggested (14,22) that the binding may occur through the aromatic N-ethyl ether grouping by attraction to the more active centre. This forms an acceptable hypothesis since the majority of systemic antioestrogens contain the N-ethylether grouping. Substitution of the NN-dimethyl group by a NN-(2-chloroethyl) group to produce ICI 79792 decreases the affinity of the compound for the oestradiol-17β receptor and decreases its antioestrogenic properties (14,23). Alternatively, since a major metabolite of tamoxifen in the rat has been shown to be a hydroxylated
derivative (24) it is possible that the hydroxyl group may be critical in the primary or secondary attachment of the oestrogen receptor. Further research is obviously warranted into this particular aspect of the mechanism of action of tamoxifen.

Analysis of [\(^{3}\text{H}\)]tamoxifen and [\(^{3}\text{H}\)]oestradiol-17\(\beta\) labelled cytosol fractions on sucrose density gradients under low-salt conditions reveals an interesting property of the antioestrogen receptor complex. Unlike the oestradiol-17\(\beta\) receptor complex which characteristically binds in the 8S region of the gradient, the tamoxifen receptor complex binds only in the 4-5S region. There are several possible explanations for this occurrence. Tamoxifen and oestradiol-17\(\beta\) may not bind to the same protein. However, this seems unlikely since tamoxifen competes efficiently for [\(^{3}\text{H}\)]oestradiol-17\(\beta\) binding components on sucrose density gradients (Fig. 4 and also ref: 14,15). Oestradiol-17\(\beta\) also specifically displaces [\(^{3}\text{H}\)]tamoxifen binding in competitive binding studies (Fig. 3) and from its 4-5S binding component (Fig. 4). Alternatively, the binding of tamoxifen to the oestradiol-17\(\beta\) receptor may produce conformational changes in the receptor protein that are different from those produced by oestradiol-17\(\beta\) and are manifested by binding in the 4-5S region of the gradient. To examine this the cytosol fractions were centrifuged through the sucrose density gradients prior to the incubation of the fractions with either [\(^{3}\text{H}\)]oestradiol-17\(\beta\) or [\(^{3}\text{H}\)]tamoxifen. If tamoxifen and oestradiol-17\(\beta\) bound to the same receptor protein then the [\(^{3}\text{H}\)]radioligands would be retained in the same region of the gradients. This was the case, and it was observed that [\(^{3}\text{H}\)]tamoxifen and [\(^{3}\text{H}\)]oestradiol-17\(\beta\) bound in the 7-8S region. The sedimentation value obtained for [\(^{3}\text{H}\)]oestradiol-17\(\beta\) binding was the same as the value obtained elsewhere (25). We believe these results to be the first reported evidence that tamoxifen, or any antioestrogen, may produce conformation changes in the oestrogen receptor protein that are different from those produced by oestradiol-17\(\beta\). Such alterations may be fundamental to the mechanism by which tamoxifen receptor complex binds to chromatin and thereby elicits its anti-tumour activity.

During the period of our current research we have noted a great variation in the receptor content of rodent mammary tumours (0 to >200 fmol/mg cytosolic protein). It was possible that the heterogeneity of receptor levels might reflect the diversity of tumour response to tamoxifen administration. Our present results substantiate
We observed a correlation between receptor content and the ability of tamoxifen to promote tumour regression. Using a cut off point of 8 fmol/mg protein no false positive or false negative values were detected. Similar results were observed after the in vivo administration of [3H]oestradiol-17β (26,27,28). The binding of [3H]oestradiol-17β to its cytoplasmic receptor protein was also found to correlate with the quality of the tumour response to tamoxifen treatment. Previously we have observed that a number of mammary tumours regressing as a result of ovariectomy or tamoxifen administration show a restimulation of tumour growth after approximately 6 weeks (23). The tumours from ovariectomised animals do not respond to tamoxifen treatment and contain low or absent levels of oestrogen binding proteins (Nicholson, manuscript in preparation). These data taken together indicate tumour autonomy and support the theory that the mammary tumour is a composite of hormone dependent and independent clones of cells, the hormone dependent portion of which regress on endocrine therapy, while independent cells progress (29). Rapidly regressing tumours could therefore be seen as containing a large percentage of receptor positive cells. Tumours containing varying proportions of receptor positive to receptor negative cells would behave in a manner integrated between growth or regression, depending on the predominant cell species. The theory, however, undoubtedly represents an over simplification since individual receptor positive cells may contain varying amounts of receptor. We await the histochemical or immunochemical localisation of oestrogen receptors within the tumour tissue to throw some light on this matter.

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L'interaction spécifique entre le Tamoxifen tritié et l'oestradiol tritié avec le receputeur oestrogénique de la tumeur mammaire de rat est d'une grande valeur dans le prédiction de la réponse de la tumeur au traitement au Tamoxifen. Ces deux composés se lient approximativement au même nombre de sites, cependant le Tamoxifen a une affinité plus faible. Contrairement au complexe receputeur oestradiol qui sédimente de façon caractéristique dans la region 8S du gradient sucrrose, quand la concentration en sel est faible, le complexe receputeur Tamoxifen sédimente seulement dans la région 4.5S. Ces résultats sont discutés en fonction du mécanisme général d'action des anti-oestrogènes.

The specific interaction of $[^3H]$Tamoxifen and $[^3H]$oestradiol-17α with the rat mammary tumour oestrogen receptor has been shown to be of some prognostic value to the determination of tumour response to Tamoxifen treatment. The compounds bind to approximately the same number of binding sites, although Tamoxifen binds with lower affinity. Unlike the oestradiol-17α receptor complex which characteristically sediments in the 8S region of a sucrrose density gradient under low salt conditions, the Tamoxifen receptor complex sediments only in the 4-5S region. These findings have been discussed in relation to the general mechanism of action of antioestrogens.
REFERENCES


28. B.G. Mobbs.: The uptake of simultaneously administered \[^3\]H\oestradiol and \[^14\]C\progesterone by dimethylbenzanthracene-induced rat mammary tumours. J. Endocr. 41: 339 (1968)

Simplified Exchange Procedure for the Determination of Receptor Bound Tamoxifen and its Principal Metabolite, Metabolite B


Tamoxifen, a nonsteroidal antiestrogen, inhibits the binding of estradiol-17β to its specific receptor by first associating with the receptor itself. In human and rat mammary tumors, tamoxifen (trans, 1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenyl-but-1-ene) binds to estrogen receptor proteins with approximately 2.5% of the affinity of that shown by estradiol-17β (\(K_a\) values, human, 3.7 \(\times\) 10^{-9} mol/l and 1.2 \(\times\) 10^{-10} mol/l, and rat, 4.34 \(\times\) 10^{-9} mol/l and 1.02 \(\times\) 10^{-10} mol/l for tamoxifen and estradiol-17β, respectively). The affinity values obtained from direct binding studies are, however, greater than those calculated from competitive binding experiments. In addition, the capacity of tamoxifen to compete with \(^{[3]H}\)estradiol-17β for estrogen receptor sites apparently decreases with increasing incubation time (Fig. 1). The effect was not so marked for the principal metabolite of tamoxifen, metabolite B (1-(4-β-dimethylaminoethoxyphenyl)-1, hydroxyphenyl, 2-phenyl-1-ene), a potent antiestrogen whose relative affinity for the estrogen receptor is approximately one-quarter that of estriol.

The relatively low binding affinity of tamoxifen for the estrogen receptor is reflected by its rapid dissociation from the receptor protein, such that following an incubation period of 24 hr at 4°C, endogenously bound tamoxifen may be specifically replaced by \(^{[3]H}\)estradiol-17β (Fig. 2). Under conditions identical to those described above, only small quantities of metabolite B and estriol were released. No substantial release of either estradiol-17β or diethylstilbestrol was observed. Elevation of the incubation temperature to 15°C resulted in the dissociation of all the compounds studied. These data are consistent with the calculated reverse rate constants for estradiol-17β and tamoxifen dissociation from the estrogen receptor (\(k_d\), 0.88 \(\times\) 10^{-4} min^{-1} and 2.0 \(\times\) 10^{-4} min^{-1}, respectively).

Application of this information to the competitive binding studies may serve to explain both the apparent time dependent variations in the affinity of tamoxifen
For estrogen receptor proteins and the affinity differences obtained between the direct and indirect methods. It is likely that during the early phase of competition tamoxifen competes readily with estrogen binding sites (forward reaction rate constants, $k_1$, $0.45 \times 10^4$ l/mol/min and $0.86 \times 10^4$ l/mol/min, respectively). At 4°C, however, tamoxifen, but not estradiol-17β, dissociates from the receptor and thus may be replaced by $[\text{H}]{\text{estradiol-17\beta}}$ at the expense of the antiestrogen. Tamoxifen can, therefore, be seen as decreasing the association rate of estradiol-17β to its
receptor protein. A similar effect of tamoxifen, nafoxidine, and dimethylstilbestrol has been observed with estrogen receptor from immature rat uteri.\(^3\)

In the light of these data it is possible that simple manipulative procedures such as those described above may form the basis of an exchange assay differentiating between receptor bound tamoxifen and metabolite B. The determination of such information is likely to be of prognostic significance where patients are pretreated with tamoxifen prior to the surgical removal of the primary tumor, or the biopsying of secondary deposits. The present investigation may also have important implications with respect to the mechanism of action of tamoxifen. It is attractive to postulate that at least part of the antiestrogenicity of the molecule resides in its rapid dissociation from the estrogen receptor.

References

The Binding of Tamoxifen to Oestrogen Receptor Proteins under Equilibrium and Non-Equilibrium Conditions

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Abstract—Data have been presented from sucrose density gradient analysis, protamine sulphate precipitation and specificity studies to show that oestradiol-17β and tamoxifen share a common binding protein. In addition, the binding of [3H]-tamoxifen to cytosol preparations from human and rat mammary adenocarcinomas and rat uteri was measured under equilibrium conditions using Scatchard analysis. The relative affinity values obtained were, however, greater than those calculated from competitive binding studies. In addition the competition between tamoxifen and oestradiol-17β for oestrogen binding sites at 4°C decreased with increasing incubation time. Based on competitive binding experiments, the principal metabolite of tamoxifen, metabolite B, associated with oestrogen receptor proteins with a higher affinity than tamoxifen. Kinetic studies indicate that these variations probably result from the relative dissociation rates of metabolite B and tamoxifen from the oestrogen receptor, tamoxifen dissociating much more rapidly than metabolite B. It is also proposed that kinetic studies may have important implications with respect to the mechanism of action of tamoxifen.

INTRODUCTION.

The antioestrogenic properties of tamoxifen [1-(4-/β-dimethylaminoethoxyphenyl) 1,2-diphenylbut-1-enl] have led to its clinical use in metastatic breast cancer in both pre- and postmenopausal women [1, 2]. Tamoxifen initiates tumour regression principally in those tumours containing oestrogen receptors [3, 4]. This applies equally to the experimental situation where the oestrogen receptor content of DMBA-induced mammary carcinomas correlates with the response of the tumours to tamoxifen treatment [5, 6]. It seems likely, that, in order to understand the mechanism by which tamoxifen influences tumour growth, the detailed nature of any interaction between the antihormone and the cytoplasmic oestrogen receptor protein should be considered. A number of metabolites of tamoxifen have also been identified in man [7] and in laboratory animals [8]. The principal compound, metabolite B [1-(4-/β-dimethylaminoethoxyphenyl) 1-β-hydroxyphenyl, 2-phenylbut-1-enl], has potent antioestrogenic activity in the rat [9] and might, therefore, provide a supportive role during tamoxifen therapy of breast cancer.

The present study examines the binding characteristics of oestradiol-17β, tamoxifen and metabolite B to cytosol preparations from human and rat mammary tumours and rat uteri. Variations in binding properties may ultimately relate to the oestrogenicity/antioestrogenicity of the molecules.

MATERIALS AND METHODS

Preparation of cytosol fractions

Tissue samples of human and rat mammary tumours and rat uteri were frozen in liquid nitrogen and pulverized in a spring-loaded plunger gun [10]. The pulverized tissue was quickly transferred to a Potter–Elvehjem homogenizer and homogenized in 10 mmole/l Tris–HCl buffer, pH 7.4, containing 5 mmole/l EDTA and 1 mmole/l dithiothreitol at 4°C (medium A: 1 g original tissue in 5 ml buffer). A high speed supernatant (cytosol) preparation was obtained by centrifugation of the homogenate in a Beckman L2-65B ultra-
fractions (200 µl) were incubated at 4°C with either [3H]oestradiol-17β (5 nmole/l) or [3H]tamoxifen (20 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled ligand (200 µl). At various time intervals the reactions were stopped by the addition of a solution (100 µl) containing an excess of unlabelled diethylstilboestrol (2 µmole/l). This was immediately followed by the addition of charcoal suspension (500 µl). The total receptor concentration was determined independently by Scatchard analysis [11].

(b) Dissociation rates of labelled oestradiol-17β and tamoxifen from oestrogen receptors. Cytosol fractions were incubated with either [3H]oestradiol-17β (5 nmole/l) or [3H]tamoxifen (20 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled ligand for 4 hr at 4°C. After the preincubation period a further excess of diethylstilboestrol (2 µmole/l) was added to the tubes containing radioactivity alone. Aliquots (400 µl) were then removed at various time intervals, and added to a charcoal suspension (400 µl). Specifically bound radioactivity was monitored in the normal manner.

(c) Dissociation rates of unlabelled compounds from oestrogen receptors. Cytosol fractions were preincubated for 4 hr at 4°C with various ligands (see "Results"). After incubation the tubes were maintained at 4°C and excess ligand removed by charcoal absorption. Aliquots (200 µl) of the charcoal free supernatant were incubated at 4° or 15°C for periods up to 24 hr with an equal volume of medium A containing 10 nmole/l phenylmethylsulphonyl fluoride together with [3H]oestradiol-17β (5 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled diethylstilboestrol. The remaining experimental procedure was as described earlier.

Chemical analyses

The protein concentrations of cytosol fractions was estimated using the method of Lowry et al. [12].

RESULTS

The data presented in Table 1 show the receptor content and dissociation constants [KD] determining for tamoxifen and oestradiol-17β binding to cytosol fractions from various oestrogen receptor containing tissues. Direct binding studies of the [3H]ligands were carried out on the same cytosol preparations to establish any relationships between the binding characteristics of the two compounds. In all tissues examined tamoxifen binds to approximately the same number of sites as does oestradiol-17β but with 2.5-5% the affinity of oestradiol-17β.

Saturation curves generated for [3H]tamoxifen and [3H]-oestradiol-17β binding to a cytosol preparation from a DMBA-induced mammary tumour clearly show a higher non-specific binding component for [3H]tamoxifen binding [Fig. 1(a)]. Nevertheless, subtraction of this value from the value determined in the presence of [3H]tamoxifen alone produced saturaibility of binding sites. Scatchard analysis [11] of the corrected data indicated a single class of binding sites for both oestradiol-17β and tamoxifen [Fig. 1 (c and d)]. The above data is representative of similar curves observed for the binding of the radioligands to cytosol preparations from human mammary tumours and also rat uteri (not illustrated).

Sucrose density gradient analysis of tamoxifen and oestradiol-17β binding is consistent with the concept of a common 7-8S binding

<table>
<thead>
<tr>
<th></th>
<th>Oestradiol-17β</th>
<th>Tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KD (nmole/l)</td>
<td>Receptor level (fmole/mg Protein)</td>
</tr>
<tr>
<td>Human mammary tumour</td>
<td>0.12 ± 0.034</td>
<td>34.1 ± 8.63(4)</td>
</tr>
<tr>
<td>Rat mammary tumour</td>
<td>0.102 ± 0.052</td>
<td>73.0 ± 24.9(5)</td>
</tr>
<tr>
<td>Rat uteri</td>
<td>0.134 ± 0.052</td>
<td>60.9 ± 13.0(3)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviations. Figures in parenthesis show the numbers per group.
Tamoxifen Binding to Oestrogen Receptors

Fig. 2. Sucrose density gradient profiles of cytosol fractions. Cytosol fractions (400 µl) prepared from DMBA-induced mammary tumours were centrifuged through linear 5–20% (w/v) sucrose density gradients under low salt conditions prior to their incubation with either [³H]oestradiol-17β (○) in the presence or absence of an excess of cold oestradiol-17β (●) or tamoxifen (△) [gradients (a) and (c)] or [³H]tamoxifen (○) with or without cold oestradiol-17β (●) [gradients (b) and (d)]. Free steroid was removed from each fraction by charcoal adsorption. Sedimentation marker (arrow) was bovine serum albumin (4.6S) in each case.

Table 2. Protamine sulphate precipitation of oestrogen and tamoxifen binding sites

<table>
<thead>
<tr>
<th>Percentage precipitation of binding sites</th>
<th>Oestradiol-17β</th>
<th>Tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat uteri</td>
<td>98.3(4)</td>
<td>96.4(4)</td>
</tr>
<tr>
<td>DMBA-induced mammary tumour</td>
<td>94.0(4)</td>
<td>95.7(4)</td>
</tr>
<tr>
<td>Human mammary tumour</td>
<td>93.1(2)</td>
<td>96.4(2)</td>
</tr>
</tbody>
</table>

Figures in parenthesis show numbers per group.
Incubation of tamoxifen (2 µM) and \([^{3}H]\text{oestradiol-17}\beta\) (5 nM) for varying periods of time with a rat uterine cytosol preparation at 4°C clearly demonstrates that tamoxifen decreases the rate of uptake of oestradiol-17\(\beta\) onto the oestrogen receptor [Fig. 5(a)]. Binding equilibrium for oestradiol-17\(\beta\) in the presence of tamoxifen was not achieved in the period up to 48 hr. Recalculation of the results as the percentage binding of \([^{3}H]\text{oestradiol-17}\beta\) [Fig. 6(a)]. Under conditions identical to those described above, only small quantities of metabolite B and oestriol were released. No substantial release of either oestradiol-17\(\beta\) or diethylstilboestrol was observed. Elevation of the temperature to 15°C resulted in the total dissociation of tamoxifen and the partial dissociation of the remaining compounds [Fig. 6(b)].

These data are consistent with the rate of

\[17\beta\] indicated that tamoxifen exhibits a biphasic inhibitory action [Fig. 5(b)]. At early (<15 min) and late (>24 hr) time points tamoxifen had little effect on \([^{3}H]\text{oestradiol-17}\beta\) binding. The maximum competition was observed at 60 min. Timed competition curves generated for metabolite B and diethylstilboestrol showed little if any secondary decrease in competition at 4°C [Fig. 5(b)]. Similar results were observed with oestrogen receptors from DMBA-induced mammary tumours (not illustrated).

The relatively low binding affinity of tamoxifen for cytoplasmic binding components is reflected by its rapid dissociation from the oestrogen receptor. Following an incubation period of 24 hr at 4°C endogenously bound tamoxifen was specifically replaced by dissociation of tamoxifen and oestradiol-17\(\beta\) from their specific binding sites (Fig. 7). At 4°C, 85% of the specifically bound \([^{3}H]\)-tamoxifen dissociates during the 48 hr experimental period \((k_2, 20 \times 10^{-4}/\text{min})\). Under similar conditions no release of \([^{3}H]\text{oestradiol-17}\beta\) was observed. Elevation of the incubation temperature to 15°C increased the dissociation rate of both tamoxifen and oestradiol-17\(\beta\) \((k_2\) values, 144 \(\times \)\(10^{-4}/\text{min}\) and 15.1 \(\times \)\(10^{-4}/\text{min}\) respectively). The association rates of \([^{3}H]\text{oestradiol-17}\beta\) and \([^{3}H]\)-tamoxifen binding were measured at 4°C only, since, at higher temperatures, the association rate was too great to determine accurately. In contrast to the reverse reaction constants, the forward rate constant for tamoxifen association with cytosolic proteins was 4 times smaller than

![Fig. 6](image_url)
Fig. 8. Association kinetics of oestradiol-17\(\beta\) and tamoxifen with oestrogen receptor proteins. DMBA-induced mammary tumour cytosol fractions were incubated with \(\beta\)-oestradiol-17\(\beta\) (●), 5 nmole/l or \([\text{H}]-\text{tamoxifen}\) (○), 20 nmole/l in the presence or absence of a 1000-fold excess of the non-radioactive ligand. Forward rate constants for the specific binding of the radioligands were calculated using the equation, \(k_1 = \frac{1}{t} \ln \left(\frac{L_o - R_o}{L - R}\right)\) assuming the reaction to be second order and where \(L_o\) > \(R\). \(L_o\), \(R_o\), \(L\) and \(R\) are the concentrations of ligand and receptor at time 0 and time \(t\) respectively. Total receptor content and dissociation constant (\(K_D\)) were determined independently by Scatchard analysis.

**Table 3.** Kinetic and equilibrium constants for oestradiol-17\(\beta\) and tamoxifen binding at 4°C to oestrogen receptors from rat mammary tumours

<table>
<thead>
<tr>
<th></th>
<th>(k_1) (10(^5)l/mole/min)</th>
<th>(k_2) (10(^{-4})/min)</th>
<th>(k_2/k_1) (nmole/l)</th>
<th>(K_D) (nmole/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol-17(\beta)</td>
<td>17.5 ± 4.34 (3)</td>
<td>—</td>
<td>—</td>
<td>0.102 ± 0.052 (5)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>4.3 ± 2.73 (3)</td>
<td>21 ± 6.7 (4)</td>
<td>4.88</td>
<td>4.34 ± 1.76 (3)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviations. Figures in parentheses indicate the number of tumours per group.

Fig. 9. Chemical structures of oestradiol, diethylstilboestrol, tamoxifen and metabolite B.
plex to the nucleus. Maintenance of relatively high concentrations of androst-5-ene, 3β,17β-diol, by implantation or continuously injecting the steroid, results in an oestrogenic effect on both mammary tumours [34] and uterus [34, 35] of the rat. Interestingly, the short term oestrogenic properties of tamoxifen in vivo are more evident when the dose of the compound is increased [9, 36, 37].

Acknowledgements—We are grateful to Dr. B. J. A. Furr of ICI Ltd. for the gift of [3H]tamoxifen and to the Tenovus Organization for its financial support.

REFERENCES


The effect of tamoxifen and stilboestrol on plasma hormone levels in postmenopausal women with advanced breast cancer


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(Accepted for publication 2 May 1978)

Plasma FSH, prolactin, testosterone and 17β-oestradiol levels were measured in postmenopausal women with advanced breast cancer before and on treatment with tamoxifen or stilboestrol. In 24 postmenopausal women results showed that tamoxifen decreased plasma FSH levels at 1, 3 and 6 months but had no effect on prolactin levels. In 16 postmenopausal women, stilboestrol had a more profound effect in suppressing FSH levels at 1, 3 and 6 months and also increased prolactin levels. Neither drug affected testosterone or oestradiol levels. These effects were present in patients with and without a clinical response to the drug.

Introduction

Both stilboestrol, an oestrogen, and tamoxifen, an anti-oestrogen, can induce remission in advanced breast cancer. There is little known regarding the biochemical effect of these hormonal regimes in patients with breast cancer, other than that stilboestrol inhibits urinary gonadotrophin excretion (Loraine & Bell, 1971) and elevates prolactin (Wilson et al., 1974). Recently, Goider et al. (1976) reported other studies on the effect of tamoxifen on plasma hormones in patients with advanced breast cancer. They found that neither prolactin nor oestradiol-17β concentrations in the plasma were altered by tamoxifen but that plasma FSH and LH concentrations were reduced, an effect similar to that found with chlomiphene. It was also noted that plasma FSH concentrations in those patients who had a clinical response to tamoxifen returned
to the pre-treatment value within 2 to 3 months; whereas in non-responders the concentrations remained significantly lower than the pre-treatment level.

The initiation of a controlled clinical trial in which tamoxifen and stilboestrol were being compared offered an opportunity to re-assess, in comparable groups of patients, the effects of these two agents on plasma hormone concentrations.

Material and methods

The studies were carried out at the Combined Breast Clinic of the University Department of Clinical Surgery and the Department of Radiotherapy at the Royal Infirmary of Edinburgh. This clinic is specifically concerned with the conduct of therapeutic trials in patients with advanced breast cancer. The women were post-menopausal and with advanced breast cancer. None had been given any previous hormone or other systemic therapy for the treatment of their disease.

Following documentation of the state of the disease, patients were entered into a double-blind crossover trial in which they received identical tablets of either tamoxifen (10 mg) or stilboestrol (3 mg), one being taken by mouth thrice daily. Prior to allocation, the patients were stratified according to the distribution of their recurrent or metastatic disease; the performance of a previous mastectomy and the recurrence-free interval. Patients attended the clinic at monthly intervals when their response to therapy was assessed. Documentation of the response was by the criteria advised by the British Breast Group (1974) and patients were classified as undergoing objective regression, an equivocal or intermediate response, and no response at 3 and 6 months.

If no response occurred or when a good response had ended, administration of the drug was stopped for 1 month before the patient was started on the second agent. Some patients, because of cardiac or respiratory problems, were considered to be unsuitable for treatment by stilboestrol. They were excluded from the trial but given elective tamoxifen therapy in the same dose as in the trial. As their pattern of hormonal changes did not differ from that of the patients included in the trial, they have been included with the patients given tamoxifen in the trial.

Before starting a drug, 10 ml of blood was withdrawn from an arm vein at 11.00 a.m. The blood was heparinized, centrifuged at −4°C and stored at −20°C. Similar samples were taken at the same time of day at 1, 3 and 6 months after starting therapy. The plasmas were sent in a batch to the chemical laboratories and assayed for FSH, oestradiol-17β, testosterone and prolactin.

Non-parametric statistical tests were used to compare the plasma hormone levels over time, and between different groups of patients. All tests were 2-sided (testing either for an increase or a decrease) carried out at the 5% level.

Hormone assays

The concentration of all hormones was estimated by radioimmunoassay. For plasma FSH and prolactin the double antibody method of Groom (1977) was used and the results expressed in terms of second IRP HMG and MRC 71/222 standards respectively.

Oestradiol-17β was determined by an R.I.A. procedure, using an antiserum raised against an oestradiol-6 (o-carboxymethyl)-oxime bovine serum albumin conjugate.
no purification procedures were incorporated in the assay since results, with and without chromatography showed no significant difference. Free and bound steroid were separated using dextran-coated charcoal and the free steroid in a 500 μl aliquot dissolved in 5 ml Aquasol (NEN Chemicals) and counted in a liquid scintillation counter. Testosterone was estimated by the method of Fahmy and Hillier (1975) employing an antisera raised against testosterone-11β-(hemisuccinyl) bovine serum albumin.

Table 1. Total number of patients on each drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>1st drug</th>
<th>2nd drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stilboestrol</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Elective tamoxifen</td>
<td>9</td>
<td>---</td>
</tr>
</tbody>
</table>

Results

The number of patients who received each drug are shown in Table 1. The effect of the drugs on plasma hormone levels were studied in all patients receiving the treatments as first or second drug; but the relationship of these hormonal changes to clinical response was restricted to those patients receiving the drug on their first treatment. Table 2 gives the results of the concentrations of plasma hormones in those patients who received tamoxifen or stilboestrol either as first or second drugs. The median increase or decrease is shown for each hormone at 1, 3 and 6 months after commencement of treatment with the significance of these changes.

For clinical reasons some of these patients were withdrawn from the hormone therapy before the 6-month period, and collection and analysis of blood samples was incomplete in some others. Thus the results at 3 and 6 months of drug therapy are based on smaller numbers than those at 1 month, so that the evidence may be insufficient to judge a considerable change in hormone level statistically significant at the later times. More seriously the patients compared at 3 and especially 6 months over-represent those who responded to treatment, and this should be borne in mind when interpreting Table 2.

The administration of tamoxifen significantly suppressed circulating levels of FSH in the plasma at 1, 3 and 6 months after its administration. It did not change the concentration of oestradiol-17β, testosterone or prolactin. Stilboestrol was more effective in depressing plasma FSH and no patient on stilboestrol had plasma FSH more than 3 mIU/ml above the limits of detection of the assay. In addition, stilboestrol significantly increased plasma prolactin at 1 month, but had no effect on oestradiol-17β or testosterone.

To relate these hormone changes with clinical response to therapy we selected those patients who had a definite clinical response at 3 and 6 months, and those with no response at either time. All those patients with a short lived or equivocal response, and those who were not fully assessed due to intolerance or other causes...
Table 2. Median values and changes with treatment at 1, 3 and 6 months. Range of values are in brackets

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Pretreatment median value</th>
<th>n</th>
<th>1 month</th>
<th>Median change</th>
<th>3 months</th>
<th>n</th>
<th>6 months</th>
<th>n</th>
<th>Significance W.S.R.T.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen as 1st or 2nd drug</td>
<td>FSH (mU/ml) 137(55-261)</td>
<td>27</td>
<td>-25(-72→+74)</td>
<td>-33(-188→+54)</td>
<td>20</td>
<td>-33(-178→+24)</td>
<td>14</td>
<td>Sig. decrease at 1/12, 3/12 &amp; 6/12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oestradiol (pg/ml) 123(0-164)</td>
<td>26</td>
<td>+1(-116→+111)</td>
<td>+10(-120→+71)</td>
<td>20</td>
<td>+1(-27→+54)</td>
<td>14</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testosterone (ng/100 ml) 40(0-62)</td>
<td>25</td>
<td>0(-36→+38)</td>
<td>0(-34→+44)</td>
<td>20</td>
<td>0(-31→+42)</td>
<td>14</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prolactin (mU/ml) 0.30(0→0.45)</td>
<td>26</td>
<td>+0.00(-0.42→+0.60)</td>
<td>-0.00(-0.37→-0.34)</td>
<td>19</td>
<td>-0.07(-0.30→+0.12)</td>
<td>13</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Stilboestrol as 1st or 2nd drug</td>
<td>FSH (mU/ml) 106(1→208)</td>
<td>16</td>
<td>-105(-203→+1)</td>
<td>-104(-203→-2)</td>
<td>8</td>
<td>-120(-69→-145)</td>
<td>3</td>
<td>Sig. decrease at 1/12, 3/12 &amp; 6/12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oestradiol (pg/ml) 30(12-55)</td>
<td>16</td>
<td>+1(-27→+183)</td>
<td>-5(-13→+12)</td>
<td>8</td>
<td>+0(-17)</td>
<td>3</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testosterone (ng/100 ml) 40(7-93)</td>
<td>16</td>
<td>0(-46→+64)</td>
<td>+1(-28→+23)</td>
<td>8</td>
<td>-7(-8→+14)</td>
<td>3</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prolactin (mU/ml) 0.12(0.07→0.46)</td>
<td>16</td>
<td>+0.30(-0.44→-0.58)</td>
<td>+0.31(-0.62→+0.78)</td>
<td>8</td>
<td>+0.13(-0.57→+0.31)</td>
<td>3</td>
<td>Sig. increase at 1/12</td>
<td></td>
</tr>
</tbody>
</table>

* W.S.R.T., Wilcoxon signed rank test.

n, number of patients
Table 3. Median changes with ranges in plasma hormone levels for responders and non-responders to tamoxifen

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Median change</th>
<th>Ranges</th>
<th>n</th>
<th>Median change</th>
<th>Ranges</th>
<th>n</th>
<th>Significance of resp. VS non-resp. on Rank Sum Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (mU/ml)</td>
<td>-26 (-63-74)</td>
<td>6</td>
<td>-15 (-71-+26)</td>
<td>9</td>
<td>N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0 (-13-42)</td>
<td>6</td>
<td>-4 (-112-+111)</td>
<td>9</td>
<td>N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (ng/100 ml)</td>
<td>-4.5 (-34-+4)</td>
<td>6</td>
<td>+4 (-35-+27)</td>
<td>9</td>
<td>N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolactin (mU/ml)</td>
<td>+0.09 (-0.41-+0.38)</td>
<td>6</td>
<td>0 (-0.37-+0.34)</td>
<td>7</td>
<td>N.S.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n, no. of patients
N.S., not significant.
were excluded. The numbers of clinical responders and non-responders to stilboestrol was insufficient for this comparison to be made. The results for tamoxifen are shown in Table 3. No relationship was detected between the changes in hormone levels and the clinical response to treatment.

Discussion

Both tamoxifen and stilboestrol reduce the concentrations of plasma FSH. In the doses used stilboestrol had a more powerful suppressing effect than tamoxifen and in addition, raised plasma prolactin.

It has been assumed that stilboestrol depresses gonadotrophin release and increases prolactin release by its oestrogenic effect on hypothalamic releasing and inhibiting factors (Meites, 1972). Tamoxifen does also have oestrogenic effects when given in large doses to animals, particularly mice (Harper & Walpole, 1967), but there is yet no evidence for this in man; the mechanism of FSH suppression by the drug is therefore unknown.

The depression of plasma FSH by tamoxifen in patients having a clinical response was identical to that in those who did not. Further, contrary to the earlier study (Golder et al., 1976) we were not able to detect any difference in pattern of FSH concentration with time in any patients. We suspect that the normalization process described in their paper may have resulted in an over-estimate of the significance of their results.

These observations provide further evidence that the nature of hormone sensitivity lies within the tumour and is not due to differences in the responsiveness of the endocrine system to treatment.

Acknowledgements

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References


BIOCHEMICAL EFFECTS OF CYTOTOXIC ESTROGENS AND CYTOTOXIC ANTIESTROGENS

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Introduction

Although it has long been recognised (Hunter, 1786) that the growth and function of the prostate gland is dependent upon the secretion of testosterone by the testis, it was only a few decades ago, following the experimental work of Huggins and his colleagues (Huggins, et al., 1940; Huggins et al., 1941 and Huggins et al., 1941) that antiandrogen therapy in the form of orchidectomy or estrogen administration was introduced for the treatment of carcinoma of the prostate. It was recognised that malignant prostatic growth can be dependent upon androgen stimulation and the administration of diethylstilbestrol has been a conventional form of treatment for the management of the disease.

The precise mechanism by which diethylstilbestrol controls prostatic growth is not yet completely established but it is generally accepted that the major antiandrogenic effect is exercised indirectly via the pituitary (Griffiths et al., 1979), suppressing LH secretion and thus decreasing the synthesis of testosterone by the testis. There is also evidence that at least part of the antiandrogenic effect of diethylstilbestrol results from a direct action on the testis (Oshima et al., 1967; Danutra et al., 1973 and Shimazaki et al., 1965). Evidence that the estrogen directly influences prostatic tissue is more equivocal although there are studies (Danutra et al., 1973; Danutra et al., 1973; Shimazaki et al., 1965; Farnsworth 1969 and Leav et al., 1971) that administration of estrogens markedly alters C₁₉-steroid metabolism by the prostate. It is generally believed that
after administration of large doses of diethylstilbestrol diphosphate (Honvan), the free and active form of the hormone is released at the appropriate site within the prostatic cells by the phosphatases present in high concentration, although to date, there is little evidence of such a concentration of the estrogen within the tissue. Experimental work from this laboratory has not been able to demonstrate an effect of diethylstilbestrol on the selective transfer of 5α-dihydrotestosterone to the nucleus of the prostate cell by the androgen receptor protein, although certain experiments have clearly indicated that after administration of [3H]-diethylstilbestrol to men with prostatic carcinoma, the radiolabeled material could be extracted from the nuclei of the removed prostatic tissue.

It is however well established that certain compounds are able to decrease the specific uptake of estradiol-17β by mammary tumor tissue. These anti-estrogens, particularly tamoxifen, the trans isomer of 1-[p-(β-dimethylaminoethoxy)phenyl]-1,2-diphenybut-l-ene, I.C.I. 46474, influence the selective translocation of estradiol by specific receptor protein molecules to the chromatin of the nucleus and thereby have a valuable role in the treatment of women with advanced carcinoma of the breast. Extensive studies by these laboratories (Nicholson et al., 1980, 1976, 1977, 1977, 1978 & 1979) have provided an insight into the biochemical mechanisms by which tamoxifen exerts its effect. It was evident from the work that tamoxifen was effective in inhibiting the growth of the DMBA-induced rat mammary tumor (Fig. 1), that the tamoxifen was responsible for the translocation of estradiol-17β receptor to the nucleus (Nicholson et al., 1976 & 1977) (Fig. 2) and over a short period of time, stimulated RNA synthesis (Nicholson et al., 1977) resulting from transcription of the DNA template by RNA polymerases. From the investigations with DMBA-induced rat mammary tumors, it has been shown that increases in RNA polymerase II (synthesising DNA-like RNA) activity from tamoxifen administration are qualitatively similar to those produced after estradiol stimulation during the early increase in RNA synthesis. This transient rise in activity is obligatory for the subsequent elevation of RNA polymerase I (synthesising ribosomal rRNA) and then a major prolonged enhancement of RNA polymerase II (Borthwick et al., 1975). Similar effects to those produced after estradiol stimulation are seen during the early phase of the second peak of activity although tamoxifen then appears unable to maintain this secondary stimulated rise (Nicholson et al., 1977).

The results established however, the initial influx of the antiestrogen
Fig. 1: The effect of ovariectomy and antioestrogens on DMBA-induced mammary tumor growth. Tumor size was measured weekly. Figs. in parentheses show the fraction of the tumors within a group that were either "growing" (——) or "regressing" (-----); (a) •, ovariectomised; ▲, sham operated. (b) Animals were injected daily for 14 days and then every 2 days for 5 weeks with 100 µg (per injection) of either I.C.I. 46,474 (■) or I.C.I. 79792 (○).

receptor complex into the nucleus and its relationship to transcriptional events. The possibility that such compounds as tamoxifen which may be selectively taken up by hormoneresponsive tumors, could be modified to carry into the nucleus a nitrogen mustard-containing grouping and localise a high concentration of this cytotoxic agent adjacent to the chromatin was considered in the early 1970's and two compounds, I.C.I. 85,966, (3,4-bis-(p-[N-bis-2-chloroethyl]carbamoyl)-phenyl)hex-3-ene) and I.C.I. 79792, (1-(4-[β-bis-(2-chloroethyl)amino]ethoxyphenyl)-trans-diphenylbut-1-enehydrochlo-
Fig. 2: Effect of in vivo administration of estradiol-17β and tamoxifen on the cytoplasmic levels of total and accessible estradiol-17β-binding sites. Rats were injected with either (a) 5 μg estradiol-17β (b) 100 μg tamoxifen (c) vehicle alone. Tumor biopsies were removed at the points indicated. Cytosol preparations from biopsies were incubated at either 4°C (accessible sites) or 15°C (total sites) for 120 mins with a saturating concentration of [3H] estradiol-17β (5.0 nmol/l) or [3H] estradiol-17β plus 1000 fold excess unlabeled estradiol-17β. Results are expressed as a % of the value obtained by incubation of cytosol with labeled steroid at 4°C (accessible binding sites at time 0) and is the mean ± SEM of 5 separate tumors.
Cytotoxic estrogens/antiestrogens

were made available to the laboratory by Dr. D. N. Richardson, I.C.I. Pharmaceuticals Ltd., Alderley Park, Cheshire. In Fig. 3 therefore are shown the structures of the nitrogen mustard-containing diethylstilbestrol and tamoxifen compounds and the following sections deal with certain aspects of their biochemistry.

Antiestrogens - effects in relation to rat mammary tumors

Mammary tumors were induced in virgin female Sprague-Dawley rats by intubation of DMBA (20 mg in 1.0 ml sesame oil). Approximately 7-12 weeks after intubation, tumors suitable for experimental purposes, (2 x 2 cm) were available. Minced tumors tissue (1 g) was incubated for 15 mins in Eagle's basal medium (10 ml/g) at 30°C in the presence of [2,4,6,7-3H]-estradiol-17β (0.5 nmol/l, sp. act. 85 Ci/mmol) and with or without the addition of the non-radioactive antiestrogen or competitor (50 or 500 nmol/l). After incubation, the minced tissue was homogenised and nuclear pellets prepared.

![Tamoxifen (ICI 46474)](image1)

![ICI 79792](image2)

![ICI 85966](image3)

Fig. 3: Structures of the various antiestrogens.
Griffiths et al. (Powell-Jones et al., 1975). The nuclear preparation was extracted with 0.6 ml KCl (0.4 mol/l) at 4°C for 30 mins and aliquots of the extract analysed by sucrose density gradient centrifugation (Davies et al., 1973).

After incubation of minced mammary tumor tissue with [3H]estradiol alone, a labeled steroid-receptor complex, sedimentation coefficient 4-5S could be extracted (Fig. 4a). Although incubation in the presence of non-radioactive estradiol, diethylstilbestrol or another stilbestrol analogue, dihydrodibutylstilbestrol (50 nmol/l) inhibited this characteristic binding of [3H]estradiol, neither tamo-
Cytotoxic estrogens/antiestrogens

xifen, I.C.I. 79792, nor I.C.I. 85966 at 50 or 500 nmol/l could produce a similar effect (Fig. 4; a and b). When minced mammary tumor tissue was pre-incubated with non-labeled competitor for 30 mins at 30°C before incubation with [3H]estradiol, tamoxifen, I.C.I. 79792 and I.C.I. 85966 inhibited the uptake of [3H]estradiol, the latter being the most effective (Fig. 4c).

These results are of interest in relation to further studies on the effect of these various compounds on the specific binding in vitro at 0°C, of [3H]estradiol (0.5 nM) by soluble supernatant cytosols prepared by centrifugation of rat mammary tumor homogenates at 100,000 g for 60 mins (Powell-Jones et al., 1975).

Sucrose density gradient analysis of such [3H]estradiol-labeled cytosol preparations showed two peaks of protein-bound radioactivity corresponding to [3H]estradiol-receptor complexes of sedimentation coefficients 4S and 8S approximately (Fig. 5a) incubation in the presence of 100-fold excess non-radioactive estradiol displaced the radioactivity of the specific 8S peak to the high-capacity 4S peak. A 100-fold excess (50 nM) of either diethylstilbestrol or dihydrodibutylstilbestrol produced a similar effect (Fig. 5b). However, whereas 100-fold excess tamoxifen and I.C.I. 79792 effectively inhibited the binding of [3H]estradiol to its receptor at a 50 nM concentration (Fig. 6a), I.C.I. 85966 had no such effect (Fig. 5a). Furthermore a 10-fold excess (5 nM) of diethylstilbestrol (Fig. 6b) and tamoxifen (Fig. 6c) also reduced the 8S-binding peak, whereas a similar concentration of I.C.I. 79792 had no effect (Fig. 6c).

The results would suggest therefore that since I.C.I. 85966 did not inhibit the specific binding of [3H]estradiol to these cytosols, the effect of the compound on the nuclear uptake of labeled estrogen by whole-cell preparations probably reflects some degree of metabolism to diethylstilbestrol. In contrast, the ineffectiveness of tamoxifen and I.C.I. 79792 under these conditions in which whole-cell preparations were used may well be caused by their slower uptake by the cells compared to diethylstilbestrol, since both were effective in displacing estradiol from cytoplasmic receptor protein, tamoxifen administration in vivo has been shown to decrease binding of [3H]estradiol by rat mammary tumor tissue (Nicholson et al., 1975) and both inhibit the growth of these DMBA-induced tumors when administered in vivo (Fig. 1). Possibly higher doses of I.C.I. 79792 could produce even more effective inhibition of growth and further such studies may well be rewarding.
Fig. 5: Effects of antiestrogens on the specific binding of $[^3\text{H}]$estradiol (0.5 nM) in cytosol of DMBA-induced rat mammary tumors. Sucrose density gradient centrifugation from left to right. The sedimentation marker was BSA ($S_{20,w}4.6S$).
Fig. 6: Effects of antiestrogens on the specific binding of $[^3H]$estradiol (0.5 nM) in cytosol of DMBA-induced rat mammary tumor. Sucrose density gradient centrifugation from left to right. The sedimentation marker was BSA ($S_{20,w} 4.6$ S).
Androgens as possible carriers of cytotoxic agents

Androgenic steroids have been used in the treatment of advanced breast cancer and there are various reports relating to this (Jones, 1979; Hayward, 1970). The effects of androgens on rat mammary tumor growth has also been considered (Teller et al., 1968). In relation to this, early studies at the Institute and in other centres clearly established the paraendocrine behavior of human breast cancer and the capacity of such tissue to metabolise steroids. In particular, the studies in these laboratories showed the conversion of the adrenal secretory product dehydroepiandrosterone sulphate to various metabolites including androstene-5-ene-3β,17β-diol and the 5α-androstanediols, of interest, since it has subsequently been shown that such C19 steroids competed effectively with [3H]estradiol-17β for the cytoplasmic estradiol receptor protein isolated from DMBA-induced mammary tumors (Fig. 7) (Nicholson et al., 1978; Poortman et al., 1975). Furthermore, it was also shown (Nicholson et al., 1978) that androst-5-ene-3β,17β-diol facilitated the nuclear translocation of the estrogen receptor although the transferred complex was found to have a relatively short nuclear retention time (Fig. 8). Later investigations in the Institute in which the C19-steroid concentration of a series of human breast cancers was determined by high resolution gas chromatography-mass spectrometry (Maynard et al., 1976) indicated relatively high levels of both dehydroepiandrosterone and the androstenediol in certain of the tumors.

The possibility therefore exists to exploit the ability of the estradiol-17β receptor protein to transfer certain C19-steroid-nitrogen mustard derivatives to the nucleus of human breast cancer cells.

Cytotoxic estrogens and the prostate gland

Preliminary investigations at the Institute on the possible biochemical role of diethylstilbestrol and certain of its analogues suggested a possible inhibitory effect of such compounds on DNA nucleotidyltransferase (DNA polymerase) (Fahmy et al., 1968; Harper et al., 1970 & 1970). Similar studies established to investigate the biochemical effect of the androgen-receptor complex on isolated prostatic DNA-dependent RNA nucleotidyltransferase (RNA polymerase) (Davies et al., 1973 & 1972) indicated that the stilbestrol analogues also influenced the activity of the enzyme system (Davies et al., 1973) (Table I), and that I.C.I. 85,966 was equally as effective an inhibitor as diethylstilbestrol.
<table>
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<th>Compound Added</th>
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<td>ICI-85966</td>
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</table>
Concentration of competitor (nM)

Fig. 7: Effects of various C19-steroids on the binding of [3H]estradiol in cytosol of DMBA-induced rat mammary tumors. E217β - estradiol; Δ4βΔandrost-4-ene-3β,17β-diol; Δ5αΔandrost-5-ene-3α,3β-diol; Δ5βΔandrost-5-ene-3β,17β-diol; ααβ, 5α - androstane-3α,17β-diol; αβα, 5α - androstane-3β,17α-diol; ααα, 5α - androstane-3β,17α-diol: A-dione, androstenedione; Epi-T, epitestosterone; DHEA, dehydroepiandrosterone; T, testosterone; DHT, 5α - dihydrotestosterone.
Cytotoxic estrogens/antiestrogens

Fig. 8: Effect of in vivo administration of androst-5-ene-3β,17β-diol on cytoplasmic and nuclear estradiol-17β binding sites. Rats were injected with 100 μg (△) or 1 mg (○) at time 0 and tumor biopsy samples removed at varying time intervals thereafter. (a) Cytosol preparations from biopsies were incubated at 4°C for 16 h with a saturating concentration of [3H]estradiol-17β (3 nM) or [3H]estradiol-17β plus 1000-fold excess of diethylstilbestrol. (b) Nuclear fractions were incubated for either 4 hours at 15°C (●, ×) or 16 hours at 4°C (○, △) with saturating concentrations of [3H]estradiol-17β (20 nM), with or without 1000 fold excess of diethylstilbestrol.

Extensive studies over a number of years have failed to indicate that diethylstilbestrol has any effect on the 5α-dihydrotestosterone-receptor interaction in prostatic tissue, although it does affect the metabolism of testosterone (Danutra et al., 1973 & 1973), and it is still not yet evident that diethylstilbestrol can be selectively taken up by prostatic tissue. There is evidence of an estrogen receptor in prostatic tissue (Wagner et al., 1975; Hawkins et al., 1975) but recent studies from the Institute (Chaisiri et al.) indicate that this may be localised in stromal tissue and not with epithelial cells.

Whether diethylstilbestrol plays any specific biochemical role in the prostatic cancer cell, which would influence tumor growth still requires elucidation and therefore the potential of such compounds
as I.C.I. 85966 is uncertain. Administration of these compounds to rats showed that they influenced androgen-dependent tissues (Figs. 9 and 10) and plasma hormone levels (Fig. 11), but whether the effect of I.C.I. 85966 relates to its metabolism to diethylstilbestrol and its consequent inhibiting action in the pituitary is unclear. Further mass spectrometric analysis of prostatic tissue from animals given I.C.I. 85,966 would seem reasonable. Certainly hydrolysis of the estradiol-nitrogen mustard derivative Estracyt occurs after

![Fig. 9: Effect of diethylstilbestrol and I.C.I. 85,966 (100 µg/day for 10 days) on organ weights (mg/g initial body weight). Differences for diethylstilbestrol were significant (p < 0.005).](image)

![Fig. 10: Effect of diethylstilbestrol and I.C.I. 85,966 (500 µg/day for 10 days) on organ weights (mg/g initial body weight). Differences for diethylstilbestrol were significant (*p < 0.005 : + p < 0.050).](image)
Cytotoxic estrogens/antiestrogens

Fig. 11: Effect of diethylstilbestrol and I.C.I. 85,966 (100 μg/day for 10 days) on plasma prolactin and testosterone. (+ p < 0.005; * p < 0.050).

Prostatic cancer patient J.W. Age 71. T3 M1 420mg t.d.s.

Fig. 12: Effect of Estracyt administration (420 mg t.d.s.) to a patient with prostatic cancer on plasma hormone levels.
administration to men with prostatic cancer (Fig. 12) and the analysis of intracellular components of the tumor tissue could offer increasing and valuable data.

Acknowledgements

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References

Huggins, C. and Hodges, C. V. (1941). Cancer Res. 1, 293.
 Alpha Omega Publishing, Cardiff.
Cytotoxic estrogens/antiestrogens


Discussion

Catane: I think we should try to concentrate on why such promising agents like the nitrogen mustard derivatives of tamoxifen were relatively ineffective. We may be able to design more effective drugs by discussing why they were ineffective. Possibilities are that the drug hydrolyzes or is metabolized before arriving at the cell or maybe the compound doesn’t enter into the cell. Dr. Griffiths, do you have any idea why this agent was ineffective?

Griffiths: We don’t. We can imagine that compound 79792 does not bind to the receptor as well as does tamoxifen and that is why you would get a less effective action. At the present time we are trying to find out what the metabolism is of all these compounds.

Katzenellenbogen: You mentioned the blood levels of tamoxifen and its metabolites. Can you determine the tissue levels of these compounds?

Griffiths: Yes, the tissue levels are being determined but we don’t have the tissue levels of the metabolites at the present time. The data, which I gave you, the 50,000/1 ratio in the tumor and in the
The Biochemistry of Tamoxifen Action

R.I. Nicholson and K. Griffiths

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INTRODUCTION

Carcinoma of the breast is the most common cause of death from cancer in women, producing annually throughout the world some quarter of a million fatalities (Logan, 1975). The fact that the disease is to some extent estrogen-dependent has been exploited in its treatment by the surgical ablation of the ovaries, the adrenal glands, and the hypophysis (Stoll, 1969; Burn, 1974), which decreases plasma estrogen concentrations. It is, however, well established that such endocrine therapy is clinically effective in only approximately 30% of these patients, the proportion varying depending upon the stringency of the criteria used to assess response (Stoll, 1978). Many of the patients are therefore subjected to surgical risk, trauma, and treatment morbidity without obtaining substantial benefit in terms of disease control. It is not
Figure 1. Cellular mechanism of estrogen action. E$_2$ indicates estradiol-17$.\beta$.

surprising that an effective, nontoxic and noninvasive means of controlling the growth of breast cancer has been sought.

There is now good evidence to indicate that at the molecular level the phenotypic response of breast tumor cells to estrogens is related to the presence of cytoplasmic receptor proteins, which bind to incoming estradiol with selective high affinity (Figure 1; Jensen et al., 1974). It is thought that the binding of the hormone to the receptor then facilitates the transfer of the receptor complex to, and its retention within, the nucleus; and that it thereby increases transcription of the DNA template. The process results in the production of components essential for cell maintenance and division. This model system for the action of estradiol in breast tumor tissue implies that any drug interfering with the ability of the tissue, or more specifically the receptor protein, to bind the estrogen or process its message is theoretically capable of causing tumor regression and ultimately tumor cell death.

A number of studies have clearly indicated that certain types of compounds, based on triphenylethylene (Figure 2 a), influence the biological activity of estradiol, possibly by competing with the hormone for its binding site on the receptor protein (Korenman, 1970; Rochefort
and Capony, 1972; Skidmore, Walpole, and Woodburn, 1972; Black and Kraay, 1973; Cidlowski and Muldoon, 1976). Indeed, earlier reports of two such antiestrogens, MER-25 and clomiphene (Solmsen, 1945; Kistner and Smith, 1959), showed that they were capable of causing tumor regression, although their association with relatively unpleasant clinical side effects prevented their widespread use in the management of breast cancer.

More recently, however, ICI Pharmaceuticals Ltd., of the United Kingdom, introduced a drug that has these necessary antiestrogenic properties and has the capacity to antagonize the biological effects of estrogens but has a reported low incidence of such side effects (Patterson and Baum, 1978). The structure of this antiestrogen, tamoxifen, trans-1-(4-B-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene, known also as Nolvadex, is given in Figure 2 b. Clinical trials have established its value in the treatment of advanced breast cancer in both pre- and postmenopausal women: it has produced, as have other forms of endocrine therapy, ablative and additive, an objective clinical response in approximately 20–40% of patients (Cole, Jones, and Todd, 1971; Ward, 1973; Goldet et al., 1976; Mouridsen et al., 1978), with an average remission interval of about 12 months (Ward, 1973). In addition, tumor regression occurred primarily in patients whose tumors contained estrogen receptor proteins (Hawkins, 1976; Lerner et al., 1976; Manni et al., 1976; Morgan et al., 1976; Westerberg et al., 1978). Tumors lacking receptors had a much lower response rate to tamoxifen. Similarly, tamoxifen was shown to inhibit the initiation (Jordan, 1975 a, 1976) and the growth of dimethyibenz(a)anthracene-induced (DMBA) rat mammary adenocarcinomas containing estrogen receptors (Jordan, 1975 a; Nicholson, Davies, and Griffiths, 1978 a).

The clinical efficacy of tamoxifen and its widespread use in the management of breast cancer has now promoted a marked interest in its mode of action, and a wider understanding of its chemistry and pharmacokinetics is being sought. The present communication deals with our experience with this compound in relation to the current research literature on the subject.

GENERAL PHARMACOLOGICAL PROPERTIES OF TAMOXIFEN

Before describing the direct interaction of tamoxifen with breast tumor cells, and, more specifically, with any estrogen receptor proteins, it is informative to examine certain of the general pharmacological properties
of tamoxifen in relation to other estrogen-responsive tissues (Furr et al., 1979). Care must be taken when considering such biological effects, since appreciable species variation has been reported. For example, tamoxifen demonstrates all the normal characteristics of an antiestrogen in both the rat (Harper and Walpole, 1967) and the chicken (Sutherland, Mester, and Baulieu, 1977), inhibiting the normal physiological responses of estrogen target tissues to estradiol; but in the mouse, it acts as a potent estrogen and shows no antagonistic activity toward the steroid (Terenius, 1970, 1971). Such differing biological characteristics have obvious value, however, in helping to understand the various factors determining the estrogenic and/or antiestrogenic properties of the molecule.
Tests based on the gain in uterine weight of immature rats (Harper and Walpole, 1967) or ovariectomized ones (Jordan, 1976; Marois and Marois, 1977) have shown that tamoxifen, in addition to its antagonistic properties toward estradiol, also possesses weak estrogenic activity (Figure 3 b). However, unlike the more conventional partial agonists of estradiol—for example, estriol (Anderson, Peck, and Clark, 1975)—repeated administration of high doses of tamoxifen to rats failed to elicit a full uterotrophic response in this species, producing only half the maximal response shown to estradiol.

It is of interest that a more detailed examination of the earlier effects of the drug, within the first 24 hr after its administration to ovariectomized animals, clearly indicates that tamoxifen promotes an increase in uterine weight (Figure 3 a) identical to that produced by a single injection of estradiol (Koseki et al., 1977; Davies, Syne, and Nicholson, 1979). This weight increase results from hypertrophy of the luminal epithelial cells (Ljungkvist and Terenius, 1972). In similar studies by Cowan and Leake (1979), tamoxifen and estradiol were administered at their respective optimal doses to immature rats. When assessments were made 24 hr after administration, it was confirmed that the drugs were equally effective not only in promoting uterine growth but also in stimulating DNA synthesis.

In contrast to these earlier events, the longer-term effects (> 24 hr) observed after multiple injections of tamoxifen and estradiol were markedly different. It has been shown that whereas estradiol continued to promote further cell division and hyperplasia (Lan and Katzenellenbogen, 1976), tamoxifen failed to maintain its initial estrogenic potential (Harper and Walpole, 1967; Koseki et al., 1977). It was only during the later phase of the action of the drug that its antagonistic properties toward the estradiol-stimulated increase in uterine weight (Harper and Walpole, 1967; Marois and Marois, 1977; Jordan et al., 1977 a; Cowan and Leake, 1979) and DNA content (Jordan et al., 1977 b; Cowan and Leake, 1979) became evident (Figure 3 b). Similar observations have been made regarding the long-term action of tamoxifen on vaginal epithelial-cell proliferation and also on the release of luteinizing hormone from the pituitary of ovariectomized rats (Nicholson, 1979).

In the mouse, tamoxifen causes stimulation of the uterus in ovariectomized animals equal to the maximum response to estradiol (Terenius, 1970, 1971) and produces full cornification of the vagina (Harper and Walpole, 1966, 1967; Jordan, 1975 b). No antagonism was demonstrated between tamoxifen and estradiol in either of these tissues.
Figure 3. Effect of tamoxifen and estradiol on rat uterine wet weight. Seven-day ovariectomized animals were killed at the indicated times after the daily injection of vehicle, estradiol (0.5 μg), tamoxifen (300 μg), or estradiol (0.5 μg) plus tamoxifen (300 μg). Uterine wet weight is expressed in relation to total body weight. Each point represents the mean ± S.D. of eight determinations. (* = p V Control < 0.05; † = p V estradiol-treated group < 0.05.)
In view of the above studies, it would appear that the antagonistic properties of tamoxifen in estrogen-responsive tissues of the rat reside in the ability of the drug to promote a full long-term estrogenic response. As stated earlier, a degree of estrogen dependency has been implicated in the growth of both human (Stoll, 1969) and rat (Dao and Sinha, 1972) mammary tumors. If breast tumor cells therefore require a prolonged estrogenic stimulus for their continued growth, and if tamoxifen cannot maintain that stimulus for their continued growth, and if tamoxifen cannot maintain that stimulus, then tumor regression will occur. The early agonistic properties, however, equivalent to those of estradiol (Figure 3a), do not support a model system for the mode of action of the drug whereby the antiestrogenic and antitumor properties reside solely in a weak estrogenic activity.

An important problem, therefore, to which research had to be directed was the reason why tamoxifen ultimately failed as an estrogen. Clues to this were to be found in a greater knowledge of the interaction of the drug with estrogen receptor proteins and the subsequent transcriptional events occurring at the level of the genome.

**INTERACTION OF TAMOXIFEN WITH ESTROGEN RECEPTOR PROTEINS**

The antagonistic properties of the triphenylethylene type of nonsteroidal antiestrogens were originally thought to reside primarily in their ability to interfere with the high-affinity binding of estradiol to its specific cytoplasmic receptor protein (Korenman, 1969, 1970), a feature common to this group of compounds (Rochefort and Capony, 1972; Skidmore et al., 1972; Black and Kraay, 1973; Cidlowski and Muldoon, 1976). Subsequently, however, this has been shown to represent only the first step in a more complicated series of events. Nevertheless it seems likely that in order to understand the mechanism by which tamoxifen influences tumor growth, the detailed nature of any interaction between the antihormone and the cytoplasmic estrogen receptor protein should be examined.

**STRUCTURAL CONSIDERATIONS**

Sucrose density gradient analysis has shown (Figure 4) that tamoxifen can compete with estradiol for its specific 4S or 8S binding protein (Jordan and Prestwich, 1977; Jordan and Koermer, 1975; Powell-Jones et al., 1975a, b; Nicholson, Davies, and Griffiths, 1978b). The drug associates with approximately the same number of binding sites as does estradi-
Figure 4. Sucrose density gradient profiles of cytosol fractions—I. Cytosol fractions (200 μl) prepared from DMBA-induced mammary tumors were incubated (2 hr at 4°C) with a single saturating concentration of 3H estradiol (5 nmol/l, •) in the presence or absence of a 100-fold excess of tamoxifen (△) or diethylstilbestrol (○). Excess ligands were removed by charcoal adsorption and the cytosols centrifuged through linear 5-20% (w/v) sucrose density gradients under low-salt conditions. Gradients were fractionated by upward displacement with sucrose. Sedimentation markers (arrows) were bovine serum albumin (BSA) (4.6S) and γ-globulin (7.7S).

ol, but with only about 5-10% of it affinity (Capony and Rochefort, 1978; Nicholson et al., 1978 b; Nicholson et al., 1979 b). Since the receptor proteins for tamoxifen and estradiol were commonly precipitated by protamine sulfate (Capony and Rochefort, 1978; Nicholson et al., 1979 b) and ammonium sulfate (Capony and Rochefort, 1978) and showed a similar specificity of binding (Capony and Rochefort, 1978; Nicholson...
et al., 1978 b; Nicholson et al., 1979 b), the experimental data provide good evidence of a common binding protein, and possibly of a common binding site for both estradiol and tamoxifen on the estrogen receptor protein.

Hahnel, Twaddle, and Ratajczak (1973) postulated that the attachment of estradiol to its receptor probably occurs via two groups, the phenolic C-3 hydroxyl group binding to a highly specific site and thereby facilitating the attraction of the C-17β-hydroxy function to a less specific binding site. The mechanism by which tamoxifen might bind to the estrogen receptor is less easy to define.

The general structure of tamoxifen, although devoid of hydroxyl groups, can be depicted in a manner similar to that of estradiol of diethylstilbestrol (Figures 2 f, 2 g). It is also of interest that the introduction of a single hydroxyl group in the para position of the C-1 phenyl of tamoxifen, forming 1-(4-β-dimethylaminoethoxyphenyl)1-4-hydroxyphenyl,2-phenylbut-1-ene, a derivative referred to as Metabolite B (Figure 2 c), produces a modification that not only increases its structural resemblance to diethylstilbestrol but increases its affinity for the receptor (Jordan et al., 1977 a; Nicholson et al., 1979 b). This latter phenomenon appears to result from a lowered rate of dissociation of Metabolite B from the binding protein (Nicholson et al., 1979 b). It is therefore attractive to postulate that the incorporation of the hydroxyl group into the tamoxifen structure strengthens the binding of the molecule to the site normally occupied by one of the hydroxyl groups of either estradiol or diethylstilbestrol.

The aminoethoxy side chain of tamoxifen would also seem to be intimately involved in binding the molecule to the receptor and in determining the antiestrogenic properties of the compound. Incorporation of two methyl groups ortho to the ether oxygen, alterations that restrict the spatial positions that the side chain can adopt, reduce its ability to compete with estradiol for mouse uterine estrogen receptor proteins (Abbott, Clark, and Jordan, 1976). Furthermore, substituting for the N,N-dimethyl group of the drug an N,N-(2-chloroethyl) group, to produce the nitrogen mustard derivative of tamoxifen, ICI 79792 (1-(4-β-(bis(2-chloroethyl) aminoethoxyphenyl))1,2-diphenylbut-1-ene (Figure 2 e) decreases the affinity of the compound for the estrogen receptor and reduces its antiestrogenic and antitumor properties (Nicholson and Golder, 1975). It remains to be resolved whether the side chain of tamoxifen is involved in the attachment of the molecule to the site normally occupied by either the 3- or the 17β-hydroxyl groups of estradiol, or whether it binds to a site, completely distinct, on the estrogen receptor protein. Whatever the mechanism, it is noteworthy that the
parent triphenylethylene is estrogenic (Robson, Schonberg, and Fahim, 1938), whereas the addition of the side chain to the molecule confers antiestrogenicity, observed when the compound is administered to certain species (Harper and Walpole, 1967).

Alternatively, it has been proposed that these nonsteroidal antiestrogens such as tamoxifen are allosteric, noncompetitive inhibitors of the binding of estradiol to the receptor (Hahnel et al., 1973). This proposal stems from indirect studies in which the binding of 13H estradiol to the receptor in the presence of unlabeled tamoxifen was examined. It is obviously essential to distinguish between these two possible mechanisms for the attachment of tamoxifen to the estrogen receptor, not only because such knowledge is fundamental to our understanding of the subsequent events concerned with its action, but also because it provides a sound scientific basis for the design of a second generation of antiestrogens. Studies associated with this topic are currently in progress.

PLASMA LEVELS OF TAMOXIFEN AND ESTRADIOL

Having established that tamoxifen is capable of binding to the estrogen receptor in vitro, it is pertinent from a mechanistic point of view to ascertain the levels of tamoxifen relative to estradiol available to the tumor tissue. Following the in vivo administration of the drug at a dose (300μg/day) capable of causing regression of estrogen-receptor-positive DMBA-induced mammary tumors, plasma tamoxifen levels rapidly increased in the treated animals, reaching 40 ng/ml in 1 hr (Nicholson et al., 1979a; Nicholson, 1979). This value was approximately three orders of magnitude higher than the corresponding concentration of estradiol, which remained fairly constant at about 30 pg/ml throughout the experimental period (cf. Nicholson and Golder, 1975). Moreover, a considerable amount of the drug was present in plasma 24 hr after the previous injection, resulting in a progressive increase in the basal plasma levels of tamoxifen to 30 ng/ml on successive injections. A somewhat similar situation was also observed in postmenopausal women with advanced breast cancer, treated orally with tamoxifen, 20 mg b. d. (Nicholson et al., 1979a; Daniel et al., 1979). Again plasma concentrations of the drug increased rapidly to 5–20 ng/ml during the first few hours of treatment, contrasting strongly with the much lower plasma concentrations of estradiol (30–50 pg/ml). On continued daily administration basal plasma tamoxifen concentration rose steadily within the first 40 days of treatment, reaching values between 150 and 250 ng/ml—
approximately 3 to 4 orders of magnitude higher than the corresponding estradiol levels, which were unaffected by the treatment (Golder et al., 1976; Nicholson et al., 1979 a; Daniel et al., 1979).

Clearly in the postmenopausal women, and also in the rat, tamoxifen was continually available to estrogen-responsive tissues and was at all times present in concentrations considerably in excess of those of estradiol. These dose levels, therefore, produce concentrations of tamoxifen in plasma that are consistent with the model for the action of the drug, in which the antiestrogen acts on the target tissue by association with estrogen receptor proteins. Preliminary data on the tumor tissue levels of tamoxifen (Nicholson and Daniel, unpublished data) and estradiol (Maynard and Griffiths, 1979) are also consistent with this concept.

RECEPTOR TRANSFORMATION AND TRANSLOCATION

Following the association of estradiol with its cytoplasmic binding protein, the receptor complex undergoes a temperature-dependent conformational change resulting in the formation of a product that has a higher molecular weight and a sedimentation coefficient of 5S (Jensen et al., 1969). It has been suggested that this step increases the affinity of the estrogen receptor for chromatin (DeSombre, Mohla, and Jensen, 1975) and results in the activation of RNA polymerases (Jensen and DeSombre 1973). Furthermore, Clark and Peck (1976) postulated that the prolonged occupation (approximately 6 hr) of high-affinity nuclear acceptor sites by estrogen receptor complexes was obligatory for the true growth of estrogen-responsive tissues.

Although little is known about the mechanism by which these events are induced, estrogen receptor translocation has also been demonstrated with a wide range of steroids other than estrogens, including the C19-steroids, androstane-3ß,17ß-diol, androst-5-ene-3ß,17ß-diol, testosterone (Nicholson et al., 1976 b, 1978 a), and 5a-dihydrotestosterone (Ruh, Wassilak, and Ruh, 1975; Rochefort and Garcia, 1976). In addition, nonsteroidal antiestrogens such as nafoxidine (Clark, Anderson, and Peck, 1973), CI628 (Katzenellenbogen and Ferguson, 1975), enclomiphene (Ruh and Baudendistel, 1977), and tamoxifen (Figure 5; Jordan et al., 1977 b; Nicholson et al., 1976 a; Nicholson, Davies and Griffiths, 1977 a) also appear capable of promoting the translocation of the estrogen receptor. It would appear reasonable to suppose, therefore, that the requirement for eliciting the translocation process is the ability of a molecule to associate with the estrogen-binding site of the receptor. Steroids such as androstenedione and epitestosterone, which do not
Figure 5. Effect of tamoxifen on the cellular distribution of estrogen-binding proteins in rat mammary tumors: 1. Early effects. Animals were injected intramuscularly with tamoxifen (300 μg in sesame oil) and the concentration of accessible cytoplasmic and total nuclear estrogen receptors (ER) measured in tumor biopsies (Nicholson et al., 1977). Results are the mean ± S.D. of six tumor biopsies per time point (From Nicholson et al., 1979 b).

compete with estradiol for the estrogen-binding protein (Davies et al., 1978; Nicholson et al., 1978 a), are unable to effect the translocation process (Nicholson et al., 1978 a).

NUCLEAR BINDING CHARACTERISTICS

Studies with the C19-steroid-transferred estrogen receptor protein indicate that it is different from the estrogen-transferred receptor complex. It has been shown that it has a short nuclear retention time and also an ability to extensively bind 3H estradiol at 4°C (Nicholson et al., 1978 a). In this respect, therefore, its characteristics are similar to those of the estrogen receptor translocated by estriol, a steroid whose weak estrogenic activity has been associated with its short nuclear retention time (Anderson et al., 1975; Clark and Peck, 1976). When estriol was continually presented to a target tissue, either by multiple injections (Anderson et al., 1975) or by chemical modification of the compound
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(Lan and Katzenellenbogen, 1976), thus maintaining the nuclear concentration of the receptor complex, it acted as a potent estrogen without antagonistic properties. Similarly, maintenance of relatively high concentrations of androst-5-ene,3β,17β-diol—by implantation or by continually injecting the steroid—produced an estrogenic effect in mammary tumors (Nicholson, unpublished data) and also in the uterus (Huggins, Jensen and Cleveland, 1954) of the rat.

Nonsteroidal antiestrogens, however, do not behave in this manner. After a single injection of nafoxidine, nuclear antiestrogen receptor complexes have been observed in immature rat uteri for periods of up to 19 days (Clark et al., 1973). Indeed, prolonged nuclear retention of the antiestrogen receptor complex appears common to tamoxifen in the rat uterus (Jordan et al., 1977 b) and the rat mammary tumor (Nicholson et al., 1979 a), and CI628 (Katzenellenbogen and Ferguson, 1975) and enclomiphene (Ruh and Baudendistel, 1977) in the rat uterus; yet each of these compounds was unable to promote a full uterotrophic response. It has been suggested that this paradoxical situation arises from the existence of different nuclear binding sites for estrogen and antiestrogen receptor complexes or from an atypical binding of the antiestrogen receptor complex to chromatin.

Following the studies of Baudendistel and Ruh (1976), Jordan et al. (1977 b) examined the salt extractability of the tamoxifen receptor complex from rat uterine nuclear preparations. They determined that all the antiestrogen receptor complexes were extractable by 0.4 M KCl, whereas only 80% of the estrogen receptor complexes were extracted by the identical procedure. Thus a small proportion (approximately 20%) of the nuclear estrogen receptor binding may be classified as high-affinity; and it has been proposed that it is these sites that regulate the true long-term growth of estrogen-responsive tissues (Clark and Peck, 1976). Antiestrogen receptor complexes, by their failure to associate with these sites or by their association with a lower affinity are unable to promote a full estrogenic action. Unfortunately, the observation that enclomiphene the cis isomer of the antiestrogen enclomiphene, and a compound capable of eliciting a full uterotrophic response, is also totally extractable by 0.4 M KCl (Ruh and Baudendistel, 1977) provides evidence contrary to this concept. Furthermore, such a hypothesis is not fully consistent with the observed early (0–24 hr) agonistic properties of antiestrogens when they exhibit a complete estrogenic response.

EFFECTS ON RNA POLYMERASES

As stated earlier, it is now widely accepted that estrogen-dependent tissues respond rapidly to estradiol with increased levels of RNA
synthesis, resulting from transcription of the DNA template by RNA polymerases (Gorski, 1964; Hamilton, Widnall, and Tata, 1968). These events are manifested by a transient rise in RNA polymerase II activity (nucleoplasmic enzyme), obligatory for the subsequent elevation of RNA polymerase I (nucleolar enzyme) and a major prolonged enhancement of RNA polymerase II (Glasser, Chytil, and Spelsberg, 1972; Borthwick and Smellie, 1975). RNA polymerase I is associated with the production of ribosomal RNA (rRNA; auxiliary RNA), whereas RNA polymerase

![Figure 6](image-url)

**Figure 6.** Alterations in activity of RNA polymerases resulting from the administration of estradiol. Mammary-tumor-bearing rats were given a single intravenous dose of 5 µg estradiol 10% ethanol in NaCl (0.15 mol/l) by intravenous injection, and biopsy samples were removed at various times up to (a) 60 min or (b) 24 hr after treatment. RNA polymerase I (□) and RNA polymerase II (■) were measured under conditions that precluded the activity of other species (Nicholson et al., 1977). Control (100%) activities, in terms of 3H UMP incorporation, were 5.4 ± 0.6 pmol/100 µg DNA for RNA polymerase A and 18.3 ± 1.4 pmol/100 mg DNA for RNA polymerase B. Values are the mean ± S.E.M. of five tumors per group. (From Nicholson et al., 1979 b.)
II results in the formation of informational messenger RNA (mRNA) (cf. Chambon, 1975; Roeder, 1976).

From investigations with DMBA-induced rat mammary tumors, it has been shown that increases in RNA polymerase II activity resulting from the administration of tamoxifen are qualitatively similar to those produced after estradiol stimulation during the first peak of activity and also during the early phase of the second peak, although tamoxifen then appears unable to maintain this secondary stimulated rise in the activity of the enzyme (Figures 6, 7; Nicholson, Davies, and Griffiths, 1977 b). The effects of tamoxifen on RNA polymerase I are, however, quantitatively inferior to those initiated by estradiol. Identical findings have been observed with the rat uterus (Davies et al., 1979).

These results may point to some basic inefficacy of the tamoxifen receptor complex in the rat, especially with regard to the eventual production of auxiliary ribosomal RNA. Such a concept is supported by subsequent studies with mouse uterus, a tissue in which tamoxifen elicits a full estrogenic effect and in which identical increases in the activity of

![Figure 7. Alterations in activity of RNA polymerases resulting from the administration of tamoxifen. Experimental design and conditions were as described in Figure 6 except that animals were given a single intravenous injection of 100 μg tamoxifen 10% ethanol in 0.15 mol/l NaCl. (From Nicholson et al., 1979 b.)](image-url)
RNA polymerase I and II are brought about by tamoxifen and estradiol (Syne, Davies, and Nicholson, unpublished observations). The informational mRNA, resulting from tamoxifen administration, also appears partially functional: early increases (e.g., within 24 hr) have been observed in the level of cytoplasmic progesterone receptor, an end product of estrogen action (Milgrom et al., 1972); and these increases are similar to increases elicited by estradiol (Nicholson et al., 1979a). Similarly, tamoxifen also seems capable of stimulating small, transient increases in the activity of certain enzymes associated with the glycolytic pathway and the hexose monophosphate shunt (Table 1).

Thus the initial influx of the antiestrogen receptor complex into the nucleus does bring about early biochemical effects leading to the stimulation of transcriptional activity and to the possible elaboration of some products such as those described above—even to the promotion of DNA synthesis (Cowan and Leake, 1979). The continued nuclear presence of the tamoxifen receptor complex (Nicholson et al., 1979a) is, however, unable to fulfill the necessary interactions to maintain turnover of essential cellular materials. Since the effects on extranuclear chromatin, manifested by increases in RNA polymerase II, may be effected directly, the tamoxifen receptor complex may satisfactorily accomplish these effects at least initially; but they may not result in correct or sufficient cellular products to prolong an efficient response at the nucleolar level, and hence to maintain the levels of auxiliary RNA

Table 1. Effect of tamoxifen on enzyme activities in rat mammary tumors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme activity (units/g protein)</th>
<th>Tamoxifen-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1 day</td>
</tr>
<tr>
<td>G6PDH + 6PGDH</td>
<td>187 ± 24.0</td>
<td>236 ± 13.3c</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>1,872 ± 360</td>
<td>2,299 ± 109c</td>
</tr>
<tr>
<td>Aldolase</td>
<td>73.2 ± 12.72</td>
<td>90.3 ± 10.70c</td>
</tr>
</tbody>
</table>

* Groups of intact animals were given daily intramuscular injections of tamoxifen (100 μg/day) in 0.1 ml sesame oil for 7 days. Tumor biopsies (200 mg tissue) for enzyme analysis were removed on days 0, 1, and 7 of treatment. The results are the mean ± S.D. of six tumors per group.

*b G6PDH; Glucose 6-phosphate dehydrogenase; 6PGDH; 6 Phosphogluconate dehydrogenase.

c p<0.05. Enzyme levels were assayed as described in Nicholson and Colder (1975).
essential for maintaining estrogen-dependent tissue growth. Indeed, Hamilton (1968) has shown that estradiol stimulates the protein-synthesizing activity of individual ribosomes, which increases for 12–14 hr and then falls, even after an additional injection of estradiol. The total number of ribosomes, however, as polyribosomes, continues to rise and thereby maintains constant high levels of protein synthesis in the estradiol-treated uterus. Following tamoxifen administration, early mRNAs may then be translated on pre-existing ribosomes, whereas those formed much later would accomplish this process less efficiently. In this respect, it is of interest that the profile of progesterone receptor stimulation by tamoxifen, in rat uteri from ovariectomized animals, increased considerably during the early action of the drug and then fell to prestimulation values (Figure 8). Furthermore, prolonged administration of tamoxifen to ovariectomized rats for 14 days reduced the activity of uterine nuclear RNA polymerases I and II below both control and estradiol-stimulated levels (Table 2). These data on RNA polymerase activity in the nuclei of rat mammary tumors and also rat uteri (Davies et al., in press) are consistent with the general properties of tamoxifen outlined earlier, when it was observed that during the early phase of the action of the drug it promotes tissue effects identical to estradiol but that it subsequently fails to maintain its initial estrogenic potential in terms of growth.

In the rat uterus, both tamoxifen and estradiol apparently increase RNA polymerase II activity by making more initiation sites available rather than by affecting the size of RNA product (Davies et al. in press). Furthermore, workers using chick oviduct (Tsai et al., 1975), have shown that the number of initiation sites is dependent upon the concentration of the nuclear estrogen receptor complexes. This also appears to be the case in the rat uterus, but for estradiol and not tamoxifen, since the nuclear concentration of the tamoxifen receptor complex is maintained after the number of initiation sites returns to prestimulation levels (Davies et al., 1979). This therefore casts further doubt on the biochemical efficacy of the antiestrogen receptor complex.

In the light of the recent report by Bichon and Baynard (1979), it would be naive, however, to interpret all data regarding alteration in RNA polymerase activity as representing a tamoxifen inhibition of all species of RNA. These workers examined the effects of a tamoxifen and estradiol, singularly and in combination, on the progesterone receptor level and the plasma renin substrate (PRS) content of immature rat uterus. It was shown that during the course of tamoxifen treatment, over a 7-day period, the drug can act (a) as a partial agonist of estradiol, as
Figure 8. Effects of tamoxifen on the concentration of cytoplasmic progesterone receptor in rat uteri from ovariectomized animals. Animals were killed at the indicated times after the injection of tamoxifen (300 μg/day, ○; 3 mg/day, ⋄), and the amount of 3H progesterone specifically bound in the cytoplasm was determined as described in Davies et al. (1979). Results are the mean of four uteri per group.

indicated by uterine growth; (b) as complete agonist, as demonstrated by an increase in progesterone receptor content; or even (c) as a more potent agonist, as observed after measuring the increase of PRS. Rather, the heterogeneity of response to tamoxifen suggests a selective failure to produce a key component or components necessary for the continued growth of the tissue. Such a regulatory process could be effective in either a positive or a negative manner.
Table 2. Long-term effects of tamoxifen and estradiol on RNA polymerases

<table>
<thead>
<tr>
<th>RNA polymerase (pmol 3H UMP incorporation/mg DNA)</th>
<th>Control Group I</th>
<th>Control Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0, 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.98 ± 0.079</td>
<td>0.99 ± 0.079</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.99 ± 0.079</td>
<td>3.06 ± 0.158</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>2.07 ± 0.086</td>
<td>2.99 ± 0.264</td>
</tr>
<tr>
<td>Estradiol + tamoxifen</td>
<td>0.34 ± 0.031</td>
<td>4.13 ± 0.259</td>
</tr>
<tr>
<td>Control</td>
<td>0.33 ± 0.065</td>
<td>1.49 ± 0.386</td>
</tr>
<tr>
<td>Estradiol</td>
<td></td>
<td>1.60 ± 0.412</td>
</tr>
</tbody>
</table>

*Ovariectomized animals were killed at the times indicated after the daily injection of vehicle, estradiol (0.5 μg), tamoxifen (300 μg), or estradiol plus tamoxifen. RNA polymerase I and II activities were measured as described in Davies et al. (1979). The results are the mean ± S.D. of 4 determinations (2 uteri per determination) performed in duplicate.*
EFFECTS ON DNA SYNTHESIS

It has previously been stated that tamoxifen fails to maintain DNA synthesis in the rat uterus after an early stimulatory effect (Cowan and Leake, 1979). Thus on continued administration estradiol initiates both hyperplasia and hypertrophy, whereas tamoxifen appears capable only of initiating the latter process (Ljungkvist and Terenius, 1972). In cell cultures of the estrogen-responsive human breast tumor (MCF 7), tamoxifen inhibits thymidine incorporation into DNA to below control levels (Lippman, Bolan, and Huff, 1976). Indeed, if cells are cultured with antoestrogen alone for more than 3 days, these inhibitory effects become irreversible, and cells detach from the surface of the culture vessel and die. These events, which are only observed in cell lines containing estrogen receptors, were interpreted by the authors as suggesting that in the absence of tamoxifen, mRNA transcripts coding for growth-regulating proteins are synthesized at a rate sufficient to support slow cell growth. However, in the presence of the antoestrogen receptor complex, these events are inhibited below control values—possibly to a level incompatible with cell viability. The inhibitory action of tamoxifen on rat uterine RNA polymerases (Table 2) is consistent with this hypothesis, although it appears to be a late rather than an early event.

It is also of some interest that $^3$H thymidine incorporation into uterine DNA in rats chronically treated with estradiol (1 μg/day) has been shown to be stimulated at 24 hr but then depressed to control levels within 24 hr after the last of three daily injections (Stormshak et al., 1975). A similar sequence of events was also observed for estrogen-induced effects on protein synthesis and oxidation of glucose. Furthermore, estriol is also able to promote this refractory state, provided that the dose level administered is sufficiently high to maintain a prolonged nuclear presence of the estrogen receptor. These data were interpreted as indicating that the refractoriness to estrogens resulted from the accumulation of certain products limiting the ability of the cells to respond to estrogens and blocked their passage from a resting (Go), or presynthetic (G1) stage of the cell cycle to the period of DNA synthesis. An effective stimulation by antiestrogens of the processes producing such factors, resulting from the continual presence of the antiestrogen receptor complex in the cell nucleus, might form one mechanism by which these compounds inhibit DNA synthesis. Certainly, it has been proposed by Lippman and his colleagues (1976) that tamoxifen can arrest estrogen-responsive MCF 7 human mammary tumor cell lines in a uniform state of the cell cycle. Obviously, further research is necessary to increase our understanding of these complex events.
RECEPTOR REPLENISHMENT

For estrogen-responsive tissues to maintain their responsiveness to the hormone, cytoplasmic estrogen receptors are replenished after translocation. This process is thought to be achieved by a combination of receptor resynthesis and recycling (Sarff and Gorski, 1971). It has been suggested that the prolonged and possibly atypical binding of anti-estrogen receptor complexes to sites on chromatin resulted in a concomitant failure to derepress the region on the genome ultimately responsible for the production of the estrogen receptor protein (Clark et al., 1973). This theory arises from observations regarding the cellular distribution of estrogen-binding proteins following the in vivo administration of high doses of antiestrogens (Clark et al., 1973; Clark, Anderson, and Peck, 1974; Katzenellenbogen and Ferguson, 1975; Nicholson et al., 1976; Jordan and Dowse, 1976; McNabb and Jellinck, 1976; Ruh and Baudendistel, 1977) and is illustrated with regard to the action of tamoxifen in the DMBA-induced mammary tumor in Figure 9. Within 5 days of tamoxifen administration (300 μg/day), virtually no specific binding of 3H estradiol can be determined in cytoplasmic preparations, although nuclear receptor is evident. Sucrose density gradient analysis of cytosol fractions at this time reveals the complete abolition of the 8S 3H estradiol-binding peak (not illustrated). Since the replenishment process and the subsequent restoration of cytoplasmic estrogen receptor (ER) levels have been implicated in the ability of the tissue to respond to further estrogen stimulation (Clark et al., 1973, 1974; Mester and Baulieu, 1975), failure to achieve these processes would break the sequence of events by which estrogens act. Indeed, experiments performed at a time when estrogen receptor levels are low can be interpreted as agreeing with this hypothesis, since it can be shown that under these conditions tamoxifen substantially reduces the total tissue uptake of radioactive estradiol into DMBA-induced mammary tumors (Nicholson, in press; Nicholson et al., 1979a), the percentage of nuclear-bound estradiol, and the formation of the nuclear-bound estradiol, and the formation of the nuclear 3H estradiol receptor complex (Nicholson et al., 1979a). Similar observations have been made in vitro (Powell-Jones, Davies, and Griffiths, 1975a; Powell-Jones et al., 1975b) and in vivo (Jordan, 1975a) by other workers. Moreover, at this time the tissue appears refractory to exogenously administered estradiol (Nicholson et al., 1977a).

More recent studies have, however, made this hypothesis untenable, since tumor regression (Figure 10) and a decrease in uterine weight (Koseki et al., 1977; Jordan et al., 1977b) have been observed following
Figure 9. Effect of tamoxifen on the cellular distribution of estrogen-binding proteins in rat mammary tumors: late effects. Experimental design was as described in Figure 5. (From Nicholson et al., 1979 b.)
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Figure 10. Effect of concurrent administration of estradiol and tamoxifen on tumors regressing as a result of tamoxifen treatment. Mammary-tumor-bearing animals were given daily intramuscular injections of tamoxifen (10 μg) in sesame oil (100 μl) for either 21 days (○) or 49 days (●). Control animals received vehicle for 49 days (△). Animals injected for 21 days were then injected with a combined daily regime of estradiol (1 μg) plus tamoxifen (10 μg) for the remaining experimental period. Tumor growth patterns were recorded as changes in tumor column and are the mean ± S.D. of six tumors per group. (* = bVa < 0.05.)

Indeed, Jordan et al. (1977 b) have recorded a small net increase in the total uterine concentration of estrogen receptors 48 and 72 hr after the daily administration of 4 μg tamoxifen, a minimum antagonistic dose, thus suggesting that estrogen receptor synthesis had occurred. It now seems more likely that the inability to detect estrogen receptor after administration of high doses of tamoxifen may be due to the long half-life
Figure 11. Estrogen receptor content of mammary tumors following the administration of tamoxifen and estradiol, singularly or in combination. Biopsy samples (approx 200 mg) were removed from tumors described in Figure 10 on day 21 (group A, Cytosol fractions; B, nuclear fractions) and then 4 h (group C, nuclear fractions) after the subsequent injections. Groups A and B: control saline injected; tamoxifen (10 μg/day) injected. Group C, as in Groups A and B, then received 1 μg estradiol concurrently with 10 μg tamoxifen. Biopsies were then assayed for accessible cytoplasmic and total nuclear estrogen receptors (Nicholson et al., 1977 a). Results are the mean ± S.D. of six tumors per group.

of the drug in plasma (Fromson, Pearson, and Bramah, 1973 a, b); thus, high levels of tamoxifen (Nicholson et al., 1979 a; Daniel et al. submitted for publication) are continually available to translocate newly synthesized estrogen receptor. This would result in a dynamic pool of receptor whose nuclear concentration at any moment in time is integrated between the rate of entry of the receptor complex (governed by the rate of synthesis of the receptor) and its destruction or inactivation. Moreover,
other evidence indicates that reduced cytoplasmic estrogen receptor levels are not necessarily a function of the antiestrogenicity of tamoxifen; reduced receptor levels are also observed in mouse uterus (Nicholson et al., 1979 a), under conditions where tamoxifen behaves as a full estrogen (Harper and Walpole, 1967) and also in rat mammary tumors following the daily administration of high doses of either estradiol or diethylstilbestrol (Nicholson and Golder, 1975).

Interestingly, at low dose levels of tamoxifen (10 ug/day), the tumor regression produced by the drug has been partially reversed in certain tumors by the simultaneous administration of estradiol (1 ug) (Figure 10). Such a procedure elevates the nuclear concentration of the estrogen receptor (Figures 11 b, 11 c)—a product, however, of both tamoxifen and estradiol action. Taken together, such data suggest that competition between the tamoxifen receptor complex and the estrogen receptor complex is closely concerned with the primary antitumor effects of tamoxifen action, as has also been postulated for the antiuterotrophic action of the drug (Jordan et al., 1977 b). Thus the occupancy of key sites on chromatin by a complex unable to satisfy the essential requirements necessary for the continued growth of the cell would be consistent with the tumor regression observed in tamoxifen-treated animals and patients.

On the basis of this model, recalcitrance to estradiol at high dose levels of tamoxifen could be seen as a consequence of the competition between the high concentrations of tamoxifen relative to low endogenous estradiol levels (Nicholson, in press; Nicholson et al., 1979 a; Daniel et al., submitted for publication) in the presence of only limited amounts of receptor. These conditions would favor the formation of the antiestrogen receptor complex.

POSSIBLE FACTORS RELATING TO THE ANTIESTROGENIC PROPERTIES OF THE TAMOXIFEN RECEPTOR COMPLEX

In 1970, Korenman demonstrated that a series of nonsteroidal antiestrogens shared the common characteristic of having a low affinity for the estrogen receptor. It was supposed that this resulted in a rapid dissociation of the antiestrogen from the receptor protein prior to its initiating a full estrogenic response. Although it is true that tamoxifen has a high dissociation rate from human and rat mammary tumor estrogen receptor preparations (Nicholson et al., 1979 b), there are a number of arguments against this hypothesis. For example, such a concept is difficult to reconcile with the early uterotrophic action of the drug where it shows
no antagonism toward estradiol (Figure 3a). In addition, the dissociation rate constant \( k_2 \) determined for the dissociation of tamoxifen from the estrogen receptor is higher in mouse uteri, a tissue where the drug acts as a potent estrogen, than in rat uteri, where it displays antiestrogenic properties (Table 3). Furthermore, the cis isomer of tamoxifen, ICI 47699, binds to the receptor with a much lower affinity than tamoxifen (Skidmore et al., 1972; Terenius, 1971) yet is fully estrogenic.

<table>
<thead>
<tr>
<th>Uterus type</th>
<th>( k_2 ) (10^{-4}/min)</th>
<th>( K_D ) (n mole/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat uterus</td>
<td>20 ± 7.3 (4)</td>
<td>4.63 ± 1.67 (4)</td>
</tr>
<tr>
<td>Mouse uterus</td>
<td>46 ± 10.3 (3)</td>
<td>10.33 ± 1.96 (3)</td>
</tr>
</tbody>
</table>

*Dissociation rate constants and \( K_D \) values were determined as described in Nicholson et al. (1979a). Results are the mean ± S.D. of the numbers of determinations shown in parentheses.

Alternatively, the antiestrogenic properties of tamoxifen may relate to conformational changes in the tertiary or quaternary structure of the receptor in response to the association of the drug with its binding site—changes different from those produced by estradiol. In this way the characteristics of the nuclear binding of receptor complex to sites on chromatin may be impaired, thereby promoting atypical transcriptional events. Whereas no direct evidence exists to substantiate this hypothesis, it is equally true that none exists to refute it. On conventional sucrose density gradient analysis (centrifugation time 16 hr) of rat mammary tumor cytosol fractions, \(^3\)H estradiol binds specifically with a sedimentation coefficient of 8S (Powell-Jones et al., 1975a; Jordan and Dowse, 1976; Nicholson et al., 1978b), whereas \(^3\)H tamoxifen gives a value of 4–5S (Nicholson et al., 1978b). Using the vertical tube rotor (centrifugation time, 1.25 hr), however, Jordan and Prestwich (1977) have found 8S binding components for both estradiol and tamoxifen and have suggested that the 4S binding peak is not a different conformational form of the receptor but is an artifact associated with the rapid dissociation rate of tamoxifen from the receptor protein on its passage through the gradient. Such a finding does not in itself exclude differences between the receptor complexes. Rather, it may emphasize the relative limitations of the techniques available. The purification of estrogen
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receptor from rat mammary tumor tissue (Al-Nuaimi, Davies, and Griffiths, 1979) and the subsequent structural characterization of the receptor in the presence or absence of either tamoxifen or estradiol may aid our understanding of the mode of action of the drug and provide a screening tool for compounds of potential antiestrogenic value.

RELEVANCE OF METABOLITES TO THE ACTION OF THE DRUG

The studies previously described in this chapter have illustrated a series of events that have been attributed to the direct action of tamoxifen. Indeed, such a concept is reinforced by experiments using the MCF 7 human mammary tumor cell line where the characteristic properties of the molecule are recorded in the absence of drug metabolism (Horwitz, Koseki, and McGuire, 1978). However, in the intact animal and in patients a number of metabolites of tamoxifen have been identified following the in vivo administration of 14C tamoxifen (Fromson et al., 1973 a, b), and it is feasible that they may influence the action of the parent drug.

The principal metabolite described by Fromson et al. (1973 a, b) was a monohydroxylated derivative, metabolite B (Figure 2 c). This compound is thought to be formed from tamoxifen by aromatic hydroxylation in the liver and then to undergo enterohepatic recirculation. It is present in plasma of postmenopausal patients undergoing tamoxifen therapy (10 or 20 mg b.d.) for advanced breast cancer, reaching some 5-10 ng/ml by day 20 and subsequently reaching a plateau (Nicholson, in press; Daniel et al., submitted for publication). This concentration of metabolite B is approximately 100 times higher than that of estradiol, but only 2% that of tamoxifen. More recently, a desmethyl derivative of tamoxifen, metabolite X, or 1-(4-B-methylaminoethoxyphenyl)1,2-diphenylbut-1-ene (Figure 2 d), has also been identified (Adam, Douglas, and Kemp, 1979). In plasma of women undergoing tamoxifen therapy, it is present in amounts equivalent to or higher than tamoxifen itself (Adam, Daniel, and Nicholson, unpublished data).

Competitive binding studies from this laboratory indicated that metabolites B and X, like tamoxifen, bind to cytoplasmic estrogen receptor preparations and displace radioactive estradiol from its specific 4S and 8S binding protein (Figure 12). An approximate 50% inhibition of 3H estradiol binding (5 nmol/l) is achieved by 50 nmol/l metabolite B, 150 nmol/l metabolite X, and 500 nmol/l tamoxifen. Similar data have
Fraction number

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BSA & -Globulin

<table>
<thead>
<tr>
<th>Radioactivity cpm</th>
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<tbody>
<tr>
<td>900</td>
</tr>
<tr>
<td>700</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>300</td>
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<tr>
<td>100</td>
</tr>
</tbody>
</table>

Fraction number

Figure 12. Sucrose density gradient profiles of cytosol fractions—II. Experimental design and conditions were as described in Figure 4 except that the competitors were tamoxifen (500 nmol/l, △), metabolite B (500 nmol/l, ▲) and metabolite X (500 nmol/l, ◊).

been recorded for tamoxifen and metabolite B in tissue from human mammary tumor (Nicholson et al., 1979 b) and also from rat uterus (Jordan et al., 1977 a; Nicholson et al., 1979 b). Moreover, metabolite B, like tamoxifen, is capable of eliciting the translocation of the estrogen receptor to the rat uterine nucleus, although its nuclear retention profile is closer to that of estradiol than to that of tamoxifen (Jordan et al., 1978).

The possibility therefore exists that metabolites B and X may effectively sequester available estrogen receptor and play a supportive, or possible primary, therapeutic role for tamoxifen in the treatment of breast cancer. Indeed, Jordan et al (1977 a) indicated that tamoxifen
administration, at a dose level at which it was inactive in animals when administered by the subcutaneous route, was effective as an antiestrogen when given orally, implying that drug metabolism had taken place. Furthermore, in vivo administration of either metabolite B or metabolite X reduced uterine weight in intact animals and caused tumor regression in estrogen-receptor-positive mammary tumors (Nicholson, in press). In each case, however, tumor regression was less marked than with tamoxifen. Jorden and Naylor (1978) have presented similar data with respect to metabolite B administered at a lower dose level.

OTHER DIRECT MECHANISMS
At the tissue level the only well-documented evidence of direct interference of the drug with the action of hormones relates to its inhibitory effect on the binding of estradiol to its receptor protein. It has been suggested, however, that tamoxifen may also influence the binding of androgens to their receptors (Tormey et al., 1976). Further, it is of interest that an early report of Harper and Walpole (1976) implied that tamoxifen could be androgenic in rats.

TAMOXIFEN EFFECTS ON PLASMA HORMONE LEVELS
Although the data described in the earlier sections of this review strongly suggest a direct action of tamoxifen on breast tumor tissue, part of its clinical effect could be due to its influence on either the hypothalamic-pituitary-ovarian or the hypothalamic-pituitary-adrenal axes. In practice, however, few convincing data exist for any regulatory effects of tamoxifen therapy on plasma hormone levels that would be likely to influence mammary tumor growth. In postmenopausal women undergoing tamoxifen therapy for advanced breast cancer, the drug is without substantial effect on plasma estradiol or C17,20-steroid levels (Willis, 1975; Golder et al., 1976; Willis et al., 1977; Kiang and Kennedy, 1977), although Kiang and Kennedy (1977) noted low response rates in patients who had low estrone levels, as did Willis et al. (1977) in hyperprolactinemic patients. In premenopausal women, tamoxifen treatment elevates plasma estradiol levels in women with breast cancer (Pearson et al., 1978) and without it (Groom and Griffiths, 1976) and influences plasma prolactin levels (Groom and Griffiths, 1976), suggesting a feedback effect on pituitary prolactin release in patients identical to that observed in the rat (Jordan, Koerner, and Robison, 1975). But unlike the case of the rat, where prolactin plays a major role in stimulating
mammary tumor growth (Manni, Trujillo, and Pearson, 1977), any
effect of tamoxifen on plasma prolactin levels in women must be of
doubtful significance in relation to the treatment of carcinoma of the
breast, since it has yet to be shown that prolactin has any significant role
in human breast cancer and since, moreover, ergot derivatives, known to
effectively inhibit prolactin secretion, have been said to be of little value
in the treatment of the disease (European Breast Cancer Group, 1972;
Pearson and Manni, 1979). Tamoxifen is also without significant effect
on serum growth hormone concentrations (Pearson et al., 1978) and
other experimental studies have indicated that tamoxifen induces rat
mammary tumor regression without affecting circulating prolactin or
estradiol levels (Nicholson and Golder, 1975).

CONCLUSIONS

It is very obvious, therefore, from this review of the biochemistry and
endocrinology relating to the action of the antiestrogen tamoxifen
(Nolvadex), that considerable progress has been made in our under¬
standing of the therapeutic role of the drug in the treatment of carcinoma
of the breast. It is also evident that there are still fundamental questions
remaining unanswered, and attention has been directed to these in this
review. In particular, research into the differences between the physico-
chemical characteristics of the antiestrogen and estradiol receptor
complexes in relation to their effect on the molecular events occurring at
the level of the genome will be invaluable to our understanding of the
action of the drug and in the development of a more effective treatment
regime.

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LITERATURE CITED

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THE BIOCHEMISTRY OF TAMOXIFEN THERAPY OF BREAST CANCER

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ABSTRACT
Experiments have been undertaken to examine the mode of action of tamoxifen in the treatment of breast cancer. Three mechanisms appear to be involved: 1) tamoxifen inhibits the formation of the oestrogen receptor complex; 2) the drug substitutes a receptor complex of lower intrinsic biological activity than the oestrogen receptor complex; 3) on continued administration of tamoxifen, the concentration of cytoplasmic oestrogen receptor sites decreases. It is suggested that the third factor may not result from an inability to resynthesise oestrogen receptors after tamoxifen therapy, but may be artifactual, in the sense that limited receptor synthesis may occur, but be masked by the high and maintained concentrations of tamoxifen observed during the period of therapy. Such a model would be consistent with the known weak oestrogenic activity of tamoxifen in the rat.

Studies on the mechanism of action of the antioestrogen, tamoxifen [1-(4-β-dimethylaminophenyl)-1,2-diphenylbut-l-ene], also known clinically as nolvadex, and, originally, as ICI 46474, have shown that the compound ultimately causes regression of hormone dependent human and rat mammary tumours, despite initially producing effects in the rat similar to those produced by oestradiol. Regressions have been observed principally in those tumours containing oestrogen receptor proteins. Tumours lacking oestrogen receptors show a much lower response rate to the drug. It is supposed, therefore, that tamoxifen acts primarily through the oestrogen receptor system, although the precise sequence of events which leads to the expression of its antioestrogenic activity has not been fully defined. Compounds which, however, interfere with the ability
of the tissue to bind oestradiol, process its message, or bind to
the receptor and produce a new message, are theoretically capable
of inducing cellular atrophy or cell death.

A consideration of the structures of tamoxifen and oestradiol
does not reveal many similarities (Fig. 1). Nevertheless several
groups of workers have demonstrated that tamoxifen can interfere
with the high affinity binding of oestradiol to its specific
cytoplasmic receptor protein in both human \(^{15,16}\) and rat mammary
tumours \(^{17}\). The antioestrogen apparently competes with oestradiol
for its specific 8S binding protein \(^9,17\), associating with approxi-
mately the same number of binding sites as does oestradiol, but
with 5-10% of its affinity \(^9,17,18\). The compounds also show a
similar specificity of association \(^{17,18}\), suggesting a common
binding site for the ligands.

![Chemical structure of oestradiol and tamoxifen](image)

Fig. 1. Chemical structure of (a) oestradiol and (b) tamoxifen

These data clearly indicate that tamoxifen has the potential
to reduce the formation of the oestrogen receptor complex \textit{in vivo}.
This concept is reinforced by studies examining the uptake of
radioactive oestradiol into oestrogen receptor-positive dimethyl-
benzanthracene (DMBA)-induced mammary tumours in the presence or
absence of tamoxifen (Fig. 2a). Administration of the drug
(300\textmu g) 2 h before a single subcutaneous injection of \(^{3}\text{H}\)oestradiol
(0.06\textmu g), substantially reduced the tissue uptake of the radio-
ligand. In addition, tamoxifen inhibited the formation of the
nuclear \(^{3}\text{H}\)oestradiol-receptor complex of characteristic sedi-
mentation coefficient 4-5S (Fig. 2b). Similar experiments performed after 4 daily injections of tamoxifen produced a more exaggerated response. Thus, if one accepts that nuclear bound oestrogen receptor is a stimulatory factor for cell growth in hormone responsive mammary tumours, then it is not difficult to envisage how tamoxifen, possibly through its competitive ability at high concentrations, might at least initially act. Indeed, Terenius suggested that such a phenomenon might form the molecular basis of antioestrogenicity. More recent studies have,
however, indicated that, although the initial association of the antioestrogen with the oestrogen receptor is of prime importance, it forms only part of a complex sequence of events by which tamoxifen affects tumour growth. It can be demonstrated that tamoxifen, in common with oestradiol, once bound to the cytoplasmic oestrogen receptor is also capable of instigating the translocation phenomenon. Thus when the cellular distribution of oestrogen binding proteins was followed in rat mammary tumours after a single intramuscular injection of 300μg tamoxifen, there was a progressive decrease in cytoplasmic oestrogen receptor levels, associated with a concomitant increase in nuclear anti-oestrogen receptor complex (Fig. 3).

![Diagram](image.png)

**Fig. 3.** Effect of tamoxifen on the cellular distribution of oestrogen binding proteins in rat mammary tumours: I. Early effects. Animals were injected intramuscularly with tamoxifen (300μg in sesame oil) and the concentration of accessible cytoplasmic and total nuclear oestrogen receptors measured in tumour biopsies.

Furthermore, the translocated complex also appears functional, causing early stimulation of RNA polymerase activity which are qualitatively similar, if quantitatively inferior to those produced by a much lower dose level of oestradiol. As seen in
Figures 4 and 5, an increase in activity of RNA polymerase B was associated with the transference of ligand-bound receptor into nuclei. This effect was more rapid after the administration of oestradiol (5µg) reaching maximum 10-30 min after injection (Fig. 4). Tamoxifen (100µg) induced a more gradual increase in activity of RNA polymerase B, attaining highest levels after 40 min (Fig. 5). Activity of RNA polymerase B was at control levels 60 min after the administration of either substance. At this time, a rise in RNA polymerase A activity in mammary tumour nuclei of oestradiol treated rats was observed, but in mammary tumour nuclei of tamoxifen treated rats the activity of RNA polymerase A was not
significantly higher than controls. Oestradiol-induced stimulation of RNA polymerase A activity was greatest 2h after injection and the effect was maintained at these high levels over the 24h period. Increases caused by tamoxifen were lower than those brought about by oestradiol, reached a maximum 4h after injection and soon returned to the control level. Both compounds, however, produced large secondary increases in RNA polymerase B activity, although oestradiol was able to maintain increased activity, whereas, tamoxifen could not.

![Graph](image)

**Fig. 5.** Alterations in activity of RNA polymerases resulting from administration of tamoxifen. Experimental design and conditions were as described in Fig. 4 except animals were given a single intravenous dose of 100μg tamoxifen [10% ethanol in NaCl (0.15 mol/l)].

In addition, tamoxifen (300μg/rat/day) produced a significant stimulation in cytoplasmic progesterone receptor levels, which increased from 150 to 250 fmol/mg protein by day 2 of treatment, although again the weakness of response was evident when compared to the high and sustained effects produced by oestradiol (5μg/rat/day) over a similar period (Fig. 6).
Fig. 6. Effect of oestradiol and tamoxifen on cytoplasmic progesterone receptor levels in rat mammary tumours. Tumour-bearing animals were injected daily with either (a) tamoxifen (300µg) or (b) oestradiol (5µg). Tumour biopsies (approximately 100mg tissue) were removed on the days indicated, 24h after the last injection. Progesterone receptor levels were determined by incubating cytosol fractions with either (1) a receptor saturating concentration of [3H]progesterone (10nmol/l; 1α,2α(n) 3H2-progesterone; 47 Ci/mmol) or (2) [3H]progesterone plus 2.5µmol/l R5020 (17,21-dimethyl,19nor, 4,9-pregnadiene 3,20-dione). Specific progesterone binding was obtained by subtraction of (2) from (1). Results are the mean ± S.E.M. of five tumours/group * p<0.05.

It would seem reasonable to assume that the secondary events observed in the cytoplasm after tamoxifen administration are a direct consequence of an interaction of the tamoxifen receptor complex with chromatin, resulting in the production of mRNA species coding for specific proteins, including the progesterone receptor. Quantitative differences between responses produced by tamoxifen and oestradiol could then be traced to their relative efficiencies at maintaining the transcriptional processes and to the inherent weak oestrogenic nature of the tamoxifen receptor complex. Moreover, antioestrogenicity could therefore be seen as a failure to maintain a full oestrogenic response and tumour remission as reflecting the requirement of tumour cells for a complete oestrogenic stimulus. Negatively, tumours could be seen as regressing because they do not receive a sufficient stimulus to maintain growth.
Implicit in such a view, is, however, the concept that the anti-oestrogen receptor complex has the potential to stimulate transcription for all the gene products normally regulated by oestrogens, but is only able to carry out the processes in an inefficient manner. Although there are many examples cited in the literature on the stimulation of various oestrogen-mediated events by antioestrogens\textsuperscript{21,22,23}, the one observation made in 1973 by Clark et al\textsuperscript{21}, and subsequently by many other groups\textsuperscript{6,7,22,23,24,25}, that high dose levels of antioestrogens deplete the cytoplasm of oestrogen binding sites for prolonged periods while maintaining the nuclear concentration of antioestrogen receptor complex, would at first sight appear to invalidate such a model, since one would have an apparent failure to synthesise the oestrogen receptor, an end-product of oestrogen action.

Fig. 7 illustrates this observation with reference of the cellular distribution of oestrogen binding proteins in rat mammary tumour tissue. Within 5 days of tamoxifen administration (300\mu g/rat/day) virtually no specific binding of \textsuperscript{3}H\textsuperscript{}oestradiol may be determined in the cytoplasm. Sucrose density gradient analysis of cytosol fractions at this time revealed only 4S non-specific binding components (not illustrated). Fig. 7 also shows that basal nuclear levels of the antioestrogen receptor complex were maintained at approximately 400 fmol/mg DNA after day 1.

These data have been previously interpreted as indicating that the anti-tumour properties of tamoxifen do not stem solely from a failure to promote a full oestrogenic response but from a prolonged and atypical binding of the antioestrogen receptor complex to sites on chromatin, associated with an accompanying failure to synthesise oestrogen receptors\textsuperscript{7}, depicted in Fig. 8a as interrupting the sequence of events by which oestrogens act. Results of experiments performed at the time of low cytoplasmic oestrogen receptor levels can be interpreted as agreeing with such a hypothesis, since one can demonstrate a failure to take up (Fig. 2) and respond to exogenously administered hormone.

More recent information, however, on the plasma levels of tamoxifen and oestradiol in animals and patients during tamoxifen therapy have lead us to reappraise this model. Following the in vivo administration of the drug (300\mu g) plasma tamoxifen levels rapidly increased in treated animals reaching 40ng/ml by 1h.
Fig. 7. Effect of tamoxifen on the cellular distribution of oestrogen binding proteins in rat mammary tumours: II. Late effects. The experimental design was as described in Fig. 2.

Fig. 8. Theoretical models of tamoxifen action. E indicates oestradiol; ER, oestrogen receptors; TAM, tamoxifen; AE, antioestrogen.
This value was approximately 3 orders of magnitude higher than the corresponding concentration of oestradiol which remained fairly constant at about 30pg/ml throughout the experimental period (Fig. 9a,b). The considerable amounts of drug present in plasma 24h after its injection resulted in a progressive increase in the basal plasma levels of tamoxifen up to day 14 at which time a maximum of 30ng/ml was reached (Fig. 9b).

Fig. 9. Plasma concentrations of tamoxifen and oestradiol: I. Intact mature female Sprague Dawley rats. Animals received either (a) a single intramuscular injection of tamoxifen (300μg in sesame oil) or (b) daily injections of the same dose level of the drug for 28 days. Plasma samples were removed at the times indicated in (a) or just prior to the next injection in (b) and assayed for their tamoxifen (•) and oestradiol (○) content.

A somewhat similar situation was also observed in postmenopausal women treated orally with 20mg tamoxifen b.d.. Again plasma concentrations of the drug increased rapidly during the first few hours of treatment and contrasted strongly with the much lower plasma concentrations of oestradiol (Fig. 10a). On continued daily dosing of patients the basal plasma tamoxifen levels rose steadily within the first 40 days of treatment to values ranging
between 150-250ng/ml, approximately 3-4 orders of magnitude higher than the corresponding oestradiol levels (Fig. 10b). Clearly in both postmenopausal women and also in rats, tamoxifen is continually available to oestrogen responsive tissues and it is, at all times, present in considerable excess over oestradiol.

Fig. 10. Plasma concentrations of tamoxifen and oestradiol: II. postmenopausal women. Patients were treated orally with 20mg tamoxifen/day for periods up to 164 days\textsuperscript{27}. Plasma samples were treated as in Fig. 9.

In the light of these data it seems equally plausible that the reduction in cytoplasmic binding sites observed in mammary tumours after tamoxifen administration (Fig. 7) may not be due to a blockage of some specific gene function by the tamoxifen receptor complex (Fig. 8a), but may be artifactual, in the sense that limited receptor synthesis may be undetectable because of the high and maintained levels of tamoxifen (Fig. 8b). Thus newly synthesised receptor would be sequestered by tamoxifen and subsequently transferred to the nucleus, thereby fulfilling the stated characteristics of an antioestrogen, that is the maintenance of the nuclear con-
centration of the tamoxifen receptor complex and a depletion of the cytoplasmic pool of receptor. In this model recalcitrance to oestradiol could be seen as a consequence of the competition between the high concentrations of tamoxifen relative to oestradiol in the presence at only limited amounts of receptor.

Furthermore, the inability of tamoxifen to maintain cytoplasmic oestrogen receptor levels is not a unique characteristic of anti-oestrogenicity. Reduced receptor levels were also observed in mouse uterus (Fig. 11), a tissue in which tamoxifen behaves as a full oestrogen and also in rat mammary tumours following the daily administration of high dose levels of either oestradiol or diethylstilboestrol. Such data must lead one to question whether a failure to detect cytoplasmic oestrogen receptors reflects an inability to synthesise the receptor or the high plasma concentrations of these compounds. Obviously further research into

![Graph](image-url)

Fig. 11. Effect of tamoxifen on uterine weights and cytoplasmic oestrogen receptor levels in ovariectomised mice. Seven day ovariectomised mice were administered tamoxifen (50μg/day in sesame oil) by intraperitoneal injection. Uteri were removed on the days indicated, weighed and assayed for their accessible cytoplasmic oestrogen receptor content. Results are the mean ± S.E.M. of three uteri/group.
this particular aspect of tamoxifen action is warranted. In addition, the interaction with retention of the antioestrogen receptor complex by chromatin and its transcription capacity must be further investigated before the differences in the two models can be truely resolved.

In summary, Fig. 12 shows a simplified and as yet theoretical cycle of events which might lead to tumour regressions in hormone dependent breast cancer but which is consistent with both the experimental data presented and also the known weak-oestrogenic properties of tamoxifen in the rat. According to this model three mechanisms operate. Firstly, the antioestrogen, possibly through its competitive ability, reduces the nuclear concentration of oestrogen receptor complexes, secondly that it substitutes an inferior system, the tamoxifen receptor complex, and thirdly, that at high dose levels of the drug the concentration of available oestrogen receptor sites decreases. It is possible that the synergistic action of these factors may adequately account for all the

![Fig. 12. Theoretical Cycle of events leading to the anti-tumour properties of tamoxifen.](image-url)
anti-tumour properties of tamoxifen. However, no evidence exists to eliminate either an indirect effect of tamoxifen on mammary tumour growth or other direct mechanisms; in fact, they may go some way to resolve the apparent anomalies of oestrogen receptor positive tumours failing on tamoxifen treatment or oestrogen receptor negative tumours undergoing a remission.

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REFERENCES

CELLULAR ASPECTS OF NON-STERoidal ANTIOESTROGEN ACTION

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Introduction

During the last 25 years a series of synthetic antioestrogens based on or derived from triarylethylene have become clinically available for the treatment of a variety of endocrine-related conditions, including cancers of the breast and prostate, certain cases of infertility, endometrial hyperplasia and inhibition of lactation. They are usually employed where oestrogens have a detrimental effect on patient health or welfare and appear to act by interfering directly with tissue responsiveness to this hormone (1).

Although a large number of compounds have been screened for their potential antioestrogenic activity, only four are currently in major clinical or endocrinological use; these are tamoxifen, clomiphene, CI628 and nafoxidine, and only the first of these (Nolvadex®, trans isomer of 1-(p-β-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene) is used extensively in cancer therapy (2). Where studied they have been shown to be primarily effective in those tissues, both normal and neoplastic, which contain oestrogen receptor proteins (1,3,4).

This information plus the early observation that antioestrogens bind to the oestrogen receptor (5) has given rise to a concept for their mode of action which is directly linked to their association with these proteins and to an interference with the cellular mechanisms whereby oestrogens cause a proliferative response within sensitive tissues (Reviewed in 1).
Biochemical and biological consequences of antioestrogens binding to oestrogen receptor proteins

Sedimentation analysis has shown that antioestrogens can compete with oestradiol for its specific binding protein (6). These drugs associate with approximately the same number of binding sites as does oestradiol, but generally with a lower affinity. Since the receptor proteins for the antioestrogen tamoxifen and oestradiol are commonly precipitated by protamine sulphate and ammonium sulphate (6,7) and show a similar specificity of binding (6), the experimental data provide good evidence for a common binding protein and possibly of a common binding site for both oestradiol and antioestrogens on the oestrogen receptor. Such a concept has recently been reinforced by the observation that tamoxifen, like oestradiol, can elute the oestrogen receptor from an affinity matrix.

![Graphs showing association between the anti-uterotrophic properties of tamoxifen and the cellular distribution of oestrogen receptors](image)

**Fig. 1.**

Association between the anti-uterotrophic properties of tamoxifen and the cellular distribution of oestrogen receptors

Groups of intact animals were treated daily for periods up to 14 days with either 1 µg (●), 2.5 µg (○), 10 µg (■), 15 µg (□), 25 µg (▲), 50 µg (▲) or 100 µg (◆) tamoxifen in sesame oil. Animals were killed at the times indicated, their uteri removed, weighed (a) and assayed for cytoplasmic (b) and nuclear (c) oestrogen receptors as described in reference 6.

used in its purification, comprising oestradiol-17β, 17 hemi-
succinyl-poly-(L-lysyl-DL-alanine) Sepharose, each ligand receptor complex being recovered in a 5S form (8).

Fig. 2.
Association between the antitumour properties of tamoxifen and the cellular distribution of oestrogen receptors

Groups of intact animals bearing DMBA-induced mammary tumours were treated daily for periods up to 28 days with either 2.5 µg (○), 10 µg (■), 25 µg (□) or 50 µg (▲) tamoxifen in sesame oil. Control (●) animals received vehicle alone. Mammary tumour size (a) was recorded as the mean of two perpendicular diameters. With the exception of the control group, where all changes in tumour size are illustrated, the results in (a) show only those tumours which at the end of the treatment period were less than their original size and represent 13, 30, 50 and 55% of tumours in the 2.5 µg, 10 µg, 25 µg and 50 µg tamoxifen/day groups respectively. The cellular distribution of oestrogen receptors was determined as is described in reference 6.

The association of antioestrogens with oestrogen receptor proteins produces two inter-related events. Firstly, because these compounds are often present in plasma (9,10) and tissues (11,12) in much higher concentrations than oestradiol, they suppress the formation of the oestrogen receptor complex and lower its nuclear concentration (13). In addition, antioestrogens instigate translocation (14). Thus, like oestradiol, the administration of these drugs in vivo results in a fall in the level of cytoplasmic oestrogen receptor proteins and a concomitant and stoichiometric rise in the nuclear anti-oestrogen-receptor pool. Furthermore, in the case of tamoxifen, the absolute concentration of antioestrogen receptor complexes
present in the nuclei of both rat uteri (Fig. 1) and also
dimethylbenzanthracene-induced mammary tumours (DMBA, Fig. 2)
correlates with the biological properties of the molecule.
Thus, at low doses of the drug (< 2.5 µg/day) where little or
no appreciable influence of tamoxifen is observed on the size
or growth of these tissues, no substantial effect of the anti-
oestrogen is seen on the cellular distribution of oestrogen
receptor proteins, while at higher doses of the drug (> 10 µg/
day) a dose related association is evident between its anti-
uterotrophic or antitumour properties and its ability to cause
the nuclear accumulation of antioestrogen receptor complexes.

Influence of antioestrogens on transcriptional and related
cellular events

Oestrogen dependent tissues respond rapidly to oestradiol with
increased levels of RNA synthesis, resulting from transcription
of the DNA template by RNA polymerases (15,16). These
early alterations, essential for subsequent protein and DNA
synthesis (17), begin with a rise in RNA polymerase B activity,
an enzyme associated with the production of informational
messenger RNA.

From investigations with DMBA-induced rat mammary tumours (18)
and also rat uteri (19) it is now evident that tamoxifen,
during the first few hours after its administration, produces
increases in RNA polymerase B activity that are quantitatively
similar to those produced by oestradiol. Moreover, the stimu-
ulatory effect of tamoxifen on this enzyme is dose related and
correlates very closely with the concentration of nuclear
antioestrogen receptor complexes (12,18,19). It is now also
clear that the RNA produced in response to the antioestrogen
is at least partially functional and stimulates a number of
cellular events similar to those elicited by oestradiol. These similarities include early increases in the cytoplasmic concentration of progesterone receptors (1,20) and elevations in the activity of certain enzymes of the hexose monophosphate shunt and glycolytic pathway (1). In addition, other authors have reported elevations of uterine plasma renin substrate and peroxidase activity (21,22), following antioestrogen treatment. The influx of the antioestrogen into the nucleus may, therefore, initiate early biochemical events leading to the elaboration of such products, an effect consistent with the known hypertrophic activity of antioestrogens (20,23).

Fig. 3.
Influence of tamoxifen on luminal epithelial cell height

Groups of 1-week ovariectomised rats were treated daily for periods up to 14 days with oestradiol (0.5 µg, ●), tamoxifen (3 mg, ○) or a combination of oestradiol and tamoxifen (■). Animals were killed at the times indicated, their uteri carefully removed, fixed in Bouins preservative and processed for their histology. Results are the mean ± SD on 20 cell height measurements in each of 5 uteri per time point.

In contrast, however, antioestrogens in general, and tamoxifen in particular, are poor inducers of DNA synthesis and cell replication (23,24,25) and ultimately cause the regression of oestrogen primed tissues (26). For example, in uteri from ovariectomised rats tamoxifen, while stimulating a marked increase in the height of luminal epithelial cells, reflecting its ability to affect cellular hypertrophy (Fig. 3), does not produce an increase in cell numbers (Fig. 4a) or mitotic activity (Fig. 4b). Indeed, when administered concurrently
Fig. 4.
Influence of tamoxifen on uterine luminal epithelial cell numbers and their mitotic activity.

Groups of one-week ovariectomised rats were treated daily for periods up to 14 days with oestradiol (0.5 μg, ●), tamoxifen (3 mg, ○) or a combination of oestradiol and tamoxifen (■).

(a) Animals were killed at the times indicated and their uteri treated as in Fig. 3. (b) As in (a) except animals were administered (iv) vincristine (1 mg/kg body weight) 6 hours prior to sacrifice (2000 luminal cells counted in each of 5 transverse sections of uteri).

with oestradiol the antioestrogen reduces the oestradiol-stimulated increases in these latter parameters to the level observed with the antioestrogen alone. Tamoxifen is therefore acting as a full agonist with respect to cell hypertrophy (Fig. 3), as manifest also by amino acid incorporation into cytoplasmic proteins (27), but as a complete antagonist towards oestradiol in relation to epithelial cell numbers and mitotic activity (Fig. 4). Furthermore, when administered (3 mg/day) to intact rats, the drug rapidly reduces the mitotic activity of both uterine luminal epithelial cells (Fig. 5a) and the epithelial cell components of DMBA-induced mammary tumours (Fig. 5b) although inhibition is achieved more quickly in the former tissue.

In this light, it is also of interest that tamoxifen, in both rat mammary tumours and rat uteri, appears to be a poor inducer of RNA polymerase A activity, an enzyme associated with the
production of ribosomal RNA. Since it has recently been suggested that RNA polymerase A might be involved in the initiation of DNA synthesis (28), it is conceivable that the inability of tamoxifen to stimulate this enzyme may relate to its anti-oestrogenic and antitumour properties. Circumstantial support for such a concept has arisen with subsequent studies with mouse uterus, a tissue where tamoxifen elicits a full oestrogenic effect (28), including hyperplasia, and in which identical increases in the activity of RNA polymerase A are brought about by tamoxifen and oestradiol (12). Furthermore, the location of this enzyme in the cell nucleolus, that is at sites normally considered distinct from those occupied by receptor complexes (29), suggests that the stimulation of this enzyme can not be brought about directly but requires the production or activation of other growth regulatory substances.
Although the control of the pathways leading to tissue hypertrophy and hyperplasia are understood only incompletely, one explanation of the differential effects of oestrogens and antioestrogens would relate to the inability of the antioestrogen receptor complex to influence all the intranuclear domains normally regulated by the oestrogen receptor complex, especially those concerned with the stimulation of components associated, however indirectly, with cell division. To investigate this possibility, the ability of the oestrogen receptor protein, purified from DMBA-induced rat mammary tumours, when complexed with either oestradiol or tamoxifen, to associate with tumour chromatin and to alter its transcriptional characteristics has recently been examined (8). Oestrogen receptor complexes and tamoxifen receptor complexes occupy chromatin sites and stimulate RNA polymerase B activity to similar extents, although high concentrations of the tamoxifen receptor complex were required to produce these effects. It was not possible in these studies to determine whether chromatin acceptor sites have a decreased affinity for the tamoxifen receptor complex, or whether they may process the tamoxifen receptor complex more rapidly. Limited digestion of chromatin by micrococcal nuclease and subsequent fractionation localised oestrogen and tamoxifen receptor complexes primarily in transcriptionally active areas. Interestingly, competitive studies between $[^{3}H]$ oestradiol receptor complexes and non-labelled tamoxifen or oestrogen receptor complexes for chromatin sites indicated that although oestrogen and anti-oestrogen receptor binding sites may be numerically similar and equally proportionately distributed throughout chromatin they may not be mutually exclusive and thereby occupy different chromatin loci. How these findings relate to the pharmacological properties of the molecules remains to be elucidated but provides some evidence for physicochemical differences between oestrogen and antioestrogen receptor complexes.

In the rat uterus, both tamoxifen and oestradiol apparently
Fig. 6.
Influence of tamoxifen on the prolactin stimulated increase in size of DMBA-induced mammary tumours

Groups of tumour-bearing animals were treated daily for 28 days with either ovine prolactin (20 I.U./day, □), tamoxifen (3 mg/day, ●), a combination of prolactin and tamoxifen (■) or sesame oil vehicle (O). Tumour size was recorded as the mean of two perpendicular diameters and is expressed ± SD of 7, 12, 10, and 10 tumours in the above groups respectively.

increase RNA polymerase B activity by making more initiation sites available rather than by affecting the size of the RNA product (12,19). Similarly, the number of initiation sites in chick oviduct chromatin is dependent upon the concentration of the nuclear oestrogen receptor complexes (30). This also appears to be the case in rat uterus, but for oestradiol and not tamoxifen, since the nuclear concentration of the tamoxifen receptor complex is maintained after the number of initiation sites returns to prestimulation values (12,19). This therefore casts further doubt on the biochemical efficacy of the antioestrogen receptor complex.

Finally, it is now becoming evident that the growth promoting hormone in mammary tissue need not be oestradiol for antioestrogens to act. Tamoxifen (3 mg/day) partially reverses the stimulation of growth of some DMBA-induced mammary tumours by daily administration of prolactin, although the regressions
are delayed and not as pronounced as those elicited by the
drug alone (Fig. 6). Rather than conflicting with an action
of antioestrogens on cell proliferation through the oestrogen
receptor system, this serves to illustrate the complexities
involved in the growth regulation of hormone sensitive tissues.
These aspects of antioestrogen action are the subject of
continuing research.

Summary

Considerable evidence favours the hypothesis that the action
of antioestrogens results from their direct association with
intracellular oestrogen receptor proteins. Moreover, the
antagonism of these compounds towards oestradiol and also
prolactin would seem to reside in the nuclear properties of
the antioestrogen receptor complex which either fails to
produce or blocks cell replication. Where measured, anti-
eostrogens achieve a considerable excess over endogenous
oestradiol in both plasma and target tissues, thereby
favouring both the formation of the antioestrogen receptor
complex and reducing, through competition, the nuclear con-
centration of oestrogen receptor complexes. The continual
nuclear presence of a receptor complex unable to instigate
or maintain the necessary environment for cell proliferation
would be consistent with the regressions observed in primed
tissues.

References

1. Nicholson, R.I., Griffiths, K.: In 'Advances in Sex Hormone
   Research'. Vol. 4 (Eds. J.A. Thomas, R.L. Singhal)
   74, 3162 (1977).
23. Clark, J.H., McCormack, S.A., Padykula, H., Markaverich, 
   B., Hardin, J.W.: In 'Effects of Drugs on the cell Nucleus' 
   (Eds. H. Busch, S.T. Crooke, Y. Daskal) Acad. Press, New 
   York, p381 (1979).
   (1975).
27. Syne, J.S.: Ph.D. Thesis, Welsh National School of 
   Medicine, U.K.
   75, 5931 (1978).
30. Thrall, C.L., Webster, R.A., Spelsberg, T.C.: In 'The 
   Cell Nucleus' (Ed. Busch, H.) No. 4, Acad. Press, p461 
   (1978).
31. Tsai, S.Y., Tsai, M.J., Schwartz, R., Kalimi, M., Clark, 
   72, 4228 (1975).
II. INVESTIGATIONS RELATING TO CANCER OF THE BREAST.

F. STEROID RECEPTORS AND PROGNOSIS.
ENDOCRINE ASPECTS OF PRIMARY BREAST CANCER

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In the period since Jensen (1) originally considered the possibility that oestradiol-17β receptor content of human metastatic breast tumours may offer a means of predicting a patient's response to endocrine therapy, the large amount of data which has accumulated (2,3,4) clearly indicates the value of such assays in the clinical management of breast cancer. It has yet to be firmly established, however, that receptor analysis of the primary tumour will provide an effective predictor for response of the subsequently developed advanced disease although a preliminary communication suggests it will (2). This report deals with certain aspects of the endocrinology of primary breast cancer, work undertaken in the Tenovus Institute in association with a number of breast clinics in the U.K. and considers among other things receptor analysis in relation to various clinical prognostic features.

In a co-operative study (5) with Mr. R.W. Blamey, at the Nottingham City Hospital, 200 patients with primary, operable breast cancer underwent simple mastectomy as their surgical treatment. Biopsies of lymph nodes were taken from low axilla, the apex of the axilla and the internal mammary chain in the second intercostal space (triple node biopsy). Patients were staged as follows: stage A, tumour confined to breast; stage B, axillary node involved; and stage C, apical or internal...
mammary node involved. Tumours were histologically assessed and graded (by Dr. C.W. Elston, Nottingham) according to Bloom and Richardson (6) - grade I (least malignant) to grade III. Blood was also routinely collected from the patients for plasma oestradiol assay and a portion of the tumour was frozen in liquid nitrogen for steroid-receptor analysis.

Oestradiol-17β receptor levels were determined using multiple ligand concentrations and estimating the binding site concentration by a computerised analysis (5) which covered the comments recently made by McGuire (7) regarding the errors in routine receptor assays.

The mean levels of receptor in positive tumours from premenopausal women was significantly lower (44.4 fmol/mg protein) than in tumours from postmenopausal women (123.0 fmol/mg protein) possibly due to higher concentration of circulating oestrogens. However, there was no difference between the concentrations of oestradiol-17β in plasma taken immediately prior to operation, in patients with oestrogen receptor positive tumours and those with receptor negative tumours, either in premenopausal women (97.3 and 100.3 pg/ml respectively) or in postmenopausal women (18.5 and 17.5 pg/ml respectively). Furthermore, there was no correlation between the levels of receptor in receptor positive cases and plasma oestradiol within either of the two groups of patients.

Table 1 shows the relationship between the oestradiol-17β receptor status of the tumours and histological grading. It is striking that few of the grade I and II

<table>
<thead>
<tr>
<th>GRADE</th>
<th>PRE-MENOPAUSAL</th>
<th>POST-MENOPAUSAL</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>-</td>
<td>5</td>
<td>11</td>
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Oestrogen-receptor content related to histological grade of tumour. From 200 patients, data on receptor content, tumour grade and menopausal status were obtained from 164.
Months follow-up

Fig.1. Relationship between receptor status and recurrence
tumours from postmenopausal women lacked receptor, whereas the majority of the most dedifferentiated tumours were found to be oestrogen receptor negative. The study therefore offers some of the first data to clearly indicate a relationship between tumour histology and receptor status.

Although data on the response to treatment of those patients in whom the disease eventually recurs is now starting to accumulate, it is interesting to note that 'receptor-negative patients' tend to recur faster than receptor-positive (Fig. 1), possibly related to the grade III histological status of receptor-negative tumours. This trend is clearer in the postmenopausal group, although it is obviously the lymph-node staging which provides the best prognostic index (Table 2).

<table>
<thead>
<tr>
<th>% PATIENTS STUDIED</th>
<th>MENOPAUSAL</th>
<th>PRE-ER</th>
<th>POST-ER</th>
<th>PRE-ER Positive</th>
<th>POST-ER Positive</th>
<th>LYMPH NODE STAGE</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DISEASE RECURRENCE</td>
<td>16.1</td>
<td>16.0</td>
<td>16.0</td>
<td>16.0</td>
<td>16.0</td>
<td>16.0</td>
<td>16.0</td>
<td>16.0</td>
<td>16.0</td>
</tr>
<tr>
<td>DISEASE FREE</td>
<td>83.9</td>
<td>83.2</td>
<td>83.2</td>
<td>83.2</td>
<td>83.2</td>
<td>83.2</td>
<td>83.2</td>
<td>83.2</td>
<td>83.2</td>
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</table>

Relationship between receptor status, lymph-node staging and recurrence
All the results are being analysed by a multivariate technique using canonical variate analysis (8) to assess the inter-relationship between the various biochemical and clinical parameters. An example of the use of the procedure is seen when the histological grade of the tumour is used as a basis for sub-population assignment. Fig. 2 shows the mapping of the canonical variates together with their 90% confidence region for the separation of the postmenopausal patients into two groups, tumour grades I and II versus grade III. Separation actually occurred at the 97.5% confidence level with the principal components of the separation being age (53%) and oestrogen receptor levels (40%), the mean ages for grades I/II and III being 65.11 and 60.17 years respectively and the mean receptor levels being 127.1 and 44.9 fmol/mg protein.

**POST-MENOPAUSAL**

**HISTOLOGICAL GRADE (I+II) vs (III)**

![Fig. 2. Mapping of the canonical variates together with their 90% confidence region. The first and second canonical variates are plotted on the abscissae and ordinates respectively.](image-url)
**TABLE 3.**

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>% RECOVERY of ORIGINAL RECEPTOR</th>
<th>PURIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. (NH₄)₂SO₄ Precipitation</td>
<td>60%</td>
<td>3-5 Fold</td>
</tr>
<tr>
<td>II. Affinity Chromatography</td>
<td>49%</td>
<td>7860 Fold</td>
</tr>
<tr>
<td>III. Iso-electrical Focussing</td>
<td>&gt;50,000 Fold</td>
<td></td>
</tr>
</tbody>
</table>

Brief summary of oestradiol receptor protein purification from DMBA induced rat mammary tumours

In order to study the oestrogen receptor further, purification of the receptor protein from DMBA-induced mammary tumour from rats is being undertaken at the Institute.

Table 3 indicates the progress made to date, and it is conceivable that a purified preparation will be invaluable in the assay of receptor proteins and also in examining the finer details of the mechanism of action of the receptor. Furthermore, since the presence of progesterone-receptor may reflect the functional integrity of the complete oestrogen response system (9) levels of this protein in primary breast tumours are being routinely determined. From the Institute's work (10), only 30% of the oestradiol-receptor positive tumours contained progesterone receptors and of those which lacked oestradiol-receptor, 10% contained progesterone receptor.

Although the relationship between C₁₉-steroids and early breast cancer has been extensively studied, particularly by Bulbrook and his colleagues (10), there is still little information on the role of endogenous androgens within the tumour cells. In the late 1960s, the Institute (12), concurrently with other laboratories, Dao (13) in Buffalo and Adams (14) in Sydney, drew attention to the paraendocrine function of breast tumour tissue. Enzymes for the conversion of C₁₉-steroids to oestrogens were found in breast tissue, DHA sulphate was metabolised to androst-5-ene-3β,17β-diol (androstenediol) and to various 5α-androstanediols and it was suggested(15) that the C₁₉-steroids may directly influence oestradiol-
17β action. Certainly, competition studies undertaken in the Institute by Dr. Nicholson, using the DMBA-ii rat mammary tumour oestradiol-17β receptor (Fig. 3) indicated that a localised concentration of C19-steroids could affect the oestrogenic stimulus to the cell. Poortman and Thijssen have made similar observations. The concentration of such steroids in breast tumour was however, unknown.

Using high resolution gas chromatography-mass spectrometry with single-ion monitoring, the concentrations of various steroids in homogenates of primary breast tumour tissue have now been determined. For this tumours were received from Professor R. Shields, Department of Surgery, University of Liverpool. Fig. 4 the levels of DHA, androstenediol and oestradiol-17 primary breast tumour tissue.

The range of DHA concentrations, 2.5 - 466 ng/obviously greater than could be expected from plasma contamination, although there was a significant correlation with age. There was no correlation between concentrations and oestradiol-receptor status except

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**Fig. 3. Specificity of [3H]oestradiol-17β binding.**

Cytosol was incubated for 2 h at 4°C with a saturating conc. [3H]oestradiol-17β (5 nM) in the presence or absence of increasing concn. potential antagonist. After incubation, free steroid was removed by charcoal adsorption.
that in 7 from 9 tumours with DHA concentration > 100 ng/g there was no measurable cytoplasmic receptor. Approximately 60% of the tumours contained > 1 ng/g androstenediol with a range up to 46 ng/g. Again, when the concentration was high ( > 20 ng/g) in 6 tumours, there was no measurable receptor. Oestradiol-17β concentrations were high and related neither to receptor levels nor to plasma oestradiol. Obviously, studies to determine the steroid levels in nuclear preparation are underway.

It is now well accepted that prolactin has many various effects (17), some of which are concerned in controlling breast physiology. The effects of prolactin on experimental mammary tumours are well documented and its relevance to human breast cancer has recently been reviewed (18). Our own studies of human breast cancer patients have however shown similar basal plasma prolactin levels at various stages of the disease and in control subjects (19,20) and these and other reports (21,22) suggest that if plasma prolactin concentrations are different in breast cancer patients, the differences will be small. A co-operative investigation with Professor A.P.M. Forrest, Edinburgh, in which sequential samples of blood were taken at 15 min intervals throughout the day from individual patients with breast cancer and from controls matched for age, years since menopause and parity has been undertaken (23). Again, no significant differences were found in prolactin levels between cancer patients and controls throughout this period of time, although plasma testosterone concentrations were found to be higher in the women with breast cancer in this more careful study (Fig. 5). It is possible that now, with the availability of sensitive radioimmunoassays, patients with breast cancer may well be found to display different hormone profiles relating to the various biorhythms, daily or monthly. The physiological role of prolactin during the human menstrual cycle is poorly understood and the monthly profile of prolactin levels has given rise to controversy (24). Although the human breast undergoes cyclical changes in normally menstruating women, it is at present difficult to assess whether the cyclical nature of many breast complaints can be related to abnormal prolactin secretion.
Fig. 4. Hormone levels in breast tumours.
Fig. 5. Plasma prolactin and testosterone concentration in patients with breast cancer and controls. Prolactin: 5 patients and controls, mean values 0.17 and 0.13 respectively. Testosterone: 6 patients and controls, mean values 30.8 and 18.3 respectively. Data from (23): fig. previously published in Lancet.

In a study with Professor R.A. Sellwood (University Hospital of South Manchester), serum prolactin profiles were determined through complete menstrual cycles of women with two clinically different benign breast diseases (25). Eleven patients were classified as having 'benign cystic disease of the breast' and for 19 others, the term 'fibroadenosis' was used to denote a diffuse condition of painful lumpy breasts. Normal women (32 subjects) were studied for comparison together with 11 women, who had undergone mastectomy for primary carcinoma of the breast, but had since resumed regular menstrual cycles (26). Fig. 6 shows some of the data from the study.
Fig. 6. Weekly pattern of serum prolactin in normal women, patients with benign breast disease and in breast cancer patients. Subjects are grouped according to age or selected as matched controls. Geometric means with their 95% confidence limits are shown for results which were pooled over 5-day intervals in the menstrual (M), follicular (F), periovulatory (O) or luteal (L) phases of the menstrual cycle.

Whereas the median prolactin concentration in 550 sera from 24 normal cycles was 0.10 mU/ml, in 19 women with 'fibroadenosis', the median of 500 values was 0.15, and was 0.27 mU/ml for 337 sera from 12 cycles of women who had had breast cysts aspirated. Abnormally elevated prolactin profiles were found in patients with benign breast disease, especially in women over 30 years of age. The use of prolactin-suppressive therapy would seem reasonable to treat the condition. Although the
incidence of breast cancer in patients with gross cystic disease has been reported to be 4 times higher than expected (26), a role for prolactin in carcinogenesis does not necessarily follow. Indeed the patients, who had a previous mastectomy, were found to have only minor abnormalities of their serum prolactin profile (26) with higher levels of prolactin around the periovulatory period.

It is noteworthy, with the current interest in the use of tamoxifen for the adjuvant treatment of women with primary breast cancer that although treatment of normal premenopausal women with tamoxifen eliminated the mid-cycle peak of prolactin (Fig. 7), possibly expected from its anti-oestrogen action, the treatment produced a concomitant rise in plasma oestradiol-17β in the luteal phase of the cycle (28).

![Fig. 7 Mean plasma hormone levels during the menstrual cycle. (a) Normal control cycle (n=6) and (b) tamoxifen (20 mg/day) given to 3 of the same subjects for 10 days during the follicular phase. Stippled area indicates ± S.D.]
A great deal of data has already been accumulated on the endocrine aspect of breast cancer but new techniques are being developed which will further our understanding. For example, development of nuclear receptor assays, the measurement of other hormones in the primary tumour and quantitative immunohistochemistry, particularly at the electron microscope level, are all being extensively studied at the Institute. However, a few more years are necessary to be able to relate the data obtained on primary tumours to the outcome of the disease.

ACKNOWLEDGEMENTS

The authors are grateful to the Tenovus Organisation for their generous financial support. They also acknowledge the expert technical assistance of Mr. A. Pike of the Institute's Mass Spectrometry Unit.


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28. Groom, G.V. & Griffiths, K.: Effect of the anti-oestrogen Tamoxifen on plasma levels of luteinising hormone, follicle-stimulating hormone, prolactin, oestradiol, and progesterone in normal pre-menopausal women: J. Endocr. 70, 421, 1976
ENOCRINE FACTORS IN PRIMARY TUMOURS IN RELATION TO PROGNOSIS

The relationship between a number of biochemical and clinical parameters has been examined in a series of primary breast cancer cases. In particular the oestradiol receptor content has been compared with clinical and pathological factors, and notably it was found that in post-menopausal patients the less differentiated tumours rarely contained measurable receptor. Very few tumours which did not contain oestradiol receptor possessed measurable amounts of progesterone receptor, although about 30% of oestradiol receptor positive. The data has also been analysed by a multivariate technique using for example, lymph node involvement or histological grading for sub-group assignment. Level of various endogenous steroids - oestradiol-17β, DHA and androst-5-ene-3β,17β-diol - in breast tumours have also been determined by mass fragmentography. This data will be described in relation to plasma hormone levels in patients with breast cancer together with studies on the isolation of oestradiol-receptor protein.
RELATIONSHIP BETWEEN OESTROGEN-RECEPTOR CONTENT AND HISTOLOGICAL GRADE IN HUMAN PRIMARY BREAST TUMOURS

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Summary.—A series of 300 patients presenting consecutively with primary operable breast cancer has been studied. A significant correlation was found between oestrogen-receptor (ER) content and histological grade: the better-differentiated tumours rarely lacked receptor. This correlation was significant only in women defined as post-menopausal. Data on early recurrence of disease indicate a worse prognosis for women in whom primary tumours are ER—.

There is considerable evidence that the assay of oestrogen receptor (ER) in metastatic breast tumours helps in predicting the likelihood of a favourable response to endocrine therapy (McGuire et al., 1975). Since cells normally dependent on oestrogen contain a similar receptor (Jensen et al., 1968; Gorski et al., 1968) the presence of this protein may reflect the degree of differentiation of the cells. If this is true, there should be a correlation between ER content and other parameters which assess differentiation, and in particular with assessment of tumour grade. A number of reports have variously stated that there was no significant correlation (Johanssen et al., 1970; Rosen et al., 1975) or that, in a small number of cases, there may be such a relationship (Heuson, 1975).

One of the objectives of the present study was to test this hypothesis, and another was to determine whether ER content of the primary tumour is as valuable in estimating likelihood of early recurrence as histological grade is reported to be (Bloom & Richardson, 1957).

MATERIALS AND METHODS

Patients and Clinical follow-up.—In this study we have, to date, examined a series of 300 female patients, aged 27–70, presenting consecutively to the Breast Clinic at the City Hospital, Nottingham, with primary operable breast cancer. In general, these patients had tumours < 5 cm in diameter, not fixed locally, and with no clinical evidence of metastatic spread, thus generally equating to TNM Stage I and II breast cancer. At mastectomy a lymph node was removed from the low axillary group, from the apex of the axilla and from the internal mammary chain via the second intercostal space. In all cases the primary tumour and lymph nodes were examined histologically, and in most cases part of the primary tumour was immediately immersed in liquid N2 and stored for subsequent ER assay.

Patients were followed up in a post-mastectomy clinic by the two surgeons (R.W.B. and C.J.D.); attendance was at 3-month intervals to 18 months and then at 6-month intervals. Blood analyses of haemoglobin, white-cell count, erythrocyte sedimentation rate, liver-function tests and serum calcium were carried out every 6 months. Bone scans with skeletal surveys were performed shortly after mastectomy and then annually.

Recurrence for the purpose of this study has been defined as:

(i) major local recurrence in the wound flaps or axillary node enlargement requiring radiotherapy. Axillary node enlargement was not treated unless
Estrogen Receptor Assay in Primary Breast Cancer and Early Recurrence of the Disease

P. V. Maynard, R. W. Blamey, C. W. Elston, J. L. Haybittle, and K. Griffiths

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Abstract

Estrogen receptor assays of primary breast tumors have been related to early recurrence of the disease. A significantly longer disease-free interval was found in women whose primary tumor was estrogen receptor positive. Although there was no relationship of receptor content to stage of disease at mastectomy, the greatest difference between recurrence rates was found when the tumor had spread to the lymph nodes, especially to those in the apex of the axilla or in the internal mammary chain. Presence of estrogen receptor is closely related to histologically well-differentiated tumors, but it was found that poorly differentiated estrogen receptor-negative tumors recurred earlier than poorly differentiated receptor-positive tumors and had a very unfavorable prognosis.

Introduction

It has been suggested that women bearing metastatic breast tumors that contain estrogen receptor are likely to survive longer than those with estrogen receptor-negative tumors (8). It was of interest, therefore, to determine whether the estrogen receptor content of the primary breast tumor was of any value in predicting the disease-free interval of the disease, as well as in indicating an appropriate therapy for immediate use upon recurrence. A recent brief report (3) stated that the estrogen receptor content of the primary tumor is of value in this respect and that it is independent of factors such as tumor size or age, of patient, or degree of nodal involvement.

We have shown2 that, although estrogen receptor content does not correlate with degree of spread of tumor to the lymph nodes, there is a highly significant relationship to histological grade of tumor, and the significance of this finding to prognosis was also examined.

Materials and Methods

Patients. The data reported are from 300 female patients (ages 27 to 70) who presented consecutively to the Breast Clinic at the City Hospital, Nottingham, England, with primary breast cancer. Stage of disease was assessed after biopsy of lymph nodes from the low axilla, from the apex of the axilla, and from the internal mammary chain taken via the second intercostal space. Patients with no tumor evident clinically and histologically in any node were classified as Stage A, those with tumor only in nodes from the low axilla were classified as Stage B, and patients with tumor cells in high lymph nodes were designated Stage C.

Details of follow-up procedure and definition of recurrence will be published. Briefly, patients were seen at a postmastectomy clinic at 3-month intervals to 18 months and thereafter at 6-month intervals, and recurrence was defined essentially as objective evidence of tumor deposits requiring a major change of treatment.

Primary tumor tissues were graded histologically on the basis of Bloom and Richardson (1) from 1, well-differentiated, to III, poorly differentiated.

Estrogen Receptor Assay. Details of the method used routinely have been published (6) and consist of incubating portions of a high-speed supernatant with different concentrations of $[^3]H$estradiol ranging from 90 to 5000 pmol/liter for 16 hr at $4\degree$C. Free and bound estradiol was separated by dextran-coated charcoal, and the binding site concentration was estimated by the Newton-Raphson iterative curve-fitting technique using equations derived from the Law of Mass Action (2). Tumors were considered positive only when they contained more than 5 fmol specific estradiol binding per mg cytosol protein.

Statistical Analysis. The curves shown in Charts 1 to 5 are derived from life table analysis of the data at each follow-up time period. Comparison between the curves in each chart are made with techniques described by Mantel (4) and Mantel and Haenzel (5), an approach that evaluates differences between the whole curves rather than between individual points on the curve.

Results

From the 300 patients thus far admitted to the study, complete data with regard to menopausal status of the woman, receptor status, lymph node staging, histological grade, and size of tumor were available in 248 cases. It was reported previously that there is no relationship between receptor content of the tumor and the degree of spread to the lymph nodes in this series of patients, although stage plays a large part in estimating the possibility of early recurrence (Chart 1).

Chart 2 shows the overall disease-free intervals for women with receptor-positive and receptor-negative tumors, and a significant advantage was seen for patients whose tumors contained receptor in measurable amounts. When the data were broken down further and receptor status within each stage group was considered (Chart 3), there was no apparent value of receptor assays in determi-
nentiation of the possibility of early recurrence in node-negative patients. All of these patients had a good prognosis. In node-positive cases, however, women with receptor-negative primary tumor presented with recurrences much earlier, prognosis being particularly poor if tumor was evidenced in the higher nodes (Chart 3, B and C).

Since we believe that receptor content estimates only the degree of "normality" of the epithelial cells and relates very closely with histological assessment of differentiation, it is valuable to compare recurrence rates within the different grades. Chart 4 shows the appropriate disease-free interval curves, and whereas there was no significant difference between recurrence rates in the well and moderately differentiated tumors (Grades I and II), there was a large difference in the poorly differentiated Grade III cases.

When receptors were not taken into account, there was no significant difference in prognosis between women with Grade I and Grade II tumors, nor between those with Grade II and Grade III tumors (Chart 5).

Discussion

The data from this study demonstrate the value of estrogen receptor assays in the primary breast tumor in a number of differing ways. The early recurrence rates (Chart 2) between women with estrogen receptor-positive and

![Chart 1. Recurrence-free intervals of primary breast cancer patients.](image1)

![Chart 2. Recurrence-free intervals of primary breast cancer patients.](image2)

![Chart 3. Recurrence-free intervals of primary breast cancer patients.](image3)


is encouraging enough to justify further efforts to define
the long-term potential and the risks of this novel
approach.

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Health.

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REFERENCES

Am. med. Ass. 1974, 228, 117.

2. Griinzig, A. Diphokutan transilzumale Rekanalisation chronischer Arter-


suppl. ii, 213 (abstract 829).


Med. 1975, 293, 216.

ESTROGEN RECEPTORS AND PROGNOSIS IN
EARLY BREAST CANCER

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Summary
In a study of the role of estrogen-receptor
analysis in early breast cancer the
estrogen-receptor content of the tumour was estimated in
286 patients undergoing mastectomy. These patients
were followed for up to 39 months, and the recurrence
disease was noted in relation to the presence or
absence of estrogen receptor. Recurrence-rates were sig-
nificantly higher in patients whose tumours did not con-
tain receptors than in those whose tumours did. This
same relationship was seen when women with and with-
out axillary metastases were considered separately.
The highest rates of recurrence were in women with axillary
lymph-node involvement whose tumours lacked estrogen receptors.
Women without axillary-node involvement whose tumours lacked estrogen receptors showed the same high rate of recurrence as all women
with axillary-node involvement. The estrogen-receptor
content of a primary breast cancer appears to be an
independent guide to early recurrence of the disease.

Introduction
The presence or absence of estrogen receptors in
tumours and metastases in patients with advanced
breast cancer is a useful guide in predicting response to
docrine therapy. Few reports have been published on
the role of estrogen receptors in the management of
early breast cancer and their relationship to prognosis
and response to therapy. Two reports have shown that
tumours lacking estrogen receptor are associated with
early recurrence, but the numbers of patients are small and the results need further evaluation. This study
was designed to evaluate the determination of estrogen-
receptor content of breast tumours at an early stage of
the disease. This investigation is part of a larger study
of several aspects of breast cancer, in which 15 surgeons
in hospitals are participating.

Patients and Methods

Patients
Samples of tumour tissue were collected immediately after
excision from nearly all the patients with primary breast
cancer under the care of the participating surgeons. This paper
presents details of the first 286 patients in the study.

The patients were staged clinically according to the interna-
tional I.N.M. system. The presence or absence of metastatic
disease was confirmed by skeletal survey or bone scan and in
some cases by urinary hydroxyproline estimations. Only pa-
tients with operable breast cancer \( T_1, T_2, N_1, M_0 \) were included
in this study.

All patients were treated with some form of mastectomy which included either an axillary-node biopsy or an axillary
dissection. The diagnosis of breast cancer was confirmed histo-
logically, and the presence or absence of axillary metastases
was determined by microscopic examination of the axillary
contents. The clinical staging was modified after the histologi-
cal examination.

After discharge the women were followed up for 3–39
months (average 19 months) at the hospital at which the
original treatment was carried out. Follow-up data were
recorded on standard forms. Recurrent local or nodal disease
was diagnosed only after histological examination of the
excised tissue and distant bony or visceral metastases were diag-
osed by unequivocal radiological evidence. Women receiving
adjuvant systemic cytotoxic therapy or hormonal therapy were
excluded.

Oestrogen-receptor Assay

Biopsy specimens of tumours were placed on ice at the time
of mastectomy and stored in liquid nitrogen until assay for
receptor proteins. For this purpose the tumours were homog
ised in ice-cold "tris"-HCl buffer and centrifuged at
100 000 g for 60 min. Samples of the resulting supernatant
were incubated with an equal volume of tris-HCl buffer con-
taining tritiated oestradiol (specific activity 96 Ci/mmol) in
amounts ranging from 10 to 500 pg for 18 h. Free and un-
bound \(^3\)H-oestradiol were separated by dextran-coated char-
coal, and the binding-site concentration was estimated by the
Newton-Raphson iterative curve fitting technique. Tumours
were considered to contain estrogen receptors only if they con-
tained more than 5 fmol of specific oestradiol binding per mg
of cytosol protein.

Statistical Methods

Graphs were derived from life-table analysis of the data at
each follow-up time period. Comparisons between the two
curves on each graph were made by the log rank test, which
evaluates differences between the entire curves rather than
between individual points on the curve.

Results

Estrogen receptors were present in 144 (51%) of the
286 breast cancers studied. Of the 75 premenopausal pa-
...tions only 29 (36%) had tumours with receptors, compared with 115 (55%) of 211 postmenopausal women. There did not seem to be any relationship between the size of tumour and the presence of oestrogen receptors.

The presence or absence of oestrogen receptors was then related to the rate of recurrence of breast cancer. Of 144 women whose tumours had receptors, the rate of recurrence was significantly lower (p < 0.001) than in 142 women whose tumour did not contain oestrogen receptor (fig. 1). This difference in rates of recurrence was significant in both premenopausal and postmenopausal women.

The rate of recurrence of breast cancer in women with and without metastatic disease in axillary lymph-nodes was then related to the presence or absence of oestrogen receptors. Of the 110 patients with involved axillary lymph-nodes at the time of presentation, 67 had tumours which did not contain oestrogen receptor. The rate of recurrence was higher for these patients (p < 0.05) than for the 33 women whose tumours had oestrogen receptors (fig. 2). Indeed, the highest rate of recurrence of any group studied was found in the 67 patients without receptors in their tumours who had axillary-node metastases.

Among the women who did not have axillary-lymph-node involvement the 74 whose tumours did not contain oestrogen receptors showed a significantly higher rate of recurrence (p < 0.01) than for the 92 who had receptors in their tumours (fig. 3). Despite the more favourable prognosis usually attributed to women without axillary lymph-node metastases, the absence of oestrogen receptors was associated with the same high rate of recurrence as that found in all the women who had lymph-node metastases (fig. 4).

Discussion

The place of oestrogen-receptor analysis has been established in the treatment of advanced breast cancer, particularly in identifying those tumours which may respond to endocrine therapy. Two studies have suggested that the presence or absence of oestrogen receptor in the primary tumour may act as an independent guide to prognosis.

The present study shows that tumours with oestrogen receptors are associated with a significantly lower rate of recurrence than tumours without receptors, and that the presence of oestrogen receptor is independent of other prognostic factors such as site of tumour and involvement of axillary lymph-nodes.

Usually a patient is considered to have a good prognosis if lymph-nodes are not involved. We have shown, however, that if oestrogen receptors are not present in the primary tumours the rate of recurrence is high. The prognosis of women without lymph-node involvement and without receptors in their tumours was in fact similar to that of women who were found to have metastases in axillary lymph-nodes at operation. This study, by observing the outcome of breast cancer in unselected patients, truly reflects the disease as it occurs in a highly populated industrial area.

We have shown that patients with both poor prognostic factors—absence of receptors and axillary-node involvement—are at high risk. This is in agreement with Knight, who found that 50% of a similar group had developed recurrence within 18 months. With the use of...
both axillary-node status and oestrogen-receptor analysis it may be possible to predict with greater accuracy the likely outcome for individual patients, and the precise definition of the factors which influence the biological behaviour of individual breast cancers may lead to a more rational approach to treatment selection. The results of the present study suggest that oestrogen-receptor analysis in early breast cancer will be helpful in this respect.

We thank the many surgeons on Merseyside who cooperated in this study. The tumours were collected by Mrs Patricia Clark, who also gave invaluable secretarial assistance. This investigation was supported by a grant from the Tenovus Institute for Cancer Research.

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REFERENCES

CHLORPROPAMIDE ALCOHOL FLUSHING AND DIABETIC RETINOPATHY

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Summary. "Mason-type" diabetics (mild diabetes which is dominantly inherited) are relatively free of retinopathy. Alcohol almost invariably causes facial flushing in these patients when they are given chlorpropamide (chlorpropamide alcohol flush, C.P.A.F.). 291 non-insulin-dependent diabetics were examined to see whether there was a difference in frequency of retinopathy between C.P.A.F. positive and negative cases who were of comparable age and duration of diabetes. Retinopathy was commoner and often severe in C.P.A.F.-negative patients. Blindness from retinopathy was almost confined to C.P.A.F.-negative cases. Lens opacities, on the other hand, were equally common in both groups. Since C.P.A.F. is an inherited trait, retinopathy in non-insulin-dependent diabetics is to a considerable extent, although not entirely, determined by genetic factors.

Introduction

Patients with non-insulin-dependent diabetes often have facial flushing after alcohol when they are on chlorpropamide (chlorpropamide alcohol flushing, C.P.A.F.)*. This reaction occurs almost invariably in "Mason-type" diabetes, which is a non-insulin-dependent type of diabetes inherited as a mendelian dominant trait. A striking feature of this condition is its comparative freedom from diabetic retinopathy. Because of the association of C.P.A.F. with Mason-type diabetes we wondered whether there was an association between C.P.A.F. and freedom from retinopathy in non-insulin-dependent diabetics generally.

Materials and Methods

Patients

We have examined 291 non-insulin-dependent diabetics. Their selection from among patients attending the diabetic clinic at King's College Hospital was random in all respects except one—we have studied as many as possible of the non-insulin-dependent diabetics who have had diabetes at least 15 years from the nominal roll of clinic attenders we estimate that we have included at least 75% of those who have had non-insulin-dependent diabetes for 15 years and who are currently attending the clinic.

C.P.A.F.

Patients were given a single challenge test of chlorpropamide 250 mg and 40 ml of sherry.†

Ophthalmoscopic Examination

This was carried out by one of us after the patients' pupils had been dilated. The examiner did not know whether the patient was C.P.A.F. positive or negative. Patients with dense bilateral cataracts which interfered with fundus examination were excluded.

Retinopathy

This was classified as absent, mild, or severe. Mild retinopathy denotes background retinopathy not encroaching on the macula to cause impairment of visual acuity. Background retinopathy causing impairment of visual acuity and all cases of proliferative retinopathy were categorised as severe.

Blindness was defined as visual acuity of 6/60 or less in either eye.

The 6 patients whose lens opacities in one eye prevented full examination were categorised according to the appearances in the other eye. "Lens opacities" were recorded as being present when more than a single opacity was observed in either eye.

All results were analysed by x2 test.

Results

On the 291 patients, 191 were C.P.A.F. positive, and 100 were C.P.A.F. negative. The age at diagnosis was similar in the two groups (mean age for C.P.A.F.-positive group, 47±7 years; for C.P.A.F.-negative group, 51-7 years). The groups were also comparable for duration (mean±S.E.M.) of diabetes (C.P.A.F. positive, 11±0-6 years; C.P.A.F. negative, 9±0-7 years).

Retinopathy was commoner and more severe in the C.P.A.F. negative than in the positive patients (table I). 54 (54%) of the 100 C.P.A.F.-negative patients had

| TABLE I—NUMBERS WITH RETINOPATHY IN C.P.A.F. POSITIVE (+) AND NEGATIVE (−) DIabetics IN RELATION TO DURATION OF DIABETES |
| Duration (yr) | Retinopathy |
|              | Nil | Mild | Severe |
| 0-5          | 28  | 10   | 0      | 3     |
| 6-10         | 46  | 21   | 1      | 3     |
| 11-15        | 37  | 12   | 4      | 4     |
| 16-20        | 15  | 2    | 5      | 8     |
| 21+          | 18  | 1    | 2      | 6     |
| Total        | 144 | 46   | 39     | 20    | 8     | 34    |
INTRODUCTION
Ever since the first demonstration by Beatson (2) that some advanced breast cancers regressed following oophorectomy there has been a vast amount of research undertaken to try to determine which metastatic breast tumours are hormonally dependent. One of the first real possibilities of achieving such an identification came with the development of urinary steroid assays and the derivation of the Bulbrook discriminant function (4). Although the usefulness of this discriminant was variously supported (18, 32) and in some degree refuted (5, 31) it did give a clear indication that hormone dependence was not solely confined to oestrogen action, but that androgens may, in some way, also play an important role.

The more recent concept of an oestrogen receptor (10, 16) has encouraged the development of assays using tumour tissue itself (26) and very convincing data from America suggest that assays for oestrogen receptor protein are invaluable in assessing response potential to endocrine manipulation.

It has been argued that even at the primary stage of breast cancer a small number of cells have broken from the main mass of the tumour and are forming sub-clinical metastases. Thus it seems likely that the majority of breast cancer patients have disseminated disease, and the detailed study of the primary tumour, which is usually available in reasonable amounts, should indicate the nature of the metastatic deposits and assist in defining the prognosis. It may also help in deciding therapy, either as an adjuvant at the time of mastectomy or later when overt metastases are present but not available for biopsy.

In the present joint studies with the Departments of Surgery at the City Hospital, Nottingham and at the University of Liverpool, we have attempted to study the oestrogen receptor in some depth and to correlate its presence in the primary tumour with a number of clinical and biochemical features. The clinical study was, amongst other considerations, undertaken to investigate the possible relationship of oestrogen receptors to prognosis and eventual course of the disease, whereas the studies relating to biochemistry were intended to attempt to understand more of the factors which influence the assay of oestrogen receptors and which may control their function within the tumour tissue.

A. RELATIONSHIP OF OESTROGEN RECEPTOR CONTENT TO OTHER CLINICAL FINDINGS

Tissues.
Two hundred patients with operable primary breast cancer presenting consecutively at the Breast Clinic in Nottingham and nearly 100 women at a number of hospitals in the Liverpool district underwent mastectomy as their surgical treatment. In all cases where available, part of the tumour was removed and deep frozen as soon as possible either at -25°C or in liquid nitrogen. This tissue, for biochemical analysis, was transported to the Tenovus Institute on dry ice and processed within 2-3 weeks.

Patients in the Nottingham series routinely underwent triple lymph node biopsy, nodes being taken from the lower region of the axilla; the internal mammary chain at the second intercostal space. The tissue was examined histologically and patients were classified as follows:
Stage A - no tumour present in any lymph node examined;
Stage B - tumour cells found only in nodes removed from the lower region of the axilla;
Stage C - tumour tissue found in nodes either from the apex of the axilla or from the internal

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mammary chain with or without evidence of tumour in the lower axillary nodes.

Patients from the Liverpool series were staged, according to the T, N, M system of classification, as I to IV with increasing size of tumour and degree of spread of disease.

In the Nottingham series a part of the tumour was sent to Dr. C. Elston of the Pathology Department for grading of tumour. Sections were taken in three different planes and assessed on the Bloom and Richardson scale (3) by two pathologists independently, and tumours graded I, II or III with increasing loss of differentiation. Intraduct tumours were classified separately.

Assay for Oestradiol Receptor.

Tumour tissue was powdered in the frozen state with a Thermovac tissue pulveriser and a 10-20% (w/v) homogenate prepared in 10mM tris-HCl (pH 7.4) containing 1 mM EDTA and 3mM sodium azide using a glass-glass hand homogeniser. The cytosol was obtained following centrifugation for 60 minutes at 100,000 x g and its protein content estimated (20). A temperature of less than 4°C was maintained throughout all procedures of the assay.

Portions of the cytosol (200µl) were incubated with an equal volume of 10mM tris-HCl buffer, pH 7.4 containing [2, 4, 6, 7-³H] oestradiol (specific radioactivity 96 Ci mmole) in amounts ranging from 10-500 pg for 18h. A suspension (400µl) of Norit A charcoal (0.5% w/v) in a solution of Dextran T-70 (0.05% w/v) in tris-HCl buffer containing gelatin (0.1% w/v) was then added and the tubes agitated for 90 minutes. The charcoal was precipitated by centrifugation and the radioactivity in 50μl of the supernatant determined.

Estimates of the binding parameters were made by use of the Newton-Raphson iterative curve fitting technique using equations derived from the Law of Mass Action (9). The cytosol was considered as oestradiol receptor positive only when the association constant was greater than 1 x 10¹⁰/M mole and the binding site concentration was greater than five fmole/mg cytosol protein. From a series of 250 tumours assayed, three had suppressible binding but a Kₐ less than 1 x 10⁹/M mole and were thus classified as oestradiol receptor negative.

The range of values for oestradiol receptor content is shown in Fig. 1 for the two series of primary tumours. As with many other series (6, 12, 14, 15, 19, 27) the mean level of oestrogen receptor is lower in tumours from pre-menopausal patients than in tumours from older women. This has been attributed (12, 14, 19) to the higher levels of oestriol present in the plasma of pre-menopausal women and therefore presumably available to the receptor, since the assay as used routinely only allows assessment of free cytoplasmic receptor.

It can also be clearly seen from Fig. 1 that tumours from women over the age of 65 years rarely lack oestradiol receptor, although there is no significant correlation between receptor levels and age in either series.

One notable difference between the two series is that the percentage of oestradiol receptor positive cases, particularly in the post-menopausal group, is higher in the Nottingham series (61%) than in the Liverpool series (49%). Although it is accepted that the two populations may differ one from the other, it is more likely that this difference is due to various methods of tissue collection and storage. In the Nottingham series the tissue for receptor analysis is taken in the operating theatre and placed in a liquid nitrogen storage container held in the theatre suite. With the Liverpool series, however, tissue is collected on ice, transported to the Department of Surgery and stored at -25°C. Such difference merely emphasises the point made elsewhere in this volume that, when fresh tissue cannot be assayed immediately, storage in liquid nitrogen as soon as possible is the best alternative.

No correlation of oestrogen receptor content with stage of disease was apparent in either group of patients, although numbers were small in the Liverpool series (Tables 1 and 2). In the Liverpool patients roughly equal numbers of oestrogen receptor positive and negative tumours were found within each stage. Sufficient numbers were available in the Nottingham series to split the data according to menopausal status, but again the ratio of positive to negative tumours was the same in each stage as in the whole group. It is possible that there
Table 1.  
Relationship between oestrogen receptor content and stage of disease in the Liverpool series of patients.

<table>
<thead>
<tr>
<th>Oestrogen receptor</th>
<th>Stage</th>
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<tbody>
<tr>
<td>+ ve</td>
<td>I</td>
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<td></td>
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<td>IV</td>
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</table>

Negative < 5 f.mole/mg protein

were an excessive number of tumours lacking receptor in Stage A patients of the pre-menopausal group but numbers are too small for this to be regarded as significant (Table 2). Similar findings have been made by others (26).

The availability of good histological grading of the tumours from the Nottingham series has allowed the relationship of the oestrogen receptor content to the tumour grade to be examined. Fig 2 shows this data and it can clearly be seen that, whilst there is little correlation in the pre-menopausal patient group, there is a good correlation of oestrogen receptor content with tumour grade in tumours from post-menopausal women (Grades I and II versus Grade III, p < 0.001). Tumours which have retained normal characteristics histologically also appear to possess measurable cytoplasmic oestrogen receptor. Tumours which are least differentiated lack receptor in many more cases. Whilst such a correlation is not unexpected few other workers have found such convincing data (13) and others have not
Table 2.

Relationship between oestrogen receptor content and stage of disease in the Nottingham series of patients.

<table>
<thead>
<tr>
<th></th>
<th>Stage</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Pre-menopause</td>
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<tr>
<td>Oestrogen receptor</td>
<td>+ ve</td>
<td>14</td>
<td>11</td>
<td>8</td>
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<tr>
<td></td>
<td>- ve</td>
<td>24</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Post-menopause</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oestrogen receptor</td>
<td>+ ve</td>
<td>45</td>
<td>23</td>
<td>14</td>
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<tr>
<td></td>
<td>- ve</td>
<td>23</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

Negative <5 fmole/mg protein

Fig 2. Oestrogen receptor content compared with histological grade of tumour - Nottingham series.
been able to demonstrate any relationship whatsoever (17, 30). It is possible that lack of such a correlation in the pre-menopausal patients may be due to "false negative" values when circulating oestradiol levels are high.

The relationship of receptor content with grade might well have been predicted from much earlier and well established evidence that tumours which are more rapidly growing and thus have a shorter disease-free interval (probably the poorly differentiated tumours) are less likely to respond to endocrine therapy (that is they are less likely to contain receptor). However this is the first time that this relationship has been shown to exist with such a large number of primary tumours.

B. RELATIONSHIP OF OESTROGEN RECEPTOR CONTENT TO PROGNOSIS AND DISEASE-FREE INTERVAL

With the increased realisation that even at the primary stage systemic chemotherapy may be of definite benefit, the receptor content of the primary tumours of patients from the Nottingham series has been examined in order to try to define more precisely the prognosis of individual patients. The clinical-pathological features of stage and of histological grade are well known to be of value in predicting prognosis, and Figs. 3 and 4 show the disease-free interval curves for patients when grouped by such classical criteria. The degree of spread of the cancer cells to the lymph nodes gives by far the best indication of likelihood of recurrence within the time period of the study (Fig. 3). When the grade of the primary tumour was considered (Fig. 4) there was no difference in recurrence rates between patients with grade II and grade III tumours, although both did considerably worse than patients with a highly differentiated tumour.

![Disease free interval of women classed by lymph node stage](image)

Fig. 3 Disease free interval of women classed by lymph node stage: ● - stage A, ▲ - stage B, ■ - stage C. Nottingham series. Figures in parentheses indicate number of patients.

When the data was analysed similarly but with respect to receptor content it is quite clear (Fig. 5) that patients whose primary tumour was receptor positive were likely to have a longer disease-free interval than those with receptor negative tumours.

Such a difference between the two groups may only have been a reflection of clinical
Fig. 4. Disease free interval of women classed by histological grade. ● - grade I, ▲ - grade II, ■ - grade III. Nottingham series. Figures in parentheses indicate number of patients.

Fig. 5. Disease free interval of women classed by oestrogen receptor content. ● - receptor positive, O - receptor negative. Nottingham series. Figures in parentheses indicate number of patients.

Factors influencing prognosis and it is interesting that there was no correlation of receptor content and stage at mastectomy. Indeed the distribution of receptor negative tumours in the pre-menopausal group is acting against this separation (Table 2).

The data was then analysed to see if the presence or absence of cytoplasmic receptor could define more accurately the prognosis within each stage group, and the curves are shown in
Fig 6 Node negative patients (stage A) show no difference in disease-free interval between receptor positive and negative tumours, although there is a tendency for receptor negative patients to do slightly better, probably reflecting the higher numbers of stage A receptor negative tumours in the pre-menopausal patients.

However in patients with positive nodes there was a significant difference between receptor positive and negative tumours. Stage B patients with receptor positive tumours did almost as well as stage A patients, whereas those with receptor negative tumours did almost as badly as stage C patients. Although all stage C patients were in a very poor prognostic group it is clear that those with receptor positive tumours were in a more favourable position whereas those with receptor negative tumours were in an extremely dire situation.

Does the prognostic value of receptor content merely reflect the histological grade of the primary tumour? Fig. 7 shows the curves when the data is analysed for grade and receptor content. Within grades I and II there is little extra benefit to be obtained from using the receptor status in defining prognosis, although it must be emphasised that the number of receptor negative tumours in these two groups is very small. However, within the poorly differentiated grade III tumours there is again an obvious advantage in having a receptor positive tumour. It thus seems very likely that receptor content is capable of providing an extra dimension to the two more well-established prognostic determinants of stage and grade.

C. RELATIONSHIP OF OESTROGEN RECEPTOR CONTENT WITH STEROID LEVELS IN PLASMA AND TISSUE

It is obvious that the levels of oestradiol in vivo, both in the tissue and in the circulating plasma, may play a very important role in the final analysis of the oestrogen receptor content of the tumour. It has been argued, for example, that the lower levels of cytoplasmic receptor in tumours taken from pre-menopausal women is due to higher plasma levels of oestradiol which would be available to translocate the receptor to the nucleus (12, 14, 19). It is also possible that high endogenous levels of oestradiol could combine with the receptor without translocation and thus render the receptor non-detectable in the routine assay. Furthermore
such receptor-steroid complex may combine to the particulate material during preparation of the cytosol fraction and again would not be measured in the assay.

In addition to the effects of oestradiol the possible interference of androgens with the oestradiol binding to the receptor has been described (8, 29) and may influence the measurable amount of available receptor in tumour cytosols as well playing an important role in the control of the receptor in vivo. To attempt to understand more of the relationship of endogenous steroids to receptor levels we have looked at oestradiol levels in plasma and in tumour cytosols and pellets, and also at the amounts of unconjugated dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) (DHA) and androstenediol (5-androstene-3β, 17β-diol) in primary breast tumours.

**Plasma oestradiol and receptor levels.**

Oestradiol was measured in plasma samples by a radioimmunoassay technique using an antiserum raised against oestradiol-6-0-carboxymethyl oxime linked to bovine serum albumin. The cross-reaction with other oestrogens was less than 1% and assays were performed on non-chromatographed plasma extracts. Fig. 8 shows the plasma level of oestradiol plotted against receptor content, the data being combined from both the Nottingham and Liverpool series of patients. It is quite clear that in the post-menopausal woman there is no correlation whatever between plasma oestradiol and receptor content. In the pre-menopausal group there is no statistically significant relationship between the two parameters although it would appear that when available receptor protein is present the actual amount is negatively correlated with plasma oestradiol levels. Nevertheless when plasma oestradiol is very high some tumours are still found to be receptor positive. These findings tend to agree with other workers who found that there was no correlation of receptor levels with plasma oestradiol (6) and also with Maass et al (21) who showed that when the plasma oestradiol was greater than 300 pg/ml all tumours were oestradiol receptor negative.

It can be argued that because the plasma was taken on the day of operation rather than at the precise time of tumour removal such data is not convincing. However a recent publication
(7) indicates that there is little change in plasma oestradiol levels during anaesthesia. It is possibly important in the younger group of women to have a plasma oestradiol assay performed in order to estimate the degree of reliability which can be placed on the oestrogen receptor result.

![Diagram](image)

Fig. 8. Oestrogen receptor content and plasma oestradiol values. □ - post-menopausal patients. ■ - pre-menopausal patients. Nottingham and Liverpool series.

**Tissue oestradiol and receptor levels.**

Of even greater importance than plasma oestradiol content is the quantity of this steroid within the tumour itself, and particularly within the cytosol preparation. In order to study this we have adapted the radioimmunoassay used for the plasma estimation to examine tissue levels. Because of interfering compounds a thin-layer chromatography step was included which has been shown to make the assay accurate and reproducible (24).

The levels of oestradiol found in the cytosols so far examined are shown in Fig. 9. It is clear from this that the amount of oestradiol in the cytoplasm is very low and is unlikely to affect in any substantial degree the assay of receptor. Even at the level of 200 pg/g tissue a 20% homogenate would contain only 40 pg/ml cytosol. Since the assay uses only 200μl of cytosol (containing eight pg oestradiol) and the amounts of oestradiol used to determine the number of binding sites range from 20 to 500 pg per assay tube, the error from endogenous oestradiol content is less than the error involved in drawing a Scatchard plot.
Fig 9. Oestradiol content of breast tissue cytosols. ● - tumour, receptor positive, ○ - tumour receptor negative (< 5 fmole/mg protein), △ - "normal" tissue (see text).

Although the numbers of tissues so far examined are small it is interesting to find that those tumours with measurable receptor are those cytosols which tend to contain more oestradiol, even though the assay does not estimate binding sites filled with endogenous steroid. The few samples of "normal" tissue (taken from mastectomy samples and confirmed histologically) also contained significant quantities of oestradiol in the cytosol preparations.

The pellets from the high speed centrifugation have also been examined for oestradiol content. When the pellets were extracted directly the quantity of oestradiol found was high, ranging up to 300 pg/g tissue. However when the pellets were extracted after washing with low ionic strength tris buffer much of this oestradiol was lost. The amount of residual steroid, possibly reflecting the receptor-steroid complex, is shown in Fig. 10, levels ranging up to only 60 pg/g tissue. Interestingly those tumours with measurable cytoplasmic receptor also contained higher amounts of oestradiol in the washed pellets, and "normal" tissue also contained measurable amounts of the steroid.

From this data it seems that breast tumours do contain considerable amounts of oestradiol (and there is no difference in content between tumours taken from pre- or from postmenopausal women) most of which is associated, albeit weakly, with the particulate material. The levels of oestradiol in the cytosol are in all cases so far examined very low and would not appear to be high enough to cause a significant error in the estimation of oestradiol binding sites by preventing the binding of \(^3\)H-oestradiol.
Tissue androgens and receptor levels.

Of many androgens and C19-steroids examined only those possessing a 3β- and a 17β-hydroxy function have any significant effect on displacing oestradiol from the receptor (8). In particular androstenediol has the greatest effect and the levels of this steroid and of its immediate precursor DHA have been estimated in tumour tissue. The method used was one of gas chromatography-mass spectrometry, the details of which have been published (23, 25). It must be emphasised that the method is extremely specific and estimates only non-conjugated steroids.

It can be seen from Fig. 11 that all the tumours examined contained measurable quantities of DHA and that there was a good correlation of the amount found with the age of the patient from whom the primary tumour was taken (correlation coefficient, r = -0.39, p < 0.001). Although these levels cannot be accounted for by plasma contamination with free DHA in the plasma (22) it is likely that the tissue DHA content reflects the levels of plasma DHA sulphate which is present in considerable amount but which is lower in women who have experienced the menopause (22). The data suggest that most, if not all, breast tumours possess a fairly active sulphatase enzyme system, supporting evidence from previous studies using tracer precursors both in vitro and in vivo (1, 11).

There was no relationship of DHA levels with oestradiol receptor content and tumours containing substantial amounts of DHA were found to be both oestrogen receptor positive and negative.

By contrast, only about one half of the tissues studied contained significant amounts of
androstenediol, and there was no significant correlation with the age of the patient (Fig. 12). Although those tumours which contained the highest levels of this steroid were oestrogen receptor negative no correlation between steroid levels and receptor content was found.

It must be emphasised that these assays for steroid content were carried out on total homogenates and, although the data is only preliminary, some evidence has been obtained on the amounts of DHA and androstenediol in cytosol and pellets of these primary tumours. DHA was measurable in all material with no substantial difference between the cytosol and the pellet. Quantities of androstenediol in the pellets and cytosols were considerably lower than DHA and approached the limit of sensitivity of the assay but again a similar distribution between cytosol and pellet was found. In a number of cases the amount of androstenediol in the cytosol reached levels where interference of oestradiol binding in the assay may have been influenced (8, 29). Furthermore these levels would presumably have been high enough to have had some effect on the activity of the receptor in vivo.

GENERAL DISCUSSION

It is clear that a detailed study of primary breast tumours can provide much valuable information concerning the endocrinological aspects of the disease. In particular the estimation of receptor not only indicates those tumours which are most likely to benefit from adjuvant endocrine therapy, but also adds a new, independent dimension to the assessment.
of prognosis for the individual patient. Although there is a very significant relationship between receptor content and grade of the tumour, even within the grades there is a positive advantage to the patient if the tumour contains measurable receptor. The usefulness of the receptor assay in post-menopausal women is obvious, but in tumours from younger women the value is not so clear. Perhaps the circulating oestrogens should be taken into account when assessing the receptor results from such tissues but it seems unlikely that the quantity of oestradiol within the cytosol itself is sufficient to cause a great error in the assay. Although translocation of the receptor to the nucleus may well have occurred in vivo it was of interest to find that amounts of oestradiol tightly bound to the nuclear material were not greater in tumours taken from pre-menopausal women. It may be that in these women the higher levels of DHA, an androgen precursor, may have affected the quantity of measurable receptor protein, and although the most likely metabolite androstenediol does not appear to be present in greater amounts in the tumours of younger women, the levels of androstenediol measured might have had some effect on the receptor and possibly have caused translocation to the nucleus (28).

As can be appreciated such an extended study as this is an ongoing project and it is hoped that even more convincing data will become available in the next few years, both with respect to longer term prognostic value of oestrogen receptors, and also from the development of further assays for nuclear receptors and from the knowledge of other androgens contained in the primary tumour.
ACKNOWLEDGEMENTS.
The authors would like to thank Professor R. Shields of the University of Liverpool, Mr. R. W. Blamey and Dr. C. Elston of the City Hospital, Nottingham and their colleagues for their cooperation and careful evaluation of the clinical and pathological aspects of the work. We also wish to acknowledge the great help from the staff of the Tenovus Institute for their advice and criticism and to the technicians and students who have assisted in the project. Not least we would like to thank the Tenovus Organisation in Cardiff for their generous financial support, and the MRC for grant No 974/125/C.

REFERENCES

Preliminary Communications

RELATIONSHIP OF OESTROGEN-RECEPTOR STATUS TO SURVIVAL IN BREAST CANCER

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Summary The oestrogen-receptor (E.R.) content of the primary tumour was measured in 133 postmenopausal women with operable breast cancer. 79 (59%) were positive for E.R. and 54 (41%) negative. Curves of life survival show that women with E.R.-positive tumours live longer than those with E.R.-negative tumours.

INTRODUCTION

Oestrogen-receptor (E.R.) status has been shown to be a useful predictor of response to endocrine treatment in breast cancer. We have demonstrated that postmenopausal women whose breast cancers were E.R. positive had a longer disease-free interval than postmenopausal women whose tumours were E.R. negative. As yet, however, survival after mastectomy has not been clearly shown to be influenced by the E.R. status of the primary tumour.

PATIENTS AND METHODS

Between 1973 and 1977, 250 consecutive women, aged between 27 and 75 years, with primary operable breast cancer, presenting to one surgeon (R.W.B.) underwent a simple mastectomy and triple lymph-node biopsy only. Of these 250 women, 148 were postmenopausal, and E.R. status was measured in 133 of these. All the patients have so far been followed up for at least 2 years.

No adjuvant therapy was used. When distant metastases became symptomatic, patients were treated with tamoxifen, and locally with radiotherapy to particular sites when indicated. Patients who failed to respond to tamoxifen were treated with combination chemotherapy, as were patients who had relapsed after a response.

The survival curves on these 133 postmenopausal women are derived from life-table analyses of the data at each follow-up time. Comparison between the curves are made with techniques described by Mantel, an approach that evaluates differences between the whole curves rather than between individual points on the curve.

Premenopausal women have not been considered in this study. A significant effect of E.R. status on disease-free interval could not be shown in premenopausal women. Possibly E.R. assay in premenopausal women is distorted by endogenous oestrogens.

RESULTS

Of the 133 postmenopausal women, 79 (59%) were E.R. positive and 54 (41%) E.R. negative. To date 39 have died. Fig. 1 shows the survival curve for patients with E.R.-positive tumours against that for patients with E.R.-negative tumours. Patients with E.R.-positive tumours survive longer than those with E.R.-negative tumours, and this difference is statistically significant (p<0.025).

When these patients are analysed according to tumour stage (fig. 2), the effect of E.R. status is seen only in patients who have lymph-node invasion at the time of mastectomy. Lymph-node staging is derived from histological examination of triple node-biopsy specimens—stage A is no node involvement, stage B low axillary node involvement, stage C internal mammary or apical node involvement.

DISCUSSION

In postmenopausal women with breast cancer the disease-free interval after mastectomy has been shown to be

<table>
<thead>
<tr>
<th>Tumour size (cm)</th>
<th>E.R. status</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>2-5</td>
<td>Positive</td>
</tr>
<tr>
<td>&gt;5</td>
<td>Negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E.R. status</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
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</tbody>
</table>

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The Relationship of Oestradiol Receptor (ER) and Histological Tumour Differentiation with Prognosis in Human Primary Breast Carcinoma


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Abstract—In a series of 273 primary breast carcinomas oestradiol receptor content (ER) has been measured by the dextran coated charcoal method, and tumour differentiation assessed histologically. Fifty-two per cent of the tumours were ER positive. There was a strong correlation between ER status and histological grade, the better differentiated tumours tending to be ER positive. This correlation was highly significant in post-menopausal women. Both histological grade and ER status were related to prognosis, better differentiated tumours and those which were ER positive giving a better survival.

INTRODUCTION

The Nottingham Breast Cancer Study was established to examine the feasibility of producing a prognostic index so that future adjuvant therapy could be given in a more rational way. The approach has been multidisciplinary, and a preliminary report has demonstrated the value of an index based on tumour size, pathological lymph node involvement and histological differentiation in identifying a group of patients with a poor prognosis (1). A further aspect of the study has been the assessment of oestradiol receptor (ER) status and its correlation with the other prognostic factors (2,3). This paper examines in particular the relationship between ER status and histological tumour differentiation, since previous reports have been at variance, one recording a positive correlation (4) but others suggesting that there is no significant relationship (5,6,7,8). Preliminary aspects of the correlation of ER status and histological differentiation with prognosis will also be considered.

MATERIALS AND METHODS

1. Clinical Details

The study is based on a single surgical team and data are currently available on a consecutive series of 344 female patients (aged 27-70 years) with primary operable breast cancer. Clinically, tumours were less than 5 cm in diameter, freely mobile and there was no clinical evidence of metastatic spread (i.e. correlation with TNM Stage I and II breast cancer). A simple or subcutaneous mastectomy was performed in all cases, with biopsy of lymph nodes from the low axilla, the apex of the axilla and the internal mammary chain via the second intercostal space. Details of follow-up procedure and definition of recurrence have been published elsewhere (1,2).

In brief, patients were seen at a post mastectomy clinic at three monthly intervals to 18 months and thereafter at six monthly intervals. Recurrence was defined as objective evidence of tumour deposits requiring a major change in treatment policy (e.g. major local recurrence in wound flaps or symptomatic axillary lymph nodes requiring radiotherapy, bone metastases seen on radio¬graphs, enlarged liver with raised alkaline phosphatase).

2. Histopathology

In most cases specimens were examined in the fresh state immediately after operation, and representative portions of tumour tissue were immersed in liquid N₂ for subsequent assay. All mastectomy specimens were fixed in 10% per cent buffered formalin. Depending on tumour size, 1 to 4 tumour blocks were selected for histological examination, having regard to adequate sampling. Paraffin sections were cut at 4-6 μm and stained with Ehrlich’s haematoxylin and eosin. Where necessary multiple sections were examined.

Histological tumour differentiation was assessed according to the method described by Bloom and Richardson (9). The degree of tubular differentiation, the variation in size and shape of tumour nuclei and the number of mitotic figures were each scored from 1 to 3, in ascending order of abnormality. Each tumour was therefore given a composite...
score of 3-9, which is arbitrarily divided as follows:

Grade I (well differentiated) 3, 4, 5.
Grade II (moderately differentiated) 6, 7.
Grade III (poorly differentiated) 8, 9.

The assessments were carried out by the two pathologists (C.W.E. and J.J.) independently, and without knowledge of clinical details or ER results. Agreement on the initial assessment was reached in 90 per cent of cases. In 5 cases final agreement was reached by consensus. In none of the cases did the original assessments differ by more than one subgroup.

3. Oestradiol Receptor Assay

Details of the assay used routinely have been published previously (2,10). Tumours were considered as positive only when they contained more than 15 fmol of specific oestradiol binding per mg. cytosol protein, and negative if they contained less than 5 fmol; results between 5 and 15 fmol were regarded as equivocal.

RESULTS

Of the 244 consecutive patients in the series ER assay was not carried out in 47 (in most of these cases tissue was not available for ER assay because the diagnosis of carcinoma had been established at prior excision biopsy). A further 20 patients were excluded from the analysis because the ER result was in the equivocal range, and in 5 patients the tumour was intra duct in type.

Table 1. Relationship between oestradiol receptor status and histological differentiation in 273 patients with primary breast carcinoma.

<table>
<thead>
<tr>
<th>Histological Grade</th>
<th>Oestrogen receptor status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>I</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>II</td>
<td>64</td>
<td>37</td>
</tr>
<tr>
<td>III</td>
<td>45</td>
<td>82</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>130</td>
</tr>
</tbody>
</table>

$X^2 = 29.0, 2$ d.f., $p < 0.0005$.

It is of importance to establish the effect of menopausal status on these results, and this is shown in Tables 2 and 3.

Table 2. Relationship between oestradiol receptor status and histological differentiation in 92 premenopausal patients with primary breast carcinoma.

<table>
<thead>
<tr>
<th>Histological Oestradiol receptor status Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>50</td>
</tr>
</tbody>
</table>

$X^2 = 2.56, 2$ d.f., $p > 0.3 > p < 0.2$.

Table 3. Relationship between oestradiol receptor status and histological differentiation in 179 postmenopausal patients with primary breast carcinoma.

<table>
<thead>
<tr>
<th>Histological Oestradiol receptor status Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>58</td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>80</td>
</tr>
</tbody>
</table>

$X^2 = 32.9, 2$ d.f., $p < 0.0005$.

In one patient menopausal status could not be determined. In the premenopausal patients, the relationship does not reach significance, but in postmenopausal patients there is a highly significant correlation between ER status and histological differentiation.

Survival data are at a preliminary stage in this study, but there is a minimum follow up of two years for the first 205 patients whose tumour ER status had been determined.

Using life table analysis and the Mantel test (11) Fig. 1 shows that there is a significant relationship between the degree of histological differentiation and survival, patients with Grade I tumours having a better survival than those with Grade II and III tumours. Oestradiol receptor status also correlates with survival (Fig. 2) and postmenopausal women who are ER positive have a significantly more favourable prognosis.

DISCUSSION

Previous investigations into the relationship between histological tumour differentiation and ER status have yielded equivocal results. Although Heuson et al. (4) found
Furthermore, a higher percentage of the graded tumours was ER positive than in the overall series (85 per cent compared with 73 per cent). Johansson et al. (5) could find no correlation between ER content and histological grade in a study of 31 breast cancers, and similar negative findings have been reported by Rosen et al. (6,7) in 120 and 177 primary breast carcinomas respectively, and in a Japanese series of 326 breast carcinomas (8).

The present study is thus the first large scale series in which an unequivocal correlation has been shown between histological grade and oestriadiol receptor status. This relationship reaches a high level of significance in post-menopausal women, and the results suggest that ER status provides another measure of tumour differentiation, parallel to that which can be demonstrated histologically. This is in accord with recent dynamic studies (12) showing that when differentiation was measured by thymidine labelling, breast carcinomas with high ER status had higher rates of replication, and thus presumably less well differentiated, tended to be ER negative.

Assessment of tumour differentiation only assumes practical importance when it can be shown to correlate with prognosis. Histological grade has been shown by several authors to be of prognostic significance (9,13,14) and although follow-up data are at a preliminary stage this study has already confirmed this correlation between histological differentiation and prognosis, patients with Grade I tumours having a significantly better survival than those with Grade II or III tumours. This emphasises the potential usefulness of histological grade as a prognostic indicator.

Oestriadiol receptor positive tumours have been shown to respond better to endocrine therapy than receptor negative patients (15), and it is therefore important to determine whether estimation of the ER status of primary tumours is of value in predicting prognosis. A report on a small number of patients stated that ER content of primary tumours is an independent prognostic factor, patients with ER negative tumours tending to suffer earlier recurrence than those with ER positive tumours (36). The present study confirms and extends this finding in a larger series of patients, for both recurrence-free interval (3), and survival are poorer in patients with ER negative tumours.

REFERENCES
Estrogen Receptors in Early Breast Cancer

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Abstract—In a prospective study the oestrogen receptor content of early breast cancers from 421 patients was related to the development of disease recurrence. The patients were followed for a mean of 21.6 months. Fifty-two per cent of the tumours contained oestrogen receptors, but tumours in premenopausal women were less likely to contain receptors than those in postmenopausal women. The presence or absence of oestrogen receptors was independent of tumour size and axillary lymph node histology. Tumours with oestrogen receptors were associated with significantly longer disease-free periods, irrespective of menopausal status. Patients without lymph node metastases whose tumours did not contain oestrogen receptors were found to have the same poor prognosis as those with lymph node involvement. Levels of oestrogen receptor in tumours that had re-occurred were similar to those of tumours that remained disease-free.

INTRODUCTION

Estimation of the oestrogen receptor content of advanced carcinomas of the breast is now widely used in the prediction of response to endocrine therapy (1). Much less is known of the role of oestrogen receptor analysis in early breast cancer. Recent reports have suggested that the presence or absence of receptors for oestrogen is a guide to prognosis in early cancer of the breast (2,3,4). These studies indicate that the presence of oestrogen receptor was associated with a more favourable prognosis, and that this effect was independent of other prognostic factors (2).

The aim of the present study was to investigate the role of oestrogen receptor analysis in primary cancer of the breast, with particular reference to prognosis following local treatment.

MATERIALS AND METHODS

This investigation is part of a larger study of several aspects of cancer of the breast which is being carried out in the Liverpool area and receives patients from 15 contributing surgeons.

A total of 421 patients with primary cancer of the breast were studied. All tumours were staged clinically by the International TNM system. Distant metastases were screened for by skeletal survey or bone scan, chest X-ray and liver function tests. Only patients with clinically localised cancer of the breast (T1-3N0-1M0) were included in this study.

Clinical data was obtained pre-operatively and listed on a standard pro-forma. All the patients were treated by mastectomy with either axillary biopsy or dissection. The diagnosis of breast cancer was confirmed by histology, as was the presence or absence of axillary metastases and the clinical staging was reviewed in the light of this information.

Regular postoperative review of all patients was carried out at the hospital in which they received their original treatment, and follow-up data was recorded on pro-formas. The women have been followed up for periods between 3 and 42 months with an average of 21.6 months. The emergence of local or nodal recurrence was confirmed by biopsy and the presence of distant metastases only accepted on unequivocal radiological evidence.

1. Oestrogen Receptor Assay

Tumour biopsies were placed on ice at the time of mastectomy and were subsequently stored in liquid nitrogen prior to assay for oestrogen receptors. All the procedures of the assay were carried out at a temperature not exceeding 4°C.

Frozen tumour tissue was powdered in a Thermovac tissue pulveriser and homogenised 10-20% (w/v) in 10 mM-tris HCl buffer (pH 7.4) containing 1 mM EDTA and 3 mM sodium azide. A cytosol was obtained by centrifugation at 100,000 g for 60 min and its protein content was determined (5).

Eight 200 µl aliquots of the cytosol were incubated with an equal volume of tris HCl buffer containing tritiated oestradiol (specific activity 96 Ci/m mol) in amounts ranging...
from 10 to 500 pg for 18 hours. Four hundred microlitres of a suspension of charcoal (0.5% w/v) in tris HCl buffer containing gelatin (0.1 w/v) and dextran 770 (0.05 w/v) was then added and the tubes agitated for 90 mins. The charcoal was precipitated by centrifugation and the radioactivity in 50 μl of the supernatant determined.

An estimate of the binding site concentration was made by the Newton-Raphson iterative curve fitting technique (6). Non-specific binding was accounted for by the inclusion of a saturating concentration of 3 (H) oestradiol in one tube and this was used as a correction to the other points. Tumours were considered positive for oestrogen receptors only when the association constant was higher than 109/mmol and the binding site concentration was greater than 5 fmol/mg cytosol protein.

2. Statistical Methods

The follow-up data was analysed by life table methods and is presented in the form of graphs. The curves on each graph were compared by the log rank test, which evaluates differences between entire curves rather than individual points on the curves (7). The presence or absence of receptors for oestrogen in the primary tumour was related to the rate of recurrence, first for all patients studied, secondly for patients sub-divided by menopausal status, and lastly for patients with or without axillary lymph node metastases.

RESULTS

A total of 421 patients with primary cancer of the breast were studied. Of the 421 tumours, 219 (52%) contained receptors for oestrogen, and 202 (48%) did not. The patients were followed up for periods between 3 and 42 months with a mean follow-up of 21.6 months. To date 84 (202) of the 421 patients have developed proven recurrence of their disease. Of the 84 patients with recurrence 58 (69%) had tumours which did not contain receptors for oestrogen.

1. Tumour Stage and the Presence of Oestrogen Receptors

No correlation was found between the presence or absence of receptors for oestrogen and either the clinical stage of the disease, or the histology of the axillary lymph nodes. The proportion of cancers with or without receptors for oestrogen was similar in all groups when patients were separated by clinical staging (7). In the same way, of 171 patients with histological evidence of lymph node metastasis 56 had receptors for oestrogen in the primary tumour and 85 did not.

1. Menopausal Status and the Presence of Oestrogen Receptors

The presence or absence of receptors for oestrogen in the primary tumour was affected by the menopausal status of the patient. Tumours in pre-menopausal women were less likely to contain receptors for oestrogen than those in patients who were past the menopause. Of 114 tumours in pre-menopausal women, 47 (42%) contained receptors compared with 171 (36%) of 307 tumours in post-menopausal women.

3. Rates of Recurrence and the Presence of Oestrogen Receptor

The rate of recurrence was affected by the presence or absence of receptors for oestrogen. In 219 patients whose tumours contained receptors, the rate of recurrence was significantly lower (P < 0.001) than that in 202 patients whose tumours did not contain receptors (Fig. 1). This relationship remained true when patients were sub-divided by menopausal status or by the presence or absence of axillary lymph node metastases.

Fig. 1. Rates of recurrence in tumours with and without oestrogen receptors.

4. Menopausal Status

Although the number of tumours which contained receptors for oestrogen was lower in pre-menopausal patients than post-menopausal ones, the rate of recurrence for tumours with or without receptors was not influenced by menopausal status. In both pre- and post-menopausal patients the rate of recurrence for tumours which contained receptors for oestrogen was significantly lower (P < 0.001) than that for those which did not contain receptors (Figs. 2 and 3).

5. Axillary Lymph Node Histology

Of the 171 patients with axillary lymph node metastases at the time of mastectomy, 85 had tumours which did not contain receptor for oestrogen. Although the rate of recurrence for these patients did not differ significantly from that for the 86 patients with receptors for oestrogen (Fig. 1) they had the highest rate of recurrence of any of the groups studied.
patients whose tumours did not contain receptors for oestrogen (Fig. 5). Despite the more favourable prognosis usually attributed to patients without axillary lymph node metastases, the absence of oestrogen receptors in such patients was associated with an unexpected high rate of recurrence. The rate of recurrence for the 116 patients without axillary node metastases and without receptors for oestrogen was equal to that of the total group of 171 patients with axillary node metastases (Fig. 6).

Of the 250 patients without axillary lymph node involvement, the rate of recurrence in the 134 patients whose tumours contained receptors for oestrogen was significantly lower (P < 0.001) than that in the 116
The results of this study indicate that tumours which contain receptors for oestrogen are associated with a significantly lower rate of recurrence than tumours without such receptors. No association was found between the presence or absence of oestrogen receptors and other well-established prognostic factors such as tumour size, and axillary lymph node metastases. For this reason the oestrogen receptor content of a tumour appears to act as an independent prognostic guide, and when used in combination with other prognostic factors it allows clearer definition of the likely outcome of local treatment.

Pre-menopausal patients were less likely to have tumours which contained receptors for oestrogen than patients who were past the menopause. Despite this, the rate of recurrence of receptor positive and receptor negative cancers was the same in both groups and suggests that patients have either receptor positive or receptor negative disease irrespective of menopausal status.

In patients with axillary lymph node metastases, there was no significant difference, as reported previously (2), between tumours with or without receptors for oestrogen. However, those patients with lymph node involvement and receptor negative tumours had the highest rate of recurrence of all the groups studied. This finding is in agreement with that of Knight (4), who reported that 50 per cent of a similar group of patients had developed recurrence within eighteen months.

In patients without axillary lymph node metastases, a group usually thought to have a good prognosis, the rate of recurrence was unexpectedly high for those patients whose tumours did not contain receptors for oestrogen. For this group the rate of recurrence was similar to that for all the patients who had axillary node metastases. The high rate of recurrence in patients without lymph node involvement and without receptors suggests that such patients should be considered for inclusion in trials of adjuvant chemotherapy.

Jenson et al. (8) has reported that in patients with advanced carcinoma of the breast there is a group of tumours which have low levels of oestrogen receptor, and these behave in the same way as receptor negative cancers. In the present study no difference was found in the levels of receptor protein when oestrogen receptor positive tumours with and without recurrence were compared. However, the number of receptor positive tumours which have recurred is low, and longer follow-up is required.

This study confirms previous reports that the presence or absence of oestrogen receptors in primary cancer of the breast is an independent guide to prognosis (3, 4).

The combination of oestrogen receptor analysis and axillary lymph node histology allows prognosis to be determined more precisely, and this may be of benefit in the selection of patients for adjuvant therapy (9).

REFERENCES


8. E. V. Jensen, T. Z. Polley, S. Smith, E. Block, D. J. Ferguson, and E. R. Desombre, Prediction of hormone depending

chapter 43

The Prognostic Value of Estrogen Receptor Content of Human Primary Breast Tumors

P. V. Maynard, C. J. Davies, C. W. Elston, R. W. Blamey, and K. Griffiths

As part of an extended study on a series of 300 consecutive patients presenting with primary operable breast cancer, the relationship of estrogen receptor content to disease-free interval has been analyzed. In the analysis, receptor content has also been combined with stage of the disease at mastectomy and with histological grade of the tumor.

When all cases were considered (Fig. 1) there was a significant advantage to women whose primary tumors were estrogen receptor positive, such patients presenting with recurrence after a longer time period. At 18 months followup this was significant (t-test, \( p < 0.05 \)). Since it was possible that this difference may have been due to an uneven distribution with the most significant clinical prognostic feature, that of stage at presentation, it was interesting to find no such correlation (Table I). Indeed there was a higher proportion of receptor-negative tumors in patients with no node involvement, particularly in the premenopausal group of patients.

Tenovus Institute for Cancer Research, Heath, Cardiff, U.K., and City Hospital, Nottingham, U.K.
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The early prognosis for node-negative patients was good for both receptor-positive and negative tumors (Fig. 2a). However, when low axillary nodes (Fig. 2b) or apical or internal mammary nodes (Fig. 2c) were involved, there was a very marked difference between the disease-free intervals of women bearing receptor-positive or negative primary tumors. These findings are similar to a recent publication by Knight et al.6

There was a very significant correlation of receptor content with histological grade of the tumor, the more differentiated tissues (grades I and II)1 being more receptor positive, whereas many of the receptor-negative tumors were very poorly differentiated (grade III) (Table II). The difference in recurrence rates between receptor-positive and receptor-negative groups was not significant within grades I and II (Fig. 3a, b),

<table>
<thead>
<tr>
<th>Grade</th>
<th>ER positive</th>
<th>ER negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>35</td>
<td>11</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>31</td>
</tr>
<tr>
<td>III</td>
<td>48</td>
<td>64</td>
</tr>
</tbody>
</table>

*ER-positive tumors contained < 5 fmol/mg cytosol protein.

Fig. 2. Recurrence-free intervals of patients with receptor-positive and receptor-negative primary tumors. (a) Patients with no nodal involvement; (b) patients with low axillary nodes only involved; (c) patients with apical nodes or internal mammary nodes involved.

Fig. 3. Recurrence-free intervals of patients with receptor-positive and receptor-negative primary tumors. (a) Well-differentiated tumors; (b) moderately differentiated tumors; (c) poorly differentiated tumors.
due principally to the small number of receptor-negative tumors, but within the grade III tissues there was again a worse recurrence rate for women with receptor-negative tumors (Fig. 3c).

Thus, it seems that the estrogen receptor content of the primary tumor is a further feature, independent of clinical parameters, but closely related to the degree of the differentiation of the tumor, which enables a more accurate prognosis for an individual, as well as a more sensible choice of a possible adjuvant therapy.

References

Relationship between Primary Breast Tumor Receptor Status and Patient Survival

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For a minimum of 30 months, 250 women who underwent mastectomy for primary breast cancer have been followed up. ER status has had a pronounced effect upon disease-free interval and survival: in patients with node involvement ER-positive (ER+) tumors carry a better prognosis.

Of patients with ER-positive primary tumors, 43% underwent objective response to their secondaries for a minimum period of six months. This compares with a response of only 18% for ER-negative (ER−) tumors.

In patients who had previously received endocrine therapy and on relapse were treated with cytotoxic chemotherapy, objective response rate to chemotherapy was better in patients in whom the primary tumor had been ER−, but not significantly so.


In recent years, the potential value of estrogen receptor (ER) analysis on primary breast tumor tissue has been realized. Women whose primary tumors are ER-positive (ER+) have a significantly longer disease-free interval in the absence of any systemic therapy than those who have ER-negative (ER−) tumors and a significantly longer survival.1 When ER status is combined with the stage of the disease, both parameters act together to provide an accurate means by which the time of symptomatic recurrence may be predicted.5 The present study extends these initial observations to examine the prognostic value of ER measurements in the primary tumor. In addition, ER analysis of primary breast tumor specimens has been examined with respect to the responses of the secondary disease to endocrine therapy and to cytotoxic chemotherapy.

Materials and Methods

Patients

Between 1973 and 1977, 250 women between 27 and 75 years of age were seen. Each patient had primary operable breast cancer and underwent a simple mastectomy and triple lymph-node biopsy procedure. Age, menopausal status, tumor size, tumor histologic grade (CWE), tumor lymph node stage, and tumor ER status were measured, but have been completed for only 206 cases. All patients have now been followed for a minimum of 30 months. A patient was judged to be postmenopausal if she had ceased menstruation and the level of follicle-stimulating hormone was greater than 50.

At mastectomy, lymph node biopsy specimens were removed from the lower axilla, from the apex of the axilla, and from the internal mammary tumor chain at the second intercostal space. Patients with no tumor histologically evident in any node were classified as Stage A; those with tumor only in nodes from the low axilla were classified as Stage B; and patients with tumor in lymph nodes at the apex of the axilla or in the second intercostal space were designated Stage C. Patients were followed up at a postmastectomy clinic at three-month intervals, up to 18 months, and thereafter at six-month intervals; they have not received any form of adjuvant therapy.

Survival curves were derived from life-table analyses of the data at each follow-up time. Comparison between the curves were made with techniques described by Haybittle and Freedman4—an approach which evaluates differences between the whole curves rather than individual points on the curve.

In this paper, recurrence is defined as the development of symptomatic distant metastases confirmed by x-ray, abnormal liver function tests, or brain scan.
Results

ER Status and Disease-Free Interval

In Figure 1, it may be seen that, overall, patients with ER+ tumors fare no better in terms of disease-free interval than do patients with ER- tumors. However, when tumor stage is taken into account, the effect of ER status is very pronounced in the patients with lymph node involvement (stages B and C) (Fig. 2), in that patients with ER+ tumors have a longer disease-free interval.

ER Status and Survival

When time to survival, rather than disease-free interval, is considered, the situation is similar. Patients with ER+ tumors survive longer than those with ER- tumors (Fig. 3). This effect was very marked in the lymph node-positive cases when tumor stage was taken into consideration (Fig. 4).

ER Status and Response to Endocrine Treatment

Table 1 shows tumor response rates to endocrine therapy in patients with recurrent breast cancer where ER status of the primary disease was known. Of the 57 patients examined, 20 were premenopausal and underwent oophorectomy; 37 were postmenopausal and received tamoxifen (Nolvadex, ICI 46474). The overall response rate was 31.6%. Separate response rates to oophorectomy and tamoxifen were 30% and 32%, respectively. Of 20 premenopausal women, 11 were ER-positive; of these, 10 responded to oophorectomy and 2 to tamoxifen. Of nine premenopausal patients who were ER-negative, two responded to oophorectomy. In the postmenopausal group, 19 of 37 were ER-positive; of these,
in the assessment of prognosis. This synergism has been demonstrated both on disease-free interval and on survival.

The overall response rate of 18% to primary endocrine therapy (oophorectomy or tamoxifen therapy) in patients with ER− primary tumors is higher than might have been expected, and the response rate of ER+ tumors (43%) lower: however, numbers are at present small. Furthermore, the study is in its early stages, and is dealing with tumors of short free interval that are therefore less likely to respond to endocrine therapy. No clear relationship between ER status of the primary tumor and response to cytotoxic chemotherapy has been demonstrated.

A further period of observation is now required for the clinician to fully assess the value of ER measurements in primary breast cancer tissue. This is of obvious importance, in that tissue from primary tumors is always accessible to the surgeon and is of better quality than secondary tumor tissue available to the biochemist.

REFERENCES

Estrogen Receptors and Breast Cancer
by R. I. Nicholson,* K. Griffiths,* R. W. Blamey,† H. M. Bishop,‡ and J. L. Haybittle†

Estrogen receptors have been assayed in a series of primary breast cancers from postmenopausal women; 59% of which were estrogen-receptor positive. These patients survived for a significantly longer period of time than those whose tumors were estrogen-receptor negative. The effect of estrogen-receptor status was only seen (and then markedly accentuated) in patients who had lymph-node invasion at the time of mastectomy. Such determinations also appear to be of value in preselecting those patients who, on recurrence, will benefit from tamoxifen therapy.

Introduction

One of the most striking features about breast cancer is that there appears to be at least two distinct practical categories of the disease. There are those tumors which are hormone-responsive and which will regress following the removal of, or interference with, their hormonal environment; objective breast tumor regressions often with extensive remission intervals are observed in approximately 30% of patients following the surgical removal of their ovaries, adrenal glands, or pituitary gland, or after the addition of pharmacological amounts of hormones or antiestrogens; there are other tumors whose growth appears to be independent of any substantial hormonal association and which derive little measurable benefit from these endocrine treatments. Although little is known about how these types of disease arise from the normal epithelium of the breast and indeed, what agents both endogenous and environmental, act to initiate and regulate their development, growth and interrelationships with one another, nevertheless it is apparent that they do represent extreme biological variants, and anecdotal evidence suggests that they are reflected in other fundamental and highly variable characteristics of the tumor, such as their degree of differentiation, their growth rates, and possibly even their invasiveness.

Currently, the most widely and successfully used method for the determination of the hormone responsiveness of breast tumors is the measurement of the intracellular concentration of a protein referred to as the estrogen receptor (1). This protein is present in the cytoplasm of hormone-responsive tumor cells and binds incoming estradiol with selective high affinity. The binding of the hormone to the receptor (ER) is then thought to facilitate the transfer to and retention of the receptor complex within the nucleus and thereby increase transcription of the DNA template, a process essential for cell maintenance and division. Clinical studies indicate that while the presence of ERs in secondary recurrent breast cancer is associated with a 50-60% objective breast tumor response rate to endocrine measures, in their absence only 5-10% of patients respond to these treatments (2). Such data have led to the routine use of ER measurements in breast cancer specimens in preselecting patients most likely to derive benefit from these endocrine therapies.

Furthermore, it has recently become evident that women whose primary tumors are ER-positive have a significantly longer disease-free interval, in the absence of any systemic therapy, than those who have ER-negative tumors, and that when ER status is combined with the lymph-node staging of the disease, both parameters act together to provide an accurate means by which early recurrence may be predicted (3, 4). The present report extends...
these initial observations to examine the value of such ER measurements in relation to the survival of the patient. In addition, ER analysis on primary breast tumor specimens has been examined with respect to the response of the secondary disease to endocrine therapy.

Materials and Methods

Patients

Between 1973 and 1977, 250 women, aged between 27 and 75 years, with primary operable breast cancer, who consecutively presented to one surgeon (RWB), underwent a simple mastectomy and triple lymph-node biopsy (5). Of these women, 148 were postmenopausal and ER status was measured on 133 of these. All patients have been followed up for at least 2 years.

At mastectomy, lymph node biopsies were removed from the lower axilla, from the apex of the axilla and from the internal mammary tumor chain at the second intercostal space. Patients with no tumor histologically evident in any node were classified as Stage A; those with tumor only in nodes from the low axilla were classified as Stage B and patients with tumor in lymph nodes at the apex of the axilla or in the second intercostal space were designated Stage C. Patients were followed up at a postmastectomy clinic at 3-month intervals to 18 months and thereafter at 6-monthly intervals and have not been subjected to any form of adjuvant therapy.

Survival curves were derived from life-table analyses of the data at each follow-up time. Comparison between the curves were made with techniques described by Haybittle and Freeman (6)—an approach which evaluates differences between the whole curves rather than individual points on the curve.

For this study recurrence was defined as the development of symptomatic distant metastases confirmed by x-ray, abnormal liver function tests, or brain action. Once symptomatic recurrence was diagnosed, the first line of endocrine treatment in premenopausal women was oophorectomy and in postmenopausal patients tamoxifen therapy (10 mg b.d.). In addition, radiotherapy was given to particular sites (e.g. vertebral metastases) when indicated. Patients who failed to show objective response to endocrine treatment when assessed six months after initiation of therapy were treated with combination chemotherapy, as were patients showing obvious tumor progression two months after the initiation of endocrine therapy. Patients who showed objective response after six months endocrine therapy received secondary endocrine therapy in the form of adrenalectomy, once the response had ended.

Estrogen Receptors

At operation a representative portion of the primary tumor was frozen in liquid nitrogen and stored at -70°C before being transported in Dry Ice to the Tenovus Institute for subsequent ER assay. Details of the assay have been previously reported (3). Tumors were considered positive only when they contained more than 5 fmole specific estradiol binding/mg cytosol protein.

Results

ER Status and Survival

Of the 133 postmenopausal women, 79 (59%) were ER positive and 54 (41%) ER negative. To date 39 have died. Figure 1 shows the survival curve for patients with ER-positive tumors against that for patients with ER-negative tumors. Patients with ER-positive tumors survive longer than those with ER-negative tumors (p < 0.025). Furthermore, it was observed that there was no additional advantage for those patients whose primary tumor contained receptor levels in excess of

Environmental Health Perspectives
were premenopausal and underwent oophorectomy and 37 were postmenopausal and received tamoxifen (Nolvadex, ICI 46474). The overall response rates to oophorectomy and tamoxifen were 30 and 32%, respectively. Eleven out of 20 premenopausal women were ER-positive and of those 4 (36%) responded to oophorectomy. Of the nine premenopausal patients who were ER-negative, 2 (22%) responded to oophorectomy. In the postmenopausal group 19 out of 37 (51%) were ER-positive and of these 9 (47%) responded to tamoxifen. Out of the 18 postmenopausal patients who were ER-negative, 3 (17%) responded to tamoxifen.

**Discussion**

The present data clearly demonstrate that postmenopausal women with ER-positive primary breast tumors survive significantly longer than those with ER-negative tumors. These data are consistent with our previous findings in which the length of the disease-free interval was found to correlate with tumor ER status (3). The fact that these relationships were established on the absence of adjuvant therapy suggests that the ER status of the primary tumor is a measure of the natural biology of the tumor. This concept is reinforced by the finding that ER status is related to tumor histological grade, well differentiated tumors rarely lacking ER proteins (5). Interestingly, the absence of a relationship between tumor stage (by lymph node status) and ER status makes these two prognostic factors synergistic in the assessment of prognosis. This synerigism has been demonstrated by our analysis of the effect in disease-free interval (3) and survival (7) (Fig. 3).
In addition to the prognostic value of ER measurements in primary breast tumor tissue, such analyses also appear to be of some value in predicting the response of metastatic disease to tamoxifen therapy in postmenopausal women. In patients with ER-positive tumors the response rate of 47% is "similar" to that observed when ER measurements are carried out on metastatic deposits (8). This effect was not however, observed in premenopausal women undergoing oophorectomy where only 36% (4/11) of patients with ER positive tumors underwent an objective breast tumor remission, a value only slightly higher than the overall response rate (30%) for this group. Conversely, the response rates of 23% (2/9) and 17% (3/18) to oophorectomy and tamoxifen therapy respectively in patients with ER-negative primary tumors are higher than would have been predicted on the basis of ER measurements carried out on secondary breast tumor tissue (2). The numbers are at present, however, small. Also the study is in its early stages and is dealing at present with tumors of short disease-free interval. A further period of observation is now required to truly assess the value to the clinician of ER measurements in primary breast cancer tissue in relation to the subsequent response of the secondary disease to endocrine therapy. This is of obvious importance in that primary tumor tissue is always accessible to the surgeon and is of better quality for the biochemist than is secondary tissue.

The authors wish to acknowledge the generous financial support of the Tenovus Organization.

REFERENCES

STEROID RECEPTORS IN EARLY BREAST CANCER: VALUE IN PROGNOSIS

R. I. NICHOLSON, F. C. CAMPBELL†, R. W. BLAMEY‡, C. W. ELSTON*, D. GEORGE‡ and K. GRIFFITHS
Tenovus Institute for Cancer Research, Cardiff;
City Hospital, Nottingham† and Royal Liverpool Hospital, Liverpool‡, U.K.

SUMMARY

The relationship of prognosis to oestradiol-17β receptor (ER) status of primary breast tumours has been studied (a) in 550 patients who presented consecutively to one surgeon (RWB) and (b) 421 patients dealt with by 15 surgeons, coordinated by DG. Patients in (a) were staged after a triple-node biopsy according to node involvement, those in (b) by the TNM system. All patients were followed-up without treatment until recurrence. In group (a), 82 patients have had local or regional recurrence, 42 of whom also developed distant metastases. 42 developed distant metastases without prior local recurrence. ER status was related to tumour grade, disease free interval and survival. Furthermore ER status, with stage and grade, can be used to select good or bad pronostic groups. The site of metastases, a clinical factor of prognostic significance also related to ER status, ER+ tumours tending to recur to bone, ER- to the viscera. In group (b), ER status also related to recurrence and survival. ER++, lymph-node negative (LN−) patients had a good prognosis. Conversely ER− LN− had a poor prognosis. Of particular interest in the group (b) study, ER − LN− patients could be identified as a high risk group with a survival rate similar to all LN+ patients.

INTRODUCTION

Although for many years, endocrine therapy has been advocated for the treatment of advanced breast cancer, it is generally accepted that the objective response rate is only of the order of 30%. Investigations directed to the means of predicting which tumours would respond to endocrine manipulation, manifest for example in the urinary discriminant function [1] have generally been unsuccessful. It was clear however that the analysis of the oestradiol receptor (ER) content of breast tumour tissue might provide such a predictive test for the selection of patients for therapy and the pioneering work of Jensen and his colleagues [2,3] did a great deal to substantiate this concept. The collected results from many centres [4,5,6] have provided unequivocal evidence that the receptor analysis of metastatic tissue is of value in the management of patients with advanced disease.

Despite the reported success of such assays not all metastatic deposits are either readily or advisably biopsiable and it was very clear that often, tumour tissue obtained at mastectomy would in many instances be the only material available for receptor analysis. Because of this therefore, two major collaborative programmes of research were established in 1973 between the Tenovus Institute for Cancer Research, Cardiff and Breast Cancer Clinics in Nottingham (Professor R. W. Blamey) and in Liverpool (co-ordinated by Mr D. George). This communication reports the data emerging from these co-operative studies regarding the relationship between the ER status of primary breast tumours and the natural history of the disease.

MATERIALS AND METHODS

Patients

In Nottingham, 550 patients presented consecutively to one surgeon (RWB) between 1973–1979. All had tumours of less than 5 cm dia. and there was no evidence of distant metastases. A simple or subcutaneous mastectomy was undertaken. At mastectomy, lymph node biopsy samples were removed from the lower axilla, from the apex of the axilla and from the internal mammary chain. Patients without tumour histologically evident in any node were classified as Stage A. Those with tumour only in the nodes from the lower axilla were Stage B and Stage C if apical axillary or internal mammary nodal involvement had occurred. Histology was the responsibility of Dr C. Elston, University of Nottingham and for tumour grading [7,8] according to Bloom and Richardson[9]; grade I, the most differentiated, grade III the least. All patients were followed up at 3 month intervals for 18 months and 6 monthly thereafter. Treatment was withheld until recurrence, no patient receiving any form of systematic adjuvant therapy.

The Liverpool patients presented to one of fifteen surgeons participating in the study. They were staged clinically according to the international TNM system and were treated by some form of mastectomy which included either an axillary node biopsy or an axillary dissection. Excised nodes were histologically examined for metastatic tissue. A total of 421 patients with operable breast cancer (T1−3, N0−1, M0) have been included in this study. Again, as with the Nottingham project, systemic treatment was withheld until recurrence of the disease.
Receptor analysis

In both studies, tumour tissue was rapidly frozen after removal and stored in liquid nitrogen before transportation in dry ice to Cardiff where ER-status was determined within 2-3 weeks.

Oestrogen receptor levels were determined using a saturation analysis technique which involved incubation of high-speed supernatant with 10 concentrations of [3H]-oestradiol-17β ranging from 200–5000 pmol/l for 16 h at 4°C. Similar incubations were carried out in the absence of cytosol to determine the inefficiency of separating free from bound label using charcoal. Non-specific binding was estimated at each concentration of oestrogen used in the incubation, from binding data obtained by addition of a 100-fold excess of diethylstilboestrol to the medium at three selected oestradiol concentrations. Estimates were calculated from a linear regression analysis of non-specific binding and oestradiol-17β concentration.

A computer program was used to calculate the ‘true’ specific binding and the concentration of label that was available to react with the receptor. This data was then used to estimate the level of receptor contained in the cytosol preparation and involved fitting a law of mass action model governing the interaction between a single species of univalent ligand and a single species of univalent binding site to the ‘corrected’ binding data. The method of computation is based on the method of least squares and uses a Newton–Raphson iterative technique described by Feldman[10].

The fiducial limits of the receptor site concentration were also computed to provide the minimum detectable concentration that can be distinguished from zero with a given probability [11], which is dependent on the precision of the receptor measurement which can vary with time. Consequently, it is inadvisable to associate an oestrogen receptor positive result with a minimum cut-off value unless this level is high enough to ensure that the probability of detection is adequate for the assay method. Since receptor levels are usually expressed with reference to cytosol protein, then errors of measurement must be considered when this cut-off value is chosen. This value is currently of the order of 5 fmol of specific binding per mg of cytosol protein. Since the equilibrium association constant is a thermodynamic property of the system, its calculated value can then be used to assess the validity of estimates of receptor site concentration. No receptor site value is considered valid if the value for the equilibrium association constant is less than 10⁸ litres/mol.

RESULTS AND DISCUSSION

ER-status and clinical parameters

The ER-concentrations shown in Fig. 1 indicate the profile of values obtained from the first 200 patients presenting to the Nottingham Clinic and is typical of those previously reported [4], with approximately 60% of the tumours containing measurable amounts of receptor and levels in the postmenopausal women being generally higher than in the premenopausal group. Approximately 50% of the tumours from the Liverpool study contained receptor.

Data from both centres clearly indicate that there is no relationship between ER-status and the stage of the disease, nor with tumour size. From the Nottingham results, a significant correlation was seen between ER-status and histological tumour differentiation [7, 8], with a larger proportion of the undifferentiated grade III tumours being ER-negative (Fig. 2). This correlation however was not statistically evident from the data accumulated from the Liverpool group and may result from the more specialised breast cancer pathology available in Nottingham. Interestingly, the anomalies observed between these two

Fig. 1. Oestrogen receptor content of the first 200 primary tumours removed from women presenting to the Nottingham Clinic.

Fig. 2. Oestrogen receptor status and histological grading Data from the first 179 postmenopausal women presenting to the Nottingham Clinic.
since the correlations are not sufficiently strong to allow the prediction of recurrence to be accurately assessed for an individual patient [20]. It is necessary therefore to consider prognostic value of ER-status of the primary tumour in association with other established clinical factors.

**Nottingham study**

The assessment of histological grade has been shown previously to be of prognostic significance [9, 21, 22] and the careful, detailed assessment of tumour differentiation in the investigation now reported confirms the correlation between tumour grade and prognosis (Fig. 4). Survival curves were derived from life-table analysis of data from each follow-up time and comparison between curves were made by techniques described by Haybittle and Freedman[23], which evaluate differences between curves rather than individual points on the curve.

For the purpose of this study, local recurrence was defined as multiple symptomatic or progressive metastases in mastectomy flaps which require major treatment, while regional recurrence was defined as symptomatic metastases in axillary or supraclavicular nodes, judged to require major therapy. Patients with asymptomatic nodal enlargement or single 'spot' recurrence in mastectomy flaps were not included in the analysis. Sites of distant metastases were confirmed by clinical examination, appropriate X-rays, brain scan, liver function tests and biopsy.

Stage A patients that is, those with no apparent nodal involvement at mastectomy formed a 'good prognosis' group with an 85% overall survival at 4 years regardless of the receptor status. Patients with tumour cells found in regional lymph-nodes at mastectomy and ER-negative tumours had a particularly bad prognosis (Fig. 5a); 70% of those with metastases in the higher nodes (Stage C) died in 3 years. In comparison, patients with nodal involvement and ER-positive tumours, have, as a group, a more favourable prognosis, nearly 70% surviving 3 years (Fig. 5b and 5c). Of these patients, those with grade III tumours form the 'poor prognosis' group (Fig. 6), the recurrence rate and survival rate being similar to those of
all patients with ER-negative tumours and with nodal involvement (Fig. 7). Node positive ER-positive patients with grade I and II tumours have a good prognosis and do as well as patients without nodal involvement (Stage A patients) (Fig. 8).

Liverpool study

Patients in this investigation were followed up at the Hospital where the original treatment was carried out. Recurrent disease, local or nodal, was diagnosed only after histological examination of excised tissue and visceral or distant bone metastases were diagnosed by unequivocal radiological evidence. Treatment of recurrent disease was at the discretion of the various surgeons involved in the study, a situation that can be considered appropriate to that of any large industrial city. Generally patients were initially treated with tamoxifen (Nolvadex) and those who failed to respond or relapsed again, were treated with combination chemotherapy. Of this group of patients studied, 50 have died from recurrence of their breast cancer.

Assessment of data from the 421 patients studied, indicated that 219 (52%) had tumours with measurable levels of receptor. Of 171 patients with axillary node involvement, 86 had ER-positive tumours and

Fig. 5. Oestrogen receptor status and prognosis in Nottingham patients with nodal involvement.

Fig. 6. Relationships between tumour grade and prognosis in patients with nodal involvement and ER-positive tumours.

Fig. 7. Prognosis of patients with nodal involvement: comparison of patients with ER-negative tumours and those with ER-positive, grade III tumours.
Steroid receptors and breast cancer

85 were negative. As with the Nottingham study, patients with ER-negative tumours recurred early, the differences being significant in both pre- and postmenopausal women [24, 25].

Of particular interest was the group of 116 patients without nodal involvement but with ER-negative tumours, who despite the favourable prognosis generally attributed to this group, unexpectedly recurred at the same rate as all women with axillary nodes involved. Furthermore, when survival rates were considered, it was again clear that this group fared badly (Fig. 9). Patients with ER-positive tumours, without axillary node involvement formed a good prognosis group. Generally the rate of survival was significantly longer (P < 0.001) in patients with ER-positive tumours (Fig. 9a).

Receptor status: site of recurrence

It has long been recognised that the anatomical site of distant metastasis provides a useful prognosis factor that relates both to response to therapy [26] and also to survival [27, 28]. Patients with visceral metastases have a less favourable prognosis than those with skeletal secondaries, who respond better to endocrine therapy.

Examination of the results from the Nottingham study indicated that major local or regional recurrence occurred in 82 patients of whom 42 had developed distant metastases and a further 82 had clinical

<table>
<thead>
<tr>
<th></th>
<th>ER Status</th>
<th>Histological grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>No recurrence</td>
<td>244</td>
<td>166</td>
</tr>
<tr>
<td>Recurrence</td>
<td>20</td>
<td>29</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 6.3; 1 \text{ d.f.} \]
\[ P < 0.05. \]

\[ \chi^2 = 21.1; 2 \text{ d.f.} \]
\[ P < 0.001. \]
evidence of distant metastases without prior local recurrence. The development of a single “spot” recurrence in the mastectomy flaps was reported in 44 patients.

ER-status did not relate to major local recurrence, nor to the incidence of “spot” recurrence. The incidence of symptomatic regional recurrence was however significantly greater in patients with poorly differentiated (grade III) or ER-negative tumours, although the observed association was more significant in the former (Table 1). The total incidence of distant metastases was unrelated to ER-status but was significantly greater in those with poorly differentiated, grade III cancer (Table 2). The site of the first distant metastasis related to both factors, with ER-positive tumours showing a significant tendency to metastasise initially to bone, whereas ER-negative, undifferentiated cancer showing an affinity for distant spread to viscera. This relationship between ER-status and a selective pattern of spread complements other studies in which a similar trend is emerging [29].

**Table 2.**

<table>
<thead>
<tr>
<th>Site of first distant recurrence</th>
<th>ER Status</th>
<th>Histological grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Bone</td>
<td>42</td>
<td>13</td>
</tr>
<tr>
<td>Viscera</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td>Combined</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Total recurrence</td>
<td>69</td>
<td>55</td>
</tr>
<tr>
<td>No recurrence</td>
<td>195</td>
<td>140</td>
</tr>
</tbody>
</table>

Receptor status of primary tumour and response to therapy on cancer

Information is accumulating to suggest that primary tumour receptor analysis will be of value in determining endocrine responsiveness of metastatic disease [15]. There is little evidence of marked changes in receptor phenotype during the period between mastectomy and disease recurrence and Jensen and his colleagues [30] have indicated that primary tumour ER-status relates well to the results of endocrine therapy for metastatic disease. Data from Nottingham on patients who have recurred and are treated by either oophorectomy, if premenopausal or with tamoxifen if postmenopausal provided further support for this. Of those patients (55) with ER-positive primary tumours treated with endocrine therapy 17/55 (31.8%) responded, whereas only 2/40 (5%) of the ER-negative group have been confirmed as responders by external assessment. Furthermore, Fig. 10 also indicates that the response rate increases with increasing levels of oestrogen receptor in the primary tumour.

Overall, it is becoming clearly more obvious that on the basis of a receptor analysis together with the clinical prognostic parameters that are available to a breast clinic, a more rational effective approach can be made by the clinician concerned with the management of women with breast cancer, for the selection of patients, as individuals, for various treatment regimes.

Acknowledgements—The authors are grateful to the Tenovus Organisation for generous financial support.

**REFERENCES**

QUANTITATIVE OESTRADIOL RECEPTOR VALUES IN PRIMARY BREAST CANCER AND RESPONSE OF METASTASES TO ENDOCRINE THERAPY

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Summary
Quantitative values of oestradiol receptor were determined in the primary breast cancer of 526 patients, 106 of whom have so far required hormonal therapy for metastatic disease (premenopausal patients—oophorectomy; postmenopausal patients—tamoxifen 20 mg twice daily). The rate of response to treatment was significantly higher in patients with receptor-positive primary cancers, and the likelihood of response further increased in proportion to the measured receptor concentration. Preliminary data also suggest that patients with high receptor values in the primary tumour will enjoy a longer remission than those with low measured values. Assays of oestradiol receptor were also carried out on biopsy specimens of accessible metastases, largely skin and lymph-node recurrences, in 24 patients who were treated mainly with local measures. Receptor status was the same in both the primary and the secondary tumour in only 18 patients.

Introduction
20 to 40% of women with advanced breast cancer will respond to hormonal therapy, which may induce a complication-free, often lengthy, remission. The relationship between oestrogen-receptor (ER) status of breast cancer and response to hormonal treatment is well established, but in most published series receptor status has been determined on biopsy material from metastatic tumour taken immediately before the start of treatment. A considerable number of patients with advanced breast cancer will have metastases which are inaccessible for biopsy. Metastatic tumour could be obtained for receptor analysis by surgical intervention, but authorities such as the British Breast Group believe that major surgery to obtain tissue for assay is not justifiable. The primary cancer provides an ample source of tissue for receptor analysis. The object of this study is to investigate the relationship between ER status of the primary tumour and response of the secondary cancer to endocrine therapy and also to relate response to measured receptor values in the primary tumour. In addition, this study investigates the stability of receptor status between the primary tumour and the metastases.

Patients and Methods
Patients
620 consecutive patients with primary operable breast cancer, aged between 28 and 75 years, presented to one surgeon (R. W. B.) between 1973 and 1980. All patients had tumours which were judged clinically to be less than 5 cm diameter, and none showed any evidence of distant metastases at presentation. All patients were treated with simple or subcutaneous mastectomy and were followed up in a post-mastectomy clinic at 3-month intervals for 18 months and at 6-month intervals thereafter.

A programme of adjuvant chemotherapy was started late in the study and was given to patients who were identified on the basis of nodal status and other criteria as having a very poor prognosis. 20 patients who received this treatment were subsequently excluded from the analysis. All other patients at this centre have been followed without any treatment until the development of recurrence.

On recurrence endocrine therapy was the first-line choice for systemic treatment and was administered to women with histologically confirmed skin-flap or lymph-node metastases which were unresponsive to radiotherapy or to patients with distant metastases confirmed by clinical examination, plain radiographs, liver-function tests, brain or liver scans, or biopsy. Premenopausal patients were treated with surgical oophorectomy, whereas postmenopausal women were given tamoxifen, in a dose of 10 mg twice daily in the early years of the study but later increased to 20 mg twice daily. Menopausal status was assessed on the basis of history and serum-follicle-stimulating-hormone (FSH) levels. Women were judged to be postmenopausal if they were no longer menstruating and had an FSH value in excess of 50 tu/l.

106 patients have received endocrine therapy for advanced disease. 11 patients (5 who received adjuvant chemotherapy, 4 who had so-existant second primary cancers of another organ, and 2 with bilateral tumours of different ER status) were excluded, leaving 95 patients assessable for response to treatment. The distribution of metastases was as follows: local or regional recurrence, 9; distant metastases 86 (bone 39, lung/pleura 27, liver 9, brain 6, multiple organs 5). Response to therapy was assessed along the guidelines recommended by the UICC Programme on Clinical Oncology, but we observed the British Breast Group recommendation that any objective benefit must last a minimum of 6 months to be considered a response. Categories of response were defined thus:

1. Complete response (CR)—Disappearance of all known disease. This includes lytic bone metastases, which must be shown radiologically to have calcified.

2. Partial response (PR)—A 50% or more decrease of the sum of the products of the largest diameters of measurable lesions and objective improvement in evaluable but non-measurable lesions without the development of new lesions.

In this paper both complete and partial responses are judged as "response".

Oestradiol Receptor Assay
Receptor analyses were carried out on samples of the primary cancer taken at mastectomy in 526 patients and biopsy specimens of accessible metastatic deposits in 24 patients. Specimens were frozen immediately and stored in liquid nitrogen at −70°C before being transported on dry ice to the Tenovus Institute, Cardiff, where assays were done with the dextran-coated charcoal method. Tumours were considered to be receptor positive when they contained more than 5 fmol specific oestradiol binding per milligram of cytosol.

Results
318 of the studied patients (60%) had ER-positive primary tumours. In this group, specific oestradiol binding ranged from 5 to 1475 fmol/mg cytosol protein, with a median value of 55 fmol.

Of the 95 patients assessable for response to treatment, 19 (20%) responded to treatment. Response rates were similar in premenopausal patients treated with oophorectomy and postmenopausal women treated with tamoxifen (table I). Patients with ER-positive primary cancers had a significantly higher rate of response than those with receptor-negative primaries (table II). The measured value of oestradiol receptor was
TABLE I—RESPONSE TO ENDOCRINE THERAPY

<table>
<thead>
<tr>
<th>No. of patients treated</th>
<th>No. responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postmenopausal</td>
<td>66</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
</tr>
</tbody>
</table>

TABLE II—ER STATUS OF THE PRIMARY TUMOUR AND SUBSEQUENT RESPONSE OF SECONDARIES TO ENDOCRINE THERAPY

<table>
<thead>
<tr>
<th>ER status</th>
<th>No. developing metastases</th>
<th>No. responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER positive</td>
<td>55</td>
<td>17 (34%)</td>
</tr>
<tr>
<td>ER negative</td>
<td>40</td>
<td>2 (5%)</td>
</tr>
</tbody>
</table>

\( \chi^2 = 8.2, \ p < 0.005 \)

directly related to the likelihood of response of metastases to hormonal treatment (fig. 1).

Primary tumours which were ER positive have also been arbitrarily categorised as either receptor-high (>60 fmol/mg cytosol) or receptor-low (<60 fmol/mg cytosol). The median duration of remission in patients who had a recurrence was 26 months for those with receptor-high cancers and only 12 ± 2 months for those with receptor-low cancers (fig. 2).

Biopsy specimens of metastatic tumour were taken from accessible sites in 24 patients (17 skin, 5 lymph node, 1 omentum, 1 liver). A median interval of 24 months elapsed between the mastectomy and submission of a biopsy sample of metastasis for receptor analysis. ER status was unchanged from that of the primary cancer in 18 patients; 3 who had ER-positive primaries developed receptor-negative secondaries; and 3 whose primary growth lacked receptor had measurable amounts in their secondary tumour. Where the status changed measured values in either primary or secondary cancer tended to be low (table III).

TABLE III—CHANGE OF ER STATUS

<table>
<thead>
<tr>
<th>Case no.</th>
<th>ER concentration (fmol/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Negative</td>
</tr>
</tbody>
</table>

\*ER values in the six tumours in which ER status was changed in the secondary from that in the primary.

Discussion

In this study, 20% of patients with metastatic breast cancer who had received no systemic therapy before the development of recurrence responded to hormonal therapy. This rate of remission is lower than that quoted by other centres, but the method of assessment of response and the minimum duration set are strict in this series. The median duration observed in this study (15 months) is thus longer than that of other series (9 months) where the apparent response rate is higher. 3,4

The relationship of receptor status of the primary cancer to observed rates of objective response of daughter metastases to endocrine manoeuvres is of obvious importance, and preliminary data from two studies support this relationship. 5,6 Patient numbers in these studies were small, and treatment methods lacked uniformity in that variety of endocrine ablative procedures had been used and some patients had received prior adjuvant chemotherapy. The present study confirms the relationship, however, and demonstrates a direct association between the measured value of receptor in the primary tumour and the likelihood of response.

Our initial findings also indicate the possibility that receptor-rich cancers (>60 fmol/mg cytosol) may respond longer than those with lower values.

Receptor status remains constant between the primary cancer and the daughter metastases in the majority of instances. Any change of status of either the primary or secondary tumour appears likely to be associated with the lower range of positive receptor values, which in their turn show a lower sensitivity to hormonal methods.

In summary, the likelihood (and possibly the duration) of remission of subsequent metastases to endocrine therapy may be reliably predicted in the individual patient on the basis of the oestriadiol receptor value in the primary malignancy at the time of mastectomy; absolute level of ER is important in addition to ER status.
ASSOCIATION OF SPINOCEREBELLAR DISORDERS WITH CYSTIC FIBROSIS OR CHRONIC CHILDHOOD CHOLESTASIS AND VERY LOW SERUM VITAMIN E

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J. SCOTT*
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Summary
Neurological syndromes similar to those associated with atebaliproteinemia or Friedreich's ataxia developed in four patients with chronic steatorrhea, two of whom had cystic fibrosis and two chronic cirrhosis of childhood. Serum concentrations of vitamin E were virtually undetectable in all four patients. Substantial clinical improvement occurred in one patient after restoration of normal vitamin E levels by parenteral therapy. The findings suggest that spinocerebellar degeneration may be secondary to severe and prolonged vitamin E deficiency.

Introduction
CONCENTRATIONS of vitamin E are undetectable from birth in patients with atebaliproteinemia and it has been reported that the neurological disease associated with this condition may be prevented or improved by vitamin E therapy.1,2 We describe four patients who had spinocerebellar disorders and chronic severe vitamin E deficiency (see accompanying table) secondary to fat malabsorption.

Case-reports

Case 1
This patient,3 whose diagnosis of cystic fibrosis was established by repeated high sweat sodium concentrations, had, on presentation at the age of 19 years, ophthalmoplegia with slight nystagmus, absent reflexes, an extensor right plantar response, and ataxia on finger-nose and heel-knee testing. Cutaneous sensation was normal. Facial fat was 38 g/24 hours (normal, 80 g/24 hours), and the duodenal bile acid concentration after an overnight fast and stimulation with cholecystokinin was 2·4 mmol/l, which is below the critical micellar concentration. Prothrombin time was prolonged by 7 s, but was corrected by parenteral vitamin K; serum vitamin A was 9 ng/ml (normal, 20–50); vitamin E was undetectable; and 25-OH cholecalciferol was 3·8 ng/ml (normal, 9–40). Liver biopsy showed no cirrhosis, but extensive fibrosis with sheets of dense collagen, proliferating bile ducts, and small amounts of inspissated bile in a few ducts were seen. Nerve conduction studies were consistent with a mild but definite peripheral neuropathy.

The patient was treated with vitamins A, D, K, and E. Dietary fat intake was reduced and pancreatic enzyme supplements were given, but there was no change in the neurological symptoms. Details of vitamin E supplements and serum vitamin E concentrations are given in the table. After oral vitamin E therapy (200 mg/day) for nearly 3 years, he complained that his hand tremors had worsened, so that he had difficulty drinking from a cup and had lost his job because of writing difficulty. He was given a flat emulsion intravenously, and vitamin E supplements intramuscularly (100 mg/week). 4 months later, the serum concentration of vitamin E was 18·7 pmol/l, and the patient reported a marked improvement. This improvement has been maintained, so that the patient has been able to complete a college course successfully. Neurological testing 2 years later shows a distinct improvement in his neurological state.

Case 2
Born in 1951, this White male was noted to be jaundiced at 17 days of age. The jaundice persisted, and laparotomy and open liver biopsy at 2 years confirmed the presence of cirrhosis, but the aetiology of the liver disease remained unexplained. The extrahepatic biliary system was patent. Cholestatic jaundice persisted, with associated malabsorption and severe growth retardation. By 1966, when he was 15, he had symptoms and signs of a cerebellar disturbance (ataxic gait and ataxia on finger-nose and heel-knee testing), ophthalmoplegia (failure of upward gaze of both eyes), and proprioaxis. He had almost complete absence of tendon reflexes, and vibration sense was absent in all limbs. Proprioception and light touch sensation were normal. He had a soft precordial murmur suggestive of mild pulmonary stenosis. Investigations for right hypochondrial pain in 1961 revealed a grossly dilated gallbladder without gallstones and confirmed that the intrahepatic and extrahepatic biliary systems were patent. Wedge liver biopsy done at the time of cholecystectomy showed cirrhosis with many multinucleated giant-cell transformations and heavy pigment deposits with the staining properties of copper.

The vitamin E status was assessed repeatedly by measuring serum concentrations of the vitamin and by in-vitro tests of red cell haemolysis3 (see table). When seen recently, at the age of 29 years and 11 months, he complained of increasing difficulty with walking. He had discontinued vitamin E therapy and serum concentrations were undetectable.

Case 3
Born in 1961 of parents who were second cousins, this Greek male has had recurrent epistaxes severe enough to require transfusion since the age of 2 years. In May, 1964, hepatosplenomegaly was noted, and a liver biopsy showed inactive cirrhosis. In 1969 a tremor of the right hand developed, and the diagnosis of hereditary ataxia was considered. The liver was palpable 7 cm below the costal margin, and the spleen 5 cm. There was no retinitis pigmentosa, ophthalmoplegia, or nystagmus. The limbs were hypotonic, deep tendon reflexes were absent, and both plantar responses were extensor. Ataxia of the limbs was evident on finger-nose and heel-knee testing. There was bilateral pes cavus, and Romberg's test was positive. The feet had diminished vibration sense. Investigations showed no acanthocytes and a normal blood film. Three experienced neurologists agreed that the clinical picture was that of a hereditary ataxia and not of Wilson's disease. Subsequently his walking deteriorated, his hands became more ataxic, and titubation developed. He walked with a waddling, wide-based gait and an exaggerated lumbar lordosis. The ataxia, which affected all four limbs, became evident not only with intentional movements, but also during maintenance of posture. His abnormal reflexes and depressed vibration sense remained unchanged. A liver biopsy sample had a copper concentration of 424 μg/g dry weight, and
OESTROGEN-RECEPTOR STATUS AND SITES OF METASTASIS IN BREAST CANCER

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Summary.—The oestrogen receptor (RE) status of the primary tumour has been assessed in 466 of a consecutive series of 550 patients with primary operable breast cancer.

All patients were followed up (without treatment) until the development of recurrence or metastases. Distant metastases have so far occurred in 124 patients and 82 have had symptomatic local or regional recurrence.

A significant correlation exists between the RE status of the primary tumour and subsequent patterns of metastasis.

Symptomatic metastases to regional lymph nodes are more common with RE-cancers. There is no significant difference in either time of onset or total incidence of distant metastases between patients with RE+ and RE− tumours. Distribution of distant metastases is influenced by RE status: RE+ tumours tend to recur in bone, RE− tumours show affinity for viscera.

In patients with advanced breast cancer, the anatomic site of distant metastases is an important clinical factor which relates both to response to endocrine therapy (Baum, 1980) and survival (Shimkin et al., 1954; Papaioannou et al., 1967; Cutler et al., 1969). Patients with skeletal metastases have a higher response rate to endocrine therapy (Taylor, 1962) and a more favourable overall prognosis (Shimkin et al., 1954; Papaioannou et al., 1967; Cutler et al., 1969) than those with visceral secondaries.

Factors influencing the distribution of distant metastases in breast cancer remain unclear.

The oestrogen receptor (RE) status of human breast cancer has also been shown to relate to response to endocrine therapy (McGuire et al., 1975; Roberts et al., 1978) and survival (Bishop et al., 1979).

This study is a search for any relationship between RE status of the primary breast tumour and subsequent incidence and distribution of secondary metastases.

PATIENTS AND METHODS

The Nottingham Tenovus series of 550 female patients aged 28-75 years with primary operable breast cancer presented to one surgeon (R.W.B.) between 1973 and 1979. In all cases, tumours were judged clinically to be less than 5 cm in diameter, and patients with distant metastases at the time of presentation were excluded from the study.

A simple or subcutaneous mastectomy was carried out in all cases. The Nottingham staging procedure has been previously described (Maynard et al., 1978) but briefly one lymph node is taken at the time of mastectomy, each from the lower axilla, the apex of the axilla and the second intercostal space. Patients were categorized as Stage A if all nodes are histologically tumour-free, Stage B for low axillary involvement and Stage C for involvement of either apical axillary or internal mammary nodes. All patients are
followed up at 3-monthly intervals to 18 months, and at 6-monthly intervals thereafter. No patient receives any treatment before the development of recurrence.

For the purpose of this study, categories of recurrence are defined thus:

“Spot” recurrence: A small discrete skin metastases which is confirmed histologically.

Local recurrence: Multiple, symptomatic or progressive metastases in mastectomy flaps which are confirmed histologically.

Regional recurrence: Symptomatic metastases in axillary or supraclavicular nodes, which are confirmed histologically.

Distant recurrence: Any distant metastases, confirmed by clinical examination, abnormal liver-function tests, appropriate X-rays, liver or brain scans or biopsy.

Asymptomatic but palpable axillary nodes are not regarded as recurrences unless histological proof is available.

Oestradiol receptor assay.—Oestradiol-receptor status of the primary breast cancer has so far been evaluated in 466 patients. Receptor data were not obtained in 84 patients, either because all the tumour at mastectomy was used for frozen section or paraffin histology or because specimens were lost. Tumour samples taken at mastectomy were frozen and stored in liquid N₂ before being transported on dry ice to the Tenovus Institute, Cardiff, where the assay is performed by the dextran-coated-charcoal method (Maynard et al., 1979).

Tumours are considered to be RE⁺ when they contain >5 fmol specific oestradiol binding per mg cytosol protein.

Seven patients, 4 of whom had co-existent primary tumours of another organ, 2 who were referred elsewhere for follow-up, and one with 2 simultaneous tumours of different RE status, are excluded from the analysis, leaving 459 evaluable patients.

RESULTS

Of the 459 evaluable patients, 264 (58%) have RE⁺ primary breast cancers.

Local recurrence: major local recurrence has so far appeared in 33 patients, while a further 44 have developed single “spot” recurrence in mastectomy flaps. RE status is not significantly related to either major local recurrence (Table 1) or to the total of major local and “spot” skin metastases (Table II).

Regional recurrence: 49 patients have developed symptomatic recurrence in axillary or supraclavicular nodes. The incidence of this complication is significantly greater in patients with RE⁺ cancers (Table III).

Distant recurrence: distant metastases have so far appeared in 124 patients. RE status is related to neither time of onset of distant metastases after mastectomy (Fig. 1) nor to the total incidence of distant metastases (Table IV). The RE status is, however, related to the anatomic site of distant metastases: RE⁺ tumours tend to metastasise initially to skeleton, whilst RE⁻ cancers show affinity for initial distant spread to viscera (lung, liver, intra-abdominal organs and central nervous system) (Table IV). Survival of
patients in whom the first distant metastases appear in bone is significantly longer than that of patients whose initial

distant recurrence develop in viscera (Fig. 2).

The disease stage, as assessed by the degree of lymph-node involvement at the time of mastectomy, which may be related to the total incidence of regional and distant metastases, is not significantly related to RE status (Table V).

**Table V.** Oestrogen receptor status and disease stage at mastectomy

<table>
<thead>
<tr>
<th>Stage*</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>134</td>
<td>116</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>46</td>
</tr>
<tr>
<td>C</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>264</td>
<td>193</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 3.9; \ 2 \text{ d.f.}; \ 0.02 > P > 0.10, \text{N.S.} \]

*For method of staging see text.

**DISCUSSION**

RE status of breast cancer is an important prognostic factor which is related to both tumour-free interval (Maynard et al., 1978) and survival (Bishop et al., 1979). Patients with RE− cancers fare worse on both these counts than those whose tumours are RE+. This study demonstrates that patients with RE− primary tumours are more likely to develop symptomatic recurrence in regional lymph nodes than are those with RE+ primaries (Table III) despite a similar incidence of involved lymph nodes in both groups of patients at mastectomy (Table V). RE− breast cancers tend to be poorly differentiated (Elston et al., 1980) and have a rapid rate of cellular replication (Meyer et al., 1977), and it is possible that these differences of clinical expression as regards tumour-free interval, survival and symptomatic nodal recurrence between RE− and RE+ tumours may be related to a more rapid growth of the latter.

The results of this study also demonstrate a significant relationship between the RE status of the primary breast cancer and sites of distant metastases: RE+ cancers tend to metastasize to bone while RE− tumours are more likely to
recur in viscera. These findings are in agreement with those of Wate et al. (1976) and Stewart et al. (1981) but contrary to those of Hahnel et al. (1979) in whose series sites of secondary metastases were unrelated to RE status.

Mechanisms governing the distribution of metastases in breast cancer to different sites are unclear. This study demonstrates that RE breast cancers favour the bony skeleton as a site of recurrence. It is conceivable that oestrogenic hormones, acting via receptors on the RE+ cancer cells, could play some part in governing the preferential growth of metastases at this site. Possibly the hormone-cell interaction could, by some unknown pathway, alter the environment in bone (metabolic or otherwise) to favour growth of these cancer cells. RE- cancer cells, however, would not be subject to these hormonal influences but, having a more rapid rate of proliferation (Meyer et al., 1977) and possibly being more virulent, would grow at whichever site they happened to come to rest. Exact mechanisms, however, remain uncertain.

Other authorities have reported previously that patients with predominantly bony secondaries survive significantly longer after recurrence than those with visceral metastases (Cutler et al., 1969) and our findings agree with that conclusion (Fig. 2). While survival will obviously be influenced by treatment, no attempt has been made to take this factor into account in this study. It is clear that the longer survival of patients with RE- cancers (Bishop et al., 1979) is related not only to a greater likelihood of response to endocrine therapy (McGuire et al., 1975) but also to their less rapid natural growth rate; it may also be that the distribution of their metastases to less lethal secondary sites plays some part.

REFERENCES


A PROGNOSTIC INDEX IN PRIMARY BREAST CANCER

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From the City Hospital, Nottingham and the †Tenovus Institute for Cancer Research, Cardiff

Received 11 September 1981 Accepted 6 November 1981

Summary.—From a multiple-regression analysis of prognostic factors and survival in a series of 387 patients with primary breast cancer, a prognostic index has been constructed, based on lymph-node stage, tumour size and pathological grade. This index is more discriminating than lymph-node stage alone, and enables a larger group of patients to be identified with a very poor prognosis.

Many studies of prognostic factors in breast cancer have been reported in the literature. In some of these, only one factor has been studied in isolation. In others, more than one factor has been investigated by breaking the data down into subgroups, each having the same combination of factors. If more than 3 factors are being studied the number of possible subgroups becomes large and the numbers of patients in each subgroup diminishes correspondingly (Myers et al., 1966). To overcome this problem some form of multivariate analysis can be used to deal with the simultaneous effect of several factors on prognosis (Myers et al., 1966; Freedman et al., 1979; Alderson et al., 1971; Wallgren et al., 1976), and the multiple regression technique described by Cox (1972) has been used in a number of cancer studies (Wilkinson et al., 1979; Gehen et al., 1976; Palmer et al., 1980; Lanzottie et al., 1977). It can make use of all the data from a group of patients having a wide range of follow-up times, and is a powerful technique which makes no assumptions about the form of the survival curve. It has been used to obtain the results reported below.

In the Nottingham Breast Cancer Study, members of a consecutive series of operable patients have all had a number of prognostic factors recorded and have received the same primary treatment. In 1979 we reported preliminary findings in 228 patients who had been followed up for at least 18 months, and we identified by use of stage, size and grade, a group of patients with a very poor prognosis (Blamey et al., 1979). This paper reports the next stage in our attempts to combine factors into a prognostic index.

PATIENTS AND METHODS

The patients for this study were taken from the first 500 consecutive female patients with primary operable invasive carcinoma of the breast seen and treated, under the care of a single surgeon, by simple mastectomy and triple-node biopsy at the Nottingham City Hospital. The prognostic factors selected for investigation were age, menopausal status (a premenopausal woman being either still menstruating or having a plasma sample containing <50 i.u./l of FSH), tumour size measured in the fresh mastectomy specimen, lymph-node involvement judged by histology, tumour grade, cellular reaction, presence of sinus histiocytosis in lymph nodes, and oestrogen-receptor (RE) content of the primary.

Lymph-node involvement, based on biopsy of a lower axillary node, an apical axillary

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Request for reprints to Professor R. W. Blamey, Department of Surgery, City Hospital, Nottingham.
node and a node from the internal mammary chain, was classified as:

Stage A: Tumour absent from all 3 nodes sampled
Stage B: Tumour in low axillary node only
Stage C: Tumour in apical and/or internal mammary node.

Histological grade (I to III) was determined by a method based on the criteria of Bloom & Richardson (1957). Cellular reaction was scored in 4 categories as described by Black et al. (1953). The absence or presence of sinus histiocytosis in lymph nodes was scored 1 and 2 respectively, whilst cases where all available lymph nodes were replaced by tumour were scored zero. RE content was assayed by the method described by Maynard & Griffiths (1979) and tumours were classified as RE+ if they contained >5 fmol specific oestradiol binding per mg cytosol protein.

A group of 387 patients for each of whom all these factors were recorded has been used for the main analysis. The 113 patients excluded were accounted for by 69 with no RE result, 10 with no cell-reaction score, 11 with non-invasive cancer (i.e. intra-duct or Paget's), and 23 excluded for a variety of reasons such as previous cancer of the breast, operation not being a simple mastectomy, inadequate clinical details and no follow-up at all.

Following the previous work on a prognostic index (Blamey et al., 1979) a decision was made, after the first 250 patients had been entered in the main study, that patients in the poor-prognosis group (Stage C, size >2 cm, Grade II or III) would be given adjuvant chemotherapy. This policy continued until Patient 370 in the main series, but was discontinued thereafter. Fifteen of the 387 patients were given adjuvant chemotherapy during this period, and it was necessary to take this into account in the analysis.

The first patient in the series was treated just over 6 years before the time of analysis, the last patient just over 1 year before. With this length of follow-up available, it was decided to use survival time as a measure of the outcome of treatment.

To assess the relative importance of the prognostic factors, a series of analyses using the method due to Cox (1972) has been carried out. The simplest use of the method, as reported in this paper, assumes a "proportional hazards" model; i.e., that the relative contribution of each factor to the risk of dying remains constant over the period covered. A more detailed analysis (Freedman & Haybittle, in preparation) using time-dependent variables has shown that in this particular set of data there are no significant departures from such a model.

The Cox method is a multiple regression technique which allows each variable to be evaluated independently, taking into account the effects of all other variables. The coefficients (β values) produced by the analysis show how much each factor contributes to the hazard, which is inversely related to survival. A positive value of β therefore indicates a poorer survival time as the given variable increases. Table I shows the coding used for the various prognostic factors in our analysis. Survival curves have been calculated using the life-table method with the time divided into 6-monthly intervals.

**RESULTS**

These are first presented for the analyses made on the group of 387 patients in whom all the factors were recorded. One patient who died from a road traffic accident without recurrence 4 months after treat-

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Codes used in Cox analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>In years</td>
</tr>
<tr>
<td>Menopausal state</td>
<td>0 = premenopausal</td>
</tr>
<tr>
<td></td>
<td>1 = postmenopausal</td>
</tr>
<tr>
<td>Size</td>
<td>In cm</td>
</tr>
<tr>
<td></td>
<td>1 = A</td>
</tr>
<tr>
<td></td>
<td>2 = B</td>
</tr>
<tr>
<td></td>
<td>3 = C</td>
</tr>
<tr>
<td>Tumour grade</td>
<td>1 = I</td>
</tr>
<tr>
<td></td>
<td>2 = II</td>
</tr>
<tr>
<td></td>
<td>3 = III</td>
</tr>
<tr>
<td>Cellular reaction</td>
<td>1 = marked</td>
</tr>
<tr>
<td></td>
<td>2 = moderate</td>
</tr>
<tr>
<td></td>
<td>3 = slight</td>
</tr>
<tr>
<td></td>
<td>4 = none</td>
</tr>
<tr>
<td>Sinus histiocytosis</td>
<td>0 = nodes completely</td>
</tr>
<tr>
<td></td>
<td>replaced by tumour</td>
</tr>
<tr>
<td></td>
<td>1 = absent</td>
</tr>
<tr>
<td></td>
<td>2 = present</td>
</tr>
<tr>
<td>Oestrogen receptor (RE)</td>
<td>0 = negative</td>
</tr>
<tr>
<td></td>
<td>1 = positive</td>
</tr>
<tr>
<td>Adjuvant therapy</td>
<td>0 = none</td>
</tr>
<tr>
<td></td>
<td>1 = therapy given</td>
</tr>
</tbody>
</table>
ment was counted as a withdrawal at the time of death. All other deaths were included.

Table II shows the \( \beta \) values obtained when all factors were included in the Cox analysis. The last column gives the \( Z \) values, which are the ratios of the absolute values of the \( \beta \)s to their standard errors. If \( Z > 1.96 \), \( \beta \) is significantly different from zero at the 5% level in a two-tailed test. Stage, size, and grade all fall within this category. The other coefficients are not significantly different from zero. This should not be taken to mean that these factors have no effect on prognosis, but only that any effect is too small to be shown up at the 5% level of significance with the number of patients in this study.

Adjuvant chemotherapy did not have a significant effect, though it tended to reduce the hazard, as shown by the negative coefficient in Table II. Since this was applied to a highly selected group (patients in Stage C with tumours > 2 cm, and in Grades II and III) its beneficial effect, if any, in the series will be confined to those patients, and tend to reduce the gradation of prognosis with size, stage and grade. This was borne out by an analysis with adjuvant therapy excluded, which produced slightly lower coefficients: 0.169, 0.723 and 0.805 for size, stage and tumour grade respectively. For the formation of a prognostic index (see below) it will therefore be preferable to use the coefficients obtained from the analysis when adjuvant therapy was included (viz. those in Table II).

### A prognostic index

The coefficients produced by the Cox analysis can be used to derive a prognostic index for each patient (Palmer et al., 1980). Only the 3 prognostic factors found to be significant in Table II have been used, and their coefficients reduced to 2 significant figures. The index \( I \) for each patient is then:

\[
I = (0.17 \times \text{size}) + (0.76 \times \text{lymph-node stage}) + (0.82 \times \text{tumour grade}).
\]

The larger the value of \( I \), the worse the prognosis for that patient.

We have investigated the application of this index in a subset of the data which excluded the period during which poor-prognosis patients were treated with adjuvant therapy. 298 cases, in which all factors were recorded, were available from Patients 1-250 and 371-500 in the main series, and the results presented below apply to survival in this group. The index was computed for each patient, and the patients then arranged in order of decreasing values of \( I \).
We have first compared the performance of the index with that of lymph-node stage (the most significant single factor) alone. The patient group consisted of 154 Stage A, 95 Stage B and 49 Stage C patients. Fig. 1 shows the survival curves for these subgroups (dashed lines) together with those for subgroups containing the same numbers of patients but selected according to their I value (viz. the 154 with the lowest values, the 49 with the highest values and the 95 in between). It is evident that I gives a better discrimination. The 49 patients with the highest I value do worse than the 49 Stage C patients, and the separation between the best and the worst prognostic groups is greater.

Our second comparison was with our earlier criteria for poor prognosis, namely Stage C, size >2 cm, Grades II or III. Twenty-five patients in the group satisfied these criteria, and their survival is compared in Fig. 2 with those of the 65 patients with the highest I values. The 2 curves are almost identical, and the new index was thus able to identify a larger group of poor-prognosis patients.

It was also of interest to look at a group of 64 patients with the lowest I values. Their survival is also shown in Fig. 2, and compared with the expected survival in a normal population of the same age distribution. It can be seen that patients with I values <2.8 constituted a very good prognosis group.

Lastly we have examined the performance of the new index as a predictor of 5-year survival. 137 patients were treated at least 5 years before the assessment date, and their status at 5 years in the

![Fig. 2. Survival of 64 patients with indices of 2.8 or less compared with the survival of an age-matched population free of breast cancer (-----). Survival of 65 patients with index >4.4, compared with the survival of 25 patients identified as having a poor prognosis by a previous index (-----). Also shown is the survival of 109 patients with intermediate indices (2.8-4.4).](image)

3 index ranges used for the graphs of Fig. 2 are given in Table III.

**DISCUSSION**

The index derived has selected out 2 groups of patients; 1 with an exceedingly poor prognosis, the other with an apparently very good prognosis. If the index is used to predict 5-year disease-free survival. Table III shows that, of the 51 patients in these two groups, 44 (86%) have been correctly assigned according to the index. Nearly two-thirds of the patients in Table III have intermediate I values. It may be found after longer follow-up that a further subdivision of this group will be valuable in predicting disease-free survival at 10 years.

Some measure of the extra contribution of grade and stage to prognostic prediction

---

**Table III.**—Performance of Index in 387 patients followed up for at least 5 years

<table>
<thead>
<tr>
<th>Index value</th>
<th>Alive and recurrence-free (%</th>
<th>Alive with recurrence (%)</th>
<th>Dead (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (&gt;4.4)</td>
<td>2 (7)</td>
<td>1 (3)</td>
<td>26 (90)</td>
<td>29</td>
</tr>
<tr>
<td>Medium (2.8-4.4)</td>
<td>46 (54)</td>
<td>7 (8)</td>
<td>33 (38)</td>
<td>86</td>
</tr>
<tr>
<td>Low (&lt;2.8)</td>
<td>17 (77)</td>
<td>2 (9)</td>
<td>3 (14)</td>
<td>22</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>137</strong></td>
</tr>
</tbody>
</table>
over stage alone can be obtained from the log likelihood values produced by the Cox analysis. These give an indication of how well the model predicts the actual survival, the greater the log likelihood the better being the model's performance. The increase in log likelihood when another prognostic factor is included shows how much extra that factor is contributing, and can be tested for statistical significance by comparing twice the increase with the $\chi^2$ distribution for one degree of freedom. The inclusion of grade in addition to stage alone increases the log likelihood by 18-8 ($P<0-0005$). The further inclusion of size increases the log likelihood by 3-7 ($P<0-01$).

None of the other factors, when included in the model, significantly increases the log likelihood. When age, menopausal status, sinus histiocytosis and RE content (for all of which the $Z$ values in Table II were non-significant but greater than 1.0), were incorporated in the index, the changes from the results shown in Figs 1 & 2 and Table III were only marginal.

In the past we have demonstrated RE content to be a significant prognostic factor in Stage B and C patients (Bishop et al., 1979; Blamey et al., 1980). RE is not a significant factor in the current analysis because it is strongly correlated with tumour grade (Maynard et al., 1978; Elston et al., 1980). When the Cox analysis was repeated with tumour grade excluded, the coefficient for RE content was $-0-523$ and had a significant $Z$ of 2-47. Thus, in the absence of reliable histopathological assessment of tumour grade, RE would give useful prognostic information and could be used to build an index:

$$I = (0.18 \times \text{size}) + (0.68 \times \text{stage}) - (0.52 \times \text{RE})$$

where RE is coded as in Table I.

The day-to-day use of our size, stage and grade index may be cumbersome because of the calculation involved. The factors for stage and grade are similar and, if these are both made equal to unity and the multiplying factor for size scaled up accordingly, we can arrive at a simpler index of the form:

$$I = 0.2 \times \text{size} + \text{stage} + \text{grade}.$$  

This simpler index gives very similar results to those obtained with the more complex formula. For example, the curves in Fig. 2 are reproduced almost exactly if the divisions are made at index values of 3-4 and 5-4 instead of at 2-8 and 4-4.

The values of the coefficients found in our analysis are such that they obtain the best discrimination on the particular set of data from which they are derived, since they give the best fit of the model to the data. The performance of the index might be different on another set of data, and we therefore plan to study its effectiveness in the patients admitted to the Nottingham Breast Cancer Study from Patient 501 onwards.

The estimation of prognosis in the individual is clearly important for determining her treatment and follow-up, for example in making decisions regarding adjuvant chemotherapy. It is equally important at the present time in the evaluation of therapies by controlled trials, where proper stratification of patients might be greatly improved by the application of indices based on a number of significant factors, each given their appropriate weight.

REFERENCES


Studies with Steroid–Fluorescein Conjugates on Oestrogen Target Tissues

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Abstract—Steroid–fluorescein amine and steroid–BSA–fluorescein–isothiocyanate conjugates have been prepared and their ability to bind to oestrogen receptors assessed in competitive binding studies. The binding of all the fluorescent conjugates to uterine cytosol proteins was low when compared with either oestradiol or diethylstilbestrol. A comparative study was carried out to assess the relationship between oestrogen receptor content, determined biochemically, and histochemical localisation of the oestrogen binding components on thin sections of rat uteri, DMBA-induced mammary tumours and also human breast tumour tissue taken at mastectomy. The data indicate that in thin sections of tissue all of these conjugates appear to bind not to the classical oestrogen receptor moiety but to other oestrogen binding proteins.

INTRODUCTION

Numerous studies have been reported relating to the potential value of oestrogen–fluorescein conjugates to assess the oestrogen receptor content of tissue sections [1–4]. It has been envisaged, for example, that the use of these compounds may eventually complement, or even replace, the standard biochemical assay for the determination of oestrogen receptor status of breast tumours as a means of selecting patients for appropriate forms of therapy. Potentially the histochemical utilisation of oestrogen–fluorescein conjugates has two obvious advantages over the currently available biochemical assay involving the association of radiolabelled ligands to tumour cytosol preparations of the receptor protein in vitro. Firstly, since there is no a priori reason to believe that all cells of ER-positive breast tumours should contain oestrogen receptor protein, a histochemical assay would provide an assessment of the proportion of cells lacking receptors to be made. Secondly, when receptors were present, their intracellular location could be directly identified. Such information might prove not only a useful prognostic parameter, but might also provide valuable information relating to a better understanding of the endocrinology of tumour growth and development.

A wide spectrum of these conjugates have been assessed, including oestradiol–fluorescein amine [5], oestradiol/oestrone linked at varying positions and through different bridging groups to fluorescein [3, 6] and oestradiol linked to bovine serum albumin–fluorescein isothiocyanate (BSA–FITC). The structures of these various conjugates are illustrated in Fig. 1. Despite the apparent successful application of these conjugates [7, 8], their efficacy in ‘localising’ oestrogen receptors has been the subject of some controversy [9].

The present study, initiated three years ago, provides the experience from this laboratory on this subject and deals with some of the more controversial factors associated with the use of these fluorescent compounds. Special emphasis has been given to their specificity, purity and relative binding affinities (RBA) for oestrogen receptor preparations. Similarly, the histochemical data obtained after the treatment of oestrogen target tissues with both oestradiol-17β and the non-steroidal antioestrogen, tamoxifen, have been compared with the cellular location of oestrogen receptor proteins as determined using standard [³H]oestradiol exchange assays. The information gained from these studies has also been applied to the in-
terpretation of the binding of fluorescent conjugates to a series of oestrogen receptor-positive and negative human breast tumours.

MATERIALS AND METHODS

Materials
All reagents and organic solvents were analytical grade with the exception of dioxan, dimethylformamide and tributylamine, which were distilled and stored over molecular sieves (4A) until required. Fluorescein isothiocyanate (FITC), fluorescein amine (Isomer I) and hexamethylenediamine (HMD) were purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset. Bovine serum albumin (BSA, Cohn Fraction V), oestradiol-3-17β-diacetate, oestradiol-17β-hemisuccinate and 11α-hydroxyprogesterone were all obtained from Sigma Chemical Corp. Ltd., Poole, Dorset; all other steroid derivatives, unless stated otherwise, were purchased from Steraloids Inc., Wilton, NH. Isobutyl chloroformate was purchased from Eastman-Kodak Ltd., Kirkby, Liverpool, and Sephadex G-25 (fine) and G200 from Pharmacia Ltd., Hounslow, Middlesex. Thin-layer (TLC) precoated silica chromatography plates (20 × 20 cm) were obtained from Camlab Ltd., Cambridge. Preparative TLC plates were prepared using Kieselgel HF254+346 (E. Merck, Darmstadt, W. Germany) and a Corning platemaker.

Animals
Mature virgin female Sprague-Dawley rats, bred in the Tenovus Institute animal unit, were used throughout the study. The animals were housed in a 12 hr light/12 hr dark environment and were fed food and water ad libitum. Two types of tissue were used: (a) uteri from 7-day ovariectomised animals to which were administered i.v. either (i) saline vehicle (100 µl), (ii) oestradiol-17β (5 µg) 2 hr prior to sacrifice or (iii) tamoxifen (300 µg) 16 hr prior to sacrifice; (b) dimethylbenzanthracene (DMBA)-induced mammary tumours from intact animals treated as in (a). Immediately after sacrifice, a representative portion of either mammary tumour tissue or uterus was taken for histochemistry. The remaining tissue was separated into cytosol and nuclear fractions [10] and assayed for oestrogen receptor content. The methods used in the determination of total and accessible oestrogen receptor sites together
with competitive binding procedures have been described previously [11].

Conjugate preparation

(a) Steroid–BSA–FITC conjugates. (i) Oestradiol-17β–BSA–FITC. Oestradiol-17β–BSA–FITC was prepared by a modification of Erlanger’s mixed anhydride procedure [12] developed by Gaetjens and Pertschuk [13]. This method produced low molar incorporation ratios of steroid: BSA. Briefly, oestradiol-17β-hemisuccinate (60 μmol) was dissolved at 11°C in anhydrous dioxan (1000 μl), tributylamine (60 μmol) and isobutyl chloroformate (60 μmol) and added to BSA (3.9 μmol) in 50% (w/v) aqueous dioxan (32 ml). The steroid–protein conjugate was then dialysed against 10mM ammonium bicarbonate and freeze-dried. When required, aliquots of the conjugate (50 mg) were dissolved in carbonate buffer (0.05 M, pH 9.5) at 4°C, FITC (2 mg) added and the mixture allowed to stand at 4°C for 7 hr. The resulting conjugate was dialysed extensively against ammonium bicarbonate and then purified by gel-exclusion chromatography on Sephadex G-25. Fractions containing the steroid–BSA–FITC conjugate were pooled, freeze-dried and stored at 4°C until required. The incorporation of steroid and FITC into the BSA was determined by spectrophotometry, as described by Gaetjens and Pertschuk [13]. The ratio of steroid to protein was 3.5:1 whilst the incorporation of FITC was 2.9:1. A derivative to progesterone-11α-hemisuccinate was prepared in the same manner.

(ii) Oestradiol-17β-6-(O-carboxymethyl)-oxime–BSA–FITC. Oestradiol-6-(O-carboxymethyl)-oxime was prepared by the method of Lee [2]. Oestradiol-3-17β-diacetate was oxidised in glacial acetic acid with chromium trioxide and the 6-keto derivative saponified in methanolic potassium hydroxide and coupled to aminoxyacetic acid hemihydrochloride to produce the 6-(O-carboxymethyl)-oxime derivative. The oxime was then coupled to BSA and FITC as described above. The incorporation of both oestradiol-6-(O-carboxymethyl)-oxime and FITC were determined as described above. The ratio of steroid: protein and FITC: protein were 9.4:1 and 3.5:1 respectively.

(b) Steroid–fluorescein conjugates. Fluorescent-labelled derivatives of oestradiol-17β-hemisuccinate, oestradiol-6-(O-carboxymethyl)-oxime, ethynyl oestradiol-6-(O-carboxymethyl)-oxime, testosterone-11α-hemisuccinate, testosterone-17-hemisuccinate, 5α-dihydrotestosterone-17-hemisuccinate, 5α-dihydrotestosterone-1-carboxyethylthioether and 11α-hydroxy progesterone-11-hemisuccinate were prepared using a modification of the method described by Dandliker et al. [14]. Briefly, aliquots of steroid derivative (100 μl, 4 mg) in dry dimethylformamide were mixed with fluorescein amine (80 μl, 50 mg/ml), dicyclohexylcarbodiimide (40 μl, 100 mg/ml) and acidified acetone (1 ml) and maintained at 4°C for 48 hr. The products were purified [4] by preparative TLC using the solvent, chloroform: ethanol: water (54:12:1 v/v), eluted and stored in ethanol at 4°C until required.

(c) Steroid–hexamethylene–fluorescein. Oestradiol-17β-hemisuccinate was linked to FITC, incorporating a hexamethylenediamine ‘bridging’ group at position C-17 by a modification of the method of Daxenbichler et al. [5]. Briefly, a 50-molar excess of hexamethylenediamine (560 mg) was reacted with FITC (40 mg) in dimethylformamide (15 ml) at 0°C. The supernatant was decanted and the precipitate washed with dimethylformamide (10 ml), dissolved in water (10 ml) and acidified to pH 5.5 with dilute hydrochloric acid. The precipitate (13.5 mg) was then linked to oestradiol-17-hemisuccinate (6.6 mg) by the mixed anhydride procedure of Erlanger et al. [12]. The final product was obtained after addition of water (5 ml) and dilute hydrochloric acid (to pH 5.5) to the reaction mixture, producing a precipitate which was extracted with ethyl acetate (2×5 ml) and then lyophilised. The product was re-dissolved in methanol and purified by TLC as described by Daxenbichler et al. [5].

(d) Steroid–hydrazone–fluorescein. Oestrone-17β–FITC [4, Fig. 1] was prepared by the method of Dandliker et al. [6].

(e) Additional purification of conjugates. ‘Free’ steroid in the BSA conjugates was removed by treatment with dextran-coated charcoal (4°C, 20 min). Non-steroidal impurities were removed by re-purifying the conjugates using gel-exclusion chromatography on Sephadex G200–120 (55 × 1.5 cm column). The effect of these additional purification steps on the characteristics of the BSA conjugates was determined in the cytosol assay and histochemical procedure.

Histochemical procedures and fluorescence assessment

All tissues were routinely mounted in Tissue-Tek II O.C.T. Mountant (Miles Labs. Inc., IL), frozen on solid carbon dioxide and stored at −70°C until required. Sections (4 μm) were cut onto subbed [16] slides using a cryostat at ap-
approximately −25°C. The frozen sections were processed by two methods.

(1) Conjugates were prepared in PBS (0.01 M, pH 7.4) containing 10% ethanol (v/v) and incubated (2 h, 25°C) with the tissue sections in a humidity chamber. After incubation the sections were rinsed thoroughly in PBS at 4°C, mounted in buffer and examined for fluorescence (λ = 490 nm) on a Leitz Orthoplan microscope (Leitz Instruments, W. Germany) fitted with a mercury lamp and a Pleomopak incident beam fluorescence unit.

(2) Conjugate solutions were prepared in Earle’s balanced salt medium [17] and incubated (30 min, 25°C) with the tissue sections in a humidity chamber. After rinsing thoroughly in buffer (4°C) the sections were mounted and examined for fluorescence.

Transparencies were taken with a Leitz Orthomat-W camera attachment on 160 ASA tungsten Ektachrome film uprated to 320 ASA using an automatic exposure system.

Fluorescence on the tissue sections was assessed 'semi-quantitatively' on a scale of intensity from negative to positive (+ to 5+) at a specific magnification of ×440. Specificity of fluorescence was determined by incubating in the presence and absence of various competitors (50-fold molar excess), including diethylstilboestrol (DES), tamoxifen, certain naturally occurring steroids and also in the presence of a number of 'non-specific' controls, comprising N-acetyl-fluorescein amine, BSA-FITC (without steroid attached), steroid BSA conjugates (without FITC) and buffer alone.

Competitive binding studies

A selection of oestrogen conjugates, listed in Table 1, were incubated as competitors to [3H]-

Table 1. Relative binding affinities (RBA) of various oestrogen–fluorescein conjugates in the rat uterine cytosol assay for oestrogen receptor

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Relative binding affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol-17β</td>
<td>100</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>94</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>5.0</td>
</tr>
<tr>
<td>Oestrone-17-FITC</td>
<td>2.8</td>
</tr>
<tr>
<td>Oestradiol-17-FA</td>
<td>1.4</td>
</tr>
<tr>
<td>Ethynyl oestradiol-6-FA</td>
<td>0.6</td>
</tr>
<tr>
<td>Oestradiol-17-HMD-FA</td>
<td>0.6</td>
</tr>
<tr>
<td>Oestradiol-6-FA</td>
<td>0.2</td>
</tr>
<tr>
<td>Oestradiol-6-BSA-FITC</td>
<td>0.01</td>
</tr>
<tr>
<td>Oestradiol-17-BSA-FITC</td>
<td>0.01</td>
</tr>
</tbody>
</table>

FA, Fluorescein amine; HMD, hexamethylenediamine; BSA-FITC, bovine serum albumin–fluorescein isothiocyanate.

RESULTS

Competitive binding studies

Table 1 provides data on the relative binding affinity of the various fluorescein-labelled oestrogens for the cytoplasmic oestrogen receptors from rat uterus. Although all conjugates tested displayed some degree of competition with [3H]-oestradiol for the receptor protein, their binding affinity, however, was low in comparison with either unlabelled oestradiol or DES. This was especially evident with those fluorescein conjugates linked to oestradiol via BSA molecules.

Introduction of a hexamethylenediamine bridging group at C-17 of oestradiol, a modification designed to increase the distance between fluorescein and the oestradiol molecule, reduced the affinity of the conjugate for the receptor protein. Ethynyl oestradiol-6-FA bound to the oestrogen receptor with approximately three times greater affinity than oestradiol-6-FA. Of the conjugates with fluorescein directly linked to the oestrogen molecule, oestrone and oestradiol-17-FA showed the highest affinity for the receptor protein, with BSA’s of 2.8 and 1.4% respectively.

Table 2. Relative binding affinities of various androgen and progesterone–fluorescein conjugates in the oestrogen cytosol receptor assay

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Relative binding affinity(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol-17β</td>
<td>100</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>94</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>5</td>
</tr>
<tr>
<td>Testosterone-11α-FA</td>
<td>0.01</td>
</tr>
<tr>
<td>Testosterone-17-FA</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone-17-FA</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone-1-CET-FA</td>
<td>0.01</td>
</tr>
<tr>
<td>Progesterone-11α-FA</td>
<td>0.001</td>
</tr>
<tr>
<td>Progesterone-11α-BSA-FITC</td>
<td>0.001</td>
</tr>
</tbody>
</table>
studies with Steroid–Fluorescein Conjugates on Oestrogen Target Tissues

Table 3. The effect of dextran-coated charcoal treatment on the RBA of oestradiol and progesterone–BSA–FITC conjugates

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Relative binding affinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−DCC</td>
</tr>
<tr>
<td>Oestradiol-6-BSA–FITC</td>
<td>0.01</td>
</tr>
<tr>
<td>Oestradiol-17-BSA–FITC</td>
<td>0.01</td>
</tr>
<tr>
<td>Progesterone-11α-BSA–FITC</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

tively (Table 1). The affinity of molecules in which fluorescein had been conjugated to testosterone, 5α-dihydrotestosterone or progesterone was low compared to oestradiol (Table 2), as might be expected from the known specificity of this receptor protein [18, 19]. Pretreatment of the BSA conjugates with dextran-coated charcoal immediately prior to their use in the competitive binding assays decreased the apparent affinity of the receptor for the conjugates (Table 3), suggesting the presence of noncovalently bound steroid. Re-purification of both the steroid–BSA–FITC and steroid–fluorescein amine conjugates by gel exclusion chromatography and thin-layer chromatography respectively had no appreciable effect on their affinities with respect to the receptor protein or upon the pattern of fluorescence observed in the histochemical procedure.

Cellular localisation of oestrogen receptors and steroid–fluorescein binding components

Characteristically, when assayed in vitro, preparations of uteri removed from non-treated intact rats contained high levels of cytoplasmic oestrogen receptor protein and low concentrations of nuclear oestrogen binding sites (Table 4). When thin sections of the same samples of tissue used in the biochemical assay were incubated with the oestrogen–fluorescein conjugates listed in Table 1, only a predominant cytoplasmic fluorescence was evident, primarily localised in epithelial elements (both glandular and luminal) (Fig. 2a, b). The fluorescence was only partially displaceable by a 50-fold molar excess of DES. This particular fluorescent pattern was achieved with 0.5–2 μM concentrations of conjugates directly linked to oestradiol or oestrone and 10–25 μM for those linked via BSA or HRP. These patterns were also observed with tissue from rat mammary tumours, with fluorescence being mainly localised in the cytoplasm of epithelial cells. Of interest was that incubation of thin sections of either rat uteri or DMBA-induced mammary tumours with fluorescein-labelled androgens or progesterone also showed this pattern of cytoplasmic binding.

Treatment of experimental animals with tamoxifen 16 hr prior to removal of mammary tumours or uteri resulted in a shift in the cellular distribution of oestrogen binding components from the cytoplasm to the nucleus, a change that was monitored by the biochemical assay (Table 4). Incubation of these same tissues with the oestrogen–fluorescein conjugates showed a differential response. Only those conjugates linked through BSA produced nuclear fluorescence (Fig. 3a, b), the remainder binding preferentially to cytoplasmic components. Furthermore, increasing the concentration of the conjugates 4-fold failed to produce any appreciable nuclear binding, merely enhancing the background and cytoplasmic staining. Similar, although often less intense, patterns of fluorescein binding were observed in tissue from animals pre-treated with oestradiol. Interestingly, incubation of thin sections of rat uteri from animals treated with either tamoxifen or oestradiol with progesterone linked to fluorescein via BSA also showed nuclear fluorescence similar to that observed with the oestrogen–BSA–fluorescein conjugates. Inclusion of dithiothreitol in the incubation mixtures markedly decreased the fluorescein binding to all sections.

The results obtained using Earle's balanced salt medium were strictly comparable with those observed with the PBS, although with the

Table 4. Cellular distribution of oestrogen receptors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytoplasm (fmol/uterus)</th>
<th>Nucleus (fmol/uterus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>815 ± 133</td>
<td>205 ± 56</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>233 ± 41</td>
<td>774 ± 73</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>329 ± 43</td>
<td>532 ± 49</td>
</tr>
</tbody>
</table>
latter, noticeably less fluorescence was present in the stromal elements and in necrotic tissue.

Incubation of thin sections of a series of human primary breast tumours with either oestradiol-BSA-FITC or oestradiol-fluorescein showed only cytoplasmic binding, and no nuclear fluorescence was evident in the tumours examined (Figs. 4–7). The distribution of binding components was also more variable than observed in either rat mammary tumour or rat uterus, and no real correlation was evident between oestrogen receptor values determined from the biochemical assay and the fluorescent patterns in thin sections. The data obtained using the BSA conjugate indicated a 38% correlation (n = 26), whereas with the oestrone-17-FITC and oestradiol-17-FA conjugates the correlations were 65 (n = 20) and 29% (n = 14) respectively with respect to the cytosol receptor assay. These data clearly indicate a lack of correlation between the cytoplasmic oestrogen receptor assay and this fluorescence localisation method. It was noted, however, that taking a separate piece of tissue for receptor analysis than that used for histochemical localisation occasionally gave anomalous results, the two pieces being assessed as one receptor-positive and the other receptor-negative. Processing the same piece of tissue through the receptor assay after cutting sections for the histochemical procedure eliminated these anomalies and improved the correlation by between 2 and 4%, a relatively small change, leaving the overall conclusion unaltered.

**DISCUSSION**

The potential value of fluorescein-labelled oestrogens as agents for assessing the oestrogen receptor status of human breast tumour tissue has received much attention in recent research literature [3, 7, 9]. Invariably it has been stated that such conjugates bind to oestrogen proteins [7, 8] and translocate these proteins from the cytoplasm to the nucleus of oestrogen target cells [4]. Significant correlations have been reported between receptor content and fluorescein-labelled oestrogen binding to thin sections of breast tumours [7, 8], the inference being that these derivatives localise oestrogen receptors. The present study, however, casts some doubt on the generality of these conclusions. Whilst all of these fluorescein-linked compounds apparently bind to the cytoplasmic oestrogen receptor protein in competitive binding studies in vitro, only the BSA conjugates bind to the nuclei of tissue sections associated with receptor translocation by tamoxifen and oestradiol, as demonstrated biochemically. Similarly, this nuclear binding was also localised by progesterone-11α-BSA-FITC, but was inhibited by the reducing agent dithiothreitol, a compound normally used to 'activate' oestrogen receptor proteins in standard biochemical assays [20].

These data, together with the ability of oestradiol-17-FA and oestrone-17-FITC to bind within the cytoplasm, under conditions whereby oestrogen receptors have been largely translocated to the nuclei of both rat mammary tumour and rat uterine tissues, implies that fluorescein-labelled oestrogens do not specifically localise oestrogen receptors. It now seems likely that these ligands bind differentially to other cellular proteins similar to the type II oestrogen binding proteins reported by Eriksson et al. [21]. Type II binding sites have been reported to be present in cytoplasmic and nuclear fractions, with the nuclear form being stimulated by oestradiol in rat uterine luminal epithelial cells [22]. Moreover, these proteins, unlike oestrogen receptors, are inhibited by thiol reagents such as dithiothreitol and have a high capacity for oestrogens.

Treatment of the steroid–BSA–FITC conjugates with a suspension of charcoal, whilst markedly reducing the relative binding affinities and suggesting the presence of non-covalently bound steroid, did not affect either the intensity or pattern of fluorescence produced in the tissues from rats treated with tamoxifen or in sections of human breast tumours.

The relatively poor correlation observed between oestrogen receptor status and the histochemical localisation of fluorescein-labelled oestrogen binding components in sections of both rat and human tissue samples, coupled with the very low relative binding affinities observed with these conjugates, emphasises the need to exercise extreme caution when trying to assess 'receptor status' with this technique. A real evaluation of the clinical usefulness of such assays may well have to await the preparation of a conjugate with a reasonably high RBA. At present, any critical assessment of the value of such available fluorescent conjugates should be made in relation to not only the biochemical receptor analysis, but also to the clinical status of patients with breast cancer and the relationship between the histochemical pattern and the subsequent course of the disease.
Fig. 2a, b. Cytoplasmic localisation of oestrogen binding components in sections of rat uteri from control animals using the oestradiol-17-FA conjugate. (magnification ×440).

Fig. 3a, b. Nuclear localisation of oestrogen binding components in sections of rat uteri from animals treated with tamoxifen using the oestradiol-17-BSA-FITC conjugate (magnification ×440).
Figs. 4–7. Cytoplasmic localisation of oestrogen binding components in sections taken from human breast tumours (magnification ×440) using the oestradiol-17-FA (Figs. 4 and 5) conjugate and oestradiol-17-BSA–FITC conjugate (Figs. 6 and 7).
REFERENCES


Short Communication

Tumour epithelial cellularity and quantitative oestrogen receptor values in primary breast cancer


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The clinical value of oestrogen receptor (RE) determinations in breast cancer has been established (McGuire et al., 1975; Maynard et al., 1978) and a recent study has demonstrated the further importance of quantitative measurements (Campbell et al., 1981). However, errors in measurement of receptor value may occur, as shown by inter- and intra-laboratory variation in values with samples of the same tumour (Raam et al., 1981). The inaccuracies of the conventional assay procedure have been emphasised (Poulson, 1981) yet a recent study has shown that much observed intra- and inter-laboratory variation could be attributable to factors other than assay conditions, such as heterogeneity of tumour samples (King, 1980).

Although the prognostic significance of RE almost certainly relates to the tumour cell fraction rather than the stromal component, receptor measurements are performed on homogenate or cytosol prepared from whole tumour. This is a further source of potential error but little information is available concerning the relationship between measured RE concentration and epithelial tumour cellularity, since previous studies have used subjective methods only (Feherty et al., 1971; Terenius et al., 1974; Rosen et al., 1975; Masters et al., 1978). The present study investigates the association between RE concentration (expressed in ng cytosol protein) and tumour epithelial cellularity (obtained by an objective histometric method of cell counting).

Oestrogen receptor assays and cellularity determinations were performed on primary breast cancers of 100 consecutive patients who presented to one surgeon (Prof. R.W. Blamey) between 1978 and 1979. Tumour samples were taken at mastectomy and all surplus adipose tissue trimmed off. Specimens were frozen and stored in liquid N₂ at −196°C before being transported on dry ice to the Tenovus Institute, Cardiff where RE assays were performed by the Dextran-coated charcoal method (Maynard et al., 1978). Tumours were considered to be RE-positive when they contained >5 fmol specific oestradiol binding per mg cytosol protein.

Adjacent tumour blocks were taken and fixed in 10% buffered formalin and paraffin sections were cut and stained with haematoxylin and eosin. Tumour epithelial cellularity was assessed by examination of multiple sections of 4–6 μm thickness on a microscope incorporating an eyepiece graticule. The graticule had an array of 25 randomly arranged points which appeared superimposed upon the field under examination (Figure 1). If N points fall upon tumour cells and M fall upon non-malignant tissue then the ratio N/(N + M) is representative of the surface area proportion occupied by tumour cells in each field. Using a magnification of 63 x, fields were counted systematically, starting with the graticule at the top left corner of the tumour edge, then moving one full field horizontally to the right and the process repeated. At the periphery of the tumours the fields examined deliberately just overlapped into adjacent non-tumour tissue in order to provide a comparable sample to that used for RE measurement. A mean of 87 fields was evaluated in 2–5 sections for each tumour (range 21–200 fields depending on tumour size). The total N/(N + M) is representative of the volume proportion of tumour cells (Delesse, 1848; Dunnill, 1968). The total ratio was expressed as a percentage and designated the tumour epithelial cellularity. A reproducibility study was performed by re-examining a random sample of 1 in 10 cases, the counting process being repeated in a vertical direction. All microscopic cell counts were performed without knowledge of the RE value for any tumour.

Oestrogen receptor value in RE-positive tumours had a lognormal distribution. Pearson's test of correlation was used and the correlation coefficient (r) was utilized for calculation of the degree of interdependence of the variables. When both receptor positive and negative tumours were considered in combination, RE measurements had a non-parametric distribution, and Kendall's rank test of correlation was used.
Sixty of the 100 patients were postmenopausal and 62 had RE-positive tumours (Table I). Measured cellularity ranged from 10%–92% (mean, 40%) (Figure 2). The reproducibility study showed a mean variation of 2.5%. Mean cellularity was 41% in RE-positive tumours and 39% in RE-negative tumours.

Receptor concentrations in RE-positive tumours ranged from 16.4–979 fmol mg⁻¹ cytosol protein. No relationship between cellularity and RE concentration was seen in the tumours of premenopausal patients, nor in the group overall. However, a significant association was observed between tumour epithelial cellularity and RE concentration in the tumours of postmenopausal women (Tau = 0.219; P < 0.05), and this relationship was particularly strong if receptor-positive tumours only were considered (Pearson r = 0.423; P < 0.01, (Figure 3)). In the Pearson test of correlation the square of the coefficient of correlation, r, equals the variance (r² = 0.185). Thus, 18.5% of the range of measured receptor concentration is due to variation in tumour epithelial cellularity.

Table I Relationship between menopausal status of patients and oestrogen receptor status of their tumours

<table>
<thead>
<tr>
<th>Menopausal status</th>
<th>Oestrogen receptor status of tumour</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-</td>
<td>21</td>
<td>19</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Post-</td>
<td>41</td>
<td>19</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>38</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Previous studies have used subjective methods to investigate the relationship between tumour cellularity and RE concentration in breast cancer. By dividing cellularity into 3 categories, high, moderate or low, some authors reported a correlation between the 2 variables (Terenius et al., 1974; Masters et al., 1978) but others could not confirm this (Feherty et al., 1971; Wittliff et al., 1971). The present study provides evidence by a reproducible and objective method that such a relationship exists; approximately one fifth of the

Figure 1 Section of a highly cellular tumour with the graticule superimposed. Note that 22/25 points fall on tumour.
measured range of RE in tumours of postmenopausal women is due to a variance of cellularity.

No relationship exists, however, between RE concentration and cellularity in tumours of premenopausal women. The range of measured receptor values is generally lower in premenopausal patients, due to high circulating levels of plasma hormone which occupy receptor sites, making them unavailable for assay (Saez et al., 1978). It is possible that this factor could conceal a relationship between cellularity and total receptor concentration.

The importance of quantitative RE values in prediction of response to therapy has recently been demonstrated (Campbell et al., 1981). However, a small proportion of patients with RE-negative cancers do respond to hormonal measures. It is conceivable in these instances that receptors were present in cancer cells, but the concentration in the cytosol was insufficiently high to be detected by conventional methods, as a result of low overall cellularity.

Figure 2 Range of cellularity values in 100 primary breast carcinomas.

Figure 3 Relationship between log RE value (fmol/mg^-1 cytosol protein) and tumour cellularity in RE-positive tumours from 42 post-menopausal women.

References


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III. STUDIES ON THE DEVELOPMENT OF QUALITY CONTROL SCHEMES FOR HORMONE ASSAYS.

A. QUALITY CONTROL SCHEMES FOR HORMONE ASSAY.
Quality control analysis in radioimmunoassays. By D. W. Wilson, K. Griffiths, A. B. J. Nix* and K. W. Kemp*. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Cardiff, CF4 4XX, and *Department of Mathematical Statistics and Operational Research, University College, Cardiff, CF1 1XL

Quality control procedures have been well established in clinical pathology (Gooszen, 1960; Amador, Bartholomew & Massod, 1968; Heilbron, Eastman & Kelly, 1974; Eilers, 1975). However, with one noteworthy exception in which inefficient quality control methods were used (Rodbard, 1974), no comprehensive literature has been published on the application of quality control theory to radioimmunoassay (RIA).

A modified ‘CUSUM’ technique has been applied to sequential RIA quality control (QC) data with particular reference to plasma oestradiol-17β. The method is approximately 50% more efficient, in detecting changes in the mean and variance of QC plasma values, than more widely used conventional quality control procedures.

The method is independent of computing facilities and statistical expertise since all computations have been reduced to the form of a nomogram and subsequent construction of a V-mask which overlays sequential QC data to assess control (Kemp, Nix, Wilson & Griffiths, 1977). The simplicity of applying the V-mask enables quality control to be undertaken by laboratory workers who perform the assays and yet may have no statistical knowledge whatsoever.

In the context of the oestradiol assay, which is used in the Supra-Regional Assay Service Quality Control Scheme, the lead distance and half angle of the V-mask were 4·23 (units of the vertical scale) and 20 degrees respectively. The scaling factor \(w\) which is the ratio of the unit of scale for batch number to that of the cumulative sum \(\frac{\sum_{i=1}^{n} (x_i - m)}{\sum_{i=1}^{n} (x_i)}\) plotted on the ordinate was such that \(w = 2\sigma\), where \(x_i\) is the \(i\)th sequential QC value and \(m\) is the target mean. Values for the average run length when the mean of \(x_i\) QC values (\(\bar{\mu}\)), normally distributed with a standard deviation \(\sigma\), were 250 and 5 for \(\bar{\mu} = m\) and \(m \pm 1·5\) \(\sigma\) respectively. In addition to monitoring the QC values, the parameters used in Rodbard’s 4-parameter equation for fitting the RIA standard curves (Rodbard & Hutt, 1974) were also assessed. Since these parameters have a physical significance in relation to a specific assay then evaluation of sequential batches with respect to these parameters can provide diagnostic information when systematic changes in the QC data are detected. The CUSUM quality control method is therefore extremely useful in determining both the source and temporal location of changes in the assay system.

This type of assay control enabled systematic changes in assay performance to be detected quickly and assay performance could be re-evaluated efficiently with a minimum of cost and testing effort.

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REFERENCES
INTERNAL QUALITY CONTROL OF RADIOIMMUNOASSAYS

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SUMMARY

A modified cumulative sum technique has been applied to radioimmunoassay quality control
data. The method is approximately 50% more efficient in detecting systematic changes in
the mean and variance of quality control values for plasma samples than more widely used
conventional methods. The salient features of the technique have been restricted to changes
in the mean quality control value of a plasma pool, but potential applications to changes
in variance and as a diagnostic aid to problems in radioimmunoassay have been evaluated.
The method is independent of computing facilities and statistical expertise since all
computations have been presented in the form of a nomogram and thus can be used by
technicians at the bench.

INTRODUCTION

The purpose of this report is to introduce into the field of radioimmunoassay, a quality
control technique which is efficient, easy to operate and, unlike many aspects of data
analysis, does not require any computer facilities other than a simple desk calculator.
Radioimmunoassay techniques have, by virtue of their high degree of specificity and
sensitivity, provided a new dimension for clinical endocrinology. Their use in clinical
diagnosis, drug trials, feedback control evaluation and metabolic and dynamic studies
serves only to illustrate the paramount importance of accuracy and precision (Holland &
Whitehead, 1974) associated with these techniques and hence the need for adequate quality
control schemes.

Although quality control procedures have been well accepted and established in clinical
pathology (Gooszen, 1960; Amador, Bartholomew & Massod, 1968; Heilbron, Eastman
& Kelly, 1974; Eilers, 1975), little has been published on their application to radioimmuno-
assay. A noteworthy exception may well be the report of Rodbard (1974), but inefficient
quality control methods were used. The modified cumulative sum (CUSUM) technique
described in this paper is more efficient than the conventional methods generally used in
endocrine laboratories and can be extended to identify the origin and temporal location of
systematic changes in radioimmunoassays within specified probability limits (Wilson,
Nix, Kemp & Griffiths, 1978).

METHODS

Radioimmunoassay procedures

Duplicate oestradiol-17β standards over the range 0–50 pg/assay tube were set up. Then
samples from the high- and low-quality control plasma pools, followed by 15 plasma
‡ Reprint requests to D. W. Wilson
samples and finally by repeat samples from the high- and low-quality control plasma pools were also set up. Quality control samples and unknown samples were assayed in duplicate. Standards and samples were not randomized within the assay, because no systematic difference in results was observed with this particular batch size in comparison with a randomized technique. The repeat samples of high- and low-quality control plasma samples at the end of the batch were introduced as a precautionary measure to ensure that systematic changes within the batch did not occur.

The assay standard curves and samples were analysed using Rodbard's four-parameter model. The statistical notation and computational techniques have been described by Rodbard & Hutt (1974).

The CUSUM technique

As a preliminary to the development of the internal quality control system, sufficient data from \( p \) consecutive batches of oestradiol-17\(^{\beta} \) assays \( (j = 1, \ldots, p) \) were collected to calculate the 'target' mean \( m \), where \( m = \frac{\sum_{j=1}^{p} x_j}{p} \) (\( x_j \) is the quality control plasma value). An estimate of the variance \( (\sigma^2) \) of the \( x_j \) is given by \( s^2 = \frac{\sum_{j=1}^{p} (x_j - m)^2}{p-1} \). Values of \( m \) and \( s^2 \) are then used to establish acceptable control procedures for subsequent quality control values.

The essential feature of the CUSUM method is that, by a cumulative process, it makes full use of all relevant information contained in the quality control assay data. Values of the cumulative sum, \( S_n = [\sum_{i=1}^{n} (x_i - m)] \) \( (i = p+1, \ldots, n) \), are plotted against \( n \) to give a CUSUM chart, and \( S_n \) is the cumulative difference between the quality control plasma value and the 'target' mean.

The profile fluctuates about a horizontal line provided the expectation of \( x_i [E(x_i)] \) is equal to the 'target' mean \( m \) (Fig. 1.). When a systematic or spurious change in the 'target' mean occurs, such that \( m \) changes to a value \( M \), then there is a marked change in the profile both in direction and in the magnitude of the slope.

The CUSUM chart was used to carry out tests to determine whether the oestradiol-17\(^{\beta} \) radioimmunoassay was within control value limits, i.e. 'in control'. These tests required consideration of certain aspects of CUSUM quality control procedures that involved the optimal scaling of the CUSUM chart, the selection of average assay run-lengths and the construction of a V-mask using a relatively simple nomogram, which has been devised from complex statistical theory relating to quality control. With regard to these three factors we made the subsequent observations.
Radioimmunoassay quality control

Optimal scaling of the CUSUM chart

For practical use in the laboratory, the visual appearance of the CUSUM chart is important. The relationship between the vertical and horizontal scales used in plotting the data must therefore be carefully considered. Since the chart can be used to investigate the reasons for systematic changes in $x_i$ values (Wilson et al. 1978) as well as in the control of these values, it is important that these scales are related, so that directional changes due to random variations in $x_i$ are minimal and visual emphasis is directed to changes in slope caused by a change in the value of $m$. Reasonable visual discrimination is achieved when the ratio of the unit of scale for $S_n$ to that of the unit of scale for $n$ (both measured in the same physical units of length) is equal to $2\sigma$. This ratio is denoted by $w$ and called the scaling factor.

Selection of average run-lengths

Using a traditional BAR-chart, it is customary to define control limit distances $\pm k\sigma$, either side of the mean quality control value. The value $k$ is determined from specified probability criteria which are required to hold when data are in control and when they are not. It is similarly necessary to select a criterion or statistic upon which to base the design of particular CUSUM procedures. Average run-length is used in these laboratories rather than probability. The average number of quality control samples assayed through a series of batches before a change in the 'target' mean value $m$ is evident is known as the average run-length (Kemp, 1961). Ideally, when an assay is in control, the average run-length $L_1$ should be large. It is equally important, however, to ensure that the average run-length $L_2$, after which the assay would be seen to be out of control, is small. In the study now described for the oestradiol-$17\beta$ assay, a value of five was chosen for $L_2$. Thus five batches of oestradiol-$17\beta$ assays would be completed on average before the assay would be judged out of control. It will become apparent from experience when applying such schemes that in many instances, rather less than five test batches will indicate an out-of-control situation. This arises from the skew (non-symmetric) shape of the run-length distribution about its mean value.

Construction and application of the V-mask

Operation of CUSUM quality control schemes is in general achieved through the use of a geometric device, termed a V-mask (Barnard, 1959; Ewan & Kemp, 1960; Kemp, 1961). In these laboratories, it was found practicable to construct the V-mask from a transparent plastic material. The two important features of the V-mask are its vertex angle $2\phi$ and the lead distance $d$, denoted by the line OP (Fig. 2) and measured in units of the vertical scale ($S_n$) of the CUSUM chart. The values of $\phi$ and $d$ are derived from the nomogram (Fig. 3) originating from this research group. The nomogram enables four quantities to be interrelated by means of a straight line. The first quantity is the ratio of the acceptance criterion ($\Delta$), usually $\pm 2\sigma$ from the mean $m$, to $2\sigma$ which in this example is denoted by $A$ (0.74). The second parameter is the value of $L_2$ denoted by $B$ (5). Extrapolation of $AB$ gives the intercept $C$ on the scale $h/\sigma$ as 3.14 and that of $L_1$ at $D$ as 250.

The symbol $h$ used here has mathematical significance but in this context is used only for convenience and $w$, as previously defined, is the ratio of the unit of scale for $S_n$ to that of $n$ both measured in the same physical units of length. Since $h = (d\Delta/2w)$, $d$ can thus be calculated when $w$ and $\Delta$ are specified. The value of $\phi$ can be calculated from the relationship $h = d\tan \phi$. The constructed V-mask is placed on the CUSUM chart, with line OP horizontal and the point O coincident with the last cumulative sum point plotted. The assay is considered to be out of control when any of the previously plotted points lie outside the limbs of the V-mask (Fig. 2).
Fig. 2. Cumulative sum of the difference between consecutive plasma pool values \( (x_i) \) and the mean value for the plasma pool \( (m) \), plotted against batch number. Diagram illustrates the application of the V-mask.

Fig. 3. Nomogram used to calculate the lead distance \( (d) \) and the angle \( (\phi) \) for the V-mask. The symbols \( \Delta, \sigma, L_1, L_2, h \) and \( w \) are defined in the text. \( h/\sigma = d\Delta/2w\sigma \).
RESULTS AND DISCUSSION

The effectiveness of the CUSUM method is illustrated in Fig. 4. The upper section (Fig. 4a) displays typical laboratory data $x_i$ which are normally distributed with a mean $m$ and standard deviation $\sigma$, and are displayed as a BAR-chart. The assay is judged out of control when values occur outside the limits $m \pm k\sigma$. The values which $k$ takes vary between laboratories, but typical values are in the range 1.5–2.0. Clearly this method fails to use information contained in those $x_i$ values which do not lie outside $m \pm k\sigma$ but which may, nonetheless, indicate a change in the value of $m$. This is demonstrated by the data in the diagram (Fig. 4a). The values of mean $m$ were deliberately changed between batches 15 and 16 from $m$ to $m + 0.5\sigma$. No definitive subsequent visual change could be observed on the chart (see point z).

Exactly the same data were used to generate Fig. 4b. Here, however, the CUSUM ($S_n$) rather than $x_i$ was plotted against batch number and the chart (Fig. 4b) shows not only that a systematic change could be recognized but also the region where this change is indicated.

A number of modifications, such as the use of both action and warning lines (Page, 1955), have been applied to the traditional BAR-charts to improve efficiency. However, Kemp (1962) has shown that the properties of such modified schemes are similar when they are optimized either to maximize their run-length when testing is in control or to minimize run-length when testing is out of control. Consequently, to compare the best traditional BAR-chart scheme with the CUSUM-chart, only one of these optimal systems needs to be considered. The scheme chosen for our present comparative purpose is the 'two-point rule', which states that if two out of any sequence of three sample points lie outside the control lines, then testing is out of control.

The subsequent numerical examples serve to illustrate to endocrine laboratories continuing to use the traditional BAR-chart methods some advantages of the CUSUM technique, already acknowledged by many in the field of clinical chemistry. In relation to radioimmunoassay, obvious advantages which become apparent and which the examples demonstrate are the additional protection given by the CUSUM method against erroneous conclusions that testing is out of control and maximum utilization of a given quantity of control plasma.
Consider a traditional BAR-chart with a control line placed symmetrically on either side of the control mean \( m \) of \( x_t \) values for a single oestradiol-17β quality control pool. Let the distance between the mean and the control lines be \( k\sigma \) and use the rule that testing is out of control when two out of any three test results lie outside these lines. Standard equations can then be employed to determine values of the average run-length \( (L) \).

If at a given mean test level the probability of obtaining a sample value \( x_t \) within the control lines is \( p \), then \( p \) can be used to calculate \( (L) \) by the equation

\[
L = \frac{(2-p^3)}{[(1-p)^2(1+p)]}.
\]  

(1)

If \( p_1 \) is the value of \( p \) when testing is in control and \( p_2 \) is that for \( p \) when testing is judged out of control, then

\[
L_1 = \frac{(2-p_1^3)}{[(1-p_1)^2(1+p_1)]}
\]

(2)

and

\[
L_2 = \frac{(2-p_2^3)}{[(1-p_2)^2(1+p_2)]}.
\]

(3)

The value of \( p_2 \) which satisfies equation (3) when \( L_2 = 5\cdot00 \) is \( p_2 = 0\cdot5242 \).

These results can be used to design a BAR-chart which makes use of the two-point rule. Suppose \( x_t \) is normally distributed with mean \( m = 2\cdot00 \) when testing is in control and that \( \sigma \) is known to be \( 0\cdot40 \). If the system is out of control then the mean testing level moves away from \( m \) by as much as \( \pm 0\cdot60 \). From tables of the normal distribution function and the value \( p_2 = 0\cdot5242 \), a BAR-chart with control lines at a distance \( 1\cdot561\sigma \) on either side of the mean \( m = 2 \) has an ‘out-of-control’ average run-length \( L_2 = 5\cdot00 \).

To illustrate the superiority of the CUSUM method, we can compute the ‘in-control’ run-length \( L_1 \) for the BAR-chart two-point rule using equation (2). From tables of the normal distribution, \( p_1 = 0\cdot881 \) which gives \( L_1 = 46 \).

The CUSUM procedure can be used to obtain the value of \( L_1 \), when \( \Delta = 0\cdot60 = 1\cdot5\sigma \) and \( L_2 = 5 \), from the nomogram by placing a ruler across Fig. 3 joining the points 075 and 5 on the scales \( \Delta/(2\sigma) \) and \( L_2 \). It is found that \( L_1 = 280 \). Therefore, an average of 280 assay batches would be tested before anomalous values of \( x_t \) would indicate an ‘in-control’ situation as out of control.

It is not necessary to labour on the significance of the comparison being made in this example, save to point out that the ‘in-control’ run-length achieved by the CUSUM method is five times that of the BAR-chart. The relative efficacy of the CUSUM and BAR-charts can also be assessed by comparing the degree of replicate testing required by the two methods to achieve schemes with common run-length values for both ‘in-control’ and ‘out-of-control’ situations. Schemes can be designed with given values of \( L_1 \) and \( L_2 \) when the mean of \( x_t \) is \( m \) and \( m+\Delta \) by adjusting the level of testing undertaken. The standard deviation \( \sigma \) of the mean \( \bar{x} \) of \( n' \) observed \( x_t \) values is \( \bar{\sigma}/n' \), where \( \bar{\sigma} \) is the standard deviation of \( x_t \). Control schemes can clearly be designed based on \( \bar{x} \) rather than single observations \( x_t \). If this is done, it is a simple matter to design schemes with particular values of \( L_1 \) and \( L_2 \) both for CUSUM schemes using Fig. 3 or for BAR-charts with ‘two-point rules’ using equations (2) and (3). Parameters for a V-mask, for example, are obtained by placing a ruler across the nomogram so that it joins the points \( L_1 \) and \( L_2 \), the values

\[
\frac{\Delta}{2\sigma} = \frac{n'^+}{2\bar{\sigma}} \quad \text{and} \quad \frac{h}{\sigma} = \frac{d\Delta}{2w\sigma} = \frac{d\Delta}{2w} \bar{\sigma}
\]

are then read off. The procedure is illustrated in the next example.

Suppose we need to control the mean testing level at 200 and want to detect changes in this level of \( \pm 0\cdot66 \). Suppose further that we know \( \bar{\sigma} = 0\cdot50 \) and require a scheme with \( L_1 = 500 \) and \( L_2 = 4\cdot0 \). From Fig. 3, we obtain

\[
\frac{\Delta}{2\sigma} = \frac{0\cdot66n'^+}{2\bar{\sigma}} = 0\cdot93 \quad \text{with} \quad \frac{d\Delta}{2w} = 2\cdot90.
\]
These values give $n' = 1.98$, $\sigma = 0.35$, $w = 0.70$, $d = 2.19$, $\phi = 25^\circ 14'$ and $h = 1.02$. Duplicate testing, together with cumulative sum control of the mean of paired test values with these V-mask parameters, will yield a scheme with the run-lengths specified.

If we use tables of the normal distribution and equations (2) and (3), we find that $n' = 3$ for the two-point rule with $L_1 = 500$ and $L_2 = 4$. For the example described, it therefore requires half as much additional sampling for the BAR-chart to achieve run-lengths equivalent to the CUSUM method described. Put another way, in these circumstances a batch of control material would last 50% longer using the CUSUM method in preference to the BAR-chart.

The arithmetic of this second example demonstrates a point which is generally valid, namely that traditional control schemes need substantially more test data than the CUSUM method to achieve comparable levels of control. Their use will therefore inevitably result in using up control plasma at an unnecessarily high rate.

**Quality control of oestradiol-17\(\beta\) assays**

A portion of the CUSUM chart used over a 12-month period, during which time 200 batches were assayed, is shown in Fig. 5. These figures represent the values from the high-quality control plasma pool. The initial 20 batches provided estimates of the ‘target’ mean $m$ and the standard deviation which were 60.0 and 7.1 pg/ml respectively. The values $L_2$ and $\Delta$ were 5 and 1.5$\sigma$ respectively, giving rise to a value of 280 for $L_1$. It is important to note in this example (Fig. 5) that the ordinate has been scaled by $\sigma^{-1}$ ($s^{-1} = s^{-1} = 1/7.1$) and so in the expressions $d\Delta/2\sigma$, $h/\sigma$ and $\Delta/2\sigma$ associated with the nomogram, the value of $\sigma$ can be taken as unity and that of $w (= 2\sigma)$ as 2.

\[\text{Fig. 5. Application of 'CUSUM' technique to radioimmunoassay of plasma oestradiol-17\(\beta\)}\]

This information was used in conjunction with the nomogram to produce a V-mask with a lead distance $d$ of 8.4 (units of the vertical scale) and equal to $20^\circ 30'$. Figure 5 illustrates the application of the V-mask when the assay went out of control on batch 140. It can be seen that previous points which were in control at this stage lie outside the limbs of the V-mask. It is considered good laboratory practice to reject the complete batch of assays if either of the high- or low-quality control charts do not conform to specified statistical criteria.

The authors are grateful to Elizabeth Buoy for technical expertise in radioimmunoassay and to the Tenovus Organization for their generous financial support.
REFERENCES


EVALUATION OF AN OESTRADIOL RADIOIMMUNOASSAY BY HIGH-RESOLUTION MASS FRAGMENTOGRAPHY

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A technique for the analysis and measurement of C19 steroids by high-resolution selected ion monitoring has been used in this laboratory to study the level of endogenous steroids in human prostatic tissue (Millington, 1975; Millington, Buoy, Brooks, Harper & Griffiths, 1975). A similar procedure has now been used as a reference method to determine the concentration of oestradiol-17β in plasma samples from a quality control scheme for the Supra-Regional Assay Service (SAS) in the United Kingdom. Results from this combined gas chromatographic-mass spectrometric (GC-MS) method were compared with those obtained from the assay of oestradiol-17β with the routine radioimmunoassay (RIA) procedure used in this laboratory (Golder, Phillips, Fahmy, Preece, Jones, Henk & Griffiths, 1976).

All gas chromatographic column packings and the reagent (N,O)trimethylsilyl acetamide were supplied by Jones Chromatography Limited, Llanbradach, Glamorgan. All solvents were of redistilled analytical grade. Precoated silica gel plates (Polygram Sil G/UV254) were obtained from Macherey-Nagel and Company, Düren, Germany.

Details of the procedure for selected ion monitoring at high resolution have been described by Millington (1975). Essentially, a Varian 2700 gas chromatograph fitted with a 2 m x 2 mm internal diameter helical glass column containing 3% OV-17 on Gaschrom Q (100-120 mesh) was interfaced to a Varian-MAT 731 mass spectrometer (MS). Samples of various plasma pools, some of which contained known amounts of added steroid, were assayed for oestradiol-17β with the routine SAS RIA and also by the selected ion monitoring procedure. For the latter method, 1 ng 2,4-dideuterio-oestradiol-17β (greater than 99.99% pure with respect to unlabelled oestradiol-17β) was added to a 10 ml plasma sample which was then allowed to equilibrate overnight at 4°C before ether extraction of the steroids. The extract was then run on prewashed thin-layer chromatography plates of silica gel in the solvent system chloroform:acetone (37:3, v/v), using [2,4,6,7(α)-3H]oestradiol-17β (83 Ci/mmol) as a means of locating non-radiolabeled oestradiol. Areas corresponding to the radioactive marker were eluted, 2-2 ng dihydroequilenin were added as internal standard and the eluates dried and placed in a desiccator containing P2O5 for 1 h. (N,O)trimethylsilyl acetamide (10 μl) was added to form the trimethylsilyl ether. Standard curves were established for oestradiol-17β concentrations ranging from 100 pg to 4-0 ng using the internal standard.

The resolution of the MS was adjusted to around 10,000, and the position of the mass scale of a particular ion was accurately pinpointed with the internal reference compound perfluorokerosene. Therefore, to assay oestadiol, the MS was tuned to m/e 416-257 corresponding to the molecular weight of oestradiol bis-trimethylsilyl ether or to the appropriate values for the two standards. The procedure for measurement, using peak height determination, has been described in detail by Millington (1975).

The peak-height ratios for the trimethylsilyl ethers of oestradiol-17β, deuterated oestradiol-17β and dihydroequilenin were used to determine the plasma oestradiol concentrations for a variety of pools and compared with those obtained by RIA. Close agreement, usually within 15%, was found between the two methods of analysis for untreated plasma pools and for those to which known amounts of oestradiol had been added. A representative set of values, all of which have a coefficient of variation of less than 11%, is shown in Table 1.

17-2
Table 1. Evaluation of an oestradiol-17β radioimmunoassay (RIA) by high-resolution mass fragmentography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of oestradiol (pg/ml)</th>
<th>GC-MS using ether extract*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RIA</td>
<td>GC-MS</td>
</tr>
<tr>
<td>Plasma pool</td>
<td>43.0</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>43.5 (45.6)</td>
<td>48.8 (52.5)</td>
</tr>
<tr>
<td></td>
<td>50.3</td>
<td>58.8</td>
</tr>
<tr>
<td>Plasma pool</td>
<td>103.6</td>
<td>102.5</td>
</tr>
<tr>
<td>+ added oestradiol-17β</td>
<td>94.0 (99.5)</td>
<td>101.3 (99.6)</td>
</tr>
<tr>
<td>(40 pg/ml)</td>
<td>108.8</td>
<td>95.0</td>
</tr>
<tr>
<td>Mean % recovery</td>
<td>135</td>
<td>118</td>
</tr>
</tbody>
</table>

* These results were obtained by taking the ether extract from the usual RIA for the gas chromatography-mass spectrometry (GC-MS) procedure. Mean values are given in parentheses.

Also shown are triplicate results obtained from one plasma pool, to which had been added in 10 μl 0.01 M-phosphate buffer the equivalent of 40 pg oestradiol/ml plasma. An assessment of experimental errors indicated that the discrepancies, such as the high recovery figures, which existed between the two methods could be accounted for on a statistical basis.

Nevertheless, despite the close agreement between the two methods, the use of mass fragmentography with steroids labelled with stable isotopes will be of considerable value in providing definitive methodology to check steroid concentrations in quality control of samples of plasma and thereby a more effective quality control scheme.

The authors thank the Tenovus Organisation for generous financial support and Mr A. W. Pike for his technical advice on the use of the GC-MS.

REFERENCES
INTERNAL QUALITY CONTROL OF RADIOIMMUNOASSAYS: MONITORING OF ERROR

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SUMMARY

A cumulative sum technique has been specially designed to monitor the error between replicate determinations made on quality control plasma for consecutive batches of assays. This procedure has played a vital role in assessing assay performance. Special consideration has been given to small sample sizes (n = 2 or 3) which is generally the rule rather than the exception in many situations. This technique has been applied to numerous steroid radioimmunoassays and has ensured that both the mean value and the standard error of hormone levels of a quality control pool were under control. Data from routine assays of oestradiol and testosterone in plasma from women are presented. Since this technique provides a sensitive measure of monitoring error, it assists the endocrinologist in elucidating statistical inferences which are a manifestation of assay performance.

INTRODUCTION

The importance of internal quality control in radioimmunoassay and other analytical methods used in endocrinology is now widely recognized (Blüttner, Borth, Boutwell, Broughton & Bowyer, 1978; Wilson & Tan, 1978). Although certain quality control procedures are well known (Wetherill, 1969; Sarkadi & Vincze, 1974) and used for radioimmunoassays (Rodbard, Rayford & Ross, 1970; Challand & Chard, 1973; Rodbard, 1974; McDonagh, Munson & Rodbard, 1977), advances in control theory (Kemp, 1967; Rowlands, 1976) have now enabled more efficient quality control methods to be developed (Kemp, Nix, Wilson & Griffiths, 1978), based on the cumulative sum (CUSUM) technique. The need for monitoring the standard error associated with replicate determinations of the hormone concentration of an internal quality control plasma pool between successive batches of assays is generally appreciated intuitively, but few techniques are currently used to control this aspect of a radioimmunoassay (Challand & Chard, 1973; McDonagh et al. 1977; Wilson, Nix, Kemp & Griffiths, 1977). A common assumption in quality control charts, which are used to control the mean, whether they be the Shewhart or cumulative sum charts, is that the inter-assay variation is nearly constant. A detailed analysis of quality control data from these laboratories indicates that this is not often the case and consequently the inherent assumptions in controlling the mean are invalid, so that false conclusions concerning assay performance could well be drawn. This report is designed to fill an important gap in processing radioimmunoassay data and provides the means by which endocrinologists and other researchers can achieve a comprehensive assessment of the temporal performance of assay systems.
From a practical viewpoint, it is important to use all quality control data as efficiently as possible, especially when the number of quality control plasma samples that can be assayed at any one time is limited by the batch size. It is appropriate to state that efficiency can be achieved by selecting a quality control technique which is sensitive to changes in the value of the parameter being controlled such as the mean or assay error. In addition, where cumulative sum techniques are used to assist quality control, V-mask parameters (Barnard, 1959; Ewan & Kemp, 1960; Kemp, 1961; Duncan, 1974; Kemp et al. 1978) such as the lead distance, \(d\), and the half angle, \(\phi\), should be chosen to maximize the sensitivity of the technique for a given control statistic. Finally, it is important to use a control statistic which is most sensitive to changes in assay performance. These criteria have been largely satisfied (Kemp et al. 1978; K. W. Kemp, A. B. J. Nix, R. J. Rowlands, D. W. Wilson & K. Griffiths, article in preparation).

Although this method has been applied to several radioimmunoassays, only examples derived from the analysis of testosterone and oestriol in samples of human female plasma are presented. These are typical examples drawn from routine steroid assays performed in the Supra Regional Assay Laboratory at the Tenovus Institute during the past 18 months.

**METHODS**

**Radioimmunoassay procedures**

Duplicate steroid standards for oestriol and testosterone over the ranges 0–500 pg and 0–150 pg respectively were set up. The standards for each assay were then followed by high-, medium- and low-titre quality control pools and then by plasma samples and finally repeat samples from high-, medium- and low-titre quality control plasma pools. The number of plasma samples processed in each assay were approximately 15 and 20 respectively. This scheme concerns only single aliquots of plasma, consequently quality control samples are performed in duplicate \((n = 2)\) since they include the repeat sample, whereas plasma samples are assayed singly. Practical considerations such as the limitation on batch size imposed by the charcoal method for separating free from bound hormone, the need for duplicate determinations made on aliquots of the organic residue from single plasma extracts, as well as the size of workload borne by the laboratory do not allow plasma samples to be assayed in duplicate. Duplicate quality control samples provide data for monitoring the mean and error. All data-processing relating to the standard curve and dose interpolation were carried out by the method of Rodbard & Hutt (1974); alternative methods are described elsewhere (Wilson & Tan, 1978).

**The CUSUM technique**

It has been reported elsewhere (Kemp et al. 1978) that efficient quality control can be achieved by the cumulative sum method rather than Shewhart charts. In particular, Kemp et al. (1978) have used the cumulative sum technique to monitor the mean value of a quality control plasma pool but, as with most other schemes, it assumes that the inter-assay variation is constant. To compensate for this deficiency, a cumulative sum technique has been used to monitor the within-assay variance for successive batches of assays, based on a small sample size (i.e. \(n = 2\) or 3). In order to apply these schemes certain concepts need to be described. In the first instance, it is not possible in most practical situations to define, in absolute terms, an in- or out-of-control situation. However, probability limits can be assigned to the distribution of the quality control plasma values that enable one to 'judge', in a statistical sense, whether a process may be in- or out-of-control. These probability limits have been mentioned by Kemp et al. (1978) wherein they have been replaced by average run-lengths for the cumulative sum scheme described in this report. The average number of quality control samples assayed through a series of batches before a change is detected, is known as the average run-length. Ideally, when an assay is in control the
average run-length, $L_a$, should be large in order to avoid unnecessary rejection of assays. It is equally important, however, to ensure that the average run-length, $L_a$, after which an assay would be seen to be out-of-control, is small.

Adopting where possible the notation of Kemp et al. (1978) it has been found and illustrated by K. W. Kemp, A. B. J. Nix, R. J. Rowlands, D. W. Wilson & K. Griffiths (article in preparation) that for a given out-of-control run-length ($L_a$), a higher average run-length for the in-control length ($L_2$) is obtained for the monitoring of error by basing a control statistic upon the sample variance rather than upon the standard deviation.

To calculate the control statistic used to monitor the error between replicate determinations on a quality control plasma sample for each batch of assays, the values of the target-variance ($\sigma_T^2$) and the control statistic $C_i$ are required. In the initial stages of setting up the scheme for a particular assay it is necessary to compute $\sigma_T^2$ from $p$ assay batches where $p$ is usually of the order of ten. If the assay values obtained in the $j$th batch are denoted by $x_{ij}$ ($j = 1, \ldots, n$), an estimate $s_j^2$ of the sample variance is given by the following equation

$$s_j^2 = \frac{\sum_{i=1}^{n} (x_{ij} - \bar{x}_j)^2}{n-1},$$

(1)

where $n$ is the number of replicate assays within each batch (i.e. $n = 2$ or $3$) and the sample mean $\bar{x}_j$, is given by the following equation

$$\bar{x}_j = \frac{\sum_{i=1}^{n} x_{ij}}{n}.$$

(2)

The target variance $\sigma_T^2$, is then calculated using the next equation

$$\sigma_T^2 = \frac{\sum_{j=1}^{p} s_j^2}{p},$$

(3)

and it is noteworthy to mention that $\sigma_T^2$, calculated in this way, is independent of possible variations in the mean value between batches. Since the value of the target variance is now known, the control statistic $C_i$ ($= s_j^2/\sigma_T^2$) can be used to monitor the error between replicate determinations of each plasma pool for subsequent batches of assays.

Cumulative values ($S_N$) of $C_i$ - 1 were calculated for successive batches of assays ($i = p + 1, \ldots, N$) using the following equation

$$S_N = \sum_{i=p+1}^{N} (C_i - 1).$$

(4)

Values of $S_N$ were plotted against $N$ to give a CUSUM chart for the control of error between replicate determinations of a quality control plasma pool for testosterone (Fig. 1b) and oestriol (Fig. 2b). It is important to emphasize that in this presentation, certain scaling factors have been used for the CUSUM error chart. These should be considered as mandatory since their adoption obviates the need for complex tables, thereby allowing this technique to be used by endocrinologists who may have only a basic knowledge of mathematical statistics. These scaling factors are $2 \sqrt{2}$ and 2 for $n = 2$ and 3 respectively. Thus for duplicate determinations, if 1 cm on the horizontal scale represents each batch number, then 1 cm on the vertical scale has a value of $2 \sqrt{2}$, provided of course that 'linear-linear' graph paper is used. It is important to note that the CUSUM values in Figs 1b and 2b have been scaled out by $2 \sqrt{2}$. These CUSUM profiles will fluctuate around a horizontal line when the assay is in control since the expectation of $C_i[E(C_i)]$ is then equal to one. When a
change occurs in the standard error between replicates, the profiles exhibit a marked change in both magnitude and direction. If the standard error changes from the target value \( \sigma_T \) to a new value \( \sigma_1 \), then a measure of this change is the value of the ratio \( \beta = \sigma_1 / \sigma_T \), henceforth called the 'tolerance level', which is analogous to the quantity \( (\Delta) \) placed on the target mean which is usually of the order of 1·5 \( \sigma \) (Kemp et al. 1978).

For a given assay, the assayist would choose a fixed tolerance level, \( \beta \), so that if a change in the standard error of more than \( \beta \sigma_T \) occurs, an out-of-control condition will be quickly diagnosed (Figs 1b and 2b). Values of \( \beta \) and \( L_2 \), the average run-length after which the assay is judged out of control (Kemp et al. 1978), are chosen by the assayist. The V-mask is constructed with appropriate parameters obtained from Table 1. The values of \( d \) and \( \phi \) in the table yield CUSUM schemes which are 'most efficient' in the sense described.

**Construction and application of the V-mask**

The V-mask was constructed from transparent plastic material as described by Kemp et al. (1978). However, unlike the control of the mean, only the lower limb of the V-mask was used for monitoring error since deterioration of assay performance was the overriding concern. The V-mask was constructed from information contained in Table 1. This table shows the relationship between average run-lengths \( L_1 \) and \( L_2 \) (Kemp et al. 1978), the tolerance level (\( \beta \)), the number of replicates (\( n \)) and optimum values for the angle (\( \phi \)) and lead distance (\( d \)) of the V-mask. In practice, duplicate determinations (\( n = 2 \)) were made on a quality control plasma pool and values for \( \beta \) and \( L_2 \) were chosen as 1·5 (i.e. a 50% increase in the standard error) and 10 respectively. Values for \( d \) (15·73 units) and \( \phi \) (9·08°)
were obtained from Table 1 and the V-mask was used to monitor the error for the radioimmunoassay of both testosterone and oestriol using these values.

![CUSUM chart](https://via.placeholder.com/150)

**Fig. 2.** Application of the cumulative sum technique (CUSUM) for control of the mean (a) and error (b) of radioimmunoassay determinations for oestriol made on a quality-control pool of plasma from women. Data indicated a systematic change in assay performance. V-Mask parameters for the control of the mean were described by Kemp et al. (1978) and those associated with the control of error were obtained from Table 1. Values for the average run-length when the assay is judged out of control ($L_2$), the target variance ($\sigma^2_2$) and the tolerance level ($\beta$) associated with duplicate determinations ($n = 2$) were 10, 5576 (nmol/l)$^2$ and 1.5 respectively.

**RESULTS AND DISCUSSION**

Experience has shown that the mean value of a quality-control plasma pool can be in control and yet the error between replicate values for each assay batch may be judged out of control. For example, in Fig. 1(b), the broken line illustrates the possibility of the error between replicates for the testosterone plasma pool going out of control. Here, the fact that the assay went out of control was probably due to a spurious result obtained from batch 17. The assay was rejected and subsequent assay batches continued to be judged in control. The corresponding data for the mean (Fig. 1a) indicated that the assay was always judged in control. An excellent example of an assay going out of control due to a change in the error between replicates when the mean remained in control is shown in Fig. 2(b). Inspection of this chart reveals that a systematic change in errors associated with replicate determinations within a particular batch of assays has probably occurred between batches 11 and 16. In this case, values from samples measured from batch 17 onwards were rejected, but again the mean value was, with the exception of batch 22, under control.

It is emphasized that the two procedures illustrated in Figs 1 and 2 are not alternative quality control schemes. One procedure monitors the mean and the other the within-assay error of quality control pools for successive batches of assays. Therefore both procedures must be used in a complementary manner to provide a more efficient overall quality control.
Table 1. The lead distance ($d$) and angle ($\phi$) of the V-mask for various values of average run-lengths, $L_1$ and $L_2$ and tolerance level ($\beta$) for the standard error of replicate ($n$) determinations of a quality control plasma pool for consecutive batches of assays are given. The average run-length is defined as the average number of quality control samples assayed through a series of batches before a change in the 'target' value of the standard error ($\sigma_T$) of replicate determinations is evident. The tolerance level $\beta$ is defined as the ratio of $\sigma_1/\sigma_T$ where $\sigma_1$ is the upper limit of acceptability for the standard error ($\sigma_T$).

<table>
<thead>
<tr>
<th>$L_2$</th>
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<tbody>
<tr>
<td>4</td>
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<table>
<thead>
<tr>
<th>$n = 2$</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$\beta$</td>
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<tr>
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</tr>
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<td>15-73</td>
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<tr>
<td>2-00</td>
<td>17-55</td>
</tr>
</tbody>
</table>

* To compensate for the scaling of cumulative sum values by factors of 2,$/2$ and 2 for duplicate and triplicate values respectively, all lead distances are expressed in units of the horizontal scale where one unit represents one batch number. Only those figures generally applicable to the control of radioimmunoassay are included.

scheme. The above-mentioned discussion of results serves to illustrate this important distinction.

The choice of values for the tolerance level $\beta$ and the average run-length, $L_2$, is dependent on the throughput of the laboratory and the level of assay precision which is considered suitable for those purposes for which the assay is intended. In a clinical situation, the change of the target error from $\sigma_T$ to $\sigma_1$ should generally comply with standards of precision whereby assays should have a coefficient of variation of less than 15%. For a specified working range and for situations where it is not practical to increase the number of replicate standards in otherwise optimized assays, the precision profile of the standard curve (Wilson, Sarfaty, Clarris, Douglas & Crawshaw, 1971) may not always allow these specifications to be met. This is especially so in situations where the value of the quality control plasma, as measured by the standard curve, approaches zero. In this situation, the assayist has usually to accept a lower standard of precision. The value of $L_2$ and the corresponding value of the average run-length when the assay is in control, $L_1$, are chosen to detect changes in assay.
performance reasonably quickly whilst at the same time the number of times the assay is judged to be out of control when in fact it is in control is reduced. In these laboratories, assays are performed twice daily and a value for $L_2$ of ten means that when the standard error changes by more than $\beta_0T$ then the assay should be judged out of control within one working week. On the other hand, Table 1 reveals that since all assays are performed in duplicate and the value of $\beta$ is 1.5, then on average only one assay in approximately 10 weeks will be rejected when the assay is in control since the corresponding value of $L_1$ is 103. Laboratories which perform assays less frequently would probably reduce the value of $L_2$ and therefore $L_1$ to achieve a more satisfactory operational system.

In practice the control of error appears to be more sensitive than the control of the mean. This necessitates choosing a value of $L_1$ for a given value of $\beta$ which suits the throughput of the laboratory. This in turn means that different values of $L_2$ for both mean and error will generally occur. Statistically the choice of $L_1$ and $L_2$ is equivalent to assessing the cost function for the scheme which varies from one assay to another and for different laboratories.

The application of this technique to radioimmunoassays will confer more rigorous internal quality control constraints for assessing deterioration in assay performance. An important use of this and other suitable internal quality control techniques may well be in the assessment of assay characteristics in those laboratories involved in external quality control, wherein temporal changes in within-laboratory assay variation should be reduced to a minimum and controlled before submitting results to the organizing laboratory for subsequent analysis.

In conclusion, a CUSUM technique has been applied to monitor the standard error in internal quality control plasma samples for successive batches of assays. It is efficient, simple to operate, requires no complicated statistics and fulfils a requirement in many experiments in endocrinology.

The authors are grateful for the generous financial support of the Tenovus Organization. They have also appreciated the constructive and helpful suggestions of Dr Diana Riad-Fahmy and the technical assistance of B. Charalambous.

REFERENCES


Kemp, K. W. (1967). Formal expressions which can be used for the determination of the operating characteristics and average sample number of a simple sequential test. Journal of the Royal Statistical Society, Series B—Methodology 29, 248–262.


DEVELOPMENT OF STATISTICAL AND ANALYTICAL TECHNIQUES FOR USE IN NATIONAL QUALITY CONTROL SCHEMES FOR STEROID HORMONES

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INTRODUCTION

National quality control (NQC) schemes are designed, inter alia, to impartially evaluate the analytical performance of individual laboratories and to assess their degree of concordance with each other. This information, together with suggestions and constructive criticisms of the co-ordinator, provides the means by which laboratories can improve or re-assess their analytical technique. Quite apart from the responsibility that each laboratory has towards users of their service, these NQC schemes may ultimately be instrumental in the mandatory certification of laboratory competence without which laboratories would not be able to practise. Therefore it is important that each laboratory can recognize and reduce known sources of error in their analytical procedure. In this situation, the statistical analysis of NQC data would then be predominantly concerned with the elucidation of those analytical reagents or procedures which would be expected to produce 'acceptable' standards of quality for a particular assay. A corollary of this would be the introduction and use of 'matched' reagents which would intuitively confer greater uniformity on the analytical performance of participating laboratories.

Adopting the rationale that the improvement of intra-laboratory performance will enable the assessment of NQC schemes to become more meaningful, this group has developed statistical and analytical techniques for the improvement of accuracy, precision and monitoring of error for the determination of steroid hormones. These developments are now described and their relevance to NQC schemes discussed.
GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) METHODS FOR STEROID HORMONES

The preparation of plasma pools with values assigned from 'accurate' analytical techniques are of particular value in NQC schemes. The choice of the GC-MS technique is favoured by many researchers for assessing steroid hormone radioimmunoassays. The principal reason is related to the high degree of specificity that can be achieved with good precision. In the development of these reference methods, several distinct stages of the technique are recognized which merit particular attention. These are the use of an internal standard to assess recovery losses, the extraction procedure for removing the steroid from the bulk of the plasma matrix, the subsequent purification stage (usually some form of chromatography), sample derivatization and then GC-MS analysis.

The incorporation of an internal standard to offset procedural losses is of crucial importance in the assessment of the precision and accuracy of the GC-MS method. Isotopic variants of the compounds to be analysed are frequently the internal standards of choice by virtue of the similarity of their physico-chemical properties. Consequently the variants that have been considered for the measurement of cortisol, oestradiol-17β, testosterone and progesterone have included \([\text{[9,11,12,12-}^2\text{H}_4\text{-cortisol, [2,4-}^2\text{H}_2\text{-oestradiol-17\beta or [16,16,17-}^2\text{H}_3\text{-testosterone and [18,18,18-}^2\text{H}_3\text{-progesterone.}}\) In practice, the internal standard contained in 10 µl of ethanol is added to an aliquot of the plasma pool and then allowed to equilibrate overnight at 20°C before extraction.

Judicious choice of extraction solvent enables a reasonable degree of specificity and high recovery of the steroid to be achieved. For example, cortisol is extracted from plasma (0.5 ml) with purified dichloromethane (10 ml) whereas oestradiol-17β which requires a less polar solvent is extracted from plasma (1.0 ml) with purified diethyl ether (8 ml).

The purification of steroid extracts is of paramount importance if ambiguous GC-MS results are to be avoided. The use of micro-columns (4 cm x 0.5 cm in diameter) of lipophilic Sephadex gels is of particular value in achieving this objective. In the oestradiol-17β method, for example, sequential chromatography on Lipidex 5000
(reversed phase) and Sephadex LH-20 (straight phase) affords the necessary sample purity. In this example, the residue from the ether extract is applied to the Lipidex 5000 column contained in chloroform/water/methanol (2:1:9) and the 0-3 ml fraction collected. The solvent is removed under nitrogen and the residue applied to a column of Sephadex LH-20 contained in methylene chloride/petroleum ether/methanol/water (100:80:15:1) and the 2-6.5 ml fraction collected for subsequent derivatization and GC-MS analysis.\(^7\)

Selective derivatization procedures may also be of value in permitting improved specificity at the preliminary purification stage. Certain steroids such as testosterone may be converted in situ (that is, prior to extraction) to the corresponding 3-oxime which, following solvent extraction, may be selectively recovered by virtue of its basicity using an ion exchange derivative of Sephadex (Axelson and Sjövall, 1976; Gaskell and Finlay, unpublished data).

Derivatisation of steroid hormones is generally essential for the improvement of subsequent gas chromatographic behaviour, such as the minimization of adsorption losses, and for the optimization of mass spectrometric properties that confer specificity and sensitivity on the analytical technique. In the oestradiol-17β method, used in these laboratories, an internal GC-MS standard, dihydroequilenin is added to the residue from the column eluate and the resultant residue dissolved in 10 μl of bis-(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (99:1). Analysis of the bis-trimethylsilyl ethers of oestradiol-17β, deuterated oestradiol-17β and dihydroequilenin allows the oestradiol-17β content of the plasma pool to be measured by a two-stage GC-MS procedure with single ion monitoring. For selected ion monitoring, tert-butyl-dimethylsilyl ethers provide a useful variant on the widely used trimethylsilyl ethers. Their value is attributable to their stability and the general property that the facile loss of C\(_4\)H\(_9\) under electron impact gives extremely intense \(\text{[M-57]}^+\) ions which generally account for a high proportion of the total ion current. These and related derivatives are currently being evaluated for use in reference methods for testosterone. In the cortisol method, purification of the extract by column chromatography is not
necessary. The residue from the dichloromethane solvent extraction is allowed to react with 30 μl of methoxyamine hydrochloride in pyridine (15 g/L) overnight at 20°C. The solvent is evaporated under reduced pressure, the residue dissolved in 10 μl of bis-(trimethylsilyl)trifluoroacetamide/trimethylbromosilane/pyridine (90:5:5 by volume) and allowed to react for 15 minutes before GC-MS analysis.

**GC-MS analysis** provides the means by which considerable specificity in the overall analytical technique can be achieved. Judicious choice of derivatives such as those described, the use of carefully selected stationary phases (1% OV-1 on Gas Chrom Q at 290°C for cortisol) and subsequent analysis using selected ion monitoring have been invaluable in assigning 'accurate' values for steroid hormones in plasma pools for use in NQC schemes.

The advantage of selected ion monitoring at high mass spectrometric resolution (m/Δm 8,500), developed and routinely used in these laboratories, is that ions with a known exact mass and therefore specific elemental composition are monitored, rather than ions of given nominal mass. In the cortisol method, two gas chromatographic-mass spectrometric analyses are performed on the derivatized extract. In the first analysis, m/z 605.3626 [(M-31)+ for cortisol-3,20-bis (methyloxime)-11,17,21-tris(trimethylsilyl ether)] and m/z 619.3782 [(M-43)+ for cortisol-3,20-bis (ethyloxime)-11,17,21-tris(trimethylsilyl ether)] were sequentially monitored. In the second analysis, ions of m/z 608.3814 [(M-31)+ for [3H] cortisol-3,20-bis(methyloxime)-11,17,21-tris(trimethylsilyl ether)] and m/z 619.3782 were sequentially monitored. The peak height ratios of m/z 605/608 for the plasma pool allowed the quantification of the cortisol content by reference to a calibration curve established for each analytical series.

Alternative means of achieving a high degree of specificity in mass spectrometric analysis have been made by metastable peak monitoring. Metastable ions are those of intermediate stability which are emitted from the mass spectrometric ion source intact but fragment before mass separation and detection. Using a double-focussing mass spectrometer, 'daughter' ions derived from these metastable ions may be detected free from 'normal' ions by adjusting the accelerating voltage. The crucial feature of the metastable analysis, from an analytical point of view, is that focussing
conditions are dependent on the masses of both the precursor and daughter ions. These techniques are expected to find applicability in the 'accurate' measurement of steroid hormones in plasma. The identification of sources of random and systematic error in GC-MS techniques and their subsequent treatment is particularly important where 'certified reference plasmas' are to be used for assessing the 'accuracy' of radioimmunoassays. A complete treatise on this topic is beyond the scope of this report but errors associated with the calibration curve and subsequent dose interpolation are worthy of mention. The calibration curve is usually a plot of the relative peak height ratios \( Y_i \) of analyte to internal standard as a function of the analyte mass \( X_i \). A physico-chemical model has been derived for GC-MS (Wilson and Gaskell, unpublished data) which is of the form

\[
Y_i = \frac{k_1}{k_2} \left( \frac{X_i + \alpha b}{\beta + \beta X_i} \right) + \epsilon_i
\]

where \( k_1, k_2, \alpha, \beta \) and \( b \) are assumed to be constant. This model reduces to

\[
Y_i = P_1 (X_i + P_2)/(P_3 + X_i) + \epsilon_i
\]

where \( P_1 = k_1/(k_2 \beta), P_2 = \alpha b \) and \( P_3 = b/\beta \).

Regression analysis of the calibration data, using the method of least squares, provides estimates for some of the physico-chemical parameters together with the fiducial limits associated with the calibration curve which are important for subsequent dose interpolation. Alternatively an empirical approach can be used for the analysis of the calibration curve and a power function of the form \( Y_i = ax_i^b \) has been found to be particularly useful for routine analysis. The choice of the function must be related to the goodness-of-fit, the number of degrees of freedom, the distribution of the signs of the residuals and the usefulness of the physico-chemical parameters in the GC-MS procedure. Whichever method is used, an estimate of the analyte concentration together with its fiducial limits must be available to potential users of this material.

These GC-MS methods, together with their attendant statistical methods, provide the means by which 'accurate' values for certain
steroid hormones can be assigned to plasma pools used in NQC schemes.

**THE PRACTICE OF STATISTICAL INference IN PROCESSING RADIOIMMUNOASSAY DATA**

Major sources of error in NQC schemes arise from inadequate analysis of radioimmunoassay data. Manual plotting and subsequent drawing of the standard curve often appear comparable with statistical methods in estimating plasma potencies. However when the calibration co-ordinates depart from an 'ideal' situation, difficulties arise from subjective assessments of the data which introduce bias into the results. Furthermore, statistical assessments of goodness-of-fit, a rationale for the rejection of outliers, and a reasonable assessment of precision are not possible with the manual method. The use of desk calculators or larger computers considerably reduces errors associated with inadequate data processing. Suggested methods for the treatment of radioimmunoassay data using computer programs have been reported elsewhere. It must be emphasized that there is no single mathematical function that is 'best' for fitting the coordinates of standard curves for all radioimmunoassays, just as there is no universal procedure for the rejection of outliers. In these laboratories, the 4-parameter logistic model has found wide applicability for many different types of assays whereas outlier rejection of standard hormone counts is based on the deviation of the individual counts from the fitted curve. The rejection of samples is based on the coefficient of variation associated with either the individual or mean coefficients of variation of the hormone concentration. The maximum acceptable value for the coefficient of variation, within the working range of the standard curve, is usually 15%. The computer program should preferably provide other information such as the minimum detectable hormone concentration, the optimizing procedure for assay conditions and graphical options for examining the standard curve and its associated precision profile. It is important that participating laboratories are able to supply this type of information to the co-ordinator of the NQC scheme and that statistical calculations (for example, the intra- and inter-assay variation), are correctly calculated. It is this type of information
which forms the background against which results from the NQC scheme are compared and inferences drawn.

EFFICIENT DESIGN OF QUALITY CONTROL PROCEDURES FOR MONITORING ASSAY PERFORMANCE

The use of inadequate internal quality control procedures for monitoring assay performance is responsible for a large proportion of the intra-laboratory error as judged from NQC data. This group has compared the three most commonly used quality control procedures used in clinical endocrinology. This section is concerned with the identification of the 'best' quality control procedure for use in hormone assays. The procedures investigated were Shewhart, modified Shewhart ('2 from N') and the Cumulative Sum (CUSUM) technique.

The assessment of each control procedure was based on the concept of average run length. Formally, the average run length, \( L \), of a given scheme is the average number of assay batches run before action is taken. Consequently it is necessary that each control procedure satisfy the following criteria. The average run length should be small when the system is out-of-control \( (L_2) \) and large when it is in-control \( (L_1) \). Since many schemes satisfy these conditions, it is important to select those which are 'optimal' in a sense to be described.

An optimal scheme with parameters denoted by \( \alpha^* \) satisfies the following equations with respect to other parameters \( \alpha \) such that for specified values of \( L_2 \)

\[
L_2(\alpha) = L_2(\alpha^*)
\]

and

\[
L_1(\alpha^*) > L_1(\alpha)
\]

Thus for a specified out-of-control run length \( (L_2(\alpha)) \), the scheme \( (\alpha^*) \) is selected which has the maximum in-control run length \( L_1 \).

Assume data for the Shewhart chart has a standardized Normal distribution when the assay is in-control and a Normal distribution with mean \( \theta \) and unit variance when out-of-control. Let \( \beta \) denote the probability of any particular observation made on the control statistic being rejected, then for Shewhart schemes the average run length is given by the reciprocal of \( \beta \).
For in-control situations

\[ \beta = \beta_1 = L_1^{-1} = 1 - \Phi(k_1) + \Phi(-k_2) \]

and for out-of-control

\[ \beta = \beta_2 = L_2^{-1} = 1 - \Phi(k_1 \pm \theta) + \Phi(-k_2 \pm \theta) \]

The \( \pm \) sign is used to denote an increase and decrease in the mean value of a quality control pool. The optimization procedure reduces to the maximization of the in-control run length \( L_1 \) where

\[ L_1 = \beta_1^{-1} = (1 - \Phi(k_1) + \Phi(-k_2))^{-1} \]

subject to the constraints

\[ L_2 = (1 - \Phi(k_1 - \theta) + \Phi(-k_2 - \theta))^{-1} \]
\[ L_2 = (1 - \Phi(k_1 + \theta) + \Phi(-k_2 + \theta))^{-1} \]

This is a constrained maximization problem with the obvious symmetrical solution \( k_1 = k_2 \), their common value being determined by the equation

\[ L_2 = (1 - \Phi(k_1 - \theta) + \Phi(-k_1 - \theta))^{-1} \]

and by a further substitution gives the maximum in-control run-length

\[ L_1(\alpha^*) = (1 - \Phi(k_1) + \Phi(-k_1))^{-1} = (2\Phi(-k_1))^{-1} \]

As can be seen, the optimizing process depends only on the availability of an expression for the average run length for a quality control procedure. In addition to Shewhart, values for \( L_1(\alpha^*) \) have been calculated for the '2 from N' and the CUSUM procedures for controlling the mean.
TABLE 1

COMPARISON OF OPTIMAL CONTROL SCHEMES FOR THE MEAN

<table>
<thead>
<tr>
<th>$L_2(\alpha^*)$</th>
<th>Control scheme</th>
<th>$L_1(\alpha^*)$</th>
<th>Acceptance Criterion $\theta$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>S</td>
<td>6.7</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>SAW</td>
<td>6.7(3)</td>
<td>16.2(3)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5.6</td>
<td>23.9</td>
</tr>
<tr>
<td>10</td>
<td>S</td>
<td>15.2</td>
<td>44.8</td>
</tr>
<tr>
<td></td>
<td>SAW</td>
<td>15.4(3)</td>
<td>53.0(4)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>18.9</td>
<td>342.7</td>
</tr>
</tbody>
</table>

Key: - S - Shewhart; SAW - Modified Shewhart; C - CUSUM; and parentheses contain optimal values of N.

For the control of the mean, the acceptance criterion, $\theta$, which is the 'change' permitted for the target mean usually expressed in standard deviation units of the assay variation, is generally about 1.5. Table 1 illustrates that for an average out-of-control run length $L_2$ equal to 5, the CUSUM method is 4-5 times more efficient. When $L_2$ is 10, a situation which can readily occur in a clinical chemistry laboratory of high through-put, the value of the average in-control run length is orders of magnitude different.

For control of the error, the permitted 'change' in the coefficient of variation could be 50%. This corresponds to a change, for example, in the coefficient of intra-assay variation from 10% to 15%. The value of $L_2$ chosen in these laboratories is usually about 10. Thus for duplicate and triplicate determinations of a quality control plasma pool the CUSUM technique is 1.4 and 3.02 as efficient respectively as the 'optimal' Shewhart scheme as shown in Table 2.

In conclusion, this group advocates the use of the CUSUM procedure for controlling the internal quality control of steroid hormone assays. These schemes have been embodied in a computer.
program (Rowlands, Nix, Kemp, Wilson & Griffiths, unpublished data) for use in the clinical endocrinology laboratory and its use is expected to produce more meaningful NQC results.

TABLE 2
COMPARISON OF OPTIMAL CONTROL SCHEMES FOR THE ERROR

<table>
<thead>
<tr>
<th>Order of Replication</th>
<th>( L_2(\alpha^*) )</th>
<th>Control Scheme</th>
<th>Tolerance Level ( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.50</td>
</tr>
<tr>
<td>2</td>
<td>( S )</td>
<td>18.3</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>( C )</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>( %RE )</td>
<td>-</td>
<td>112</td>
</tr>
<tr>
<td>10</td>
<td>( S )</td>
<td>73.5</td>
<td>250.3</td>
</tr>
<tr>
<td></td>
<td>( C )</td>
<td>103</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>( %RE )</td>
<td>140</td>
<td>260</td>
</tr>
<tr>
<td>3</td>
<td>( S )</td>
<td>37</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>( C )</td>
<td>45</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>( %RE )</td>
<td>122</td>
<td>188</td>
</tr>
<tr>
<td>10</td>
<td>( S )</td>
<td>178</td>
<td>1155</td>
</tr>
<tr>
<td></td>
<td>( C )</td>
<td>538</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>( %RE )</td>
<td>302</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: \( S \) - Shewhart, \( C \) - Cusum, \( \%RE \) - Percentage Relative Efficiency

DEVELOPMENT OF ROBUST ANALYTICAL METHODS FOR IMMUNOMETRIC ASSAY OF STEROID HORMONES IN HUMAN PLASMA

These laboratories have developed a number of simple and robust immunometric assays for the measurement of steroid hormones in plasma, the reagents for which can be despatched to area laboratories in the form of matched-reagent packs. The assay techniques satisfy 'normal' criteria for sensitivity, precision, specificity and, if GC-MS methods are available, accuracy. Consideration has
also been given to reducing the running costs and improving the overall efficiency of these analytical procedures. The term robustness is defined here as 'that quality conferred by the analytical procedure which reduces the effect of perturbations on the final result brought about by manipulative, physical and chemical processes associated with the assay system'. This is an important property if the matched-reagent packs are to be used by analysts in other laboratories who may have varying degrees of proficiency.

Two examples of matched-reagent packs currently being evaluated by laboratories in the United Kingdom are discussed.

The cortisol method is a solid phase assay performed directly on diluted unextracted plasma using conditions of low pH where the effect of endogenous binding proteins is negligible. The pack contains cortisol-3-(O-carboxymethyl)-oxime $^{125}$I-histamine radioligand, a specific cellulose linked antiserum, 3 quality control plasmas labelled with the cortisol content estimated by GC-MS and 'standard' cortisol solutions in dexamethasone-suppressed plasma. Full details for the measurement of cortisol in human plasma using this match-reagent pack have been described elsewhere. Preliminary results from reputable laboratories in the United Kingdom show a marked improvement in their inter-laboratory coefficient of variation.

The progesterone method, which has been developed for non-specialist laboratories, employs an enzymeimmunoassay procedure. This technique uses progesterone-lla-hemisuccinyl-horseradish peroxidase conjugate as the 'label' and an antiserum raised in New Zealand white rabbits, using lla-hydroxyprogesterone-ll-hemisuccinate coupled to bovine serum albumin as the immunogen. The antiserum was then coupled to microcrystalline cellulose ready for use in the solid-phase enzymeimmunoassay procedure. Details of the analytical protocol, which involves a petroleum ether extraction step, are available from the authors (B.G. Joyce & D.R. Fahmy, unpublished data). The constituents of this pack, which are routinely used to assess luteal function, have provided values for National external quality control plasma pools which are in agreement with consensus values from those laboratories using conventional radioimmunoassays. Further development of this solid-phase enzymeimmunoassay procedure now allows an assessment of 'total'
progestin during the luteal phase of the cycle directly from diluted unextracted plasma. Since preliminary data indicates that the profiles for 'total progestin' and progesterone are similar (B.G. Joyce & D.R. Fahmy, unpublished data) this improved assay method may be suitable for the assessment of luteal function directly from plasma. The advantages of solid-phase enzyme immunoassay, such as the elimination of any radioactivity hazard and time-consuming counting procedures, reduced running costs, their adaptability to standard automated systems and the long shelf-life of the enzyme label, make enzyme immunoassays likely candidates for the next generation of immunometric methods.

It is important that these and other matched reagent packs are evaluated with the same rigour as the more conventional radioimmunoassays. All reagent packs must contain comprehensive statistical information that is truly representative of the product and where possible statements that indicate accuracy of the method should be included.

NATIONAL QUALITY CONTROL SCHEMES FOR OESTRADIOL-17β AND PROGESTERONE

Data from the NQC schemes for oestradiol-17β and progesterone, organized from these laboratories, have been analysed from 1976 - 1979. The result of these studies, wherein the co-ordinator has exercised both an analytical and an educational role, has been an increase in the degree of uniformity of assay performance between participating laboratories. The availability of oestradiol-17β levels assigned to quality control plasma pools from GC-MS analysis has been an important factor in achieving this goal. GC-MS values for plasma progesterone have been more time consuming to develop but values are now becoming available.

The use of certified reference materials in external quality control programmes is considered to be a major advance in assessing laboratory performance. In the case of steroid hormones, their use obviates the need to rely solely on the consensus value of the participating laboratories.

The choice of reference material for this purpose is of crucial importance if the maximum benefits are to be gained. This material by definition is 'a substance or material one or more properties
of which are sufficiently well established to be used for the calibration of an apparatus or for the verification of a measurement method. Consequently the plasma matrix of the reference material should be representative of those plasma samples which are to be routinely analysed. For example, a certified reference plasma with abnormal steroid concentration ratios may produce biased results by virtue of different degrees of cross-reaction in a particular immunometric assay. With these reservations borne in mind, the use of certified reference plasmas are expected to play a major rôle in the validation of assay procedures especially for steroid hormones.

CONCLUSION

In this report, attention has been focussed on some of the factors necessary for improving standards of quality in immunometric assays and their relevance to laboratories participating in NQC schemes as described. These have included the 'accuracy', precision and robustness of assay procedures as well as improved methods for internal quality control.

ACKNOWLEDGEMENTS

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REFERENCES

ANALYTICAL PERFORMANCE CHARACTERISTICS AND CHRONOBIOLOGICAL CONSIDERATIONS IN QUALITY CONTROL

D. W. Wilson, A. B. J. Nix, R. J. Rowlands, K. W. Kemp and K. Griffiths

INTRODUCTION

The Tenovus Institute is engaged upon a number of multi-centre collaborative cancer projects throughout the United Kingdom and elsewhere, which involve analyses of hormones and other analytes in biological tissues and fluids.

One such project, conducted in association with the British Prostate Study Group, was the measurement of plasma hormones in patients with cancer of the prostate (1). The purpose of the plasma analyses, *inter alia*, was to relate pre-treatment hormone levels to initial staging of the disease at first presentation and then to correlate these findings with the survival rates of groups of patients classified according to primary tumour grading and metastatic status.

It soon became clear that stringent control procedures were necessary to monitor the quality of hormone assays; otherwise statistical inferences may relate to analytical performance rather than to clinical aspects of the study. Since quality control (QC) procedures investigated at that time (1976) proved inadequate, a QC study group was established with the Department of Mathematical Statistics and Operational Research, Cardiff, to study in depth problems associated with the quality of analytical results obtained by immunoassay.

The problems associated with the design of effective QC schemes became obvious whether consulting the perfective, altruistic or routine analyst. They generally required an accurate, precise, sensitive, and robust assay that was easy to operate, had a high throughput and turn-around time, and was easy to control. This report concerns some of the analytical and statistical developments made to improve the quality of service to the clinician and, consequently, health care of the patient. It would be negligent to ignore the chronobiological aspects of QC since the quality of the sample is, *inter alia*, time dependent. The practice of using the value of a single sample to compare with ‘normal’ ranges for the assessment of the patient’s health, requires extensive revision if the clinician is to interpret the results more meaningfully.

ANALYTICAL PERFORMANCE CHARACTERISTICS

Quality control can be defined as a set of operations intended to maintain or improve the quality of a product (2). In the context of clinical chemistry this generally refers to the quality of analytic determinations for biological tissues and fluids which are often a necessary aid to diagnosis and treatment of disease. This set of operations generally includes an analytical method, which is a written set of instructions completely defining the procedure to be adopted by the analyst in order to maintain the required analytic result (3, 4), an analyst, reagents, apparatus, laboratory and sample which is to be assayed. Performance characteristics (3-8) such as accuracy, precision, bias and other such parameters are not necessarily invariant properties of the analytical method. They are properties of the analytical errors, preferably referred to as the random or systematic uncertainties (9) of the method. The definition of performance characteristics of an analytical method used under a given set of experimental conditions is that they are a set of quantitative and experimentally-determined values for parameters of fundamental importance in assessing the suitability of the method for any given purpose (3). It is of crucial importance, when publishing or adopting a method designed for a particular purpose, that all the relevant details of the
analytical method, together with experimentally determined tolerance limits, are made available and performance characteristics known.

Progress in the development of analytical procedures in clinical chemistry should be directed towards improved specificity and precision, these being implicit statements of accuracy. To accomplish these objectives it is necessary to delineate random and systematic uncertainties rather than rely on the vagaries of a simple mean result (10), a task often made difficult when the Central Limit Theorem is not applicable (11).

Random uncertainties associated with the stages of the analytical procedure may be evaluated by repeated measurements. These include the uncertainties of handling, such as pipetting reference and calibration materials, inherent features of the analytical procedure and the random response of instrumentation. Random uncertainties may also be associated with the heterogeneity of materials. Provided the numbers of repeat observations are large enough \( n \geq 32 \) then tests of Normality, such as the Kolmogorov-Smirnov test, can be applied. For example, the number of observations falling within a particular class interval can be compared with those expected for a particular distribution using the \( \chi^2 \) distribution (12).

Systematic uncertainties have first to be recognized by the analyst and successful identification is often highly dependent on experience, judgement and intuition. Tolerance limits may then be imposed on these systematic uncertainties, but the allowed range may not be symmetrical about the mean. Two methods have been suggested (9, 13). If \( y = f(a, b, c \ldots) \) is a function dependent on a number of independent physical measurements denoted by \( a, b, c, \ldots \), then the systematic uncertainty \( \Delta y \) due to systematic uncertainty \( \Delta a \) is given by the partial differentiation equation

\[
(\Delta y)_a = \left| \frac{\delta y}{\delta a} \right| \Delta a
\]

Similar equations can be derived for systematic uncertainties in \( \Delta y \) for \( b \) and \( c \). The first method, which overestimates the error of \( \Delta y \), is given by the chain rule as follows:

\[
\Delta y = \left| \frac{\delta y}{\delta a} \right| \Delta a + \left| \frac{\delta y}{\delta b} \right| \Delta b + \left| \frac{\delta y}{\delta c} \right| \Delta c \ldots
\]

and the second method, which often under-estimates \( \Delta y \), is by quadrature addition of the form

\[
(\Delta y)^2 = \left( \left| \frac{\delta y}{\delta a} \right| \right)^2 (\Delta a)^2 + \left( \left| \frac{\delta y}{\delta b} \right| \right)^2 (\Delta b)^2 + \left( \left| \frac{\delta y}{\delta c} \right| \right)^2 (\Delta c)^2 \ldots
\]

often referred to as the 'propagation of errors' method (14). The statistician may derive aesthetic equations for systematic uncertainties but in practical terms these are often difficult to quantify. These uncertainties would include instability of analyte, biological matrix and reagents; changes in physico-chemical properties of the analytical procedure; bias inherent in the analytical curve; the presence of background noise; instrumental drift; method of calculation of final result and many others depending on the procedure employed.

The published analytical procedure for the measurement of any analyte in clinical chemistry should include and quantify every identifiable source of random and systematic uncertainty together with their component and overall limits associated with the final result.
IMPRECISION

When a number of identical aliquots of a stable homogeneous sample are analysed, experience has shown that they differ from each other, usually randomly, according to some frequency distribution. In order to assess the imprecision component of the analytical 'error', some measure of uncertainty must be attached to the result which is often expressed as a measure of central tendency such as the mean, mode or median or some other property of the distribution. In general, it is assumed that the observations are randomly distributed around the measure of central tendency denoted by the mean. If the variable is a centered random variate with an expectation of zero then the imprecision is defined by the variance which is the square root of the expectation of the square of the centered variate. It is often preferable to quote the relative imprecision as the ratio of standard deviation to the absolute value of the arithmetic mean.

For the homogeneous set of results, i.e. a random sample from a population, an unbiased estimate of the population variance, \( \sigma^2 \), is given by

\[
s^2 = \frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2
\]

and the coefficient of variation (CV), expressed as a percentage, is given by

\[
CV = \frac{100s}{\bar{x}}
\]

where \( x_i \) is the \( i \)th individual observation and \( \bar{x} \) is the mean of all \( n \) results.

PRACTICAL IMPLICATIONS OF IMPRECISION

(a) Sensitivity and working range of the analytical curve

Sensitivity may be defined as the minimum detectable analyte concentration (\( \Delta x \)) that can be distinguished from zero (15-21). Expressed numerically, it is that concentration of analyte (\( x \)) that changes the mean value of the response metamer at zero analyte concentration from a value \( y_1(x=0) \) to \( y_2(x=\Delta x) \) such that \( y_1(x=0) - y_2(x=\Delta x) \) may be made equal to \( \sigma_{y_1}(x=0) \). Thus \( y_1(x=0) - y_2(x=\Delta x) = \sigma_{y_1}(x=0) \).

However, the variance of the difference between \( y_1(x=0) \) and \( y_2(x=\Delta x) \) is given by

\[
\sigma_{y_1y_2}^2 = \sigma_{y_1}^2 + \sigma_{y_2}^2
\]

Let \( \sigma_{y_1}^2 = \sigma_{y_2}^2 \) (a reasonable assumption in numerous analytical situations) then

\[
y_1(x=0) - y_2(x=\Delta x) = \sqrt{2} \sigma_{y_1}
\]

or more simply expressed as \( \Delta y(x=\Delta x) = K\sigma_{y_1} \). The probability of detecting an analyte concentration above zero can be obtained from Normal probability tables (22) when the number of replicate determinations of analyte is known (23).

The working range of the analytical curve depends on its precision profile, that is to say it is related to the coefficient of variation in analyte concentration. This precision profile (21, 24) passes from infinity at \( x=0 \) and approaches infinity as the calibration curve becomes asymptomatic at larger values of \( x \). The working range of the standard curve can be located...
at a fixed value of the coefficient of variation associated with the region above the nadir of the precision profile.

The maximum sensitivity that the entire system is capable of achieving can be predicted if the analytical system is adequately described by a mathematical model, the parameters of which have real significance in relation to the physico-chemical properties of the system rather than some empirical relationship. Optimization of immunoassay by Ekins (25) using the Law of Mass Action (26), combined the slope of the calibration curve

$$\frac{dy}{dx} = \frac{\Delta y}{\Delta x}$$

where $\Delta y$ and $\Delta x$ have been previously defined (vide supra), such that

$$\Delta x = \frac{dx}{dy} \frac{\Delta y}{y}$$

It simply remains for the components of uncertainty in $\Delta y$ (21, 25) to be identified and the value of the absolute maximum sensitivity can be computed (25, 27, 29). This is accomplished using values for the equilibrium constant and binding site concentration previously calculated from a calibration curve (30, 31).

This approach has been improved mathematically to allow for the inefficiency of separation of free from bound analyte, dissociation of bound complex at different dilutions of antibody and for various concentrations of dextran-coated charcoal (D. W. Wilson, unpublished data). In this situation, experience has shown that the extra laboratory effort required to elicit additional parameters for optimization are not generally cost-effective. The preferred method of optimizing an assay for maximum sensitivity is the application of a simple optimization computer program followed by an empirical means of laboratory trial and error. This process may be repeated as many times as is felt necessary. Knowledge of the uncertainties of the components in the model allow the application of Monte Carlo methods (32, 33) to find the dispersion of CV values around the nadir of the precision profile. The Student-t test may then be applied using these results at the nadir to compare dispersions at other locations along the precision profile. When the differences between the populations are, for example, highly significant at just $p \leq 0.01$, then the upper and lower values of $x$, now defined on the calibration curve, will provide the upper and lower limits of the useful working range of the analytical curve (34).

During the past decade, clinical chemistry has witnessed an unprecedented improvement in assay sensitivity, particularly for steroid hormones which can now be measured in the femtomolar range (35, 36). Recent studies instigated at the Tenovus Institute sought to determine the limits of sensitivity brought about by the statistical uncertainties of molecular collisions. It would appear that the Law of Mass Action model is only valid above a concentration of 10⁶ molecules for each reactant, whereupon the coefficient of variation was estimated to be 2.5% (37).

(b) The calibration curve

This usually consists of repetitive values (i) of the response metamer, $y_i$, for each analyte concentration, $x_i$, where $i = 1, 2, \ldots, n$. The regions between the data points are interpolated by graphical or numerical means so that estimates of analyte concentrations can be made in samples to be assayed. The graphical method is time consuming in a high throughput laboratory and is often biased according to the analyst. The numerical approach is unbiased with regard to its mathematical curve of best fit provided, inter alia, the ‘true’
minimum sum of squares is reached, but its use may be biased, particularly at the extreme
ranges of the calibration curve where the model becomes inappropriate.

For gas chromatography-mass spectrometry techniques, the response metameter \( y_i \) at
is often linear over a substantial part of the range of analyte concentrations to be assayed.
However, certain factors may distort the extreme range of the calibration curve and a non-
linear function may well provide a better fit over the whole range of analyte concentrations
of interest. Two types of model have been used in the Tenovus Institute; one is based on the
physical properties of the system and the other is an empirical function that adequately
describes the data (38). The suitability of any function should be assessed by the goodness-of-
it (39), the randomness of the residuals using the runs and sign test (40) and the range over
which it is of practical value.

In immunoassay, the functions used to describe the sigmoid shape are almost innumerable;
the important ones that are cited in the reference list fall into those classes of models
based on the Law of Mass Action (21, 30, 31, 41-48), hyperbolics (25, 48-53), polynomials
25, 53), logarithmic (48, 54, 55), logit (56, 57), logarithmic-logit (56-58), arc sine (60), and
others (61) such as the use of the spline function (62). In our laboratories we have used almost
exclusively the methods of Rodbard and Hutt (56) and Faden and Rodbard (28) which we
have found over several years to have wide applicability for many different immunoassay
systems and results have been found to be satisfactory (63). Nevertheless, having selected the
function of ‘best’ fit and computed the fiducial limits of the analyte concentration in the
sample, one may still be presented with the problem of ‘outliers’ and non-Normal distribu-
tions. Erugation of the robustness difficulties associated with parameter estimations in
mathematical modelling are left to the more erudite statisticians, the work of Tiede (64),
Tukey (65), and Huber (66-68) being prominent since many methods relating to the theory of
parameter estimation are due to Gauss (69).

c) Outliers

Most laboratories observe values of the response metameter, \( y_i \), which do not appear to
be in consensus with the rest obtained for that particular standard, \( x_i \), or indeed with other
iliquots of the same sample assayed. If the uncertainties of the response metameter are
homogeneous for locations along the calibration curve, then Cochran’s test (70) can be
applied to the largest variance such that it may be judged to be within specified tolerance
limits.

The recent program of Faden and Rodbard (28) can be used to form bins of variances
for a given range of sample values. This being the case, Cochran’s test may be applied to
reject a set of replicate values, the rejection values being made on the dispersions associated
with samples rather than standards. Guidelines for the treatment of outliers have been docu-
mented previously (63) and procedures worth investigating for rejecting outlying values
from a small number of replicates have been suggested (71-73). It must be emphasized that
there is no universal procedure for the treatment of outliers (note the method of Fulld (73) in
this volume) due essentially to marked heteroscedasticity along the calibration curve and
among the samples themselves. Despite what has been discussed on outliers, the non sequitur
basis for outlier rejection is still some rule-of-thumb of say16-20% relating to the individual
CV or group CV for a sample of aliquots. Much effort and time will have to be expended if a
more universal system is to be developed.

(d) Internal quality control

Despite all the precautions taken to ensure that an analytical procedure will provide the
necessary results, performance of the test may temporarily deteriorate due to instability of
the analyte or sample matrix during storage, change in analyst conducting that particular assay or for a whole host of reasons that may not be apparent at the time.

To monitor the temporal performance of an analytical procedure, aliquots are taken from bulk preparations of homogeneous plasma (generally corresponding to high, medium and low analyte concentrations) and properly stored at \(-20^\circ\). These QC samples are placed in the same location \((74, 75)\) in every consecutive batch of assays and the values of their various statistics are monitored \((76)\) and computed \((77)\). Two features of industrial applications of QC \((78, 79)\) contrast with the situation in medicine. These are that the industrial plant would be shut down if the average number of batches of products that were judged to be out-of-control when they were still in-control were too high and, secondly, the number of samples that can be taken for analysis is large in comparison with clinical chemistry.

Since it has been shown that cumulative sum schemes (CUSUMs) are among those most efficient for detecting both large and small changes in the analytical system \((74, 80)\), then this is the preferred method and current work indicates that, of the schemes currently evaluated, CUSUMs appear to be superior (Rowlands, unpublished data). This has now become more widely recognized since the application of the V-mask \((81-86)\). Of the four British Standard documents on guides to data analysis and QC using CUSUM techniques being prepared, the first two are referenced \((87, 88)\). The application of CUSUM techniques to monitor analyte determinations in clinical chemistry laboratories was designed to control the mean level of 3 QC pools as well as their imprecision and drift within a batch of assays \((74-76)\). In our laboratories we have superceded the V-mask with an equivalent decision interval scheme \((86)\).

Computer programs have been developed for a main-frame computer \((77)\), together with an extremely versatile, easy-to-operate, interactive version for the PET and other micro-computers \((89)\) and the PDP11/34 mini-computer (unpublished data). The output of all these programs is designed to afford an instant assessment of the 'control' status and to provide graphical records for some or all of the QC charts, for each of the 3 statistics \((viz.\ mean,\ imprecision\ and\ drift)\) for each of the 3 QC pools. The system has recently been updated to accommodate more pools should that be necessary.

An important practical detail is that the target statistics can be modified if the initial estimates needed to initiate the scheme were imprecise. Also, when an assay is judged from the graphical records to be going out of control, new plasma pools should be placed in the subsequent batches of assays to obtain new target statistics in order to check whether it is the QC control pool that is changing or the analytical procedure itself. Occasionally, one of the duplicates from a QC is missing and so, rather than abort the run on this basis when all the other statistics are judged to be in-control, a random number is generated based on the expected imprecision of the assay. Experience will determine whether or not this is sound practice.

The applications of CUSUMs to clinical endocrinology are discussed later in this volume \((76, 79)\) but significant contributions to the literature have been made by others, some of which are cited \((90-99)\).

(e) Assay robustness

Robust assays are those in which perturbations brought about by changes in temperature, reagents, labelled ligands, antibodies, etc., are minimal. Let us suppose that an analytical procedure has \(N\) independent uncorrelated stages each with a variance \(\sigma_i^2\), then the total variance \(\sigma^2\) associated with the result can be approximated according to the Central Limit Theorem \((11)\), such that if the individual stages are linear then

\[
\sigma^2 = \sigma_1^2 + \sigma_2^2 + \ldots \sigma_N^2.
\]

It would seem logical that assays with fewer stages and comparable variances at each stage of
the analytical procedure would have lower precision *ceteris paribus*. This is true, for example, in the simple direct radioimmunoassay for plasma cortisol (100) which features a 125I-labeled ligand and a solid-phase separation technique. Unlike many conventional immunoassays, no extraction of the steroid is necessary. Incubations with label and solid-phase antibody are carried out in the same tube and, after equilibration and centrifugation, the solid-phase antibody containing the labelled bound hormone is counted in a γ-counter. Such a system has fewer stages requiring manual manipulation and therefore the widespread use of systems of this kind should be encouraged. Of particular relevance is its robustness with regard to different analysts.

(f) Analyst uncertainties

In a routine hormone laboratory with a high through-put of several types of assays, such as those performed at the Tenovus Institute, a major problem in monitoring analytical performance is due to differences between the analysts (101). Using the same assay, analysts can not only vary with respect to their imprecision but also have different biases. This is of major concern. Correction factors for each analyst have been suggested for those with comparable imprecision and this would probably work in practice. Instead, we have adopted the policy that only those analysts who produce a comparable and acceptable performance for a particular assay are allowed to do that assay. This means that the organization of the laboratory staff is restricted by the lack of interchange between analysts for all assays. Nevertheless, it is only by training, education, adopting these sorts of safeguards and developing analytical procedures have we been able to achieve consistently good performance over many years. We look forward to the time when analytical procedures will be fully automated and some of these problems will be overcome.

(g) External quality assessment schemes

Adopting the necessary technical and statistical skills (*vide supra*) the results returned by the designated laboratory analyst for external QC pools distributed by the scheme coordinator should reflect the analytical procedures used by participants. The rôle of the organizer is to analyze these results in order to assess laboratory performance. Participation should be mandatory because immunoassays are being increasingly used in diagnosis and monitoring of patient treatment. To date, the external assessments have generally relied upon a few basic statistics to achieve their objective (102, 103). These include an assessment of imprecision and bias calculated from results obtained from the same or different pools of material despatched by the organizer. It is clear, even to an empiricist, that the linch-pin of the effectiveness of an external assessment scheme is a proper evaluation of the imprecision of each laboratory (104). In practice, let us suppose that 6 pools are measured on average four times a year in each laboratory. Furthermore, if the imprecision of the data from the same pool has a CV of 10% then the 95% confidence region of CV estimates lies between 2%, and 18% and the distribution is skewed indicating a tendency to underestimate the CV. If the CV is 15%, and this is probably nearer the truth for most hormone assays, then 95% of the coefficient of variation estimates lie between 4% and 27%. At a CV of 15%, even if the number of determinations is increased from 4 to 10, then 95% of the estimates range from 8% to 22%. On what basis then can the organizer say to the participant, who has a true assay CV of 15%, that because the last month’s imprecision was 8% and this month it is 23%, then the assay imprecision is increasing? The same basic concept and criticisms can be directed towards a ‘running coefficient of variation’ and Variance Index, Score, which try to provide an overall index of laboratory performance (104). Calculations of bias for a particular laboratory in relation to the group mean for all laboratories is dependent on the imprecision of the participating laboratories (104). The basic problem does not necessarily relate to the
statistics used but is due mainly to an insufficient amount of data available to the organizer. Organizers of external assessment schemes should be cautious in reporting and offering advice to analysis of participating laboratories especially if the number of pools despatched to assess imprecision is small and particularly so if powers of sanction are brought to bear as to whether or not a laboratory should be allowed to practise (105).

The problem of inadequate data for organizers of national assessment schemes may be readily solved by preparing a large bulk of material which could be distributed to laboratories for use as internal QC material. In this way, the organizer would have sufficient data to make objective statements concerning temporal performance and degree of concordance with other participants. Rodbard (personal communication) has suggested that participants may adopt a hybrid scheme such that only one of the internal QC plasmas is that distributed by the organizer. The use of internal QC data (106) to assess laboratory performance should be accompanied by samples despatched by the organizer at frequent intervals as 'spot checks' on individual laboratories. In this way, the first mandatory statistical objective of an external quality assessment scheme may be achieved, viz., intra- and inter-assay imprecision as judged from internal QC data for each participating laboratory. Estimates of bias would then become more meaningful and analytical sources of uncertainty as well as inter-laboratory discordance revealed using appropriate statistical methods.

For analytes with a well-defined molecular structure, such as the steroid hormones, physico-chemical methods of analysis, in particular gas chromatography-mass spectrometry (38, 108-111) can be used to determine more specifically the amount of analyte in a particular material. Indeed, reference standards (112) and reference plasmas may now be made available (113). These reference plasmas with assigned values could then be distributed to participating laboratories of an external quality assessment scheme for the purposes of calibrating or evaluating a laboratory assay method.

In this report, some basic concepts have been discussed which should provide the basis for improved QC in clinical chemistry. However, there are at least three major areas which have not been discussed. These are (a) the clinical interpretation of results, (b) the usefulness of assays requested within the National Health Service of the United Kingdom and (c) the chronobiological implications in relation to QC. The first two of these are beyond the scope of this Workshop but attention is drawn in the concluding section of this paper to the implications of biological rhythms in QC.

**CHRONOBILOGICAL CONSIDERATIONS IN QUALITY CONTROL**

Claude Bernard (114, 115), the eminent nineteenth century physiologist, clearly indicated that the homeostatic viewpoint of organic life was, in principle, fallacious. He stated that life cannot be solely explained by an internal principle of action but rather exists as a conflict, not a battle, with the exterior physico-chemical conditions to which it is exposed. It thus seems logical that the integration of neural, cellular and humoral variations of substances that constitute temporal aspects of physiology is different in health and disease. Following the recognition that body rhythms are indeed manifestations of a *consensus partium in tempore* (116), it would seem that their characterization and quantification in health and disease may allow the identification of rhythmic changes that mark the progression of a 'healthy' subject to one with overt disease. In an analogous manner to a biopsy, the chronopsy (such as the haemopsy, salopsy and thermopsy which are temporal phenomena in blood, saliva and temperature, respectively) may enable the clinician to define rhythms in health, screen for disease or even assess the efficacy of a particular chronotherapeutic regimen.
These biorhythms, existing as regulatory or oscillatory systems (117), have a wide frequency range (118). In the human, for instance, the following examples may be cited: the beta waves of the brain at 50 cycles per second; the heart beat at 70 cycles per minute; 90 minute cycles in light to deep sleep; 90 minute cycles in narcolepsy; 12 hour and 24 hour periodicities in breast skin temperature; circadian (24 hr) rhythms (119) in a wide variety of physiological variables (120); circatrigintan (monthly) rhythms of physiological change, particularly evident in the premenopausal woman; circaseptan (weekly) rhythms in some endogenous and artefactual socio-ecological or religious environments (121); circaseptan rhythms of, for example, human kidney transplant rejection; circannual rhythms of hormones such as prolactin (120); the circaseptuagenarium rhythm of life existing from conception to death. In the case of the human being, it is a spectrum of rhythms that controls health and determines work efficiency, moods, psychomotor skills and other activities (122, 123). Their importance in defining health and disease, preventive health maintenance and treatment (124) cannot be ignored and it is in the context of biological QC that discussion of hormone rhythms \textit{(vide infra)} has relevance in clinical endocrinology.

It is common practice in clinical endocrinology for analytical results for a patient to be compared with a so-called ‘normal’ range. The syllepetic ambiguities of the ‘normal’ range in relation to health and statistics \textit{etc.}, require that the concepts of ‘normal’ range be re-examined. In this context, the term ‘normal’ range should be abandoned (125-127) and replaced by ‘reference’ range, which requires an explicit and precise description of the population and the techniques employed for the collection, transport, storage, method of analysis and other relevant factors concerned with QC. Reference ranges should be constructed according to whether the subjects are ambulant, supine, hospitalized, domiciled as well as being stratified according to age, body weight, obesity, intake of medicines and a wide range of physiological, pharmacological, environmental and analytical factors. Nevertheless, the concept of the ‘homme moyen’ (128-129) enunciated by Quetelet still applies, though now to a reference population. Historically, the reference group would provide only the mean value (10) together with the range bounded by maximum and minimum values (130). The statistical developments of Galton (131), Pearson (12, 132, 133), Gosset (134) and Fisher (135) provided the means by which a so-called ‘normal’ range could be constructed (136). The practicability of the 95\% range was established for ‘normal’ values (137-138) although data not Normally distributed (139) may require transformation (140). The effect of the analytical uncertainties on the ‘normal’ range is of crucial importance. Some workers have recommended the use of non-parametric statistics to overcome this (141), but strict attention to criteria of accuracy, imprecision, drift and internal QC are advocated prior to any data manipulation. Finally, chronobiological quantification of reference ranges may provide the clinician with a more useful diagnostic aid in medicine.

Circadian rhythms of physiological variables are well known and unless a sample of biological material is time-qualified, its use in constructing reference ranges, or its comparison with reference samples, is of limited value. An obvious example concerns a human plasma cortisol measurement that is not time-qualified. A patient may be diagnosed as ‘normal’ but may well also be interpreted as having Cushing’s or Addison’s disease depending on what time of day the blood sample was taken (142).

Time-qualified references ranges can be constructed such that the time span of interest is divided into a number of bins of, for example, two hour intervals over a time span of 24 hours. Values of the physiological variable or some suitable transformation of the data, obtained from a homogenous population, are tested for having a Normal distribution using the Kolmogorov-Smirnov test (143). Time-qualified reference ranges, known as chronodesms (144), can then be constructed for each bin for the time span of interest; these are known individually as monodesms and collectively over the period of interest as a
merodesm. The reference interval is calculated using the formula, $\hat{y} \pm K_s$, where $\hat{y}$ is an estimate of the mean for each bin, $s$ is the corresponding standard deviation and $K$ is a multiplier depending on the type of range chart required. These charts may be simply 95% prediction charts and values of $K$ can be obtained from tables relating to the area under the standardized Normal distribution (22). Alternatively, a tolerance chart containing a proportion of the population with a specified confidence can be constructed and values of $K$ can be found elsewhere (145). Unless otherwise stated, the chronodesm refers to the tolerance interval whereas the prediction-chronodesm refers to the prediction chart (vide supra). The use of the chronodesm is not quite so dependent on biostationarity (146) and so can be applied, for example, to reference ranges of salivary levels of progesterone during the luteal phase of the menstrual cycle (Wilson et al., unpublished data), thus enabling the clinician to assess whether or not an individual is euchronic with respect to the chronodesm describing the reference population.

Rhythmosdesms, which are tolerance bands for a particular function describing the rhythmic data, can be constructed for an individual (idio-) or for a population (pan-). The simplest model used in practice is the tolerance interval associated with the cosine function fitted to the data by the combined linear and non-linear method of least squares analysis. This cosinordesm is mathematically described elsewhere (144).

Individual reference values in clinical chemistry may be more appropriate in assessing 'health' or response to treatment, particularly when the inter- and intra-subject variability of the analyte are disproportionate in magnitude, the former predominating. Statistical methods for individual assessments made on serial measurements for the individual have been described elsewhere (147-149), although analyses involving sequential testing with correlation have been previously described (150).

The endocrine status of an individual may be more precisely defined by comparing values of the rhythm parameters of a particular mathematical function, used to fit the data, with those of a reference group, or simply by comparing the temporal changes in rhythm parameters obtained from serial measurements made on an individual. However, before proceeding to comparison of rhythm parameters, statistical tests for the presence of a rhythm and its sinusoidality must be applied (151).

Where there is a systematic change in residual values associated with the fitted function with time then correlograms, which are graphs of a particular set of autocorrelation coefficients versus some time lag $k$, can be plotted for autoregressive data and a lag may be reached after, say, 5 observations when no significant correlation exists (152). In this case, a cosine function can be used to fit selected 'uncorrelated' data of lag $k$ in a manner analogous to serial section analysis (153) devised by Halberg (personal communication). This procedure gives rise to the decimated cosinor analysis which gives a more realistic estimate of the rhythm parameters.

The comparison of rhythm parameters from a single, group and population cosinor has been described elsewhere (151). These include (i) comparison of mesor, the mean of the fitted rhythm, (ii) the joint testing of the amplitude and acrophase (the time of the crest of the fitted rhythm from some reference point), and (iii) the testing of all the rhythm parameters. Hypothesis testing for a set of parameters can be achieved using the Hotelling's $T^2$ test.

The establishment of reference ranges for individual or peer groups that are time-qualified and the possibility of comparing rhythm parameters for such groups may enable clinicians more precisely to interpret and diagnose 'health' and disease.

**SUMMARY**

In this paper, the analytical performance characteristics and chronobiological considerations in QC have been presented and may be of value to most practising clinical
chemists. It is hoped that these principles will provide the clinician and the researcher with the means by which the quality of assays can be improved. Consequently, patients should be beneficiaries of improved health care arising from these technical aspects of QC in clinical chemistry.

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REFERENCES


130. Woley, H. P. (1910) JAMA, LV, 121.
134. Gosset, W. S. — 'Student' (1908) Biometrika 6, 1.
INTRODUCTION

This paper provides an introduction to those to be presented to this Workshop which describe recent work undertaken in Cardiff on quality control (QC) (1-3). When developing techniques of the kind described at this Workshop it is important to avoid what may be described as the ‘cook-book’ or ‘do-it-yourself statistics’ approach. Many of the techniques of contemporary statistical method are based on theoretical and practical assumptions which are often not clearly stated in lay terms. There is accordingly a danger that the implications of these basic assumptions are not appreciated in a new area of application. Some methods, particularly in fields of application like QC, have been designed to achieve particular objectives in very specific circumstances. The criteria on which they are based reflect these circumstances and are not appropriate to direct application to other situations in the same field without significant modification. This is indeed the case for clinical and endocrinological control testing.

To justify this contention, this paper indicates (a) the basis on which much industrial QC is founded, (b) the need to establish new criteria relevant to clinical testing and (c) the scope for adjusting different features of control charts to achieve such criteria.

Once certain essential differences between clinical and industrial QC are realized, it becomes clear that comparisons between the various available techniques need assessment in the context of assay testing per se. This principle should become apparent from several papers presented at this Workshop.

The Cardiff approach to internal QC

The successful adoption of QC schemes in practice is by no means just dependent on getting the theory right. Essential features of the procedures need to be presented in terms easily understood and interpreted by technical personnel unfamiliar with the mathematical principles on which they are based. This aspect of implementation cannot be overemphasized. To ensure the smooth introduction and effective use of new test methods it is clearly important to involve those responsible for the organization and running of routine assay testing. To achieve a required degree of simplification it may be necessary to carry out intricate computer programming. Such objectives can be most easily achieved with the formation of a team of individuals representing the various kinds of expertise needed. In this context it should be recognized that the efficient use of small amounts of test data will probably involve consideration of techniques more recently developed and more sophisticated than simple Shewhart schemes.

In summary, new testing procedures should be devised and their utility assessed by a team of individuals able to provide a broad background of knowledge and experience. Areas of expertise which are particularly important are (a) statistical theory, (b) computer programming with particular reference to automatic computation and visual presentation, (c) the technology of the assay tests used and (d) the day-to-day in-house problems of laboratory routine testing.

This is the method we have adopted in Cardiff. Other papers in this Workshop (1-5) demonstrate the necessity and benefit of the combined approach.
The statistician's rôle

Justification of the need for areas of expertise denoted by (b), (c), and (d) above is hardly necessary. The specific involvement of the statistician calls for further comment.

As the application of statistical method has become more widespread, there has been a tendency to use it uncritically. An example was the application of early methods of experimental design to the study of industrial production processes. Many of the designs first used, planned ways in which agricultural field trials should be carried out to ensure meaningful analysis of data. Trials were designed to assess associations between factors such as crop yield and different kinds or levels of fertilizer. Minimizing the influence of fertility gradients across fields used for experimentation was an important built-in feature of the designs formulated. The relevance of such considerations when examining the performance, for example, of knitting machines in a textile mill is clearly open to question! One would expect design criteria in these two areas of investigation to be very different. It would not be unreasonable therefore to suppose that the design procedures developed for one application may need substantial modification before application to the other.

What, one may ask, has this example to do with the design of clinical test control procedures? It is important to realize the techniques we are discussing were developed primarily for the purpose of industrial QC. It is unrealistic to suppose that the criteria on which they are based can be taken as immediately relevant to clinical testing. The remarks which follow indicate that they are not. The methods we describe are based on different and more relevant criteria. In addition, it may not be justifiable to conclude that industrial QC procedures are the best that can be devised for clinical testing. It should be recognized that technical facilities available on the factory floor are often not as sophisticated as those generally available in clinical laboratories. Certain control procedures used in industry are used for no better reason than that they are simple to operate. Extra costs incurred by testing quite large samples are frequently secondary considerations.

Nature of work reported

To obtain data which can be used for comparative as well as diagnostic purposes, two areas of clinical testing need attention, namely, within laboratory consistency (internal QC) and between laboratory consistency (external QC). Papers presented at this Workshop by the Cardiff group report work we have been doing with regard to internal QC and relate to schemes for the control of the mean level and variability of the measurement of analyte in a QC pool.

COMPARISON OF INDUSTRIAL AND CLINICAL QC

The significant distinguishing factor between clinical testing and quality assessment in manufacturing industry is the difference in routine test levels normally used. In much assay testing, control material, such as a plasma pool, is expensive to prepare. Testing is frequently complex, expensive and not easily stabilized. For these and other technical reasons, once a plasma pool has been introduced it is essential to maximize its life whilst testing is in-control. One way of doing so is to limit the number of replicate tests (r) carried out when control samples are inserted into routine testing. In clinical endocrinology, the number of replicate estimations of analyte concentration in a QC pool performed within an assay batch is usually 2 or 3. For much routine industrial testing, the economics associated with sampling is relatively unimportant. Limiting the size of r does not therefore appear as a factor which needs to be seriously taken into account when formulating criteria appropriate to the design of industrial control charts. In contrast, the aim in clinical testing must be to obtain as much information as possible from limited data. For industrial applications, the required properties
of control schemes are usually obtained both by choice of technique and sample size adjustment.

The design of control schemes in clinical endocrinology needs to be rather different in that the achievement of desirable test characteristics must be attained by means other than minimal sample size adjustment.

Assessment parameters

Assessment of control procedures over recent years has tended to be based on the average run length (L). The average run length of a given scheme is the number of control batches which will be tested before the scheme indicates that testing is out of control. When the system being monitored is out of control the average run length (L₂) should be small and the corresponding run length when the system is in control (L₁) should be large.

New criteria

For schemes suitable for clinical testing which have been used in an industrial context we can enquire on what basis they were designed for the latter. Values for L₁ and L₂ are usually determined from cost considerations relating to production and the consequences of marketing sub-standard material or goods. Schemes are then designed to achieve appropriate values of L₁ and L₂, determined on the basis of these kinds of economic considerations, by adjusting the level of replication, r. A few calculations quickly show that such a free choice of L₁ and L₂ cannot be made in practical clinical testing where r is as small as 2 or 3. In these circumstances, what should be the basis for the control scheme? The criteria we have used is as follows. For the highest permissible number of replicates, r, determine from the laboratory the highest value of L₂ which can be tolerated. For these values of r and L₂ obtain the control scheme which maximizes L₁.

CHOICE OF QC TECHNIQUES IN CLINICAL CHEMISTRY

Available choice

If we cannot achieve values of L₁ which are large enough for practical use by sample size adjustment, what other options are available? We can:

(i) make a choice between testing techniques;
(ii) consider the selection of parameter values;
(iii) consider different functions of test data;
(iv) examine different sampling intervals.

(i) Testing techniques

There appear to be three possibilities, viz (a) Shewhart-type control charts, (b) cumulative sum (CUSUM) tests (with straight line decision boundaries), and (c) a combination of (a) and (b).

There is a fourth possibility which we are examining, namely CUSUM testing with curvilinear decision boundaries. The use of curved masks instead of V-masks to assess the significance of cumulated sums is a generalization of (c) which would make a combination of (a) and (b) easier to operate from a practical point of view. It would probably yield even more sensitive tests than the ones we have described so far in circumstances where test results are correlated with one another.

(ii) Choice of parameter values

(a) Shewhart charts. The procedure is well known. For a given value of L₂ we can maximize L₁ by adjusting (1) the position of the warning and action lines and (2) the number of points
between action and warning lines or above the action line, which are used as the criteria that testing is out of control.

(b) CUSUM testing. Let \( X_i \) be the control value obtained for the \( i \)th batch of control tests. The mean values of \( X_i \) when testing is in control and out of control are denoted by \( m_1 \) and \( m_2 \) respectively. We carry out the CUSUM procedure in one of two equivalent ways. (1) We can choose a value \( k \) (called the reference value) which lies between \( m_1 \) and \( m_2 \) and compute

\[
s_n = \sum_{i=1}^{i=n} (X_i - k)
\]

recalculating \( s_n \) whenever it becomes \(<0\) and concluding that testing is out of control on the first occasion that \( s_n > h \), (where \( h \) is the decision interval). (2) Alternatively, we can compute

\[
S_n = \sum_{i=1}^{i=n} (X_i - m)
\]

without restriction, plotting values of \( S_n \) against \( n \) and using a V-mask. It is concluded that testing is out of control when the path of plotted values crosses a limb of the V-mask.

Whichever method for carrying out the test is used, \( L_1 \) can be maximized by adjusting the two parameter values, \( h \) or \( k \), for (1) or, equivalently, the lead distance \( d \) of the V-mask and its semi-vertex angle \( \theta \) for (2).

(c) Combined use of Shewhart and CUSUM. If both techniques are used simultaneously, six parameters can be adjusted and, in addition, the number of points between and above action lines varied.

Choice of parameters under the above headings should provide sufficient emphasis for the need to involve statisticians capable of performing the theoretical evaluation necessary to identify maximal schemes. The problem is clearly one of constrained maximization. For a given out-of-control value (\( m_2 \)), we introduce a search procedure to locate the maximum \( L_1 \) (at \( m_1 \)) and identify \( h \) and \( k \) for the CUSUM procedure or the position of warning and action lines, etc., for the Shewhart-type charts.

To computerize the process requires a subroutine which permits accurate computation of \( L_1 \) and \( L_2 \). There are serious theoretical difficulties for the simulation approach. Computation of \( L_1 \) and \( L_2 \) for Shewhart charts presents no particular difficulty from the theoretical point of view although the computer programming required is not particularly straightforward. This has not, however, been the case for the CUSUM procedure. Tests we obtained have been formulated to monitor variability and mean levels of analyte in a QC pool. The latter has required considerable mathematical effort to obtain expressions which yield values for \( L_1 \) and \( L_2 \) with sufficient accuracy for this purpose.

(iii) Function of test data

To illustrate the element of selection available to increase \( L_1 \) by using test data in different ways take, for example, the control of variability. Suppose this is to be achieved by using schemes designed to control the test standard deviation, \( \sigma \).

We denote the target value of \( \sigma \) which we need to achieve when testing is in control by \( \sigma_1 \), and that when testing is judged out of control by \( \beta \sigma_1 \) (\( \beta > 1 \)). We fix \( L_2 \) when \( \sigma = \beta \sigma_1 \) and
seek schemes which give us the largest values of $L_i$ when $\sigma = \sigma_1$.

There are a number of alternative sample statistics related to $\sigma$ which can be used. It was not obvious to us which of them would give optimal schemes in the above sense. We have considered just two, namely $S_i = S/\sigma$ and $S_i^2 = S^2/\sigma^2$ where

$$i = r$$

$$S_i^2 = \frac{1}{r} \sum_{i=1}^{r} (x_{ni} - \bar{x}_n)^2$$

$x_{ni}$ is the test value obtained for the $i$th sample of the $n$th batch of $r$ replicates. The calculations reported in Tables 1-3 are based on the assumption that all $x_{ni}$ are Normally distributed with common standard deviation, $\sigma$.

Values of $L_i$ and $L_j$ were computed for Shewhart schemes where the points plotted are the values of $S_i$ or $S_i^2$, and for cumulative sum procedures when $[S_i - (reference\ value)]$ or $[S_i^2 - (reference\ value)]$ are cumulated. Our calculations indicate that there is a substantial advantage to be gained by using $S_i^2$ rather than $S_i$ for both Shewhart and CUSUM charts (2). A brief summary of some of the values computed is also given below.

(iv) Sampling interval

In a number of clinical assays an important feature of the control test is the average number of samples likely to be processed when testing is out of control. Suppose there are $b$ samples between successive QC determinations. It is usual to call $b$ the sampling interval. For a scheme with out-of-control average run length $L_2$, the average number of routine tests dealt with during the out-of-control period before an indication that testing is indeed out of control, $B_2$, will be $bL_2$. The corresponding quantity when testing is in control, $B_1$, will be $bL_1$. Additional maximization of $L_i$ or $B_i$, for the same life of a plasma pool, can be achieved by increasing the value of $r$, proportionately increasing $b$ and decreasing $L_2$. Thus if the sampling interval is increased to $\frac{5}{3}b$ and $L_2$ is decreased to $\frac{2}{3}L_2$, the resulting value of $B_2$ is unchanged. Suppose the total number of control samples that can be obtained from a plasma

<table>
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*Non-entry signifies values are too small for practical interest.
pool is N. The life of this pool in terms of batches which can be tested is clearly \( l = (N_b/r) \). Obviously \( l \) does not change if \( b \) and \( r \) are adjusted by the same proportion. We can increase \( r \) from 2 to 3 and increase \( b \) to \( 3b/2 \) without affecting \( l \). In view of these considerations we asked the following question. In circumstances where \( B_2 \) is the appropriate parameter value to fix, can better test features in terms of \( L_1 \) or \( B_1 \) be achieved by inserting control samples less frequently into routine testing and proportionately increasing replication?

Examples and tables given in the following section show that substantial improvement in the properties of maximal schemes can be achieved in this way. From a practical point of view it is obviously necessary to reach a compromise between the achievement of increased values of \( L_1 \) or \( B_1 \) and the consequences likely to arise from decreasing the sampling frequency.

**Table 2**

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**Table 3**

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<th>( \text{CUSUM schemes} )</th>
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*Non-entry signifies values are too large for practical interest.
COMPARISONS OF APPROACHES TO THE CONTROL OF TEST ERROR

Some of the calculations we have carried out for the control of test error are shown in Tables 1 and 2. They show the maximum values of $L_1$ which can be achieved for $r = 2$, according to the control test statistic being used. Table 1 shows the kind of increase in $L_1$ which can be obtained by choosing one statistic rather than another. It shows comparisons between various optimal CUSUM schemes, illustrating that $(S^2/\sigma^2)$ is better than $S/\sigma$. Our calculations revealed that this was also the case for Shewhart schemes. Table 2 shows typical comparisons between optimal Shewart and CUSUM schemes using $(S^2/\sigma^2)$. It is clear that for the values of $L_2$ and $\beta$ shown, the CUSUM is uniformly better than the Shewhart. The increase in $L_1$ which can be obtained by increasing $r$ from 2 to 3 for CUSUM schemes is shown by comparing Table 1 and Table 3.

An example of the differences in $B_1$ which can be achieved by increasing $r$ and the sampling frequency is also illustrated by Tables 1 and 3. Taking the values $\beta = 1.75$, $L_2 = 6$ and $L_1 = 81$ in Table 1, it is clear that $B_1 = 81b$ and $B_2 = 6b$ when the number of routine tests between control insertions is $b$. If control samples are inserted every $\frac{1}{2}b$ tests the equivalent value of $B_1$ is given if we take the scheme which relates to the first row of Table 3. For this scheme, $B_1 = 142.5b$. As a second illustration, take $\beta = 2$, when (Table 1) $L_2 = 6$. $B_2$ is $6b$ and $B_1$ is $303b$. From Table 3 it is clear that the corresponding value of $B_1$ when $r = 3$ is $838b$.

In view of our earlier comments about the limited size of plasma pools or control material, values of $B_1$ as large as $838b$, for example, could be somewhat academic. There may then be a value of $B_1$ which is large enough for practical purposes. In these circumstances there could be situations where we should consider minimizing $B_1$ for a fixed $B_2$. It follows from our last examples that this should be possible by using optimal schemes of Tables 1 and 3 and adjusting $r$ and the sampling interval $b$. Suppose, for example, that $B_1$ is large enough once it reaches $400b$. Interpolation from our calculations shows that if we achieve a value of $B_1 = 400b$ using $r = 2$ and sampling interval $b$, then $B_2 = 9.07b$. The optimal scheme with $r = 3$ and sampling interval $3b/2$ has the smaller value, $B_2 = 6.6b$.

ACKNOWLEDGEMENTS

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REFERENCES

1. Wilson, D. W., Nix, A. B. J., Rowlands, R. J., Kemp, K. W. and Griffiths, K. this volume, p.5.
APPLICATION OF CUSUM TECHNIQUES TO MONITOR ERROR IN HORMONE ASSAYS


INTRODUCTION

The need for efficient internal quality control (QC) procedures in clinical endocrinology and related disciplines is now well recognized (1). It is becoming evident from recent literature that the search for more efficient control procedures is synonymous with developing new control schemes and decision rules. There has been very little effort directed towards finding the 'best' control scheme for a given control procedure. This report is not concerned with proposing yet another control procedure but rather with a comparison of some

Fig. 1. Three control procedures used for comparison. (a) Ordinary Shewhart chart which gives an out-of-control signal if any point falls in the rejection zones bounded by $K_1$ and $-K_2$. $m$ and $\sigma$ are the predetermined mean and standard deviation of replicate (n) assays of the QC pool. $\bar{x}$ is the mean concentration of analyte measured in the same pool for successive batches of assays. (b) Modified Shewhart or '2 from N' rule which gives an out-of-control signal if any point falls in the rejection zones bounded by $K_1$ and $-K_2$ or if any 2 out of the last N points falls between action and warning lines bounded by $K_1$ & $K_2$ and $-K_1$ & $-K_2$. (c) The CUSUM chart which gives an out-of-control signal if any part of the CUSUM trace crosses the lines of the V-mask when the cross-wire of the mask is superimposed on the most recent point. $d$ is the lead distance of the mask of semi-vertex angle $\theta$. 
commonly used control procedures in a way which requires knowledge of what the optimal schemes are within a given procedure. The purpose of this investigation was to find an efficient QC procedure that was easy to operate in any laboratory practising internal QC.

Basic concepts of internal QC

Whether operator-, material- or equipment-based, the determined response of any assay method is subject to error fluctuations. In clinical chemistry it is common practice to monitor this variability by placing QC material in the assay system such that the analyte levels are in the lower, middle and upper part of the working range of the standard curve. When the predicted analyte level of a QC material is of known variation, the control mechanism becomes one of deciding whether an observed QC analyte level is statistically 'unlikely' under the null hypothesis that assays are in-control (i.e. there is no systematic inaccuracy and there is acceptable precision).

The method which is commonly employed to make this decision is the Shewhart chart. For example, suppose n replications are performed for each QC pool and that \( \bar{x} \) is the mean of these observations, then the data could be plotted on the chart shown in Figure 1a, where \( m \) and \( \sigma \) are the 'known' mean and standard deviation of the analyte in the QC pool used. The statistic plotted is \( (\bar{x} - m) \) and the decision that the system is out of control is reached when an observation falls more than K standard deviations \( (\sigma / \sqrt{n}) \) away from the target mean, \( m \). If the factor K is 2, then the probability of false rejection for a given observation is approximately 0.05 when the system is in control. In other words, the average number of observations needed before an out-of-control situation is registered, when in fact the system is in control, is 20. If this average number of observations is too small, or, equivalently, the rejection probability too high when the system is in control, the action lines should be moved further out or, equivalently, the factor 2 increased. Whatever factor is used there are three points to note. (a) \( \sigma \) is assumed constant throughout time. (b) The symmetry of the rejection lines is assumed. (c) Only the current observation is used in the decision process.

Bearing these points in mind it seems necessary at least to control the mean analyte level and the within-assay variability of the QC pool and perhaps to consider schemes not possessing symmetry.

We have investigated the properties of three procedures, shown in Figure 1, for the monitoring of the mean level of analyte concentration in a QC pool and associated within-assay variability.

The optimization procedure

The question arises as to how one can assess and compare objectively the performance characteristics of different control procedures. The way it should be done is to specify a cost function for the given situation, i.e. assess the costs involved in making wrong decisions. However, our investigations lead us to the conclusion that the costs involved are difficult to assess properly and would be highly laboratory dependent, so making any conclusions drawn from such a cost function only valid for that particular laboratory.

Bearing this in mind we adopt the commonly used concept of average run length (2) to assess control schemes, believing this to have much wider applicability. Graphically the concept of average run length is illustrated in Figure 2.

The functions shown vertically alongside the charts are illustrative of the probability distributions of the scores for the in- and out-of-control situations. For the in-control situation (Fig. 2a), the greater part of the probability distribution is concentrated around the target level and so one would expect that many observations would be needed before a value was found in the rejection region, i.e. in the tail of the distribution. On the other hand, if the distribution of scores is as shown in Figure 2b, then relatively few observations would be needed before a point fell in the rejection region. Formally, the average run length (ARL) is
Fig. 2. Illustration of the concept of average run length, showing typical distribution of the series for (a) in-control and (b) out-of-control situations. Probability distributions for the scores are shown to the right of each chart. For the in-control situation, the mean of the distribution coincides with the target level.

The average number of observations required before action is taken. From the reasoning presented above, it would seem necessary to satisfy the following requirements. Firstly, the average run length should be small when the system is out-of-control (denoted by \( L_2 \)) and secondly the average run length should be large when the system is in-control (denoted by \( L_1 \)).

While these two criteria are necessary, they are not precise enough to allow comparisons to be made rigorously. However, the essential points of the two requirements are embodied in the optimization procedure (3) developed by this group and formalized below.

If \( \alpha \) denotes a set of parameters for the control scheme in question (e.g. for Shewhart schemes this would be the positions of the action lines \( K_1 \) and \(-K_2\)), a scheme with parameters \( \alpha^* \) is said to be optimal if \( L_1 (\alpha) \leq L_1 (\alpha^*) \) for all other schemes \( \alpha \) such that \( L_2 (\alpha) = L_2 (\alpha^*) \) (specified). That is, we select from amongst all of those schemes having the same out-of-control average run length that scheme which has the maximum in-control average run length.
Table 1

Control of the mean

Comparison of optimal values for the in-control average run length \( L \), for specified values of the out-of-control average run length \( L \), for different values of deviation from the target mean.

<table>
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*S: Shewhart control scheme; SAW: Shewhart scheme with action and warning lines; C: CUSUM.
†Figures in parentheses are optimal values of \( N \) for the '2 from \( N \)' rule. See Figure 1.

Application of the optimization procedure

It is obvious from the form of the optimization procedure that, before it can be used, expressions for the average run lengths must be obtained. The average run length for the Shewhart procedure is the reciprocal of the rejection probability and that for the modified Shewhart procedure has been obtained by Page (4). It is only recently that fast approximations for calculating the CUSUM average run lengths have been available. Consequently, we are now in a position to compare properly the three control procedures shown in Figure 1. The results of the comparisons are shown in Tables 1-3.

For the control of the mean analyte concentration in an internal QC plasma pool, Table 1 shows that for most practical purposes (i.e. an acceptably large in-control ARL, \( L_{ij} \)) encountered in clinical chemistry, the CUSUM technique is superior to other methods investigated.

Tables 2 and 3 show the comparison between the Shewhart chart with action lines only and the CUSUM technique for the control of error. Suppose most routine assays operate...
INTERNAL QUALITY CONTROL

Table 2

Control of error
Comparison of optimal values of the average run length for the in-control situation $L_1$, for various values of the out-of-control average run length $L_2$, for Shewhart and CUSUM procedures at three tolerance levels for duplicate determinations of QC pools.

<table>
<thead>
<tr>
<th>$L_1$</th>
<th>Control scheme for error*</th>
<th>Optimal $L_1$ at specified tolerance level (factor $\beta$)</th>
<th>1.50</th>
<th>1.75</th>
<th>2.00</th>
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</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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*S: Shewhart; C: CUSUM. %RE: relative efficiency of CUSUM/Shewhart schemes given by quotient of values of $L_1$.

with a coefficient of variation of samples and QC plasmas of 10-15%, then in practice one can allow the value of the standard deviation to rise by a factor of 0.5 or even 1.0 of the target level before remedial action is taken to rectify the analytical procedure. It is seen in Table 2 that for all values of $L_1$ greater than 5 and for values of $\beta$ (the tolerance factor (5)) greater than 1.5, the CUSUM technique is more efficient. For a value of $L_1$ equal to 10, the CUSUM technique is shown to be 40, 160, and 720% more efficient than the Shewhart scheme for values of $\beta = 1.5$, 1.75 and 2.00, respectively. The differences are more pronounced for triplicate determinations, as shown in Table 3. It should be pointed out that the ARLs for CUSUM schemes are calculated from approximations and, as such, very large ARLs should be viewed only as reasonable estimates.

It is to be emphasized, once again, that it is important to select control schemes, whether Shewhart or CUSUM, which have a reasonably large in-control run length. For example,
Comparison of optimal values of the average run length for the in-control situation $L_1$ for various values of the out-of-control average run length $L_2$ for Shewhart and CUSUM procedures at three tolerance levels for triplicate determinations.

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<th>$L_2$</th>
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<td></td>
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</table>

*S: Shewhart; C: CUSUM. %RE: relative efficiency of CUSUM/Shewhart schemes given by quotient of values of $L_1$.

during an analytical run of length 50, for the control of the error the probability of false rejection is 0.64 when $L_1$ is taken to be 50, whereas it is only 0.095 if $L_1$ is 500.

**DISCUSSION**

Since no control scheme should be operated with a very small value of $L_1$, it is apparent from the Tables that for most practical purposes encountered in clinical chemistry, the CUSUM technique is more efficient than either the Shewhart with action lines or the modified Shewhart with both action and warning lines. Our work leads us to believe that modified Shewhart schemes which are generally better than CUSUM schemes do not exist.

Development of control schemes for error have been necessary for two reasons. In the first instance, it is often fallacious to assume that within-assay variation is constant, this
assumption being essential for conclusions based on the control of the mean to be valid. Secondly, by monitoring error we have a direct means of observing whether the precision is at an acceptable level.

We are aware of reasoning which has led others (6) to consider mixed schemes for contingencies where neither the modified Shewhart nor even the CUSUM with straight decision boundaries is adequate. If a combination of Shewhart and CUSUM schemes is more efficient than either Shewhart or CUSUM alone, then the results of this paper suggest that the combination of two CUSUM schemes should be even more efficient. Combining schemes is equivalent to superposing the V-masks corresponding to each constituent scheme. This being so, we are suggesting that more efficient schemes can be obtained using polygonal masks. Carrying this line of argument further, it is relatively easy to see that for observations plotted at discrete points along the horizontal axis (batch number) a polygonal mask is equivalent to a curvilinear mask. We conclude that most efficient schemes may be obtained by using curvilinear control masks, an idea already remarked upon by Barnard (7).

In conclusion, it is the ease of use and the visual information contained in a control scheme that are the all-important factors from the analyst’s point of view, just as much as the detailed statistical properties of the device being used. The CUSUM excels in both of these areas; its use merely depends upon accumulating quantities of the form \((C_i - m)\) where \(C_i\) is the \(i\)th value of the control statistic and \(m\) is the target value. It also gives immediate information on which way the mean of the control statistic is moving and at approximately what point in time it started to change. If, as mentioned above, more efficient schemes can be obtained by using curvilinear boundaries, then this means only a change of shape for the decision mask and not a change in the control procedure used. It is important to note here that, if the volume of data being processed is high, a computerized form of the control scheme should be used (8).

ACKNOWLEDGEMENTS

The authors are grateful to the Tenovus Organisation for their generous financial support.

REFERENCES

INTRODUCTION

Numerous internal quality control (QC) procedures are available for monitoring assay performance (1, 2) but recent developments have shown that the cumulative sum (CUSUM) technique is generally superior (3, 4). Although the internal monitoring of performance by means of this technique entails only simple routine calculations, there is a strong case for computerization in laboratories which have a high throughput and rapid turn-round time.

The use of a computer relieves laboratory staff of the time consuming and repetitive task of plotting CUSUM charts and eliminates the possibility of arithmetic error. Mistakes are inevitable in manual systems and, moreover, the frequency of such errors often increases with time. Furthermore, when a number of different assays are run routinely the existence of an automatically updated and easily accessed status file enables those responsible for QC to survey the current situation without delay; a quick scan of the file reveals any assay which is judged out-of-control.

The control of precision is as important as the control of bias if the laboratory is to provide consistent results. This paper describes a computer program which has been developed to monitor efficiently not only the mean, but also the precision of analyte determinations in immunometric assays.

A TYPICAL LABORATORY PROCEDURE FOR DATA PROCESSING AND QC

Figure 1 shows the *modus operandi* of a typical laboratory procedure for assessing the quality of assay results. Many of the facets relating to laboratory procedure, computation of

![Diagram](image-url)
standard curve and dose interpolation, and choice of QC scheme are described in this Workshop proceedings. Data in the form of radioactive counts, absorbance of light at a specified wavelength etc., are produced from standard hormone concentrations used to construct a calibration curve, QC pools and samples contained within the analytical batch. This information is then used to fit a representative function to the calibration data and, by suitable transformation of the fitted function, allows the hormone concentrations in the samples and QC pools to be calculated. QC information is then used as input data for the computer program for the monitoring of analytical performance.

THE STRUCTURE OF THE COMPUTER PROGRAM

Data input

The program described in this report can handle up to ten different assays run routinely when three QC plasma pools are used per assay or up to fifteen if there are only two QC pools. It reads one QC data record per QC sample, where each record contains the following information: (a) the assay and QC pool identifier, (b) the sample size, \( n \) \((= 1, 2, 3)\) and (c) the \( n \) replicate QC determinations made.

Any number and mixture of records can be submitted at the same time, the only restriction being that the order of submission within each assay and QC pool identifier should follow the temporal order in which the samples were measured.

![Flow chart of the interactive monitoring system](image)

**Fig. 2.** Flow chart of the interactive monitoring system illustrating the central role played by the CUSUM module and its various print options in the typical decision making processes carried out by the QC co-ordinator.
Structure of the monitoring system
Whenever a sequence of QC data records is submitted, the QC determinations are tested for lack of control by the CUSUM module using information stored in a History file. When the analysis of the latest data sequence is complete the processed data are added to the History file and a Status file is updated, as shown in Figure 2. The Status file lists the current state of each assay, indicating whether it can be assumed to be running in-control or not.

If lack of control is indicated, a Plot file can be accessed but when the conclusion is that the out-of-control message was caused by a spurious observation, the relevant record can be deleted and the CUSUM plots reset accordingly. On the other hand, if the decision is that lack of control exists, a systematic search for its cause can be undertaken, aided by the information contained in the Plot file.

Fig. 3. Flow chart of CUSUM module (n>1). \( x_1, x_2, \ldots, x_n \) are the \( n \) replicate QC determinations

\[ \bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i \]

\[ S^2 = \frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2 \]

are the sample mean and sample variance, respectively. \( m \) is the target mean and \( \sigma^2 \) is the target variance. UQM and LQM are the respective upper and lower cumulations for the control of the mean. QV is the cumulation for the control of the variance. E and L are the reference value and decision interval for the mean. K and H are the corresponding parameters for the error. The current values of the work variables D, E, F are stored in the History file. Initially, \( D = E = F = O \).
he CUSUM module

The module searches for the presence of systematic bias relative to a pre-assigned target value by running a CUSUM check on successive sample means. This is supplemented by a check which flags exceptionally large deviations from target. One reason for the inclusion of this feature is that those CUSUM schemes which are especially efficient at detecting moderate degrees of bias react slightly less quickly to large changes than Shewhart schemes with a comparable false-rejection rate. Such combined schemes, which have long been used in practice but have only recently been discussed in the literature (2, 5), will also detect a decrease in precision if it occurs. However, when the QC determinations are replicated \( n > 1 \), the CUSUM of sample variances (6) is a more efficient scheme for detecting an increase in the error. Consequently, this scheme has also been programmed, as shown in Figure 3. It should be noted that to every V-mask scheme there corresponds an equivalent decision interval scheme (7) and, for convenience, it is the decision interval versions which are used in the computer program.

itialization

The program must be supplied with a target mean, \( m \), and a target variance, \( \sigma^2 \), for each assay and QC pool, together with the Shewhart parameter denoted by \( K_1 \), the values of the acceptance criterion, \( \Delta \), and the out-of-control average run length, \( L_2 \), for the control of the mean (8). In addition, when \( n > 1 \), the values of the tolerance level, \( \beta \) (6), and the out-of-control average run length, \( L'_2 \), for the control of the variance must be specified. The CUSUM of mean values will then take an average of \( L_2 \) samples to detect a systematic deviation from the target mean of size \( \Delta \), while the CUSUM of variances will detect an increase of \( (\beta - 1) \sigma^2 \) in the standard deviation of the determinations after an average delay of \( L'_2 \) samples. The desirability of a low false rejection rate must always be borne in mind when selecting \( L_2 \) values and therefore a table of in-control average run lengths is consulted before the final choice is made.

The responsibility for choosing \( \Delta \), \( \beta \) and the \( L_2 \) values rests with the laboratory but, when the program is given these specifications and the target values, it selects the corresponding internal optimal scheme parameters automatically. Consequently, there is no need to use a nomogram in order to set up the scheme.

he Plot file

This file contains numerical plot data for each assay starting from the most recent initialization. It can be read on a line printer but, if the facilities are available, it can be displayed on a visual display unit or plotted by a graph plotter. The program provides the following plot options: (a) a plot of successive deviations from target (Shewhart); (b) a continuous CUSUM plot (V-mask); (c) a decision interval plot.

To assist with the interpretation of the data the file also contains a list of the points among which it is likely that the shift or drift first occurred. The problem of constructing a confidence interval for the change point is unsolved and appears to be intractable (9). Instead, the program calculates the relative likelihood of each possible point of change and lists the leading contenders in order of merit.

An example of data output from the Plot file is shown in Table 1. The contents relate to a sequence of radioimmunoassay determinations for oestriol made on a QC plasma pool. These data have been discussed elsewhere (6) in connection with the control of error using a V-mask. The sample size in this application is two, one QC determination being made at the beginning of each batch of patient samples and the other at the end. This design ensures that any serious within-batch drift will be detected. Taking the columns from left to right, the output lists the standardized deviations of the sample means from their target value (zero), the deviations from unity of the standardized sample variances, the lower and upper decision
Table 1

Data in the computer print-out from the internal quality control program for monitoring the mean and variance of values from a quality control pool for plasma oestradiol

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Deviation from target mean</th>
<th>Deviation from target variance</th>
<th>Lower CUSUM for mean</th>
<th>Upper CUSUM for mean</th>
<th>Upper CUSUM for error</th>
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Likelihood analysis of change point

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<th>Likelihood</th>
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<td>0.0000D 00</td>
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<td>16</td>
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</tr>
<tr>
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<td>15</td>
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<td>15</td>
<td>-0.1617D 01</td>
</tr>
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<td>8</td>
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<td>-0.1652D 01</td>
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<td>-0.1652D 01</td>
</tr>
<tr>
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<td>3</td>
<td>-0.1654D 01</td>
<td>3</td>
<td>-0.1654D 01</td>
</tr>
</tbody>
</table>
interval CUSUMs for the mean, the decision interval CUSUM for the variance and, finally, the continuous CUSUMs of the sample means and variances. The likelihood analysis for the change points appears beneath these columns.

The program was initiated by setting \( \Delta = \beta = 1.5 \) and the \( L_2 \) values for the mean and variance equal to 5 and 10, respectively. The respective reference values \( k \) and \( K \) and decision intervals, \( h \) and \( H \), for the decision interval schemes were then 0.75, 0.45 and 3.14, 7.11. In addition, the Shewhart parameter \( K_1 \), was set equal to 2.5. As can be seen by examining the fourth, fifth and sixth columns of Table 1, the CUSUM for the error goes out-of-control for the first time at batch 18, since the last entry in column six exceeds the decision interval \( H = 7.11 \), whereas the mean remains in-control throughout.

Since it is the error which is out-of-control, it is the likelihood analysis for the error which is of interest in this case. If the plot of successive standardized batch or sample variances is examined (Fig. 4b) it might be thought that batch 15 is most likely to be the last in-control batch. However, the analysis reveals that batch 11 is the most likely candidate. Reading down the list in Table 1, it is seen that batch 15 is the 2nd most likely change point, while batch 16 is third, followed by batches 13 and 10. The most likely inference, then, is that the determinations on patient samples are of poor quality from batch 12 onwards. The number beside each batch number is the natural logarithm of the relative likelihood of that batch. With this scoring system, the most likely change point always receives a score of zero while the other candidates receive negative scores. The more negative the score, the less likely it is that this batch is the change point.

---

![Fig. 4. Plots of (a) standardized individual batch means, \((x-m)/(\sigma^2/\sqrt{2})\), and (b) within-batch variances, \(S^2/\sigma^2\), for radioimmunoassay determinations of oestriol. Shewhart action lines are placed at (a) \( \pm 2.9 \) and (b) 6.64.](image-url)
The same scoring system is used for the change point analysis of the mean. In the present example, the likelihood analysis strongly suggests that if an undetected shift or drift in the mean has occurred, then it has only just taken place.

The in-control average run lengths of the CUSUM schemes for the mean and error used in this example are 270 and 103, respectively. It is of interest to note that the corresponding Shewhart schemes with the same in-control average run lengths fail to detect the change in error at or before batch 18, as is shown in Figure 4. Of course, if the action lines for the mean had been placed at ±2 rather than at ±2.9, or if the action line for the variance had been placed at 5.9 rather than 6.64 then the change would have been detected by these schemes. However, such changes would increase the false rejection rate and it can be calculated by means of Page's reciprocal rule (10) that the in-control average run length of the two Shewhart schemes taken together is already only 74, i.e. one or other of the two schemes will falsely reject the assay on average once in every 74 batches when the assay is in-control.

In conclusion, a computer program has been developed for monitoring and analysing QC data in a routine analytical laboratory.

ACKNOWLEDGEMENTS

The authors are grateful to the Tenovus Organisation for their generous financial assistance and appreciate the constructive criticisms of Dr. D. Riad-Fahmy and Mr. B. G. Joyce.

REFERENCES

ADVANTAGES OF CUSUM TECHNIQUES FOR QUALITY CONTROL IN CLINICAL CHEMISTRY

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Summary

The performance of routine analytical laboratories is assessed, inter alia, by the use of appropriate internal quality control techniques. Despite evidence that the cumulative sum technique is generally superior to Shewhart-type control charts and many others, its use has been limited by the popular misconception that it is inferior to Shewhart's in detecting large variations and/or outliers in quality control data. The application of computer simulation methods has enabled us to answer this criticism and has provided the basis for further improvements in the design of the appropriate control scheme for general use in clinical chemistry.

Introduction

Although the cumulative sum (CUSUM) quality control technique is well established in fields such as chemicals production engineering [1] its advantages have yet to be fully appreciated in clinical chemistry. In order to promote the CUSUM technique, Westgard et al. [2] have advocated the use of the "decision limit" procedure in preference to the equivalent "V-mask" CUSUM technique, because the former employs the control limit concept familiar to users of Shewhart or Levy-Jennings charts. The present paper rectifies an important misconception, which exists concerning the use of CUSUM schemes of any kind, namely that, although they may be more efficient at detecting small systematic changes, Shewhart charts are preferable for larger ones.

* To whom correspondence should be addressed.
Comparison of Shewhart and CUSUM schemes

Definitions and notation

(a) Average run length
The average run length (ARL) of any continuous inspection scheme is defined as the average number of quality control (QC) samples taken before lack of control is indicated. Its value depends on the parameters of the scheme and on $\theta$, the deviation of the current mean of the control statistic from its target value. The ARL of any sensible scheme must be large when the assay is in control ($\theta = 0$), but small when it is not ($\theta \neq 0$). The possibility that the assay will be falsely rejected cannot be completely eliminated when the standard deviation $\sigma$ of the determinations is non-zero. The probability that false rejection will occur during an analytical run containing $N$ control samples is given approximately in terms of the ARL by the formula

$$1 - \left(1 - \frac{1}{\text{ARL}}\right)^N$$

For example, if the in-control ARL of a continuous inspection scheme is 250, the probability that it will falsely reject an assay which is in control during an analytical run containing 30 control samples is approximately 0.11.

The relative merits of rival schemes can be assessed by comparing their respective rejection probabilities or run length distribution functions* at selected values of $\theta$, but it is more convenient to examine graphs of their average run lengths plotted against $\theta$. Consider the ARL functions shown in Fig. 1a. The in-control ARL's of the two schemes A and B are the same, but scheme B is preferable to A because its ARL is always less than the ARL of A when $\theta \neq 0$, as discussed by Roberts [3]. However, the choice is not always so easy, as shown in Fig. 1b. The ARL curves cross at $\theta = \theta_0$ so that neither scheme is strictly preferable to the other over the whole range of possible values of $\theta$. In this case more information is needed before a proper choice can be made. For example, if the main concern is the detection of departures from target which exceed $\theta_0$ then C is preferable to D. On the other hand, if the crossover point is so far from the origin that both ARL functions are effectively equal to unity beyond $\theta_0$ then D should be used in preference to C.

(b) Control schemes
One-sided and symmetrical two-sided decision limit or decision interval schemes are specified by two parameters called the decision interval and references value, respectively. These schemes have been described extensively in the literature [1,2,4]. The two-sided decision limit CUSUM scheme with decision interval $h$ and reference value $k$ will be denoted by C($h$, $k$).

*The run length distribution function is the probability that the scheme will take, at most, $N$ samples to reject the assay plotted against $N$. Westgard et al. present various run length distribution functions for a number of selected schemes.
The Shewhart scheme \* with action limits located at \( \pm K \) will be denoted by \( S(K) \).

CS\((h, k K)\) will denote the scheme which is a combination of the schemes \( C(h, k) \) and \( S(K) \), whereas the scheme which is a combination of the two CUSUM schemes \( C(h_1, k_1) \) and \( C(h_2, k_2) \) will be denoted by \( CC(h_1, k_1 | h_2, k_2) \).

**Results**

When individual QC determinations in an assay system are normally distributed and the control statistic is the arithmetic mean \( x \) (if each sample consists of a single control measurement \( x \), then \( x = x \)) of the QC scheme, the schemes \( S(2.5) \) and \( C(3.3, 0.5) \) have the same in-control ARL of 81, but Table I shows that the CUSUM will detect a shift of \( 2\sigma \) or less more quickly on average than the Shewhart scheme. On the other hand, \( S(2.5) \) responds sooner to shifts in excess of \( 2\sigma \). Which scheme is preferable depends on the application of interest and if it is only large shifts from target which have clinical significance, then \( S(2.5) \) is preferable to \( C(3.5, 0.5) \). Among CUSUM schemes whose in-control ARL is 81, \( C(3.5, 0.5) \) is most efficient when \( \theta = \sigma \), but if rapid detection is not of interest at such small values of \( \theta \) then \( C(3.5, 0.5) \) is not the CUSUM scheme to use. The in-control ARL of \( C(1.17, 1.4) \) is also 81, but this scheme is most efficient when \( \theta = 2.8\sigma \) and so is better suited to detecting large shifts. In fact, inspection of Table I shows that it is better than \( S(2.5) \) for all values of \( \theta \) up to \( 4\sigma \). Beyond this point there is nothing to choose between the two schemes since their average run lengths are both effectively equal to unity. Therefore, of the schemes considered, it is the CUSUM scheme \( C(1.17, 1.4) \) which is preferable when the only concern is the detection of systematic changes of size \( 2\sigma \) or more.

The above example is typical. Given any Shewhart scheme and any value of \( \theta_0 \), a CUSUM scheme with the same in-control ARL can be found, which is

\* Control charts with action and warning limits are not considered in this paper. Warning limits are used to increase the Shewhart chart's sensitivity to small systematic changes, but if it is important to detect such changes quickly then a CUSUM scheme should be used [4].
TABLE I

AVERAGE RUN LENGTHS OF VARIOUS SCHEMES FOR CONTROLLING A NORMAL MEAN AS A FUNCTION OF \( \theta \), THE DEVIATION OF THE MEAN OF \( \bar{X} \) FROM THE TARGET VALUE

\[(a = \sigma_1 / \sqrt{n} \text{ where } n \text{ is the number of control measurements per sample and } \sigma_1 \text{ is the standard deviation of each determination. ARL values of C(3.3, 0.5) were obtained from the contour nomograms of Goel and Wu [5] and ARL values of C(1.17, 1.4) from the table of Chiu [6].}\]

<table>
<thead>
<tr>
<th>Control scheme</th>
<th>( \delta / \sigma )</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
<th>4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>S(2.5)</td>
<td></td>
<td>81</td>
<td>41.5</td>
<td>14.9</td>
<td>6.3</td>
<td>3.24</td>
<td>2.00</td>
<td>1.45</td>
<td>1.23</td>
<td>1.11</td>
</tr>
<tr>
<td>C(3.3, 0.5)</td>
<td></td>
<td>81</td>
<td>20.0</td>
<td>7.0</td>
<td>4.0</td>
<td>2.88</td>
<td>2.28</td>
<td>1.92</td>
<td>1.64</td>
<td>1.42</td>
</tr>
<tr>
<td>C(1.17, 1.4)</td>
<td></td>
<td>81</td>
<td>35.0</td>
<td>11.3</td>
<td>5.0</td>
<td>2.64</td>
<td>1.82</td>
<td>1.40</td>
<td>1.19</td>
<td>1.08</td>
</tr>
<tr>
<td>CC(3.7, 0.5/1.17, 1.6)</td>
<td></td>
<td>81</td>
<td>21.4</td>
<td>7.3</td>
<td>4.0</td>
<td>2.71</td>
<td>1.93</td>
<td>1.50</td>
<td>1.25</td>
<td>1.11</td>
</tr>
<tr>
<td>CS(3.7, 0.5/2.74)</td>
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<td>81</td>
<td>21.7</td>
<td>7.4</td>
<td>4.1</td>
<td>2.74</td>
<td>2.01</td>
<td>1.53</td>
<td>1.25</td>
<td>1.11</td>
</tr>
</tbody>
</table>

better at detecting every size of shift up to and including one of size \( \theta_0 \), by taking \( k \) large enough. Beyond \( \theta_0 \) the Shewhart scheme will be more efficient, but when \( \theta_0 \) is large this advantage is only of academic interest since both schemes will have average run lengths practically equal to unity. Consequently, when only large changes are of clinical significance, it is still advantageous to use the appropriate CUSUM scheme rather than a Shewhart chart. This is a fundamental conclusion which is rarely considered in choosing a suitable control scheme by practising clinical chemists.

Returning to the example, what scheme should be used when it is important to detect small systematic changes as well as large ones? The scheme C(1.17, 1.4) is still better than S(2.5) in this case, but should C(3.3, 0.5) now be preferred? The latter is considerably more efficient in the neighbourhood of \( \theta = \sigma \), but at the price of being slightly less responsive to large shifts. In fact, this question can be answered only by taking into account the economic and other costs associated with the use of these schemes in the laboratory. Such costs can be difficult to assess, and an alternative is to compromise by using a combined scheme in which two control schemes are run simultaneously; one to detect small changes and the other to detect the larger ones.

**Combined schemes**

The ARL of a combined scheme is defined as the average number of QC samples taken before one or other of the component schemes indicates a lack of control. The combined CUSUM scheme CC(3.3, 0.5/1.17, 1.4), not included in Table I, is certainly efficient at detecting both large and small changes, but its ARL at \( \theta = 0 \) is considerably less than 81, the in-control average run length of its component schemes. In order to find a combined CUSUM scheme whose in-control ARL is 81, a simulation study was undertaken and the scheme CC(3.7, 0.5/1.17, 1.6) was found to have this property. The results are presented in Table I. It can be seen that it is almost as efficient as C(3.3, 0.5) at detecting small deviations, and responds nearly as quickly as C(1.17, 1.4) when the shift is large. An alternative procedure, which has received some attention in the literature [2], is to combine a single CUSUM scheme with a suitably chosen Shewhart scheme, though some loss of efficiency can be expected. For
example, it was found that the scheme CS(3.7, 0.5|2.74) has an in-control ARL of 81, but it can be seen from Table I that CC(3.7, 0.5|1.17, 1.6) performs slightly better throughout the working range.

Discussion

If one is interested in detecting changes in \( \theta \) over a limited range, then the procedure to adopt is to select the appropriate optimal CUSUM scheme [7,8]. If, however, one is interested in detecting shifts of any size, then combined schemes should be considered as these are more efficient overall at detecting both small and large changes in the control statistic being monitored than either of the component schemes alone.

Consequently the incorporation of the CUSUM technique into the quality control program would improve laboratory performance and contribute further to health care assurance.

Acknowledgements

The authors are grateful to the Tenovus Organisation for their generous financial support.

References

The Control of Performance in Immunoassays


INTRODUCTION

Immunological assays are inherently imprecise when compared with many of the chemical and physical techniques used in clinical biochemistry. Even when optimized, they are subject to errors arising from the instability of reagents and the involvement of complex manipulations as, for example, in assay separation. Nevertheless, the increasing involvement of immunoassays in the diagnosis of disease and the monitoring of patient treatment emphasizes the need for the adoption of control procedures, such that assay performance is maximized and then maintained within acceptable limits.

In order to assess performance of an assay it is, of course, necessary to understand the major sources of error in immunoassay systems. Errors may be random as in the case of pipetting errors. Alternatively they may be systematic. Within-batch drift can be produced by factors affecting the equilibrium of the reaction (e.g. temperature change) whereas between-batch variability may result from changes of methodology or reagents. Random errors result in poor precision while the systematic errors lead to the production of biased results. It is therefore necessary to monitor several aspects of assay performance if accurate results are to be produced consistently. During recent years a number of Department of Health and Social Security (DHSS) sponsored schemes have been developed to assess laboratory performance at a national level. The objectives of such schemes are not clearly
defined. However, by distributing samples to laboratories for assay at regular intervals, the organizer seeks to gain information which will enable him to identify poor performance, and also monitor changes in performance with time. Such information should thus be of value to the laboratories concerned, as well as indicating where there exists a need for advice or further action from an objective assessor. In this chapter we draw attention to the relative insensitivity of schemes in operation at present and describe how simple procedures can be used within a laboratory to monitor several aspects of assay performance.

ASSESSMENT OF PERFORMANCE BY EXTERNAL MONITORING

A fundamental difficulty facing the organizer of an external monitoring scheme is that he is forced to base his assessment on extremely limited data. In practice, up to five samples of serum pools are distributed at monthly intervals in such a way that approximately six pools will be measured on average four times by each laboratory. Individual performance is assessed by obtaining a coefficient of variation (CV) for each pool (the standard deviation of the observations divided by the mean value). The limitation of this procedure is illustrated in Figure 13.1a, which plots the distribution of values for CV which would be obtained for a laboratory with a true CV of 10%, when that estimate is based on only four observations. Not only is the distribution wide, 95% of the estimates ranging from 2 to 18%; it is also skewed indicating a tendency to underestimate. As illustrated in the lower portion of Figure 13.1, the situation is noticeably improved by increasing the number of observations to 10. It is unfortunate, however, that for some assays CV is frequently greater than 10%.

Figure 13.2a shows the distribution of CV estimates again based on four observations for a laboratory with a true CV of 15%. The distribution is wide, 95% of the observed values falling between 4 and 27%, and once again it is skewed. As before the situation is improved by increasing the number of observations to 10, though here the estimate still ranges from 8 to 22%.

The statistical confidence limits on a CV derived from 4 or 10 observations are illustrated in Figure 13.3. Based on only four observations, the 95% confidence limits on an estimated CV of 10% are 4–36%. Based on 10 observations these limits are 6–18%.

In an attempt to derive an index of overall performance of a laboratory, it has been common practice to combine the data obtained from all pools estimated during a 6-month period, thus yielding a 'running CV'. Since the pools assayed during that period cover a wide range of analyte concentrations, this approach is only valid if the error of the analyte estimation is proportional to the dose of analyte present. Calculation of precision profiles indicates that this is not so, relative error being greater at the extremes of the dose range and in some cases constant only over a limited portion of the dose-response curve.
It is interesting to note that, in a situation where CV is dose-dependent, the distribution of the running CV based on a limited number of observations can be similar to that observed when the true CV is constant. Figure 13.4 shows the effect of combining data from three pools where the CV differs on the distribution of calculated CV.

An alternative approach to external assessment of certain steroid immunoassays has been the use of a variance index (VI), as developed by Whitehead\(^3\) for application to monitoring in clinical chemistry laboratories. The principle of the method is to relate each observation to the method mean as given by the formula

\[
\frac{x - x_m}{x_m} \times 100
\]

where \(x\) is the observed value, and \(x_m\) the method mean.
The VI is obtained by dividing this value by a chosen target coefficient of variation (CCV). Thus a high VI is taken as an indication of poor performance. In order to deal with the problems of wide fluctuations in VI based on individual observations, Whitehead introduced the principle of a running mean VI based on the most recent 40 observations. In this way long-term fluctuations could be distinguished from the background 'noise' level.

We have used computer-simulated data to study the performance of a relatively poor laboratory as assessed by calculation of VI. The data were simulated for a laboratory with a true CV of 23% when the CCV is 15%.

Figure 13.5 shows a plot of 600 observations obtained for this laboratory using a running mean of 40. It is apparent that overall there is oscillation...
about the expected VI of 122. It is important to note, however, that these simulated data exhibit spurious trends. It would be tempting, for example, to interpret the early pattern (observations 40–70) as indicating an improvement in assay performance. Subsequently there appears to be a static period (observations 70–130) followed by an apparent deterioration in performance (observations 130–170). In fact there is no interpretation, since the true CV is constant at 23%.

However, the real danger in this system becomes apparent when, because of the limited observations available to organizers of external assessment schemes, a smaller running mean is used. Figure 13.6 shows the initial portion of the data from Figure 13.5 plotted using a running mean of 10. The marked downward trend in VI from 130 to 70 (observations 10–16) followed by a reversal (observations 17–26) implies dramatic improvement followed by equally dramatic deterioration when the system has in fact remained static. When the same data are plotted using a running mean of only four observations (Figure 13.7) these fluctuations assume ludicrous proportions. The ability to identify changes in laboratory performance against this background noise is clearly limited.
Figure 13.4  Simulated sampling distribution of running CV (four estimates on each of six pools) for the situation where true CV is constant at 15% (a) or varied (b). In the latter case pools were assigned a CV as follows: 2 at 10%, 2 at 15%, and 2 at 20%.

Figure 13.5  Simulated variance index for a laboratory with a true CV of 23% using a chosen CVV of 15%. The data plotted are derived using a running average of 40 observations (ROMVIS).
When assays are available at a limited number of centres, it is desirable that the results obtained in different centres agree as closely as possible. It is standard practice to use the relationship of observed values to the group mean.
Table 13.1: Sample of values obtained for 22 laboratories for control pool of oestradiol-17\beta.

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Mean (pml/l)</th>
<th>CV (%)</th>
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<td>4</td>
</tr>
<tr>
<td>2</td>
<td>210</td>
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<td>3</td>
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<td>7</td>
<td>260</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>22</td>
<td>180</td>
<td>4</td>
</tr>
</tbody>
</table>

as an indication of the bias of a particular laboratory. In this situation, too, the limitations imposed by small samples are apparent. Table 13.1 lists data obtained for 22 laboratories on a pool used in the assessment of performance in the measurement of oestradiol-17\beta. We have used these data to study the effect on the performance of laboratory 1 of other laboratories. Relative to the group of the first eight laboratories (mean value 269), laboratory 1 shows a distinct negative bias. This bias disappears when the next seven laboratories are included since the group mean now becomes 238. The position remains similar when the final seven laboratories are included (mean 240). Because of the variation in estimates on the pool (CV = 4\% for laboratory 1), the calculated bias will follow a distribution. Figure 13.8 illustrates the distribution of bias for laboratory 1 expressed as VI (CCV = 15\%) when that laboratory is considered as part of a group of 8, 15 or 22 laboratories.

Assessment of bias by this procedure is thus limited by the problem of imprecision. When precision is low the calculated bias shows a wide distribution. This is illustrated in Figure 13.9, which plots the distribution of bias values calculated for laboratory 13 (CV = 18\%) in relation to the overall group mean. Thus the effect of other laboratories in a group on individual performance, and the large error involved in calculating bias, emphasize the problems facing the organizer of a scheme which attempts to monitor changes in the performance of an individual laboratory.
INTERNAL MONITORING OF ASSAY PERFORMANCE

It is universally accepted that if a laboratory wishes to maintain a satisfactory level of performance on a day-to-day basis, some form of internal monitoring system is essential. Ideally, it should be possible not only to recognize the out-of-control situation, but also to identify the nature of change which produced that situation and the point at which the change occurred. Such a procedure can be carried out by a system involving sequential monitoring based on established analyte pools. Suitable criteria for pool selection are:

1. they should resemble as near as possible the type of sample used in the assay (e.g. patient serum);
2. they should contain concentrations of analyte which correspond to patient values where critical interpretation is required; and
3. they should be available in sufficiently large quantities for continuous monitoring over a reasonable period of time (i.e. for several months).
We have adopted the following scheme for monitoring the performance of an assay for human \( \alpha \)-fetoprotein (AFP), used as a screening test on maternal blood for the antenatal diagnosis of neural tube defects. Three parameters were chosen for monitoring: (i) assay precision, (ii) batch variance (drift), and (iii) between-batch variation. Assessment was based on three sample pools prepared by adding purified AFP to 300 ml volumes of fresh sheep serum. Ideally, of course, analyte-free human serum should be used, so that care was taken to establish that the sheep serum produced no detectable interference in the assay. The procedure used was a radioimmunoassay which involved a 5 h reaction between rabbit antibody (Hoechst Pharmaceutical Company, Hounslow, Middlesex, UK), \([^{125}\text{I}]\)AFP (supplied by Dr J. Young, Ninewells Hospital, Dundee, UK) and patient samples or standard (NIHSC. 72/227). Precipitation of the bound antigen was carried out overnight using sheep (anti-rabbit IgG) antibody. All assays were carried out using a computer-controlled, fully automated immunoassay system (Kemtek 3000, Kemble Instruments Limited, Burgess Hill, Sussex, UK). Sample pools were placed after the standard curve and then after each batch of 20 patient sera. All samples were assayed in duplicate.
Precision was monitored by calculating the error in each pair of observations at low, medium and high analyte concentrations. The batch variance was monitored by calculating the error between the mean concentration of each pool at the beginning and end of each assay. Drift was thus identified when the error exceeded that arising from random variation. Monitoring of between-assay variance was based on the mean value obtained for each pair of pool estimations. This type of analysis is considerably more sensitive to variation than that based on monitoring mean values for the complete assay. Sequential analysis of the data was provided by plotting each parameter in the form of a CUSUM. The application of this type of monitoring to internal control of immunoassay performance has been described previously. The formulae used to derive values for each of the parameters are listed in Appendix I. Each pool was assayed ten times in duplicate in order to derive target values for precision and mean analyte concentrations. The V-masks used in monitoring were designed as described previously with the objective of identifying a shift in excess of 1.5 times the standard deviation of the target values within an average of five estimations. Such a scheme would yield a false-positive rate of 1 in 200 observations.

The data derived from 17 sequential assays of α-fetoprotein can be used to illustrate the unique advantages of this type of monitoring system. Figure 13.10 shows a CUSUM plot of assay precision as indicated by the low pool (50 µg/l). The tendency for this sequential plot to rise indicates that the error obtained in repeated assays is marginally higher than the target value, though
only once (observation 45) is an out-of-control situation identified. This situation was also identified by the medium pool, and it was noticeable that several samples in that region of the assay showed rather poor duplication. This problem, which occurred near the end of an assay, was traced to malfunction of a pump in the automated system.

Figure 13.11 shows a CUSUM plot for the batch variance based on data derived from the medium pool (79 μg/l). Each point is based on the difference between first and last estimates of that pool in each assay. No drift was detected up to assays 11 and 12 which were both flagged as out of control. There was no doubt that substantial drift had occurred in both these assays which were rejected on that basis. The problem appeared to be associated with overheating of equipment during assay separation. After correction of this problem no further drift occurred.

![CUSUM plot](image)

Figure 13.11  CUSUM plot for batch variance of serum AFP assays based on the difference between duplicate determinations of a medium pool placed at the beginning and end of each batch

A third type of out-of-control situation is illustrated in Figure 13.12 which shows a CUSUM plot based on mean values for the high pool (151 μg/l). Observations 36–40 show the appearance of a systematic error which produced high values for this pool within a single assay. The acute deviation of this CUSUM plot was flagged as out of control using the V-mask by the third
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observation within that assay. The error which produced this marked positive bias was traced to a batch of serum used as diluent for standards in this assay. The assay was repeated using a different batch of serum diluent and was subsequently in control. It is important to note that the situations illustrated in Figures 13.11 and 13.12 reflected systematic error, there being no loss of precision on either occasion.

DISCUSSION

These studies on control of α-fetoprotein assays serve to emphasize the fact that even with relatively straightforward immunossay techniques there still exist many potential sources of error. By adopting a simple monitoring procedure, we have demonstrated how it is possible to identify some of these errors and treat them accordingly. Of particular value is the ability to distinguish the random errors caused by machine or operator failure from the more systematic errors introduced by methodological or reagent variability. Monitoring of precision, independent of other parameters, is of overwhelming importance for if precision is poor it may be impossible to identify other sources of assay error.

A major drawback of external assessment schemes in operation at present is that they do not adequately assess assay precision. For example, as illustrated in Figure 13.9, there is little point in attempting to identify bias when the poor
performance of a laboratory makes such identification impossible. Moreover, it is apparent that attempts to monitor the performance of a laboratory based on limited statistical data are only capable of identifying extreme situations, and are certainly not appropriate for assessing fluctuations in individual performance.

The computer simulation studies as shown in Figures 13.5–13.7 illustrate the problem that it is not possible to assume that a monitoring scheme, which has been applied successfully in certain areas of clinical biochemistry, is necessarily appropriate to the assessment of performance in immunoassay laboratories. In such cases the inherently high imprecision and infrequency of sampling combine to render VI monitoring virtually useless as a guide to assay performance.

If immunoassay performance in UK laboratories is to be improved, it can only be by attacking the sources of error directly. It is first necessary to understand the nature of the error in individual assay systems. Where these can be traced to reagent variability, a central supply of standardized reagents should be considered. Subsequent monitoring of the assay performance can be made only through effective internal control. We feel that the means for such control should be provided centrally in the form of advice, computer programs for data-handling and also serum pools for use in each assay. The data generated by such a scheme would enable an organizer to assess assay performance in a far more meaningful way than through existing schemes with their high inertia and low sensitivity. This direct approach to identification and elimination of error could only improve the quality of immunoassay techniques in this country.

Acknowledgments

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APPENDIX 1

Precision

Target standard deviation \( (\sigma_T) \) was calculated from 10 pairs of estimations on each pool using the formula:

\[
\sigma_T = \sqrt{\frac{\sum (x_2 - x_1)^2}{2n}}
\]

where \( x_2 \) = observation 2

\( x_1 \) = observation 1

of each pair

and \( n = \) number of pairs (10)
The value used for the CUSUM plot of precision was then calculated from the formula:

\[
\frac{S_p^2}{\sigma_T^2} - 1 \text{ } \frac{2}{\sqrt{2}}
\]

(2)

where \( S_p^2 \) = \( \frac{(x_i - \bar{X})^2}{2} \)

(3)

**Batch variance**

Target standard deviation (\( \sigma_T \)) for each pool was calculated by the formula:

\[
\sigma_T = \sqrt{\frac{\Sigma x^2 - n(\bar{X}^2)}{n-1}}
\]

(4)

where \( x \) = each single estimation

\( \bar{X} \) = mean of \( n \) estimations (target mean)

and \( n \) = number of single estimations (20).

The value used for the CUSUM plot of batch variance was calculated from the formula:

\[
\frac{S_b^2}{\sigma_T^2} - 1 \text{ } \frac{2}{\sqrt{2}}
\]

(5)

where \( S_b^2 \) = \( (x_e - x_b)^2 \)

(6)

\( x_e \) = mean of pair at the end of the batch

\( x_b \) = mean of pair at the beginning of the batch.

**MEAN**

Target standard deviation (\( \sigma_T \)) for each pool was calculated from 10 pairs of observations from the formula:

\[
\sigma_T = \sqrt{\frac{\Sigma x^2 - n(\bar{X}^2)}{n-1}}
\]

(7)

where \( x \) = individual estimation

\( \bar{X} \) = mean of \( n \) estimations (target mean)

and \( n \) = number of single estimations (20).
The value of the CUSUM used for the mean of each pair of observations on each pool was calculated from the formula:

\[
\frac{(x_i - \bar{x})}{2\sigma_x}
\]

where \(x_i\) = mean of each pair of estimations
\(\bar{x}\) = target mean

Note: these formulae were derived such that when the data are plotted one unit on the ordinate scale is one CUSUM unit, and an identical unit on the abscissa represents one observation of each parameter. The V masks used in each case were designed as described previously4.

References