THE ASSAY OF LUTEINIZING HORMONE AND ITS
CLINICAL APPLICATION

Angelo D. Papanicolaou, M.D.

Thesis presented for the Degree of Doctor of Philosophy
at the University of Edinburgh

April 1969
# CONTENTS

<table>
<thead>
<tr>
<th>SUMMARY</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

## SECTION A

### GENERAL INTRODUCTION

- Pituitary Gonadotrophins: 2
- Human Urinary Gonadotrophins from Non-pregnant Sources: 8
- Human Chorionic Gonadotrophin: 9
- Pregnant Mare's Serum Gonadotrophin: 11
- Methods of Purification and Extraction: 12
- Extraction of human pituitary gonadotrophins from urine: 16
- Standard Preparations: 18
- Methods claimed to be specific for the assay of human pituitary gonadotrophins: 20
  - A. Biological Methods: 20
    - 1. Bioassays for FSH: 20
    - 2. Bioassays for LH: 23
  - B. Immunological Methods: 28
    - 1. Immunoassays for FSH: 28
    - 2. Immunoassays for LH: 29
- Aim of the Studies: 33

## CHAPTER 1

### Studies on the Ventral Prostate Weight (VPW) Test in Hypophysectomised Rats

- A. Comparison of the VPW and OAAD tests in the assay of LH in urine: 34
- B. Investigation of the dose-response relationship: 39
| C. Study on the temporal relationship between hypophysectomy and the duration of the assay procedure in the VPW test | 43 |
| D. Completeness of hypophysectomy | 48 |
| E. Further investigation of the dose-response relationship - Modified method | 51 |
| F. Control of toxicity: Effect of cortisone acetate on the increase in weight of the ventral prostate following the administration of Pergonal and urinary extracts | 54 |
| G. Application of the modified VPW method to clinical problems | 59 |
| Summary | 61 |

### CHAPTER 2

<p>| Studies on the Ovarian Ascorbic Acid Depletion Test in Rats | 62 |
| Modifications to the OAAD Test | 69 |
| Strain of animals | 69 |
| Control of Toxicity | 74 |
| Application of the Modified OAAD Method to estimate Urinary LH Level in Toxic Urinary Extracts | 79 |
| Analysis of Results obtained with the OAAD Test incorporating the Modifications in the Assay Procedure | 81 |
| Contamination of Extracts with Corticosteroids excreted in the Urine | 83 |
| Seasonal Variations in Ascorbic Acid Content of Pretreated Rat Ovaries following Pergonal Administration | 84 |
| Discussion | 86 |
| Summary | 89 |</p>
<table>
<thead>
<tr>
<th>CHAPTER 3</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studies on the Purification of Urine prior to the Extraction of Gonadotrophins</td>
<td>90</td>
</tr>
<tr>
<td>Collection of Urinary Samples with a Bacteriostatic Agent</td>
<td>96</td>
</tr>
<tr>
<td>The Effect of Dialysis of Urine prior to Extraction on Urinary LH</td>
<td>101</td>
</tr>
<tr>
<td>Comparison of the VPW and OAAD Tests in the Assay of LH in Urine</td>
<td>109</td>
</tr>
<tr>
<td>Summary</td>
<td>112</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SECTION B</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>113</td>
</tr>
<tr>
<td>I. Normal menstrual cycle and normal men</td>
<td>113</td>
</tr>
<tr>
<td>II. Perimenopausal and postmenopausal period</td>
<td>117</td>
</tr>
<tr>
<td>III. Oral contraceptives</td>
<td>120</td>
</tr>
<tr>
<td>IV. Clomiphene</td>
<td>125</td>
</tr>
<tr>
<td>V. 47,XXY Chromosomal abnormality</td>
<td>126</td>
</tr>
<tr>
<td>VI. Turner's syndrome</td>
<td>127</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 1</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocrine Function in Perimenopausal Women</td>
<td>132</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocrine Function in Postmenopausal Women</td>
<td>146</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation on the Effect of Oral Contraceptives on Excretion Values for Luteinising Hormone</td>
<td>156</td>
</tr>
</tbody>
</table>
CHAPTER 4

Studies on the Mechanism of Action of Clomiphene in Women with Secondary Amenorrhoea

CHAPTER 5

Urinary Luteinising Hormone Excretion in Men with 47,XYY Chromosomal Constitution

CHAPTER 6

Turner's Syndrome

REFERENCES

APPENDIX

List of published papers included in the Thesis

Acknowledgements
SUMMARY

1. The aim of the present studies was to investigate the urinary excretion of luteinising hormone (LH) in human subjects in normal and pathological conditions using two bioassay systems, the ventral prostate weight (VPW) and the ovarian ascorbic acid depletion (OAAD) tests.

2. A number of difficulties were encountered in the performance of these assays. These included lack of sensitivity, low precision and high toxicity of urinary extracts. An attempt was made to improve the reliability criteria of the tests and at the same time extraction methods from urine were re-investigated.

3. With reference to the VPW test the most important modifications introduced involved (a) the administration of cortisone acetate to the experimental animals thus considerably reducing the mortality rate of the experimental animals, and (b) the reduction of the interval between hypophysectomy and assay proper thus increasing the sensitivity of the overall procedure. Minor modifications were concerned with the strain of animal used and the diet employed.

4. Modification to the OAAD method included (a) a reduction of the time interval between the injection of the test materials and the sacrifice of the animals from 4 to 3 hours, (b) restriction of weight range of experimental animals between 35 and 45 g. and (c)
reduction of log dose interval from $\log_{10}^5 (0.69897)$ to $\log_{10}^4 (0.60206)$. Cortisone acetate affected the response of the animals when administered simultaneously with LH, but when large amounts of corticosteroids were added to urine and extracted no effect on LH activity was found.

5. The urine collected routinely for clinical studies was found to be heavily contaminated with bacteria, and this resulted in marked toxicity of the urinary extracts. The collection of urine to which Hibitane had been added inhibited such growth and reduced toxicity. Moreover, dialysis of the urine prior to extraction served as a first step of purification, removing from the urine materials which might affect the mode of action of LH in bioassay procedures.

6. The clinical conditions investigated using the modified assay procedures included the perimenopause, the postmenopause and the effects of oral contraceptives and Clomiphene on pituitary gonadotrophic function. In addition, studies were made on urinary LH levels in male subjects with a $47,XYY$ chromosomal karyotype and in patients suffering from Turner's syndrome.

7. In four subjects late in their reproductive life urinary LH was grossly elevated and in two of them ovulation as judged by urinary steroid assays occurred normally in the presence of high LH readings; in one abnormally high LH and oestrogen excretion values co-
existed. No significant difference in overall LH excretion was noted between the perimenopausal subjects and a series of four postmenopausal women.

8. Urinary LH levels were estimated in four women during and following long term therapy with oral contraceptives administered in the "classical" or sequential forms or as a "pure" gestagen. In three subjects there was definite evidence for the resumption of ovulation in the first post-treatment cycle; none of the four patients showed significant suppression of urinary LH activity as a result of medication. In a fifth woman the administration of chlormadinone acetate continuously in low dosage for a short period of time had no significant effect on either LH or oestrogen output; ovulation as judged by urinary pregnanediol assays may have inhibited.

9. Clomiphene did not exhibit any significant effect on urinary LH excretion when administered to a series of five patients suffering from abnormal gynaecological conditions associated with infertility. It was concluded that the site of action of Clomiphene was on the ovaries rather than on the pituitary-hypothalamic axis.

10. LH levels in three phenotypic males with a 47,XXY chromosomal constitution were found to be grossly elevated, being significantly higher \((P < 0.001)\) than those found in normal male subjects.

11. In Turner's syndrome LH values were variable in individual subjects, being either within or below the
postmenopausal range; no apparent correlation existed between the chromosomal karyotype and the LH output. In one of the four patients studied a bilateral ovarian transplantation was performed; this being associated with a marked rise in LH excretion immediately after the operation.
The possible existence of an extra-ovarian factor capable of regulating ovarian activity was postulated by Heape in 1897. Fox (1901) observed rapid follicular growth and development in immature ovaries when grafted into adult animals. As early as 1912, Aschner (1912) was able to demonstrate atrophic changes in the 'genital organs' of dogs following hypophysectomy. Enlargement of the ovaries and formation of numerous corpora lutea in normal rats treated with extracts of pituitary tissue were reported by Evans during the early 1920s (Evans, 1924). At about the same time Smith (1926) and Zondek and Aschheim (1927) demonstrated that pituitary extracts or implants were capable of influencing the gonad.

In 1931, Fevold et al. produced evidence to support the hypothesis that gonadal development was influenced by two hypophyseal hormones, which were called follicle-stimulating hormone (FSH) and luteinising hormone (LH) or interstitial cell stimulating hormone (ICSH). Techniques involving fractional precipitation with organic solvents and inorganic salts combined with pH adjustment, have demonstrated that extracts of sheep and swine pituitary glands contain these two gonadotrophin principles (Fevold et al., 1931; Van Dyke and Wallen-Lawrence, 1933; Bates et al., 1934). Meanwhile
evidence has been presented to indicate that the hypophysseal factor responsible for luteal function was not one of the known gonadotrophins. Dresel (1935) and Lahr and Riddle (1936) showed that purified hormone preparations would inhibit oestrous cycles in normal adult mice and rats and found that the ovaries of such animals contained a number of large corpora lutea. Astwood and Fevold (1939) put forward the suggestion that while LH is responsible for the conversion of follicles into corpora lutea, a third hypophysseal gonadotrophin termed prolactin or luteotrophic hormone (LTH) is concerned with luteal function. The Third International Conference on the Standardisation of Hormones, held in Geneva in 1959, decided to adopt the term "gonadotrophin" for the various gonad-stimulating principles.

1. Pituitary Gonadotrophins

Research into the cellular origins of the pituitary gonadotrophin hormones has not been easy, nor in many cases have the results been clear-cut. During the past decade, however, advances have resulted from the introduction of histochemical and biophysical techniques. It is now believed that prolactin is produced by the acidophil cells of the anterior pituitary (Lacour, 1950; Purves and Griesbach, 1952; Hymer et al., 1961; Barnes, 1962) while FSH and LH are secreted by the basophil cells
Some 25 years ago Marshall (1942) suggested that the hypothalamus exerted a controlling influence over the secretion of the pituitary gland. It has been demonstrated that the pituitary stalk connecting the median eminence of the tuber cinereum with the anterior pituitary gland contains a minute portal system of blood vessels (Popa and Fielding, 1930, 1933). These workers postulated that nerve tracts in the hypothalamus probably release humoral substances into the portal vessels and that these substances are transported in this way into the anterior pituitary itself. Regulation of hormone secretion is therefore controlled by chemotransmitters stimulating the cells of the anterior pituitary.

Evidence to support a hypothesis that secretion of luteotrophin may be controlled by a different mechanism has been presented by Meites et al. (1963). These workers claim that the secretion of prolactin may be depressed by the hypothalamus and have shown that it increases when the connection between the hypothalamus and the anterior pituitary is severed. The existence of a follicle-stimulating releasing factor (FSH-RF) is not yet well established, but there is some evidence to suggest that the secretion of FSH might be dependent upon hypothalamic control (Igarashi and McCann, 1964a; Igarashi et al., 1964; McCann et al., 1964). Evidence
is accumulating which supports the existence of an LH releasing factor (Taleisnik and McCann, 1961; Halasz and Gorski, 1967; Dosono et al., 1967). Recently McCann et al. (1968) in an extensive review concluded that the basal secretion of LH is mediated via the basal tuberal region of the hypothalamus and that, in females, a more rostral region which includes the suprachiasmatic area is responsible for the 'ovulatory burst' of LH.

The biological activities of the gonadotrophins have not been completely elucidated mainly due to insufficient purity of the available hormones. Evans et al. (1939) observed that the ovary of the hypophysectomised rat is much more sensitive to FSH than to LH, and that administration of FSH to hypophysectomised, immature rats caused follicular growth without luteinisation or corpus luteum formation. This observation was confirmed by Simpson (1961) and Smith and Bradbury (1963). This specific effect of FSH led to the development of a bioassay method for its determination (Evans et al., 1939; Simpson, 1961). However, Lestroh (1965) reported that in the hypophysectomised rat, highly purified sheep FSH and LH were by themselves ineffective in causing any oestrogenic effect on the uterus, stimulating follicular development or affecting ovulation. It was found that as little as 0.02 μg. of LH added to FSH (3 μg.) resulted in oestrogen secretion as evidenced by uterine enlarge-
ment; 0.2 µg. produced a maximum stimulation, 1.0 µg. initiated luteinisation of large follicles, and a final dose of 3.0 µg. LH two days later brought about ovulation. These data suggest that FSH alone cannot induce follicular stimulation, either as judged by its capacity to initiate follicular development or to promote the secretion of gonadal hormones. Both phenomena appear to be stimulated by an FSH-LH mixture, although the ratio between the two hormones in the mixture does not seem to be critical (Carter et al., 1961). The largest body of data on gonadotrophin action on the ovary of different species, *in vitro*, is that of Savard and his co-workers (Savard et al., 1965; Marsh et al., 1966; Marsh and Savard, 1966). The steroidogenic effect of FSH observed by them was thought to be due to contamination by LH.

Several investigators have clearly demonstrated that the presence of the pituitary gland is required for the maintenance of spermatogenesis (Smith, 1930; Greep and Fevold, 1937). The subject has been reviewed by Greep (1961) and Albert (1961). Albert (1961) in his review concluded that spermatogenesis is regulated entirely by pituitary gonadotrophins which exert direct supervision over the rate of both mitotic and meiotic activity and indirect control over spermatid maturation or spermiogenesis mediated by the activity of the Leydig
Nevertheless, Cutuly and Cutuly (1940) and Clermont and Morgentaler (1955) stated that the first steps of spermiogenesis may proceed in the absence of hypophyseal hormones.

While most authors agreed that LH acts on the seminiferous epithelium indirectly through the androgens produced by the Leydig cell (see review, Greep, 1961), the role of FSH remains the object of divergent opinions. Nelson (1952) suggested that FSH might be responsible only for the proliferation of spermatogonia. On the other hand Greep (1961) stated that FSH acts on the spermiogenic cells in the testes with the same selectivity that it exhibits for germinal tissues in the female gonad. Recently Clermont and Harvey (1967) investigated the effect of testosterone propionate, FSH and LH on the maintenance of spermatogenesis in hypophysectomised rats. They concluded that testosterone was the hormone immediately affecting the seminiferous epithelium, LH presumably stimulated the Leydig cells to secrete androgens, which in turn acted on the seminiferous tubules. The effect attributable to FSH was most probably due to the contamination of the preparation injected by LH. Dorfman et al. (1967) reported that pretreatment of immature male rats with human chorionic gonadotrophin (HCG) produced intense stimulation of the conversion of cholesterol to pregnenolone. This effect
could not be elicited by either FSH or LH alone, but was evident when the rats were treated with a mixture of FSH and LH.

These diverse opinions regarding the effects of gonadotrophins on the gonads are probably due to a number of factors. Undoubtedly the lack of purity of the hormones utilised is largely responsible for many findings that are contradictory in nature. Moreover there is a remarkable variability of the response to injected hormones in different species, strains and even individual animals (Lostroh et al., 1963). The complexity of the target tissues involved in gonadotrophic stimulation makes it difficult to interpret experimental findings with any great certainty. For example, spermatogenesis is not a simple phenomenon, it includes an elaborate system of proliferating and self-renewing spermatogonia, a long and intricate mechanism of cell division (meiosis) and a complex series of cytological changes when spermatids metamorphose into spermatozoa.

The effect of LH on the ovary is somewhat better established. Thus LH in the absence of the pituitary gland and of FSH, acts chiefly on the interstitial tissue of the ovary (Baillie and Griffiths, 1964; Boss et al., 1965; Deane and Rubin, 1965; Rubin et al., 1965; Balogh et al., 1966). The mechanism of action of LH appears to lie in its effect on the enzymes of the ovary.
leading to the induction of steroidogenesis (Balogh et al., 1966). Savard and his colleagues (Savard et al., 1965; Marsh et al., 1966; Marsh and Savard, 1966) have also shown by means of acetate incubations of human and bovine corpora lutea and by human ovarian stroma that LH and HCG cause an overall increase in the biosynthesis of steroids in vitro.

2. Human Urinary Gonadotrophins from Non-pregnant Sources

It has been generally assumed that the gonadotrophic material excreted in the urine of non-pregnant human subjects is derived from the pituitary gland. Relatively small amounts of gonadotrophic material have been found in urine from normal adult male and from non-pregnant female subjects, while smaller amounts have been measured in the urine of young children of both sexes. The highest levels of activity have been found in the urine of menopausal and post-menopausal women. Crude concentrated extracts of urinary gonadotrophin show both FSH and LH activities in varying proportions.

In 1960, Lunenfeld et al. demonstrated that gonadotrophins derived from the urine of menopausal and post-menopausal women, designated as human menopausal gonadotrophin (HMG) was effective in inducing ovarian stimulation. Subsequent work carried out by Lunenfeld (1963)
and other investigators demonstrated that HMG, when given in combination with HCG, could cause ovarian stimulation and ovulation (Grooke, 1964; Passeto and Montanino, 1964; Diczfalusy, 1965; Neuwrith et al., 1965).

3. Human Chorionic Gonadotrophin

In 1927 a gonad-stimulating substance, present in comparatively large amounts in the blood and urine of pregnant women, and capable of producing ovarian follicular maturation, luteinisation and haemorrhage of the ovarian stroma in immature female mice, was demonstrated by Aschheim and Zondek (1927). Following this important discovery there was much speculation regarding the source of this material. Aschheim and Zondek (1927) suggested that the gonadotrophic factor was derived from the pituitary. Subsequently it became apparent that, like the adenohypophysis, the chorionic tissue also was capable of elaborating gonadotrophic substances. Many convincing reports have now been presented which demonstrate that the gonadotrophic principle present in the body of the pregnant women is derived from chorionic tissue and not from the pituitary (Engle, 1929; Collip, 1930, 1935; Deanesly, 1935). Production of gonad-stimulating material in vitro was shown to take place in placental tissue (Sannicandro, 1934; Gay et al., 1938;
Jones and Bucher, 1943; Stewart et al., 1948). Histochromatic studies by a number of workers (Wislocki and Bennett, 1943; Zilliacus, 1953), including intraocular transplantation experiments (Stewart, 1951), confirmed the placental production of the gonadotrophic substance. The human placental gonadotrophin is generally designated as human chorionic gonadotrophin (HCG).

The role of HCG during pregnancy has not been entirely clarified. However, it appears that the hormone plays a significant role in maintaining the function of the corpus luteum during the first weeks of gestation. In a review of the subject, Lunenfeld and Donini (1966) concluded that during the later stage of pregnancy, HCG might contribute to the proper functioning of certain enzyme systems that are responsible for progesterone and oestrogen biosynthesis in the feto-placental complex. The specific action of LH and HCG on steroidogenesis in human corpora lutea of the menstrual cycle and of ectopic pregnancy has been described by Savard et al. (1965).

The administration of HCG to normally menstruating women, depending on dosage and timing, will induce luteinisation of their cells (granulosa cells and follicles). In the presence of fresh corpora lutea, HCG may have a trophic effect, causing a prolongation of its functional life. When HCG is administered to women
with anovulatory cycles it may, in combination with HMG or PMSG or human pituitary FSH, promote luteinisation of follicular cysts, follicular rupture and ovulation (for references see Loraine and Bell, 1968).

4. **Pregnant Mare's Serum Gonadotrophin (PMSG)**

Zondek (1930) and Cole and Hart (1930) independently discovered the presence of a potent gonadotrophic material in the serum of pregnant mares. The erroneous concept that the gonadotrophic material present in the serum of pregnant mares is not excreted in the urine of these animals has been so prevalent (Albert, 1961a) that the term "serum gonadotrophin" has been widely used in connection with this particular gonadotrophic substance. However, Zondek (1931) and Schmidt-Elmendorff et al. (1962a) have demonstrated the presence of this material in the urine as well as in the serum of these animals.

The mode of action of PMSG on the gonads is not well understood. Engle and Hamburger (1935), in experiments on Rhesus monkeys, observed a pure follicle-stimulating action. Experiments on human subjects were soon undertaken during which the hormone was given intramuscularly and several days later laparotomy was performed. Thus Watson et al. (1938) observed not only formation of follicular cysts but also luteinisation on the theca and, in some cases, of the granulosa too. Furthermore, Davis
and Koff (1938) were able to induce ovulation and formation of corpora lutea by the intravenous injection of PMSG. These observations were confirmed by Siegler and Fein (1939). During the last two decades, PMSG has been extensively used for the induction of ovulation, and this subject has been reviewed by Rydberg (1966).

Methods of Purification and Extraction
(a) FSH: The extraction of crude pituitary powders in mixtures of ethanol and acetate buffers has proved to be a most useful method for the purification of gonadotrophins (Koenig and King, 1950). In addition, the technique leads to a virtually complete separation of gonadotrophins from other hormones, notably growth hormone, corticotrophin and prolactin. Since FSH, alone of the pituitary hormones, is not precipitated by 50% saturated ammonium sulphate, the purification of pituitary gonadotrophin was originally achieved by this salting-out procedure. Later, the use of ion-exchange celluloses were described (Steelman et al., 1959); from solutions of low ionic strength only LH is adsorbed on to carboxymethyl cellulose (CM-C), while it is eluted before FSH from columns of diethylaminoethyl cellulose (DEAE-C). Other methods used include electrophoresis on starch, polyacrylamide gels or ethanolized cellulose, gel filtration and chromatography (Li et al., 1960; Butt
et al., 1961; Butt et al., 1963; Reichert and Parlow, 1963a; Roos and Gemzell, 1964). Fractional precipitation with ammonium sulphate has been employed for the purification of ovine FSH and the product has been further purified by chromatography on DEAE-C and other ion exchange materials (Ellis, 1958; Woods and Simpson, 1960; Reichert and Parlow, 1963) and by gel filtration on Sephadex G-100 (Courrier, 1964). It is difficult to compare the biological potencies of the different preparations obtained since a variety of standard preparations and several bioassays have been used. According to Butt (1968), who converted the reported FSH potencies into international units (i.u.), the preparation of Woods and Simpson (1960) had the highest relative potency per mg. pituitary tissue (830-1170 i.u.). Although the potency of the preparation obtained by Reichert and Parlow (1963) was relatively low (36 i.u./mg.), contamination of LH appeared to be at a minimum (0.3 i.u./mg.).

Preparations of human FSH of high potency have been obtained by Roos and Gemzell (1964), Parlow et al. (1965) and Butt (1968). According to Butt (1968) the preparation of Roos and Gemzell (1964) has a potency of 3,900 to 5,200 i.u./mg.; however, the LH contamination was expressed as i.u. HCG (6.0 i.u./mg.) which cannot be converted to i.u. LH. The preparation of Parlow et al.
(1965) had a relative potency of 1930-2440 i.u./mg. but the investigators did not report the LH contamination. The preparation of Butt (1968) contains 450 to 600 i.u. FSH/mg. and 250-500 i.u. LH/mg. It is stable almost indefinitely at room temperature if kept dry; in solution at -15°C it retains its potency for at least a year. The two components can be separated by gel electrophoresis, and the FSH fraction has a potency of 120 i.u./mg. with less than 3.0 i.u. LH/mg.

(b) LH: Ovine pituitary LH preparations were obtained over twenty years ago by fractionation with ammonium sulphate; the preparations appeared to be homogeneous according to the physical and biological criteria of purity then available (Li et al., 1940, 1942). Later methods have employed ion exchange resins and cellulosics as in the purification of FSH (Squire and Li, 1959; Woods and Simpson, 1961; Ward et al., 1961). Reichert and Parlow (1963a) concentrated the LH by fractionation with metaphosphoric acid and ethanol followed by gel filtration using Sephadex G-100. Butt (1968), who converted the reported potencies to i.u. per mg., found that the most potent material (6,300 - 6,800 i.u./mg.) was prepared by the method of Reichert and Parlow (1963a). This material also had the lowest contamination with FSH (0.3 i.u./mg.).

Methods used for the preparation of human LH are
based on the extraction either with ammonium acetate plus ethanol (Hartree et al., 1964) or with ammonium sulphate (Reichert and Parlow, 1964), but in each, ion-exchange celluloses are employed for subsequent purification. According to Butt (1968) the preparation of the former investigators had a relative potency of 3,200 i.u./mg. against 4,800 i.u./mg. of the latter workers; however, Hartree's material was practically free from contamination by FSH (0.14 i.u./mg.) or TSH (6 x 10^{-3} i.u./mg.).

Since FSH and LH have not been isolated completely free from other pituitary hormones or impurities, it has not so far been possible to determine their precise chemical composition. They appear to be proteins containing carbohydrate (Butt, 1968). According to Morris (1955) the molecular weight of ovine FSH is 67,000 and its isoelectric point is 4.5, while that of porcine FSH is 29,000 with an isoelectric point lying between 5.1 and 5.2 (Steelman and Segaloff, 1959).

In contrast to FSH, LH appears to be more stable in a highly purified form, and no losses have been reported following freeze-drying. It has been reported that purified LH fractions, even when contaminated with proteinase activity, undergo no loss of potency on storage after lyophilization (Reichert and Parlow, 1964a).
Extraction of human pituitary gonadotrophins (HPG) from urine

Many methods have been proposed for the extraction of gonadotrophins from urine, to facilitate the assay of the material present in the urine of patients under investigation. These procedures fall into three main groups.

(a) Ultrafiltration through collodion membranes (Gorbman, 1945): Following filtration the membrane, together with the residue, was removed from the filter and placed in an alcohol-ether solution. The alcohol-ether dissolved the collodion membrane and the gonadotrophins were precipitated.

(b) Adsorption methods: These involve adsorption on kaolin followed by acetone precipitation. Two main variants of this technique have been proposed.

(i) The method of Albert (1955, 1956). The pH of urine is adjusted to 4.5 prior to adsorption of HPG on to kaolin. Elution is performed by NH₄OH, the eluate is adjusted to pH 5.5 and the HPG is precipitated with acetone.

(ii) The method of Loraine and Brown (1959) in which HPG is adsorbed on to kaolin at pH 4, eluted by NaOH at pH 11.3 and the active material is precipitated at pH 4 with acetone.

Crude kaolin extracts have been purified by Albert
et al. (1961) using ammonium acetate and ethanol. This procedure produces materials of higher specific activity; it also reduces toxicity but generally results in a loss of variable amounts of biological activity ranging from 10 to 40 per cent (Albert et al., 1961a; McArthur et al., 1967).

(c) Precipitation methods: Techniques depending on precipitation are preferred in some laboratories because the extracts are of a relatively low toxicity. The reagents include tannic acid (Johnsen, 1958), and zinc acetate (Courrier, 1964).

The method of Johnsen (1958) has been widely used by many investigators, and the extracts prepared are less toxic to experimental animals than those obtained by the majority of other extraction techniques (see Borth and Menzi, 1964; McArthur et al., 1967a). Borth and Menzi (1964), who studied pooled urine obtained from normal subjects, used as the end point of their bioassay the mouse uterus test. The extraction methods compared were (i) the alcohol precipitation-dialysis method of Klinefelter et al. (1943), (ii) the kaolin-acetone method of Albert (1956) as modified by Borth et al. (1961), (iii) the benzoic and tungstic acids method of Butt (1958), (iv) the tannic acid method of Johnsen (1958), and (v) the kaolin-acetone method of Loraine and Brown (1959). Toxicity of the extracts was noted in
varying degree with all extraction methods except those prepared by the method of Johnsen (1958). The accuracy of this technique has been examined recently by Herbst et al. (1967) who conducted recovery experiments involving the addition of Pergonal (a standard preparation obtained from human menopausal urine) to pools of normal male urine. It was found that approximately 100 per cent of the LH activity, but only 50 per cent of the FSH activity, was recovered. McArthur et al. (1967) reported that they obtained a much more satisfactory recovery with the tannic acid procedure than with kaolin adsorption (Albert, 1955) especially in toxic urines. Albert et al. (1965) and Albert and Rosemberg (1965) reported that tannation of Pergonal appeared to increase the LH potency 2.4-fold but did not affect the FSH potency. This observation was not confirmed by McArthur et al. (1967). In fact the latter group of investigators found that their recovery of activity by the tannic acid method showed a loss of activity slightly greater for LH than for FSH.

Standard Preparations

Pituitary FSH and LH

Some of the earliest preparations of ovine pituitary FSH and LH were produced by Armour Laboratories (Chicago) and were termed FSH 264-151X and LH 227-80 respectively.
Later preparations available from the American National Institute of Health have been designated NIH-FSH-S1-S8 and NIH-LH-S1-S8. At the time of writing international standard preparations of human pituitary FSH and LH are not available.

**Urinary Gonadotrophins**

In the past kaolin adsorption was employed for the purification of urinary gonadotrophin of pituitary origin (Dekanski, 1949). The product was designated HMG-24 (Human Menopausal Gonadotrophins-24) and it was selected as the first International Reference Preparation (IRP) (Bull. W.H.O. 1960).

Another product designated Pergonal-23 was prepared by Donini (Serono-Rome) from post-menopausal urine by a method depending on kaolin adsorption followed by chromatography. Rosenberg and Engel (1961) and Schmidt-Elmendorff et al. (1962) have shown that Pergonal-23 has a higher specific activity than HMG-24 and was probably a more suitable choice for an International Reference Preparation. The Expert Committee on Biological Standardisation selected Pergonal-23 as the Second International Reference Preparation for Human Menopausal Gonadotrophin (2nd IRP-HMG). A potency of 40.0 international units (i.u.) of FSH and LH activity was assigned to each ampoule of this urinary preparation.

**HCG preparations:** The first international standard
was established in 1938, when 0.1 mg. of a preparation of HCG containing lactose was defined as the unit. The second international standard has recently been introduced, is of medium potency and relatively impure. Collaborative assays showed that each ampoule (approximately 6.8 mg.) contained 5,300 i.u. (Butt, 1968).

**PMSG preparation:** This preparation was introduced in 1939 and the unit was defined as the activity of 0.25 mg. of the reference standard (Bull. W.H.O. 1939).

**Methods claimed to be specific for the assay of human pituitary gonadotrophins**

A. Biological Methods

1. Bioassays for FSH

   a) **Follicular growth in hypophysectomised immature female rats** (Evans et al., 1939; Simpson, 1961): In this method immature rats are hypophysectomised at 26-27 days of age. An interval of seven to eight days is allowed for regression of follicles below antrum size and for interstitial cells to become deficient. The material for assay is then administered to the animals daily for 3 days, and the animals killed 72 hours later. Simpson (1961) reported that the method is specific, reproducible, and, when compared with the "augmentation test" (see below), gave parallel results. In view of the observation of Lostroh (1965) (see p. 4) that highly
purified FSH was unable to induce follicular development in hypophysectomised rats, the specificity of the method must be questioned. Furthermore, it is unlikely that this method would be suitable for FSH determinations in extracts prepared from urine because the animals would be unable to tolerate the large doses of urine which would have to be administered. The method is tedious and laborious; it is unsuitable for clinical application and is no longer used.

b) Augmentation tests in rats and mice: The principle of this assay was first defined by Bates and Schooly (1942). They concluded that when HCG with its luteinising action is present in excess, any further increase in ovarian weight over the plateau of response attributable to LH must be due to FSH. Steelman and Pohley (1953) described an ovarian augmentation assay system for FSH in rats based on this early work. They found that intact immature rats were more satisfactory than hypophysectomised animals and that the assay was specific for FSH. Brown (1955) adapted the Steelman-Pohley assay for use in mice, and Schmidt-Elmendorff et al. (1962) reported that this latter modification had a satisfactory degree of precision (generally below 0.2). Subsequently, Brown and Wells (1966) concluded from the assay of mixtures of purified human pituitary FSH and LH assayed against FSH alone that there was no evidence
that the assay system used was not specific for FSH.

c) Tests depending upon the uterus:

(i) Igarashi and McCann (1964) reported a technique for the estimation of FSH, which was claimed to be specific. The method is based on the synergism between FSH and a small amount of HCG on the uterus of intact immature mice. The test material mixed together with HCG is injected into intact immature female mice. The method suffers from two disadvantages, firstly the precision is low ($\lambda = 0.34 \pm 0.04$) and secondly that large amounts of LH appear to interfere with the response. These disadvantages were pointed out by the originators and were confirmed by Ross and Brown (1967).

(ii) The method described by Lamond and Bindon (1966) also makes use of the increase in uterine weight of immature mice treated with FSH and HCG. In this procedure hypophysectomised rather than intact animals are used. Ross and Brown (1967) investigated the specificity of this method and compared it with the augmentation test in mice. The addition of LH in the Lamond-Bindon assay resulted in differences in slope between the responses obtained following the administration of pure FSH and for FSH and LH mixtures. If comparisons were made between pure FSH and a mixture consisting of FSH + $1/3$ LH there was a significant difference in slope ($P < 0.05$). However, probably due to the limited number
of assays performed they were unable to show complete lack of specificity.

2. Bioassays for LH
   
a) The repair of interstitial tissue in hypophysectomised immature female rat test (Evans et al., 1939; Simpson, 1961): The animals and experimental procedure used in the assay for LH are the same as those used in the assay for FSH (see page 20) except that injections in this method are given intraperitoneally rather than subcutaneously. There is convincing evidence that LH exerts a specific effect on the interstitial tissue of the ovary (see page 7) and for this reason this assay seems to be highly specific. However, the technique is no longer used because of its laborious nature.

   b) Rat Ovarian Hyperaemia test (Ellis, 1961a, b): In this assay standard and test materials are injected intravenously in immature rats together with radio-iodinated serum albumin. The content of radio-iodinated serum albumin in the ovaries is then used to assess the extent of hyperaemia. The technique is claimed by its originator to be specific for LH. Parlow and Reichert (1963) showed that the hyperaemia test is 0.4 times as sensitive as the ovarian ascorbic acid depletion assay, and evidence was presented suggesting that sheep FSH
augmented the response to sheep LH.

c) Tests depending on superovulation in rats and mice (Zarrow et al., 1958; Soliman, 1960; Cunningham, 1962): Brown and Wells (1965) showed that the method of Cunningham (1962) is not specific for LH, since FSH also induces a response.

d) The ovarian cholesterol depletion test (Bell et al., 1964): The technique follows a similar procedure to that for the ovarian ascorbic acid test (see page 26), but the depletion of cholesterol rather than ascorbic acid is measured. According to Skosey and Goldstein (1966), the dose-response curve of the assay is multiphasic rather than linear, and is independent of the strain of animal used (Wistar and Holtzman). It was concluded that the test is not suitable for use in clinical studies.

e) The ventral prostate weight test (VPW) in immature hypophysectomised rats (Greep et al., 1941): This method has been widely used for the determination of LH activity in both pituitary tissue and urinary extracts (for references see Rosenberg et al., 1964; Albert et al., 1965; Loraine and Bell, 1966; Christiansen, 1967; De Groot, 1967a; Hutchinson et al., 1968).

The specificity of the method has been questioned by a number of investigators (Pasqualini, 1953; Segaloff et al., 1956; Grayhack et al., 1955) who have claimed
that weight increase is a form of growth and that growth can be caused by a number of factors other than LH, including growth hormone itself. However, while bovine growth hormone seemed to be active by itself in that it caused a dose-dependent growth reaction of the ventral prostate, van Rees et al. (1962) showed that after destruction of LH without an effect being produced on growth hormone, no prostatic growth was found. Paesi et al. (1956) have demonstrated that non-specific growth of the ventral prostate is almost negligible.

It has also been suggested (Chase et al., 1957; Lostroh et al., 1958) that prolactin might sensitise the prostate to the action of LH. This observation was not confirmed by Diczfalusy and Loraine (1958), McArthur et al. (1958) or van Rees et al. (1962).

Furthermore, Parlow (1963) and Parlow and Reichert (1963a) stated that FSH, the one hormone tested and considered free from interference by Greep et al. (1941) and McArthur et al. (1958), induced non-specific potentiation of the effect of LH in the VPW test. However, Rosenberg et al. (1965) and Christiansen (1968) convincingly demonstrated that such an interference did not occur.

f) Ovarian Ascorbic Acid Depletion test in immature rats (OAAD) (Parlow, 1958): The end point of this assay depends on the depletion of ascorbic acid by LH from the
ovaries of intact immature rats treated with PMSG and HCG. Since the method was described it has been used by many investigators to determine the LH activity of both pituitary tissue and urinary extracts. A variety of modifications have been used and have included varying the strain of rat, the age of the experimental animals, the schedules of pretreatment, the route of administration of standard and test material to be assayed, and the use of one or both ovaries for the estimation of the ascorbic acid (McCann and Taleisnic, 1960; Schmidt-Elmendorff and Loraine, 1962; Sakiz and Guillemin, 1963; Bell et al., 1965; Bogdanove and Gay, 1967).

Instead of intact animals, the possibility of using hypophysectomised rats has been explored (McCann et al., 1960; Baird et al., 1961). These workers showed that, following hypophysectomy, the concentration of ascorbic acid in the luteinised ovary falls markedly and the response to LH diminishes. However, the ovarian ascorbic acid responsiveness could be maintained if the hypophysectomised rats were supplied with an ectopic pituitary graft (Baird et al., 1961). Similarly, Guillemin and Sakiz (1963) found that treatment with exogenous prolactin could substitute for the pituitary graft.

The specificity of the method has been studied by a number of investigators. In the opinion of McCann and
Taleisnik (1960), Parlow (1961), Schmidt-Elmendorff and Loraine (1962), Sakiz and Guillemin (1963) and Rosenberg et al. (1965) the method is specific for LH activity. However, Pelletier (1964) and De Groot (1967) claimed that the OAAD test gave spurious results when the assay animal was injected with plasma obtained from hypophysectomised rats. It would, however, be anticipated that in the plasma of such animals LH releasing factor (LH-RF) might be present; this has also been shown to induce depletion of the ovarian ascorbic acid (Nallar and McCann, 1965). It has been reported that there are a number of substances, e.g. pitressin, vasopressin, lysine and supernatant suspensions from homogenised starch gel used in electrophoresis, which have significant activity in the OAAD test (McCann and Taleisnik, 1960; Gibson et al., 1965).

Our knowledge of the control of secretion of LH has been based mainly on the VPW and OAAD assays, which are the two most widely used tests. There are, however, a number of facts related to these two methods that still require to be explained. When the relative potencies of different preparations of LH are compared by the OAAD and VPW assays, using NIH-LH-S1 as standard, the ovine and bovine preparations have similar potencies, whereas the rat, equine and human preparations have apparently higher specific activities in the VPW assay (Parlow,
1963a; Rosenberg et al., 1964; De Groot, 1967; Christiansen, 1967; Hutchinson et al., 1968).

An explanation for these findings remains difficult. Since the test solutions are administered subcutaneously in the VPW assay, whereas intravenous administration has been the preferred route for the OAAD test, Parlow (1963a) attributed the discrepancies to differences in the biological half-life of LH from various species. However, Reichert (1966) has shown that the discrepancies are still apparent when the OAAD method is compared with the ovarian hyperaemia method, using intravenous administration in both cases. Probably, therefore, factors other than biological half-life must contribute to these different estimates of potency. It should, however, be emphasised that when urinary extracts are assayed by these two methods in terms of a urinary standard (2nd IRP-HMG) the index of discrimination (Gaddum, 1955) is approximately unity and it is reasonable to conclude that the active principle measured by the two tests is identical (Rosenberg et al., 1964; Rosenberg, 1967).

B. Immunological Methods

1. Immunoassays of FSH

Immunoassays of FSH are at present undergoing development. There are, however, two main problems to be overcome. Firstly, highly purified preparations of
human FSH are not readily available and, secondly, the majority of antisera raised to FSH cross react with LH and in many cases with thyroid stimulating hormone (TSH). In the radioimmunoassay there arise difficulties in preparing $^{131}$I-labelled FSH of sufficient purity to compete with unlabelled antigen in the assay system (Faiman and Ryan, 1967; Franchimont, 1968; Rosen et al., 1968).

2. Immunoassays for LH

It is well established that antisera raised to HCG will block the biological activity of LH in experimental animals. Mougdal and Li (1961) showed that LH and HCG have an antigenic pattern in common, an observation which has been confirmed by other workers (Franchimont, 1966; Faiman and Ryan, 1967). The antisera are usually produced in rabbits by the administration of HCG or LH.

Three types of immunological methods have been employed for the determination of LH, i.e. the complement fixation test, the haemagglutination-inhibition test and radioimmunoassay.

a) Complement fixation test: This technique was first applied by Brody and Carlstrom (1960). It depends upon the ability of the antigen-antibody system to fix complement as a linear function of antigen concentration.
For the assay proper, antigen and antisera are incubated. As an indicator of whether or not complement has been fixed, sensitised sheep red blood cells are added, i.e. red cells mixed with an appropriate amount of antibodies (haemolysin) and the extent of haemolysis is measured colorimetrically after a further period of incubation. In the absence of complement no visible reaction is seen between the erythrocytes and haemolysin. On the other hand, when free complement is present, haemolysis will occur. Brody (1967) pointed out that the specificity of the method is based upon antibody homogeneity. However, hormones presently available are not sufficiently purified to enable specific antisera to be raised.

b) **Haemagglutination-inhibition test:** The method of Wide and Gemzell (1960) depends on the ability of a standard or test preparation of HCG to inhibit the agglutination reaction between "stable" HCG-coated sheep red blood cells and HCG antiserum. "Stabilisation" of the red blood cells is effected by treatment with formaldehyde; they are subsequently treated by tannic acid and finally sensitised by treatment with HCG. However, the criticism with such methods arises when one is dealing with an impure antigen. Hunter (1967) has emphasised that in these techniques red blood cells will be sensitised with contaminants as well as with the hormone, and when the reaction is inhibited with
biological fluids, a response will occur to the contami-
nants which cannot be distinguished from that due to the
hormone.

c) **Radioimmunoassays**: Franchimont (1968) reported
the development of a specific system for the measurement
of LH. In his technique HCG or LH preparations are
labelled with $^{131}$I, and the denaturated fractions of the
labelled hormone are removed by filtration on Sephadex
G200. For the separation of labelled hormone from that
bound to antibody, starch gel electrophoresis is
employed. Urinary material assayed by this technique
must first be extracted to avoid non-specific inter-
ference. As Franchimont (1968) has pointed out, the
method requires numerous precautions especially in
determining the specificity of the immunological system
employed.

The specificity of a radioimmunoassay is defined
primarily by the nature and homogeneity of the hormone
preparation used for labelling with radiiodine.
Although there are available highly purified LH prepara-
tions, in an LH-anti-LH system, cross-reaction with FSH
still remains the main problem (Hunter, 1969). There
is evidence that a few carefully selected antisera to
HCG show cross reaction with LH but not with FSH, and
specific assays for LH may be established when these are
available (Franchimont, 1968). The radioimmunoassays
are much more sensitive as compared with bioassays and should make it possible to measure gonadotrophins in small amounts of human fluids. However, as pointed out by Wide and Gemzell (1962), if HCG is heated to 100°C, its biological activity is rapidly destroyed but its immunological potency remains. Thus it is probable that the antigenic groups are not the same as those essential for biological activity.
Aim of the Studies

The aim of these studies was to investigate the urinary excretion of LH in the human subjects in normal and pathological conditions.

As an essential prerequisite for this work two bioassay systems, the OAAD and the VPW tests, claimed to be specific for the measurement of LH, were examined. Many difficulties were encountered in their performance including lack of sensitivity, low precision and high toxicity of routinely prepared urinary extracts.

Section A of this thesis describes methodological studies carried out on the two assay systems together with an examination of techniques used in the extraction of gonadotrophins from urine.

In Section B results obtained when the modified assay procedures were applied to clinical problems are described. The clinical conditions investigated included a study of the perimenopause and postmenopause, and the effect of oral contraceptives and of Clomiphene (Clomid; MRL-41), on pituitary gonadotrophic function. In addition, studies have been made on urinary LH levels in male subjects with a 47,XYY chromosomal karyotype and in patients suffering from Turner's syndrome.
CHAPTER 1

STUDIES ON THE VENTRAL PROSTATE WEIGHT (VPW) TEST
IN HYPOPHYSECTOMISED IMMATURE RATS

It has been convincingly demonstrated that the VPW method is specific for the estimation of LH activity and that there is no interference by FSH (Creep et al., 1941; McArthur et al., 1958; Rosenberg et al., 1965; Christiansen, 1968), by prolactin (Dicsfalussy and Loraine, 1958; McArthur et al., 1958; van Rees et al., 1962) or by growth hormone (Passi et al., 1956; van Rees et al., 1962).

A. Comparison of the VPW and OAAD tests in the assay of LH in urine

Introduction

Since Creep and his colleagues described the VPW test (Creep et al., 1941), the technique has been used extensively for the measurement of LH activity, in both pituitary tissue and urinary extracts. Different laboratories have introduced their own modification of the original method, and these are listed in Table 1. However, it has been reported that when measurements of LH were carried out on pituitary tissue obtained from different species, values tended to be higher with the
<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain of Animal</th>
<th>Age of hypophysectomy (days)</th>
<th>Body Weight (g.)</th>
<th>P/operative treatment (day)</th>
<th>Duration of treatment (days)</th>
<th>Number of s.c. injections/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greep et al. (1941)</td>
<td>?</td>
<td>21 - 22</td>
<td>35 - 47</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Loraine &amp; Brown (1954)</td>
<td>Wistar</td>
<td>?</td>
<td>40 - 50</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Paesi &amp; de Jongh (1954)</td>
<td>?</td>
<td>24</td>
<td>29 - 35</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Albert et al. (1961)</td>
<td>Sprague-Dawley</td>
<td>21</td>
<td>50 ± 2</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Rosenberg &amp; Engel (1961)</td>
<td>&quot;</td>
<td>?</td>
<td>?</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Lostroh et al. (1963)</td>
<td>&quot;</td>
<td>21</td>
<td>?</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>McArthur et al. (1964)</td>
<td>&quot;</td>
<td>?</td>
<td>45 - 55</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Christiansen (1967)</td>
<td>S.S. Wistar</td>
<td>21 ± 1</td>
<td>35 - 45</td>
<td>3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>De Groot (1967a)</td>
<td>?</td>
<td>?</td>
<td>35 - 40</td>
<td>1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Hutchinson et al. (1968)</td>
<td>Sprague-Dawley</td>
<td>20</td>
<td>?</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>
VPW than with the OAAD test (see page 27). Thus when an ovine preparation (NIH-LH-S1) was used as a standard of reference, it was found that rat, equine and human LH preparations possessed higher specific activities in the VPW assay than in the OAAD test (Parlow, 1963a; Rosemberg et al., 1964; De Groot, 1967a; Christiansen, 1967; Hutchinson et al., 1968). However, Rosemberg et al. (1964) and Rosemberg (1967) demonstrated that when urinary extracts are assayed in terms of a urinary standard using both methods of assay, the index of discrimination (Gaddum, 1955) was approximately unity. It has been suggested (Gaddum, 1955) that an index of discrimination of unity gives some indication of the confidence to be placed on the results obtained by two assay techniques both measuring the same active principle; a result substantially greater or smaller than unity implies that the activities estimated in the different techniques might be due to different principles.

Since both techniques were to be employed to measure the activity of urinary LH extracts in patients (see Section B, page 113) it was important to confirm that the index of discrimination approximated to unity.

Materials and Methods

(1) Bioassay Procedure

(a) VPW test: The assay procedure adopted was
that described by Loraine and Brown (1954), except that Sprague-Dawley instead of Wistar animals were used.

The rats, 21 days old and weighing between 35 and 45 g., were hypophysectomised using the parapharyngeal approach. These animals were obtained from the Hormone Assay Laboratories Inc., Chicago, and arrived in the Unit the day after the operation. Throughout the assay procedure, they were fed a mixture of wholemeal bread, milk and glucose together with sliced oranges and were provided with 0.9% saline solution to drink.

The assay procedure was started on the 4th or 5th postoperative day, continued for 5 days and concluded on the 9th or 10th postoperative day. The regimen consisted of administering the assay material, standard (S) or unknown (U), subcutaneously daily for four days, in a volume of 0.25 ml. of normal saline. The animals were killed by ether on the 5th day, i.e. 9 or 10 days postoperatively, and the accessory sex organs were removed and fixed in Bouin's fluid. The completeness of hypophysectomy of the assay animals was checked at this time by examination of the sellae turcicae. After a minimum period of 24 hours in fixative the ventral prostate was dissected free from connective tissue, blotted and weighed on a torsion balance to the nearest 0.5 mg.

A total of 152 animals were used in the experiment and four animals were employed at each dose level of "S"
or "U". All assays were of 4 point design (Finney, 1952), and the log dose interval used was \( \log_{10} 5 \) (0.69897).

(b) OAAD test: The procedure adopted in this test was that of Bell et al. (1965) and is described on page 64. In this experiment a total of 220 animals were employed and 5 animals were used at each dose level of S or U.

(2) Material for Assay

(a) Urine: A patient suffering from secondary amenorrhoea, in whom LH excretion was expected to be high, collected complete 24 hour urine samples throughout a period of 35 days. The urine was pooled as 48 hour specimens and the gonadotrophins extracted in the manner described on page 92. Each 48 hour extract was divided into two equal parts and each part assayed by either the OAAD or the VPW method.

(b) Standard of reference: In the present study and in all the studies reported in this thesis, except when otherwise stated, the standard preparation used was Pergonal-23, designated as the 2nd IRP-HMG. Assay results are expressed as international units (i.u.) of the 2nd IRP-HMG per 24 hours.

Results

Forty-eight per cent and forty-two per cent of the
animals used for the OAAD and VPW assays respectively, died during the experiments. The majority of dead animals came mainly from groups injected with the high dose of the urinary extracts. It was also observed that most of the animals employed in the VPW test suffered from diarrhoea.

As a result of the high incidence of mortality the majority of estimations were calculated as 3 point assays. In only seven OAAD and three VPW assays was it possible to test for parallelism. Five of the seven OAAD, but none of the VPW, assays deviated significantly from parallelism.

The working range for the VPW method lay between 3.0 and 15 i.u. while that of the OAAD assay extended from 0.4 to 2.0 i.u.

The results of measuring urinary LH extracts by the two bioassay techniques used are illustrated in Figure 1. The mean index of precision calculated from s/b (\( \lambda \)) for the VPW assays was 0.22 ± 0.02 (± Standard Deviation (S.D.)) while the corresponding value for the OAAD estimations was 0.35 ± 0.04.

The mean LH value obtained from the VPW estimations was 160.7 ± 18.9 (± S.E.) i.u. per 24 hours and the corresponding figure for the OAAD assays was 150 ± 55.5 i.u. per 24 hours. When these two mean values were compared using the Student's "t" test they did not differ
Figure 1

Comparative study between OAAD and VPW test

I = fiducial limits (P = 0.95) of individual estimations
significantly. However, when the results obtained by both methods on individual samples are compared, significant discrepancies can be seen. This is represented by the calculation of the index of discrimination shown in Figure 2. The values calculated range from 0.03 to 12.4.

Discussion

The unacceptably high rate of mortality resulting in the calculation of relative potencies on a 3 point bases, the relatively low sensitivity and precision of both methods used and the lack of correlation between the VPW and OAAD techniques, suggested that neither method was satisfactory under the conditions employed. It was therefore decided that the individual steps in both techniques should be re-examined.

Methodological studies performed on the VPW technique will be presented in this chapter, while those carried out in relation to the OAAD test will be presented in Chapter 2.

B. Investigation of the dose-response relationship

Introduction

In the previous investigation it was observed that the VPW method gave rather unsatisfactory results. The majority of animals suffered from diarrhoea and a high
Figure 2
proportion of the animals died during the assay procedure. It was also found that the method did not possess adequate sensitivity.

Before any attempt was made to modify the method it was of interest to re-investigate the characteristics of the dose-response relationship and to assess the sensitivity and the optimum working range of the procedure. For this reason the following experiment was designed.

Material and experimental design

The assay procedure used was identical to that described above (p. 35).

Forty-one hypophysectomised animals were allotted to eight treatment groups. The total dose-levels of Pergonal injected per animal were 0, 0.375, 0.75, 1.5, 3.0, 6.0, 12.0 and 24.0 i.u. Each animal received the material over a period of four days, one injection being given per day in a volume of 0.25 ml. saline.

Results

It was again observed that the majority of animals were unhealthy during the assay procedure and suffered from diarrhoea. Fifty per cent died during the test with the greater mortality occurring on the last day of treatment.

The results obtained are shown in Table 2 and
<table>
<thead>
<tr>
<th>Control</th>
<th>i.u. 2nd IRP-HMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.375</td>
</tr>
<tr>
<td>Ventral</td>
<td>7.5</td>
</tr>
<tr>
<td>Prostate</td>
<td>7.5</td>
</tr>
<tr>
<td>Weight</td>
<td></td>
</tr>
<tr>
<td>(mg.)</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± S.E.**

7.5 ± 0 7.5 ± 0 7.5 ± 0 7.8 ± 1.1 10.2 ± 1.1 11.0 ± 1.9 17.0 ± 1.9 15.0 ± 0

* Dead animal  ** Standard error

Regression of the Dose-Response Curve (1.5 - 12.0 i.u.)

Sample regression coefficient (b) = 0.81 mg./i.u. LH
Regression equation (y = \bar{y} + b(x - \bar{x})) = 7.6 + 0.81x
Sample standard deviation of the regression coefficient (s_b) = 0.14
Test of significance of b: t = b/s_b = 5.79
Degree of Freedom (D.F.) = n - 2 = 7
P < 0.001
Figure 3. The linear portion of the dose-response curve can be seen to lie between 1.5 and 12.0 i.u. and the optimum working range from 3.0 to 12.0 i.u. The mean index of precision ($\lambda$) was 0.217.

The regression of prostate weight ($y$) on the $\log_{10}$ dose ($x$) was calculated according to the method described by Snedecor and Cochrane (1967); the sample regression coefficient ($b$) was calculated to be 0.81 mg./i.u.

Figure 4 illustrates the coefficient of variation of the responses associated with each dose level of Pergonal. As can be seen, in the absence of prostate weight increase (viz. control, 0.375 and 0.75 i.u.) the coefficient of variation was low and constant. However, the corresponding values associated with increased concentrations of LH that induce growth of the ventral prostate with the exception of the highest level (24 i.u.) are much higher ranging from 16 to 25 per cent.

Discussion

One of the major points to emerge from this and the previous experiment was the poor state of health of the experimental animals. This is shown by the high proportion of animals suffering from diarrhoea and the extremely high rate of mortality which reached its maximum during the last day of treatment.

It was suspected that this colony of animals had a
Figure 3

The vertical lines represent the standard errors of individual means.
THE EFFECT OF PERNONAL ON THE VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS USING THE ORIGINAL METHOD

Figure 4
relatively poor resistance to the experimental treatment involving hypophysectomy and their resistance was not improved by feeding a high concentration of carbohydrate in the post-operative diet. The percentage of dead animals in this experiment was higher than in the previous one, although the animals in the former experiment were treated with relatively crude urinary extracts.

The method was again insensitive and unsuitable in its present form for the assay of urinary extracts that might prove toxic. The precision calculated in this experiment was below optimal especially in circumstances in which a purified material was being assayed.

In well planned bioassays the variation of the response measured at each dose level must be independent of the expected response (Finney, 1964; Snedecor and Cochrane, 1967a). In the present experiment, as can be seen, the coefficient of variation rose sharply with the lowest effective dose of Pergonal and remained so within the working range of the method.

The findings of both experiments described above strongly suggested that the VPW method should be re-examined.
C. Study on the temporal relationship between hypophysectomy and the duration of the assay procedure in the VPW test

Introduction

Since the VPW test was first described, many modifications have been introduced in an attempt to increase both sensitivity and precision. The modifications previously reported are inserted in Table 1. Some workers used Sprague-Dawley animals while others preferred Wistar rats. The age on the day of hypophysectomy ranged in different centres from 20 to 24 days. The weight range of the animals used was reported by the originators of the method to be 12 g. This has been restricted by some investigators to 5 g. The day on which the assay was started after hypophysectomy also varies from one centre to another (1 to 5 days) while the duration of treatment has ranged from 4 to 7 days. Finally, a few workers reported that they obtained better results when the regimen of treatment consisted of two injections daily. It should, however, be noted that none of these investigators gave evidence to support the modifications described.

Since, in the experiments reported herein, the majority of animals suffered from diarrhoea and since it was considered that this might be due to the high concentration of carbohydrate in the post-operative diet,
it was decided to examine a diet of a different constitution. Moreover, since the animals themselves appeared to be unsuitable, it was decided to purchase rats from a different source.

In the previous experiments it was observed that the majority of animals died during the last day of treatment, i.e. the 4th day. It was thought that, due to rather long periods after hypophysectomy (8 to 9 days), the animals became unduly insensitive and died.

The aim of the present study was to investigate the optimal duration of treatment and the optimal time interval from operation until the commencement of the assay procedure. These studies were carried out in the three separate experiments described below.

Materials and Methods

Rats (Sprague-Dawley) were obtained from the Charles River Breeding Laboratories, Boston, U.S.A. The animals weighed between 45 and 50 g. at 21 days of age, when hypophysectomy was performed. They were received at the laboratory within 30 hours of operation, and, except for the loss of an occasional animal in a consignment of 75, they appeared extremely healthy. Postoperatively all animals received the standard diet of rat cake used in the C.E.R.U. and were given normal saline to drink.
Design of Experiments

Experiment I

Thirty-two animals were allotted at random into eight groups. Again at random, four groups were allotted to an assay to be performed over 3 days, while the remainder were used in an assay carried out over 4 days. All groups received the same dose levels of Pergonal, 0, 1.0 and 4.0 i.u. LH and in the same total volume of 1.5 ml. per animal, administered subcutaneously once daily during the 3 or 4 days of the assay.

Experiment II

Sixty-four animals were allotted at random amongst 16 treatment groups (4/group) and the groups randomly allotted to 4 intervals of time between hypophysectomy and the start of the assay procedure. The time intervals selected were 2, 4, 6 and 8 days postoperatively. In all instances the assay, using 3 dose levels of Pergonal in each case, was carried out over a three-day period. The log dose interval used was \( \log_{10} 3 \) (0.47712).

Experiment III

In this experiment thirty-two animals were randomly allocated amongst 8 treatment groups, 4 of which were allotted to an assay to be carried out over a period of 3 days, and four groups over a period of 6 days. Three dose levels of Pergonal were selected, i.e. 0.4, 1.6 and
6.4 i.u., and they were administered to the selected groups of animals in both types of assay in a volume of 0.5 ml. per injection.

Results

In the first experiment the results obtained from the two schedules of treatment, i.e. for 3 and 4 days, are shown in Figure 5. No animals died during the assay in any of the groups, and the optimum working range of the dose–response curve obtained with a 4-day treatment period ranged from 1.0 to 4.0 i.u. LH. When the results of the assays performed over 3 and 4 days respectively were analysed (see Table 3) it was found that the dose–response curves were not parallel ($P < 0.001$) and that the effect of LH was significantly lower ($P < 0.01$) in the assay carried out over the 4-day period.

In the second experiment none of the animals died during the assay procedure completed on the 5th post-operative day. However, in the groups killed on the 7th, 9th and 11th postoperative days, the percentage of dead animals in the respective assays was 25%, 33% and 58% (see Table 4). Figure 6 shows the results obtained from each assay in which the ventral prostate weights are plotted along the ordinate and the dose levels of Pergonal along the abscissa. The regression equations
THE EFFECT OF PREGNANOL ADMINISTERED FOR 3 or 4 DAYS ON VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS

DOSE RESPONSE RELATIONSHIP

3 Days Treatment

4 Days Treatment

VENTRAL PROSTATE WEIGHT (mg.)

Control 1.0 2.0 4.0

Control 1.0 2.0 4.0

i.u. LH (Second IRP—HMG)

Figure 5
TABLE 3
THE EFFECT OF PERTHONAL ADMINISTERED FOR 3 OR 4 DAYS
ON VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS

<table>
<thead>
<tr>
<th>Dose 1.u.</th>
<th>Response</th>
<th>VPW (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
<td>4 days</td>
</tr>
<tr>
<td>2nd IRP-HMG treatment</td>
<td>Mean ± SE</td>
<td>treatment</td>
</tr>
<tr>
<td>1.0</td>
<td>8.5</td>
<td>8.0</td>
</tr>
<tr>
<td>2.0</td>
<td>10.5</td>
<td>9.3</td>
</tr>
<tr>
<td>4.0</td>
<td>18.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Analysis of variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>Degree of freedom (D.F.)</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>V.R. (Variance Ratio)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>15.65</td>
<td>15.65</td>
<td>13.99</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>206.74</td>
<td>206.74</td>
<td>184.87</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>14.06</td>
<td>14.06</td>
<td>12.57</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Curvature</td>
<td>2</td>
<td>25.64</td>
<td>12.82</td>
<td>11.46</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>262.09</td>
<td>52.42</td>
<td>46.87</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>16.77</td>
<td>1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>278.87</td>
<td>13.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose i.u.</td>
<td>2nd IRP-HMG</td>
<td>5th P.O.</td>
<td>7th P.O.</td>
<td>9th P.O.</td>
<td>11th P.O.</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>0 (saline)</td>
<td></td>
<td>10.0</td>
<td>7.5</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.9</td>
<td></td>
<td>17.0</td>
<td>9.5</td>
<td>9.0</td>
<td>6.0</td>
</tr>
<tr>
<td>2.7</td>
<td></td>
<td>22.5</td>
<td>14.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>8.1</td>
<td></td>
<td>40.0</td>
<td>14.0</td>
<td>12.0</td>
<td>14.5</td>
</tr>
</tbody>
</table>

* Dead animal
Regeneration calculated from the 3 dose levels of LH

<table>
<thead>
<tr>
<th></th>
<th>5th P.O. day</th>
<th>7th P.O. day</th>
<th>9th P.O. day</th>
<th>11th P.O. day</th>
</tr>
</thead>
<tbody>
<tr>
<td>$y = \bar{y} + b(x - \bar{x})$</td>
<td>16.6 + 2.86x</td>
<td>9.5 + 0.7x</td>
<td>9.0 + 0.27x</td>
<td>5.11 + 0.96x</td>
</tr>
<tr>
<td>b</td>
<td>2.86</td>
<td>0.7</td>
<td>0.27</td>
<td>0.96</td>
</tr>
<tr>
<td>$s_b$</td>
<td>±0.26</td>
<td>±0.156</td>
<td>±0.107</td>
<td>±0.231</td>
</tr>
<tr>
<td>t</td>
<td>11.0</td>
<td>4.55</td>
<td>2.523</td>
<td>4.156</td>
</tr>
<tr>
<td>D.F.</td>
<td>10.0</td>
<td>9.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
<td>N.S.</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>
THE EFFECT OF Pergonal administered over a period of 3 days on the ventral prostate weight of hypophysectomised rats killed 5, 7, 9 and 11 days postoperatively

Dose response relationship

Figure 6
\( y = \bar{y} + bx \), calculated from the three dose levels of LH, are 16.6 + 2.9x, 9.5 + 0.7x, 9.0 + 0.3x and 5.1 + 1.0x for the assays performed on the 5th, 7th, 9th and 11th postoperative days respectively. As it is clearly shown in Figure 6, the 4 regression lines are not parallel. The most satisfactory assay was that obtained from animals killed on the 5th postoperative day \( b = 2.9 \text{mg./i.u.}, P < 0.001 \) (Table 4).

In the third experiment the effect of the 3 dose levels of Pergonal administered for either 3 or 6 days on the ventral prostate weight is shown in Figure 7. The analysis of the results is shown in Table 5. Although both dose-response curves are parallel, there is a significant difference between the ventral prostate weight of animals treated for 3 days when compared with those treated for the 6-day period. This is shown by the significant difference between preparations \( P < 0.05 \) shown in the analysis of variance (Table 5). Also the estimated potency \( 0.375 \text{ i.u.} \) calculated from the 6-day schedule, instead of unity indicated a loss of sensitivity of approximately 60% when this period was used.

**Discussion**

The results obtained with the experiments reported above support the view that in hypophysectomised animals the mortality rate increases proportionally after the
THE EFFECT OF Pergonal ADMINISTERED FOR 3 OR 6 DAYS ON VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS
DOSE RESPONSE RELATIONSHIP

Figure 7
TABLE 5
THE EFFECT OF PREGONAL ADMINISTERED FOR 3 OR 6 DAYS
ON THE VPW OF HYPOPHYSECTOMISED RATS

<table>
<thead>
<tr>
<th>Dose 1.u. 2nd IRP-HMG</th>
<th>Response 3 days treatment Mean ± SE</th>
<th>VPW (mg.) 6 days treatment Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>13.0</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>11.5 ± 0.7</td>
</tr>
<tr>
<td>1.6</td>
<td>20.0</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>19.5</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.5 ± 1.6</td>
</tr>
<tr>
<td>6.4</td>
<td>32.0</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>35.0</td>
<td>28.6 ± 3.0</td>
</tr>
</tbody>
</table>

Analysis of variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>148.656</td>
<td>148.656</td>
<td>8.58</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>795.018</td>
<td>795.018</td>
<td>45.86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>19.680</td>
<td>19.680</td>
<td>1.14</td>
<td>N.S.</td>
</tr>
<tr>
<td>Curvature</td>
<td>2</td>
<td>27.363</td>
<td>13.681</td>
<td>1.27</td>
<td>N.S.</td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>990.717</td>
<td>198.143</td>
<td>11.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>260.021</td>
<td>17.335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>1,250.738</td>
<td>62.537</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Preparation Relative Potency Fiducial Limit
6 days treatment 0.375 0.147 - 0.769
7th postoperative day. Also, the first and third experiments clearly show that the activity of the ventral prostate to increase in weight, after the administration of LH, decreases progressively after hypophysectomy. The findings of these experiments do not support the suggestion of Paesi and de Jongh (1954), Christiansen (1967) and De Groot (1967a) who claimed that 6 or 7 days treatment gives better results. The results presented herein demonstrated that a 3-day schedule gives better results than a treatment period of 4 or 6 days; they also showed that the optimal day on which to commence treatment is the 2nd postoperative day. The latter finding supports the suggestion of Paesi and de Jongh (1954), Albert et al. (1961), Lostroh et al. (1963) and De Groot (1967a) that the best period to start treatment is the 1st or 2nd day after hypophysectomy.

The general conclusion to be drawn from these experiments was that the optimal time to begin an assay is the 2nd postoperative day and that the duration of hormone administration should be 3 days.

D. Completeness of hypophysectomy

It was pointed out earlier (see page 35) that the completeness of hypophysectomy in all animals used in the VPW assay was checked, at the conclusion of the assay, by inspection of the sella turcica. However,
during the performance of the assays it was observed that several animals had spuriously high prostatic weights suggesting that hypophysectomy was not complete, although no pituitary tissue could be observed in their sellae turcicae. It was considered that some readily available method for verifying the completeness of hypophysectomy should be established. Christiansen (1967) and Hutchinson et al. (1968) reported that completeness of hypophysectomy could be checked by extraction of body weights, adrenal weight and the inspection of the sella turcica. The following experiment was designed to investigate the possibility of using these criteria in the assay procedure.

Material and Experimental Design

Thirty-five animals were marked, weighed and allocated amongst 7 treatment groups (5/group) immediately prior to the commencement of treatment. The animals in each treatment group received a total dose of 0, 0.4, 0.8, 1.6, 3.2, 6.4 or 12.8 i.u. LH. The hormone was administered subcutaneously once daily for 3 days. The treatment started on the 2nd postoperative day and lasted for 3 days. On the 5th postoperative day the animals were weighed and killed under ether. The sellae turcicae were examined histologically for remnants of pituitary tissue. At autopsy the adrenals were
removed, dissected free from adhering tissue and weighed on a torsion balance to the nearest 0.50 mg.

Results

On histological examination remnants of pituitary tissue were observed in the sellae turcicae of three animals and these animals were discarded. The mean (± S.E.) initial and final body weights of the remaining 32 animals were 47.3 ± 2.8 g. and 51.1 ± 2.8 respectively. Within a period of 4 days the average increase in weight was 5 g. The mean adrenal weight (both glands) was 7.5 ± 0.2 mg.

Discussion

In order to recognise and eliminate animals that have been incompletely hypophysectomised the value of measuring the increase in body weight and adrenal weight in addition to inspecting the sellae turcicae offers a number of advantages. If an animal gained more than 5 g. from the 2nd postoperative day to the day of autopsy it seemed probable that hypophysectomy was incomplete. Moreover, rats having a combined adrenal weight exceeding 7.5 mg. should be discarded even although their sellae turcicae were found to be free from pituitary tissue. This scheme of recognising and eliminating animals that have been incompletely hypo-
physsectomised has been followed routinely in the subsequent performance of all the VPW assays.

The Charles River C.D. rats appeared much healthier then and the change of postoperative diet reduced the incidence of diarrhoea. The high mortality rate was considerably reduced and only the occasional animal died. The commencement of assay on the 2nd postoperative day and the performance of the assay over a period of three days resulted in an increase in sensitivity and precision of the method. Finally, the establishment of a satisfactory system for the elimination of animals not completely hypophysectomised enabled spuriously high values to be discarded.

E. Further investigation of the dose-response relationship

Modified method

In order to confirm the effectiveness of the modifications introduced into the VPW method and in order to compare it with the original technique, the effect of Pergonal on the ventral prostate weight was re-investigated.

Material and Methods

Twenty-eight animals of the Charles River C.D. strain were hypophysectomised in Boston at an age of 21
days. The body weight of the rats ranged from 45 to 50 g. They arrived in the laboratory the next evening, and the assay procedure started the following morning. The animals were marked and the body weights recorded. The animals were allocated to 7 treatment groups and the doses of Pergonal selected were 0, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 i.u. \((\log_{10} 2/\text{dose intervals 0.30103})\). Treatment started immediately and lasted 3 days. One injection was given daily in a volume of 0.5 ml. On the fifth postoperative day the animals were again weighed and sacrificed. The completeness of hypophysectomy was checked as previously described (p. 49). The accessory sex organs were removed and fixed in Bouin's fluid. Twenty-four hours later the ventral prostate was dissected out, dried by blotting and weighed on a torsion balance to the nearest 0.5 mg.

Results

The results are shown in Table 6 and Figure 8. The linear range of the dose-response curve extended from 0.4 to 6.4 i.u. LH. The optimum working range of the method appears to lie between 0.4 and 3.2 i.u. LH. In Figure 9 are shown the coefficients of variation from which it can be seen that the corresponding values in dose levels ranging from 0.4 to 3.2 i.u. LH are low and constant ranging from 4% to 9%. Thereafter the co-
THE EFFECT OF PREGNAL ON THE VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS

DOSE RESPONSE RELATIONSHIP

Figure 8
THE EFFECT OF PERGONAL ON THE VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS USING THE MODIFIED METHOD

Figure 9
### TABLE 6

**The Effect of Pergonal on the Ventral Prostate Weight of Hypophysectomised Rats**

<table>
<thead>
<tr>
<th></th>
<th>Control saline</th>
<th>0.4</th>
<th>0.8</th>
<th>1.6</th>
<th>3.2</th>
<th>6.4</th>
<th>12.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral prostate</td>
<td>7.0</td>
<td>14.0</td>
<td>17.0</td>
<td>20.0</td>
<td>23.0</td>
<td>32.0</td>
<td>21.0</td>
</tr>
<tr>
<td>weight (mg.)</td>
<td>6.3</td>
<td>12.5</td>
<td>14.0</td>
<td>16.5</td>
<td>21.5</td>
<td>22.5</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>13.5</td>
<td>17.0</td>
<td>19.5</td>
<td>23.0</td>
<td>25.0</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>11.5</td>
<td>17.0</td>
<td>20.0</td>
<td>23.5</td>
<td>35.0</td>
<td>28.5</td>
</tr>
</tbody>
</table>

Mean ± S.E. 6.7 ± 0.2 12.9 ± 0.6 16.3 ± 0.7 19.0 ± 0.8 22.8 ± 0.5 28.6 ± 3.0 26.0 ± 2.2

Regression of the dose-response curve (0.4 - 6.4 i.u.)

\[
b = 3.42 \text{ mg./i.u.}
\]

\[y = 9.5 + 3.4x\]

\[s_b = 0.73\]

\[t = b/s_b = 4.67 \quad D.F. = 22 \quad P < 0.001\]

Comparison between the sample regression coefficients obtained from the original and modified method, student's "t" test

\[t^* = \frac{3.42 - 0.81}{0.14 + 0.73} = 3 \quad D.F. = 24 + 9 - 2 = 31\]

\[P < 0.01\]
efficient of variation rises and the corresponding value is 21%.

The regression of VFW on log dose was also calculated and the results are shown in Table 6. The sample regression coefficient \( b \) is 3.42 mg./i.u. LH \( (P<0.001) \). The index of precision was calculated to be \( \lambda = 0.121 \).

**Discussion**

The results of the present experiment together with those obtained earlier (see page 43) show that the modification performed in the VFW assay method has led to improvements in both sensitivity and precision.

When the sample regression coefficient of the original technique was compared with that estimated by the modified method (see Table 6) there was a significant increase in slope in the modified technique \( (P<0.01) \). Moreover, sensitivity was increased eightfold and the precision was doubled.

The variance of response associated with each dose level was much more uniform and lower in the modified technique (see Figures 4 and 9) and, while there was no measurable heteroscedasticity in the responses obtained by both techniques, the lower degree of variation encountered in the modified method is a significant improvement.
The establishment of a more satisfactory routine for the elimination of animals suspected of being incompletely hypophysectomised enables many of the spuriously high responses to be eliminated without the time-consuming procedure of carrying out a histological examination of the sella turcica.

F. Control of toxicity

Effect of cortisone acetate on the increase in weight of the ventral prostate following the administration of Pergonal and urinary extracts

Introduction

Despite the improvements made to the VPW method the problem of toxicity in relation to the assay of urinary extracts still remained. It was not unusual to find that many of the assay animals which died had been treated with urinary extracts obtained from one particular patient.

McArthur et al. (1964, 1967) reported that during their assay procedure cortisone was administered to experimental animals simultaneously with crude urinary extracts. However, these workers did not provide evidence to support this practice nor did they present data to show that cortisone itself had no effect on the weight of the ventral prostate.

Hypophysectomised animals are used in the assay
system and accordingly it is reasonable to anticipate that following this operation a progressive degeneration of the adrenal cortex occurs. This would have the effect of reducing the resistance of the animals to the stress produced by the injection of materials. In order to test this hypothesis the effect of administering cortisone to the animals, alone or in combination with urinary extracts, during the assay procedure was examined.

Material and Method

In the first experiment thirty-six hypophysectomised animals were randomly allocated amongst 9 treatment groups. Three groups of animals were scheduled to receive either 0, 0.4, or 1.6 i.u. LH only. A further 3 groups were to receive the identical dose levels of LH but in addition each animal was treated with 0.1 mg. cortisone acetate administered simultaneously with the LH. The remaining 3 groups again received similar dose levels of LH but the concentration of cortisone administered simultaneously was increased to 1.0 mg.

In Experiment II 76 animals were allocated at random amongst 19 groups each consisting of 4 animals. Three groups were selected at random and received 0, 0.5 and 1.5 i.u. LH, while the remaining 16 groups were used to assay 4 urinary extracts. The urinary extracts were
prepared from 96-hour pools divided into two equal parts and each part was administered to one of the 16 groups of rats selected at random either with or without the addition of cortisone (1.0 mg./day/rat) at two dose levels. The low (L) dose levels employed were equivalent to 74.9 ml. urine and the high dose (H) was equivalent to 224.7 ml. urine.

In the third experiment the upper range of the dose-response curve was extended by the inclusion of a dose-level of 3.6 i.u.; two urinary extracts were injected. They had previously been shown to be toxic in that when equivalents of 30 and 90 ml. of urine were administered over the normal period of 3 days, between 40% and 60% of the animals receiving the low dose and all those receiving the high dose had died. In the present experiment this toxic urine was administered at dosages equivalent to 68.7 (L) and 206.0 (H) ml. urine; these were given with cortisone acetate 1.0 mg./day.

All animals treated with cortisone acetate were given tap water instead of saline to drink.

Results

The effect of 3 levels of cortisone acetate, 0, 0.1 and 1.0 mg., on the dose-response curve obtained in the VPW test following the administration of 3 levels of LH, 0, 0.4 and 1.6 i.u. respectively, are illustrated in
THE EFFECT OF CORTISONE ON THE INCREASE IN VENTRAL PROSTATE WEIGHT OF HYPOPHYSECTOMISED RATS AFTER THE ADMINISTRATION OF PERNONAL

**Figure 10**
<table>
<thead>
<tr>
<th>Doses</th>
<th>( 0.1 \text{ mg.} )</th>
<th>( 1.0 \text{ mg.} )</th>
<th>( 0.4 \text{ i.u.} )</th>
<th>( + )</th>
<th>( LH )</th>
<th>( 1.6 \text{ i.u.} )</th>
<th>( + )</th>
<th>( LH )</th>
<th>( 1.6 \text{ i.u.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>11.0</td>
<td>11.0</td>
<td>12.3</td>
<td>12.0</td>
<td>10.0</td>
<td>10.5</td>
<td>28.0</td>
<td>28.5</td>
<td>21.5</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>11.5</td>
<td>12.0</td>
<td>9.0</td>
<td>10.5</td>
<td>10.5</td>
<td>13.0</td>
<td>18.0</td>
<td>19.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>9.0</td>
<td>14.0</td>
<td>11.0</td>
<td>12.5</td>
<td>14.5</td>
<td>13.0</td>
<td>20.5</td>
<td>18.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>9.0</td>
<td>11.0</td>
<td>12.5</td>
<td>15.0</td>
<td>13.5</td>
<td>11.0</td>
<td>21.0</td>
<td>16.0</td>
<td></td>
</tr>
</tbody>
</table>

**Mean \( \pm SE \):** 10.1 \( \pm \) 0.6, 12 \( \pm \) 0.7, 11.2 \( \pm \) 0.8, 12.5 \( \pm \) 1.2, 12.1 \( \pm \) 1.1, 11.8 \( \pm \) 0.7, 22.0 \( \pm \) 3.4, 21.7 \( \pm \) 2.5, 17.8 \( \pm \) 1.7

### Analysis of Variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>Variance Ratio</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doses</td>
<td>8</td>
<td>695.9</td>
<td>87.0</td>
<td>11.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LH</td>
<td>2</td>
<td>641.0</td>
<td>320.5</td>
<td>42.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>533.9</td>
<td>533.9</td>
<td>70.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>107.1</td>
<td>107.1</td>
<td>14.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cortisone</td>
<td>2</td>
<td>18.7</td>
<td>9.3</td>
<td>1.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>10.3</td>
<td>10.3</td>
<td>1.3</td>
<td>N.S.</td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>8.4</td>
<td>8.4</td>
<td>1.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Interaction</td>
<td>4</td>
<td>36.2</td>
<td>9.1</td>
<td>1.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>26</td>
<td>198.0</td>
<td>7.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>893.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10. The analysis of variance carried out on this data (Table 7) showed that there was no significant effect attributable to cortisone administration.

Figure 11 illustrates the results obtained in the second experiment in which the effect of cortisone added to urinary extracts was examined. When the results were analysed statistically (see Table 8), it was found that the first two dose-response curves with cortisone ($U_1$ and $U_2$) were parallel to $S$, and that the preparations did not differ significantly; the two slopes ($U_1$ and $U_2$ without cortisone) were not parallel. In the case of $U_3$ both slopes were parallel to that of $S$ but the response of the group of animals to which cortisone had been administered was greater than the untreated group. The difference between the two preparations of $U_3$ was highly significant ($P<0.001$). The slope for $U_4$ with cortisone showed a significant deviation from parallelism whereas that without cortisone did not. In this experiment the urinary extract was not toxic and accordingly none of the animals in the groups not receiving cortisone acetate died.

The results of the third experiment are shown in Figure 12. Statistical analysis of the data (Table 9) indicated that the curves obtained for $U_1$, $U_2$ and the standard over the working range of the dose-response curve did not deviate significantly from parallelism and
THE EFFECT OF CORTISONE ON THE VENTRAL PROSTATE WEIGHT ASSAY OF URINARY EXTRACTS

---

**Figure 11**

---

Control

<table>
<thead>
<tr>
<th>0.5 IU LH (2nd IRP - HMG)</th>
<th>L</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>U1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U4</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 8

**The Effect of Cortisone on the VPW Assay of Urinary Extracts**

<table>
<thead>
<tr>
<th>2nd IRP-HMG</th>
<th>U₁*</th>
<th>U₁ + C**</th>
<th>U₂</th>
<th>U₂ + C</th>
<th>U₃</th>
<th>U₃ + C</th>
<th>U₄</th>
<th>U₄ + C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD*</td>
<td>HD†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 i.u. i.u.</td>
<td>LD*</td>
<td>HD†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral</td>
<td>11.5</td>
<td>18.5</td>
<td>11.5</td>
<td>15.0</td>
<td>10.5</td>
<td>18.0</td>
<td>9.5</td>
<td>19.0</td>
</tr>
<tr>
<td>Prostate</td>
<td>10.0</td>
<td>18.5</td>
<td>10.5</td>
<td>13.0</td>
<td>11.0</td>
<td>16.0</td>
<td>12.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Weight (mg.)</td>
<td>10.0</td>
<td>18.0</td>
<td>10.5</td>
<td>12.5</td>
<td>9.0</td>
<td>16.3</td>
<td>12.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

| Mean | 10.4| 18.3 | 11.0| 12.7   | 10.4| 16.8   | 12.0| 15.6   |
| SE   | 0.4 | 0.1  | 0.2 | 1.1    | 0.5 | 0.5    | 1.5 | 0.6    |

---

* Unknown
** Cortisone acetate
† Low dose
‡ High dose
<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>23.766</td>
<td>21.63</td>
<td>3.48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>92.641</td>
<td>92.641</td>
<td>84.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>37.516</td>
<td>37.516</td>
<td>34.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>153.922</td>
<td>51.307</td>
<td>46.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>13.187</td>
<td>1.099</td>
<td>0.582</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>167.109</td>
<td>11.141</td>
<td>14.417</td>
<td></td>
</tr>
</tbody>
</table>

**Analysis of Variance**

Unkn. + Cortisone Acetate
<table>
<thead>
<tr>
<th>Variations</th>
<th>D.P.</th>
<th>Unknown₂</th>
<th></th>
<th>Unknown₂ + Cortisone Acetate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sum of squares</td>
<td>Mean square</td>
<td>V.R.</td>
<td>P</td>
</tr>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>1.102</td>
<td>1.102</td>
<td>2.51</td>
<td>N.S.</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>131.102</td>
<td>131.102</td>
<td>47.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>18.490</td>
<td>18.490</td>
<td>6.68</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>150.695</td>
<td>50.232</td>
<td>18.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>33.205</td>
<td>2.767</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>183.900</td>
<td>12.260</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 8 (contd.)
Analysis of Variance (contd.)
<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum of squares</th>
<th>V.R.</th>
<th>Mean square</th>
<th>V.H.</th>
<th>Mean square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>12.250</td>
<td>2.47</td>
<td>5.000</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>162.562</td>
<td>32.72</td>
<td>162.562</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>9.000</td>
<td>9.000</td>
<td>9.000</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>185.625</td>
<td>61.87</td>
<td>61.87</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>59.625</td>
<td>4.969</td>
<td>4.969</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>243.457</td>
<td>16.229</td>
<td>16.229</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Analysis of Variance (contd.)**

<table>
<thead>
<tr>
<th>Unknown 3 + Cortisone Acetate</th>
<th>Unknown 2</th>
<th>Unknown 1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variations</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Sum of squares</td>
<td>12.250</td>
<td>162.562</td>
<td>185.625</td>
</tr>
<tr>
<td>V.R.</td>
<td>2.47</td>
<td>32.72</td>
<td>61.87</td>
</tr>
<tr>
<td>Mean square</td>
<td>5.000</td>
<td>162.562</td>
<td>185.625</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Note:** The table continues with further analysis and statistical significance levels.
TABLE 8 (contd.)
Analysis of Variance (contd.)

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.P.</th>
<th>Unknown\textsubscript{4}</th>
<th></th>
<th></th>
<th>Unknown\textsubscript{4} + Cortisone Acetate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sum of squares</td>
<td>Mean square</td>
<td>V.R.</td>
<td>P</td>
<td>Sum of squares</td>
</tr>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>37.516</td>
<td>37.516</td>
<td>4.22</td>
<td>N.S.</td>
<td>319.516</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>293.266</td>
<td>293.266</td>
<td>32.99</td>
<td>&lt;0.001</td>
<td>656.641</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>1.891</td>
<td>1.891</td>
<td>0.21</td>
<td>N.S.</td>
<td>97.516</td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>332.672</td>
<td>110.891</td>
<td>12.47</td>
<td>&lt;0.001</td>
<td>1,073.672</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>106.687</td>
<td>8.891</td>
<td></td>
<td></td>
<td>69.812</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>439.359</td>
<td>29.291</td>
<td></td>
<td></td>
<td>1,143.484</td>
</tr>
</tbody>
</table>
THE EFFECT OF CORTISONE ON THE VENTRAL PROSTATE WEIGHT OF TOXIC URINARY EXTRACTS

Figure 12
<table>
<thead>
<tr>
<th>TABLE 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>THE EFFECT OF CORTISONE ON THE VENTRAL PROSTATE WEIGHT OF TOXIC URINARY EXTRACTS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>i.u. 2nd IRP-HMG</th>
<th>Unknown&lt;sub&gt;1&lt;/sub&gt; + Cortisone</th>
<th>Unknown&lt;sub&gt;2&lt;/sub&gt; + Cortisone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>1.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Ventral prostate weight (mg.)</td>
<td>12.0</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>11.9 ± 0.8</td>
<td>16.8 ± 0.5</td>
</tr>
</tbody>
</table>

**Analysis of Variance**

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>2</td>
<td>58.951</td>
<td>29.475</td>
<td>28.06</td>
<td>0.001</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>371.297</td>
<td>371.297</td>
<td>353.42</td>
<td>0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>2</td>
<td>1.359</td>
<td>0.679</td>
<td>0.65</td>
<td>N.S.</td>
</tr>
<tr>
<td>Curvature</td>
<td>1</td>
<td>1.500</td>
<td>1.500</td>
<td>1.43</td>
<td>N.S.</td>
</tr>
<tr>
<td>Between doses</td>
<td>6</td>
<td>433.107</td>
<td>72.184</td>
<td>68.71</td>
<td>0.001</td>
</tr>
<tr>
<td>Error</td>
<td>21</td>
<td>22.062</td>
<td>1.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>455.170</td>
<td>16.858</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Common slope = 11.659
= 0.088

**Potencies**

<table>
<thead>
<tr>
<th>Relative Potency</th>
<th>Fiducial Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown&lt;sub&gt;1&lt;/sub&gt;</td>
<td>6.80 i.u.</td>
</tr>
<tr>
<td>Unknown&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5.12 i.u.</td>
</tr>
</tbody>
</table>
that a valid estimate of potency could be obtained.

Discussion

These experiments demonstrated that cortisone acetate does not exert any significant effect on the ventral prostate weight test and that cortisone can be administered simultaneously with urinary extracts to the experimental animals. Moreover, cortisone administration decreased the mortality rate of the animals when urinary extracts were assayed. Three out of four extracts given simultaneously with cortisone gave valid results, whereas half of the assays in which urinary extracts were administered alone were invalid due to lack of parallelism between the dose-response curves of S and U. The results obtained with the third urinary extract (Fig. 11) indicate that the effect of urinary LH is greater on the ventral prostate weight when cortisone is administered simultaneously ($P < 0.001$).

With respect to $U_4$ it will be noted that the dose-response curve of the extract along with which cortisol was not administered to the animals was parallel to $S$, whereas the slope of the extract administered in association with cortisol was considerably steeper ($P < 0.001$). This finding might suggest that the relative potency obtained with the extract without cortisone acetate represents an under-estimate of the "true" activity.
The difference between the slope of $U^*_4$, with and without cortisone acetate, cannot be accounted for readily. Finally, the administration of cortisone acetate appeared to be useful in the assay of LH when highly toxic extracts were estimated. An equivalent of 30 ml. of urine given to the animals without cortisone acetate was sufficient to kill 50%, whereas a concentration 9 times higher of the same urine sample when injected along with 1 mg. cortisone per day was not associated with any mortality among the animals.

G. Application of the modified VPW method to clinical problems

Using the modified method, 22 assays were performed for the determination of urinary LH. In the course of these assays 1672 animals were used and 176 urinary extracts were tested.

Figure 13 shows a frequency polygon of the distribution of lambda values obtained in the 22 VPW assays. The figures lie between 0.05 and 0.2 and appear to be normally distributed. It will be noted that two of the three measures of central tendency, i.e. the mean and median, coincide although the mode does not differ greatly.

Figure 14 shows the frequency polygon of values calculated for the common slope obtained in the 22 assays.
Figure 13
FREQUENCY POLYGON OF COMMON SLOPE VALUES OBTAINED USING THE MODIFIED V.P.W. METHOD

Figure 14
Again the values appear to be normally distributed and the three measures of central tendency differ very little from one another.

Discussion

Since Greep and his colleagues (1941) described the VPW assay, the method has been used in many laboratories (see Table 1 for references) for the estimation of LH in biological fluids.

In different centres the assay procedure starts on different postoperative days (Table 1), and, according to McCann (1962), the method is insensitive when the original procedure is used. During the present study it was found that the second postoperative day was optimal for the commencement of the assay. Unfortunately it was impossible to start the assay on the first postoperative day because the animals only arrived from the U.S.A. 30 hours after the operation. Assays commencing on the second postoperative day were of sufficient sensitivity for use in clinical situations.

Paesi and de Jongh (1954), Christiansen (1967) and De Groot (1967a) reported that by extending treatment period to six or seven days the assay became more sensitive. This hypothesis was investigated in the course of the present study and it was found that a 3 day treatment period gave better results than a 6 day regime.
It was found that the administration of cortisone acetate along with toxic urinary extracts resulted in an increase in the tolerance of the experimental animals and thus enabled valid results to be obtained.

De Groot (1967) reported that, when the body weight range of the experimental animals was restricted to 5 g., this improved the reliability criteria of the assay. This suggestion was adopted during the present study.

The modifications which have been described herein have resulted in an improvement in the precision of the test together with an eightfold increase in sensitivity. The mortality of the experimental animals injected either with saline or with standard preparations was markedly reduced, while the use of cortisone acetate has virtually eliminated mortality arising from toxicity.

Summary

Some of the problems associated with the VPW assay for LH have been discussed.

Modifications in the original method have been described which increase precision and sensitivity. In particular, the administration of cortisone acetate along with urinary extracts has virtually eliminated the mortality rate of the experimental animals.
CHAPTER 2

STUDIES ON THE OVARIAN ASCORBIC ACID DEPLETION TEST IN RATS

INTRODUCTION

Ascorbic acid is widely distributed in the animal organism, the adrenal cortex, pituitary and corpus luteum having the highest concentration (Ravavenstein, 1943-45). The early observation of Sayers et al. (1944) that the amount of ascorbic acid in the adrenals decreases in response to ACTH stimulation raised the problem of the physiological importance of ascorbic acid in endocrine tissues. Later, a similar observation was made with respect to the action of LH on the ovary (Claesson et al., 1949; Hokfelt, 1950). However, at the time of writing the function of ascorbic acid in ovarian physiology is poorly understood and a number of conflicting observations have been reported. Kahnt et al. (1952) and Staudinger et al. (1955) presented evidence of a stimulatory effect of ascorbic acid on steroidogenesis. On the other hand, Hayano et al. (1956) showed that ascorbic acid inhibited the conversion of cholesterol to progesterone in vitro.

The existing relationship between LH and ovarian ascorbic acid concentration has served as a method of LH
assay (Parlow, 1958). This test was an advance in the bioassay of LH because it avoids the use of hypo-
physaectomised animals. It gives satisfactory results with highly purified preparations derived from pituitary tissue, but when applied to the bioassay of LH in urine a number of difficulties are encountered. In urinary assays different investigators have reported relatively poor precision, toxicity of routinely prepared extracts (Parlow, 1961; Schmidt-Elmendorff and Loraine, 1962; Hutchinson and Worden, 1964), and lack of a reproducible dose-response relationship (Zarrow, 1961).

Nevertheless several investigators have used the OAAD test for the bioassay of LH in urinary extracts. Thus Rosenberg and Keller (1965) and Stevens and his colleagues (for reference see Stevens and Vorys, 1967) found that the method was satisfactory for the determination of urinary LH levels in normally menstruating women. The latter group of investigators, together with Roy et al. (1963) and Bell et al. (1967), reported the results obtained when the OAAD test was applied to the measurement of urinary LH in patients treated with drugs affecting fertility.

The aim of the present study was to apply the OAAD test to the measurement of urinary LH activity in several clinical situations. These included secondary amenorrhoea treated with Clomiphene, the postmenopause,
and the effects of therapy by oral contraceptives on pituitary gonadotrophic function.

MATERIALS AND METHODS

The assay procedure employed was that described by Schmidt-Elmendorff and Loraine (1962) and Bell et al. (1965).

Pretreatment of animals: Intact immature Wistar rats from the Clinical Endocrinology Research Unit (C.E.R.U.) colony were used. They varied in age from 21 to 23 days and in weight from 35 to 50 g. Each animal received a subcutaneous injection of 50 i.u. PMSG (Gestyl - Organon) and 72 hours later a subcutaneous injection of 25 i.u. HCG (Pregnyl - Organon). Both preparations were dissolved in saline and the injections were administered in a total volume of 0.5 ml./animal. As a result of this pretreatment the animals showed heavily luteinised ovaries, ranging in weight from 70 to 140 mg. The animals were housed in groups of three according to the specifications of Chance (1956), the advantages of this design having recently been confirmed by Brown et al. (1968).

Bioassay proper: This was performed between the 5th and 9th day after the injection of HCG. Animals were allocated at random in groups of five.

The standard (S) (Pergonal) and unknown (U)
materials were administered intraperitoneally in 0.5 ml. of normal saline. The assays were of four-point design. One group of animals was also used as control and was injected intraperitoneally with saline (0.5 ml. per animal). The log dose interval was \( \log_{10} 5 \) (0.69897) and the high dose of \( (U) \) usually represented the equivalent of 125 ml. of urinary extract per animal. The animals were killed 4 hours \( \pm \) 10 minutes after the intraperitoneal injections by dislocation of the cervical spine, and the two ovaries were removed using a dorsal approach. The individual ovaries were rapidly cleaned and weighed on a torsion balance to the nearest 0.5 mg. Each ovary was then homogenised in a mortar and pestle with 2.5 ml. of 2.5\% metaphosphoric acid (freshly prepared) and a trace of sand. After thorough grinding, a further 7.5 ml. of 2.5\% metaphosphoric acid was added and the homogenate was filtered through Whatman No. 2 filter paper. The ascorbic acid content of the filtrate was then estimated according to the method described by Mindlin and Butler (1938) as follows: 2.0 ml. of the clear metaphosphoric acid extract was mixed with 2 ml. of standardised 2,6-dichlorophenolindophenol solution and read immediately in a spectrophotometer (SP-600). The 2,6-dichlorophenolindophenol solution was prepared in the following way: 12.0 mg. of the dye were dissolved in 40.0 ml. of distilled water which was then mixed with
280.0 ml. of sodium acetate solution (18.16 g. of sodium acetate in 400 ml. of distilled water - pH of the solution adjusted to pH 7.0 using glacial acetic acid and N/10 sodium hydroxide solution). The dye solution was freshly prepared on the day on which it was to be used and the concentration of the dye was adjusted by dilution with the sodium acetate solution.

The amount of reduced ascorbic acid was determined from the calibration curve prepared with known amounts of pure ascorbic acid solution in 2.5% metaphosphoric acid. The regression of \( X \) (ascorbic acid) on \( Y \) (optical density) was calculated according to the method described by Snedecor and Cochrane (1967) and the regression equation \( y = 450.25 - 2.2x \) (Table 10, Figure 15).

The ascorbic acid concentration of each ovary was expressed as \( \mu g. \) per 100 mg. ovarian tissue and the mean value of both ovaries from the same animal was used as one observation, according to the suggestion of Borth (personal communication). The mean concentration of ascorbic acid in the group treated with the low dose of S was compared with that found in the control group by using the Student's "t" test. If there was no significant depletion of ascorbic acid in the former group the assay was regarded as invalid.

A total of 58 experiments was carried out, during
TABLE 10
CALIBRATION CURVE RELATING ASCORBIC ACID
CONCENTRATIONS TO CHANGES OF COLOUR
MEASURED AT A WAVELENGTH OF 520 µm.

<table>
<thead>
<tr>
<th>Ascorbic Acid (µg./10 ml. 2.5% Metaphosphoric acid)</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>233</td>
</tr>
<tr>
<td>229</td>
<td></td>
</tr>
<tr>
<td>228</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>345</td>
</tr>
<tr>
<td>339</td>
<td></td>
</tr>
<tr>
<td>336</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>396</td>
</tr>
<tr>
<td>395</td>
<td></td>
</tr>
<tr>
<td>399</td>
<td></td>
</tr>
</tbody>
</table>

Regression

\[ b = -2.197 \]

\[ y = \bar{y} + b(x - \bar{x}) = 450.25 - 2.2x \]

\[ s_b = 0.01 \]

\[ t = b/s_b = 220 \]

\[ D.F. = n - 2 = 10 \]

\[ P < 0.001 \]
CALIBRATION CURVE RELATING ASCORBIC ACID CONCENTRATIONS TO CHANGES OF COLOUR MEASURED AT A WAVELENGTH OF 520 m.µ.

\[ y = 450.25 - 2.2x \]

Figure 15
which 2,610 animals were used and the LH content of 174 urine extracts were assayed.

RESULTS

During these experiments 1,740 animals were injected with urinary extracts; a total of 644 animals (37%) died during the assays. The majority of these came from groups injected with the high dose of the urinary extracts. One hundred and twenty-seven samples were assayed; 47 of these (27%) were rejected due to their toxic effect on the experimental animals. Fifty-two of the assays (41%) had to be rejected because of significant deviation from parallelism between $S$ and $U$. Valid results were obtained from only 75 samples (43% of the total number).

The precision of the method was poor. The mean value calculated for $\lambda$ was $0.44 \pm 0.15$ (± S.D.). The frequency polygon of the distribution of these values is shown in Figure 16 from which it will be noted that values range from 0.13 to 0.78; these were not normally distributed, the three measures of central tendency differing from each other.

DISCUSSION

During these studies the results obtained were unsatisfactory. Only 43% of the total number of urinary
FREQUENCY POLYGON OF LAMBDA VALUES OBTAINED USING THE ORIGINAL OAAD METHOD

Figure 16
samples tested resulted in valid four-point assays. Lack of parallelism was more prevalent in these experiments than in previously published reports by Schmidt-Elmendorff and Loraine (1962) and Hutchinson and Worden (1964). According to these two groups of workers the percentage of invalid assays due to significant deviation from parallelism between the dose response curves of S and U was approximately 20%. However, in the experience of Herbst et al. (1967) the percentage of invalid results for the same reason rose to 58%. The precision of the method during the present series of studies was less satisfactory than that obtained by Schmidt-Elmendorff and Loraine (1962), Hutchinson and Worden (1964) and Herbst et al. (1967); the figures for § reported by these investigators were generally below 0.3. Moreover Herbst et al. (1967) found that the problem of toxicity was less severe when the tannic acid method was used for the extraction of urinary gonadotrophins. In their limited experience the percentage of dead animals was 4.5 per cent. However, a high incidence of mortality appeared in the present study. Thirty-seven per cent of the animals injected with urinary extracts died during the assays. In addition the results obtained in animals which survived treatment after injection of the urinary extracts were unreliable. Accordingly, 27% of the assays were rejected.
The experiments reported herein confirm the previous observation (page 34) that the OAAD test does not give satisfactory results when urinary gonadotrophins are assayed. It was therefore essential to re-examine the assay procedure in an attempt to improve the reliability criteria and reduce the unacceptably high rate of mortality of the experimental animals.

MODIFICATIONS TO THE OAAD TEST

**Strain of animals**

Facing these obvious difficulties it was considered that the animals bred in the C.E.R.U. were unduly sensitive to the toxic effect of the urinary extracts and for this reason the results obtained were generally unsatisfactory. Accordingly, an attempt was made to investigate whether animals from other sources of the same or of different strains could be used. It was, however, important to ascertain that the sensitivity of such animals compared favourably with the C.E.R.U. colony.

Several investigators reported differences in the reaction between strains of animals used for the OAAD test (Koed and Hamburger, 1965, 1968), and others have suggested that the optimal time for performance of the assay is from the 5th to the 7th day after the HCG injection (Hutchinson and Worden, 1964; Bogdanove and
Gay, 1967). The latter suggestion has been adopted and the OAAD tests have been carried out on the 6th or 7th day after the administration of HCG.

Materials and Methods

Five assays were conducted in which animals obtained from two separate commercial sources were compared with the C.E.R.U. rats. Three assays were conducted using Sprague-Dawley animals and one with Wistar rats, both obtained from commercial sources.

Fifty animals were used for each assay using Sprague-Dawley animals, while 30 and 25 rats respectively were employed in the case of Wistar animals obtained from a commercial source and from the C.E.R.U.

The assay method was the same as that described above (see page 64), the only difference being that the bioassay proper was carried out on the 6th or 7th day after HCG administration.

In the three assays in which Sprague-Dawley animals were used, the standard preparation injected was NIH-LH-S6. In the first, the doses selected ranged from 0.012 to 3.0 μg. and the dose interval was \( \log_{10} 2 \) (0.30103) while in the second and third they ranged from 0.032 to 48.83 μg. with a dose interval of \( \log_{10} 2.5 \) (0.39794).

Both groups of Wistar animals were treated with
Pergonal. The doses selected for the animals obtained from the commercial source ranged from 0.1 to 62.5 i.u. and the log dose interval was \( \log_{10} 5 \) (0.69897) whereas the doses for the C.E.R.U. rats ranged from 0.3 to 2.4 i.u., the dose interval being \( \log_{10} 2 \) (0.30103).

**Results**

The results obtained are shown in Figures 17, 18 and 19 and in Table 11.

An analysis of variance of the results obtained for the first Sprague-Dawley group showed that dose levels of LH up to 1.5 \( \mu g \), including controls, did not produce any significant change in the ascorbic acid concentration. When, in the analysis of the results, the upper range of the dose-response curve was extended to include the dose level of 3.0 \( \mu g \), LH, a slight depletion was shown to have occurred \( (P < 0.05) \). When the concentrations of ascorbic acid at each dose level were compared with the control value, using Student's "t" test, no significant difference could be shown. Similar results were obtained when the data of the second and third Sprague-Dawley groups were analysed. Again dose levels of LH up to 1.25 \( \mu g \) and 0.5 \( \mu g \) for the second and third groups respectively did not produce any significant effect on the ovarian ascorbic acid concentration. The ascorbic acid depletion was initiated at a dose level of
THE DEPLETION OF ASCORBIC ACID FROM PRETREATED SPRAGUE-DAWLEY RAT OVARI<br> Following the administration of NIH-LH-S6

Figure 17
THE DEPLETION OF ASCORBIC ACID FROM PRETREATED WISTAR RAT OVARIES FOLLOWING THE ADMINISTRATION OF Pergonal

Figure 18
THE DEPLETION OF ASCORBIC ACID FROM PRETREATED C.E.R.U. RAT OVARIIES AFTER THE ADMINISTRATION OF PERNONAL

DOSE RESPONSE RELATIONSHIP

Figure 19
TABLE II
THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARIES FOLLOWING THE ADMINISTRATION OF LH

<table>
<thead>
<tr>
<th>NIH-LH-S6 (µg.)</th>
<th>1st Group</th>
<th>2nd Group</th>
<th>3rd Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-LH-S6 (µg.)</td>
<td>O.A.A.* Concentration (µg./100 mg. tissue)</td>
<td>Mean ± S.E.</td>
<td>O.A.A. Concentration (µg./100 mg. tissue)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>110.4 ± 1.5</td>
<td>Saline</td>
<td>104.0 ± 21.6</td>
</tr>
<tr>
<td>0.01171</td>
<td>111.4 ± 10.0</td>
<td>0.032</td>
<td>125.9 ± 14.9</td>
</tr>
<tr>
<td>0.0234</td>
<td>124.7 ± 14.0</td>
<td>0.080</td>
<td>113.6 ± 10.5</td>
</tr>
<tr>
<td>0.0468</td>
<td>113.9 ± 15.9</td>
<td>0.20</td>
<td>116.1 ± 10.9</td>
</tr>
<tr>
<td>0.0937</td>
<td>123.6 ± 12.5</td>
<td>0.50</td>
<td>122.7 ± 17.6</td>
</tr>
<tr>
<td>0.1875</td>
<td>112.1 ± 15.0</td>
<td>1.25</td>
<td>100.5 ± 17.8</td>
</tr>
<tr>
<td>0.375</td>
<td>115.5 ± 7.4</td>
<td>3.1250</td>
<td>73.2 ± 19.9</td>
</tr>
<tr>
<td>0.75</td>
<td>115.8 ± 19.3</td>
<td>7.8125</td>
<td>70.3 ± 9.7</td>
</tr>
<tr>
<td>1.50</td>
<td>113.8 ± 13.7</td>
<td>19.53</td>
<td>58.5 ± 6.2</td>
</tr>
<tr>
<td>3.00</td>
<td>92.9 ± 10.2</td>
<td>48.83</td>
<td>54.0 ± 8.9</td>
</tr>
</tbody>
</table>

* Ovarian ascorbic acid.
| 2nd IRP-HMG i.u. | COMMERCIAL | | WISTAR RATS | | O.A.A. Concentration (µg./100 mg. tissue) Mean ± S.E. | 2nd IRP-HMG i.u. | O.A.A. Concentration (µg./100 mg. tissue) Mean ± S.E. |
|-----------------|------------|------------------|------------------|
| Saline          | 75.3 ± 11.4| Saline           | 130.2 ± 3.4      |
| 0.1             | 85.8 ± 13.2| 0.3              | 110.6 ± 1.4      |
| 0.5             | 62.0 ± 13.8| 0.6              | 104.1 ± 2.3      |
| 2.5             | 59.2 ± 8.9 | 1.2              | 89.2 ± 2.2       |
| 12.5            | 65.3 ± 13.7| 2.4              | 85.8 ± 1.5       |
| 62.5            | 39.9 ± 12.4|                  |                  |
### TABLE II (contd.)

**Analysis of Variance**

#### SPRAUCG-DAWLEY

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>9</td>
<td>3,386.91</td>
<td>376.32</td>
<td>2.567</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Error</td>
<td>39</td>
<td>5,717.20</td>
<td>146.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>9,104.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>8</td>
<td>1,044.70</td>
<td>130.59</td>
<td>0.873</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>35</td>
<td>5,236.24</td>
<td>149.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>6,280.94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2nd Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>9</td>
<td>27,910.41</td>
<td>3,101.16</td>
<td>6.908</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>16,160.73</td>
<td>448.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>44,071.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>664.03</td>
<td>132.81</td>
<td>0.211</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>23</td>
<td>14,475.32</td>
<td>629.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>15,139.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3rd Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>9</td>
<td>35,893.06</td>
<td>3,988.12</td>
<td>30.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>38</td>
<td>5,010.97</td>
<td>131.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>40,904.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>4</td>
<td>1,588.82</td>
<td>397.21</td>
<td>2.09</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>3,791.80</td>
<td>189.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>5,380.62</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**TABLE 11 (contd.)**

Analysis of Variance (contd.)

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WISTAR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 62.5 i.u. (2nd IRP-HMG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>6,008.4</td>
<td>1,201.7</td>
<td>2.654</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Error</td>
<td>23</td>
<td>10,411.6</td>
<td>452.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>16,420.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 12.5 i.u. (2nd IRP-HMG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>4</td>
<td>2,279.8</td>
<td>569.9</td>
<td>0.545</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>19</td>
<td>19,848.6</td>
<td>1,044.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>22,128.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.E.R.U.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 2.4 i.u. (2nd IRP-HMG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>4</td>
<td>8,430.08</td>
<td>2,107.52</td>
<td>14.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>2,950.30</td>
<td>147.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>11,380.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 0.6 i.u. (2nd IRP-HMG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between doses</td>
<td>2</td>
<td>1,624.28</td>
<td>812.14</td>
<td>7.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>1,312.80</td>
<td>109.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>2,937.08</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.125 and 1.25 μg. LH in the second and third group respectively and became pronounced by inclusion of dose levels up to 48.83 μg. LH (P < 0.001). As before, the results obtained for individual dose levels of hormone were compared with their respective controls using the "t" test.

In the second group, no significant effect could be demonstrated. However, in the last group LH dose levels of 19.53 and 48.83 μg. had a significant effect (P < 0.02).

Analysis of the results obtained following the administration of Pergonal to Wistar rats from a commercial source indicated that dose levels of LH up to 12.5 i.u. did not produce any significant alteration in ascorbic acid concentration. When a dose level of 62.5 i.u. LH was included in the analysis a slight effect was noted (P < 0.05). Individual comparisons with the control value, using Student's "t" test, showed no significant difference.

The results obtained in C.E.R.U. animals showed that the ascorbic acid concentrations, following the administration of a range of dose levels of Pergonal from 0.3 to 2.4 i.u., including the control differed significantly (P < 0.001). This effect was also noted for dose levels of LH up to 0.6 i.u. (P < 0.001). Using Student's "t" test, as before, it was shown that even the lowest dose
level of LH (0.3 i.u.) caused a significant depletion of ovarian ascorbic acid (P<0.01).

**Discussion**

The results obtained in these experiments, in relation to the Sprague-Dawley animals, show that the minimal dose of LH required to produce ovarian ascorbate depletion was 3.0, 3.125 or 1.25 μg. LH in the first, second and third groups respectively.

In Wistar animals, from a commercial source, the slight depletion of ascorbic acid occurred only at a dose level of 62.5 i.u. LH (P<0.05).

The data obtained from the last experiment showed that the animals of the C.E.R.U. colony were more sensitive and the variation in response at each dose level was less than that observed for the other groups.

Rosenberg and Lewis (1966), using the OAAD assay, reported that 1,000 μg. of NIH-LH-S1 was equivalent to 500 i.u. LH (2nd IRP-HMG). Using this conversion figure, assuming NIH-LH-S1 and S6 to be equipotent, the relative sensitivities of the different assays were compared in terms of the 2nd IRP-HMG. In the Sprague-Dawley animals the minimal effective doses were equivalent to either 0.66, 1.5 or 1.55 i.u. LH, while in the C.E.R.U. colony a significant depletion occurred at a dose level of 0.3 i.u. The Wistar animals from a
commercial source were less sensitive than those in the other groups, a dose level of 62.5 i.u. LH being required to produce a response.

The present study showed that the rats of choice for the OAAD assay were those of the C.E.R.U. colony. With these animals the method was reasonably sensitive and the optimal working range of the dose-response curve (Figure 19) lay between 0.3 and 1.2 i.u. Except where otherwise stated, this $\log_{10} 4$ dose interval (0.60206) has been adopted for all the OAAD assays, instead of that originally used ($\log_{10} 5$, 0.69897).

**CONTROL OF TOXICITY**

**Introduction**

The previous experiments demonstrated the suitability of the animals from the C.E.R.U. colony in the OAAD test. Nevertheless when the technique was applied to the measurement of LH in urinary extracts the results obtained, as mentioned previously, were disappointing. It was considered that the experimental animals might give more satisfactory results if they were protected from the stress effect induced by the toxic urinary extracts.

An attempt was made to control toxicity of urinary extracts in a similar manner to that which has been satisfactorily employed in the VPW test (see page 54).
Thus it appeared of interest to investigate whether cortisone acetate could be used and whether a shorter time interval between the administration of S or U and measurement of ascorbic acid concentration might be beneficial.

The aim of the following four experiments was therefore to investigate whether cortisone acetate exerted any significant effect on the OAAD test and if a three-hour interval was sufficient for the induction of a significant depletion of ovarian ascorbic acid by LH (Pergonal).

**Materials and Methods**

The assay procedure followed that described on page 64, the only difference being that the body weight of the animals was restricted from 35 to 45 g., the 10 g. weight range being selected as a result of the findings of Hutchinson and Worden (1964).

In the following four experiments, the division of animals into groups and the allocation of treatments to these groups was carried out on a random basis.

**Experiment I:** Forty-five rats were divided into nine treatment groups. Three groups of animals received either 0, 0.4 or 1.6 i.u. LH alone. A further three groups were treated with the identical dose levels of LH, but, in addition, each animal was treated with 10 μg.
cortisone acetate administered simultaneously with the LH. The remaining three groups again received similar dose levels of LH, but the concentration of cortisone administered was increased to 100 μg.

**Experiment II:** Five groups of five rats were used. One group was treated with saline and served as control. The other four were divided into two main groups, one to be killed 3 hours and the other 4 hours after the administration of LH. The doses selected were 0.4 and 1.0 i.u. per animal for the low and high doses respectively.

**Experiment III:** Eighty-two animals were divided into 9 groups. Groups of rats were killed either 3 or 4 hours after injection of 0.3125, 0.625, 1.25 or 2.5 i.u. LH. The remaining group acted as control.

**Experiment IV:** Ninety-five animals were allotted to 11 groups. One group served as control, 5 groups were treated with 0.4 i.u. and 5 groups with 2.0 i.u. LH. One group of animals was killed at varying time intervals following the injection of either dose level of hormone and the ascorbic acid concentration determined. The intervals selected were 2 hours 40 mins., 3 hours, 3 hours 20 mins., 3 hours 40 mins. and 4 hours.

**Results**

The results of the first experiment are shown in Table 12 and Figure 20. An analysis of variance
EFFECT OF CORTISONE ON THE DEPLETION OF ASCORBIC ACID FROM THE PRETREATED RAT OVARY BY LH (PERGONAL)

Figure 20
### Table 12

THE EFFECT OF CORTISONE ON THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARY BY LH

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Cortisone acetate 10 µg.</th>
<th>Cortisone acetate 100 µg.</th>
<th>LH 0.4 i.u.</th>
<th>Cortisone acetate 10 µg. + LH 0.4 i.u.</th>
<th>Cortisone acetate 100 µg. + LH 0.4 i.u.</th>
<th>Cortisone acetate 10 µg. + LH 1.6 i.u.</th>
<th>Cortisone acetate 100 µg. + LH 1.6 i.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian ascorbic acid concentration (µg./100 mg. ovarian tissue)</td>
<td>119.7</td>
<td>101.4</td>
<td>112.8</td>
<td>120.2</td>
<td>116.8</td>
<td>117.6</td>
<td>86.2</td>
<td>95.3</td>
</tr>
<tr>
<td></td>
<td>120.6</td>
<td>113.8</td>
<td>113.5</td>
<td>118.6</td>
<td>114.0</td>
<td>117.9</td>
<td>87.7</td>
<td>93.0</td>
</tr>
<tr>
<td></td>
<td>106.2</td>
<td>107.6</td>
<td>117.5</td>
<td>122.2</td>
<td>114.9</td>
<td>114.8</td>
<td>79.7</td>
<td>91.8</td>
</tr>
<tr>
<td></td>
<td>115.0</td>
<td>109.2</td>
<td>118.6</td>
<td>119.7</td>
<td>120.5</td>
<td>86.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>123.9</td>
<td>111.9</td>
<td>113.0</td>
<td>112.2</td>
<td>86.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean: 117.1 108.8 115.0 120.3 116.4 116.6 85.1 93.4 104.2

<table>
<thead>
<tr>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
<th>±</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E.</td>
<td>3.4</td>
<td>2.4</td>
<td>1.2</td>
<td>0.9</td>
<td>1.3</td>
<td>1.6</td>
<td>1.7</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Variations</td>
<td>Degree of Freedom</td>
<td>Sum of Squares</td>
<td>Mean Square</td>
<td>Variance Ratio</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------------</td>
<td>----------------</td>
<td>-------------</td>
<td>----------------</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doses</td>
<td>8</td>
<td>5,309.08</td>
<td>663.63</td>
<td>27.01</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>2</td>
<td>4,265.90</td>
<td>2,132.95</td>
<td>86.81</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear (L)</td>
<td>1</td>
<td>2,881.20</td>
<td>2,881.20</td>
<td>117.26</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic (Q)</td>
<td>1</td>
<td>1,384.70</td>
<td>1,384.70</td>
<td>56.36</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisone</td>
<td>2</td>
<td>357.28</td>
<td>178.63</td>
<td>7.30</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>197.53</td>
<td>197.53</td>
<td>8.04</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>159.73</td>
<td>159.73</td>
<td>6.50</td>
<td>&lt; 0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>4</td>
<td>685.90</td>
<td>171.47</td>
<td>7.00</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH_L v. C_L</td>
<td>1</td>
<td>528.42</td>
<td>528.42</td>
<td>21.51</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH_L v. C_Q</td>
<td>1</td>
<td>64.71</td>
<td>64.71</td>
<td>2.63</td>
<td>N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH_Q v. C_L</td>
<td>1</td>
<td>92.73</td>
<td>92.73</td>
<td>3.77</td>
<td>N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH_Q v. C_Q</td>
<td>1</td>
<td>0.04</td>
<td>0.04</td>
<td>0.001</td>
<td>N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>33</td>
<td>810.74</td>
<td>24.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>6,119.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
carried out on these data showed that there was a highly significant effect attributable to cortisone administration \((P < 0.01)\), and that it was linearly related to the dose level injected \((P < 0.01)\). Furthermore, the interaction between LH (linear) and cortisone acetate (linear) was highly significant \((P < 0.001)\), demonstrating that as the effect of LH on ascorbate depletion increases with dose, the effect of higher doses of cortisone acetate becomes more pronounced and progressively more inhibitory.

The results obtained in the second experiment are shown in Figure 21. The dose-response curves obtained at the two time intervals are clearly not parallel. The sample regression coefficients \((b)\) were calculated (see Table 13); at 3 hours \(b = -62.23\) and was highly significant \((P < 0.001)\), whereas at 4 hours \(b = -17.42\) and was not significant.

Figure 22 and Table 14 show the results obtained from the third experiment. In the analysis of variance it was found that the effect of LH on ovarian ascorbic acid did not differ significantly at either time interval. The dose-response curves were both parallel and linear.

The results of the fourth experiment are shown in Figure 23. The 2 hour 40 mins. interval was sufficient to induce significant depletion and at 3 hours the effect
The depletion of ascorbic acid from pretreated rat ovaries 3 or 4 hours after the administration of pergonaI.

Dose Response Relationship

Ovarian ascorbic acid concentration (μg/100 mg tissue)

Control 04 10

i.u. LH (second IRP-HMG)

3 hours

4 hours

Figure 21
### Table 13

**The Depletion of Ascorbic Acid from Pretreated Rat Ovaries 3 or 4 Hours After the Administration of Pergonal**

<table>
<thead>
<tr>
<th></th>
<th>3 Hours</th>
<th></th>
<th>4 Hours</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4 i.u. LH</td>
<td>1.0 i.u. LH</td>
<td>0.4 i.u. LH</td>
<td>1.0 i.u. LH</td>
</tr>
<tr>
<td>0.4 i.u. LH</td>
<td>124.22</td>
<td>87.77</td>
<td>94.52</td>
<td>106.04</td>
</tr>
<tr>
<td>1.0 i.u. LH</td>
<td>117.44</td>
<td>75.62</td>
<td>108.14</td>
<td>90.32</td>
</tr>
<tr>
<td>0.4 i.u. LH</td>
<td>124.91</td>
<td>91.19</td>
<td>126.32</td>
<td>120.34</td>
</tr>
<tr>
<td>1.0 i.u. LH</td>
<td>116.40</td>
<td>80.98</td>
<td>111.88</td>
<td>108.98</td>
</tr>
<tr>
<td>0.4 i.u. LH</td>
<td>122.64</td>
<td>83.37</td>
<td>128.94</td>
<td>91.84</td>
</tr>
</tbody>
</table>

121.12 ± 1.50  83.79 ± 2.99  113.96 ± 6.61  103.50 ± 5.76

**Regression**

<table>
<thead>
<tr>
<th></th>
<th>3 Hours</th>
<th></th>
<th>4 Hours</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>-62.23</td>
<td></td>
<td>-17.42</td>
<td></td>
</tr>
<tr>
<td>y = y̅ + b(x - x̅)</td>
<td>141.66 - 62.23x</td>
<td>120.92 - 17.42x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s_b</td>
<td>±5.36</td>
<td></td>
<td>±14.06</td>
<td></td>
</tr>
<tr>
<td>t = b/s_b</td>
<td>11.61</td>
<td></td>
<td>1.239</td>
<td></td>
</tr>
<tr>
<td>D.F.</td>
<td>8</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td></td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>
THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARIAN 3 OR 4 HOURS AFTER THE ADMINISTRATION OF LH (PERGONAL)

DOSE RESPONSE RELATIONSHIP

Figure 22
**TABLE 14**

THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARIES

3 OR 4 HOURS AFTER THE ADMINISTRATION OF PEROCONAL

<table>
<thead>
<tr>
<th></th>
<th>Three Hours</th>
<th></th>
<th></th>
<th></th>
<th>Four Hours</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>i.u. LH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>i.u. LH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3125</td>
<td>0.625</td>
<td>1.25</td>
<td>2.5</td>
<td>0.3125</td>
<td>0.625</td>
<td>1.25</td>
<td>2.5</td>
</tr>
<tr>
<td>114.81</td>
<td>97.62</td>
<td>96.18</td>
<td>77.70</td>
<td>106.89</td>
<td>96.96</td>
<td>84.54</td>
<td>80.04</td>
</tr>
<tr>
<td>109.31</td>
<td>86.90</td>
<td>89.61</td>
<td>71.52</td>
<td>91.88</td>
<td>101.40</td>
<td>89.23</td>
<td>79.42</td>
</tr>
<tr>
<td>108.69</td>
<td>98.79</td>
<td>81.83</td>
<td>80.40</td>
<td>95.11</td>
<td>102.96</td>
<td>79.61</td>
<td>80.40</td>
</tr>
<tr>
<td>101.79</td>
<td>99.78</td>
<td>83.02</td>
<td>74.29</td>
<td>102.00</td>
<td>98.37</td>
<td>73.46</td>
<td>82.55</td>
</tr>
<tr>
<td>104.48</td>
<td>91.28</td>
<td>71.52</td>
<td>78.02</td>
<td>108.21</td>
<td>91.33</td>
<td>85.27</td>
<td>82.90</td>
</tr>
<tr>
<td>106.89</td>
<td>95.41</td>
<td>81.47</td>
<td>76.28</td>
<td>112.10</td>
<td>92.24</td>
<td>85.94</td>
<td>76.61</td>
</tr>
<tr>
<td>108.21</td>
<td>93.95</td>
<td>78.95</td>
<td>74.45</td>
<td>108.19</td>
<td>105.59</td>
<td>90.08</td>
<td>75.43</td>
</tr>
<tr>
<td>112.78</td>
<td>89.43</td>
<td>96.25</td>
<td>67.05</td>
<td>110.33</td>
<td>92.93</td>
<td>83.58</td>
<td>79.17</td>
</tr>
<tr>
<td>110.50</td>
<td>96.18</td>
<td>91.13</td>
<td>73.02</td>
<td>126.22</td>
<td>104.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>103.81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>108.13</td>
<td>94.37</td>
<td>85.55</td>
<td>74.75</td>
<td>108.63</td>
<td>98.42</td>
<td>83.96</td>
<td>79.56</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.34</td>
<td>1.45</td>
<td>2.77</td>
<td>1.50</td>
<td>3.53</td>
<td>1.60</td>
<td>2.06</td>
<td>0.93</td>
</tr>
</tbody>
</table>
### TABLE 14 (contd.)

Analysis of Variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>117.323</td>
<td>117.323</td>
<td>2.91</td>
<td>N.S.</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>10,261.921</td>
<td>10,261.921</td>
<td>254.87</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>11.537</td>
<td>11.537</td>
<td>0.287</td>
<td>N.S.</td>
</tr>
<tr>
<td>Curvature</td>
<td>4</td>
<td>192.988</td>
<td>48.247</td>
<td>1.20</td>
<td>N.S.</td>
</tr>
<tr>
<td>Between doses</td>
<td>7</td>
<td>10,583.768</td>
<td>1,511.967</td>
<td>37.55</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>64</td>
<td>2,576.882</td>
<td>40.264</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>13,160.650</td>
<td>185.361</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Relative Potency (i.u. 2nd IRP-HMG)</th>
<th>Fiducial Limit (i.u. 2nd IRP-HMG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours interval</td>
<td>0.88</td>
<td>0.72 - 1.07</td>
</tr>
</tbody>
</table>
COMPARISON OF DIFFERENT TIME INTERVALS BETWEEN THE ADMINISTRATION OF Pergonal AT 2 DOSE LEVELS AND SUBSEQUENT OVARIAN ASCORBIC ACID DEPLETION

Figure 23
<table>
<thead>
<tr>
<th></th>
<th>2.40' Hours</th>
<th>3.0' Hours</th>
<th>3.20' Hours</th>
<th>3.40' Hours</th>
<th>4.0' Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4 i.u. LH</td>
<td>2.0 i.u. LH</td>
<td>0.4 i.u. LH</td>
<td>2.0 i.u. LH</td>
<td>0.4 i.u. LH</td>
</tr>
<tr>
<td>78.80</td>
<td>79.95</td>
<td>82.61</td>
<td>74.66</td>
<td>107.53</td>
<td>77.25</td>
</tr>
<tr>
<td>79.10</td>
<td>74.24</td>
<td>95.45</td>
<td>77.11</td>
<td>82.61</td>
<td>87.19</td>
</tr>
<tr>
<td>90.17</td>
<td>70.40</td>
<td>90.26</td>
<td>69.99</td>
<td>95.45</td>
<td>90.83</td>
</tr>
<tr>
<td>89.68</td>
<td>91.31</td>
<td>70.69</td>
<td>69.39</td>
<td>90.26</td>
<td>111.43</td>
</tr>
<tr>
<td>93.50</td>
<td>78.07</td>
<td>90.97</td>
<td>70.42</td>
<td>79.69</td>
<td>83.98</td>
</tr>
<tr>
<td>87.85</td>
<td>84.83</td>
<td>88.71</td>
<td>67.57</td>
<td>105.27</td>
<td>95.30</td>
</tr>
<tr>
<td>94.41</td>
<td>65.93</td>
<td>95.44</td>
<td>68.79</td>
<td>112.67</td>
<td>71.41</td>
</tr>
<tr>
<td>99.33</td>
<td>75.98</td>
<td>92.61</td>
<td>70.44</td>
<td>108.09</td>
<td>80.16</td>
</tr>
<tr>
<td>78.70</td>
<td>73.35</td>
<td>95.32</td>
<td>75.91</td>
<td>91.12</td>
<td>73.76</td>
</tr>
<tr>
<td>104.48</td>
<td>75.86</td>
<td></td>
<td>96.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
|                  | 2.65        | 2.85       | 1.77        | 0.85        | 3.70       | 4.50        | 5.0         | 2.50        | 4.12
### Analysis of Variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>Variance ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>4</td>
<td>2,102.202</td>
<td>525.550</td>
<td>5.10</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>4,168.448</td>
<td>4,168.448</td>
<td>40.48</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>4</td>
<td>149.486</td>
<td>37.371</td>
<td>0.36</td>
<td>E.S.</td>
</tr>
<tr>
<td>Between doses</td>
<td>9</td>
<td>6,420.136</td>
<td>713.348</td>
<td>6.93</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Error</td>
<td>85</td>
<td>8,753.138</td>
<td>102.978</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>15,173.274</td>
<td>161.418</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Relative Potency (i.u. 2nd IRP-HMG)</th>
<th>Fiducial Limit (i.u. 2nd IRP-HMG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0' hour interval</td>
<td>1.29</td>
<td>0.57 - 3.11</td>
</tr>
<tr>
<td>3.20' hour interval</td>
<td>0.36</td>
<td>0.15 - 0.82</td>
</tr>
<tr>
<td>3.40' hour interval</td>
<td>0.34</td>
<td>0.12 - 0.77</td>
</tr>
<tr>
<td>4.0' hour interval</td>
<td>0.40</td>
<td>0.15 - 0.87</td>
</tr>
</tbody>
</table>
was maximal. Thereafter, at 3 hours 20 mins., the concentration of ovarian ascorbic acid increased and remained constant after this time. The analysis of variance (see Table 15) also showed that the effect of LH on ovarian ascorbic acid varied in relation to the time at which animals were killed \((p < 0.05)\). If the results obtained at 2 hours 40 mins. were used as a reference preparation then the responses obtained at 3, 3.20, 3.40 and 4 hours showed that the highest activity was obtained at 3 hours. The estimated potency of 1.29 i.u. is some 80% higher when compared with the other three (Table 15).

**Discussion**

The results obtained in the first experiment show that cortisone acetate undoubtedly affects ascorbic acid depletion. Therefore it was not possible to consider the use of cortisone as a method of reducing the toxic effect of urinary extracts upon the experimental animals, in the same manner as described earlier for the VPW assay.

The lack of significant regression of the dose-response curve in the second experiment from the group killed four hours after the administration of the hormone, was probably due to the phenomenon occasionally seen with the OAAD assay in which the animals do not
react and there is no depletion of the ovarian ascorbic acid when small doses of LH are given.

The results obtained from the third and fourth experiments are in agreement with those of Hutchinson and Worden (1964) who also demonstrated that a 3-hour interval between hormone administration and measurement of ascorbate depletion produced a more acceptable dose-response relationship.

The results obtained in the fourth experiment indicated that the time course of ascorbic acid depletion is such that it became maximal 3 hours after the administration of both dose levels of LH. Ascorbate depletion was less marked after 3 hours 20 mins. and this might suggest that ascorbic acid may be repeated at these later time intervals. The optimal time interval between hormone administration and ascorbic acid measurement is 3 hours, and at this time the difference in response to the two dose levels of hormone is also maximal. In particular when urinary extracts are to be assayed, the 3-hour interval is the procedure of choice, since the experimental animals are exposed to the effect of toxicity for a shorter period of time.

APPLICATION OF THE MODIFIED OAAFD METHOD TO ESTIMATE URINARY LH LEVEL IN TOXIC URINARY EXTRACTS

As a result of the experiments carried out in the
assay procedure, it was decided to incorporate the following modification:

1. The dose interval was reduced to \( \log_{10} 4 \) (0.60206). In addition, the finding of Hutchinson and Worden (1964) regarding the initial body weight range was adopted.

2. The time interval between administration of the hormone and estimation of ovarian ascorbic acid was reduced to 3 hours.

The modified OAAD assay was then applied to the estimation of LH in urinary extracts.

Three pilot experiments were performed in which urinary extracts of proven toxicity were assayed by the modified technique in an attempt to assess the suitability of the method for the routine estimation of urinary LH.

Materials and Methods

Thirty-five animals were used in each of three assays. In each experiment rats were randomly allocated to 7 groups, one of which served as control. The assays were of four-point design. Six urinary extracts, previously tested and proved to be toxic, were used.

Results

Of the 60 animals treated with urinary extracts 10
died during the assay procedure (17%). Two out of six estimations had to be rejected because of significant deviation from parallelism between the S and U preparations. The index of precision (\(\lambda\)) of the method was 0.24 ± 0.09 (± S.D.).

When the results are compared with those obtained using the original method, it is clear that some improvements have been made by reducing the interval from 4 to 3 hours. The mortality rate was reduced from 37 to 17 per cent and the index of precision was reduced from 0.44 ± 0.15 to 0.24 ± 0.09 but despite these improvements approximately 1/3 of the assays had to be rejected due to lack of parallelism between "S" and "U" preparations.

These results demonstrated that the extraction procedure should perhaps be re-examined and this is discussed in Chapter 3.

**ANALYSIS OF RESULTS OBTAINED WITH THE OAAD TEST INCORPORATING THE MODIFICATIONS IN THE ASSAY PROCEDURE**

With the improvements made to the OAAD method and to the techniques of extraction from urine (see Chapter 3), 38 assays were performed during which 228 samples were tested; 2,850 Wistar C.E.R.U. rats were used.

**Results**

During these assays the mortality rate following
the administration of urinary extracts was significantly reduced. Five assays (13%) were invalid due to lack of response of the animals treated with the standard preparation. In 34 out of 198 samples significant deviation from parallelism occurred between the dose-response curves of S and U.

The mean index of precision (± S.D.) was 0.206 ± 0.101. Figure 24 shows the frequency polygon of the distribution of values obtained in the 33 valid assays, and the figures appear to be normally distributed. Two of the three measures of central tendency, i.e. mean and median, coincided, while the mode differed only very slightly.

The frequency polygon of values for the common slope (b) obtained in 33 assays is shown in Figure 25. Again the values appear to be normally distributed and the three measures of central tendency differed very little from one another.

Discussion

The results obtained when improvements were made to the OAAD assay and the extraction procedure indicated that the method became more satisfactory and was suitable for the estimation of LH in urinary extracts. Seventy-two per cent of the assays performed were valid.
FREQUENCY POLYGON OF LAMBDA VALUES OBTAINED USING THE MODIFIED OAAD METHOD

Figure 24
FREQUENCY POLYGON OF COMMON SLOPE
VALUES OBTAINED USING THE MODIFIED OAAD METHOD

Figure 25
CONTAMINATION OF EXTRACTS WITH CORTICOSTEROIDS EXCRETED IN THE URINE

Introduction

The observation that cortisone acetate exerts a non-specific effect on the OAAD assay made it necessary to ascertain whether the urinary extracts prepared by the tannic acid procedure (see page 92) were contaminated with corticosteroids. In order to investigate this hypothesis the following experiment was designed.

Material and Method

The OAAD method used was identical to that described above. Two thousand ml. fresh postmenopausal urine was split into two equal parts. To one of them were added prednisolone tertiary butylacetate (100 mg.), hydrocortisone (100 mg.) and cortisone acetate (100 mg.); subsequently both pools were extracted by the tannic acid procedure.

Results

The results obtained are shown in Figure 26 and in Table 16. The analysis of variance (Table 16) demonstrated that corticosteroids added prior to the extraction procedure did not exert any effect on the OAAD assay. The two preparations did not differ significantly; the slopes of the dose-response curve were
THE EFFECT OF CORTICOSTEROIDS ADDED TO URINE PRIOR TO EXTRACTION ON THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARY BY LH

Figure 26
TABLE 16

THE EFFECT OF CORTICOSTEROIDS ADDED TO URINE PRIOR TO EXTRACTION
ON THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARY BY LH

<table>
<thead>
<tr>
<th>2nd IRP-HMG</th>
<th>U₁</th>
<th>U₁ + C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3125 i.u.</td>
<td>1.25 i.u.</td>
<td>L.D.</td>
</tr>
<tr>
<td>Ovarian ascorbic acid concentration</td>
<td>116.4</td>
<td>90.5</td>
</tr>
<tr>
<td>107.4</td>
<td>82.3</td>
<td>108.4</td>
</tr>
<tr>
<td>105.4</td>
<td>84.5</td>
<td>106.4</td>
</tr>
<tr>
<td>(µg./100 mg. ovarian tissue)</td>
<td>104.9</td>
<td>85.5</td>
</tr>
<tr>
<td>102.5</td>
<td>76.5</td>
<td>95.5</td>
</tr>
</tbody>
</table>

Mean ± S.E. | 107.3 ± 2.7 | 83.5 ± 2.7 | 104.9 ± 3.0 | 85.8 ± 1.9 | 104.1 ± 2.2 | 84.8 ± 2.2 |

* Prednisolone tert. butylacetate 100 mg.

Hydrocortisone 100 mg.

Cortisone acetate 100 mg.
TABLE 16 (contd.)

Analysis of Variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>2</td>
<td>5.717</td>
<td>2.858</td>
<td>0.115</td>
<td>N.S.</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>3,234.408</td>
<td>3,234.408</td>
<td>130.62</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>2</td>
<td>35.933</td>
<td>17.966</td>
<td>0.726</td>
<td>N.S.</td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>3,276.058</td>
<td>655.211</td>
<td>26.46</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>594.276</td>
<td>24.761</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>3,870.334</td>
<td>133.460</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Potencies

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Relative Potency i.u. 2nd IRP-HMG</th>
<th>Fiducial Limit i.u. 2nd IRP-HMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown&lt;sub&gt;1&lt;/sub&gt;</td>
<td>125.42</td>
<td>91.84 - 171.31</td>
</tr>
<tr>
<td>Unknown&lt;sub&gt;1&lt;/sub&gt; + Corticosteroids</td>
<td>133.18</td>
<td>97.70 - 182.33</td>
</tr>
</tbody>
</table>

Index of discrimination: \( I = U_1 + C/U_1 = 1.06 \)
parallel and the index of discrimination was 1.06.

Discussion

These results showed that when urine is extracted by the tannic acid procedure, the corticosteroids are not precipitated with the gonadotrophic hormones. Although the levels of corticosteroids excreted in urine, even in pathological conditions such as Cushing's syndrome, are seldom higher than 10 mg./24 hours (Cost, 1963a, b), the amounts added to urine in this study were 30 times greater. The results obtained demonstrated that the urinary extracts were free from corticosteroid contamination and it could therefore be concluded that when the OAAD assay is performed under these conditions a specific estimation of urinary LH can be obtained.

SEASONAL VARIATIONS IN ASCORBIC ACID CONTENT OF PRE-
TREATED RAT OVARIIES FOLLOWING PREGNANAL ADMINISTRATION

Stevens et al. (1964) reported the occurrence of a diurnal variation in the ascorbic acid content of ovaries pretreated with PMSG and HCG, and emphasised the importance of performing the assay at the same time of the day. In their experience, the best time of day to administer S and U was 7 a.m.
Material and Method

In order to investigate whether the initial concentration of ascorbic acid in pretreated rat ovaries is critical, the slopes obtained from standards used in 55 assays performed over a period of 12 months were plotted. The results are shown in Figure 27.

Results and Discussion

The initial level of ascorbic acid in the ovaries of pretreated rats is probably not critical in order to obtain a satisfactory slope for the assay. For example, in the assay at the end of January, although the level of ascorbic acid in the control group was low (70 μg. ascorbic acid/100 mg. tissue), the depletion was marked and the slope was satisfactory (-23.3). On the other hand, in the next assay, although the level of ascorbic acid in the control group was similar, the depletion was small and the slope was flat (-12). In April and May four assays were performed. In all of these the levels of ascorbic acid in the control groups were high (137, 136, 146 and 130 μg. of ascorbic acid/100 mg. of tissue respectively). The depletion of ascorbic acid in the first 3 assays, following a dose level of 0.4 i.u. of Pergonal, was much larger than that occurring between 0.4 and 2.0 i.u. Since the total depletion of ascorbic acid from the ovary appears to be limited, the depletion
VARIATION IN ASCORBIC ACID CONTENT OF PRETREATED RAT OVARIRES FOLLOWING PERGONAL ADMINISTRATION.
ASSAYS PERFORMED OVER A PERIOD OF 12 MONTHS

Figure 27
between 0.4 and 2.0 i.u. was very small and the slope of the dose-response curve was flat. In the fourth assay, although the initial level of ascorbic acid was slightly lower, the depletion produced with the same quantities of Pergonal gave a much steeper slope. Similar changes were noted at other times during the year.

In the two assays performed at the beginning of July, the initial levels of ascorbic acid in the pre-treated ovaries were similar. However, 0.4 i.u. of Pergonal produced the same effect on the depletion of ascorbic acid in the first assay as was produced by five times that amount in the second. Thus the first assay was invalid because the slope was flat (-9) while the second assay was successful because the slope was very steep (-40).

During this period, 17.5% of the assays were invalid due to a lack of response following administration of the standard preparation.

DISCUSSION

The OAAD method remains one of the two bioassay procedures of choice for the estimation of LH in body fluids. Several investigators have reported that there are important strain differences causing variation in the precision and sensitivity of the assay and, according to Parlow (1961) and Koch and Hamburger (1968), rats of the Holtsman strain give the most satisfactory results. The present study demonstrated that Wistar animals
derived from the C.E.R.U. colony are reasonably sensitive to LH administration and also result in the production of assays of relatively high precision.

Hutchinson and Worden (1964), who also used rats of the Wistar strain, found that the maximum depletion of ascorbic acid occurred within three hours after the administration of LH either intravenously or intraperitoneally. These results are in agreement with those found in the present investigation. When the time interval between the injections and sacrifice of the animals was reduced from 4 to 3 hours, the mortality of experimental animals, due to toxicity of crude material injected, was reduced from 37 to 13 per cent.

In addition, an improvement in the performance of the assay was noted as a result of the modifications which have been incorporated. These include:

1. The body weight of the rats was restricted to between 35-45 g.

2. The log dose interval adopted was \( \log_{10} 4 \) (0.60206) instead of \( \log_{10} 5 \) (0.69897).

3. The assay procedure was performed on the 6th or 7th day after HCG treatment.

When the modified assay method was applied to clinical problems (Section B) along with the improvements made in the extraction procedure, the mean index of precision of the method was 0.206 and only 17% of the
assays were rejected due to significant deviation from parallelism between the S and U dose-response curves.

The lack of reaction in 15% of the groups treated with standard preparation cannot be explained.

The suggestion of Stevens et al. (1964) that the initial concentration of ascorbic acid is critical has not been shown during the present study. The initial level of ascorbic acid in the rat ovary does not appear to exert any significant effect on the results.

Throughout the whole study, when urinary extracts were tested, the 2nd IRP-HMG was used as standard reference preparation, merely because it was thought essential that the standard reference preparation and the tested material should be of the same origin. Recently, Koch and Hamburger (1967), who compared the dose-response curves for LH of ovine (NIH-LH-S8) and human (2nd IRP-HMG) origin, found that the two slopes differed significantly \((P<0.01)\) because the latter curve was flatter.

The observed non-specific effect of cortisone acetate on the OAAD test does not invalidate the results when urinary extracts are used, since it has been shown in this study that the product extracted by the tannic acid method is not contaminated with corticosteroids. This finding is also supported by the fact that corticosteroids are excreted in the urine either free or
conjugated with glucuronic or sulphuric acids (Butt, 1968a). Since they are not conjugated with proteins it is unlikely to be precipitated with tannic acid and they should therefore appear in the filtrate during the extraction procedure.

**SUMMARY**

1. Modifications to the OAAD method for the estimation of LH in urinary extracts have been described. These include:

   (a) Reduction of the time interval between the injection of S and U and the sacrifice of animals from 4 to 3 hours.

   (b) Restriction of body weight to a range between 35 and 45 g.

   (c) Reduction of the log dose interval used in the assay from \( \log_{10} 5 \) (0.69897) to \( \log_{10} 4 \) (0.60206).

2. Using these modifications the precision of the OAAD method was improved and the mortality rate following the administration of urinary extracts was reduced.

3. Cortisone acetate affected the response of the experimental animals but when large amounts of corticosteroids were added to urine and extracted, no effect on LH levels were found.

4. The initial level of ascorbic acid in the rat ovary did not influence the results of the assay.
CHAPTER 3

STUDIES ON THE PURIFICATION OF URINE PRIOR TO
THE EXTRACTION OF GONADOTROPHINS

INTRODUCTION

Two methods for the extraction of urinary gonadotrophins depending upon precipitation by tannic acid (Johnsen, 1958) and adsorption to kaolin acetate (Albert, 1955, 1956, 1961) have been widely used in the estimation of gonadotrophic activity.

Borth and Menzi (1964) reported that Johnsen's method yields extracts less toxic than those produced by the kaolin-acetone method and it is to be recommended when urinary gonadotrophins estimations are being performed (see General Introduction, page 17). This observation is in agreement with that reported by Wells (1965). The latter investigator compared the effectiveness of several extraction methods, by means of the mouse ovulation test (Canningham, 1962). The methods selected for comparison were the benzoic acid of Katzman and Doisy (1934), the benzoic and tungstic acids of Butt (1968), the kaolin acetone methods of Brown (1959), Loraine and Brown (1959) and Borth et al. (1961) and the tannic acid method of Johnsen (1959). It was concluded that the tannic acid method gave extracts with the least
insoluble material and with a significantly higher yield on three out of six occasions.

When the tannic acid procedure was employed for the extraction of urinary gonadotrophins, in earlier studies reported herein (page 79), the percentage of dead animals, among those injected with urinary extracts, was still high and modifications to the OAAD technique itself did little to improve this mortality (13%).

In his original paper Johnsen (1958) pointed out that as the method extracts all proteins it is possible that the concentration of bacterial protein might result in urine preparations that were toxic. Since urine represents a very good medium for bacterial growth, it was of interest to investigate the possibility that such contamination could occur when urine was collected routinely, and to examine the effect of bacterial growth in the urine upon subsequent extraction and bioassay procedures.

MATERIAL AND METHOD

(1) Urine

Twenty-four hour urinary samples were collected from a normal menopausal woman who had no symptoms of urinary tract infection. When the specimen arrived in the laboratory an aliquot of 20 ml. was obtained for bacteriological study and the remainder of the sample
was extracted by the tannic acid method.

(ii) Extraction procedure (Johnsen, 1958)

(a) Acidification of urine: The pH of each 24-hour urine sample is adjusted to 4 ± 0.3 by the addition of glacial acetic acid.

(b) Precipitation: Following acidification, sodium chloride (10 ± 2 g./litre) and hyflo-supercel (10-12 g.) are added to the urine and stirred. A solution of 10% tannic acid (20 ± 2 ml.) is then added and stirred continuously for 5 minutes. Dissolved gases produced as a result of acidification of the urine is removed by applying a reduced pressure to the flask. This prevents subsequent breakdown of the filter cake.

(c) Formation of filter cake: Hyflo-supercel (2 ± 0.5 g.) is suspended in 100 ml. water and poured into a Buchner funnel lined with filter paper. The precipitated urine is then added slowly to the Buchner funnel in such a way that any disturbance to the 'filter cake' of supercel is avoided.

When empty the urine flask is rinsed with 225 ± 25 ml. 0.1% tannic acid to which is added 2 ± 0.5 g. of hyflo-supercel and the contents added to the Buchner funnel. During filtration through hyflo-supercel 'cake', suction is applied to the Buchner flask and is continued through the next stage of the extraction.
(d) Differentiation: 225 ± 25 ml. of alcohol (96%) is added to the funnel before the filter cake becomes dry. This is followed by the addition of 225 ± 25 ml. of 10% ammonium acetate (pH 11) in 80% alcohol.

(e) Elution: The Buchner funnel containing the filter cake of hyflo-supercel is next transferred to a clean receiving vessel and 10% ammonium acetate (pH 11) in 40% alcohol is added to the funnel and mixed with the cake. Suction is then applied to the receiving vessel and 30-40 ml. of solution is collected. The fluid is then allowed to drain slowly through the 'cake' for approximately 20 minutes and the remainder of the filtrate is then collected under reduced pressure.

(f) Neutralization and repricipitation: The eluate is transferred to a 750 ml. flask, and is cooled for 3-4 minutes in ice-water. Then, during vigorous shaking, 10 ± 0.5 ml. glacial acetic acid is added. Subsequently the volume is made up to 600 ± 50 ml. by the addition of absolute alcohol mixing carefully by rotating the flask. The flask is stored in a refrigerator overnight.

(g) Collection of precipitate: The precipitate is collected by centrifugation at 2,000 r.p.m. for 10 minutes. The supernatant is discarded and the precipitate resuspended in 15-20 ml. absolute alcohol before re-centrifugation. The alcohol supernatant is discarded,
and the precipitate washed with 15-20 ml. of ether. After centrifugation the extract is dried in a vacuum desiccator and is then stored in a refrigerator at 4°C until assayed.

(iii) **Bioassay**

The bioassay procedure was the OAAD as described earlier (Chapter 2).

Thirty animals were randomly allocated to 6 treatment groups. One group was treated with saline (control) while the remaining groups were injected either with the (S) or (U) preparations. The doses selected for the standard were 0.3 and 1.2 i.u. LH, while the three dose levels of the urinary extracts administered were equivalent to 8.75, 35.0 or 140 mls. of urine in each group respectively.

**RESULTS**

The results of the bacteriological study are shown in Table 17. The urine was heavily contaminated with gram negative bacilli, and to a lesser extent with gram positive bacilli, while the contamination with gram positive cocci was the least. Following culture, a heavy growth developed consisting mainly of *Escherichia coli* and enterococci.

The results of the assay are shown in Table 18.
### TABLE 17

**BACTERIOLOGICAL STUDY OF URINE**

**SELECTED WITHOUT PRESERVATIVE**

<table>
<thead>
<tr>
<th>Microorganism and Cocci</th>
<th>Degree of contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Film</strong></td>
<td></td>
</tr>
<tr>
<td>Debris</td>
<td>+</td>
</tr>
<tr>
<td>Gram positive cocci</td>
<td>+</td>
</tr>
<tr>
<td>Gram negative bacilli</td>
<td>+++</td>
</tr>
<tr>
<td>Gram positive bacilli</td>
<td>++</td>
</tr>
<tr>
<td><strong>B. Culture</strong></td>
<td>More than 10⁵ organisms per ml.</td>
</tr>
<tr>
<td>A heavy mixed growth</td>
<td></td>
</tr>
<tr>
<td>consisted mainly of</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> and <em>enterococci</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>113.60</td>
</tr>
<tr>
<td>Ovarian ascorbic</td>
<td>102.80</td>
</tr>
<tr>
<td>acid concentration</td>
<td>110.58</td>
</tr>
<tr>
<td>(µg./100 mg. tissue)</td>
<td>106.50</td>
</tr>
<tr>
<td></td>
<td>120.13</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>110.7±3.3</td>
</tr>
</tbody>
</table>

* Dead animal.
The percentage of dead animals in the groups treated with urinary extracts was 27%. The assay was invalid because of the slope reversal in the dose-response curve of the unknown preparation. A concentration of 8.75 equivalent millilitres of urine induced a highly significant depletion, similar to that obtained in the group of animals receiving 1.2 i.u. LH. When these two mean values were compared using Student's "t" test no significant difference could be demonstrated (t = 0.393, D.F. = 8, P = N.S.). No depletion of ovarian ascorbic acid was noted in animals treated with 35.0 or 140.0 equivalent millilitres of urine, the readings being similar to those obtained in the control group.

DISCUSSION

This experiment indicated that when urinary samples are collected routinely, the possibility of bacterial contamination is great. As the bacteriological study demonstrated (see Table 17), the contamination of the urine was such that the tannic acid extraction procedure could not reduce the toxicity of the urine to an acceptable level.

When a very small concentration of urinary extract was injected, no deaths occurred and a significant depletion of ovarian ascorbic acid was noted. However, with higher concentrations of urinary extracts (35 and
140 equivalent millilitres of urine) no depletion of ascorbic acid occurred. This could be due to the effect of toxic material on the experimental animal or to unknown inhibitory factors.

It was concluded that the conditions of collection of urinary samples should be re-examined.

**COLLECTION OF URINARY SAMPLES WITH A BACTEROIDSTATIC AGENT**

**Introduction**

Since the urine was so heavily contaminated, it was decided to add a bacteriostatic agent to the container, prior to collection, to inhibit bacterial growth.

The agent selected was a solution of chlorhexidine gluconate (Hibitane concentrate 5%, I.C.I. Pharmaceuticals). This is normally a liquid miscible with water and alcohol. Several investigators have shown that chlorhexidine is one of the most effective antiseptics (Calman and Murray, 1956; Lawrence, 1960) and it is usually employed as a preservative for eye-drops and for the pre-operative disinfection of the skin.

Although it is unlikely that chlorhexidine is extracted by the tannic acid procedure it was necessary to investigate whether it had any effect on the OAAD assay.

The aim of the following experiments was to
determine:

1. The concentration of hibitane which was required to inhibit bacterial growth.

2. The effect of hibitane on the OAAD test, and

3. Its effect on the gonadotrophin extraction and bioassay of urine.

In view of the fact that, in clinical studies, gonadotrophins and steroids are routinely assayed in the same sample, it was also necessary to determine whether the addition of hibitane to urine would affect the steroid analysis.

Materials and Methods

Experiment I: In 7 clean containers, of one litre capacity each, were added either 1, 2, 4, 8, 16, 32 or 64 mls. of hibitane concentrate 5%, and urinary samples were collected from the same subject used for the previous experiment. When a collection of 1,000 ml. was completed, the container was kept at a temperature of 4°C. When all collections were completed a specimen of 20 ml. of urine from each container was removed for bacteriological study.

Experiment II: Forty-five pretreated rats for the OAAD test were randomly allocated amongst 9 treatment groups. Three groups of animals received either 0, 0.6 or 3.0 i.u. LH alone. A further three groups were
treated with identical dose levels of LH but in addition each animal was treated with 0.2% hibitane administered simultaneously with the LH. The remaining three groups again received similar dose levels of LH but the concentration of hibitane administered simultaneously was increased to 2.0%.

The assay procedure in this and in the following experiment was the OAAD test as it has been described in Chapter 2, page 79.

Experiment III: A 24-hour urinary sample was collected from a normal healthy menopausal woman. During the collection special precautions were taken to avoid, as far as possible, contamination of the urine with bacterial (i.e. the subject washed the external genitalia with soap and water prior to urination and during the 24-hour period of collection the urine was kept in the refrigerator at 4°C). When the sample arrived in the laboratory the urine was split into two equal volumes (800 ml. each) and to one of them was added 16 ml. hibitane concentrate 5%. Subsequently both volumes were extracted as described earlier.

Forty pretreated animals for the OAAD test were allocated at random amongst 8 groups. Four groups received 0, 0.4, 1.6 and 6.4 i.u. LH, while the remaining four groups were used to assay the two urinary extracts. The low (L) dose levels employed were equivalent to 40 ml.
and the high doses (H) were equivalent to 160 ml. of urine.

**Experiment IV:** A pool of 3,000 ml. of freshly collected urine was obtained from several healthy young female technicians and was divided into two equal parts. One hundred and fifty ml. of hibitane concentrate 5% or an equal volume of distilled water was added to each. Subsequently, both specimens were divided into six equal parts (275 ml.) and these were individually assayed for oestrogens and pregnanediol.

**Results**

In the first experiment the bacteriological studies performed on the seven urinary samples to which different concentrations of hibitane had been added showed that no contamination occurred with 0.4% hibitane or above (Table 19). Only scanty debris appeared on the film and no growth occurred on culture.

The effect of three dose levels of hibitane on the dose-response curve for LH is shown in Figure 28. No consistent effect of hibitane was noted at any of the levels of LH used. The analysis of variance carried out on this date (Table 20) showed that there was no significant effect attributable to hibitane administration.

The results of the third experiment are shown in
**Table 19**

**Bacteriological Study of Urine**

**Selected with Hibitane**

<table>
<thead>
<tr>
<th>Microorganism and Cocci</th>
<th>Degree of Contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Film</strong></td>
<td></td>
</tr>
<tr>
<td>Debris only</td>
<td>Scanty</td>
</tr>
<tr>
<td><strong>B. Culture</strong></td>
<td></td>
</tr>
<tr>
<td>No growth</td>
<td></td>
</tr>
</tbody>
</table>


EFFECT OF HIBITANE ON THE DEPLETION OF ASCORBIC ACID FROM THE PRETREATED RAT OVARY BY LH (PREGONAL)

Figure 28
The effect of Hibitane on the depletion of ascorbic acid from pretreated rat ovary by LH

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Hibitane 0.2%</th>
<th>Hibitane 2.0%</th>
<th>Hibitane 0.6 i.u. + LH</th>
<th>Hibitane 0.6 i.u. + 2.0%</th>
<th>Hibitane 3.0 i.u. + LH</th>
<th>Hibitane 0.2% + 3.0 i.u.</th>
<th>Hibitane 2.0% + 3.0 i.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian ascorbic acid</td>
<td>90.27</td>
<td>93.94</td>
<td>88.12</td>
<td>87.93</td>
<td>83.76</td>
<td>68.02</td>
<td>67.57</td>
<td>62.79</td>
</tr>
<tr>
<td>Ovarian concentration</td>
<td>102.92</td>
<td>93.43</td>
<td>84.53</td>
<td>83.36</td>
<td>79.63</td>
<td>66.81</td>
<td>70.20</td>
<td>62.16</td>
</tr>
<tr>
<td>(µg./100 mg. ovarian tissue)</td>
<td>102.25</td>
<td>91.96</td>
<td>81.49</td>
<td>96.20</td>
<td>85.09</td>
<td>59.95</td>
<td>61.92</td>
<td>62.33</td>
</tr>
</tbody>
</table>

Mean ± S.E.:
- Ovarian ascorbic acid: + 2.4
- Ovarian concentration: + 1.3
- Ovarian tissue: + 1.6

*Note: The table provides a comparison of ascorbic acid concentration in ovarian tissue samples treated with different concentrations of Hibitane and LH. The values represent percentage depletion of ascorbic acid. The mean is given with its standard error (S.E.).
<table>
<thead>
<tr>
<th>Variations</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>Variance Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doses</td>
<td>8</td>
<td>6,833.79</td>
<td>854.22</td>
<td>47.80</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LH</td>
<td>2</td>
<td>6,717.39</td>
<td>3,358.69</td>
<td>187.95</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hibitane Linear</td>
<td>1</td>
<td>6,439.55</td>
<td>6,439.55</td>
<td>360.35</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hibitane Quadratic</td>
<td>1</td>
<td>277.84</td>
<td>277.84</td>
<td>15.55</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>K.S. Linear</td>
<td>1</td>
<td>53.54</td>
<td>53.54</td>
<td>3.46</td>
<td>0.19</td>
</tr>
<tr>
<td>K.S. Quadratic</td>
<td>1</td>
<td>3.46</td>
<td>3.46</td>
<td>0.19</td>
<td>0.88</td>
</tr>
<tr>
<td>Interaction</td>
<td>4</td>
<td>62.86</td>
<td>15.71</td>
<td>17.87</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>643.30</td>
<td>17.87</td>
<td>7,477.09</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>7,477.09</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
THE EFFECT OF HIBITANE ADDED TO URINE PRIOR TO EXTRACTION ON THE DEPLETION OF ASCORBIC ACID FROM PRETREATED RAT OVARY BY LH (Pergonal)

Figure 29
### TABLE 21

**The Effect of Hibitane Added to Urine Prior to Extraction on the Depletion of Ascorbic Acid from Pretreated Rat Ovary by LH**

<table>
<thead>
<tr>
<th>2nd IRP-HMG</th>
<th>(U_1) Equivalent mls. of urine</th>
<th>(U_1 + H^*) Equivalent mls. of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 i.u.</td>
<td>1.6 i.u.</td>
<td>6.4 i.u.</td>
</tr>
<tr>
<td>40.0</td>
<td>160.0</td>
<td>40.0</td>
</tr>
<tr>
<td>116.64</td>
<td>97.38</td>
<td>77.88</td>
</tr>
<tr>
<td>86.35</td>
<td>91.76</td>
<td>80.38</td>
</tr>
<tr>
<td>80.14</td>
<td>90.88</td>
<td>74.46</td>
</tr>
<tr>
<td>114.92</td>
<td>84.66</td>
<td>85.94</td>
</tr>
<tr>
<td>91.69</td>
<td>74.57</td>
<td>76.81</td>
</tr>
<tr>
<td>66.27</td>
<td>90.34</td>
<td>74.89</td>
</tr>
<tr>
<td>((\mu g./100) mg.</td>
<td>80.65</td>
<td>86.31</td>
</tr>
<tr>
<td>79.26</td>
<td>80.85</td>
<td>74.89</td>
</tr>
<tr>
<td>110.24</td>
<td>91.45</td>
<td>69.10</td>
</tr>
<tr>
<td>80.65</td>
<td>89.37</td>
<td>76.67</td>
</tr>
<tr>
<td>72.33</td>
<td>85.94</td>
<td>76.81</td>
</tr>
<tr>
<td>74.57</td>
<td>86.31</td>
<td>74.89</td>
</tr>
<tr>
<td>113.9</td>
<td>93.63</td>
<td>70.79</td>
</tr>
<tr>
<td>85.0</td>
<td>89.3</td>
<td>73.6</td>
</tr>
<tr>
<td>74.5</td>
<td>76.4</td>
<td>73.6</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>2.1</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Hibitane 2.0%*
### TABLE 21 (contd.)

**Analysis of Variance**

<table>
<thead>
<tr>
<th>Variations</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>Variance Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>2</td>
<td>625.53</td>
<td>312.76</td>
<td>18.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>5,062.22</td>
<td>5,062.22</td>
<td>296.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>2</td>
<td>54.62</td>
<td>27.31</td>
<td>1.60</td>
<td>N.S.</td>
</tr>
<tr>
<td>Curvature</td>
<td>1</td>
<td>283.36</td>
<td>283.36</td>
<td>16.59</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Between doses</td>
<td>6</td>
<td>6,025.73</td>
<td>1,004.29</td>
<td>58.79</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>28</td>
<td>478.28</td>
<td>17.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>6,504.01</td>
<td>191.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Potencies**

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Relative Potency i.u. 2nd IRP-HMG</th>
<th>Fiducial Limit i.u. 2nd IRP-HMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown₁</td>
<td>33.62</td>
<td>25.86 - 44.38</td>
</tr>
<tr>
<td>Unknown₁ + Hibitane 2%</td>
<td>41.47</td>
<td>31.77 - 55.29</td>
</tr>
</tbody>
</table>

*Index of discrimination: \( U₁ + H/U₁ = 1.23 \)
Figure 29 and Table 21. The dose-response curves obtained from the urinary extracts, in one of which 2% of hibitane concentrate 5% was added, were both parallel to the standard curve. The estimated potencies of the two unknowns were 33.6 and 41.2 i.u. LH and the index of discrimination was 1.23.

The results of the last experiment, in which estimations of oestrone, oestradiol, oestriol and pregnane-diol were performed, are shown in Table 22. The mean figures obtained for individual steroids were compared between groups using Student’s "t" test. No significant effect attributable to hibitane administration was observed.

Discussion

When hibitane concentrate 5% was added at a concentration of 0.4% or above, the growth of micro-organisms in urinary samples was inhibited.

It is unlikely that the tannic acid procedure will extract hibitane from urine, since it is soluble in water and alcohol and will presumably be removed during the initial filtration step. On the other hand, if the urinary extracts did contain hibitane then no effect of this material on LH-induced ascorbate depletion could be demonstrated.

The results of the third experiment support the
### Table 22

**The Effect of Hibitane Added to Urine as a Preservative Prior to Estimation of Oestrogens and Pregnanediol**

<table>
<thead>
<tr>
<th>Oestrone</th>
<th>Oestradiol (µg./1000 ml)</th>
<th>Oestriol (mg./1000 ml)</th>
<th>Pregnanediol (µg./1000 ml)</th>
<th>Oestrone</th>
<th>Oestradiol (µg./1000 ml)</th>
<th>Oestriol (mg./1000 ml)</th>
<th>Pregnanediol (µg./1000 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7</td>
<td>1.5</td>
<td>8.5</td>
<td>0.36</td>
<td>5.2</td>
<td>2.7</td>
<td>8.6</td>
<td>0.43</td>
</tr>
<tr>
<td>4.4</td>
<td>1.8</td>
<td>8.9</td>
<td>0.50</td>
<td>4.8</td>
<td>1.8</td>
<td>9.4</td>
<td>0.58</td>
</tr>
<tr>
<td>4.3</td>
<td>1.9</td>
<td>6.9</td>
<td>0.80</td>
<td>5.2</td>
<td>2.5</td>
<td>7.9</td>
<td>0.56</td>
</tr>
<tr>
<td>3.9</td>
<td>1.7</td>
<td>8.5</td>
<td>1.06</td>
<td>4.0</td>
<td>2.0</td>
<td>8.5</td>
<td>0.72</td>
</tr>
<tr>
<td>3.7</td>
<td>1.7</td>
<td>9.3</td>
<td>0.54</td>
<td>4.0</td>
<td>2.5</td>
<td>8.2</td>
<td>0.73</td>
</tr>
<tr>
<td>4.7</td>
<td>0</td>
<td>9.6</td>
<td>1.10</td>
<td>5.0</td>
<td>2.6</td>
<td>10.0</td>
<td>0.62</td>
</tr>
</tbody>
</table>

"t" Student's Test

<table>
<thead>
<tr>
<th></th>
<th>Mean ± Standard Error</th>
<th>t</th>
<th>D.F.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With Hibitane</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestrone</td>
<td>4.12 ± 0.23</td>
<td>1.160</td>
<td>10</td>
<td>N.S.</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>1.43 ± 0.43</td>
<td>1.437</td>
<td>10</td>
<td>N.S.</td>
</tr>
<tr>
<td>Oestriol</td>
<td>8.62 ± 0.64</td>
<td>0.134</td>
<td>10</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pregnanediol</td>
<td>0.73 ± 0.17</td>
<td>0.500</td>
<td>10</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
finding that the addition of hibitane to urine as a preservative has no effect on urinary LH levels, both dose-response curves obtained from the "U" preparations with and without hibitane being identical.

No effect on steroids analyses could be demonstrated when higher concentrations of hibitane in urine were employed.

These experiments show that it is possible to avoid contamination of urinary samples with bacteria by the addition of hibitane to the container prior to collection. Hibitane, at the concentrations used, had no effect on the OAAD test or on urinary gonadotrophins. The estimations of urinary oestrogen and pregnanediol were similarly unaffected.

THE EFFECT OF DIALYSIS OF URINE PRIOR TO EXTRACTION ON URINARY LH

Introduction

Since Graham (1861) described the technique of dialysis as a procedure for the purification of colloidal dispersions, the method has been widely used for the separation of substances in true solution from those forming colloidal mixtures. The method utilises the fact that the majority of substances in true solution can pass through a membrane while colloidal particles are retained. These particles diffuse very slowly, due
to their relatively large dimensions, whereas the small molecules and ions in true solution diffuse much more rapidly (Gladstone and Lewis, 1964).

The following experiments were designed to investigate whether dialysis of urinary samples prior to extraction could improve the procedure.

**Materials and Method**

**Experiment I:** Two litres of urine were collected from a normal postmenopausal woman, in a container to which 20 mls. hibitane had been added. When the urine arrived in the laboratory it was divided into two equal parts (1,000 ml. each). The first was stored in the cold-room while the second was dialysed at 4°C. The urine was contained in a cellulose bag (Visking, Hudes-Co.) and was suspended in a vessel containing 2 litres of distilled water. The dialysing fluid was changed every four hours over a period of 16 hours and was then dialysed overnight after the fourth change, the whole procedure being concluded within 24 hours. Subsequently the gonadotrophins from both urinary samples (dialysed and non-dialysed) were extracted as described previously (see page 92).

For the estimation of LH the OAAD test was employed (Chapter 2). Fifty animals were divided into 10 treatment groups and four groups were selected at random to
receive either 0, 0.46875, 1.875 or 7.5 i.u. LH, while the remaining 6 groups were used to assay the two urinary extracts. The three dose levels employed were equivalent to 11.25, 45.0 and 180 ml. of urine respectively.

**Experiment II**: A total of four litres of urine was collected from the same subject, pooled and divided into four 1 litre fractions. Three of these were dialysed as described above. The dialysates were pooled and retained for further processing. After dialysis, 750 ml. of each of the three urine fractions, together with 750 ml. of the undialysed urine, were extracted separately by the tannic acid procedure.

The dialysate, totalling 24 litres, was divided into two equal portions. No precipitate was formed when one of these was extracted by the tannic acid procedure. The remainder of the dialysate was concentrated in a rotary evaporator, but when the total volume of dialysate was reduced to 30 ml. no further concentration was possible because of the viscous nature of the material. The concentrate was then centrifuged at 3000 r.p.m. for 20 mins. It was assumed that the supernatant and the precipitate contained the materials which had been removed, by dialysis, from 1.5 litres of urine. Both fractions were split into two equal parts.

A control dialysis experiment was also carried out
in order to determine the recovery of an added quantity of LH. One ampoule of Pergonal (75.0 i.u. LH) was
dissolved in 10 mls. saline and was dialysed against a
volume of 20 mls. of distilled water, the procedure
being the same as that described in Experiment I.

The bioassay employed was the VPW test, carried out
as described in Chapter 1. Eighty-five hypophysectomised
animals were randomly allocated amongst 17 treatment
groups (5/group). Three groups were selected at random
to receive 0, 0.9 and 3.6 i.u. LH, while the remaining
14 groups were used to assay the following preparations
(see also Figure 30):

1. \textbf{Preparation A:} Dialysed Pergonal at dose levels
presumed to be 0.9 and 3.6 i.u. respectively.

2. \textbf{Preparation B:} Fraction I of the dialysed and
extracted urine.

3. \textbf{Preparation C:} The non-dialysed urine extract.

4. \textbf{Preparation D:} The first fraction of the super-
natant obtained after centrifugation of the concentrated
dialysate.

5. \textbf{Preparation E:} A combination of fraction II of
the dialysed extracted urine combined with the second
supernatant fraction.

6. \textbf{Preparation F:} The first fraction of the precipi-
tate obtained after centrifugation of the concentrated
dialysate.
EFFECT OF DIALYSIS ON URINARY LH ACTIVITY ESTIMATED BY VPW ASSAY

EXPERIMENTAL DESIGN

PERGONAL (75 i.u.)

\[ \downarrow \]

DIALYSED

\[ \downarrow \]

ASSAYED (PREPARATION A)

\[ \downarrow \]

1000 ml.

DIALYSED

\[ \downarrow \]

ASSAYED (PREPARATION B)

\[ \downarrow \]

1000 ml.

DIALYSED

\[ \downarrow \]

FRACTION I

\[ \downarrow \]

ASSAYED

\[ \downarrow \]

FRACTION II

\[ \downarrow \]

ASSAYED

\[ \downarrow \]

FRACTION III

\[ \downarrow \]

ASSAYED (PREPARATION C)

\[ \downarrow \]

DIALYSATE (24 LITRES)

\[ \downarrow \]

12 LITRES

EVAPORATED (30 ml.)

\[ \downarrow \]

CENTRIFUGED

\[ \downarrow \]

SUPERNATANT equally divided

\[ \downarrow \]

ASSAYED (PREPARATION D)

\[ \downarrow \]

RECOMBINED with FRACTION II

\[ \downarrow \]

ASSAYED (PREPARATION E)

\[ \downarrow \]

RECOMBINED with FRACTION III

\[ \downarrow \]

ASSAYED (PREPARATION F)

\[ \downarrow \]

RECOMBINED with FRACTION III

\[ \downarrow \]

ASSAYED (PREPARATION G)

\[ \downarrow \]

Figure 30
7. **Preparation G:** The second half of the precipitate combined with fraction III of the dialysed extracted urine.

The two dose levels employed for all the urinary extracts were equivalent to 28.13 mls. and 112.5 mls. respectively, while the doses selected for the supernatant as well as for the precipitate obtained after centrifugation of the concentrated dialysate represented the equivalent substances removed by dialysis from 28.13 and 112.5 ml. of urine respectively. Throughout the assay procedure all experimental animals received cortisone acetate (1 mg. per day).

**Results**

The weights of the urinary extracts in the first and second experiments were as follows: 54.5 and 58.3 mg. per litre of the non-dialysed and 16.7, 23.6, 17.0 and 20.1 mg. per litre of the dialysed urinary samples. The corresponding mean figures were 56.4 ± 2.39 and 19.35 ± 1.68 for the non-dialysed and the dialysed urine respectively. When these two mean values were compared by Student’s "t" test it was found that the difference was highly significant (t = 9.103, D.F. = 4, P < 0.001). When the powders were dissolved in saline prior to injection it was observed that those which came from non-dialysed urine were less readily dissolved than those
### Table 23

**The Effect of Dialysis on Urine Prior to Extraction (OAAD Test)**

<table>
<thead>
<tr>
<th></th>
<th>1.u. (Second IRP-HMG)</th>
<th>Non-dialysed Equivalent millilitres of urine</th>
<th>Dialysed Equivalent millilitres of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.46875 1.875 7.5</td>
<td>11.25 45.0 180.0</td>
</tr>
<tr>
<td>Ovarian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ascorbic acid</td>
<td></td>
<td>96.04 83.76 72.27</td>
<td>99.45 98.68 81.68</td>
</tr>
<tr>
<td>concentration</td>
<td></td>
<td>95.24 79.63 56.53</td>
<td>89.30 97.48 80.88</td>
</tr>
<tr>
<td>(μg./100 mg.)</td>
<td></td>
<td>93.71 85.09 55.28</td>
<td>106.50 88.75 78.15</td>
</tr>
<tr>
<td>ovarian tissue</td>
<td></td>
<td>101.07 87.22 68.32</td>
<td>92.99 83.65 72.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>105.60 88.04 65.24</td>
<td>101.83 84.70 86.11</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>98.3 84.7 63.5</td>
<td>98.0 90.6 79.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± ± ±</td>
<td>± ± ±</td>
</tr>
</tbody>
</table>

<p>|                | 2.3 1.6 3.3 | 3.3 2.9 2.7 | 2.7 3.2 2.0 |</p>
<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Non-dialysed Urine</th>
<th>Dialysed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sum Sq.</td>
<td>Mean Sq.</td>
<td>V.R.</td>
</tr>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>397.71</td>
<td>397.71</td>
<td>11.16</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>3,515.23</td>
<td>3,515.23</td>
<td>98.65</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>343.54</td>
<td>343.54</td>
<td>9.64</td>
</tr>
<tr>
<td>Curvature</td>
<td>2</td>
<td>58.81</td>
<td>29.40</td>
<td>0.82</td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>4,315.28</td>
<td>863.06</td>
<td>24.22</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>855.22</td>
<td>35.63</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>5,170.50</td>
<td>178.29</td>
<td></td>
</tr>
</tbody>
</table>
EFFECT OF DIALYSIS ON URINARY LUTEINISING HORMONE ACTIVITY ESTIMATED BY THE OVARIAN ASCORBIC ACID DEPLETION TEST

Figure 31
Figure 32
prepared from the dialysed extracts.

The results from the first experiment, in which the OAAD test was employed, are shown in Table 23 and Figure 31. The analysis of variance calculated individually for each "U" against the "S" preparation demonstrated that dialysed urine gave a dose-response relationship which was identical to that of the standard. The dose-response curve of the non-dialysed urine was not parallel to that of the standard ($P < 0.01$) and the overall difference in the responses obtained for both preparations was significantly different ($P < 0.01$) (Table 23).

The results obtained in Experiment II, using the VPW test, are shown in Table 24 and Figure 32. A statistical analysis of these results showed that in hypophysectomised rats no significant difference in potency was observed between the dialysed (Preparation A) or non-dialysed Pergonal (Table 24).

The dose-response curves obtained for the bioassay of preparations B, C and G were all parallel to the standard. Significantly lower ventral prostatic responses were observed following the administration of preparations B ($P < 0.01$), C ($P < 0.001$) and G ($P < 0.001$), in relation to those obtained following treatment with the standard of reference. A comparison between the responses obtained for preparations B and C (Table 25) demonstrated that the dose-response curves were parallel,
### Table 24

The Effect of Dialysis on Pergonal and on Urine Prior to Extraction (VPW Test)

<table>
<thead>
<tr>
<th>Standard Preparation</th>
<th>Urine</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undialysed</td>
<td>Dialysed</td>
<td>Undialysed-Extracted</td>
<td>Dialysed-Extracted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9 i.u. 3.6 i.u.</td>
<td>0.9 i.u. 3.6 i.u.</td>
<td>L.D.</td>
<td>H.D.</td>
<td>L.D.</td>
</tr>
<tr>
<td>Ventral prostate</td>
<td>21.0 36.0</td>
<td>13.0 21.5</td>
<td>12.0 16.0</td>
<td>17.5 19.5</td>
<td></td>
</tr>
<tr>
<td>Prostate weight (mg.)</td>
<td>23.0 24.0</td>
<td>14.0 32.0</td>
<td>11.0 16.0</td>
<td>16.0 24.3</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>20.4 27.8</td>
<td>16.0 28.1</td>
<td>10.8 16.1</td>
<td>16.6 22.5</td>
<td></td>
</tr>
</tbody>
</table>

S.E. values:

| S.E. | 1.2 2.5 1.5 3.3 0.6 1.5 0.4 1.1 |
|----------------|-----------------------|-----------------------------------------|-----------------------|-----------------------------------------|
| Ventral        | 7.0  | *    | 16.0 | *    | 11.0 | 12.0 | 14.5 | 21.0 |
| prostate       | 8.0  | *    | 16.0 | *    | 11.0 | 13.0 | 13.0 | 18.5 |
| weight (mg.)   | 8.0  | *    | 17.0 | *    | 11.0 | 12.0 | 15.0 | 21.0 |
| (mg.)          | 8.0  | *    | 15.5 | *    | 11.0 | *    | 13.0 | 17.0 |
| Mean           | 7.8  |      | 16.1 |      | 11.0 | 12.3 | 13.9 | 19.4 |
| S.E.           | 0.2  |      | 0.4  |      | 0    | 0.3  | 0.5  | 1.0  |

* Dead animal.
TABLE 24 (contd.)

Analysis of variance of the valid 4-point assays

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Dialysed Pergonal</th>
<th>Dialysed-Extracted Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sum Sq.</td>
<td>Mean Sq.</td>
</tr>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>21.01</td>
<td>21.01</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>475.31</td>
<td>475.31</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>27.61</td>
<td>27.61</td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>523.94</td>
<td>174.66</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>413.20</td>
<td>25.82</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>937.14</td>
<td>49.32</td>
</tr>
</tbody>
</table>
## TABLE 24 (contd.)

### Analysis of Variance (contd.)

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Non-Dialysed-Extracted Urine</th>
<th>Precipitate of D/sate + Dial.-Extr. Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sum Sq.</td>
<td>Mean Sq.</td>
</tr>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>567.11</td>
<td>567.11</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>201.62</td>
<td>201.62</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>5.51</td>
<td>5.51</td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>774.24</td>
<td>258.08</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>193.00</td>
<td>12.06</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>967.24</td>
<td>50.91</td>
</tr>
</tbody>
</table>
TABLE 25

COMPARISON OF THE RESULTS BETWEEN THE
DIALYSED-EXTRACTED AND NON-DIALYSED-EXTRACTED
PREPARATIONS

STANDARD: Dialysed-extracted urine
UNKNOWN: Non-dialysed-extracted urine

Analysis of Variance

<table>
<thead>
<tr>
<th>Variations</th>
<th>D.F.</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between preparations</td>
<td>1</td>
<td>184.81</td>
<td>184.81</td>
<td>75.13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Common regression</td>
<td>1</td>
<td>155.71</td>
<td>155.71</td>
<td>64.34</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Parallelism</td>
<td>1</td>
<td>0.39</td>
<td>0.39</td>
<td>0.16</td>
<td>N.S.</td>
</tr>
<tr>
<td>Between doses</td>
<td>3</td>
<td>340.91</td>
<td>113.64</td>
<td>46.96</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>39.43</td>
<td>2.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>380.34</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
but that the effect of the undialysed preparation (C) was significantly less ($P<0.001$).

Preparation D was extremely toxic and all animals injected with the high dose died within a few minutes of the injection. A similar effect was noted in the group of animals injected with the high dose of preparation E. Nevertheless all but one of the animals survived when the lower doses of these two preparations were administered. The low dose of fraction D had no effect on the weight of the ventral prostate. However, when preparation E was assayed, a significant increase in prostatic weight was noted, the figure being similar to those obtained with the low dose of preparations A, B and G. Preparation F was less toxic and only 15 per cent of the animals injected either with this preparation alone or in combination with dialysed extracted urine (Preparation G), died during the assay procedure. The results obtained when preparation F was administered showed no significant difference between doses although the mean values (11.5 ± 0 and 12.3 ± 0.3 mg. respectively) when compared with the control using Student's "t" test were both significantly higher ($P<0.001$).

Discussion

Dialysis of urine prior to extraction resulted in a highly significant reduction in the weight of powder
produced \((P<0.001)\). These powders were also much more soluble than those obtained from undialysed urine. It was demonstrated that dialysis of Pergonal did not result in a significant loss of activity as estimated by the VPW test.

The significantly lower effect induced by the non-dialysed urine (Preparation C) in the OAAD test \((P<0.01)\), as well as in the VPW test \((P<0.001)\), suggests that the dialysis procedure removes a material which inhibits the effect of LH in both assay procedures. Assays of combined dialysed-extracted urine with either the supernatant or precipitate of the concentrated dialysate (Preparations E and G) were compared with the results obtained for the non-dialysed extracted urine (Preparation C). The supernatant of the centrifuged concentrated dialysate (Preparation D) was extremely toxic and all the animals injected with the high dose died. The low dose of the precipitate of the concentrated dialysate (Preparation F) induced a highly significant increase in the weight of the ventral prostate, but the high dose did not increase this effect. When the combined materials (Preparation G) was assayed it produced a dose-response curve which was parallel to that induced by the dialysed-extracted urine (Preparation B).

Several workers have claimed the existence of a factor inhibiting the action of LH (Futterweit et al.,
However, the urine used by these workers was initially heated to 100°C for one hour in order to destroy any LH activity. Under such conditions it is difficult to assume that this inhibitor is a physiological substance, since it may well be an artifact due to heating. Rosenberg et al. (1965) reported that they were unable to find a gonadotrophin-inhibiting material in human urine and suggested that toxicity may account for the inhibitory properties of certain extracts. Ota et al. (1968) re-investigated the problem and they demonstrated that the degree of toxicity was not related to an inhibitory factor.

The experiments presented herein demonstrated that dialysis of the urine prior to extraction improves the methods. However, the reason for this effect remains obscure.

COMPARISON OF THE VPW AND OAAD TESTS IN THE ASSAY OF LH IN URINE

Introduction

An attempt was made to compare the VPW and OAAD tests in the measurement of urinary LH and the results have been reported and discussed earlier (see page 34). Subsequently methodological studies were carried out on both bioassay techniques (see Chapters 1 and 2) which resulted in considerable improvements in the reliability
of both procedures. However, the toxicity of urinary extracts following administration was still severe and approximately 13% of the animals used in the OAAD test that were injected with crude material died. This suggested that the extraction procedure should also be re-examined with a view to eliminating toxicity as a problem. It has been demonstrated earlier (see page 96) that hibitane used as a preservative during the collection of urine inhibits the growth of microorganisms and cocci without affecting the bioassay procedure. Moreover, dialysis of urine immediately on arrival in the laboratory led to a smaller yield of extract without adversely affecting the results obtained by both bioassay techniques. The results obtained in assays in which all these modifications were applied routinely to clinical studies have been previously reported in Chapter 2 (page 81).

The aim of the present study was to measure the urinary excretion of LH by both the OAAD and VFW tests and to compare the indices of precision obtained with those calculated for a similar study described earlier (page 34). This would serve to indicate the effect of the various modifications made to both bioassay systems.

**Material and Method**

Three patients with an abnormal karyotype were
selected. The first patient (Mr P) had a 47,XYY sex chromosome constitution while those of Misses E.T. and L.B. were 47,XXX and 45,X/46,XX respectively.

The 48-hour urinary extracts were split into two equal parts and assayed by both the VPW and OAAD tests using the modification described in Chapters 1 and 2.

**Results**

The results obtained are shown in Figure 33. The mean LH value obtained for Mr P by the VPW test was 39.2 ± 8.5 and by the OAAD assay was 56.7 ± 18.7. The mean LH readings for Miss E.T. were 46.1 ± 12.2 and 45.2 ± 4.2 for the OAAD and VPW tests respectively, the corresponding figures for Miss L.B. being 14.5 ± 0.7 and 12.0 ± 1.6 i.u. per 24 hours. One LH value for Miss L.B., estimated by the OAAD test, could not be calculated because the animals treated with standard preparation did not respond. The mean indices of precision were 0.21 ± 0.02 (± S.D.) and 0.08 ± 0.04 for the OAAD and VPW tests respectively.

The index of discrimination (Figure 33) between the OAAD and VPW estimations ranged from 0.5 to 2.0.

**Discussion**

The results obtained from the comparative study between the OAAD and VPW tests, unlike those reported in
COMPARATIVE STUDY BETWEEN THE OVARIAN ASCORBIC ACID DEPLETION ASSAY AND THE VENTRAL PROSTATE WEIGHT TEST

Mr. P  Miss E.T.  Miss L.B.

OAAD

IOO-
50-
O-
150-

VPW

INDEX OF DISCRIMINATION

DAY OF INVESTIGATION

Figure 33
Chapter 1, are similar. The mean LH values obtained by the OAAD and VPW assays for Mr P did not differ significantly and the mean readings obtained by the two methods for the second and third patients were almost identical. The values obtained are within the range found in patients with sex chromosomal abnormalities (see Section B, Chapters 5 and 6).

Finally the index of discrimination did not deviate markedly from unity. It is therefore concluded that the active principle measured by the two tests was identical.

**SUMMARY**

It was observed that urine collected routinely for clinical studies was extremely heavily contaminated with bacteria which resulted in toxicity of the urinary extracts. The collection of urine with the bacteriostatic agent Hibitane inhibited such growth. Dialysis of the urine prior to extraction serves as a first step of purification, removing from it materials which may affect the mode of action of LH in bioassay procedures.

The tannic acid method for the extraction of urinary gonadotrophins appeared to be satisfactory, when sample is collected with a preservative and purified, prior to extraction, by dialysis. The extracts do not appear to be toxic and valid results can be obtained with both the OAAD and VPW bioassay methods for LH.
I. Normal menstrual cycle and normal men

Much of the understanding of human pituitary and gonadal function is based upon measurements of urinary gonadotrophins and steroid excretion.

The generally accepted view of oestrogen output in women during the menstrual cycle has been described by Brown and his colleagues (Brown, 1960; Brown and Matthew, 1962). The amount of the three main oestrogens, i.e. oestrone, oestradiol and oestriol, rises and falls together. Usually the quantity of oestriol is greater than or equal to that for oestrone while oestradiol is present in the smallest amount. The output of oestrogens is lowest during the first week of the cycle and then rises to a well-defined peak, termed the "ovulatory" peak, which occurs on or about the 13th day. The oestrogen excretion then falls and rises again to a second maximum termed the luteal maximum. This peak is usually of smaller magnitude than the "ovulatory peak" and in some women may be ill-defined. During the last few days of the cycle, oestrogen excretion falls and menstruation occurs.

Another hormone which also shows a characteristic pattern of values during the ovulatory cycle is progesterone; in addition, changes in its chief urinary
metabolite, pregnanediol, are also well recognised. It has been shown that progesterone is secreted from the pre-ovulatory graafian follicle in small amounts; it starts to rise at ovulation with a maximum level 5 days after ovulation (Forbes, 1950; Zander, 1958; Mikhail et al., 1963). Pregnanediol does not derive uniquely from progesterone (Tait, 1963) but its excretion pattern is similar to that for blood progesterone. Thus in the follicular phase of the cycle pregnanediol output is low, but, following ovulation, a rise in its excretion occurs with a peak 4 to 5 days after the appearance of the ovulatory peak of oestrogen output (see Klopper, 1957; Brown and Matthew, 1962; Loraine and Bell, 1966, 1968).

At the time of writing the literature on urinary LH excretion, measured by bioassay methods, in normal menstruating women is still relatively scanty but is greater than the corresponding information in male subjects.

There is reasonably good agreement among investigators with regard to the pattern of urinary LH excretion in normally menstruating women. This is characterised by low and relatively constant levels during the follicular phase; an increase at about midcycle and low levels is the trend toward the luteal phase (McArthur, 1959; Fukushima et al., 1964; Becker and Albert, 1965; Stevens et al., 1965; Rosenberg and Keller, 1965; Steven and Vorys, 1967). Detailed studies include those
of Becker and Albert (1965) and Rosenberg and Keller (1965). The former group, using the VPW method, calculated their mean urinary LH levels from serial estimations performed throughout the menstrual cycle in seven women. The results were expressed in "rat units". They were converted into mg. equivalent of NIH-LH-S1 and subsequently to i.u. (2nd IRP-HMG) using factors described by Becker and Albert (1965) and Rosenberg and Lewis (1966) respectively. The mean LH output was 16.6 ± 1.5 (M.S.E.) i.u. per 24 hours (10 observations); the corresponding value extracted from the data of Rosenberg and Keller (1965) was 4.3 ± 0.56 i.u. 2nd IRP-HMG per 24 hours (83 observations). When these two mean values were compared, using Student's "t" test, there was a highly significant difference between them (P<0.001). This discrepancy between Becker and Albert's (1965) and Rosenberg and Keller's (1965) results may be due to differences in the design of the studies. Thus Becker and Albert (1965) did not mention the age of the subjects studied, but they reported that the cycles varied from 24-36 days; they also emphasised that in their study they included women who had previously shown high gonadotrophin levels as measured by the mouse uterus test. Probably in this way they incorporated subjects with abnormally high LH levels in contrast to the group described by Rosenberg and Keller (1965) which
consisted of girls aged from 18 to 20 years and with menstrual cycles quite similar in length (27 days). Moreover, the use of factors to convert the results of Becker and Albert (1965) from "rat units" to i.u. 2nd IRP-HMG might alter their data. Because of the scanty and conflicting information, it is obvious that at the time of writing a definite conclusion cannot be drawn regarding the range of LH output in normally menstruating women and further work in this area is obviously required.

Becker and Albert (1965) performed isolated urinary LH estimations in 9 normal men ranging in age from 22 to 39 years. They used a kaolin-acetone method of extraction (Albert, 1955) and estimated LH by the VPW test. The results were expressed in "rat units" and were converted, in the same manner as described above, to i.u. 2nd IRP-HMG. The mean (M ± S.E.) LH based on 9 observations was 15.6 ± 2.7 i.u. 2nd IRP-HMG per 24 hours.

Rifkind et al. (1967), using Albert's kaolin-acetone technique (Albert, 1955; Albert et al., 1961) and the same bioassay method, have studied urinary LH excretion in normal men (10 observations). They found lower readings for LH which were approximately four times lower than those reported by Becker and Albert (1965), their mean (M ± S.E.) LH value being 4.7 ± 0.6 i.u. per
24 hours. The lower estimate reported by Rifkind et al. (1967) cannot be explained solely from the fact that they purified their urinary extracts to a greater extent than did Becker and Albert (1965), since it has been shown that this purification step results only in a loss of from 10 to 40 per cent of the biological activity (Albert et al., 1961a; McArthur et al., 1967). The significant higher value (P<0.01) found by Becker and Albert (1965), as compared with that reported by Rifkind et al. (1967), may possibly have been due to factors used to convert the readings from rat units to mg. NIH-LH-S1 and subsequently to i.u. 2nd IRP-HMG.

II. Perimenopausal and postmenopausal period

At present there is a great lack of information regarding LH activity in women late in their reproductive life and at or beyond the menopause. Many years ago the very obvious alterations of gonadal function in the aged female raised the question of whether they represented the cause of ageing in general. However, when in the second and third decades of this century the leading role of the pituitary in controlling endocrine function, and especially that of the gonads, became well known, the idea that ageing might be caused primarily by a decrease of pituitary function began to gain credence.

Gross morphological changes of the anterior
pituitary with ageing have been known for many years and the weight of the gland at different ages has been intensively studied. There is evidence to support the view that the human female pituitary gland increases in weight until the age of 39 and from then onwards becomes smaller (Simonds, 1914; Roessle and Roulet, 1932). Furthermore, it was found that it is mainly the anterior lobe which shows a progressive decrease after the 40th year (Shanklin, 1953). Similar observations have been made in dogs on the basis of which it was reported that in senile animals the pituitary weight was decreased (White and Faust, 1944; Francis and Mulligan, 1949).

The histology and cytology of the pituitary during ageing were studied between 1930 and 1950. Castration or menopausal cells have been found in the pituitary of aged males and females; these are basophil in type and are aggregated especially in small adenomata. The cells contain large granules and have chromatin rich nuclei and large vacuoles (Korenchevsky, 1961). Certain conclusions have been drawn as to the functional activity of the pituitary gland on the basis of the morphological changes associated with age, but their validity remains uncertain unless a change of hormone content or production can be demonstrated.

Early attempts were made to elucidate this problem by several investigators who measured the gonadotrophic
activity of the pituitary gland in different animal species. Thus, Lauson et al. (1939) studied the pituitaries of rats of different ages and measured gonadotrophic activity by the uterine weight assay. Throughout sexual life activity remained at the puberal level despite continued increases in pituitary size (from 5.2 to 16.1 mg.). However, in three senile female rats (2½ years of age) a remarkable increase in gonadotrophic hormone content was found.

Assay of human pituitaries has not shown any striking differences in the gonadotrophin content at different ages. Thus, Bahn et al. (1953) using the interstitial cell repair test in hypophysectomised female rats found that in postmenopausal women the pituitary LH concentration was similar to that found in males and in women during reproductive life.

Another approach to this problem has been made by measuring the urinary excretion of gonadotrophins at different ages. Heller and Shipley (1951), using the mouse uterus test, reported that no significant correlation could be established between excretion values and age. However, several workers found that the high gonadotrophin excretion after the menopause persisted for the remainder of the subject's life (Heller and Heller, 1939; Albert et al., 1958; Johnsen, 1959; Apostolakis and Loraine, 1960; Rosenberg and Engel,
A small number of investigators have attempted to estimate urinary LH in ageing females. One study in this field is that by Sherman and Woolf (1959) who were unable to demonstrate any increase in LH output in postmenopausal women when compared with levels found in normally menstruating subjects. On the other hand, McArthur et al. and Yahia et al. (1964) found a marked increase in LH excretion during the postmenopausal era. Becker and Albert (1965), Albert and Mendoza (1966) and Keller (1966) also reported high LH levels in postmenopausal women, but these results do not agree from a quantitative point of view.

III. Oral contraception

Since 1955 when Pincus reported some effects of steroidal compounds on reproductive processes in humans, the probable mode of action of oral contraceptives has been repeatedly studied (see MacLeod and Tietze, 1964; Diczfalusy, 1965; Mears, 1965a, 1965b; Pincus, 1965; Borell, 1966; F.D.A., 1966; Greenblatt et al., 1966; Haller, 1966; Maas, 1966; Rudel and Kincl, 1966; Starup, 1966; W.H.O., 1966; Lauritzen, 1967; Diczfalusy, 1968; Loraine and Bell, 1968). However, the mechanism of action of these compounds still remains obscure. There are seven general sites in the female
organism which might be involved. Thus the compounds might act

(1) on the centres in the central nervous system concerned with the production of gonadotrophin-releasing or inhibiting factors;

(2) on the anterior pituitary directly to inhibit the secretion of FSH, LH or both hormones;

(3) on ovarian tissue affecting the rates of steroidogenesis and the maintenance of the corpora lutea;

(4) on the fallopian tubes by altering the nature of the tubal contractions and the composition of tubal secretions;

(5) on the uterus myometrium and endometrium, affecting growth and contractibility in the former and growth and secretory processes in the latter;

(6) on implantation sites in the uterine endometrium; and

(7) on the cervical mucus rendering the latter more hostile than previously to sperm penetration.

Animal experiments have shown that administration of medroxy-progesterone acetate causes depletion of pituitary LH stores while norethisterone and chlormadinone induce storage of LH in the pituitary gland (Brown et al., 1965; Labhsetwar, 1966, 1967, 1968). Schally et al. (1968) investigated the effect of anti-ovulatory compounds on the response to luteinising hormone
releasing factor (LRF). Ovariectomized rats were injected with large doses of oestrogens, gestagens and combination of the two hormones. Plasma LH was measured by the oaaD method and it was found that the above steroids exerted a suppressive effect. However, administration of 0.4 μg. LRF to rats treated with the above compounds caused a highly significant elevation of plasma LH. This might suggest that the oral contraceptives do not act directly on the pituitary but on the hypothalamus or on higher centres.

During the past few years numerous studies have been carried out in order to investigate the effect of contraceptive steroids on human pituitary gonadotrophic function. In the earlier studies, gonadotrophic activity was estimated using non-specific methods, mainly the mouse uterus test, and different opinions have been expressed concerning the effect of progestogens alone or combined with oestrogens on the excretion of "total gonadotrophic activity" in urine. Some investigators, including Heller (1957), Smith and Albert (1958), Brown et al. (1960), Matsumoto et al. (1960), Paulsen et al. (1960), Albert and Smith (1961), McArthur et al. (1961), Moore et al. (1961), Loraine et al. (1961), Brown et al. (1962), Loraine et al. (1963), Fuchs et al. (1964), Loraine (1964), Sas et al. (1964), Loraine et al. (1965), Paulsen (1965), Apostolakis et al. (1966) and
Starup (1967), did not find any significant reduction of gonadotrophic activity and concluded that the steroids used might not affect pituitary gonadotrophic function but might exert a direct action on steroid biosynthesis in the ovary. However, other investigators have shown that progestogens, oestrogens, or progestogen-oestrogen mixtures, when administered to fertile women, produce a distinct decrease in excretion values for "total gonadotropic activity" (Crooke and Butt, 1953; Garcia and Rock, 1960; Matsumoto et al., 1960; McArthur et al., 1961; Buchholz et al., 1962; Kaiser, 1963; Brown et al., 1964; Demol and Ferin, 1964; Erb and Keller, 1964; Lin et al., 1964; Østergaard, 1964; Taylor, 1964; Walser et al., 1964; Buchholz and Nocke, 1965; Paulsen, 1965; Stevens et al., 1965; Brown et al., 1966; Dapunt, 1966; Lauritzen, 1966; Østergaard et al., 1966; Pincus, 1966; Ryan et al., 1966; Schmidt-Elmendorff and Kopera, 1966; Starup, 1966; Daume and Kaiser, 1967; Keller, 1967; Starup, 1967). It must however be stressed that this suppression was often not statistically significant when the mean of serial estimations carried out in the control cycle was compared with the corresponding value in the treatment cycle. It seems probable that if contraceptive compounds exert a suppressive effect on gonadotrophin excretion then this might only become significant in relation to the mid-
cycle peak: studies in which specific assays for LH have been conducted have tended to confirm this conclusion (see Table 33 for references).

Another attempt was made by several workers to investigate the mode of action of oral contraceptives by joint administration of a steroid hormone and a gonadotrophin. It was argued that if steroids inhibit ovarian function directly, then exogenous administration of gonadotrophins would probably fail to overcome the ovarian blockade; if, however, the steroids directly inhibited pituitary function then the exogenous administration of gonadotrophins should compensate for the pituitary inhibition by directly stimulating the ovary. As a result of these types of studies some authors inferred a central effect because ovarian inhibition by steroids was abolished by exogenous gonadotrophins (Bettendorf, 1962; Johannisson et al., 1965; Taymor and Rizkallah, 1965; Starup and Østergaard, 1966), whilst others assumed a peripheral effect, because administration of gonadotrophins did not restore ovarian function (Diczfalusy, 1961, 1962; Lunenfeld et al., 1962, 1963; Arguelles et al., 1964; Hecht-Lucari, 1964; Lunenfeld, 1964). It should, however, be noted that only the results of Starup and Østergaard (1966) are based on observations made in normally menstruating women (22 subjects); the other investigations were made
on patients who suffered from amenorrhoea or the Stein-Leventhal syndrome, and therefore these results should be accepted with reserve.

It is obvious that the mechanism of action of oral contraceptive steroids is still incompletely understood. This is in part due to the fact that many of the physiological mechanisms involved in the regulation of the human reproductive processes have not yet been elucidated. The paucity of information on normal endocrine regulation of these processes renders it difficult to evaluate some of the pharmacological effects induced by contraceptive steroids. Another source of difficulty arises from the fact that the various types of compound, and even the same compound at various dose levels, may have different sites and modes of action.

IV. Clomiphene

This compound was initially shown on the basis of experiments in rodents to have contraceptive properties (Holtkamp et al., 1961). Early studies by Greenblatt and his colleagues, summarised in a review by Greenblatt and Mahesh (1965), indicated that the effects of Clomiphene in human subjects were very different from those in rodents. These workers noted that the compound lacked anti-ovulatory activity in women and observed that, when it was administered to patients with unduly
short menstrual cycles, the latter reverted to a normal length. Greenblatt and his associates (Greenblatt, 1961; Greenblatt et al., 1961) subsequently showed that Clomiphene stimulated ovulation in a proportion of sterile women.

The mode of action of Clomiphene is not yet known with certainty. Several workers claimed that it has a direct effect on the ovaries while others believe that it acts through the anterior pituitary gland (see Table 37 for references).

V. 47,XYY* Chromosomal abnormality

The first male patient with an XYY chromosome constitution was reported by Sandberg et al. (1961) and Hauschka et al. (1962). Subsequent chromosomal examination of randomly selected newborn males, randomly selected adult males, and males examined for a variety of reasons, revealed that the XYY abnormality was rare and was calculated to occur in approximately one out of 2,000 subjects (Court-Brown et al., 1964). Until the identification of this type of patient in a State Hospital (Jacobs et al., 1965), only 12 males had been described with a sex chromosome complement of this type among the general population in various countries.

* Throughout the following chapters the nomenclature proposed at the Chicago Conference on Cytogenetics (1966) will be used.
Jacobs et al. (1965) and Price et al. (1966) were the first to indicate that there may be characteristics common to patients with the XYY sex chromosome abnormality. They showed that in these subjects there were tendencies to mild mental retardation, aggressive behaviour which often resulted in criminal or violent acts, and increased height.

Price et al. (1966) reported that in eight out of nine XYY males the external genitalia were normally developed. On this evidence they concluded that there was no reason to suppose that under different circumstances the XYY patients would have been subfertile.

In spite of this observation clinical findings suggest that the incidence of hypogonadism is high in this type of sex chromosomal abnormality (Balodimos et al., 1966). To the best of the knowledge of the author no information regarding pituitary gonadotrophic function in males with an XYY karyotype has so far been reported.

VI. Turner's Syndrome

In 1938 Turner described in seven females a syndrome of sexual infantilism, webbed neck and cubitus valgus, associated with short stature. At that time it was thought that these patients had diminished ovarian function secondary to a hypopituitary state or a defect
in the pineal gland. Sharpey-Schafer (1941), however, took the view that the ovaries were defective. When tests for pituitary gonadotrophins in urine were developed, it was found that the syndrome was associated with high levels of "total gonadotrophic activity" (Albright et al., 1942; Varney et al., 1942; Schneider and McCullagh, 1943; Wilkins and Fleischmann, 1944; Amorose et al., 1966). The increased levels of gonadotrophins in adult patients with gonadal dysgenesis were presumed to reflect the lack of the normal ovarian inhibition of pituitary gonadotrophin production.

In 1954 Polani et al. and Wilkins et al. simultaneously demonstrated that individuals with Turner's syndrome were chromatin-negative. Polani et al. (1956), using evidence from nuclear sexing and colour-blindness inheritance, suggested that such individuals possessed a 45,X sex chromosome constitution; this was later confirmed by Ford et al. (1959). Since these original studies, chromosomal analyses have been carried out in a large number of subjects with gonadal dysfunction and a variety of karyotypes have been reported. The possible variations comprise (1) the presence of a structurally abnormal sex chromosome (iso-chromosome); (2) mosaicism; and (3) a combination of (1) and (2) (Court-Brown et al., 1964). The 45,X karyotype represents the largest entity, accounting for
roughly half the cases; about one-third of the cases are mosaics, and the isochromosome of the long arm of the X-chromosome represents the commonest structural abnormality (Mittwoeh, 1967). An isochromosome is a chromosome in which the arms on either side of the centromere are physically identical and bear the same gene loci. There are two possible mechanisms by which an isochromosome may be formed. If the two homologous acrocentric chromosomes fracture at their centromeres and the long arms reunite, an isochromosome is produced which consists of two homologous long arms. This mechanism is called "centric fusion". An isochromosome may also follow "centric fusion" involving two meta-centric chromosomes. In theory, such an exchange between homologous metacentric chromosomes should produce two isochromosomes but not infrequently two of the fragments produced are acentric and become lost. A second mechanism by which an isochromosome may arise is the following: a metaphase chromosome is composed of two identical longitudinal halves which are united at a pale or unstained area. Each half is called a chromadit, and the pale point of union is the centromere. If transverse fission through the centromere of a metaphase chromosome occurs, and the fractured chromatids reunite with their adjacent (sister) strands, one or sometimes two isochromosomes are produced. This is called
"sister strand reunion" (Egger, R.R., 1965).

Non-disjunction of the sex chromosomes in spermatogenesis which may give rise to Klinefelter's syndrome may also result in Turner's syndrome. It might be anticipated that Turner's syndrome might occur more frequently than Klinefelter's syndrome because the former condition can arise from the loss of a chromosome which would go into the polar body. However, the opposite has been found to be the case. Thus the incidence of Klinefelter's syndrome at birth has been estimated to be approximately 2 per 1,000 whereas that of Turner's syndrome is roughly 4 per 10,000 (Mittwoch, 1967). The most likely explanation for this apparent discrepancy may be found in the results of chromosomal studies on aborted embryos. Carr (1965) reported that among 200 spontaneously aborted embryos, eleven had a 45,X chromosome constitution, while none showed 47,XXY karyotype. This suggests that the frequency of Turner's syndrome at birth may be only a few per cent of that at conception and it appears possible that the abnormalities of the heart and blood vessels associated with this syndrome may result in a high mortality before birth.

The classical belief has existed for many years that the primary defect in Turner's syndrome lies in the ovaries. However, certain observations exist in the literature which may necessitate some modification of
this view. It has been reported that children with Turner's syndrome showed grossly elevated urinary HPG (Silver, 1951; Silver and Kempe, 1953; Silver and Dodd, 1957). Moreover, normal menstrual function, sometimes for several years, has been reported in proved cases of the syndrome (Hoffenberg et al., 1957; Stewart, 1960; Bahner et al., 1960). Evidence of this type suggests that the classical view regarding the aetiology of Turner's syndrome may represent an over-simplification of the true situation. In attempts to elucidate this problem the estimation of total gonadotrophic activity does not yield sufficient information. Accordingly, in order to give more insight into the aetiology of the condition, the excretion values for LH and FSH must be studied separately, in order to investigate whether the pituitary plays an important role in the pathogenesis of Turner's syndrome.
CHAPTER 1

ENDOCRINE FUNCTION IN PERIMENOPAUSAL WOMEN

INTRODUCTION

The term menopause (emmena = menses; paieis = cessation; Greek term) is used to describe a transitional phase of a woman's life which bridges the gap between normal reproductive function and ovarian senescence. It usually occurs between the ages of 45 and 50, the average time of onset being 47 years (see MacGregor, 1963).

The symptoms which arise at the menopause are often of a psychological nature; in addition, there is frequently evidence of vasomotor instability. Many of the clinical features are believed to result from a decline and eventual failure of ovarian function and a number of theories have been adduced relating endocrine function to the symptomatology of the menopause. The evidence for and against such hypotheses has been evaluated by various investigators including Loraine (1958) and Fluhmann (1964). One of the most widely accepted theories (see Albright, 1936) postulates that the symptoms are caused directly by the overproduction of human pituitary gonadotrophins, while another, that of "oestrogen lack", proposes that the clinical features
arise from a decrease in the levels of circulating oestrogens (Heller et al., 1944).

Over the past three decades much new information has been accumulated on hormone excretion patterns in women during reproductive life. However, there is a paucity of similar information in women at or about the menopause, and, in the absence of objective data of this type, an authoritative opinion cannot be given regarding the validity of the hypotheses mentioned above.

The aim of this study was to investigate the interrelationship between LH and oestrogen output in the perimenopausal period, and to find out if any significant correlation exists between hormonal excretion pattern and symptoms that appear during the menopause. The study was then extended to examine the effect of exogenous administration of HCG on ovarian function in one of these subjects.

Subjects Studied

Patients 2, 3 and 4 were selected either because of their age or due to the presence of menopausal symptoms. Patient 1 was discovered in the course of a study designed to examine the effect of a low dosage progestogen (Chlormadinone) on endocrine function; the data shown in Figure 34 represent the pretreatment cycle in the investigation.
Patient 1 - Mrs M.B., aged 37 years, para 3 + 0

The menarche occurred at 14 years of age. Menstruation was regular, the duration of the cycles being 28 days with bleeding lasting 5 days; there were no menopausal symptoms. One year following the hormone assay study the subject's menstrual periods were still regular. Her chromosomal karyotype was determined and was found to be normal (46, XX).

Patient 2 - Mrs O.K., aged 42 years, para 6 + 0

The menarche occurred at 15 years, and at the age of 19 the right ovary was removed on account of a suspected neoplasm. Until the age of 38 menstruation was regular (5/28); thereafter spells of oligomenorrhoea alternated with periods of excessive bleeding. For some 20 months prior to the investigation, the patient had complained of hot flushes and depressive attacks; these symptoms were well controlled by the administration of large doses of Chlorpromazine, the drug being discontinued immediately prior to the commencement of the present investigation. On the 37th day of the study bleeding commenced, and this subsequently developed into substantial flooding. Dilatation and curettage were performed on day 49; the biopsy showed a non-functional endometrium with loose stroma and scanty mitoses. The intermittent bleeding continued and accordingly it was decided to perform a hysterectomy. At operation the
remaining ovary was removed. It measured 24 x 15 mm. and contained three cysts all with recognisable luteal cells; no corpora albicantia were observed and there were no developing follicles. The cervical epithelium was normal and the endometrium proliferative in type.

Patient 3 - Mrs A.G., aged 48 years, para 6 + 0

The menarche was at age 15. Menstruation was regular (6/28) and her previous health good. In the months immediately preceding the study the subject had complained of dyspareunia, hot flushes and irritability. The patient was selected for the stimulation test with HCG (Pregnyl, Organon), the results of which are shown in Figure 38.

Patient 4 - Mrs E.F., aged 51 years, para 1 + 0

The menarche was at age 16. Cycle length had always tended to be slightly irregular, varying from 34 to 36 days with menstruation lasting 3-5 days. At the time of the study there were no menopausal symptoms. Menstruation ceased one year after the end of the study.

METHODS

All subjects collected complete 24 hour urine samples throughout the period of investigation. Assays were conducted on 48 hour pools of urine, results being expressed as i.u. (2nd IRP-HMG) per 24 hour sample.

LH was extracted from urine by the tannic acid
procedure of Johnsen (1958) and assayed by the OAAD test (see Section A, Chapter 2, page 79). A total of 75 assays was performed; 28% of the assays had to be discarded either because of significant deviation from parallelism between the slopes of the dose-response curve of standard and unknown preparations (17.3%) or due to failure to obtain any slope with the standard preparation (10.7%). The mean index of precision ($\lambda \pm S.D.$) for the LH assays was $0.15 \pm 0.05$.

Assays of the three classical oestrogens (oestrone, oestradiol and oestriol) were performed by the method of Brown et al. (1957) while pregnanediol was measured by the procedure of Klopper et al. (1955).

RESULTS

Patient 1 (Fig. 34)

The investigation extended over a period of 33 days. Levels of oestrone, oestradiol and oestriol were within the normal range for a woman during reproductive life. They rose gradually during the time of observation reaching an ovulatory peak on day 15; thereafter there was a luteal phase rise in oestrone and oestradiol output on days 22 and 23. Pregnanediol excretion was also typical of the ovulatory cycle, levels being at their maximum in the luteal phase on days 22 and 23.

The pattern of LH output was grossly abnormal,
Figure 34

Hormone excretion pattern in patient 1.

Hatched areas indicate duration of menstrual bleeding.

I = fiducial limits (P = 0.95) of individual assays.
readings being very high at the beginning of the cycle, falling at midcycle and rising again slightly in the luteal phase. The first three readings — 383, 227 and 199 i.u. per 24 hours — are some 15 times higher than would be anticipated in women during reproductive life (see Table 26). In this subject, ovulation, as judged by oestrogen and pregnanediol assays, probably occurred in the presence of the lowest LH excretion values.

Patient 2 (Fig. 35)

The study continued for a period of 46 days. Oestrogen excretion was quite atypical for a woman during reproductive life, patterns for oestrone, oestradiol and oestriol all being dissimilar. Oestradiol levels were generally higher than in normally menstruating women and fluctuated irregularly throughout. Oestrone values were low at the commencement of the investigation, attained their maximum from days 29 to 34 and thereafter decreased slightly. A small peak of oestriol output occurred on days 32 to 34, some 6 days before the commencement of bleeding. Pregnanediol levels were all low, being within the postmenopausal range, and, on the basis of the urinary steroid analyses, there was no evidence to indicate that ovulation had occurred.

Throughout the whole period of study, LH levels were elevated as compared with women during reproductive life.
Mrs. C.R.: AGE 42: PARA 6.0

MENOPAUSAL SYNDROME

Figure 35

Patient 2. Notation as in Figure 34.
However, the pattern of LH output bore some resemblance to that of normally menstruating women, showing higher levels towards the middle of the cycle and lower readings elsewhere.

**Patient 3 (Fig. 36)**

The duration of the investigation was 51 days. It included the second half of an ovulatory cycle and a complete menstrual cycle.

The first cycle terminated with menstruation on day 15. The ovulatory peak of oestrogen excretion occurred from days 1 to 4 and the luteal phase maximum of pregnanediol was present from days 7 to 10. LH levels were abnormally high for women during reproductive life, ranging from 69 to 173 i.u. per 24 hours.

The second cycle had a duration of 28 days. The pattern of oestrogen excretion was reasonably typical of an ovulatory cycle, and there was a luteal phase rise in pregnanediol output maximal from days 34 to 36. LH levels were high, ranging from 80 to 272 i.u. per 24 hours and fluctuated in an irregular manner.

**Patient 4 (Fig. 37)**

The study occupied a period of 30 days. Ovulation as judged by pregnanediol assays did not occur, urinary levels of this steroid remaining consistently below 0.6 mg. per 24 hours. Oestrone readings rose gradually reaching a maximum of 9.0 ug. per 24 hours from days 22
Mrs. A.G.: AGE 48; PARA 6.0; MENOPAUSAL SYNDROME

Figure 36

Patient 3. Notation as in Figure 34
Mrs. E.F.: Age 51, Para 1+0

Menopausal Syndrome

Figure 37

Patient 4. Notation as in Figure 34.
to 24; a similar increase in oestriol output took place, the maximum value of 9.0 ug. per 24 hours being encountered just after the oestrone peak. LH levels were consistently high, ranging from 71 to 144 i.u. per 24 hours.

Comparison of results between perimenopausal and normally menstruating women

Mean values for steroid and LH excretion in normally menstruating women abstracted from the literature are shown in Table 26, while the corresponding values for the four perimenopausal subjects studied appear in Table 27. The comparison between the two groups is illustrated in Table 28. Figures which were lower in the perimenopausal women are indicated by a minus sign while those which are higher are designated by a plus; significant differences are also shown. In all four patients the mean LH output was much higher than in normally menstruating women. Patient 1 showed levels of oestrogens and pregnanediol which were essentially normal. In Patient 2 readings for oestrone and oestradiol were significantly higher than in women during reproductive life while pregnanediol values were significantly lower. In Patient 3 levels of oestrone, oestradiol and pregnanediol were reasonably normal, but the output of oestriol and "total oestrogens" was
<table>
<thead>
<tr>
<th>Substance Estimated</th>
<th>Mean/24 hours</th>
<th>No. of Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>$3.1 \pm 0.3^*$ µg.</td>
<td>27</td>
<td>Brown &amp; Matthew (1962)</td>
</tr>
<tr>
<td>Oestrone</td>
<td>$9.2 \pm 0.7$ µg.</td>
<td>27</td>
<td>Do.</td>
</tr>
<tr>
<td>Oestriol</td>
<td>$13.8 \pm 1.2$ µg.</td>
<td>27</td>
<td>Do.</td>
</tr>
<tr>
<td>&quot;Total oestrogens&quot;</td>
<td>$26.1 \pm 2.15$ µg.</td>
<td>27</td>
<td>Brown et al. (1959)</td>
</tr>
<tr>
<td>Pregnanediol</td>
<td>$1.72 \pm 0.13$ mg.</td>
<td>82</td>
<td>Klopper (1957)</td>
</tr>
<tr>
<td>LH</td>
<td>$4.3 \pm 0.56$ i.u.</td>
<td>83</td>
<td>Rosenberg &amp; Keller (1965)</td>
</tr>
</tbody>
</table>

* $\pm$ Standard error
<table>
<thead>
<tr>
<th>Patient</th>
<th>Oestrogens (µg./24 hours)</th>
<th>Pregnanediol (µg./24 hours)</th>
<th>LH (m. U./24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestradiol</td>
<td>Oestrone</td>
<td>Oestriol</td>
</tr>
<tr>
<td>1</td>
<td>2.7 ± 0.7(13)</td>
<td>8.5 ± 1.2(13)</td>
<td>13.3 ± 1.9(13)</td>
</tr>
<tr>
<td>2</td>
<td>7.5 ± 1.2 (16)</td>
<td>13.9 ± 1.5(16)</td>
<td>16.1 ± 1.6(16)</td>
</tr>
<tr>
<td>3</td>
<td>3.3 ± 0.4 (25)</td>
<td>7.6 ± 0.6(25)</td>
<td>7.3 ± 0.7(25)</td>
</tr>
<tr>
<td>4</td>
<td>7.5 ± 0.3 (15)</td>
<td>4.8 ± 0.7(15)</td>
<td>3.1 ± 0.7(15)</td>
</tr>
</tbody>
</table>

* Mean ± Standard error
+ Number of observations
<table>
<thead>
<tr>
<th>Patient</th>
<th>Oestrogens (µg./24 hours)</th>
<th>Pregnanediol (mg./24 hours)</th>
<th>LH (1.u./24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestradiol</td>
<td>Oestrone</td>
<td>Oestriol</td>
</tr>
<tr>
<td>1</td>
<td>-0.4</td>
<td>-0.7</td>
<td>-0.4</td>
</tr>
<tr>
<td>2</td>
<td>+4.4</td>
<td>+4.7</td>
<td>+2.3</td>
</tr>
<tr>
<td>3</td>
<td>+0.2</td>
<td>-1.5</td>
<td>-6.5</td>
</tr>
<tr>
<td>4</td>
<td>-1.6</td>
<td>-4.4</td>
<td>-10.7</td>
</tr>
</tbody>
</table>

* P < 0.05
** P < 0.01
*** P < 0.001
Patient 4 showed values for pregnanediol and the three classical oestrogens which were significantly lower than in normally menstruating women.

**Stimulation tests with HCG**

The results shown in Table 28 suggest that in women late in their reproductive life the reciprocal relationship normally existing between pituitary gonadotrophin production and ovarian steroid secretion was no longer operative. Accordingly, it appeared important to investigate whether in the perimenopausal group the ovaries were working at maximum capacity or were still capable of stimulation by gonadotrophic hormones. For this reason stimulation tests with HCG were conducted in one of the patients (No. 3) in whom levels of urinary oestrogens and pregnanediol indicated the presence of continuing ovarian function. The stimulation tests were performed one year after the initial investigation, it having been established that at that time the subject's oestrogen and pregnanediol output were within the range normally anticipated for a woman during reproductive life. In view of the apparently normal ovarian function it was decided that the total dosage of HCG administered to the patient should not exceed 9,000 i.u. in order to avoid the possibility of hyperstimulation.

The results of the test are shown in Figure 38.
Mrs. A.G. Age 48yrs: PARA 6+0  
Menopausal Syndrome

![Graph showing the effect of HCG on urinary steroid output in patient 3.](image)

Figure 38

*Effect of HCG on urinary steroid output in patient 3.*
Days 1 to 9 served as a control period and on day 10 3,000 i.u. HCG were given intramuscularly. This had no effect on oestrogen output and, because of this, two further injections, each of 3,000 i.u., were administered on days 15 and 16; again there was no effect on oestrogen excretion. It should, however, be noted that a rise in pregnanediol output occurred as a result of HCG administration.

**DISCUSSION**

The constant feature in the four perimenopausal patients described herein was the presence of very high levels of urinary LH throughout the period of investigation. The highest LH readings were encountered in Patients 1, 3 and 4, but even in the case of subject 2, in whom excretion values were significantly lower than in Patient 3 ($P < 0.05$) and 4 ($P < 0.01$), the mean output was still considerably higher than that reported by Rosenberg and Keller (1965) and Becker and Albert (1965) in women during reproductive life.

Furuhjelm (1966) has reported that in perimenopausal women the excretion of "total gonadotrophic activity" is often normal while Funnell et al. (1951) have stated that in menopausal subjects LH output is low. These results conflict with the observations reported in the present study and with much of the published work on
this subject. It must, however, be emphasised that the reliability criteria of the assay methods used by Furuhjelm (1966) and Funnell et al. (1951) were not entirely satisfactory and for this reason the data which they report must be accepted with reserve.

Subject 1 in the present investigation had no menopausal symptoms and had a normal chromosomal karyotype (46, XX). Nevertheless, her LH output generally remained very high throughout the period of study, suggesting that an increase in pituitary LH secretion had antedated the onset of the clinical menopause by some years; this interesting possibility is at present being further explored. A further feature to note in subject 1 was the fact that ovulation as judged by urinary steroid output occurred in association with the lowest LH levels encountered during the investigation, this being the opposite of the situation normally obtaining in women during reproductive life.

In Patient 2, who had been treated with Chlorpromazine until just before the commencement of the investigation, abnormally high LH levels coincided with oestrogen readings which were above the normal range. It appears unlikely that this pattern of hormone excretion resulted from Chlorpromazine therapy since this drug is known to depress pituitary gonadotrophic activity in animals (Barraclough and Sawyer, 1959) and
to produce signs of oestrogen deficiency in women in association with amenorrhoea and galactorrhoea (Polishuk and Kulesar, 1956).

The major finding to emerge from the present investigation is that ovulation as judged by urinary steroid output can occur in an apparently normal manner in the presence of very high LH levels. This is well illustrated by the results in Patients 1 and 3, while in Patient 2 abnormally high LH and oestrogen levels co-existed. These observations suggest that in women towards the end of their reproductive life the reciprocal relationship or "feed back" mechanism believed to exist between the pituitary and the gonads is disturbed.

The symptoms which arise during the perimenopausal period are generally treated with oestrogens either because of the alleged oestrogen deficiency or to suppress the hyperactivity of the anterior pituitary gland. From the present study none of these hypotheses can be supported. Only in the last patient (E.F. - Table 28) was a deficiency found in oestrogen output. On the other hand, on subject 2 (C.R.) oestrogen excretion was significantly higher than that found in normally menstruating women. It should be pointed out, however, that this patient had experienced the most severe symptoms of the menopause. The first (M.B.) and the third (A.G.) patients showed normal oestrogenic
It is not yet clear whether in perimenopausal women the ovaries are under maximum stimulation by the pituitary or whether they are still capable of responding to stimulation with gonadotrophic hormones. Preliminary results obtained in Patient 3 (Fig. 38) would tend to suggest the former hypothesis, but the reason for the rise in pregnanediol output in this subject in the absence of any effect on oestrogen excretion remains to be explained. The possibility exists that the total amount of HCG administered (9,000 i.u.) may not have been adequate. Gemzell et al. (1958) reported similar hormone excretion patterns (increase in pregnanediol output without any effect on oestrogen excretion values) in two amenorrhoelic women with fairly normal excretion of oestrogens who were treated with a total dose of 36,000 i.u. HCG within a period of six days. On this evidence, together with endometrial biopsy, these authors concluded that ovulation occurred following the administration of HCG. On the other hand, recently it has been shown that the ovaries of postmenopausal women, even though twenty years beyond the menopause, are still capable of functioning under the stimulatory effect of HCG and to secrete much higher levels of oestrogens than those found in normally menstruating women (Poliak et al., 1968). It should be pointed out, however, that the
total dose of HCG given by the above investigators was 50,000 i.u. within a period of 5-10 days. The latter evidence suggests that the ovaries even though of post-menopausal women are capable of responding to gonadotrophic (HCG) stimulation.

**SUMMARY**

Serial assays of urinary oestrogens, pregnanediol and LH have been performed in four subjects late in their reproductive life, and the results have been compared with those in normally menstruating women.

In all the subjects LH levels were grossly elevated being much higher than those normally encountered in women during reproductive life.

In two patients ovulation as judged by urinary steroid assays occurred normally in the presence of elevated LH readings, while in one abnormally high LH and oestrogen levels coexisted.

Stimulation test with HCG was conducted in one patient, which had no effect on oestrogen output but a rise in pregnanediol excretion occurred as a result of it.

The evidence presented suggests that in women late in reproductive life the reciprocal relationship believed to exist between pituitary gonadotrophic function and ovarian oestrogen secretion is no longer operative.
CHAPTER 2

ENDOCRINE FUNCTION IN POSTMENOPAUSAL WOMEN

INTRODUCTION

At the present time a considerable volume of literature exists with respect to endocrine function in normally menstruating women (see Loraine and Bell, 1966, 1968, for references). However, there is a great dearth of similar information in subjects beyond the menopause.

Heller and Shipley (1951), using the mouse uterus test, reported that no significant correlation could be established between urinary excretion of gonadotrophins and age. However, Heller and Heller (1939) found previously that the high gonadotrophin excretion after the menopause persisted permanently. Johnsen (1959) claimed that the mean HPG excretion was found to be higher in postmenopausal women aged from 50 to 64 than from 65 to 87 years. Albert et al. (1958), Rosenberg and Engel (1960) and Apostolakis and Loraine (1960) found high HPG activity in the urine of postmenopausal women, and the first two groups of investigators also noticed a day-to-day fluctuation.

Further attempts were made by several workers to estimate the urinary LH or FSH and LH using methods
claimed to be specific. Thus Sherman and Woolf (1959) were unable to find any increase in LH output in postmenopausal women when compared with the levels found in normally menstruating women. On the other hand, McArthur et al. (1958) and Yahia et al. (1964) found a marked increase in LH excretion during the postmenopausal period.

The aim of the present study is to report comparable data in the postmenopausal women by describing the results obtained when serial assays of LH and oestrogens in urine were conducted in four such subjects.

MATERIAL AND METHODS

Patients

Details of the women who volunteered for the study are shown in Table 29. All were normally active during the period of investigation, and with one exception (subject 2) none gave any previous history of endocrine abnormalities. Subject 2 had been treated for thyrotoxicosis by subtotal thyroidectomy at the age of 40. Since then she had been maintained on thyroxine at a dose level of 0.2 mg. per day orally; she was euthyroid at the time of the investigation.

Hormone assay methods

All subjects collected complete 24 hour urine
TABLE 29

CLINICAL DETAILS OF SUBJECTS STUDIED

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Parity</th>
<th>History</th>
<th>Years beyond the menopause</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>6+0</td>
<td>Normal</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1+0</td>
<td>Subtotal thyroidectomy for thyrotoxicosis; treated with thyroxine</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>2+1</td>
<td>Normal</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>2+0</td>
<td>Normal</td>
<td>18</td>
</tr>
</tbody>
</table>
samples throughout the period of investigation. Assays were conducted on 48 hour pools of urine, results being expressed as i.u. (2nd IRP-HMG) per 24 hour sample.

LH was extracted from urine by the tannic acid procedure (see Section A, Chapter 3). The bioassay for the hormone was the OAAD test as has been described in Chapter 2, p. 79. A total of 44 assays were performed; three assays (7%) had to be discarded due to significant deviation from parallelism between the dose response curves of standard and unknown preparations.

In three women (1, 2 and 3) estimations of the three classical oestrogens - oestrone, oestradiol and oestriol - were performed by the method of Brown et al. (1957). Pregnanediol was measured in the urine of one subject (1) using the technique of Klopper et al. (1955).

RESULTS

The results of LH and oestrogen determinations are illustrated in Figures 39, 40, 41 and 42.

The mean index of precision (± S.D.) for the LH assays was 0.18 ± 0.06.

Subject 1 (Fig. 39)

The study continued for 35 days. The pattern of excretion of oestrone and oestriol was similar to that observed in women during reproductive life with a well-defined peak at the middle of the investigation and a
Mrs. B.M. : AGE 50 : PARA 6+0 : POSTMENOPAUSAL

Figure 39

LH and steroid excretion in Subject 1
second peak for oestrone on days 31 and 32; oestradiol levels fluctuated in an irregular manner showing no consistent pattern. Pregnanediol readings remained low, and there was no evidence that ovulation had occurred.

Excretion values for LH were elevated throughout. Readings were lowest at the beginning and at the end of the study; they were at their maximum in the middle, coinciding with the peak of oestrogen output. The last three LH estimations were significantly lower than that of days 14, 15, 18 and 19 as indicated by the lack of overlap of the fiducial limits of estimated potencies.

Subject 2 (Fig. 40)

Two periods of investigation, one of 7 and the other of 22 days duration, separated by 63 days, were carried out. Oestrogen levels remained low throughout, the figure for "total oestrogens" not exceeding 10 μg. per 24 hours. LH readings were high and fluctuated in an irregular manner; the range was from 22.4 to 240.0 i.u. per 24 hours.

Subject 3 (Fig. 41)

The investigation was conducted over a period of 9 days. Levels of oestrogen remained low, the figure for "total oestrogens" not exceeding 9.0 μg. per 24 hours. LH readings were lower than in the other postmenopausal subjects ranging from 11.0 to 50.7 i.u. per 24 hours.
Mrs A.B: AGE 50 yrs: PARA 1+0

POST MENOPAUSAL

Figure 40

LH and oestrogen excretion in Subject 2
Mrs. E.T. \text{AGE 61 YRS: PARA 2+1} \quad \text{POSTMENOPAUSAL}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure41}
\caption{LH and oestrogen excretion in Subject 3}
\end{figure}
Subject 4 (Fig. 42)

As with subject 2, this woman was studied twice, the time of investigation being separated by 3½ months. The first period lasted for 7 days and the second, which is shown in Figure 42, for 22 days.

In the first study the mean (M.S.E.) total oestrogen excretion (12 estimations) was \(5.2 \pm 1.4\) μg per 24 hours (Table 30) and the corresponding figure for LH (4 estimations) was \(149 \pm 21.4\) i.u. for 24 hours. In the second study (Fig. 42) only LH assays were performed. All readings were much higher than those found in women during reproductive life. From days 1 to 14, levels ranged from 55.7 to 132 i.u. per 24 hours; however, the fiducial limits \((P = 0.95)\) of all 6 estimates overlapped, indicating the absence of any significant differences between the individual readings. The value on days 15 and 16 (300.7 i.u. per 24 hours) was significantly higher than the previous readings, the fiducial limits showing no overlap.

In Table 30 is shown the mean LH and oestrogen output in the four subjects studied and Table 31 presents a comparison of the results in postmenopausal women with those in women during reproductive life (see Chapter 1, Table 26). Figures which are lower in the postmenopausal group are indicated by a minus sign and those which are higher by a plus sign; significant differences
Mrs. E. Mc. I., AGE 63, PARA 2 + 0
POSTMENOPAUSAL

Figure 42
LH excretion in Subject 4
<table>
<thead>
<tr>
<th>Subject</th>
<th>Oestrogens (μg. per 24 hours)</th>
<th>LH (i.u. per 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestrone</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>1</td>
<td>4.3 ± 0.6* (16)</td>
<td>1.3 ± 0.3 (16)</td>
</tr>
<tr>
<td>2</td>
<td>1.4 ± 0.4 (11)</td>
<td>2.5 ± 0.6 (11)</td>
</tr>
<tr>
<td>3</td>
<td>1.6 ± 0.9 (4)</td>
<td>1.8 ± 0.9 (4)</td>
</tr>
<tr>
<td>4</td>
<td>4.0 ± 0.9 (4)</td>
<td>1.2 ± 0.8 (4)</td>
</tr>
</tbody>
</table>

* Mean ± standard error
+ Number of observations
++ Undetectable
<table>
<thead>
<tr>
<th>Subject</th>
<th>Oestrogens (μg. per 24 hours)</th>
<th>LH (i.u. per 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestrone</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>1</td>
<td>-4.9***</td>
<td>-1.8***</td>
</tr>
<tr>
<td>2</td>
<td>-7.8***</td>
<td>-0.6</td>
</tr>
<tr>
<td>3</td>
<td>-7.6***</td>
<td>-1.3</td>
</tr>
<tr>
<td>4</td>
<td>-5.2***</td>
<td>-1.9</td>
</tr>
</tbody>
</table>

* P< 0.05
** P< 0.01
*** P< 0.001
are also shown. In subject 1 the output of oestrone and oestradiol was within the range encountered in the latter group. In subjects 2, 3 and 4 excretion values for oestrone and oestriol were significantly lower, but the mean figures for oestradiol did not differ significantly from those in women during reproductive life. Mean levels of LH were significantly higher in all the postmenopausal subjects.

DISCUSSION

In the four postmenopausal women studied the overall mean LH levels (± S.E.M.) was 116.8 ± 10.8 i.u. per 24 hours. This figure is almost identical with that found in a series of four subjects studied late in their reproductive life (Chapter 1, p. 132) in whom the mean LH output was 115.6 ± 17.8 i.u. per 24 hours. The similarity of these mean values lends support to the view put forward by the author that an elevation in LH output to postmenopausal levels may precede the clinical onset of the menopause (see page 142).

At the time of writing, few reports exist in the literature in which estimates of LH, expressed in i.u., have been performed in postmenopausal subjects. Becker and Albert (1965) studied a series of six women, in a proportion of whom an oophorectomy had been performed and in the remainder the menopause had occurred.
spontaneously. A total of 8 LH assays were conducted, and, when the data were converted into i.u. in the manner described by Rosenberg and Lewis (1966), the mean value (M.S.E.) (131.2 ± 14.6 i.u. per 24 hours) was reasonably similar to that reported in the present study. In a later paper Albert and Mendosa (1966) reported the results of LH assays in 2 46-year-old patient who had been oophorectomised seven years previously, under the assumption that the LH secretion is similar to that found in postmenopausal women. It is doubtful if these two conditions are similar since it has been shown by Poliak et al. (1968) that the ovaries of postmenopausal women, even though 20 years beyond the menopause, are still capable of functioning under the stimulatory effect of HCG and to secrete much higher levels of oestrogens than those found in normally menstruating women. Also, Steinberger and Duckett (1966) have shown that FSH and LH levels in surgically induced cryptorchid and in orchietomised rats, although significantly higher than those found in intact animals, the gonadotrophin values in cryptorchid rats were significantly higher than those in orchietomised animals. The mean LH output (M.S.E.) from the data of Albert and Mendosa (1966), based on 9 estimations, was significantly lower (P<0.01) than that previously reported, being 50.1 ± 5.4 i.u. per 24 hours.
Low LH values were also found by Keller (1966) who conducted single LH determinations in each of 7 postmenopausal subjects; the mean value was 28.0 and ranged from 6.2 to 51.0 i.u. per 24 hours. The foregoing data emphasises the small number of postmenopausal patients in whom reliable estimations of LH output have so far been published and stress the necessity for further research in this field in order to establish a representative normal range of excretion values.

The LH excretion of Patient 3 (Table 31) in the present study was significantly lower when compared with the mean found either in Patient 1 ($P<0.01$) or in Patient 4 ($P<0.05$). This discrepancy cannot be explained on the basis of the age of the patient. Johnsen (1959) emphasised the importance of the subject's chronological age and noted that the mean HPG excretion was higher in patients aged from 50 to 64 than from 65 to 87 years. From his data has been extracted the mean HPG output from each group which was $209.96 \pm 23.6$ (M.S.E.) (24 observations) for the group aged 50 to 64 years and $141.7 \pm 15.2$ (M.S.E.) (20 observations) for the group aged 65 to 87. However, when these two means were compared using the student's "t" test they did not differ significantly. The only possible explanation of the low LH output found in Patient 3 is that the period studied was rather short (3 estimations in 9 days) and
so higher LH readings were missed.

In contrast to LH there is now an extensive literature on urinary oestrogen output in postmenopausal subjects (see Loraine and Bell, 1966, 1968), and it is generally agreed that the main source of oestrogen production at this time of life is the adrenal cortex. In three of the women reported herein (2, 3 and 4) oestrogen levels were within the range normally anticipated in the postmenopausal period while in the fourth (subject 1) the mean excretion was above this range (Table 30). This finding is probably explained by the fact that in subject 1 cessation of menstruation had occurred only one year before the commencement of the study, and it is therefore reasonable to expect that the ovaries as well as the adrenals were contributing to the amount of hormones excreted in the urine.

It is not yet known if the human pituitary ages. Bahn et al. (1953), using interstitial-cell repair in hypophysectomised female rat test, estimated the human pituitary LH, and they found that the concentration in postmenopausal was similar to that found in males or in menstruating females. Ryan (1962) claimed that he found, using the OAAD test, an increase in LH concentration in the anterior pituitary of females with age. From the data that Ryan gave, this increase is not proven to be significant due to the overlap of the
fiducial limits of the estimated potencies.

From the present study as well as from the findings of Poliak et al. (1968) and from the findings in the perimenopausal group (Chapter 1, p. 132) there is evidence to support the view that in women it is the ovary that ages. The anterior pituitary probably works under its maximum capacity but is unable to stimulate ovarian function.

**SUMMARY**

Serial assays of LH and oestrogens have been performed in the urine of four postmenopausal women.

The mean LH output in individual cases was variable ranging from $34.6 \pm 13.6$ to $144.7 \pm 16.9$ i.u. per 24 hours; the corresponding figures for "total oestrogens" ranged from $4.5 \pm 2.2$ to $16.1 \pm 2.5$ ug. per 24 hours.

A significant difference in LH excretion was not noted between the postmenopausal subjects and a series of four women studied late in their reproductive life.
CHAPTER 3

OBSERVATIONS ON THE EFFECT OF ORAL CONTRACEPTIVES ON EXCRETION VALUES FOR LUTEINISING HORMONE

INTRODUCTION

It is now generally agreed that progestogen-oestrogen mixtures, when administered in the "classical form" on a short term basis, obliterate the midcycle peak of LH excretion and produce anovulatory cycles as judged by the pattern of oestrogen and pregnanediol output (see Table 33 for references). When given as long term therapy these compounds also inhibit ovulation on the basis of steroid excretion; however, their effects on the excretion of follicle-stimulating hormone (FSH) and luteinising hormone (LH) have been investigated to a much more limited extent and may well be dependent on such factors as the composition of the tablet, its dosage and the duration of administration (see Bell et al., 1967).

The aim of the present study is to contribute to this field by reporting results of serial assays performed in five patients treated with oral contraceptive drugs. Four of them were on long term therapy, one was treated with a progestogen-oestrogen mixture, another with a "pure" progestogen and in the remaining
two women a sequential form of contraception was administered. In the fifth patient the short term effects on endocrine function of a progestogen given continuously in low dosage was studied.

MATERIALS AND METHODS

A. Patients

Clinical details of the women studied together with the medication given are shown in Table 32. All compounds were administered by the oral route.

Subject A, Mrs K.D., aged 26 years, Para 3+0

The menarche occurred at 13 years of age and since then her menstruation has been rather regular (4-6/27-30). Previous birth control was restricted to the use of the rhythm method.

She was treated with Ovulen (G.D. Searle & Co.), each tablet containing 1.0 mg. of 17a-ethinylestrone-3-17-diol diacetate (ethynodiol diacetate) plus 0.1 mg. ethinyl oestradiol-3-methyl-ether (mestranol). During the period of time when she was taking this preparation her legs tended to swell and during the time of study she developed a deep venous thrombosis.

Subject B, Mrs M.M., aged 42 years, Para 7+1

The menarche occurred at 13 years of age and until therapy with oral contraceptives she menstruated regularly every 28 days, the duration of the bleeding
<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Parity</th>
<th>Previous Menstrual History</th>
<th>Compound administered</th>
<th>Side effects during treatment</th>
<th>Total Duration of Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Years</td>
<td></td>
<td>Duration of cycles (days)</td>
<td>Duration of bleeding (days)</td>
<td></td>
<td>Calendar months</td>
</tr>
<tr>
<td>A</td>
<td>26</td>
<td>3 + 0</td>
<td>27 - 30</td>
<td>4 - 6</td>
<td>Ovulen</td>
<td>48</td>
</tr>
<tr>
<td>B</td>
<td>42</td>
<td>7 + 1</td>
<td>28</td>
<td>5 - 6</td>
<td>Norethisterone acetate</td>
<td>67</td>
</tr>
<tr>
<td>C</td>
<td>32</td>
<td>3 + 0</td>
<td>27 - 30</td>
<td>4 - 5</td>
<td>Mestranol and Ethynodiol diacetate</td>
<td>20</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>2 + 1</td>
<td>28 - 31</td>
<td>6 - 7</td>
<td>Mestranol and Chlormadinone acetate</td>
<td>12</td>
</tr>
<tr>
<td>E</td>
<td>37</td>
<td>3 + 0</td>
<td>28</td>
<td>5</td>
<td>Chlormadinone acetate</td>
<td>None noted</td>
</tr>
</tbody>
</table>
being 5-6 days.

The oral contraceptive, 17α-ethinyl-19-nortestosterone acetate (norethisterone acetate), had been taken for 67 calendar months. During the first three cycles she took 5 mg. norethisterone acetate daily, but this was subsequently reduced to 2.5 mg. daily.

Since the administration of this oral contraceptive the patient's cycles had become irregular. The duration of her periods varied and the menstrual flow was generally reduced. Withdrawal bleeding did not occur after every treatment cycle, the longest period of amenorrhoea being 2½ months. Side effects during therapy included headaches and increase in weight.

Subject C, Mrs W.D., aged 32 years, Para 3 + 0

The menarche occurred at 12½ years of age. Menstruation was rather regular; bleeding lasted from 4 to 5 days and the duration of the cycles was from 27 to 30 days. She received a sequential preparation consisting of 10 tablets, each containing 0.1 mg. mestranol, followed by another 10 tablets containing, in addition to 0.1 mg. mestranol, 0.5 mg. ethynodiol diacetate. Virtually no side effects were noted during the 20 calendar month period of treatment apart from slight weight gain and occasional headaches.

Subject D, Mrs M.J., aged 35 years, Para 2 + 1

The menarche occurred at 11 years of age. The
length of the cycles was from 28 to 31 days and the duration of menstruation was from 6 to 7 days. Thirty months prior to the present study the patient was fitted with an intra-uterine device but had been able to wear this for 5 months only; it was removed because of heavy and painful periods and intermenstrual bleeding. Subsequently, and for 13 months, she was under treatment with a mixture of 19-nortestosterone and ethinyl oestradiol (Gynovlar). For the last 12 months until the present investigation the patient was taking a sequential treatment. This regime consisted of 15 tablets each containing 0.08 mg. mestranol followed by 5 tablets each containing a mixture of 0.08 mg. mestranol plus 2.0 mg. 17α-acetoxy-6-chloropregna-4,6-diene-3,20-dione (chlormadinone acetate). The menstrual periods of the patient became moderate while on Gynovlar and remained so while on sequential treatment. Side effects during therapy included headaches, which were at their worst during the days she was not on treatment, and increase in weight.

Subject E, Mrs M.B., aged 37 years, Para 3 + 0

The menarche occurred at 14½ years of age. Menstruation was regular, the duration of the cycles being 28 days with bleeding lasting 5 days. The patient had not previously received oral contraceptives and she started treatment with 0.5 mg. chlormadinone acetate
daily during the second cycle of the present investigation.

**General Design of Investigation**

All five women collected complete 24 hour urine samples throughout the present investigation; episodes of bleeding were also recorded.

**Subject A (Fig. 43):** Hormone assays were performed during the 52nd cycle on oral contraception and in one cycle immediately following withdrawal of medication.

**Subject B (Fig. 44):** The hormonal study started on the 67th month of medication and continued for 55 days after cessation of therapy. It should be noted that bleeding did not occur following the last treatment cycle.

**Subject C (Fig. 45):** This patient received a 10/10 sequential regime in which mestranol was given alone starting on the 5th day of the cycle, this being followed by combined treatment with ethynodiol diacetate and mestranol. Hormone assays were performed during the 24th cycle on medication and in two succeeding cycles.

**Subject D (Fig. 46):** The form of contraception administered to this patient was a 15/5 sequential regime in which mestranol was given alone starting on the first day of the cycle, this being followed by
combined therapy by chlormadinone acetate and mestranol. Assays were conducted during the 14th cycle on treatment and in the succeeding cycle after cessation of medication.

Subject E (Fig. 47): The aim of this study was to investigate the short term effect of chlormadinone acetate on the urinary hormone levels. The hormone assays were conducted on two cycles. The first served as the control period and the second was under the effect of the administered low dose progestogen (0.5 mg daily).

B. Methods

Assays were conducted on 24, 48 or 72 hour pools of urine and the results were expressed as i.u. (2nd IRP-HMG) per 24 hour sample.

LH was extracted from the urine by the tannic acid procedure (see Section A, Chapter 3). In subjects A, B, C and D the bioassay method used was the VPW test described in Section A, Chapter 1. A total of 120 assays were performed; 7.5 per cent of these were invalid because of incomplete hypophysectomy and 13.3 per cent were invalid due to significant deviation from parallelism between the slopes of the dose-response curves of S and U. In the assays in subject A only one dose level of the unknown fell within the working range of the dose-response curve of the S, making it necessary
to calculate the results on a three point basis. The
mean index of precision ($\lambda \pm S.D.$) of the VPW assays was
0.13 ± 0.05.

In subject E, LH was assayed by the OAAD test (see
page 79). A total of 30 assays were performed; 20
per cent of these were invalid either because of signifi-
cant deviation from parallelism between the slopes of the
dose-response curves of standard and unknown preparations
(16 per cent) or due to lack of slope of the standard of
reference (4.0 per cent). The mean index of precision
($\lambda \pm S.D.$) was 0.22 ± 0.07.

In subject E, estimations of the three classical
oestrogens (i.e. oestrone, oestradiol, oestriol) were
performed by the technique of Brown et al. (1957) and in
all five subjects pregnanediol was measured by the
method of Klopper et al. (1955).

RESULTS

Subject A (Fig. 43)

The study continued for a period of 70 days.
During the first 20 days, which included the last course
of treatment, excretion values for pregnanediol ranged
from 0.45 to 1.5 mg. per 24 hours, which might indicate
absence of ovulation. The third day after the cessation
of medication menstruation occurred which lasted 5 days;
two days later bleeding reappeared and lasted for 3 days.
Figure 43

Hormone excretion in Subject A
In the post-treatment cycle, which lasted for 46 days, the marked increase in pregnanediol readings, maximal from days 60 to 62 of the study, indicated that ovulation had taken place.

In both cycles urinary LH was consistently low throughout the whole period of investigation. Values ranged from 1.7 to 4.5 i.u. per 24 hours. The fiducial limits of individual estimations overlapped, indicating the absence of any significant difference between them.

Subject B (Fig. 44)

The period of investigation was 55 days. From day 4 to 23 norethisterone acetate was administered; following cessation of therapy bleeding did not occur. The treatment cycle was anovular on the basis of urinary pregnanediol assays. LH levels at this time were low or undetectable with the exception of a peak of activity of 32.6 i.u. per 24 hours on days 20 and 21.

In the post-treatment period, which lasted for 32 days, the pattern of pregnanediol output was typical of an ovulatory cycle. However, it should be noted that the highest level for this steroid (1.1 mg. per 24 hours from days 47 to 49) was still below the range encountered in the luteal phase of the normal menstrual cycle. LH readings during the post-treatment cycle remained low from days 24 to 32; thereafter, they rose, the highest reading being 26.7 i.u. per 24 hours between days 37 and 38.
Figure 44

Hormone excretion in Subject B
Subject C (Fig. 45)

The last cycle on treatment with a sequential 10/10 regime was anovular as judged by urinary pregnanediol assays. At the commencement of the study, when mestranol was administered alone, LH levels were above the range normally encountered in women during reproductive life; when ethynodiol diacetate was added to the sequential regime a significant fall in LH output occurred.

The remainder of the study consisted of two ovulatory cycles as judged by urinary pregnanediol assays. LH readings during the first post-treatment cycle ranged from 14.4 to 37.5 and in the second from 16.6 to 22.7 i.u. per 24 hours. However, the fiducial limits of all 21 estimates overlapped, indicating the absence of any significant difference between the relative potencies obtained.

Subject D (Fig. 46)

The first 20 days of the investigation consisted of the last treatment period of a sequential regime consisting of mestranol and chlormadinone acetate on a 15/5 regimen. The cycle was anovulatory on the basis of pregnanediol assays. LH output was not suppressed by the medication, levels being at the upper end of the normal range for women during reproductive life. It should, however, be noted that a midcycle peak of LH
Figure 45

Hormone excretion in Subject C
Mrs. M.J. Age 35 years: PARA 2+1

ORAL CONTRACEPTION

Figure 46

Hormone excretion in Subject D
activity was not present.

Following cessation of treatment an ovulatory cycle ensued, this being characterised by a luteal phase rise in pregnanediol output. Menstruation commenced on day 24 and lasted for 6 days. For the first 11 days of the post-treatment cycle LH levels remained low; thereafter a midcycle peak occurred from days 37 to 41. Following this peak there was a marked fall in LH output during the luteal phase, the last four readings all being below 4 i.u. per 24 hours.

Subject B (Fig. 47)

The duration of the study was 61 days. It included one control cycle and a period of 32 days during which therapy with chlormadinone acetate was administered.

In the pretreatment cycle ovulation as judged by oestrogen output probably occurred on days 15 and 16; this was followed by a luteal phase rise in excretion values for oestrone, oestradiol and pregnanediol. LH readings were grossly elevated for a woman during reproductive life. Furthermore, the pattern of excretion of this hormone was atypical, readings being high at the beginning of the cycle, low at midcycle and high again in the late luteal phase. It should be noted that, in contrast to the situation pertaining in normally menstruating women, ovulation occurred at the time of the lowest LH values.
Figure 47

Hormone excretion in Subject E
During the treatment period the pattern of oestrogen excretion was somewhat atypical, but the readings were in the same range as those encountered in normally menstruating women. Pregnanediol readings remained low ranging from 0.55 to 1.5 mg. per 24 hours, and on the basis of the urinary steroid assays a definite conclusion as to whether or not ovulation had occurred could not be reached. LH output in the treatment cycle showed a grossly irregular pattern, levels generally being considerably elevated for a woman during reproductive life.

DISCUSSION

The first four subjects in the present investigation received long term therapy with oral contraceptives. In three of these (A, C and D, Figures 43, 45 and 46) ovulation as judged by urinary pregnanediol assays occurred in the first post-treatment cycle. In the fourth (B, Fig. 44) pregnanediol readings in the post-treatment period were low, but the pattern of excretion of this steroid was typical of the ovulatory cycle. Klopper (1957) found that during the luteal phase of an ovulatory cycle, maximal urinary pregnanediol levels ranged from 2.5 to 6.2 mg. per 24 hours. However, in view of the demonstration by Erb and Ludwig (1965) and Østergaard and Starup (1968) that, in subjects treated with oral contraceptives, at laparotomy fresh corpora lutea
indicative of ovulation can coexist with urinary pregnanediol levels of less than 1 mg. per 24 hours, it appears reasonable to assume that in subject B (Fig. 44) ovulation may have taken place. Accordingly, the overall results reported herein indicate the rapid resumption of ovulatory cycles following cessation of long term medication with oral contraceptives which are similar to those previously reported by others (Loraine et al., 1963, 1965; Bell et al., 1967).

The present study has failed to provide any definite evidence that such long term therapy produces any marked effect on urinary LH output. Thus, in patient A, who was treated with the progestogen-oestrogen mixture, Ovulen, LH readings in both the treatment and post-treatment cycles were within the normal range for women during reproductive life (see Rosenberg and Keller, 1965). Furthermore, the fiducial limits of the assays in the treatment cycle overlapped completely with those in the post-treatment cycle, indicating the lack of any significant difference between individual readings. In subject B, who was treated with a "pure" 19-nor-testosterone compound (norethisterone acetate), 4 of the 5 LH assays performed in the treatment cycle were low and one was at the upper end of the normal range; following withdrawal of medication, readings in the immediate post-treatment period were low and thereafter showed a
significant increase.

In the two women who received long term sequential therapy pituitary gonadotrophic function as judged by urinary LH assays was not inhibited, LH levels in the last treatment cycle of patient C being generally above and those in patient D being within the normal range for women during reproductive life. In neither case did the readings in the treatment and post-treatment cycles differ markedly, the only point of note being the occurrence of a midcycle LH peak in the post-treatment but not in the treatment cycle of subject D. This discrepancy on the LH readings in subjects C and D might well be due to the different gestagens administered as well as to the different ratio of oestrogen to the gestagen in the two preparations used. Also this might well be due to the different regimen of sequential contraception employed in the two patients studied (10/10 in subject C and 15/5 in subject D).

Vorys et al. (1965) and Stevens and Vorys (1967) reported a composite normal cycle for FSH and LH. They also attempted to investigate the effect of various oestrogens, gestagens and the combination of both on the urinary excretion values for FSH and LH. By comparing their results with the composite cycle they found that gestagens in sufficient dosage exhibit a suppressive effect on urinary LH excretion, the exception being
norgestrol which has a stimulatory effect. They also claimed that oestrogens are stimulants of LH output and they concluded that the effect of oral contraceptives on urinary LH is dependent on the ratio of the activity of the oestrogen and gestagen in the tablets. It should, however, be pointed out that

(1) each compound was generally tested in one subject only;

(2) the quantitative bioassays for FSH and LH have a wide variation of normal range and so the effect of any treatment must be compared with a control period from the same individual and not with an artificial composite cycle; and

(3) in the measurement of gonadotrophins by bioassay, the fiducial limits of the relative potencies often overlap. Under such circumstances the estimates, although perhaps widely separated, should not be regarded as significantly different.

In Tables 33, 34, 35 and 36 is presented a summary of the literature on the effects of the "classical", "sequential" and "pure" gestagen forms of contraceptives on urinary LH output. With the exception of the papers by Flowers et al. (1966), Ryan et al. (1966) and Bell et al. (1967), short term effects on LH excretion are described. As can be seen in these Tables, the composition and dosage of the material administered have varied
<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of patients</th>
<th>Oestrogen</th>
<th>Daily dose (mg.)</th>
<th>Progestogen or 19-nor-compound</th>
<th>Daily dose (mg.)</th>
<th>Effect on LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown, P.S. et al.</td>
<td>1</td>
<td>Mestranol</td>
<td>0.075</td>
<td>Norethynodrel</td>
<td>5.0</td>
<td>Midcycle peak abolished</td>
</tr>
<tr>
<td>(1964)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stevens et al.</td>
<td>2</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Ethynodiol diacetate</td>
<td>0.5</td>
<td>Midcycle peak abolished</td>
</tr>
<tr>
<td>(1965)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Ethynodiol diacetate</td>
<td>1.0</td>
<td>Midcycle peak abolished</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Ethynodiol diacetate</td>
<td>2.0</td>
<td>Midcycle peak suppressed</td>
</tr>
<tr>
<td>Vorys et al.</td>
<td>2</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Ethynodiol diacetate</td>
<td>2.0</td>
<td>Midcycle peak abolished**</td>
</tr>
<tr>
<td>(1965)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flowers et al.</td>
<td>4</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Ethynodiol diacetate</td>
<td>0.5</td>
<td>Stimulation**</td>
</tr>
<tr>
<td>(1966)++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaiser et al.</td>
<td>1</td>
<td>Ethinyl oestradiol</td>
<td>0.05</td>
<td>Norethisterone acetate</td>
<td>4.0</td>
<td>Midcycle peak suppressed</td>
</tr>
<tr>
<td>(1966)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ryan et al.</td>
<td>2</td>
<td>Mestranol</td>
<td>0.075</td>
<td>Norethynodrel</td>
<td>5.0</td>
<td>Midcycle peak abolished**</td>
</tr>
<tr>
<td>(1966)++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bell et al.</td>
<td>1</td>
<td>Ethinyl oestradiol</td>
<td>0.05</td>
<td>Megestrol acetate</td>
<td>4.0</td>
<td>Suppression</td>
</tr>
<tr>
<td>(1967)++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>No. of patients</td>
<td>Oestrogen</td>
<td>Daily dose (mg.)</td>
<td>Progestogen or 19-nor-compound</td>
<td>Daily dose (mg.)</td>
<td>Effect on LH</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------</td>
<td>-------------</td>
<td>------------------</td>
<td>--------------------------------</td>
<td>------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Schmidt-Elmendorff et al. (1967)</td>
<td>5</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Lynestrenol</td>
<td>0.5</td>
<td>Midcycle peak abolished*</td>
</tr>
<tr>
<td>Starup &amp; Lebech (1967)</td>
<td>6</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Megestrol acetate</td>
<td>5.0</td>
<td>Midcycle peak abolished in 4 of 6 cases</td>
</tr>
<tr>
<td>Stevens &amp; Vorys (1967)</td>
<td>2</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Norethynodrel</td>
<td>2.5</td>
<td>Suppression*</td>
</tr>
<tr>
<td>Orr &amp; Elstein (1968)</td>
<td>Not stated</td>
<td>Mestranol</td>
<td>0.075</td>
<td>Norethynodrel</td>
<td>5.0</td>
<td>Midcycle peak abolished +</td>
</tr>
<tr>
<td></td>
<td>Not stated</td>
<td>Mestranol</td>
<td>0.1</td>
<td>Ethynodiol diacetate</td>
<td>1.0</td>
<td>Midcycle peak abolished +</td>
</tr>
</tbody>
</table>

* No control cycle.
+ It is not reported if a control cycle was studied.
++ Long term effect.
### TABLE 34

**EFFECT OF "SEQUENTIAL" FORM OF CONTRACEPTION ON URINARY LH EXCRETION**

<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of patients</th>
<th>Oestrogen</th>
<th>Daily dose (mg.)</th>
<th>Progestogen or 19-nor-compound</th>
<th>Daily dose (mg.)</th>
<th>Regimen</th>
<th>Effect on LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaiser et al. (1966)</td>
<td>5</td>
<td>Ethinyl oestradiol</td>
<td>0.05</td>
<td>Medroxyprogesterone acetate</td>
<td>5.0</td>
<td>10/10</td>
<td>No effect</td>
</tr>
<tr>
<td>Stevens &amp; Vorys (1967)</td>
<td>1</td>
<td>Mestranol</td>
<td>0.08</td>
<td>Chlormadinone acetate</td>
<td>2.0</td>
<td>15/5</td>
<td>Stimulation*</td>
</tr>
<tr>
<td>Do.</td>
<td>1</td>
<td>Ethinyl oestradiol</td>
<td>0.05</td>
<td>Norethindrone acetate</td>
<td>1.0</td>
<td>10/10</td>
<td>Suppression*</td>
</tr>
</tbody>
</table>

* No control cycle.
<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of patients</th>
<th>Compound</th>
<th>Daily dose (mg.)</th>
<th>Effect on LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taymor (1964)</td>
<td>3</td>
<td>Norethisterone</td>
<td>2.5</td>
<td>Midcycle peak abolished in two patients,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetate</td>
<td>5.0</td>
<td>no effect in the third*.</td>
</tr>
<tr>
<td>Yorys et al. (1965)</td>
<td>1</td>
<td>Ethynodiol diacetate</td>
<td>2.0</td>
<td>Midcycle peak abolished**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Norgestrol</td>
<td>2.5</td>
<td>Stimulation**</td>
</tr>
<tr>
<td>Bell et al. (1967)</td>
<td>1</td>
<td>Norethisterone</td>
<td>10.0</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schmidt-Elmendorff et al. (1967)</td>
<td>5</td>
<td>Lynestrenol</td>
<td>0.5</td>
<td>No effect+</td>
</tr>
<tr>
<td>Stevens &amp; Vorys (1967)</td>
<td>2</td>
<td>Ethynodiol diacetate</td>
<td>0.25</td>
<td>No effect**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
<td>No effect**</td>
</tr>
<tr>
<td>Poss et al. (1968)</td>
<td>3</td>
<td>Norgestrol</td>
<td>0.05</td>
<td>No effect in two patients. In the third,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>midcycle peak abolished?**</td>
</tr>
</tbody>
</table>

* Serial assays during the midportion of three successive cycles.

** No control cycle.

+ It is not reported if a control cycle was studied.
<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of patients</th>
<th>Compound</th>
<th>Daily dose (mg.)</th>
<th>Effect on LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stevens &amp; Vorys</td>
<td>1</td>
<td>Medroxyprogesterone acetate</td>
<td>150.0</td>
<td>Suppression</td>
</tr>
<tr>
<td>(1967)</td>
<td></td>
<td>(single dose intramuscularly)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orr &amp; Elstein</td>
<td>8</td>
<td>Chlormadinone acetate</td>
<td>0.5</td>
<td>No effect*</td>
</tr>
<tr>
<td>(1968)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* It is not reported if a control cycle was studied.
considerably from one patient to another, and, in general, a very small number of women have been treated with the same compound. Secondly, in a high proportion of the studies a control period either before or following the administration of the contraceptive was not incorporated, and in the absence of such a period it is a matter of considerable difficulty to determine whether the compound produces any significant effect on LH activity.

At the time of writing, little information exists regarding the mode of action of gestagen compounds, administered continuously in low dosage, on endocrine function. In the subject reported herein (E) chlormadinone acetate did not exhibit any significant short term effect on either LH or oestrogen output. However, the pattern of pregnanediol excretion in the treatment cycle was atypical, levels being less than 2 mg. per 24 hours throughout.

For the time being the mode of action of contraceptive drugs remains a difficult and complex subject for investigation. The effects of seemingly closely related compounds often appear to vary and the possible sites of action for the various agents may also be numerous. Systematic large scale studies on the various effects of contraceptive drugs will contribute to a better understanding of the mode of action of these compounds.
SUMMARY

Serial assays of luteinising hormone (LH) and pregnanediol in urine have been performed in four women during and following long term therapy with oral contraceptives. One subject was treated with a progestogen-oestrogen mixture, another with a progestogen alone and two with a sequential regime. In three subjects there was definite evidence for the resumption of ovulation in the first post-treatment cycle; in the fourth the evidence was less clear cut.

None of the patients showed significant suppression of urinary LH activity as a result of medication, and in all four the fiducial limits of individual assays in the treatment and post-treatment cycles overlapped to a considerable extent. One woman receiving sequential therapy showed a midcycle LH peak in the post-treatment but not in the treatment cycle.

In one subject the short term effect of chlormadinone acetate administered continuously in low dosage was investigated. The compound produced no significant effect on either LH or oestrogen output, but may have inhibited ovulation as judged by urinary pregnanediol assays.
CHAPTER 4

STUDIES ON THE MECHANISM OF ACTION OF CLOMIPHENE IN WOMEN WITH SECONDARY AMENORRHEA

INTRODUCTION

Clomiphene citrate (1-p-(diethylaminoethoxy)phenyl)-1,2-diphenyl-2-chlorethylene) is a member of the trieneethylene series of compounds to which belongs also chlorotrianisene (TACE), a potent oestrogen. Structurally, the compound bears some resemblance to stilbestrol (Fig. 48).

The effects of clomiphene in animals were first investigated by HoltKamp et al. (1961). The compound showed marked antifertility properties in female rats, being capable of preventing conception and subsequent pregnancy. The experiments of Segal and Nelson (1961) demonstrated that clomiphene reduced the sperm count and decreased fertility in male rats.

Because of its contraceptive potential, the agent was submitted to clinical trial and unexpectedly an interesting biologic activity in the field of reproductive physiology was encountered. The striking differences of biologic activity in the rat and in the human soon became evident in studies of Greenblatt and his colleagues (1961). This nonsteroidal agent could
Figure 46

Structural formulae of Stilbestrol, TACE and Clomiphene.
stimulate ovulation in a proportion of women in whom this event normally failed to occur. This activity was also shown by a compound, MER-25 (Tyler et al., 1960; Kistner and Smith, 1961), but, unlike clomiphene, proved to be too toxic for human use and was withdrawn from clinical usage.

At the time of writing and despite recent intensive studies (see Table 37 for references), neither the site nor the mode of action of clomiphene is known with certainty. Unfortunately a high proportion of our knowledge is based on the results of animal experiments carried out mostly in rodents. The completely contrary effects of the drug in humans and animals limit the usefulness of animal experiments and makes extrapolation to the human situation extremely hazardous.

Some investigators have suggested that the compound acts directly on the gonads while others believe that the effects are produced via the anterior pituitary and/or hypothalamus and some claimed that clomiphene has a suppressive effect on the anterior pituitary gland (Table 37).

The aim of the present study is to contribute to this field by reporting the results of serial LH assays in the urine of five women suffering from secondary amenorrhoea and treated with clomiphene.
MATERIALS AND METHODS

A. Patients

Patient 1. Miss H.H., aged 23 years, para 0 + 0

The menarche occurred at 12 years of age and she menstruated regularly (3/28) until the age of 19. At this time she noticed breast enlargement and galactorrhoea. She was given stilbesterol (1 mg./day) for periods of 20 days each month for eight months. There was no improvement in her mammary discomfort and at the end of this treatment she developed secondary amenorrhoea for 7 months. The patient was then treated with Enovid, 5 mg. daily for 20 days monthly for five months, which resulted in withdrawal bleeding but, after the cessation of treatment, amenorrhoea reappeared. A further course of treatment with norethisterone acetate (Norlutin-A) also resulted in withdrawal bleeding but amenorrhoea followed the cessation of treatment. On examination, except for evidence of galactorrhoea, there was no stigmata of any endocrine disturbance. Also there was no evidence to suspect the presence of a pituitary tumour since her vision was normal and, on examination, no defect could be discerned as regards the visual fields.

The present investigation started 8 months after her last bleeding.
Patient 2. Mrs W.P., aged 30 years, para 2 + 0

The menarche occurred at 13½ years of age. Until the age of 29, menstruation was regular (5/28) and she had two successful pregnancies. Over the past three years menstruation became irregular but amenorrhoea never persisted for more than two months. One year prior to the investigation the patient had been under treatment with Enovid (5 mg.) for five months, for family planning reasons, which resulted in the development of secondary amenorrhoea followed by some mammary secretion. Vaginal cytology at this period showed low oestrogen effects with a cornification index of less than 10%. On examination the pelvic organs were normal and, apart from mammary secretions and the secondary amenorrhoea, she did not show any endocrine abnormality. Ten months after the appearance of amenorrhoea the patient was treated with clomiphene.

Patient 3. Mrs M.G., aged 41 years, para 2 + 0

The menarche occurred at 13 years of age and she menstruated regularly (4-5/29) until the age of 37 years. She had two successful pregnancies but at the time of her second pregnancy, when she was 28 years of age, she suffered a severe postpartum haemorrhage, although menstruation returned to normal within the normal period of time. When she was 37 years of age, menstruation ceased and there was a progressive loss of libido. She
gradually developed symptoms of hypothyroidism and was treated with thyroxine (0.2 mg./day). This resulted in alleviation of her symptoms and her libido returned, although amenorrhoea persisted. Four years after the development of amenorrhoea the patient was treated with clomiphene because of her desire for a further conception.

**Patient 4. Mrs E.T., aged 25 years, para 0 + 0**

This patient had been married five years without any children. Her menarche occurred at 12 and she had regular periods for approximately one year, which was then followed by amenorrhoea for a year, and then she menstruated about every 9 months. When she was 22 years old a wedge resection of the ovaries confirmed clinical findings that indicated she suffered from Stein-Leventhal syndrome. The patient at this time showed hirsutism of face and abdomen. Three years following the wedge resection the patient was treated with clomiphene.

**Patient 5. Mrs I.E., aged 25 years, para 0 + 0**

Her menarche occurred when she was 18 years of age. She menstruated irregularly for about 5 years and subsequently became amenorrhoeic. Her buccal smear was normal, and in these circumstances it was not justifiable to proceed to blood culture for karyotype analysis.

Two years after the development of amenorrhoea the patient was treated with clomiphene.
B. Methods

All five patients collected complete 24-hour urine samples throughout the period of investigation. Assays were conducted on 48-hour pools of urine, results being expressed as i.u. (2nd IRP-HMG) per 24 hours.

LH was extracted from the urine by the tannic acid procedure of Johnsen (1958) (see page 92). In subjects 1, 2, 3 and 4, the bioassay used was the OAAD test (see page 79). A total of 81 assays were performed; 38.3% of these were invalid either because of significant deviation from parallelism between the slopes of the dose-response curves of standard and unknown preparations (22%) or due to lack of standard slope (16.3%). The mean index of precision (λ ± S.D.) was 0.20 ± 0.08. In patient 5 LH was assayed by the VPW test (see page 51). A total of 38 assays were performed; 5.3% of these were invalid because of incompleteness of hypophysectomy in the test animals. The mean index of precision (λ ± S.D.) was 0.14 ± 0.04.

In patient 4 during the presumptive period of gestation instead of LH, estimations of HCG were performed by the method of Loraine (1950), based on the increase in total prostatic weight in intact immature rats. The mean index of precision (λ ± S.D.) was 0.14 ± 0.04. Results for HCG were expressed in terms of the Second International Standard for HCG as i.u. per
24 hours.

All bioassays were of a symmetrical 4-point design in which two dose levels of the standard were compared with two dose levels of the test preparation. In a proportion of the OAAD assays only one dose level of the unknown fell within the working range of the dose-response curve of the standard, making it necessary to calculate the results on a three-point basis.

In patients 1, 3, 4 and 5 estimations of the three classical oestrogens, oestrone, oestradiol and oestriol, were carried out by the technique of Brown et al. (1957) while in patients 2, 3, 4 and 5 pregnanediol was measured by the method of Klopper et al. (1955).

RESULTS

Patient 1 (Fig. 49)

The investigation extended over a period of 25 days. Clomiphene was administered orally from day 5 to 9 at a dose-level of 100 mg. per day. Oestrogen levels before treatment were within the postmenopausal range; clomiphene caused a rise in oestrogen excretion, the increase in oestradiol being maximal during therapy and that of oestrone and oestriol maximal 4 days following withdrawal of medication. Thereafter urinary oestrogens output fell and from day 17 of the study readings were in the same range as those encountered during the control
Figure 49

The effect of Clomiphene on hormone excretion in Patient 1.
LH values ranged from 2.7 - 10.7 i.u. per 24 hours and the mean was 5.7 ± 0.8 (M.S.E.) i.u. per 24 hours. These values are within the range encountered in women during the reproductive life. The fiducial limits ($P = 0.95$) of individual estimates overlapped indicating the absence of a significant difference between any of the values; it was considered that clomiphene had no effect on LH excretion levels. The treatment did not produce an ovulatory cycle and menstruation failed to occur.

**Patient 2 (Fig. 50)**

The study continued for a period of 20 days. Clomiphene was administered at a dose-level of 100 mg. per day from days 3 to 8 inclusive. Pregnanediol levels were all low being within the postmenopausal range. The drug had no effect on LH excretion, readings of this hormone being consistently very low ranging from 1.0 to less than 3.2 i.u. per 24 hours. The mean LH output (M.S.E.) was 1.87 ± 0.55 i.u. per 24 hours. The fiducial limits ($P = 0.95$) for all the LH assays overlapped indicating that the individual values did not differ significantly.

**Patient 3 (Fig. 51)**

The duration of the investigation was 90 days. After the initial control period of 5 days, clomiphene
Mrs W.P: AGE 30 years, PARA 2+0

SECONDARY AMENORRHOEA
CLOMIPHENE TREATMENT

Figure 50
The effect of Clomiphene on hormone excretion in Patient 2.
was administered orally at a dose-level of 50 mg. b.i.d. per day for 5 days. Thirty-one days later another treatment of clomiphene was given again for 5 days but the dose-level was 100 mg. b.i.d. daily.

During the control period excretion values for oestrogens, pregnanediol and LH were all within the range found in postmenopausal women. The first course of treatment with clomiphene did not show any effect on endocrine function. Thirteen days after the cessation of treatment, levels of oestrone and oestriol rose gradually reaching a maximum on days 29-30 and 35-36 respectively. Thereafter they fell slightly. The second course of clomiphene was associated with a marked rise in oestrone and oestradiol output; readings of 12.2 and 6.7 µg. per 24 hours were noted respectively on days 45 and 46. Following withdrawal of medication oestrogens fell to levels found in postmenopausal women.

Pregnanediol values were consistently low throughout the period studied, readings being similar to those found during the control period.

LH levels were very high, being within the range found in postmenopausal women. The mean LH output (M.S.E.) was 75.9 ± 42.9 i.u. per 24 hours. This value does not differ significantly from the mean level found in perimenopausal and postmenopausal women. However, the fiducial limits for all the LH assays overlapped
Mrs. M.G., Age 41 Years
Para 2+0

Secondary Amenorrhoea
(Clomiphene Treatment)

Figure 51

The effect of Clomiphene on hormone excretion in
Patient 3.
indicating that the individual values did not differ significantly. It was considered that clomiphene did not exert any significant effect on LH excretion.

Patient 4 (Fig. 52)

This study extended over a period of 70 days. During the control period, which lasted for 7 days, oestrogen levels were within the range encountered in normal women during the reproductive life. On days 1 to 3 of the study pregnanediol values were high (3.74 mg./24 hours) but thereafter readings gradually fell.

LH levels were very high ranging from 170.4 to 211.5 i.u. per 24 hours. These readings are at the upper end of the limit found in postmenopausal women. The fiducial limits of individual estimations overlapped indicating the lack of a significant difference between them. Clomiphene was administered from days 8 to 14 inclusive, the dose-level being 100 mg. per day. During this time a rise in oestriol occurred but the drug had no effect on oestrone, oestradiol and pregnanediol output. The LH level on days 10 and 11 was significantly lower when compared with all the others and coincided with the oestriol rise. However, the other LH values did not differ significantly from those found during the control period; the fiducial limits of individual estimations overlapped, and it was considered that clomiphene
Mrs. E.T: AGE 25 YEARS PARA 0+0

STEIN-LEVENTHAL SYNDROME - CLOMIPHENE TREATMENT

Figure 52

The effect of Clomiphene on hormone excretion in
Patient 4.
did not exert any significant effect on LH output.

On day 40 of the study the patient passed a few clots of blood and bled for one day. It was thought that these symptoms might indicate that an abortion had occurred, and, since the urinary assays were carried out at a later period, the design of the study was changed; from the second day after the cessation of clomiphene treatment until the day of vaginal bleeding, HCG estimations were performed instead of LH assays. Pregnanediol values started to rise on the sixth post-treatment day with a maximal on day 29 of the investigation (17.3 mg. per 24 hours). A peak of HCG output of 7,520 i.u. per 24 hours coincided with the highest pregnanediol figure (29th day of the study). Oestrogen levels also rose, a figure for "total oestrogens" of 429.0 µg. per 24 hours being noted on day 30 of the study. Subsequently excretion values for HCG, oestrogens and pregnanediol gradually fell and by days 36 and 37 steroid and HCG readings were in the same range as those found in non-pregnant subjects.

A second course of treatment with clomiphene was started on day 56. After a control period of 6 days the drug was administered orally for four days at a dose-level at 200 mg./day. The medication had no effect on endocrine function readings of oestrogens, pregnanediol and LH being within the same range found during the
control period. Forty-eight hours after the cessation of treatment, on days 62-63, a rise in oestradiol output was noted; an increase in oestrone excretion also occurred, the maximum value being found immediately after the oestradiol peak. A marked rise in pregnanediol output occurred, the maximum being from days 61 to 64. Menstruation commenced on day 71 and lasted for five days. LH readings ranged from 40.5 to 206.8 i.u. per 24 hours and the mean value (M.S.E.) was 147.3 ± 52.8 i.u. per 24 hours.

**Patient 5 (Fig. 53)**

The investigation extended over a period of 75 days. During the control period, which lasted 13 days, excretion values for oestrogens were in the range normally encountered in menstruating women. Pregnanediol values fluctuated, ranging from 0.5 to 1.7 mg. per 24 hours. LH values were higher than those found in normally menstruating women, ranging from 23.8 to 65.1 i.u. per 24 hours. Clomiphene was administered at a dose level of 100 mg. per day for seven days. Treatment caused a marked effect on oestrogen output. There was a peak of oestradiol excretion from days 17 to 20, and this was followed some 24 hours later by a peak of oestrone output. Oestriol levels rose in a step-wise manner, reaching a maximum of 67.9 μg. per 24 hours on days 33 to 36; the highest oestriol figures were
Figure 53

The effect of Clomiphene on hormone excretion in Patient 5.
associated with peaks of oestrone and oestradiol and with a maximum of pregnanediol excretion. Menstruation commenced on day 40 and lasted for four days. Between days 45 and 72, readings of oestrone, oestriol and pregnanediol remained relatively constant, being in the range found in normally menstruating women during the proliferative phase of the cycle.

Clomiphene had no discernible effect on LH excretion. The readings of this hormone during the period studied ranged from 21.9 to 65.1 i.u. per 24 hours but the fiducial limits overlapped these values and it was considered that no significant difference existed between any of the relative potencies obtained. The mean LH value (M.S.E.) was 33.9 ± 10.3 i.u. per 24 hours.

DISCUSSION

The main and constant finding of the present study is the lack of any significant effect of clomiphene administration on LH excretion values.

At the time of writing much of the literature on this subject (Table 37) is unsatisfactory because

(1) non-specific methods have been used by the majority of investigators for the assay of HPG;

(2) a proportion of the results are based on isolated determinations;

(3) several conclusions are based on poorly designed
<table>
<thead>
<tr>
<th>Reference</th>
<th>Sex and number of patients</th>
<th>Diagnosis</th>
<th>Design of HPG assays</th>
<th>Effect on hormone excretion</th>
<th>Assay methods for gonadotrophins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charles et al. (1963)</td>
<td>1</td>
<td>Stein-Leventhal Syndrome</td>
<td>Serial</td>
<td>St.* N.E.**</td>
<td>MUT(a)</td>
</tr>
<tr>
<td>Heller &amp; Moore (1963)</td>
<td>4</td>
<td>Normal</td>
<td>Not stated</td>
<td>St. Sp. 50%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Paulsen &amp; Herrmann (1963)</td>
<td>8</td>
<td>Secondary ovarian failure</td>
<td>Serial</td>
<td>St. 64% St. 45%</td>
<td>Not stated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.E. 36% N.E. 55%</td>
<td></td>
</tr>
<tr>
<td>Roy et al. (1963)</td>
<td>1</td>
<td>Stein-Leventhal Syndrome</td>
<td>Serial</td>
<td>St.</td>
<td>OAT?(b); OAAAD(c)</td>
</tr>
<tr>
<td>Smith et al. (1963)</td>
<td>5</td>
<td>Various gynaecological abnormalities</td>
<td>Serial</td>
<td>St. N.E.</td>
<td>MUT; vaginal oestrus in intact immature rats</td>
</tr>
<tr>
<td>Harkness et al. (1964)</td>
<td>4</td>
<td>Normal</td>
<td>Serial</td>
<td>St. N.E.</td>
<td>MUT</td>
</tr>
<tr>
<td>Naville et al. (1964)</td>
<td>37</td>
<td>Anovulatory and isolated irregular cycles</td>
<td>Isolated</td>
<td>N.E. 62% St. 38%</td>
<td>MUT</td>
</tr>
</tbody>
</table>
## MODE OF ACTION OF CLOMIPHENE (contd.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sex and number of patients</th>
<th>Diagnosis</th>
<th>Design of HPG assays</th>
<th>Effect on hormone excretion</th>
<th>Assay methods for gonadotrophins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riley &amp; Evans (1964)</td>
<td>31</td>
<td>Primary and secondary amenorrhoea; oligomenorrhoea</td>
<td>Isolated; in St. 1 subject serial without control period</td>
<td>St. 74% St. N.E. 26%</td>
<td>MUT; OAT; OAAD</td>
</tr>
<tr>
<td>Dickey et al. (1965)</td>
<td>4</td>
<td>Oligomenorrhoea, See text anovulatory cycles</td>
<td>Sup.</td>
<td>St. ? St. ?</td>
<td>OAT; OAAD</td>
</tr>
<tr>
<td>Harkness et al. (1965)</td>
<td>5</td>
<td>Normal; Addison's disease</td>
<td>Serial</td>
<td>St. N.E.</td>
<td>MUT</td>
</tr>
<tr>
<td>Pildes (1965)</td>
<td>19</td>
<td>Various gynaecological abnormalities</td>
<td>Isolated</td>
<td>St. 57% N.E. 43%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Thompson &amp; Mellinger (1965)</td>
<td>15</td>
<td>Oligospermia; azoospermia hypogonadism</td>
<td>Isolated Sp. or N.E.</td>
<td>St. 71% St. 71% N.E. 29% N.E. 29%</td>
<td>MUT; OAT; VPW(d)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Various gynaecological abnormalities</td>
<td>Isolated St. or N.E.</td>
<td>St. 40% Sup. 27% N.E. 33%</td>
<td></td>
</tr>
</tbody>
</table>
## MODE OF ACTION OF CLOMIPHENE (contd.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sex and number of patients</th>
<th>Diagnosis</th>
<th>Design of HPG assays</th>
<th>Effect on hormone excretion</th>
<th>Assay methods for gonadotrophins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beck et al. (1966)</td>
<td>Not stated</td>
<td>Various gynaecological abnormalities</td>
<td>Isolated</td>
<td>&quot;Total gonadotrophic activity&quot; FSH LH</td>
<td>MUT</td>
</tr>
<tr>
<td>Bell et al. (1966)</td>
<td>5</td>
<td>Various gynaecological abnormalities</td>
<td>Serial; FSH St.</td>
<td>N.E. N.E.</td>
<td>MUT; OAT</td>
</tr>
<tr>
<td>Charles et al. (1966)</td>
<td>2</td>
<td>Secondary amenorrhoea</td>
<td>Serial</td>
<td>St. N.E.</td>
<td>MUT</td>
</tr>
<tr>
<td>Loraine et al. (1966)</td>
<td>2</td>
<td>Chiari-Frommel syndrome</td>
<td>Serial</td>
<td>St.? 50% N.E. 50%</td>
<td>MUT</td>
</tr>
<tr>
<td>Mellinger &amp; Thompson (1966)</td>
<td>18</td>
<td>Oligospermia; azoospermia, eunuchoidism</td>
<td>Isolated</td>
<td>St. and Sp. 55% N.E. 45% N.E. 45% N.E. 45%</td>
<td>MUT; OAT; VPW</td>
</tr>
<tr>
<td>Bret &amp; Coiffard (1967)</td>
<td>13</td>
<td>Various gynaecological abnormalities</td>
<td>Isolated; no control period</td>
<td>St. 44% N.E. 56% St. 62% N.E. 38%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Bardin et al. (1967)</td>
<td>14</td>
<td>Normal</td>
<td>Serial; no control period</td>
<td>St. N.E.</td>
<td>Radio-immuno-assay</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Hypopituitarism</td>
<td></td>
<td>N.E.</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Sex and number of patients</td>
<td>Diagnosis</td>
<td>Design of HPG assays</td>
<td>Effect on hormone excretion</td>
<td>Assay methods for gonadotrophins</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------------</td>
<td>----------------------------------</td>
<td>----------------------</td>
<td>-----------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Foss et al. (1967)</td>
<td>1</td>
<td>Klinefelter's syndrome</td>
<td>Serial</td>
<td>N.E., Sup.</td>
<td>MUT; OAT</td>
</tr>
<tr>
<td>Mishell (1967)</td>
<td>6</td>
<td>Oligomenorrhoea; Serial infertility</td>
<td>Serial</td>
<td>N.E.</td>
<td>Immunocassay (haemagglutination-inhibition)</td>
</tr>
<tr>
<td>Odell et al. (1967)</td>
<td>2</td>
<td>Normal; hypogonadism</td>
<td>Serial; no control period</td>
<td>St. or N.E.</td>
<td>Radio-immunoassay</td>
</tr>
<tr>
<td>Bell et al. (1968)</td>
<td>1</td>
<td>Secondary amenorrhoea</td>
<td>Serial</td>
<td>St.</td>
<td>MUT</td>
</tr>
<tr>
<td>Cargille et al. (1968)</td>
<td>3</td>
<td>Normal</td>
<td>Serial; no control period</td>
<td>St.</td>
<td>Radio-immunoassay</td>
</tr>
<tr>
<td>Harkness et al. (1968)</td>
<td>2</td>
<td>Adrenocortical insufficiency; castrate</td>
<td>Serial</td>
<td>N.E. 50% N.E. 50%</td>
<td>MUT; OAT</td>
</tr>
</tbody>
</table>
**MODE OF ACTION OF CLOMIPHENE (contd.)**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sex and number of patients</th>
<th>Diagnosis</th>
<th>Design of HPG assays</th>
<th>&quot;Total gonadotrophic activity&quot;</th>
<th>Assay methods for gonadotrophins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jacobson et al. (1968)</td>
<td>7</td>
<td>Anovulatory cycles; infrequent ovulation</td>
<td>Serial; limited control period</td>
<td>St.?</td>
<td>St.?</td>
</tr>
</tbody>
</table>

**Notes:**
- **St.** = Stimulation of steroidogenesis.
- **N.E.** = No effect.
- **Sup.** = Suppression.
- **Sp.** = Spermatogenesis.

**Assay Methods:**
- (a) **MUT** = Mouse uterus test.
- (b) **OAT** = Ovarian augmentation test in rats or mice.
- (c) **OAD** = Ovarian ascorbic acid depletion test in rats.
- (d) **VPW** = Ventral prostatic weight test in hypophysectomised rats.
experiments; and

(4) despite the limitations related to the precision of the assay methods used, several investigators, on the basis of an increase or decrease in the values of gonadotrophins estimated, drew the erroneous conclusion that it was significant without taking into consideration the confidence limits of the estimated potencies.

Dickey et al. (1965) reported results in four patients each of whom suffered from a different abnormal gynaecological condition. The data in two of these cases is almost impossible to interpret because of concomitant therapy by dexamethasone and the prior administration of HCG and ACTH. In the remaining two women the effect of clomiphene on FSH output was variable. In one of them FSH rose during the treatment but it is difficult to suggest that this rise was significantly higher than the control values. In the latter two patients clomiphene did not exert any significant effect on LH excretion values.

Thompson and Mellinger (1965) and Mellinger and Thompson (1966) estimated the urinary LH output in patients treated with clomiphene using the VFW test. The urinary gonadotrophins were tested against NIH-LH-31 as standard. However, since it has been shown that when the relative potencies of ovine and human preparations are assayed by the VFW test, the latter have
apparently higher specific activities (Rosemberg et al., 1964; Hutchinson et al., 1968), it is remotely possible that the results of the LH assays of the above investigators are valid.

Jacobson et al. (1968) performed serial FSH and LH assays with inadequate control observations in the plasma of sterile women who were treated with clomiphene. They observed an increase of plasma FSH and LH a few days after the cessation of treatment, and they concluded on the evidence of these observations that this was due to clomiphene treatment. The lack of observations during a control period and the absence of simultaneous urinary steroid assays make the interpretation of these results difficult.

The initial clomiphene-induced increase in urinary steroids may be followed, either during prolonged therapy or after therapy has been discontinued, by an increase in gonadotrophin excretion and it was thought that clomiphene exhibits a delayed effect. The logical interpretation of these findings is that the primary site of action of the drug is a direct stimulation of steroid biosynthesis by the gonads, with a subsequent pituitary stimulation via the gonad-hypothalamus-pituitary axis.

Although in none of the patients investigated in the present study did clomiphene exhibit any significant
effect of LH output, the findings in patients 1, 3, 4 and 5 suggest that the drug exerts a direct effect on the gonads. These results are in agreement with those reported by other investigators (see Table 37). Smith et al. (1963) have shown that the pre-operative administration of clomiphene to an anovulatory woman increased the rate of incorporation of labelled acetate into oestradiol by the ovary in vitro. Furthermore the observations by Smith and Day (1963) and Hagerman et al. (1966) lend further evidence to the premise that the primary effect of clomiphene is a direct one on the ovary. The latter investigators have shown that the precise site of action of clomiphene is to reduce the amount of available cytochrome-C reductase. Thus more TPNH is made available for the aromatization reaction converting androgen to oestrogen.

Herrmann (1963) observed a further increase in pregnanediol excretion when clomiphene was administered after ovulation had already occurred. Kistner (1965) also reported that the urinary excretion of pregnanediol is sometimes greater following ovulation induced by clomiphene than that during the luteal phase of the normal cycle. The results obtained in patients 4 and 5 do not support these views. Patient 4 when under treatment with clomiphene already had a functional corpus luteum, as judged by the pregnanediol assays;
However, the compound did not immediately increase pregnanediol excretion. In patient 5, although she ovulated under the effect of clomiphene administration, the highest pregnanediol reading was at the lower end of the range found during the luteal phase in normally menstruating women (Klopper, 1957).

The most intensive clinical studies on the effect of clomiphene are those of Johnsen et al. (1966) and MacGregor et al. (1968). According to them the clinical conditions that respond most readily to clomiphene are the Stein-Leventhal syndrome, post-contraceptive amenorrhoea, and irregular menstruation at intervals of less than 6 months. In this type of patient the ovulatory response varied from 75 to 80% and about 35% achieved pregnancy. Finally in galactorrhoea-amenorrhoea syndrome the ovulatory response was 41.6%.

Treatment with clomiphene in patient 1 of the present study, who suffered from galactorrhoea-amenorrhoea syndrome, was unsuccessful. In this patient, although the mean LH value was within the range encountered in normally menstruating women, oestrogen levels during the control period were within the post-menopausal range.

In patient 2, clomiphene treatment apparently failed to induce ovulation, probably due to pituitary insufficiency. This view is supported by the mean LH
output which was significantly lower from that found in women during reproductive life \( (P < 0.05) \) (Rosenberg and Keller, 1965).

In patient 3, values for oestrogens during the control period and for LH throughout the whole period of investigation were similar to those found during the postmenopausal life (see page 146). Probably this patient had no secondary amenorrhoea but was already post-menopausal, and clomiphene is ineffective in this type of patient (Riley and Evans, 1964).

Patient 4 suffered from Stein-Leventhal syndrome and she was probably ovulating sporadically. Pregnanediol values were raised at the beginning of the investigation, which might indicate that ovulation occurred prior to the initiation of clomiphene treatment. The patient probably conceived at or about the period of treatment as judged by steroid and HCG assays. The pregnancy was unsuccessful and at the end of the third week she aborted. The second course of clomiphene treatment induced ovulation as judged by steroid analysis but on this occasion there was no evidence that conception ensued. Menstruation appeared at the end of the study.

The urinary oestrogen levels in this patient during the control period were within the range found in women during the reproductive life. On the other hand, the
LH values were very high, being within the range found in perimenopausal and postmenopausal women (see pages 132 and 146). This observation is in agreement with the results of Taymor and Barnard (1962) who reported abnormally high LH levels in patients suffering from the Stein-Leventhal syndrome.

Another interesting feature in this subject was the occurrence of ovulation, as judged by urinary steroid assays, in the presence of high LH readings, an observation similar to that found in women late in their reproductive life (see page 132).

In the last patient the mean (M.S.E.) LH value (33.9 ± 10.3 i.u. per 24 hours) is significantly lower than that found in perimenopausal and postmenopausal women (P<0.001) (see pages 132 and 146), but is significantly higher than the comparable reading in normally menstruating women (P<0.01) (Rosenberg and Keller, 1965). In this patient the main point to note in the control period was the high LH levels in association with oestrogen readings within the normal range for a woman during reproductive life. In this subject clomiphene induced ovulation as judged by urinary steroid assays, but conception did not ensue.

**SUMMARY**

Endocrine function as judged by endogenous hormone
levels in urine has been measured before clomiphene therapy in a series of five patients with abnormal gynaecological conditions associated with infertility. In all, the response to the administration of the compound was assessed by serial hormone assays.

Although clomiphene exhibited a direct effect on the ovaries as judged by the oestrogen output, the drug had no discernible effect on LH excretion values.

Ovulation as judged by urinary steroid assays occurred in two of the subjects studied.

The evidence presented herein is compatible with the view that clomiphene acts directly on the ovaries rather than through the pituitary-hypothalamus axis.
CHAPTER 5

URINARY LUTEINISING HORMONE EXCRETION IN MEN
WITH 47, XYY CHROMOSOME CONSTITUTION

INTRODUCTION

The characteristics of phenotypic males with a 47, XYY chromosome complement have recently been described by Jacobs et al. (1965) and Price et al. (1966). These men were patients in a State Hospital and had a mild mental retardation, a tendency towards aggressive behaviour which resulted in criminal or violent acts, and a tendency towards increased height.

As was mentioned earlier (page 126), the incidence of hypogonadism is high in patients with this type of sex chromosome abnormality and no information regarding pituitary gonadotrophic function in these subjects has been reported.

The aim of this study was to measure the urinary excretion of LH in three such patients.

MATERIAL AND METHODS

Patients

Subject 1 (P), aged 16 years: His height was 181 cm. The body hair was scanty but pubic hair was normally distributed; he lacked facial hair. He did
not have gynaecomastia and the external genitalia appeared normal. The patient was of average intelligence (I.Q. 100).

**Subject 2 (G), aged 19 years:** His height was 180 cm. The body hair was scanty but of normal distribution; facial hair shaved twice per week. The external genitalia appeared normal. The patient was mentally subnormal (I.Q. 80).

**Subject 3 (McC), aged 17 years:** Height 169 cm.; axillary and pubic hair normally developed; facial hair shaved 3 days in 7; penis and scrotum normally developed; both testes of normal size; mentally subnormal (I.Q. 90).

**Methods**

Hormone assays were conducted on 48 or 72 hour pools of urine and the results were expressed as i.u./24 hours (2nd IRP-HMG). In all three subjects complete 24 hour collections were made over a period of 10 days, this being the maximum time during which it was possible to obtain the collaboration of the patients.

HPG was extracted from the urine by the tannic acid procedure (see page 92) and the bioassay of LH was carried out by the OAAD method (see page 79). All the assays were of a symmetrical 4-point design.
RESULTS

In the 14 assays performed the mean index of precision ($\frac{\lambda}{\sigma}$ S.D.) for the LH estimations was 0.16 ± 0.02.

The results of serial LH assays are illustrated in Figures

**Subject 1** (Fig. 54): Marked fluctuations in LH output were present and a consistent pattern of excretion was not observed. Only the last reading on day 9 of the study was within the normal range for male subjects, the other 4 estimations being abnormally high.

**Subject 2** (Fig. 55): All measurements were considerabably above the level expected for normal male subjects.

Although the readings of the relative potencies varied from 41.3 to 79.3 i.u. per 24 hours, the fiducial limits (95%) encompassed those values and it was considered that no significant differences existed between any of the values estimated.

**Subject 3** (Fig. 56): The first two readings were at the upper end of the normal range while the third on day 9 of the study was grossly abnormal.

The mean (± S.E.M.) urinary LH level of the three subjects was 77.34 ± 16.6 i.u. per 24 hours.
Figure 54

Urinary LH excretion in Subject 1
Mr. G. Age 19 Years  47, XYY Karyotype

Figure 55

Urinary LH excretion in Subject 2
Mr. Mc. C.

AGE 17 Years

47, XYY KARYOTYPE

LH

Figure 56

Urinary LH excretion in Subject 3
Prior to the studies of Jacobs et al. (1965) and Price et al. (1966) only isolated cases have been reported of the XYY abnormality, and knowledge of only 15 patients in the general population has been identified. Table 38 contains a summary of cases that have been reported in the literature. Examination of these 15 cases shows that XYY males have no consistent physical or mental stigmata. However, the incidence of gonadal abnormalities is high. Nine patients showed evidence of either abnormal genital development (age from 21 months to 12 years) or of hypogonadism. Only two of those 15 patients were with certainty fertile.

The measurements of LH in the three patients described herein are similar to those found in patients with primary testicular hypogonadism (Papanicolaou et al., 1967).

The mean LH value of the present study (77.34 ± 16.6) is significantly higher (P< 0.001) than that found in normal male patients (Rifkind et al., 1967) (see page 116).

It is remotely possible that the high LH values in the 47, XYY males, as compared with the normal subjects of Rifkind et al. (1967), could be explained on methodological grounds since the assay procedures used in the two investigations (the hypophysectomised prostate test by Rifkind et al. (1967) and the OAAD method in the
### TABLE 38
SUMMARY OF PREVIOUSLY REPORTED CASES OF XYY KARYOTYPE FROM THE GENERAL POPULATION

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (years)</th>
<th>Phenotype</th>
<th>Genotype</th>
<th>Abnormalities</th>
<th>Names of Investigators</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9/12</td>
<td>Male</td>
<td>XYY</td>
<td>Undescended testes</td>
<td>Uchida et al. (1964) Dunn et al. (1961)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Male</td>
<td>XYY</td>
<td>Undescended testes, foreskin deficiency</td>
<td>Fraccaro et al. (1962)</td>
</tr>
<tr>
<td>3</td>
<td>3.6/12</td>
<td>&quot;Female&quot;</td>
<td>XYY</td>
<td>Phallus, testes present; hypospadias. No internal female sex organs</td>
<td>Vignetti et al. (1964)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Male</td>
<td>XYY</td>
<td>Gross mental retardation</td>
<td>Dent et al. (1963)</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>Male</td>
<td>XYY</td>
<td>Undescended testes</td>
<td>Fraccaro et al. (1962)</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>Male</td>
<td>XYY</td>
<td>Hypospadias, small penis, small descended testes</td>
<td>Milen et al. (1964)</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>Male</td>
<td>XYY</td>
<td>Hypogonadism?</td>
<td>Verresen and van der Berghe (1965)</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>Male</td>
<td>XYY</td>
<td>Marfan's syndrome</td>
<td>Hustinx et al. (1964)</td>
</tr>
<tr>
<td>No.</td>
<td>Age (years)</td>
<td>Phenotype</td>
<td>Genotype</td>
<td>Abnormalities</td>
<td>Names of Investigators</td>
</tr>
<tr>
<td>-----</td>
<td>-------------</td>
<td>-----------</td>
<td>----------</td>
<td>---------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>Male</td>
<td>XYY</td>
<td>Right testes in the scrotum, smaller and softer than normal. Left could not be palpated in the scrotum or in the inguinal canal</td>
<td>Sandberg et al. (1963)</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>Male</td>
<td>XYY</td>
<td>Grossly mentally retarded</td>
<td>Ricci and Malacarne (1964)</td>
</tr>
<tr>
<td>11</td>
<td>23</td>
<td>Male</td>
<td>XYY</td>
<td>Hypogonadism (small penis and scrotum)</td>
<td>Court-Brown et al. (1964)</td>
</tr>
<tr>
<td>12</td>
<td>26</td>
<td>Male</td>
<td>XYY</td>
<td>Hypogonadism (penis and scrotum normal, testes smaller and softer than normal)</td>
<td>Nielsen et al. (1966)</td>
</tr>
<tr>
<td>13</td>
<td>44</td>
<td>Male</td>
<td>XYY</td>
<td>Fertile</td>
<td>Sandberg et al. (1961), Hauschka et al. (1962)</td>
</tr>
<tr>
<td>14</td>
<td>64</td>
<td>Male</td>
<td>XYY</td>
<td>Hypogonadism</td>
<td>Balodimos et al. (1966)</td>
</tr>
<tr>
<td>15</td>
<td>64</td>
<td>Male</td>
<td>XYY</td>
<td>Fertile</td>
<td>Tompсон et al. (1967)</td>
</tr>
</tbody>
</table>
present investigation) were different. However, such an explanation appears to be most unlikely in view of the observations that when parallel assays by the two techniques are performed on urinary extracts prepared by the tannic acid procedure of Johnsen (1958), similar results are obtained (Rosenberg, 1967).

Reasons for the abnormal LH excretion in males with a 47,XXX karyotype remain obscure. Findings on testicular biopsy might well throw light on the question and, at the time of writing, such data are available in two subjects (Balodimos et al., 1966; Nielsen et al., 1966). The appearance of the testes was found to be similar but not identical to that encountered in classical cases of Klinefelter's syndrome, being characterised by virtual absence of spermatogenic and Sertoli cells with relatively normal numbers of Leydig cells which were arranged in the form of small clusters and showed a moderate degree of hyperplasia.

LH is known to be the hormone which acts upon the Leydig cells of the interstitial tissue of the testes and regulates the secretion of testosterone (Johnsen, 1967; Lunenfeld, 1967; Gemzell, 1968). Several investigators who performed testosterone estimations in plasma and in urine of XYY males found the testosterone values to be within the highest normal range (Goodman and Smith, 1967; Price and van der Molen, 1967; Ismail
et al., 1968).

On the basis of these results it might be postulated that in men with a 47,XY Y karyotype the Leydig cells of the testes are relatively insensitive to stimulation by pituitary LH, and in order to overcome this refractoriness, abnormally large quantities of this hormone must be produced by the anterior pituitary leading to greater quantities being excreted in the urine. In this connection it appears worthy of comment that the normal reciprocal relationship between the pituitary and the gonads does not appear to be present in 47,XY Y males, and abnormally high LH output in these subjects is associated with urinary testosterone readings that are at the highest normal range.

From the present study it is not possible to draw any definite conclusions about the XYY syndrome because of the limited number of patients and the difficulties of co-operation with them in a more prolonged and detailed investigation due to their aggressive behaviour.

**SUMMARY**

Serial assays of luteinising hormone (LH) in urine have been performed in 3 phenotypic males with a 47,XY Y sex chromosome constitution. LH levels were grossly elevated, being significantly higher (P< 0.001) than those found in normal male subjects. The possible explanation of this finding has been discussed.
INTRODUCTION

Although the sex-chromosomal karyotypes and the clinical features of patients with Turner's syndrome have been extensively studied (see Introduction, page 127) there remains a great lack of information regarding pituitary gonadotrophic function in this condition. In the few studies reported in the literature, non-specific methods of assay for HPG were used and it was not possible to draw any definite conclusions from the data presented.

The aim of the present study was to determine urinary LH levels in four patients with Turner's syndrome. In one of these subjects an ovarian implantation was performed, and the effect of this procedure on LH output was investigated, in an attempt to determine whether the ovarian defect in this syndrome is primary or is due to abnormal pituitary gonadotrophic function.

Patients

Subject 1 (E.W.), aged 25 years: Height 138 cm.; primary amenorrhoea with underdeveloped genitalia; no breast development; axillary hair absent; pubic hair
sparse; webbing of neck; bifid left renal pelvis and left ureter; chromosomal karyotype 46,XXq?i.

**Subject 2 (J.B.), aged 18 years:** Height 132 cm.; primary amenorrhea with absence of secondary sex characteristics; chromosomal karyotype 46,XXq1.

**Subject 3 (J.M.), aged 31 years:** Height 141 cm. Menarche at the age of 13. She menstruated for one year and subsequently became amenorrhoeic. Her breasts were reasonably developed, but she had no axillary hair. There was a small uterus, but no endometrium was obtained on curettage, and the ovaries were not palpable. Gynaecography revealed no definite evidence of ovarian shadows. A chromosomal analysis showed the presence of a 46,XXq1 karyotype.

**Subject 4 (L.B.), aged 18 years:** Height 149.9 cm. with primary amenorrhea and absence of any secondary sex characteristics. The uterus was hypoplastic and the ovaries were not palpable. Radiological examination of skull was normal, and there were no abnormal haematological or biochemical findings. The chromosomal karyotype was 45,X/46,XX.

Further investigation six months after the initial presentation of subject 4 revealed at laparotomy that the cervix was infantile, the uterus hypoplastic and that the ovaries consisted of bilateral streaks along the superior aspect of the infundibular pelvic ligaments. Bilateral
ovarian transplantation was performed, the donor being her mother who had a history of regular menstruation and whose ovaries were histologically normal. The post-operative course of this patient was uneventful. Two months following the operation she developed acne affecting the face, forehead and shoulders; she also noted lower abdominal and mammary discomfort and leucorrhoea. Examination of the vaginal cytology at this time showed superficial cells with pyknotic nuclei which might indicate oestrogenic activity.

METHODS

All four patients collected complete 24-hour urine samples throughout the period of investigation. In the case of subject 4, who was resident in Pittsburg, U.S.A., samples were deep frozen before being transported to Edinburgh by air. Assays were conducted on 48-hour pools of urine, results being expressed as i.u. (2nd IRP-HMG) per 24 hour sample.

LH was extracted from the urine by the tannic acid procedure of Johnsen (1958). In subjects 1, 2 and 4 the bioassay was the OAAD test (see page 79 ). A total of 19 assays were performed; the mean index of precision ($\lambda \pm$ S.D.) was 0.22 ± 0.06. In patient 3 LH was assayed by the VPW test (see page 51 ). A total of 22 assays were performed; 27% of these were invalid either because
of significant deviation from parallelism between the slopes of the dose-response curves of standard and unknown preparations or due to incompleteness of the hypophysectomy. The mean index of precision ($\lambda \pm S.D.$) for the VPW test was $0.11 \pm 0.05$.

In patients 1 and 4 estimations of the three classical oestrogens (oestrone, oestradiol and oestriol) and of total 17-hydroxycorticosteroids (17-OHCS) were performed by the techniques of Brown et al. (1957) and of Appleby et al. (1955) respectively.

**RESULTS**

**Subject 1 (Fig. 57 )**

Levels of oestrone, oestradiol and oestriol were either low or undetectable while readings of 17-OHCS also tended to be low. LH values were abnormally high for a woman during reproductive life, ranging from 38.0 to 113.0 i.u. per 24 hours. The fiducial limits ($P = 0.95$) for all the LH assays overlapped, indicating that the individual values did not differ significantly.

**Subject 2 (Fig. 58 )**

LH levels were much above the range normally encountered in women during reproductive life, readings ranging from 103.4 to 328.6 i.u. per 24 hours. The fiducial limits ($P = 0.95$) of individual estimates overlapped, indicating the absence of a significant difference
Figure 57

Hormone excretion in Subject 1
Miss J.B: AGE 18 YEARS

TURNER'S SYNDROME
46, XXq1 KARYOTYPE
(PRIMARY AMENORRHOEA)

Figure 58

LH excretion in Subject 2
Figure 59

LH excretion in Subject 3
Miss L.B.: AGE 18 YEARS

**TURNER'S SYNDROME**

45,X/46,XX KARYOTYPE

(Primary Amenorrhoea)

---

**Figure 60**

Steroid excretion in Subject 4
Miss L.B.: AGE 18 Years

**TURNER'S SYNDROME**

45,X/46,XX KARYOTYPE

(Primary Amenorrhea –
Ovarian Transplantation)

---

**CLOMIPHENE 50mg /IV**

**OVARIAN TRANSPLANTATION**

---

**EOSTRADIOL**

**EOSTRONE**

**EOSTRIOL**

---

**DAY OF INVESTIGATION**

---

Figure 61

Effect of Clomiphene and ovarian transplantation on LH and oestrogen excretion in Subject 4.
similar to those in the control period. Some eight weeks after the operation, when leucorrhoea, mammary discomfort and acne were present, excretion values for oestrogens were low or undetectable and LH readings were in the same range as those encountered pre-operatively.

Mean hormone excretion in Turner's syndrome

This is shown in Table 39. In the two subjects in whom urinary oestrogens were estimated, levels were very low indicating the virtual absence of ovarian activity, while readings for 17-OHCS were within or at the lower end of the normal range. LH readings were very variable being within the postmenopausal range in subjects 1 and 2, at the upper end of the normal range for women during reproductive life in subject 4 and above this range in subject 3.

Comparison of results in Turner's syndrome and postmenopausal women

Table 40 shows the difference in LH levels between the patients with Turner's syndrome and a series of four postmenopausal women investigated concurrently (see Chapter 2). Figures which are lower in Turner's syndrome are indicated by a minus sign and those which are higher by a plus sign; significant differences are also shown. In patients 1 and 2, levels are in the same
<table>
<thead>
<tr>
<th>Subject</th>
<th>Chromosomal karyotype</th>
<th>Total oestrogens (µg./24 hours)</th>
<th>17-OHCS (mg./24 hours)</th>
<th>LH (i.u./24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46,XXq?i</td>
<td>0.9 ± 0.8*</td>
<td>4.8 ± 1.7</td>
<td>72.2 ± 18.2</td>
</tr>
<tr>
<td>2</td>
<td>46,XXqi</td>
<td></td>
<td></td>
<td>186.0 ± 54.7</td>
</tr>
<tr>
<td>3</td>
<td>46,XXqi</td>
<td></td>
<td></td>
<td>34.5 ± 2.3</td>
</tr>
<tr>
<td>4</td>
<td>45,X/46,XX</td>
<td>4.9 ± 0.3</td>
<td>7.1 ± 0.7</td>
<td>22.2 ± 10.3</td>
</tr>
</tbody>
</table>

* + Standard error
TABLE 40
DIFFERENCE BETWEEN MEAN LH EXCRETION IN PATIENTS WITH TURNER'S SYNDROME AND NORMAL POSTMENOPAUSAL WOMEN

<table>
<thead>
<tr>
<th>Subject</th>
<th>Chromosomal karyotype</th>
<th>LH i.u./24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46,XXq?i</td>
<td>-40.6</td>
</tr>
<tr>
<td>2</td>
<td>46,XXq1</td>
<td>+69.2</td>
</tr>
<tr>
<td>3</td>
<td>46,XXq1</td>
<td>-82.3***</td>
</tr>
<tr>
<td>4</td>
<td>45,X/46,XX</td>
<td>-94.6***</td>
</tr>
</tbody>
</table>

*** p< 0.001
range as in postmenopausal subjects, while in patients 3 and 4 they are significantly lower. The chromosomal karyotype in Turner's syndrome did not appear to influence the results.

**DISCUSSION**

One of the main findings in the present investigation was the variability in urinary LH levels in the four cases of Turner's syndrome reported. Thus in two patients, readings were within the postmenopausal range, in one at the upper end of the normal range for women during reproductive life (see Rosemberg and Keller, 1965) and in one above this range. Another point to emerge from the study was the apparent lack of correlation between the mean LH output on the one hand and the chromosomal karyotype on the other.

In the patient in whom the ovarian transplantation was performed a marked rise in urinary LH excretion immediately followed the operation. The reason for this finding is not entirely clear, although the following possibilities might be considered.

If the defect in Turner's syndrome is primarily in the ovaries, it might be expected that the transplant would function normally. This did not appear to be the case. Clinical evidence points to the view that the ovarian transplants became functional, this being
indicated by the presence of acne, leucorrhoea and oestrogenic changes in the vaginal smear at the time of examination some two months after the operation even when LH and oestrogen levels had returned to those seen during the pre-operative period.

Another possibility is that the increase in LH output, indicating pituitary hyper-activity, was due to the physiological and psychological stress to the patient occasioned by the operation. Espiner (1966) has shown that after surgical intervention there is a marked rise in the urinary excretion of free cortisol which is accompanied by an increased level of ACTH. Nevertheless, there is no evidence to support the view that in human subjects there is an effect on the secretion of HPG, either by itself or caused by the increase in levels of ACTH or corticosteroids (Mason et al., 1948; Sohval and Soffer, 1951; Crean et al., 1963, 1966; Loraine et al., 1967). Giuliani et al. (1961), using a specific method of LH assay, showed that ACTH, released as a result of stress, does not increase LH secretion in the rat. However, Charles et al. (1965) reported a rise in the output of HPG as a result of surgery in one out of six women subjected to total hysterectomy and salpingo-oophorectomy for endometrial carcinoma. It should, however, be noted that this rise in HPG was followed by a significant increase in urinary oestriol output, presumably of
adrenal origin. The fact that such a rise did not occur in the present study suggests that there may be other factors operating that affect LH excretion in these circumstances.

Silver and his colleagues (Silver, 1951; Silver and Kempe, 1953; Silver and Dodd, 1957) and Grossman (1960) have shown that in children with Turner's syndrome, ranging in age from 2½ to 9 years, urinary HPG levels may be grossly elevated, being either within or above the range encountered in normally menstruating women. These results suggest that the pituitary in Turner's syndrome may be hyperactive. Moreover, normal menstrual function with regular periods, sometimes for several years, has been reported in established cases of the syndrome. Hoffenberg et al. (1957) reported two patients with gonadal dysgenesis who had menstruated irregularly for 5 and 6 years respectively. Laparotomy was performed in both cases one year after the cessation of menses and the streak gonads, typical of Turner's syndrome, were found. These consisted of fibrous stroma without follicles or interstitial cells. Stewart (1960) described classical chromatin-negative Turner’s syndrome in a woman who had menstruated regularly for six years. Bahner et al. (1960) found two cases of chromatin-negative Turner's syndrome with regular menstruation in one of these subjects for four years. The second
patient had menstruated regularly since the age of 16, and at 31 years of age gave birth to a normal son.

Some light has been shed on ovarian development in patients with a 45,X karyotype by the recent findings of Sing and Carr (1966). Thirteen abortuses with a known chromosomal complement of 45,X were collected ranging in age from five weeks to four months. In all the specimens the gross anatomical appearance of the gonad was normal and, when examined histologically, the presence of primordial germ cells could be demonstrated. The histological sections of the 45,X gonads were compared with sections of gonads from known 46,XX abortuses of similar ages. Sing and Carr (1966) found no significant difference between the 45,X and the 46,XX gonads up to the end of the third month in utero. In the older foetuses there was a relative increase in the connective tissue of the 45,X gonads.

The cumulative evidence from all these observations suggests that the classical view regarding the aetiology of Turner's syndrome may represent an oversimplification of the true situation. Further work is obviously necessary in order to determine whether other factors, and particularly an abnormality of the pituitary-hypothalamic axis, play an important role in the pathogenesis of the condition.
SUMMARY

1. Bioassays of LH have been conducted on the urine of four patients with Turner's syndrome in one of whom a bilateral ovarian transplantation was performed.

2. LH levels were variable in individual subjects being either within or below the postmenopausal range.

3. There was no apparent correlation between the chromosomal karyotype and the LH output.

4. A marked rise in LH excretion followed the ovarian transplantation, but this was not associated with an increase in urinary oestrogen output.
REFERENCES


Brown, P.S., Wells, M., and Cunningham, F.J. (1964):
Lancet, 2, 446.
*J. Reprod. Fertil.*, 11, 481.
Nature, 205, 88.
Gynäk (Karger), 21, 148.
Geburts. Frauenheilk, 22, 923.
Butt, W.R., Crooke, A.C., and Cunningham, F.J. (1961):
Biochem. J., 81, 596.
Butt, W.R., Crooke, A.C., Cunningham, F.J., and
Cargille, C.M., Ross, G.T., and Bardin, C.W. (1968):
La Lancet, 14, 1298.

p. 251.


Evans, H.M. (1924): Harvey Lectures, 19, 212.
   Endocrinology, 54, 516.
   Fertil. and Steril., 16, 665.
   Amer. J. Physiol., 97, 291.


*Endocrinology*, 80, 378.

Herrmann, W.L. (1963): Personal communication.

Joint Meeting of German, Austrian and Swiss  
Societies of Obstetrics and Gynaecology, Zurich.

Hoffenberg, R., Jackson, W.P.U., and Muller, W.H.  


Hunter, W.M. (1967): In *Recent Research on Gonado- 
p. 319.


*Genetica*, 24, 262.

Hutchinson, J.S.M., Armstrong, D.T., and Greep, R.O.  

*J. Endocr.*, 22, 47.

Hymer, W.C., McShan, W.H., and Christiansen, R.G.  

Igarashi, M., and McCann, S.M. (1964): *Endocrinology*,  
74, 440.

Igarashi, M., and McCann, S.M. (1964a): *Endocrinology*,  
74, 446.

Igarashi, M., Nallar, R., and McCann, S.M. (1964):  
*Endocrinology*, 75, 901.
Ismail, A.A.A., Harkness, R.A., Kirkham, K.E.,
Loraine, J.A., Whatmore, P.B., and Brittain, R.P.
Jacobs, P.A., Brunton, M., Melville, M.M., and
Jacobson, A., Marshall, J.R., Ross, G.T., and
Johannisson, E., Tillinger, K.G., and Diczfalusy, E.
Johnsen, S.G. (1967): In Endocrinology of the Testis.
Edited by Wolstenholme, G.E.W., and O'Connor, M.
Jones, G.E.S., and Bucher, N.L.R. (1943):
Endocrinology, 22, 46.
Jones, G.S., and de Moraes-Ruehsen, M.D. (1967):
Acta, 34, 1790.
obstet. gynec. scand., 45, 53.
106, 125.
Li, C.H., Simpson, M.E., and Evans, H.M. (1940):
Endocrinology, 27, 803.
J. Amer. chem. Soc., 64, 367.
Arch. Biochem. Biophys., 86, 110.
Lin, T.J., Durkin, J.W. Jr., and Kim, Y.J. (1964):


Mellinger, R.C., and Thompson, R.J. (1966): Fertil. and Steril., 17, 94.


J. Endocr., 23, 413.


Silver, H.K. (1951): Paediatrics, 8, 368.


Published Work included in this Thesis


ACKNOWLEDGEMENTS

The work described in this thesis has been carried out in the Clinical Endocrinology Research Unit, (Medical Research Council), 2 Forrest Road, Edinburgh, I.

I wish to thank Dr. J.A. Loraine for his supervision and advice, Dr. K.E. Kirkham for his invaluable encouragement and criticism and Dr. P. Myerscough for his help.

I should like to express my thanks to Professor D. Charles (Pittsburg, U.S.A.), Professor J.A. Strong (Edinburgh), Dr. M.D. Casey (Sheffield), Dr. G.L. Foss (Bristol), Dr. M.T. Harrison (Glasgow), Dr. W.H. Price (Edinburgh), Dr. G.A. Dove (London), Dr. N.B. Loudon (Edinburgh) and Dr. M.C.N. Jackson (Exeter) for permission to study patients under their care.

I am grateful to Dr. L. Ganguli and Dr. S. Semple for their advice and help in the performance of the bacteriological studies reported in Chapter 3, Section A. I wish to thank Mr. H.A.F. Blair and Mr. D.N. Love for carrying out the steroid assays reported in Section B. I am grateful to the Medical Research Council Computer Service Centre (London) for performing the statistical analysis of the bioassays reported in Section B.

I am extremely grateful to Mr. S.F. Lunn for his criticism and to Miss M.A. Mackay and Miss A.V. Patek for their help in the performance of the bioassays.