STEROID METABOLISM BY INTERSTITIAL TISSUE
FROM THE OVARY OF THE FOWL
(GALLUS DOMESTICUS)

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This thesis is presented for the degree of Doctor of Philosophy of the University of Edinburgh.

1983
I declare that this thesis has been composed by myself. The work presented has not been submitted to the University in any other form.
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Abstract

This thesis reports the results of an investigation into the metabolism of $[4-^{14}C]$progesterone and $[4-^{14}C]$-testosterone by thecal and zona parenchymatosa homogenates from the ovary of the laying hen. These tissues, which constitute the interstitial component of the ovary, have been postulated as a site of androgen and oestrogen synthesis.

Thecal tissue from follicles $> 2$ mm in diameter and the zona parenchymatosa, comprising the remaining mass of smaller follicles, were collected from the ovary either 1 to 2 hours or 15 hours before an expected ovulation. The tissue was homogenized for incubation with the substrates in the presence of cofactors.

Thecal homogenates converted approximately ten times more $[4-^{14}C]$progesterone to androstenedione than did zona parenchymatosa homogenates. The latter metabolized $[4-^{14}C]$progesterone preferentially to $5\beta$-reduced derivatives, notably $5\beta$-pregnan-3$\alpha$-ol-20-one. Similarly, $5\beta$-reduced androgens were the major metabolites produced by zona parenchymatosa homogenates incubated with $[4-^{14}C]$testosterone.

Thecal homogenates, however, preferentially metabolized $[4-^{14}C]$testosterone to androstenedione. Conversion of $[4-^{14}C]$testosterone to phenolic steroids was greatest in homogenates of thecal tissue from follicles between 2 and 10 mm in diameter. It was concluded that the theca layer of developing follicles possesses a greater
17α-hydroxylase, C-17,20-lyase and 17β-hydroxysteroid dehydrogenase activity than the zona parenchymatosa.

With the exception of the largest, preovulatory follicle, the ability of thecal tissue to convert [4-14C]progesterone to androstenedione remained constant throughout the final phase of growth (from 10 mm to 35 mm in diameter). Thecal homogenates from the largest follicle (approximately 35 mm in diameter) converted significantly less [4-14C]progesterone to androstenedione 1 to 2 hours before ovulation compared with earlier in the cycle, whereas production of 20β-hydroxy-4-pregnen-3-one increased significantly. These changes were not seen in the second and third largest follicles (approximately 30 and 28 mm in diameter respectively) and may therefore be related to the ovulatory process.

In contrast, thecal homogenates from follicles between 2 and 10 mm in diameter converted significantly more [4-14C]progesterone to androstenedione when the follicles were collected 1 to 2 hours before ovulation, compared with earlier in the cycle. These findings suggest that the plasma surge of LH is responsible for stimulating thecal androgen production.
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I also wish to thank Mrs. M. Walker for dissection of the ovaries, Mrs. M. Davidson for undertaking the histological work and Mr. J. Culbert for carrying out the h.p.l.c. chromatography. The photographic work was carried out by Mr. N.J. Russell and Mr. R.K. Field.

I am grateful to the director of the Poultry Research Centre, Roslin, Midlothian for allowing me to undertake this research at the Poultry Research Centre, and to Dr. J. Manson of the Department of Agriculture, University of Edinburgh, for his help.

Finally, I wish to record my appreciation to the British Egg Marketing Board's Research and Education Trust Fund for financial sponsorship, and to Mrs. S. Hayton for typing the manuscript.
Nomenclature

1. **Systematic Names for Steroids**

   The IUPAC-IUB 1967 revised tentative rules for steroid nomenclature have been adopted as published in Steroids (1969), 13, 277.

2. **Trivial Names for Steroids**

   The following trivial names have been used throughout this thesis:
   - androstenedione - 4-androstene-3,17-dione
   - cholesterol - 5-cholesten-3β-ol
   - dehydroepiandrosterone - 3β-hydroxy-5-androsten-17-one
   - dihydrotestosterone - 17β-hydroxy-5α-androstan-3-one
   - pregnenolone - 3β-hydroxy-5-pregnen-20-one
   - progesterone - 4-pregnene-3,20-dione
   - oestrone - 3-hydroxy-1,3,5(10)-oestratrien-17-one
   - oestradiol - 1,3,5(10)-oestratriene-3,17β-diol
   - oestriol - 1,3,5(10)-oestratriene-3,16α,17β-diol
   - testosterone - 17β-hydroxy-4-androsten-3-one

3. **Anatomical Terms**

   a) The term interstitial tissue refers collectively to the zona parenchymatosa and the theca layer of developing follicles.

   b) The term zona parenchymatosa refers to that part of the ovary remaining after removal of the zona vasculosa and all follicles > 2 mm in diameter.
INTRODUCTION

The production of steroid hormones by the ovary of the hen is a fundamental requirement for egg-laying. Androgens, oestrogens and progesterone are produced by the ovary in response to the pituitary gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Hill and Parkes, 1935; Opel and Nalbandov, 1961; Senior, 1974a). These hormones are carried in the bloodstream to their target organs where they induce specific physiological responses vital for egg-formation.

The dramatic increase in weight of the immature chick oviduct in response to oestrogen has been well-documented (Munro and Kosin, 1943; Dorfman and Dorfman, 1948; Breneman, 1955; Brant and Nalbandov, 1956). This effect is largely due to the differentiation of epithelial cells in the magnum portion of the oviduct into three distinct types (Kohler, Grimley and O'Malley, 1969; Oka and Schimke, 1969a, 1969b; Palmiter and Wrenn, 1971; Laughier, Brard, Sandoz and Boisvieux-Ulrich, 1975). These are the tubular gland cells and the goblet cells, which secrete egg-white proteins, and the ciliated cells which assist in the propulsion of the egg through the oviduct.

Steroid hormones also control the secretion of egg-white proteins by the oviduct. The tubular gland cells secrete ovalbumin and lysozyme in response to oestrogen (O'Malley, McGuire and Korenman, 1967; Schimke, McKnight, Shapiro, Sullivan and Palacios, 1975; O'Malley,
Roop, Lai, Nordstrom, Catterali, Swaneck, Colbert, Tsai, Dugaiozyk and Woo, 1979), whilst progesterone stimulates the synthesis of avidin by the goblet cells (Hertz, Fraps and Sebrell, 1943; O'Malley, 1967; O'Malley and Means, 1974; Rantala, Helin and Elo, 1982). Progesterone also appears to facilitate the release of ovalbumin granules from the tubular gland cells (Laughier and Brard, 1980).

Oestrogens, produced by the ovary, are essential for egg-yolk formation by acting directly on the liver to stimulate the synthesis of egg-yolk constituents. The synthesis of vitellogenin, a precursor of the yolk proteins phosvitin and lipovitellin is under the control of oestrogen (McIndoe, 1971; Redshaw and Follett, 1972; Gruber, 1972; Lazier, 1978; Tata and Smith, 1979) as is the synthesis of transferrin (Lee, McKnight and Palmiter, 1978) and riboflavin-binding protein (Murthy and Adiga, 1978). Very low-density lipoproteins (V.D.L.P.'s) are also synthesized in the liver in response to oestrogens (Chan, Jackson, O'Malley and Means, 1976; Chan, Jackson and Means, 1978; Nadin-Davis, Lazier, Capony and Williams, 1980). V.D.L.P.'s are not susceptible to hydrolysis by lipoprotein lipase (Griffin, Grant and Perry, 1982), and are therefore transported primarily to the ovary where they are incorporated into the oocyte (Evans, Perry and Gilbert, 1979).

The steroid hormones produced by the ovary in turn exert a feedback action on the brain to control the release of gonadotrophins. Release of the preovulatory
surge of LH from the pituitary is thought to be due to the positive feedback action of progesterone (Furr and Smith, 1975; Etches and Cunningham, 1976; Wilson and Sharp, 1976; Williams and Sharp, 1978). The latter may act at the level of the hypothalamus to increase LHRH synthesis and/or release (Tanaka, Kamiyoshi and Sakaida, 1974; Fraser and Sharp, 1978). The positive feedback action of progesterone may be dependent upon oestrogen priming (Wilson and Sharp, 1976; Kawashima, Kamiyoshi, Tanaka, Hattori and Wakabayashi, 1981), possibly by increasing the concentration of hypothalamic progesterone receptors (Kawashima, Kamiyoshi and Tanaka, 1979).

Despite the many changes which are under the control of steroid hormones in the laying hen (some of which have been described above), their cellular sites of origin and pathways by which they are synthesized have not been studied extensively until recently. Much of this work has focussed on the production of progesterone by the granulosa cells of the large preovulatory follicles (Huang, Kao & Nalbandov, 1979; Wells, Gilbert and Culbert, 1980). The synthesis of androgens and oestrogens in the ovary of the laying hen has been ascribed to the interstitial cells both of the theca layer of developing follicles and the ovarian stroma (Fell, 1924; Benoit, 1926; Allen, Whitsett, Hardy and Kneibart, 1924; Marlow and Reichart, 1940; Marshall and Coombs, 1957; Boucek and Savard, 1970; Dahl, 1971a, 1971b; Peel and Bellairs, 1972). Apart from some recent studies utilizing thecal
cells from the three largest preovulatory follicles, (Huang, Kao and Nalbandov, 1979; Wang and Bahr, 1983) most of the evidence linking the interstitial cells with androgen and oestrogen synthesis has been indirect in nature (see 1:2:1). The present study was therefore designed firstly to investigate the steroidogenic capability of interstitial tissue \textit{in vitro}, and secondly to make a preliminary assessment of thecal steroidogenesis throughout follicular development.
1. LITERATURE REVIEW
Follicular development in the ovary of the domestic fowl

Ovarian development starts with the migration of the primordial germ cells to the genital ridge (Brambell, 1956; Romanoff, 1960; Marshall, 1961; Franchi, Mandl and Zuckerman, 1962). These cells eventually give rise to the primary oocytes by repeated mitotic divisions. In the domestic fowl, as in most avian species, development of the reproductive system is asymmetrical, starting with the migration of the germ cells exclusively to the left genital ridge. Subsequent development of the reproductive system results in only the left ovary and oviduct becoming functional in the adult hen, the right ovary and oviduct remaining vestigial (Franchi, 1962).

At hatching, the left ovary comprises the cortex and the underlying medulla which is highly vascular and consists mainly of connective tissue with some nerves and smooth muscle (Gilbert, 1971c; Gilbert, 1979). At this stage of development, these two regions remain anatomically distinct from one another (Narbaitz and de Robertis, 1968; Prochazkova and Komarek, 1970; Gilbert, 1979). The cortex contains numerous oocytes which have entered their first meiotic division. They remain arrested at the diplotene stage of prophase until shortly before ovulation when the first meiotic division is completed. Only a small fraction of these oocytes will undergo
complete maturation and become ovulated during adult life. The fate of the vast majority of oocytes which will start to develop is atresia (Brambell, 1956). A major wave of atresia occurs shortly after hatching (Faure-Fremiet and Kaufman, 1928; Franchi, 1962). Pearl and Schoppe (1921) estimated that there are several thousand oocytes visible to the naked eye in the ovary of the adult hen, although the total number present in the ovary probably exceeds 1 million (Hutt, 1949).

Follicular development starts a few days after hatching when the oocytes become surrounded by a single layer of granulosa cells which are thought to be derived from the embryonic germinal epithelium (Brambell, 1956; Romanoff, 1960; Franchi et al., 1962; Callebaut, 1976). By day 10, post-hatching, primary follicles have been formed (Narbaitz and de Robertis, 1968). Shortly after (days 13 to 20 post-hatching) the cortex starts to proliferate and a series of grooves are formed. During the next 40 days this process continues and the grooves become deeper and progressively invaded by medullary tissue. At the same time the follicles show considerable growth and interstitial cells are seen in the developing theca layer as well as in the interfollicular spaces (Narbaitz and de Robertis, 1968). By the time sexual maturity is reached, which occurs between 150 and 180 days post-hatching (Nalbandov and Card, 1946), the cortex and medulla are no longer
anatomically distinct from one another (Prochaskova and Komarek, 1970). Therefore the terms zona parenchymatosa and zona vasculosa have been proposed (Prochazkova and Komarek, 1970) and adopted (King, 1979) to describe these two irregular masses of tissue which comprise the ovary of the adult hen. The zona parenchymatosa, derived largely from the cortex, contains developing follicles, whilst the underlying zona vasculosa is composed chiefly of medullary tissue (Gilbert, 1979).

The ovary of the immature bird contains follicles of up to 1 mm in diameter only (Nalbandov and Card, 1946; Taber, Claytor, Knight, Gambrell, Flowers and Ayers, 1958). As sexual maturity approaches, some follicular growth occurs and the ovary may contain a number of small follicles of up to approximately 6 mm in diameter in the early stages of yolk deposition. Immediately before laying commences, some of these small follicles undergo a further increase in diameter due largely to the deposition of yolk within the oocyte. 5 to 7 large yellow-yolky follicles become suspended from the surface of the ovary (Lofts and Murton, 1973) forming a hierarchy of follicles of increasing size. The largest of these, usually termed the F1 follicle, reaches a diameter of approximately 35 mm just before ovulation. Although pituitary gonadotrophins are believed to be responsible for stimulating the development of the hierarchy of follicles (Hill and Parkes, 1935; Opel and Nalbandov, 1958; 1961; Mitchell,
1967a; 1967b), attempts to stimulate precocious follicular development in immature chicks using either mammalian or avian gonadotrophin preparations have been unsuccessful (Domm, 1931; Domm and Van Dyke, 1932; Asmundsen and Wolfe, 1935; Nalbandov and Card, 1946; Taber, 1947; Das and Nalbandov, 1955; Taber, Claytor, Knight, Gambrell, Flowers and Ayers, 1958). Avian pituitary preparations however, were slightly more effective, in that they were able to bring about an increase in the numbers of small follicles of approximately 1 mm in diameter (Taber et al., 1958). Das and Nalbandov (1955) also obtained some follicular growth of up to 9 mm in diameter, following injections of chicken anterior pituitary preparations to immature chicks.

The ovary of the laying fowl contains a large number of small developing follicles in addition to the 5 or 7 large preovulatory follicles. There may be as many as 100 or so follicles with diameters between 1 and 8 mm (Griffin, Perry and Gilbert, 1983; Gilbert, Perry, Waddington and Hardie, 1983). Within this group of follicles, the rate of atresia is high; the number of viable follicles being inversely related to follicle size (Gilbert et al., 1983). There were about 20 viable follicles with a diameter between 1 and 2 mm, but only one follicle of 7 to 8 mm in diameter. In many ovaries, follicles of 7 to 8 mm in diameter were absent. Follicles larger than 8 mm in diameter comprised the graded series of follicles
Legend to Plate 1.

The ovary of the laying hen.
destined for ovulation, and rarely became atretic. Gilbert et al (1983) have therefore suggested that atresia plays a prominent role in maintenance of the follicular hierarchy of the hen ovary.

The mechanism whereby a follicle escapes atresia and embarks upon the final phase of growth culminating in ovulation is not known. Gilbert and Woodgush (1970) have suggested that regulation of blood-borne gonadotrophins to the follicle by local nervous control could be important. FSH has been implicated since preparations of mammalian gonadotrophins rich in FSH have been shown to cause the growth of many large follicles simultaneously (Bates, Lahr and Riddle, 1935; Fraps and Riley, 1942; Nalbandov and Card, 1946) when injected into laying hens. Egg-laying usually ceases within a few days, but ovulations can be brought about by injection of LH (Fraps and Riley, 1942; Nalbandov and Card, 1946). Studies using hypophysectomised laying hens have shown that crude preparations of chicken gonadotrophins are more effective than mammalian preparations in promoting follicular growth (Opel and Nalbandov, 1958; Mitchell, 1967a; 1967b). Similar results have been obtained following injections of gonadotrophins to hens with regressed ovaries induced by either starvation (Morris and Nalbandov, 1961) or drug treatment (Imai, 1977). The latter author reported the formation of a graded series of follicles containing yellow yolk following treatment with a
chicken anterior pituitary extract. Mitchell (1967b) also obtained follicles of graded size in hypophysectomized hens with regressed ovaries after treatment with a similar preparation but none of the follicles developed to normal ovulatory size (i.e. > 30 mm in diameter). A crude preparation of avian FSH was found to be less effective (Mitchell, 1970), indicating that LH may be required in addition to FSH.

A cross section through one of the large preovulatory follicles is shown in figure 1.1. The various layers, which are not drawn to scale, are from the oocyte outwards, the perivitelline layer, the granulosa layer, the basement lamina, the theca layer and its connective tissue coat and finally a layer of epithelium. The theca layer which consists of connective tissue traversed by blood vessels and nerves (Perry, Gilbert and Evans, 1978) has become differentiated into the theca interna and theca externa. The latter, comprising the majority of the theca layer, is composed of fibroblast cells arranged in a stratified manner. This layer surrounds the theca interna, a narrower zone which is less dense in appearance (Peel and Bellairs, 1972; Perry et al, 1978). The interstitial cells form flattened patches distributed at intervals around the circumference of the theca interna and are often associated with nerve fibres (Dahl, 1970a; 1970b; Perry et al, 1978). In follicles of less than 5 mm in diameter, groups of interstitial cells appeared to be
Legend to Figure 1.1

A cross section of a large preovulatory follicle.
THECA EXTERNA
THECA INTERNA (TI)
BASAL LAMINA (BL)
GRANULOSA CELLS (GC)
PERIVITELLINE LAYER (PV)
OOCYTE PLASMA MEMBRANE (OM)
YOLK (Y)
surrounded by enclosing cells and delineated by a basement membrane (Dahl, 1970a; Peel and Bellairs, 1972). These so-called "thecal glands" were not seen by Perry et al (1978) in the large preovulatory follicles.

Follicular rupture occurs at the stigma, a pale relatively avascular area at the apex of the follicle (Nalbandov and James, 1949) which lacks the connective tissue coat surrounding the rest of the follicle (Guzsal, 1966). Immediately before follicle rupture, the stigma widens and becomes more translucent (Phillips and Warren, 1937; Neher, Olsen and Fraps, 1950; Nalbandov, 1961; Yoshimura and Fujii, 1979). The stigma tears and the oocyte is extruded from the follicular sac which collapses (Nalbandov, 1961). Although ovulation is known to occur in response to the plasma surge of LH (Fraps and Riley, 1942; Fraps, Fevold and Neher, 1947; Rothchild and Fraps, 1949; Fraps, 1961), little is known about the mechanism whereby LH is able to bring about follicle rupture. It appears that LH sets in motion a sequence of events culminating in degradation of the follicle wall at the stigma (Yoshimura and Fujii, 1979). These changes have been established by the last 1 to 2 hours prior to ovulation, since follicles removed from the ovary during this period but not earlier, are capable of ovulating spontaneously in vitro (Neher, Olsen and Fraps, 1950; Ogawa and Nishiyama, 1969).
Various proposals have been put forward to explain the mechanism of follicle rupture in the hen. These hypotheses have included an increase in hydrostatic pressure (Bartelmez, 1912), smooth muscle contraction (Phillips and Warren, 1937), nervous stimulation (Ferrando and Nalbandov, 1969) and necrosis of the stigma (Nalbandov, 1961), but do not appear entirely satisfactory (see reviews by Van Tienhoven, 1961; Gilbert, 1971a; Gilbert, 1979). It is probable that degradation of the follicle wall at ovulation is brought about by the action of proteolytic enzymes (Nakajo, Zakaria and Imai, 1973; Yoshimura and Fujii, 1979; Tojo, Fujii and Ogawa, 1982) as in other species (Espey and Lipner, 1965; Espey and Coons, 1976; Espey, 1974; Beers, 1975). However, the mechanism whereby these changes are brought about in response to LH is not understood. Prostaglandins also appear to be necessary for follicular rupture in the mammal; in the rat (Bauminger and Lindner, 1975) and the rabbit (LeMaire, Yang, Behrman and Marsh, 1973) there is an increase in follicular prostaglandin levels (PGE and PGF) before ovulation. Indomethacin, a prostaglandin synthesis inhibitor (Flower, Gryglewski, Herbaczynska and Vane, 1972) blocked ovulation both in the rabbit (O'Grady, Caldwell, Auletta and Speroff, 1972) and the rat (Armstrong and Grinwich, 1972; Tsafriri, Lindner, Zor and Lamprecht, 1972). In the hen, a rise in both PGE (Hammond, Olsen, Frenkel, Biellier and Hertelendy,
13.

and PGF (Day and Nalbandov, 1977) levels was seen in the preovulatory follicle several hours before ovulation. Day and Nalbandov (1977) concluded however, that prostaglandin synthesis may not be essential for follicle rupture since indomethacin was unable to block ovulation in the hen. There is some evidence that progesterone synthesis in response to LH facilitates follicle rupture in the hen (Tojo and Huston, 1981). Progesterone has also been implicated in the ovulatory process in other species (Rondell, 1974; Sridharen, Meyer and Karavolos, 1974; Takahashi, Ford, Yoshinaga and Greep, 1974). In the isolated perfused rabbit ovary collected after the surge of LH, addition of progesterone to the medium significantly enhanced the number of ovulations obtained in vitro (Hamada, Wright and Wallach, 1979). Similar results have been reported recently by Testart, Thebault and Lefevre (1983) using isolated rabbit follicles collected after the LH surge.

Following ovulation in the hen, the post-ovulatory follicle is not transformed into a corpus luteum (Aitken, 1966) and its 3β-hydroxysteroid dehydrogenase activity declines rapidly (Armstrong, Davidson, Gilbert and Wells, 1977). Progesterone levels fell rapidly (Dick, Culbert, Wells, Gilbert and Davidson, 1978) whilst PGF levels increased progressively reaching a maximum 24 hours later (Day and Nalbandov, 1977). The post-ovulatory follicle is believed to control the timing of oviposition since its removal (Rothchild and Fraps, 1944;
Tanaka and Nakada, 1974) results in delayed oviposition, as does removal of, or damage to, the granulosa cells of the post-ovulatory follicle (Gilbert, Davidson and Wells, 1978). Oviposition can also be delayed by indomethacin treatment (Hertelendy, 1974; Hertelendy and Biellier, 1978) or induced by exogenous prostaglandins (Hertelendy, Biellier and Todd, 1975). Although it was postulated that prostaglandins produced by the post-ovulatory follicle (Day and Nalbandov, 1977) may be responsible for acting on the shell-gland to induce oviposition, conflicting evidence was obtained by Tanake (1976) and Tanake and Goto (1976). They concluded that the substance produced by the post-ovulatory follicle which controls the timing of oviposition is neither a steroid nor a prostaglandin.

1:2 Steroidogenesis in the ovary of the hen

There are two cell types within the ovary of the hen which are thought to be responsible for synthesising steroid hormones, namely the interstitial cells and the granulosa cells. Before reviewing the evidence linking each of these cell types with steroidogenesis (see sections 1:2:2 and 1:2:3 respectively), a short account outlining the sequence of enzyme reactions involved will be given (section 1:2:1). Since few detailed studies have been undertaken regarding these enzyme reactions in hen ovarian tissue, information derived from various mammalian steroidogenic tissues...
(i.e. ovary, testis, adrenal and placenta) has been included.

1:2:1 **Enzyme reactions involved in steroidogenesis**

The first reaction in the synthesis of steroids, which is also the rate-limiting step of the pathway, is the conversion of cholesterol to pregnenolone. Unlike the subsequent transformations which take place in the endoplasmic reticulum, this reaction occurs within the mitochondrion, probably on the inner mitochondrial membrane (Tamaoki, 1973). Conversion of cholesterol to pregnenolone is catalysed by cholesterol side-chain cleavage enzyme complex. This is a mixed function oxidase which requires cytochrome P450 as the terminal oxidase (Simpson and Boyd, 1967; Mason and Boyd, 1971). Both 20α-hydroxycholesterol and 20α, 22-dihydroxycholesterol have been identified as intermediates in this reaction (Shimizu, Gut and Dorfman, 1962; Hall and Young, 1968). In the fowl, Wells, Dick and Gilbert (1981a) demonstrated the conversion of [4-14C]cholesterol to [4-14C]progesterone by granulosa cell monolayers in vitro. Aminoglutethimide also inhibited LH-stimulated progesterone production by the granulosa cells (Well et al, 1981a). Aminoglutethimide inhibits 20α-hydroxycholesterol formation, probably by preventing the binding of cholesterol to the cytochrome P450 component of the cholesterol side-chain cleavage system (McIntosh, Mitani, Uzgiris, Alonso and Salhanick, 1973).
There are two major pathways by which pregnenolone may be metabolized (Ryan and Smith, 1961; Short, 1961) within the ovary. These have become known as the \( \Delta^4 \)-pathway and the \( \Delta^5 \)-pathway respectively (see figure 1.2). In the former, pregnenolone is converted to androstenedione via the \( \Delta^4 \)-3-ketosteroids progesterone and 17\( \alpha \)-hydroxyprogesterone, whilst in the latter, the 3\( \beta \)-hydroxysteroids, 17\( \alpha \)-hydroxypregnenolone and dehydroepiandrosterone serve as intermediates. In addition, testosterone may also be produced from dehydroepiandrosterone via the formation of androstenediol. The presence of both these pathways in ovarian homogenates from the laying hen was demonstrated by Nakamura, Tanabe and Katukawa (1974). The present study however, deals only with the \( \Delta^4 \)-pathway.

The conversion of pregnenolone to progesterone is carried out by the joint action of two closely associated enzymes, namely 3\( \beta \)-hydroxy steroid NAD\(^+\) oxidoreductase and 3-oxo steroid \( \Delta^4 \)-\( \Delta^5 \)-isomerase. These two enzymes are known collectively as 3\( \beta \)-hydroxysteroid dehydrogenase. The presence of this enzyme in the ovary of the domestic fowl was demonstrated by Armstrong and Wells (1976; 1980). More detailed studies using granulosa cell homogenates from the large preovulatory follicles showed that this enzyme is located on the endoplasmic reticulum (Armstrong, 1979; 1982a). Progesterone and androstenedione acted as competetive inhibitors for this enzyme, the effect being more marked when pregnenolone was used as
Legend to Figure 1.2

Pathways of steroidogenesis in the ovary.

1. cholesterol side-chain cleavage complex
2. 3β-hydroxysteroid oxido-reductase
3. 17α-hydroxylase
4. C-17,20 lyase
5. aromatase complex
6. 17β-hydroxysteroid dehydrogenase
a substrate rather than dehydroepiandrosterone. Oestradiol also inhibited $3\beta$-hydroxysteroid dehydrogenase activity but to a lesser extent; the nature of this inhibition was not established (Armstrong, 1982a).

The conversion of progesterone to androstenedione is carried out by an enzyme complex containing 17$\alpha$-hydroxylase and C,17-20lyase (Slaunwhite and Samuels, 1956; Chaselow, Marr and Taylor, 1982). 17$\alpha$-Hydroxyprogesterone is an intermediate in this reaction which requires NADPH and involves cytochrome $P_{450}$ (Betz, Tsai and Weakly, 1975). In the rat testis, the microsomal lyase complex was stimulated by a hormone dependent regulator protein present in the cytosol (Chaselow, 1979).

The conversion of androstenedione to oestrone and of testosterone to oestradiol is catalysed by the aromatase complex (often referred to as oestrogen synthetase), a mixed function oxidase consisting of at least two components, a specific cytochrome $P_{450}$ and the flavoprotein NADPH-cytochrome-c-reductase (Thompson and Siiteri, 1974a, 1974b; Bellino and Hussa, 1982). NADPH and oxygen are required for this reaction (Thompson and Siiteri, 1974a). The mechanism of aromatization has been studied in detail using placental preparations from a number of species (Ryan, 1959; Ainsworth and Ryan, 1966; see reviews by Engel, 1973; Brodie, 1979) and probably involves three successive hydroxylation steps. 19-Hydroxyandrostenedione and
19-oxo-androstenedione have been isolated as intermediates using androstenedione as a substrate (Meyer, 1955; Longchampt, Gual, Ehrenstein and Dorfman, 1960; Hollander, 1962; Aktar and Skinner, 1968).

The reversible oxidation of oestradiol to oestrone is catalysed by $17^\beta$-hydroxysteroid dehydrogenase. This NAD$^+$ dependent enzyme has been isolated from the human placenta and is known as $17^\beta$-oestradiol dehydrogenase because of its high specificity for oestradiol as a substrate (Langer and Engel, 1958; Karavolas, Baedecker and Engel, 1970; Karavolas and Engel, 1971). An enzyme with similar properties has been isolated from the ovary of the sheep (Kautsky and Hagerman, 1970; Michel, Nicholas and Crastes de Paulet, 1975). $17^\beta$-Hydroxysteroid dehydrogenase preparations from the rabbit ovary however, utilized oestradiol and testosterone equally well as substrates (Rodway and Rahman, 1978).

From these results, it is not clear whether there is a single or two separate ovarian enzymes for catalysing the reversible oxidation of testosterone and oestradiol.

1:2:2 **Interstitial cells**

The interstitial cells of the avian ovary have been postulated as a site of steroidogenesis on the basis of a number of histological studies (Fell, 1924; Benoit, 1926; Marshall and Coombs, 1957; Narbaitz and de Robertis, 1968; Dahl, 1970a, 1970b, 1971a; Peel and Bellairs, 1972; Perry et al, 1973). These cells, which have been detected in the
ovarian medulla from about the seventh day of embryonic life onwards (Narbaitz and de Robertis, 1968), contained abundant smooth endoplasmic reticulum, mitochondria with tubular cristae, lipid droplets and cholesterol (Scheib, 1959; Narbaitz and Sabatini, 1963; Narbaitz and Adler, 1966; Simone-Santora, 1968), all features considered to be typical of steroid-producing cells (Christensen and Gillim, 1969). The presence of 3β-hydroxysteroid dehydrogenase, a key enzyme found in all steroid-producing tissues (Jacoby, 1962), has also been demonstrated histochemically (Chieffi, Manelli, Botte and Mastrolia, 1964; Narbaitz and Kolodny, 1964; Scheib and Haffen, 1968).

The appearance of interstitial cells in the ovarian medulla coincides closely with the onset of steroidogenesis in the embryonic ovary. Progesterone, androgens and oestrogens were isolated from the ovaries of 10 day old embryos onwards (Wolff, 1946; Gallien and le Fougoc, 1957; Tanabe, Nakamura, Fujioka and Doi, 1979). Other studies have shown that ovaries obtained from 7½ to 18 day old embryos can synthesize progesterone, androgens and oestrogens in vitro (Weniger, 1965, 1966; Guichard, Cedard, Mignot, Scheib and Haffen, 1977, 1979) and were also capable of converting radioactively labelled precursors, including sodium [1-14C]acetate, [3H]pregnenolone, [4-14C]progesterone and [4-14C]-dehydroepiandrosterone to oestrogens (Cedard and Haffen, 1966; Weniger, Ehrhardt and Fritig, 1967; Haffen and Cedard, 1968; Cedard, Guichard and Haffen, 1970; Weniger
and Zeiss, 1971; Guichard, Cedard and Haffen, 1973). Ovarian slices from 7, 10 and 15 day old embryos converted [4-14C]progesterone to androgens and oestrogens (Galli and Wasserman, 1972, 1973). Following treatment of embryonic ovaries with collagenase, Teng and Teng (1977; 1979) prepared a fraction believed to contain interstitial cells. These cells when grown as monolayers secreted testosterone and oestradiol. The addition of hCG enhanced steroid synthesis, possibly by a mechanism involving cAMP.

In the ovary of the adult hen, interstitial cells, derived from the embryonic medulla (Narbaitz and de Robertis, 1968), are found in the theca layer of developing follicles as well as in the ovarian stroma. These cells, like their embryonic counterparts, give a positive histochemical reaction for both 3β-hydroxy-steroid dehydrogenase and 17β-hydroxysteroid dehydrogenase (Chieffi and Botte, 1965; Wyburn and Baillie, 1966; Boucek and Savard, 1970; Peel and Bellairs, 1972) and show ultra-structural characteristics typical of steroid-secreting cells (Dahl, 1970a; Peel and Bellairs, 1972; Perry et al, 1978). Variations in the ultra-structure of the interstitial cells (Marshall and Coombs, 1957; Dahl, 1971a, 1971b) and in the intensity of the dehydrogenase reactions (Boucek and Savard, 1970; Peel and Bellairs, 1972) have also been correlated with the transition from one reproductive state to another and the associated changes in plasma steroid levels. These
and other experiments in which ovarian slices (Boucek and Savard, 1970) or ovarian homogenates (Nakamura et al., 1974) from laying hens were shown to convert radioactive precursors into androgens and oestrogens, support the hypothesis that the interstitial cells of the hen ovary are a site of androgen and oestrogen synthesis in vivo (Gilbert, 1971b).

Analysis of steroid levels within the ovary of the laying hen indicated that the small follicles together with the ovarian stroma, may be a major site of oestrogen synthesis (Senior and Furr, 1975). Nevertheless, subsequent work has concentrated on the large preovulatory follicles. Thecal tissue from these follicles contained both androgens and oestrogens in addition to progesterone (Allen, Whitsett, Hardy and Kneibert, 1924; Marlow and Richert, 1940; Shahabi, Norton and Nalbandov, 1975a; Nakamura, Tanabe and Hirano, 1979). Of the six largest follicles present in the ovary of the domestic fowl, Nakamura et al. (1979) found that oestrogen levels were highest in the third or fourth largest follicles (F3 and F4 respectively), but thereafter decreased as the follicle increased in size (Shahabi et al., 1975a; Nakamura et al., 1979). Similar changes in thecal oestrogen content have been reported in quail preovulatory follicles (Kumagai and Homma, 1974). The progressive decline in both 3β-hydroxysteroid dehydrogenase (Armstrong, 1982b) and aromatase activity (Armstrong, 1983) in thecal homogenates from the large
preovulatory follicles (F5 > F4 > F3 > F2 > F1) may account for these observations.

Collagenase-dispersed theca cells from the third largest follicle (F3) secreted greater amounts of oestradiol into the culture medium than did those obtained from the second largest follicle (F2), whilst thecal cells from the largest follicle (F1) synthesized negligible amounts of oestradiol in vitro (Huang, Kao & Nalbandov, 1979). Thecal cells from F3 follicles were also able to convert greater amounts of exogenous testosterone to oestradiol than those from F2 follicles (Huang, Kao & Nalbandov, 1979; Wang and Bahr, 1983). These results suggest that during the final period of follicular maturation, oestrogen secretion by the pre-ovulatory follicles decreases. Wang and Bahr (1983) have also measured oestradiol production by thecal cells cultured in the presence of $1 \times 10^{-6}$M testosterone at various periods of the ovulatory cycle. A progressive decrease in oestradiol production was observed by thecal cells from F3 follicles collected 24, 18, 12 and 2 hours before an expected ovulation. Oestradiol production by thecal cells collected 6 hours before ovulation however, was significantly higher than at 18 or 12 hours before ovulation, although still significantly lower compared with 24 hours before ovulation. Over the same period, basal oestradiol secretion increased slightly from 24 hours to 6 hours before ovulation and then decreased significantly 2 hours
before ovulation. These results demonstrate a possible stimulatory effect of LH on thecal steroidogenesis since in the laying hen there is a plasma surge of LH 4 to 7 hours before ovulation (Cunningham and Furr, 1972; Furr, Boney, England and Cunningham, 1973; Shodono, Nakamura, Tanabe and Wakabayashi, 1973; Wilson and Sharp, 1973; Johnson and van Tienhoven, 1980). With the exception of the first and second largest follicles (Huang, Kao & Nalbandov, 1979; Wang and Bahr, 1983), an increased synthesis of oestrogens by the interstitial cells of the developing follicles may be responsible for the plasma surge of oestrogens which occurs 4 to 7 hours before ovulation (Peterson and Common, 1972; Shodono et al, 1973; Senior, 1974b; Senior and Cunningham, 1974; Johnson and van Tienhoven, 1980).

1:2:3 Granulosa cells

The granulosa cells, unlike the interstitial cells which appear to be active from early on in embryonic life onwards (see section 1:2:2), do not begin to show a positive histochemical reaction for 3β-hydroxysteroid dehydrogenase until the follicle has reached a size of approximately 5 mm in diameter (Davidson, Gilbert and Wells, 1979). Thereafter the reaction becomes more intense with increasing follicle size (Davidson et al, 1979), being marked in the post-ovulatory follicle collected immediately after ovulation (Wyburn and Baillie, 1966; Narbaitz and de Robertis, 1968; Armstrong et al, 1979). Armstrong (1982b) using granulosa cell
homogenates from the 5 largest follicles, also obtained a positive correlation between 3β-hydroxysteroid dehydrogenase activity and follicle size. These results imply that the granulosa cells are only steroidogenically active during the final phase of rapid follicular growth prior to ovulation.

Furr (1969) first identified the large preovulatory follicles as a major site of progesterone synthesis within the ovary of the laying hen, whilst Dick, Culbert, Wells, Gilbert and Davidson (1978) and Culbert, Hardie, Wells and Gilbert (1980) subsequently showed that the granulosa layer of these follicles contains large amounts of progesterone. The rise in plasma progesterone levels 4 to 7 hours before ovulation (Cunningham and Furr, 1972; Furr et al., 1973; Shodono et al., 1975; Johnson and van Tienhoven, 1980) has been attributed to an increased synthesis of progesterone by these cells (see Culbert et al., 1980).

Recent studies have utilized pure preparations of granulosa cells isolated from the large preovulatory follicles (Gilbert, Evans, Perry and Davidson, 1977) and incubated in vitro (Gilbert, Evans, Perry and Wells, 1978; Huang and Nalbandov, 1979). These studies have shown that the granulosa cells secrete progesterone under the influence of LH (Hammond, Todd and Hertelendy, 1978; Huang and Nalbandov, 1979b; Hammond, Burke and Hertelendy, 1980; Hammond, Todd and Hertelendy, 1980; Scanes and Fagioli, 1980; Wells, Gilbert and Culbert,
1980; Zakar and Hertelendy, 1980a, 1980b; Hammond, Burke and Hertelendy, 1981). LH has also been shown to stimulate progesterone synthesis by the granulosa cells from the 5 largest preovulatory follicles in vivo (Culbert, Hardie, Wells and Gilbert, 1980). The mechanism whereby LH enhances steroidogenesis is not understood, but probably involves binding of LH to cell surface receptors (Gilbert, Davidson and Wells, 1983) and stimulation of adenyl cyclase activity in the cell membrane (Calvo, Wang and Bahr, 1981). The subsequent increase in steroidogenesis may involve increased intracellular synthesis of cAMP (Zakar and Hertelendy, 1980a, 1980b) and protein (Wells, Gilbert and Culbert, 1981b).

The available evidence suggests that the granulosa cells become increasingly responsive to LH during the final phase of maturation. Armstrong (1982b) has measured an increase in 3β-hydroxysteroid dehydrogenase activity during this period, whilst Calvo et al (1981) and Calvo and Bahr (1982) have shown that both basal and LH-stimulable adenyl cyclase activity of granulosa cells from the three largest follicles increased with follicle size (F1 > F2 > F3). These changes could be explained by an increase in granulosa cell LH receptors during the final period of follicle growth as has been shown to occur in the rat (Uilenbroek and Richards, 1979). A preliminary report by Ritzhaupt (1983) however, has indicated that in fowl preovulatory follicles the ability of granulosa cells to bind ovine LH is negatively
correlated with follicle size (i.e. greatest in F5 and lowest in F1). Should these findings prove correct, the mechanism whereby the granulosa cell adenyl cyclase system becomes increasingly sensitive to LH stimulation during the final phase of maturation remains obscure. Nevertheless, these changes ensure that by the time the follicle has reached ovulatory size, it is capable of secreting large amounts of progesterone in response to LH.

In contrast to LH, FSH does not appear to play an important role in regulating granulosa cell steroidogenesis in the large preovulatory follicles. Although FSH has been shown to exert a stimulatory action on progesterone production by granulosa cells from these follicles in vitro (Huang, Kao & Nalbandov, 1979; Scanes and Fagioli, 1980; Hammond et al, 1981), the doses used were high and exceeded the normal physiological range found in laying hen plasma. Hammond et al (1981) were able to demonstrate that the stimulatory effect of FSH on progesterone production by cultures of granulosa cells from the largest follicle was entirely due to contamination with LH. Nevertheless, FSH may play a role in enhancing progesterone secretion in the smaller follicles.

Whilst granulosa cells isolated from the large preovulatory follicles produced large amounts of progesterone in vitro, the same cultures synthesized negligible amounts of androgens and oestrogens (Huang, Kao & Nalbandov, 1979; Wells et al, 1980). It appears
that avian granulosa cells, like their mammalian counterparts (Short, 1964), are unable to convert progesterone to androstenedione due to a deficiency of 17α-hydroxylase and/or C17-20lyase. Granulosa cells from hen preovulatory follicles are also unable to aromatize exogenous androgens to oestrogens (Huang, Kao and Nalbandov, 1979). A more recent study by Armstrong (1983) has confirmed that the granulosa cells of hen preovulatory follicles lack aromatase activity. In this respect, avian granulosa cells differ from their mammalian counterparts which possess an active aromatase system (Ryan and Short, 1965; Dorrington, Moon and Armstrong, 1975; Makris and Ryan, 1975; Erickson and Ryan, 1975; Armstrong and Papkoff, 1976; Fortune and Armstrong, 1978; Erickson and Hseuh, 1978) and which are believed to aromatize androgens, derived from the theca layer (Erickson and Ryan, 1976; Fortune and Armstrong, 1977; Tsang, Moon, Simpson and Armstrong, 1979; Tsang, Armstrong and Whitfield, 1980), to oestrogens (Falck, 1959; Bjersing and Carstensen, 1964; Armstrong and Papkoff, 1976; Baird, 1977). However, in some species, notably the primates, the theca layer of developing preovulatory follicles may also make a substantial contribution to follicular oestrogen production (Channing, 1969; Channing and Coudert, 1976; see review by Ryan, 1979). In the hen follicle it is likely that oestrogen synthesis is limited exclusively to the theca layer (see 1:2:2). Indirect evidence
suggests that in some other species such as the pocket gopher, this may also be the case (Mossman and Duke, 1973). It has been postulated by Huang, Kao and Nalbandov (1979) that progesterone produced by the granulosa cells of the large preovulatory follicles may be utilized by the theca layer for oestrogen synthesis. Thus, thecal cells from the third largest follicle when cultured with equal numbers of granulosa cells, produced approximately six times the amount of oestrogen than did thecal cells cultured on their own.
2. MATERIALS AND METHODS
2:1 Management of experimental hens

Hens of commercial egg-laying hybrid strains (Shaver 288, H. & N. Nick Chick or Ross Browns) were housed individually in cages and provided with food and water ad lib. The hens were maintained on a 14 hour:10 hour light/dark cycle, with lights on at 04.00 hours and lights off at 18.00 hours. Each cage was fitted with an automatic timer which recorded oviposition times to within one minute.

Laying hens, aged between 6 and 8 months were killed with an overdose of sodium pentobarbitone (Expiral, Ceva, Southampton) at either 1 to 2 hours or approximately 15 hours before an expected ovulation. The expected time of ovulation was estimated by examining the previous egg-laying record and assuming that ovulation would occur within ± 60 minutes of oviposition (Gilbert, Davidson and Wells, 1978). The oviducts and shell glands of the hens were routinely examined for recently ovulated oocytes and for eggs in their various stages of calcification. In this way it was confirmed that all the hens selected were at the stage of the ovulatory cycle predicted by their laying records.

2:2 Collection and processing of tissue

After killing the hens, the ovary was immediately removed and placed in 0.9% saline at room temperature. In the first experiment (see section 3.1) the 5 to 7 largest follicles were collected and the remainder of the ovary discarded. These follicles ranged in diameter from approximately 12 mm to approximately 35 mm (see table 2.1).
Table 2.1 The diameter of the large yellow-yolky follicles present in the ovary of the laying hen.
Data from 5 laying hens (Ross Browns) collected shortly before ovulation.

<table>
<thead>
<tr>
<th>Follicle No.</th>
<th>Mean Diameter, mm</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>34.5 (32)*</td>
<td>33.5 - 35.9</td>
</tr>
<tr>
<td>F2</td>
<td>30.3 (30)</td>
<td>28.7 - 31.4</td>
</tr>
<tr>
<td>F3</td>
<td>27.7 (26)</td>
<td>23.9 - 29.3</td>
</tr>
<tr>
<td>F4</td>
<td>25.5 (22)</td>
<td>23.8 - 27.1</td>
</tr>
<tr>
<td>F5</td>
<td>22.1 (17)</td>
<td>20.3 - 24.1</td>
</tr>
<tr>
<td>F6</td>
<td>17.3 (12)</td>
<td>16.6 - 18.3</td>
</tr>
<tr>
<td>F7</td>
<td>12.1</td>
<td>11.5 - 12.8</td>
</tr>
</tbody>
</table>

* Corresponding data for single-comb White Leghorns (Etches, MacGregor, Morris and Williams, 1983).
Table 2.2  The weights (g) of individual follicles present in the hierarchy of the ovary of three laying hens (Shaver 288).

<table>
<thead>
<tr>
<th>Follicle Number*</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIRD 1</td>
<td>12.30</td>
<td>9.78</td>
<td>6.99</td>
<td>4.26</td>
<td>1.81</td>
<td>0.72</td>
<td>-</td>
</tr>
<tr>
<td>BIRD 2</td>
<td>14.31</td>
<td>13.07</td>
<td>9.22</td>
<td>7.45</td>
<td>4.15</td>
<td>1.64</td>
<td>0.53</td>
</tr>
<tr>
<td>BIRD 3</td>
<td>17.93</td>
<td>14.74</td>
<td>11.29</td>
<td>7.69</td>
<td>4.15</td>
<td>1.44</td>
<td>0.62</td>
</tr>
<tr>
<td>Mean Weight (g)</td>
<td>14.85</td>
<td>12.53</td>
<td>9.17</td>
<td>6.47</td>
<td>3.37</td>
<td>1.27</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* The follicles are numbered consecutively, starting with the largest which is designated as Fl.
In this study, follicles of greater than 10 mm in diameter were considered to be part of the hierarchy of follicles containing yellow yolk. Following removal from the ovary, the 5 to 7 largest follicles (F1 to F7) were weighed. The follicles were then slit across the stigma using a scalpel blade and the yolk, with its surrounding layer of granulosa cells, separated from the theca layer. By this procedure, the granulosa layer is completely separated from the theca layer (Gilbert, Evans, Perry and Davidson, 1977). Each theca was blotted dry, weighed and kept on ice to await homogenization. By subtraction of the theca weight from the original follicle weight, the weight of yolk present in the follicle was obtained. In addition, the yolk from the hard-shelled egg present in the shell-gland was weighed. For each hen, these yolk weights were compared to ensure that a normal hierarchy of follicles was present in the ovary.

Table 2.2 gives follicle weights from 3 hens which were considered to have normal hierarchies. Although there can be considerable variation between hens, successive follicle weights within individual hens usually differ by approximately 2 to 3 g. A difference of 4 g or more between follicles would indicate a gap (i.e. missing follicle) in the hierarchy which is not uncommon (Warren and Conrad, 1939; Lacassagne, 1960; Gilbert, 1972).

In subsequent experiments, concerned with steroid metabolism by the smaller ovarian follicles, the large follicles > 10 mm in diameter were removed from the ovary.
These were discarded, with the exception of the second largest follicle (F2), which was retained as a representative of this group of follicles for purposes of comparison.

Of the remaining ovarian follicles, all those with a diameter $> 2$ mm were removed and divided into two arbitrary groups according to their size. The first group consisted of follicles with a size range of $> 4$ mm to $< 10$ mm in diameter (pool 1). Deposition of yellow yolk had commenced in this group. The second group of follicles consisted of those with a size range of $> 2$ mm to $< 4$ mm (pool 2). The follicles present in each pool were then slit with a scalpel blade and the yolk removed by squeezing gently. (In one experiment (3.4) however, the follicles present in pool 2 were homogenized whole.) This procedure does not remove the granulosa layer, since previous histological experience has shown that at this stage of follicular development it remains firmly attached to the basal lamina.

After removal of the yolk from each group of follicles, the remaining tissue was blotted dry, weighed and kept on ice to await homogenization. The resulting two homogenates (pool 1 and pool 2 respectively) have been referred to as thecal homogenates throughout this thesis, although strictly speaking they should be regarded as homogenates of the follicle wall since they also contained granulosa cells. The reason for this is explained in a later section on page 133.

The zona vasculosa (Gilbert, 1979) was cut away from the rest of the ovary and discarded. The remainder of the ovary, the zona parenchymatosa (Gilbert, 1979) consists
Legend to Plate 2.1

Distribution of 3\(\beta\) -hydroxysteroid dehydrogenase in the zona parenchymatosa from two different hens. The upper picture was taken using x 25 objective and the lower picture using a x 10 objective. (Eyepiece x 17).

I - Interstitial cells.
of a mass of small follicles (of up to 2 mm in diameter). Following the removal of two representative portions for histological examination, it was blotted dry, weighed and kept on ice to await homogenization. Routine histology of the first portion confirmed that the correct tissue had been selected. The second portion was used to identify the distribution of the enzyme $3\beta$-hydroxysteroid dehydrogenase. For this purpose, it was frozen immediately in a mixture of solid carbon dioxide and isopentane and treated by the histochemical method of Pearse (1972) for the detection of $3\beta$-hydroxysteroid dehydrogenase. Sections of freshly frozen tissue were cut at 10µm and incubated for 30 minutes with dehydroepiandrosterone as substrate. The sections were then lightly counter-stained with neutral red to aid visual identification of ovarian structure. A positive reaction for this enzyme is characterized by the formation of blue-black deposits of diformazan. Typical results are shown in plate 2.1. The interstitial cells gave a positive reaction for this enzyme, whilst the granulosa cells were negative and consequently judged to be steroidogenically inactive (Tamaoki, 1973; Davidson et al, 1979).

The theca layer, pooled thecae (pool 1 and pool 2) and the zona parenchymatosa were each homogenized after the addition of 10 volumes of ice-cold sucrose ($0.25 \text{ mol/l}$) containing $\text{MgCl}_2$ ($20 \text{ mmol/l}$), $\text{KCl}$ ($20 \text{ mmol/l}$) and EDTA-tetrasodium salt ($5 \text{ mmol/l}$). Each tissue was homogenized on ice using several two second bursts with a Polytron
homogenizer at about 12,000 rpm. The resulting homogenate was used immediately either with or without centrifugation to remove nuclei and cell debris.

2:3 Estimation of protein in homogenates

The Bio-Rad assay was used to measure the protein concentration of the homogenates. This method is based on the observation that an acidic solution of the dye Coomassie Brilliant Blue G-250 has an absorbance maximum which increases from 465 to 595 nm when it binds to protein (Bradford, 1976). A stock solution of bovine serum albumin (BSA), fraction V, was made up at a concentration of 1 mg/ml in redistilled deionized water. A series of standards were prepared from this stock solution ranging from 10 to 100 µg BSA in 0.1 ml of water. After addition of 5 ml of the diluted dye reagent (1:4, v/v), the test tubes were covered with parafilm and inverted gently several times to ensure thorough mixing. The optical density was read at 595 nm (using a Pye-Unicam SP500 series 2 spectrophotometer) between 15 to 25 minutes after addition of the dye reagent. During this time the dye complex is stable (Bradford, 1976). The protein concentration of the samples was read from the resultant standard curve (see figure 2.1). The slight non-linearity of the response is due to the reagent itself (Bradford, 1976).

Using this method, it should have been possible to assay the homogenates directly but the large amounts of lipid present interfered strongly. To overcome this, protein extracts were prepared according to Lowry,
Legend to Figure 2.1

A typical standard curve obtained using the Bio-Rad assay for the determination of protein in extracts of ovarian homogenates. Bovine serum albumin was used as the standard (BSA) and the optical density (OD) read at 595 nm with a Pye-Unicam Series 2 Spectrophotometer.
Rosebrough, Farr and Randall (1951). Ice-cold 20% trichloroacetic acid was made up in ethanol:diethyl ether (1:1, v/v) and 1 ml added to an equal volume of homogenate at 2 to 3°C. After centrifugation for 10 minutes at 2°C and 2,000 rpm, the supernatant, which was cloudy due to the presence of lipid, was discarded. The precipitate was shaken with 1 volume of ethanol:diethyl ether (1:1, v/v) and recentrifuged. The residual protein pellet was dissolved in 1 ml of NaOH (0.5 mol/l). Two dilutions were prepared from each protein extract by taking either 50 μl or 150 μl, adding an equivalent volume of HCl (0.5 mol/l) and making up to 1 ml with redistilled deionized water. Addition of HCl was necessary to neutralize the NaOH, since this interferes strongly in the Bio-Rad assay. The above dilutions were chosen so that all samples gave a reading on the standard curve. Each dilution was assayed in duplicate, using a sample volume of 0.1 ml. From these results, the amount of protein in the original homogenate was calculated.

In order to determine both the intra and inter-assay variation, a standard protein extract was prepared by pooling several thecal homogenates. This extract, assayed at 6 different dilutions within one assay, gave a mean value (± standard error of the mean) of 2.2 ± 0.04 mg/ml of protein in the original homogenate, and an inter-assay coefficient of variation of 5%. Using the same standard protein extract (150μl, diluted as for samples) a mean value of 2.1 ± 0.05 mg/ml was obtained for 8 consecutive assays, giving an inter-assay coefficient of
variation of 6%. The remaining source of variation, was the procedure for extracting the protein. This was assessed by preparing 10 extracts from the same homogenate and assaying them in one assay. A mean value of $2.0 \pm 0.13$ mg/ml was obtained, with a coefficient of 20%.

2:4 Incubation and extraction of metabolites

In the first experiment (section 3:1) the thecal homogenates were centrifuged at 800 g for 10 minutes and the supernatant was used immediately. The precipitate, consisting of nuclei and cell debris, was discarded. Duplicate tubes containing [4-$^{14}$C]progesterone (1.7 nmol, specific activity 2.18 GBq/mmol) were incubated with 0.1 ml of the supernatant (which corresponded to 10 mg of fresh tissue) in a total volume of 0.5 ml phosphate buffer, pH 7.4, containing 0.5 μmol NADP+, 2.5 μmol glucose-6-phosphate and 2.5 μmol NAD+. The tubes were incubated for 1 hour in a water bath at 37°C with continuous shaking, using air as the gas phase. At the end of the incubation period, the tubes were placed on ice to stop the reaction. 20μg each of progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone and oestradiol were then added as carriers. The radioactive metabolites were extracted thrice with 5 volumes of dichloromethane and the extracts pooled. The extracts were washed three times with distilled water to remove water soluble substances before evaporating to dryness under air. Final traces of water were removed by storing the tubes in a vacuum desiccator (with anhydrous calcium sulphate as desiccant).
In all subsequent experiments, the homogenates were used immediately without prior centrifugation, and thecal or zona parenchymatosa homogenates were incubated as follows unless otherwise stated. Duplicate glass vials containing [4-\(^{14}\)C]progesterone (8.7 nmol, specific activity 2.18 GBq/mmol) or [4-\(^{14}\)C]testosterone (8.9 nmol, specific activity 2.15 GBq/mmol) were incubated with 0.2 ml of homogenate (equivalent to 20 mg of fresh tissue) in a total volume of 1 ml of phosphate buffer, pH 7.4, containing 5.0 μmol NAD\(^{+}\), 5.0 μmol glucose-6-phosphate and 1.0 μmol NADP\(^{+}\). The incubations were carried out for 3 hours in a shaking water bath at 37°C with oxygen: carbon dioxide (95%:5%, v/v) as the gas phase.

2:5 Separation of metabolites

2:5:1 Chromatography

The radioactive metabolites were separated by thin-layer chromatography (t.l.c.) using silica gel coated glass plates, 20 cm by 20 cm (Merck F\(254\)), or by instant-thin-layer chromatography (i.t.l.c.) using glass fibre sheets, 20 cm by 20 cm, impregnated with silica gel and incorporating a fluorophor (ITLC-SAF, Gelman Hawksley Ltd.). The latter has the advantage of much faster separation times, in addition it can be cut with scissors into strips. Table 2.3 shows the type of separation achieved using the following one-dimensional solvent systems:

A) i.t.l.c. systems

System 1. A partition system with propylene glycol as the stationary phase and carbon tetrachloride: light petroleum, b.p. 40 to 60°C, (9:1, v/v), as the
Table 2.3 The $R_f$ values for steroid separation achieved with various solvent systems.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>SOLVENT SYSTEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5$\alpha$-androstane-3,17-dione</td>
<td>69 72 36</td>
</tr>
<tr>
<td>5$\alpha$-androstane-3,17-dione</td>
<td>79 45</td>
</tr>
<tr>
<td>5$\beta$-pregnane-3,20-dione</td>
<td>83 50</td>
</tr>
<tr>
<td>5$\alpha$-pregnane-3,20-dione</td>
<td>59</td>
</tr>
<tr>
<td>androstenedione</td>
<td>69 27 63 62 26 51</td>
</tr>
<tr>
<td>progesterone</td>
<td>86 57 36 58</td>
</tr>
<tr>
<td>5$\beta$-androstan-3$\alpha$-ol-17-one</td>
<td>37 69 79* 50* 58*</td>
</tr>
<tr>
<td>5$\beta$-androstan-3$\beta$-ol-17-one</td>
<td>51 71* 39* 48*</td>
</tr>
<tr>
<td>5$\alpha$-androstan-3$\alpha$-ol-17-one</td>
<td>55 79* 44*</td>
</tr>
<tr>
<td>5$\alpha$-androstan-3$\beta$-ol-17-one</td>
<td>37 76* 42*</td>
</tr>
<tr>
<td>5$\beta$-androstan-17$\beta$-ol-13-one</td>
<td>33 74 54* 30* 45*</td>
</tr>
<tr>
<td>5$\beta$-androstan-17$\alpha$-ol-13-one</td>
<td>46 62* 14*</td>
</tr>
<tr>
<td>5$\beta$-pregnan-3$\alpha$-ol-20-one</td>
<td>61 74 87* 70* 21*</td>
</tr>
<tr>
<td>5$\beta$-pregnan-3$\beta$-ol-20-one</td>
<td>58 81* 59* 39*</td>
</tr>
<tr>
<td>5$\alpha$-pregnan-3$\alpha$-ol-20-one</td>
<td>73</td>
</tr>
<tr>
<td>5$\alpha$-pregnan-3$\beta$-ol-20-one</td>
<td>56 86*</td>
</tr>
</tbody>
</table>

* $R_f$ value of corresponding acetylated steroid

*continued overleaf*
Table 2.3 (continued) The $R_f$ values for steroid separation achieved with various solvent systems.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>SOLVENT SYSTEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>20β-hydroxy-4-pregnen-3-one</td>
<td>46</td>
</tr>
<tr>
<td>20α-hydroxy-4-pregnen-3-one</td>
<td>42</td>
</tr>
<tr>
<td>17α-hydroxyprogesterone</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>testosterone</td>
<td>16</td>
</tr>
<tr>
<td>5β-androstane-3α,17β-diol</td>
<td>8</td>
</tr>
<tr>
<td>5β-androstane-3β,17β-diol</td>
<td></td>
</tr>
<tr>
<td>5β-pregnane-3α,20β-diol</td>
<td>14</td>
</tr>
<tr>
<td>5β-pregnane-3α,20α-diol</td>
<td>8</td>
</tr>
<tr>
<td>oestrone</td>
<td>0</td>
</tr>
<tr>
<td>oestriadiol</td>
<td>0</td>
</tr>
<tr>
<td>oestriol</td>
<td>0</td>
</tr>
</tbody>
</table>

* $R_f$ value of corresponding acetylated steroid.
moving phase. The stationary phase was applied by dipping the glass fibre strip in a 20% solution of propylene glycol in acetone and air drying.

**System 2.** hexane:butyl acetate (5:2, v/v).

**System 3.** diethyl ether.

**System 4.** isopropyl ether:light petroleum, b.p. 40 to 60°C. (1:1, v/v), two developments.

**System 5.** dichloromethane:ethyl acetate, 98:1.5.

**System 6.** hexane:butyl acetate (70:30, v/v), three developments.

**System 7.** cyclohexane:ethyl acetate (5:2, v/v).

**System 8.** A partition system with toluene as the moving phase and propylene glycol (applied as a 20% solution in acetone) as the stationary phase.

**System 9.** hexane:butyl acetate (70:30, v/v), first development; light petroleum, b.p. 40 to 60°C.: isopropyl ether (80:20, v/v) second development.

**System 10.** hexane:ethyl acetate (2:1, v/v), two developments.

B) **t.l.c. systems**

**System 11.** chloroform:ethyl acetate (4:1, v/v).

**System 12.** cyclohexane:ethyl acetate (1:1, v/v).

**System 13.** chloroform:cyclohexane:butyl acetate (1:1:1, by volume).

**System 14.** toluene:acetone (4:1, v/v).

The radioactive areas on the chromatogram were located by using a Panax thin-layer radio-chromatogram scanner (Panax Equipment Ltd.) or by autoradiography using Xomat S XS1 18 x 24 cm 'X' ray film (Kodak Ltd.). The latter
was the method of choice when the amounts of radioactivity on the chromatogram was very low. Reference steroids and carrier steroids which absorbed in the ultra-violet region were located under ultra-violet light (250 nm). Other steroids were located by placing in iodine vapour or by spraying with an anisaldehyde-sulphuric-acetic acid spray similar to that described by Neher (1964). This spray was prepared freshly before use by adding 1 part (by volume) of concentrated $\text{H}_2\text{SO}_4$ to 40 parts of 0.5% anisaldehyde in glacial acetic acid. After spraying, the chromatograms were heated at 100°C for 5 minutes, or until coloured spots appeared.

The radioactive areas were recovered from the chromatograms by either cutting them out and eluting thrice with 5 ml of methanol (i.t.l.c. strips), or by scraping off the silica (Merck t.l.c. plates) and extracting thrice with 10 ml of toluene after the addition of 1 ml of water to deactivate the silica gel. Another quicker method which was later successfully tried, was to place the silica scrapings from the plate in a glass pipette plugged with glass wool and to elute thrice with 2 ml of ethyl acetate. The latter method was preferred since it involved less manipulations, was much quicker to perform and gave similar recoveries of radioactivity.

**2:5:2 Separation of neutral and phenolic steroids**

A counter-current distribution procedure for separating neutral and phenolic steroids was used (figure 2.2). 5 ml of toluene:light petroleum, b.p. 60 to 80°C (1:1, v/v) and 1 ml of NaOH (1M) was added to tube 1
Legend to Figure 2.2

The partition system used to separate the phenolic steroids (oestrogens) from the neutral steroids (e.g. testosterone).
PARTITION SYSTEM TO SEPARATE PHENOLIC AND NEUTRAL STEROIDS

Neutral Fraction

| 1 | 2 | 3 |

5 ml toluene:petrol.
60-80°C (1:1, v/v)

1 ml NaOH (1M)

Phenolic Fraction
which contained the radioactive extract to be partitioned. After shaking for 1 minute, the two phases were allowed to separate. The lower phase (NaOH) was transferred to another tube (tube 2) containing 5 ml of toluene:light petroleum and the process repeated. The lower phase (NaOH) was again removed and transferred to a third tube (tube 3) and partitioned with another 5 ml toluene:light petroleum, to remove any remaining neutral steroids. The NaOH layer (1 ml) was then removed from tube 3 and kept. Residual phenolic steroids were removed from the tubes containing toluene:petroleum by backwashing twice with 1 ml aliquots of NaOH. The NaOH fraction plus the two washings were combined and concentrated orthophosphoric acid added dropwise until the pH was 7.0. The neutralized solution was extracted thrice with 3 volumes of dichloromethane. After washing several times with distilled water, the phenolic extract containing the oestrogens was evaporated to dryness. The toluene:petroleum containing the neutral steroids (tubes 1, 2 and 3) was evaporated and 1 ml of distilled water added to each tube. Dilute phosphoric acid was added dropwise to adjust the pH to 7.0. The contents of each tube were then extracted thrice with 5 volumes of dichloromethane and the extracts washed several times with distilled water before evaporating to dryness. The radioactivity in the neutral extracts from tubes 1, 2 and 3 and in the tube containing the phenolic extract was measured by dissolving each extract in 2 ml acetone
and taking 100 µl for counting. When $[4^{-14}C]$testosterone (19 KBq) was subjected to this partition procedure, the percentage of radioactivity found in each of the tubes was as follows: tube 1, 95.1%; tube 2, 3.5%; tube 3, 0.4%; with 0.6% of the radioactivity present in the phenolic fraction. The neutral steroids, (as represented by testosterone), as expected, were present mainly in the epi-phase following the first partition (tube 1). $[^3H]$-oestrone (1.5 KBq) was also subjected to this partition procedure with the following results: tube 1, 1.6%; tube 2, 2.0%; tube 3, 2.7%; with 93.4% of the radioactivity being present in the hypo-phase.

### 2:6 Identification of metabolites

The radiometabolites isolated from the incubations of tissue homogenates with $^{14}C$-labelled steroid precursors, were separated initially by running in solvent system 1. This solvent system separates steroids according to their class with diketones having the highest $R_f$ values followed by mono-hydroxy-mono-ketones. Metabolites containing two or more hydroxyl groups and the oestrogens remain at or near baseline.

A tentative identification of a given radiometabolite was made when the activity coincided with a particular reference steroid after chromatography in several different solvent systems. If isopolarity of the radiometabolite and the reference steroid was still maintained after oxidation and acetylation (see section 2:6:1), a more positive identification was established by
recrystallisation to constant specific activity (see section 2:6:2). Facilities for identification of radiometabolites by gas-liquid chromatography and mass-spectroscopy were not available for this investigation.

2:6:1 Formation of derivatives

Oxidation and acetylation of metabolites were carried out according to Bush (1961). For oxidation, the metabolite was blown to dryness in a test-tube and then dissolved in 100 μl glacial acetic acid. 100 μl of CrO₃ (2% w/v in distilled water) was added and the tube stoppered, shaken and left in the dark for at least 2 hours. 1 ml of distilled water was then added before extracting the metabolite three times with 5 ml dichloromethane. The organic extract was washed with 0.5M NaHCO₃ (1 ml) followed by several washes with distilled water (1 ml) before evaporating to dryness.

Acetylations were carried out by adding a reagent consisting of equal volumes of pyridine and acetic anhydride (100 μl) and standing in the dark overnight at room temperature. The excess reagent was then evaporated in a current of air.

2:6:2 Recrystallisation of radioactive metabolites

When the identity of a metabolite had been tentatively established by isopolarity with a reference steroid in several chromatographic systems, both before and after the formation of derivates, it was recrystallised repeatedly after the addition of approximately 20 mg of the authentic steroid. The method used was a simplified
version of the procedure described by Axelrod, Matthijssen, Goldzieher and Pulliam (1965).

At first, small circles of aluminium foil were cut out and made into containers (approximately 1.5 cm in diameter), but afterwards a more convenient container was found to be the caps of the mini-counting vials. The containers were each weighed three times to 5 decimal places (using a Mettler balance) and the mean weight calculated. At least 20,000 dpm of the radiometabolite to be recrystallised was evaporated to dryness in a small clean glass test-tube. After the addition of the authentic steroid, the mixture was dissolved in 1 ml of chloroform:methanol (1:1, v/v). Approximately 75 µl of this solution was pipetted into one of the pre-weighed containers and the solvent allowed to evaporate. The container was then re-weighed (as before) to determine the weight of crystals and then placed in a vial together with scintillator and shaken thoroughly to dissolve the crystals. After counting and correcting for quenching, the activity in dpm of the crystals was calculated. The initial specific activity (dpm/mg) was obtained by dividing the observed activity by the weight of crystals.

The remainder of the chloroform-methanol mixture was then blown to dryness in air and then the radiometabolite and steroid was dissolved in a small volume (approximately 0.5 ml) of boiling acetone to obtain a saturated solution. This solution was heated in a water
bath (temperature 65°C) and some of the solvent evaporated by blowing a gentle stream of air over the end of the glass tube. In this manner, a super-saturated solution was obtained. The tube was removed from the water bath and a second solvent (see list given over the page), in which the steroid is less soluble than the first solvent, was added dropwise with shaking, in order to induce the formation of crystals. Crystal formation usually occurred after the addition of the second solvent or after the test-tube had been allowed to cool. If crystals failed to appear after cooling, more of the solvent was evaporated until crystals started to appear at the bottom of the tube. When crystals had started to appear, the test-tube was stoppered and left in a refrigerator overnight or until sufficient crystals had formed. The mother liquor of this first crop was pipetted into a preweighed container for measurement of its specific activity. The remaining crystals (1st crop) were dissolved in 1 ml of chloroform: methanol and an aliquot (75 µl) was pipetted into another preweighed container for determination of its specific activity. After evaporating the remaining chloroform: methanol, another recrystallisation was carried out as described above and the specific activities of the second crop of crystals and the second crop of mother liquor obtained. Altogether, six successive recrystallisations were carried out using different solvent pairs, e.g. acetone:hexane; acetone:cyclohexane; acetone:ethyl acetate; acetone:ethanol.
The main source of error in measuring specific activities lies in the weighing of small amounts, usually (1 to 2 mg) of crystals. The errors involved in counting the radioactivity due to steroid quenching were corrected by using a "quench calibration curve". This was prepared by adding increasing increments (10 to 140 µl) of chloroform (which simulates chemical quenching) to a series of ten tubes containing a fixed, predetermined amount of radioactivity. The values obtained are given in Table 2.4. This curve was stored in a program on the LKB-Wallac liquid scintillation counter. The program converts the values obtained for each sample from counts per minute (cpm) to disintegrations per minute (dpm), using the sample counting efficiency as calculated by interpolation of the sample's channel ratio with the calibration curve.

Constant specific activity was deemed to have been achieved when the mean change in specific activity of the crystals was less than 5% after three or more successive recrystallisations. It became obvious when the radio-metabolite under investigation was not identical to the added steroid. Under these circumstances the specific activity of the mother liquor was much higher than the specific activity of the crystals after the first recrystallisation since most of the radioactivity was present in the former. Successive recrystallisations yielded crystals whose specific activity fell progressively. When the radiometabolite was identical with the
Table 2.4 Data used to construct the quench calibration curve, using chloroform to simulate chemical quenching. Vials containing 5 ml of scintillator and 9.73 KBq of [4-14C]testosterone were counted for 60 seconds in an LKB-Wallac 1216 Rackbeta 11 Liquid Scintillation Analyser.

<table>
<thead>
<tr>
<th>% Efficiency</th>
<th>Channel Ratio (II/I)*</th>
<th>Chloroform added (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>92.90</td>
<td>1.500</td>
<td>0</td>
</tr>
<tr>
<td>92.60</td>
<td>1.001</td>
<td>10</td>
</tr>
<tr>
<td>92.15</td>
<td>0.852</td>
<td>20</td>
</tr>
<tr>
<td>91.50</td>
<td>0.653</td>
<td>30</td>
</tr>
<tr>
<td>91.25</td>
<td>0.619</td>
<td>40</td>
</tr>
<tr>
<td>90.57</td>
<td>0.425</td>
<td>60</td>
</tr>
<tr>
<td>88.48</td>
<td>0.304</td>
<td>80</td>
</tr>
<tr>
<td>87.80</td>
<td>0.257</td>
<td>100</td>
</tr>
<tr>
<td>86.08</td>
<td>0.162</td>
<td>120</td>
</tr>
<tr>
<td>84.60</td>
<td>0.127</td>
<td>140</td>
</tr>
</tbody>
</table>

* Channel II set at energy level 120-165, and Channel I set at energy level 50-120.
# Determined by calibration against external 14C standard.
**Table 2.5** Recrystallisation data following 4 successive recrystallisations of $[4^{-14}C]_{\text{testosterone}}$ with authentic testosterone.

<table>
<thead>
<tr>
<th>Recrys. No.*</th>
<th>Solvent Pair</th>
<th>Specific Activity (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crystals</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>1709</td>
</tr>
<tr>
<td>1</td>
<td>Acetone/hexane</td>
<td>1777</td>
</tr>
<tr>
<td>2</td>
<td>Acetone/cyclohexane</td>
<td>1744</td>
</tr>
<tr>
<td>3</td>
<td>Acetone/ethylacetate</td>
<td>1748</td>
</tr>
<tr>
<td>4</td>
<td>Acetone/ethanol</td>
<td>1694</td>
</tr>
</tbody>
</table>

* Recrystallisation number
carrier steroid, the dramatic drop in specific activity in successive recrystallisations did not occur. Table 2.5 shows the results obtained when authentic [4-\textsuperscript{14}C]-testosterone (16.6 KBq) was recrystallised with crystalline testosterone, following chromatography in solvent system 1.

2:7 Glassware

Glassware which had contained radioactive material was routinely decontaminated by rinsing with acetone followed by water and then by soaking overnight in Decon (0.2% v/v). After this, the glassware was washed thoroughly in hot water with Pyroneg, rinsed with tap water and then rinsed several times with distilled water. The glassware was air dried. This procedure was shown to remove any radioactivity left from the tubes. All cleaned glassware used during the [4-\textsuperscript{14}C]-testosterone experiments was silanized beforehand by soaking for 2 to 3 hours in a 0.1% (v/v) solution of dichloro-dimethyl-silane in toluene. This glassware was rinsed with methanol and allowed to dry in air before use.

2:8 Chemicals

Radioactive steroids were obtained from Amersham International (formerly the Radiochemical Centre), Amersham, Bucks. Most non-radioactive steroids, glucose-6-phosphate, NAD\textsuperscript{+}, NADP\textsuperscript{+} and bovine serum albumin (Fraction V) were supplied by Sigma Chemical Co., Poole, Dorset. Steroids which were not available from Sigma were purchased from Steraloids Ltd., Croydon, Surrey. 5\textbeta-Androstan-17\alpha-ol-
3-one was a gift from the MRC steroid reference collection. The 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) and 2,5-diphenyloxazole (PPO) were obtained from Koch-Light Laboratories, Colnbrook, Berks. Other chemicals were supplied by BDH Chemicals, Poole, Dorset. Organic solvents (Analar grade) were redistilled before use.
3. EXPERIMENTAL RESULTS
Conversion of \([4-^{14}C]\)progesterone to androstenedione by thecal tissue from the large follicles

Introduction

The thecal interstitial cells of the follicle of the domestic fowl have been proposed as a site of androgen and oestrogen biosynthesis both on the basis of their ultra-structural appearance (Dahl, 1970a; Perry et al., 1978), and the presence within the cell, of \(3\beta\)-hydroxysteroid dehydrogenase and \(17\beta\)-hydroxysteroid dehydrogenase (Wyburn and Baillie, 1966; Boucek and Savard, 1970). Other evidence linking thecal interstitial cells with steroidogenesis is reviewed in section 1:2:2. Armstrong (1982b) has also recently demonstrated that thecal tissue homogenates from the five largest follicles are capable of converting \([^{3}H]\)pregnenolone to progesterone. The results presented here show that thecal homogenates from these follicles are also capable of converting \([4-{^{14}C}]\)progesterone to androstenedione and testosterone.

Experimental procedures

Six hens (Shaver 288's) were killed 1 to 2 hours before an expected ovulation and a further 5 hens were killed approximately 15 hours before an expected ovulation. Thecal tissue homogenates from the 5 to 7 largest follicles of hens killed 1 to 2 hours before ovulation and from the 3 largest follicles of hens killed 15 hours before ovulation were prepared as described in 2:2. After centrifugation, the supernatant was incubated immediately with \([4-{^{14}C}]\)progesterone (3.7 KBq) for 1 hour in the presence
Legend to Figure 3.1

A flow-chart outlining the procedure used for incubation of the homogenates with [4-14C]progesterone and the isolation of the radiometabolites from the incubation medium.
1. theca
   ↓
2. homogenise in 10 vols 0.25M sucrose
   ↓
3. centrifuge at 800g for 10 mins
   ↓
4. 0.1 ml supernatant + \(^{14}\text{[C]}\) - progesterone (3.7 kBq)
   ↓
5. incubate 1 hr at 37°C
   ↓
6. extract 2 x 5 vols CH\(_2\)Cl\(_2\)
   ↓
7. i.t.l.c. chromatography
of NAD⁺ and NADPH-generating system. The latter consisted of NADP⁺ and glucose-6-phosphate. It was not necessary to add glucose-6-phosphate dehydrogenase to the incubation medium to ensure the generation of NADPH, since thecal homogenates have been shown to contain high levels of this enzyme (Armstrong, 1982b). A flow-chart outlining the procedures used for incubation and extraction of metabolites is given in figure 3.1. A more detailed description is given in section 2.4. The incubation time was chosen after conducting a preliminary time-study using a thecal homogenate from the sixth largest follicle of a hen killed 1 to 2 hours before ovulation. No androstenedione was produced after 20 to 30 minutes. Androstenedione was first detected after 45 minutes and reached a peak after 60 minutes. Thereafter, a similar rate of conversion of [4-¹⁴C]progesterone to androstenedione was maintained for incubation times of up to 150 minutes, but had begun to fall by 180 minutes. The chosen incubation time was 60 minutes.

After incubation of the thecal homogenates with [4-¹⁴C]progesterone as outlined above, the radioactive metabolites were extracted with dichloromethane (see section 2.4). The recoveries obtained were generally between 70% and 80%. An aliquot of each extract (40%) was chromatographed on i.t.l.c. strips in solvent system 1. The radiometabolites were separated into 5 major zones. (See plate 3.1). The first two zones corresponded to carrier progesterone and androstenedione. The fourth zone
corresponded to carrier 17α-hydroxyprogesterone and testosterone which were not always completely separated from each other. The first two zones (which corresponded to progesterone and androstenedione) were located by ultra-violet light (250 nm), cut out, and placed in glass scintillation vials containing 5 ml scintillator (0.4% PPO, 0.03% POPOP). The vials were counted in a Philips liquid scintillation analyser with an efficiency for 14C of about 90%. Quenching, due to the presence of the i.t.l.c. paper, was ignored as it was found to be very small. Addition of a similar piece of i.t.l.c. paper to a vial containing a known amount of radioactivity, decreased the count to 98% of its original value. After correction for recovery and counting efficiency, the results, in dpm, from the duplicate incubations were averaged.

Using the specific activity of the original [4-14C]-progesterone, the yield of androstenedione and the amount of progesterone left was converted from dpm to nmol. By subtracting the amount of progesterone left from the amount of progesterone at the start of the incubation, the amount of progesterone metabolized was obtained. Although the amount of protein in the homogenates was measured, the variability of the data when expressed in nmol/mg protein per hour compared to nmol/10mg fresh tissue per hour was greatly increased. This was thought to be associated with the procedure for extracting protein from homogenates (see section 2:3). In order to
Legend to Plate 3.1

Autoradiograph of i.t.l.c. strips showing the separation of metabolites obtained after chromatography in solvent system 1.

F1 - largest follicle
F2 - second largest follicle

Thecal homogenates from F1 and F2 collected 1 to 2 hours before ovulation were incubated with [4-14C]progesterone (3.7 KBq) for one hour in the presence of cofactors. The position of each Zone (I, II, III, IV and V) is indicated.
minimise the variation, the results were expressed in nmol/10 mg fresh tissue per hour. The remaining zones of the chromatogram (zone III, IV and V) were also cut out and the radioactive metabolites eluted. Each zone was pooled and kept for further identification. For 3 hens at each period of the ovulatory cycle, a further aliquot (40%) of each extract from F1, F2 and F3 only was run in solvent system 1 and the entire chromatogram cut into 2.5 mm strips which were counted, as before, by liquid scintillation analysis. From these data, the yield of metabolites in each of the five zones of the chromatogram was calculated.

3:1:3 Results

a) [4-14C]progesterone metabolism

Thecal tissue homogenates from all the follicles actively metabolized progesterone (see table 3.1). Since at the start of the incubation there was 1.7 nmol of [4-14C]progesterone, the values given in table 3.1 represent a conversion rate varying from 73% to 90%, assuming that all of the radioactivity in this zone was progesterone. In fact, it was later shown (see 3:3:3, f) that a small amount of 5β-androstenedione was also present. For each follicle, Student's t-test was used to compare the mean values at 1 to 2 hours before ovulation with those obtained 15 hours before ovulation. No significant differences in progesterone metabolism were seen by thecal tissue homogenates from F1, F2 and F3 follicles. At 1 to 2 hours before ovulation, significantly
Table 3.1 Metabolism of [4-\textsuperscript{14}C]progesterone by thecal homogenates (nmol/10 mg wet tissue per hour) at two different periods of the ovulatory cycle. Values are given as means ± SEM, \(n\) = number of follicles (in parenthesis)

<table>
<thead>
<tr>
<th>Follicle</th>
<th>1 - 2 hours</th>
<th>15 hours</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.360 ± 0.051 (5)*</td>
<td>1.289 ± 0.147 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>F2</td>
<td>1.570 ± 0.023 (5)</td>
<td>1.398 ± 0.107 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>F3</td>
<td>1.472 ± 0.093 (5)</td>
<td>1.394 ± 0.082 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>F4</td>
<td>1.600 ± 0.014 (5)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>1.562 ± 0.032 (5)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>F6</td>
<td>1.572 ± 0.037 (3)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>F7</td>
<td>1.519 ± 0.057 (3)</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* Significantly different from preceding follicle (P<0.05)

NS - No significant difference
Table 3.2  Conversion of [4-14C]progesterone to androstenedione by thecal homogenates (nmol/10 mg wet tissue per hour) at two different periods of the ovulatory cycle. Values are given as means ± SEM, n = number of follicles (in parenthesis)

<table>
<thead>
<tr>
<th>Follicle</th>
<th>1 - 2 hours</th>
<th>15 hours</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.095 ± 0.018 (6)**</td>
<td>0.236 ± 0.026 (5)</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>F2</td>
<td>0.265 ± 0.024 (6)</td>
<td>0.231 ± 0.023 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>F3</td>
<td>0.287 ± 0.019 (6)</td>
<td>0.221 ± 0.025 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>F4</td>
<td>0.281 ± 0.041 (6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>0.279 ± 0.038 (6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F6</td>
<td>0.237 ± 0.028 (4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F7</td>
<td>0.370 ± 0.076 (3)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

** Significantly different from preceding follicle (P<0.01)

NS - No significant difference
less progesterone was metabolized by thecal homogenates from F1 follicles compared with F2 follicles (P<0.05, Student's paired t-test). The difference in progesterone metabolism between thecal homogenates from F2 and F3 follicles collected 15 hours before ovulation however, was not significant.

b) Conversion of [4-14C]progesterone to androstenedione

Thecal tissue homogenates from all the follicles were able to convert [4-14C]progesterone to androstenedione (see table 3.2). With the exception of the largest pre-ovulatory follicle (F1), thecal homogenates from all the follicles collected 1 to 2 hours before ovulation (F2-F7) converted similar amounts of [4-14C]progesterone to androstenedione (average value 0.283 nmol/10 mg fresh tissue per hour). Thecal homogenates from the largest follicle (F1) however, produced significantly less androstenedione compared with thecal homogenates from F2 follicles 1 to 2 hours before ovulation (P<0.01, Student's paired t-test). This represents a drop in the percentage conversion of [4-14C]progesterone to androstenedione from 16% to 5%. This difference was not seen when thecal homogenates from F1 and F2 follicles were collected 15 hours before an expected ovulation.

Thecal homogenates from F1 follicles collected 1 to 2 hours before ovulation also converted significantly less [4-14C]progesterone to androstenedione compared with F1 follicles collected 15 hours before an expected ovulation.
Table 3.3 Metabolism of [4-14C]progesterone (nmol/10 mg wet tissue per hour) by thecal homogenates from the 3 largest follicles, F1, F2 and F3 at two different periods of the ovulatory cycle. Values are given as means ± SEM, number of follicles = 3.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Follicle</th>
<th>1 - 2 hours</th>
<th>15 hours</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ZONE I</strong> (R_F 0.75 - 0.94)</td>
<td>F1</td>
<td>0.547 ± 0.123</td>
<td>0.280 ± 0.021</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>F2</td>
<td>0.423 ± 0.100</td>
<td>0.322 ± 0.013</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>0.287 ± 0.024</td>
<td>0.349 ± 0.020</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ZONE II</strong> (R_F 0.55 - 0.75)</td>
<td>F1</td>
<td>0.109 ± 0.044*</td>
<td>0.314 ± 0.013</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>F2</td>
<td>0.304 ± 0.057</td>
<td>0.282 ± 0.006</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>0.349 ± 0.052</td>
<td>0.310 ± 0.003</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ZONE III</strong> (R_F 0.35 - 0.55)</td>
<td>F1</td>
<td>0.836 ± 0.056**</td>
<td>0.401 ± 0.105</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>20p-hydroxy-4-pregnen-3-one</td>
<td>F2</td>
<td>0.316 ± 0.048</td>
<td>0.344 ± 0.078</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>0.329 ± 0.190</td>
<td>0.217 ± 0.030</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ZONE IV</strong> (R_F 0.15 - 0.35)</td>
<td>F1</td>
<td>0.125 ± 0.047</td>
<td>0.278 ± 0.030</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>F2</td>
<td>0.213 ± 0.051</td>
<td>0.291 ± 0.015</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>0.263 ± 0.046</td>
<td>0.355 ± 0.006</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ZONE V</strong> (R_F 0.00 - 0.15)</td>
<td>F1</td>
<td>0.113 ± 0.021</td>
<td>0.497 ± 0.079</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Rest</td>
<td>F2</td>
<td>0.483 ± 0.159</td>
<td>0.534 ± 0.069</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>0.516 ± 0.106</td>
<td>0.538 ± 0.033</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Significantly different from preceding follicle (P<0.05)
** Significantly different from preceding follicle (P<0.01)
NS - No significant difference
(P<0.01, Student's t-test. Thecal androstenedione production by F2 or F3 follicles did not vary significantly according to whether they were collected 1 to 2 hours or 15 hours before ovulation.

c) **Conversion of [4-¹⁴C]progesterone to other metabolites**

Table 3.3 shows the conversion of [4-¹⁴C]progesterone to the various other metabolites present in each of the five zones of the chromatogram after chromatography in solvent system 1. The values obtained for thecal homogenates from F2 and F3 follicles were similar regardless of whether the follicles were collected 1 to 2 hours or 15 hours before ovulation. As before (see table 3.2), thecal homogenates from F1 follicles converted significantly less [4-¹⁴C]progesterone to androstenedione 1 to 2 hours before ovulation compared with 15 hours before ovulation (P<0.05, Student's t-test). With the exception of 20β-hydroxy-4-pregnen-3-one, conversion of [4-¹⁴C]progesterone to all other metabolites was also decreased, although for testosterone this was only significant at the 10% level (see table 3.3). Conversion of [4-¹⁴C]progesterone to 20β-hydroxy-4-pregnen-3-one by thecal homogenates from F1 was significantly increased 1 to 2 hours before ovulation (P<0.05, Student's t-test) compared with earlier in the cycle. This represents an increase in the conversion rate from about 26% 15 hours before ovulation to 47% just before ovulation.

A comparison of thecal progesterone metabolism by
successive follicles at each period of the ovulatory cycle revealed that F1 follicles produced significantly more 20β-hydroxy-4-pregnen-3-one than F2 follicles (P<0.01, Student's paired t-test) 1 to 2 hours before ovulation. As before (see table 3.2), F1 follicles also produced significantly less androstenedione than F2 follicles at this period of the cycle (P<0.05, Student's paired t-test). These differences were not seen when the follicles were collected 15 hours before ovulation.

The overall change in the pattern of [4-14C]progesterone metabolism by thecal homogenates from F1 just before ovulation is illustrated in Plate 3.1. This plate shows an autoradiograph of two i.t.l.c. strips (side by side) after chromatography in solvent system 1 of both homogenates from F1, 1 to 2 hours before ovulation and from F2, 1 to 2 hours before ovulation. Autoradiographs of thecal homogenates from F1, 15 hours before ovulation and all the other follicles collected in this experiment (at either 1 to 2 hours or 15 hours before ovulation) were similar to (b).

d) Identification of metabolites

The remaining aliquots of each extract were chromatographed in solvent system 1 and each of the five radioactive zones (see plate 3.1) was eluted and pooled separately to await further identification.

ZONE I (Rf 0.94 - 0.75), which coincided with carrier progesterone was chromatographed in a further two solvent systems (13 and 14) and found to contain only one radioactive
Table 3.4  Recrystallisation data for radiometabolite isolated from Zone II with androstenedione, acetylated radiometabolite from Zone IV with testosterone acetate, remainder of Zone IV (after acetylation) with 17α-hydroxyprogesterone.

<table>
<thead>
<tr>
<th>Recrys. No.</th>
<th>Solvent Pair</th>
<th>Androstenedione</th>
<th>Testosterone Acetate</th>
<th>17α-hydroxyprogesterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crystals</td>
<td>ML*</td>
<td>Crystals</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Acetone/hexane</td>
<td>3072</td>
<td>3478</td>
<td>388</td>
</tr>
<tr>
<td>2</td>
<td>Acetone/ethylacetate</td>
<td>3252</td>
<td>3085</td>
<td>343</td>
</tr>
<tr>
<td>3</td>
<td>Acetone/ethanol</td>
<td>3146</td>
<td>3119</td>
<td>303</td>
</tr>
<tr>
<td>4</td>
<td>Acetone/cyclohexane</td>
<td>3234</td>
<td>2911</td>
<td>333</td>
</tr>
</tbody>
</table>

* Mother Liquor
+ Recrystallisation Number
Table 3.5  Recrystallisation data for radiometabolite from Zone III (from F1, F2 and F3) with 5β-androstan-17β-ol-3-one.

<table>
<thead>
<tr>
<th>Recry. No.*</th>
<th>Solvent Pair</th>
<th>Crystals</th>
<th>Mother Liquor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>1646</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Acetone/hexane</td>
<td>1088</td>
<td>6105</td>
</tr>
<tr>
<td>2</td>
<td>Acetone/ethylacetate</td>
<td>747</td>
<td>2563</td>
</tr>
<tr>
<td>3</td>
<td>Acetone/ethanol</td>
<td>324</td>
<td>1118</td>
</tr>
<tr>
<td>4</td>
<td>Acetone/cyclohexane</td>
<td>172</td>
<td>LOST</td>
</tr>
<tr>
<td>5</td>
<td>Acetone/hexane</td>
<td>167</td>
<td>2557</td>
</tr>
<tr>
<td>6</td>
<td>Acetone/ethylacetate</td>
<td>88</td>
<td>2266</td>
</tr>
</tbody>
</table>

* Recrystallisation Number
Table 3.6 Recrystallisation data for oxidized radiometabolite isolated from Zone III (from F1, F2 and F3) with either progesterone or 5\(\beta\)-androstanedione. Solvent pairs as in table 3.5.

<table>
<thead>
<tr>
<th>Recrys. No.</th>
<th>Crystals</th>
<th>ML*</th>
<th>Crystals</th>
<th>ML*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1868</td>
<td>-</td>
<td>8692</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1945</td>
<td>2482</td>
<td>2128</td>
<td>26,840</td>
</tr>
<tr>
<td>2</td>
<td>1871</td>
<td>1200</td>
<td>1373</td>
<td>23,854</td>
</tr>
<tr>
<td>3</td>
<td>1942</td>
<td>1975</td>
<td>613</td>
<td>24,468</td>
</tr>
<tr>
<td>4</td>
<td>1940</td>
<td>1981</td>
<td>272</td>
<td>10,259</td>
</tr>
<tr>
<td>5</td>
<td>1971</td>
<td>4932</td>
<td>329</td>
<td>4,988</td>
</tr>
<tr>
<td>6</td>
<td>1856</td>
<td>1340</td>
<td>264</td>
<td>662</td>
</tr>
</tbody>
</table>

* Recrystallisation Number
* Mother Liquor
peak with the same \( R_F \) as carrier progesterone. After oxidation and acetylation, one radioactive peak was obtained which was coincident with progesterone in solvent systems 12 and 13. \( 5\beta \)-Androstanedione may also have been present in this zone as these solvent systems were not capable of completely separating progesterone and \( 5\beta \)-androstanedione.

**ZONE II** (\( R_F \) 0.75 - 0.55), which was coincident with carrier androstenedione, had the same \( R_F \) value as androstenedione when run in solvent systems 11, 12 and 13. This radiometabolite was then recrystallised to constant specific activity after the addition of authentic androstenedione (see table 3.4).

**ZONE III** (\( R_F \) 0.55 - 0.35) from F1, F2 and F3 contained one radioactive metabolite only. This had the same \( R_F \) as \( 5\beta \)-androstane-17\( \beta \)-ol-3-one (trivial name \( 5\beta \)-dihydrotestosterone or \( 5\beta \)DHT) in solvent systems 3, 11 and 13, but successive recrystallisations with authentic \( 5\beta \)-androstane-17\( \beta \)-ol-3-one did not yield crystals with a constant specific activity (see table 3.5). Upon oxidation a single radioactive peak was obtained which corresponded to both \( 5\beta \)-androstanedione and progesterone in solvent system 13. Successive recrystallisations of the oxidised radiometabolite with progesterone but not \( 5\beta \)-androstanedione (see table 3.6), yielded crystals with a constant specific activity. It was then found that \( 20\beta \)-hydroxy-4-pregnen-3-one (trivial name \( 20\beta \)-dihydroprogesterone, \( 20\beta \)DHP) which can be oxidised to progesterone, also had
the same R\textsubscript{F} as the radiometabolite in Zone III in solvent system 13 (R\textsubscript{F} 20\textbeta\textbeta\textbeta DHP 0.19, R\textsubscript{F} 5\textbeta\textbeta\textbeta DHT 0.19). Upon acetylation, the radiometabolite had exactly the same R\textsubscript{F} as 20\textbeta\textbeta\textbeta-hydroxy-4-pregnen-3-one monoacetate and was clearly separated from 5\textbeta\textbeta\textbeta-androst-17\textbeta\textbeta\textbeta-ol-3-one monoacetate in solvent system 4. Further confirmation that this metabolite was indeed 20\textbeta\textbeta\textbeta DHP, was obtained later (see section 3.2). Although 20\textbeta\textbeta\textbeta-hydroxy-4-pregnen-3-one was the only metabolite found in Zone III from incubations of thecal homogenates from F1, F2 and F3, incubations from the smaller follicles (F3 to F7) produced in addition, small amounts of another metabolite. This metabolite had an R\textsubscript{F} similar to 5\textbeta\textbeta\textbeta-pregnane-3\textalpha\textalpha\textalpha-ol-20-one (solvent system 13), and upon oxidation yielded a metabolite with an R\textsubscript{F} similar to 5\textbeta\textbeta\textbeta-pregnane-20-one (solvent system 13).

ZONE IV (R\textsubscript{F} 0.35 - 0.15) included the carriers 17\textalpha\textalpha\textalpha-hydroxyprogesterone and testosterone which were not always completely separated. This zone was acetylated and run in solvent system 2. Two \textsuperscript{14}C-labelled peaks were obtained. One peak had an R\textsubscript{F} identical to testosterone acetate, and after the addition of authentic steroid, was recrystallised to constant specific activity (see table 3.4). The other peak had the same R\textsubscript{F} as carrier 17\textalpha\textalpha\textalpha-hydroxyprogesterone (which under the acetylation conditions used does not form an acetate). About 80% of the activity in Zone IV was associated with 17\textalpha\textalpha\textalpha-hydroxyprogesterone. Recrystallisation with 17\textalpha\textalpha\textalpha-hydroxyprogesterone, however, did not yield crystals with a constant specific activity (see table 3.4).
ZONE V ($R_F \ 0.15 - 0.00$) was partitioned as described in materials and methods, to separate the neutral and phenolic steroids (see section 2:5:2). Over 90% of the activity was associated with the neutral fraction which consisted of a mixture of at least three or more metabolites. Oxidation of these metabolites gave predominantly $5\beta$-androstane-3, 17-dione accompanied by a small amount of $5\beta$-pregnane-3, 20-dione (solvent system 13). One of the neutral metabolites had the same $R_F$ as $5\beta$-pregnane-3$\alpha$, 20$\beta$-diol, whilst the major metabolite had an $R_F$ coincident with $5\beta$-androstane-3$\alpha$, 17$\beta$-diol (solvent systems 8 and 12). Further identification of the metabolites in the neutral fraction was not attempted. The amount of activity in the phenolic fraction was low and amounted to less than 1% of the total activity on the initial chromatogram. When run in solvent system 12, neither oestrone nor oestradiol were detected, although a $^{14}$C-labelled peak with an $R_F$ similar to oestriol was present.

3:1:4 Discussion

These results have shown that thecal tissue homogenates from all of the 5 to 7 large yolk-filled follicles present in the ovary of the laying hen can convert progesterone to androstenedione and testosterone \textit{in vitro}. This is in agreement with an earlier report in the literature by Botte, Delrio and Lupo di Prisco (1966) in which thecal minces from "growing" follicles (size not specified) were found to be capable of converting progesterone to androstenedione and testosterone. More recently, Nakamura et
al (1979) have also shown that thecal homogenates from the two largest follicles are capable of converting both \([4-^{14}C]\)progesterone and \([4-^{14}C]\)pregnenolone to androstenedione and testosterone, whilst Huang, Kao and Nalbandov (1979) have found that incubations of thecal cells isolated from follicles of the same size were able to convert exogenous progesterone to testosterone. Taken together, these results indicate that the thecal interstitial cells of the large yellow-yolky follicles possess an active 17\(\alpha\)-hydroxylase, C-17,20lyase and 17\(\beta\)-hydroxysteroid dehydrogenase (Armstrong, 1982b), and support the proposition that the theca layer is a site of androgen synthesis.

The present results show that during the final period of follicular growth, the ability of a given mass of thecal tissue to convert \([4-^{14}C]\)progesterone to androstenedione remains relatively constant, with the exception of the largest preovulatory follicle 1 to 2 hours before ovulation which produced much lower amounts of androstenedione (see table 3.2). These results imply that thecal 17\(\alpha\)-hydroxylase and/or C-17,20lyase activity remains high throughout the final stages of development until shortly before ovulation. This interpretation of the results assumes that the proportion of interstitial cells within each of the thecal homogenates remains constant. Peel and Bellairs (1972) considered that the number of interstitial cells within the thecal layer of the developing follicles

al (1979) have also shown that thecal homogenates from the two largest follicles are capable of converting both \([4-^{14}C]\)progesterone and \([4-^{14}C]\)pregnenolone to androstenedione and testosterone, whilst Huang, Kao and Nalbandov (1979) have found that incubations of thecal cells isolated from follicles of the same size were able to convert exogenous progesterone to testosterone. Taken together, these results indicate that the thecal interstitial cells of the large yellow-yolky follicles possess an active 17\(\alpha\)-hydroxylase, C-17,20lyase and 17\(\beta\)-hydroxysteroid dehydrogenase (Armstrong, 1982b), and support the proposition that the theca layer is a site of androgen synthesis.

The present results show that during the final period of follicular growth, the ability of a given mass of thecal tissue to convert \([4-^{14}C]\)progesterone to androstenedione remains relatively constant, with the exception of the largest preovulatory follicle 1 to 2 hours before ovulation which produced much lower amounts of androstenedione (see table 3.2). These results imply that thecal 17\(\alpha\)-hydroxylase and/or C-17,20lyase activity remains high throughout the final stages of development until shortly before ovulation. This interpretation of the results assumes that the proportion of interstitial cells within each of the thecal homogenates remains constant. Peel and Bellairs (1972) considered that the number of interstitial cells within the thecal layer of the developing follicles
Table 3.7  Estimation of the number of interstitial cells present in thecal homogenates

<table>
<thead>
<tr>
<th>Follicle No.</th>
<th>Thecal wet weight (g)</th>
<th>No. granulosa cells*</th>
<th>No. interstitial cells**</th>
<th>No. interstitial cells*** per 10 mg theca wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.58</td>
<td>18.6</td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>F2</td>
<td>0.51</td>
<td>17.1</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>F3</td>
<td>0.44</td>
<td>15.3</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>F4</td>
<td>0.34</td>
<td>13.0</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>F5</td>
<td>0.22</td>
<td>10.0</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>F6</td>
<td>0.14</td>
<td>7.5</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>F7</td>
<td>0.07</td>
<td>5.0</td>
<td></td>
<td>0.71</td>
</tr>
</tbody>
</table>

* Number of granulosa cells/follicle; data of Gilbert et al., (1980).

** Estimate of number of interstitial cells in each of the homogenates (10 mg wet weight used).
remained the same during the final phase of growth, and that the interstitial cells became stretched around the circumference of the follicle as it increases in size. Although the absence of mitotic figures in these cells supports this view (Perry et al., 1978), the theca layer does show a ten-fold increase in wet weight as the follicle grows from approximately 10 mm in diameter to its final size of 35 to 40 mm in diameter. The majority of this increase comes from the fibroblasts of the theca and the increase in the vascular system. At the same time, the number of granulosa cells within the follicle increase from around 5 to 18.6 million (Gilbert, Hardie, Perry, Dick and Wells, 1980). Armstrong et al. (1977) have suggested from histological observations of the post-ovulatory follicle that the interstitial cells are present in numbers equal to those of the granulosa cells. Table 3.7 was constructed assuming this to be the case for all the large preovulatory follicles (F1 to F7). According to this table it appears that the homogenates from the 4 largest follicles (F1 to F4) contained roughly the same proportion of interstitial cells whilst the smaller follicles (F5, F6 and F7) contained a higher proportion of interstitial cells. This could explain the higher values obtained for conversion of [4-14C]progesterone to androstenedione by the thecal homogenates from these follicles (see table 3.2).

The sudden decrease in conversion of [4-14C]progesterone to androstenedione by the largest preovulatory
follicle may have been brought about by exposure to the plasma surge of LH which occurs 4 to 7 hours before ovulation in the hen (Cunningham and Furr, 1972; Furr et al, 1973; Wilson and Sharp, 1973; Shodono et al, 1975; Johnson and Van Tienhoven, 1980). When the largest pre-ovulatory follicle (F1) was collected 15 hours before it was due to ovulate i.e. prior to the LH surge, thecal 17α-hydroxylase and/or C-17,20lyase activity was not decreased (see table 3.2). Thecal homogenates of F2 and F3 follicles however, showed no difference in enzyme activities according to whether they were collected at 15 hours or 1 to 2 hours before an expected ovulation. These results agree with those of Nakamura et al (1979) who collected F1 follicles at either 10 hours, 4 hours or 40 minutes before they were due to ovulate and incubated thecal homogenates with [4-14C]progesterone for 1 hour. Conversion of [4-14C]progesterone to 17α-hydroxy-progesterone and androstenedione was found to be decreased approximately 40 minutes before ovulation compared with earlier in the cycle i.e. 4 hours and 10 hours before ovulation. As in this study, the decrease in thecal 17α-hydroxylase and/or C-17,20lyase activity was not seen in F2 follicles. A similar decrease in thecal 17α-hydroxylase/C-17,20lyase activity has also been observed in thecal cell preparations isolated from preovulatory follicles of the rat following the LH surge (Hamberger, Hillensjo and Ahren, 1978). Whilst thecal 17α-hydroxylase and/or C-17,20lyase in the large
preovulatory follicle (Fl) decreased shortly before ovulation, conversion of [4-\(^{14}\)C]progesterone to 20\(\beta\)-hydroxy-4-pregnen-3-one was significantly increased (compared with earlier in the cycle i.e. 15 hours before ovulation).

These changes were not seen in F2 and F3 follicles, and may therefore be related to the ovulatory process. (For discussion see 3:2:4).

3:2 Conversion of [4-\(^{14}\)C]progesterone to 20\(\beta\)-hydroxy-4-pregnen-3-one by thecal tissue from the large preovulatory follicle 1 to 2 hours before ovulation

3:2:1 Introduction

In the previous section it was found that the pattern of [4-\(^{14}\)C]progesterone metabolism by thecal homogenates from the large, preovulatory follicle (Fl) changed as ovulation approached. Thecal homogenates from Fl follicles collected 1 to 2 hours before ovulation compared with 15 hours before ovulation converted significantly more [4-\(^{14}\)C]progesterone to a metabolite tentatively identified as 20\(\beta\)-hydroxy-4-pregnen-3-one. In order to collect sufficient material to complete the identification of this metabolite and to follow the time-course of its production, further incubations of [4-\(^{14}\)C]progesterone with thecal homogenates from Fl follicles collected 1 to 2 hours before they were due to ovulate, were carried out.

3:2:2 Experimental procedures

Fl follicles were collected from the ovaries of two hens (Ross Brown's) killed 1 to 2 hours before they were due to ovulate. Thecal homogenates were prepared (see
2:2) and pooled. Five aliquots (0.2 ml, which were equivalent to 20 mg fresh tissue) were each incubated with [4-\textsuperscript{14}C]progesterone (8.7 nmol) for 100 minutes in a total volume of 1 ml phosphate buffer (pH 7.4) containing 5 \textmu{mol} NAD\textsuperscript{+}, 5.0 \textmu{mol} glucose-6-phosphate and 1.0 \textmu{mol} NADP\textsuperscript{+}. The incubations were carried out in glass vials in a water bath at 37°C with oxygen:carbon dioxide (95\%:5\%, v/v) as the gas phase. At the end of the incubation, the glass vials were capped and frozen at -20°C until extraction. No carriers were added prior to extraction of the metabolites with dichloromethane as described in section 2:4. The extracts were chromatographed in solvent system 1 to separate the radioactive metabolites and the areas corresponding to 20\textbeta-hydroxy-4-pregnen-3-one (Zone III in section 3) were eluted and pooled to await further identification.

A time-study was also carried out using further aliquots of the pooled thecal homogenate incubated as described above. The incubates were removed from the water bath at various time intervals from 0 to 100 minutes. In addition, 4 more incubations were carried out for 100 minutes as follows:

1) in the absence of cofactors,
2) with an NADPH generating system only,
3) with NAD\textsuperscript{+} only, and
4) with NAD\textsuperscript{+} and a NADPH generating system.

After extraction from the incubation medium and the addition of carrier steroids (progesterone, 17\alpha-hydroxyprogesterone, 20\textbeta-hydroxy-4-pregnen-3-one,
androstenedione and testosterone) the metabolites were separated by i.t.l.c. chromatography (solvent system 1) and located and quantified by liquid scintillation counting as described before (see section 3:1:2).

3:2:3 **Results**

a) **Conversion of [4-\(^{14}\)C]progesterone to 20\(^{\beta}\)-hydroxy-4-pregnen-3-one**

The results of the time-study are shown in figure 3.2. With the exception of 17\(^{\alpha}\)-hydroxyprogesterone and 20\(^{\beta}\)-hydroxy-4-pregnen-3-one, the identity of the remaining radioactive metabolites isolated i.e. androstenedione and testosterone was assumed, as these steroids were previously shown to be metabolites of [4-\(^{14}\)C]progesterone by thecal homogenates from large follicles (see 3:1:3). Progesterone concentrations decreased progressively over the 100 minute period, whilst production of 20\(^{\beta}\)-hydroxy-4-pregnen-3-one rose steadily. Production of androstenedione, testosterone and a steroid similar to 17\(^{\alpha}\)-hydroxyprogesterone over this period was very low, but increased slightly. When cofactors were omitted from the incubation medium production of 20\(^{\beta}\)-hydroxy-4-pregnen-3-one after 100 minutes was also very low (reduced by 94%). With an NADPH generating system only present in the incubation medium, production of 20\(^{\beta}\)-hydroxy-4-pregnen-3-one was greatly increased but to only 60% of that produced when both NAD\(^{+}\) and an NADPH generating system were present in the incubation medium. With NAD\(^{+}\) only, production of 20\(^{\beta}\)-hydroxy-4-pregnen-3-one equalled that achieved when both cofactors were present.
Legend to Figure 3.2

Time-study showing the production of 20β-hydroxy-4-pregnen-3-one, androstenedione, testosterone and a steroid similar to 17α-hydroxyprogesterone after incubation of thecal homogenates from the largest follicle, collected 1 to 2 hours before ovulation, with [4-14C]progesterone. For details of incubation procedure see text.
- 20β-Hydroxy-4-pregnene-3-one
- Progesterone
- Androstenedione
- 17α-Hydroxyprogesterone
- Testosterone
in the incubation medium. These results indicate that the incubation system is able to generate NADH from the added NAD⁺, and that thecal 20β-hydroxysteroid dehydrogenase may utilize either NADH or NADPH as a cofactor, although under these incubation conditions it appears that NADH is utilized preferentially for the conversion of progesterone to 20β-hydroxy-4-pregnen-3-one.

b) Identification of 20β-hydroxy-4-pregnen-3-one

The pooled radiometabolite from the 5 separate incubations (carried out concomitantly with the above time study) which corresponded to 20β-hydroxy-4-pregnen-3-one was chromatographed in a high pressure liquid chromatography (h.p.l.c.) system using a 'Hypersil' M22 column, 5 x 100 mm with methanol:water (60:40, v/v) as the solvent. With this system, a single ultra-violet absorbing peak was recorded at 242 nm, confirming the presence of a conjugated system, presumably a Δ⁴-3-keto structure in the molecule. The retention time of the metabolite (5.2 minutes) was identical to that of 20β-hydroxy-4-pregnen-3-one and was clearly separated from the following Δ⁴-3-keto steroids; androstenedione (2.0 minutes), 17α-hydroxyprogesterone (2.4 minutes), testosterone (2.5 minutes), progesterone (3.7 minutes) and 20α-hydroxy-4-pregnen-3-one (4.1 minutes). (See figure 3.3). After passing through the detector, the metabolite was recovered by evaporating the solvent and recrystallised to constant specific activity after the addition of 25 mg of authentic 20β-hydroxy-4-pregnen-3-one as a final proof of identity. (See table 3.8).
Identification of the pooled radiometabolite, produced from [4-\textsuperscript{14}C]progesterone by thecal homogenates from the largest follicle (Fl) 1 to 2 hours before ovulation, by high-pressure liquid chromatography. Column: hypersil M22, length 5 x 100 mm; pressure 1600 psi; solvent 60% methanol; wavelength of detector 242 nm; absorbance range 0.1.

i denotes injection onto column of either a) or b)

a) Mixture of standards:
   1. solvent
   2. androstenedione
   3. 17\textalpha -hydroxyprogesterone
   4. testosterone
   5. 20\textalpha -hydroxy-4-pregnen-3-one
   6. 20\textbeta -hydroxy-4-pregnen-3-one

b) Radiometabolite from Fl = X
Table 3.8  Recrystallisation of radiometabolite from Fl (Zone III) with 20β-hydroxy-4-pregnen-3-one after elution from h.p.l.c. column.

<table>
<thead>
<tr>
<th>Recrys. No.*</th>
<th>Solvent Pair</th>
<th>Crystals (dpm/mg)</th>
<th>Mother Liquor (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>50,090</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Dichloromethane/hexane</td>
<td>49,227</td>
<td>47,840</td>
</tr>
<tr>
<td>2</td>
<td>Dichloromethane/hexane</td>
<td>49,964</td>
<td>46,390</td>
</tr>
<tr>
<td>3</td>
<td>Acetone/hexane</td>
<td>49,634</td>
<td>46,332</td>
</tr>
<tr>
<td>4</td>
<td>Acetone/ethylacetate</td>
<td>48,952</td>
<td>42,725</td>
</tr>
<tr>
<td>5</td>
<td>Acetone/cyclohexane</td>
<td>46,520</td>
<td>69,350</td>
</tr>
<tr>
<td>6</td>
<td>Acetone/cyclohexane</td>
<td>46,165</td>
<td>43,186</td>
</tr>
</tbody>
</table>

* Recrystallisation Number
Discussion

It has been shown that the steroid produced in large amounts from \([4^{-14}C]\)progesterone by thecal homogenates of the large, preovulatory follicle shortly before ovulation is \(20\beta\)-hydroxy-4-pregnen-3-one. As thecal homogenates from the second and third largest follicle, due to ovulate 24 hours and 48 hours later respectively, produced much lower amounts of \(20\beta\)-hydroxy-4-pregnen-3-one at this time (see section 3:1:3), a possible involvement of this steroid in the ovulatory process deserves further investigation.

The mechanism whereby LH induces ovulation of the preovulatory follicle is not known, although progesterone synthesis has been implicated (Tojo and Huston, 1981). Etches, MacGregor, Morris and Williams (1983) administered gonadotrophin-releasing hormone (GnRH) to laying hens at various times of the ovulatory cycle and found that GnRH was first able to elicit premature ovulation of the largest follicle 10 hours after the preceding ovulation, i.e. around 16 hours before it was due to ovulate. It seems that the effectiveness of exogenous GnRH, LH or chicken anterior pituitary extracts in bringing about a premature ovulation is related to their ability to stimulate a sustained rise in follicular progesterone levels similar to that normally seen following the endogenous surge of LH (Imai, 1977; Etches et al, 1983).

The second and third largest follicles are also capable of responding to the LH surge (Shahabi et al, 1975a) or to exogenous LH with an increased progesterone
production (Shahabi et al, 1975b; Imai and Nalbandov, 1979; Imai, 1977; Culbert et al, 1980) but do not normally ovulate in response to LH stimulation. As these follicles, but not the largest follicle, also produce androgens and oestrogens in response to the LH surge (Shahabi et al, 1975a), it is possible that ovulation can only take place when there is a sustained increase in thecal progesterone levels in conjunction with low levels of androgens and oestrogens. An increase in conversion of progesterone to \(20\beta\)-hydroxy-4-pregnen-3-one within the thecal tissue of the largest follicle following the surge of LH, by limiting the amount of progesterone available for conversion to \(17\alpha\)-hydroxyprogesterone and hence androstenedione via the \(\Delta^4\) pathway, may ensure that thecal androgen and oestrogen concentrations remain low in the period between the LH surge and ovulation. An inverse relationship between \(20\beta\)-hydroxy-4-pregnen-3-one production and production of androgens and oestrogens has also been observed by Galli and Wasserman (1973) in the embryonic chick ovary, after incubation of ovarian slices with \([4-^{14}C]\)progesterone in vitro. Tojo, Fujii and Ogawa (1982) have measured thecal progesterone and oestradiol concentrations during the 9 hour period preceding ovulation. Their results show a progressive increase in the ratio of progesterone to oestradiol during this period. Although it has been proposed that progesterone synthesis within the preovulatory follicle is necessary for follicle rupture (Tojo
and Huston, 1981) possibly by stimulating collagenolytic activity. Tojo et al (1982) were unable to demonstrate any change in thecal collagenase, acid protease or neutral protease activity in the 9 hour period leading up to ovulation.

Whether or not an increased production of 20β-hydroxy-4-pregnen-3-one by the theca layer of the pre-ovulatory follicle in response to the plasma surge of LH could play a direct role in the ovulatory process remains unclear. In other non-mammalian vertebrates, such as amphibians (Schuetz, 1967; Smith, Ecker and Subtelny, 1968; Reynhout and Smith, 1973; Finidori-Lepicard, Schorderet-Slatkine, Hanoune and Baulieu, 1981) and fish (Hirose, 1976; Jalabert, Bry, Szollosi and Fostier, 1973; Sundararaj and Goswami, 1977; Iwamat-su, 1978), progesterone and other C21-steroids may be responsible for inducing the final stage of oocyte maturation. In a variety of fish (including rainbow trout, brook trout, pike, yellow perch and goldfish) 17α,20β-dihydroxy-4-pregnen-3-one and 20β-hydroxy-4-pregnen-3-one were found to be particularly effective at inducing germinal vesicle breakdown and ovulation in vitro (Jalabert, 1976; Goetz and Bergman, 1978; Goetz and Theofan, 1979; Duffy and Goetz, 1980). In addition, plasma concentrations of 17α,20β-dihydroxy-4-pregnen-3-one rose dramatically during the final phase of oocyte maturation and ovulation in both the salmon and the rainbow trout (Campbell, Fostier, Jalabert and Truscott,
1980; Wright and Hunt, 1982). It is conceivable that in the hen, production of 20β-hydroxy-4-pregnen-3-one by thecal tissue of the preovulatory follicle may also induce the final stage of oocyte maturation.

[4-14C]progesterone metabolism by thecal tissue from small follicles, the second largest follicle and by the zona parenchymatosa

Introduction

The previous two sections have dealt with the metabolism of [4-14C]progesterone by thecal tissue homogenates from the 5 to 7 large hierarchical follicles only. The aim of the work presented in this section was to investigate the metabolism of [4-14C]progesterone by thecal homogenates from the smaller follicles and by homogenates of the zona parenchymatosa. Whilst these tissues have been shown to contain interstitial cells with ultrastructural features characteristic of steroid synthesis (Dahl, 1970a) and which possess the enzymes 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase (Wyburn and Baillie, 1966; Boucek and Savard, 1970; Peel and Bellairs, 1972), much of the evidence linking these tissues with steroidogenesis in the laying hen is indirect (see section 1:2:2, for review). The results presented here show that thecal homogenates from small follicles and homogenates of the zona parenchymatosa are capable of converting [4-14C]progesterone to androstenedione and testosterone. The metabolism of [4-14C]progesterone by these tissues is also compared with that of
thecal homogenates from the second largest ovarian follicle (F2) in order to make a preliminary assessment of the changes in interstitial enzyme activities during follicular development.

3:3:2 Experimental procedures

Six laying hens (Ross Brown's) were killed 15 hours before ovulation and eight hens were killed 1 to 2 hours before an expected ovulation. From each ovary, the second largest follicle, F2, was chosen as a representative of the large follicles (i.e. those follicles > 10 mm in diameter) as it had been shown previously that [4-\textsuperscript{14}C]-progesterone metabolism by thecal homogenates from these follicles was similar (section 3:1:3). The remaining large follicles were discarded. Two groups of small follicles were obtained; group 1 consisting of follicles 4 mm < 10 mm in diameter and group 2 consisting of follicles 2 mm < 4 mm in diameter. The zona pannychymatosa was also obtained (as described in section 2:2). For each hen, routine histology confirmed that this piece of tissue was indeed the zona pannychymatosa. Thecal homogenates of F2, thecal homogenates from each group of small follicles (pool 1 and pool 2) and homogenates of Z.P. were incubated with [4-\textsuperscript{14}C]progesterone as outlined in figure 3.4. Details of the incubation procedure are given in section 2:4.

Further aliquots (0.2 ml) of each homogenate were also incubated separately with [4-\textsuperscript{14}C]testosterone. For clarity however, these results are presented later in section 3:4.
Legend to Figure 3.4

A flow-chart outlining the procedure used for incubation of the homogenates with [4-$^{14}$C]-progesterone (or [4-$^{14}$C]testosterone) and the isolation of the radiometabolites from the incubation medium. For full details see section 2:4.
1. tissue

2. homogenise in 10 vols 0.25M sucrose

3. 0.5 ml homogenate + $^{14}$[C]-progesterone (18.9 kBq) or $^{14}$[C]-testosterone (19.4 kBq)

4. incubate 3h at 37°c in 95% O$_2$ : 5% CO$_2$

5. extract 3×5 vols CH$_2$Cl$_2$

6. i.t.i.c. chromatography
After extraction of the metabolites from the incubation medium (as described in section 2:2), recoveries of between 75% and 80% were obtained. Duplicate extracts were chromatographed in solvent system 1 and the radioactive metabolites located by autoradiography. As before (see plate 3.1), the metabolites were separated into 5 zones, but this time with some streaking of activity between them. This was thought to be caused by the presence of lipid in the extracts. Previously, when an aliquot of each extract was chromatographed instead of the whole extract, the amount of lipid spotted onto the chromatogram had not been sufficient to cause this problem.

After chromatography of the duplicate extracts, one set of radioactive zones was cut out, placed in plastic mini-vials, and after the addition of 4 ml scintillator (0.4% PPO, 0.032% POPOP), the vials were counted using an LKB Wallac scintillation analyser with an efficiency for $^{14}$C counting of 93%. After correction for quenching and counting efficiency, the amount of activity (dpm) in each zone was calculated and corrected for recovery. The corresponding set of radioactive zones was also cut out and the radioactive zones eluted. After purification by subsequent chromatography (see section 3:3:3 (f) the percentage of any given metabolite in each zone was calculated. Knowing this and the amount of activity in each zone, the yields of individual metabolites were obtained. As before, the results were converted from dpm to nmol (section 3:1:2) and expressed in nmol/20 mg fresh tissue per hour.
Results

a) Statistical treatment of results

For each tissue type, the data collected at different periods of the ovulatory cycle were analysed using the Wilcoxon Mann-Whitney two-sample test (Snedecor & Cochran, 1967). In this case, Student's t-test was not appropriate because of the heterogeneity of the sample variances.

b) [4-^14C]progesterone metabolism

Table 3.9 shows the amount of [4-^14C]progesterone left (nmol/20 mg wet tissue per hour). All the homogenates actively metabolized progesterone, with conversion rates of around 97%. There were no significant differences in progesterone metabolism between the two periods of the ovulatory cycle. Thecal homogenates from the second largest follicle (F2) were less active in metabolizing progesterone; on average 2 to 3 times as much progesterone remained unmetabolized at the end of the incubation period compared with the other homogenates (pool 1, pool 2 and zona parenchymatosa).

c) Conversion of [4-^14C]progesterone to 20β-hydroxy-4-pregnen-3-one

Table 3.10 shows the conversion of [4-^14C]progesterone to 20β-hydroxy-4-pregnen-3-one by the thecal homogenates (F2, pool 1 and pool 2). This steroid was not produced by the zona parenchymatosa homogenates. The latter produced large amounts of 5β-pregnan-3α-ol-20-one (shown also in table 3.10). No significant differences in conversion of [4-^14C]progesterone to either
Table 3.9  Metabolism of \([4-\text{C}]\)progesterone by ovarian homogenates (nmol left/20 mg wet tissue per hour) at two different periods of the ovulatory cycle. Median values are given with the range indicated in parenthesis.

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>1 - 2 hours</th>
<th>15 hours</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thecal, F2*</td>
<td>0.095 (0.048 - 0.253)</td>
<td>0.217 (0.101 - 0.385)</td>
<td>NS</td>
</tr>
<tr>
<td>Thecal, pool 1**</td>
<td>0.063 (0.022 - 0.145)</td>
<td>0.118 (0.035 - 0.140)</td>
<td>NS</td>
</tr>
<tr>
<td>Thecal, pool 2+</td>
<td>0.060 (0.022 - 0.092)</td>
<td>0.086 (0.050 - 0.098)</td>
<td>NS</td>
</tr>
<tr>
<td>Zona parenchymatosa++</td>
<td>0.029 (0.016 - 0.062)</td>
<td>0.060 (0.047 - 0.078)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS - No significant difference  
* second largest follicle  
** pooled thecae from follicles $>4 \text{ mm} < 10 \text{ mm}$ in diameter  
+ pooled thecae from follicles $>2 \text{ mm} < 4 \text{ mm}$ in diameter  
++ contains follicles $<2 \text{ mm}$ in diameter
Table 3.10  Conversion of [4-\textsuperscript{14}C]progesterone to A) 20\beta-hydroxy-4-pregnen-3-one and B) 5\beta-pregnan-3\alpha-ol-20-one by thecal and zona parenchymatosa homogenates respectively at two different periods of the ovulatory cycle (nmol/20 mg wet tissue per hour). Median values are given with the range indicated in parenthesis (see table 3.9 for explanatory footnotes).

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Time to ovulation</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 - 2 hours</td>
<td>15 hours</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thecal, F2*</td>
<td>0.129 (0.110 - 0.478)</td>
<td>0.324 (0.264 - 0.382)</td>
</tr>
<tr>
<td>Thecal, pool 1**</td>
<td>0.064 (0.029 - 0.104)</td>
<td>0.053 (0.024 - 0.136)</td>
</tr>
<tr>
<td>Thecal, pool 2+</td>
<td>0.091 (0.033 - 0.237)</td>
<td>0.065 (0.028 - 0.112)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zona parenchymatosa**</td>
<td>0.374 (0.190 - 0.767)</td>
<td>0.784 (0.352 - 1.049)</td>
</tr>
</tbody>
</table>
20β-hydroxy-4-pregnen-3-one (by thecal homogenates) or 5β-pregn-3α-ol-20-one (by zona parenchymatosa homogenates) were observed between the two periods of the ovulatory cycle studied.

d) Conversion of [4-14C]progesterone to androstenedione

Table 3.11 shows the conversion of [4-14C]progesterone to androstenedione by the ovarian homogenates. Thecal homogenates produced approximately 10 times more androstenedione than did zona parenchymatosa homogenates. Conversion of [4-14C]progesterone to androstenedione by thecal homogenates of the second largest follicle (F2) and zona parenchymatosa homogenates did not differ significantly according to the period of the ovulatory cycle. Thecal homogenates from both pools of small follicles (pool 1, 4 to 10 mm in diameter and pool 2, 2 to 4 mm in diameter) however produced significantly more androstenedione 1 to 2 hours before ovulation compared with earlier in the cycle (15 hours before ovulation).

e) Conversion of [4-14C]progesterone to testosterone

Table 3.12 shows the conversion of [4-14C]progesterone to testosterone by the ovarian homogenates. No significant differences in testosterone production by any of the homogenates was observed between the two periods of the ovulatory cycle studied except for thecal homogenates from F2 which produced significantly more testosterone 1 to 2 hours before ovulation compared with earlier in
Table 3.11 Conversion of [4-$^{14}$C]progesterone to androstenedione (nmol/20 mg wet tissue per hour) by ovarian homogenates at two different periods of the ovulatory cycle. Median values are given with the range indicated in parenthesis (see table 3.9 for explanatory footnotes).

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>1 - 2 hours</th>
<th>15 hours</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thecal, F2*</td>
<td>0.362 (0.283 - 0.631)</td>
<td>0.282 (0.097 - 0.516)</td>
<td>NS</td>
</tr>
<tr>
<td>Thecal, pool 1**</td>
<td>0.354 (0.143 - 0.778)</td>
<td>0.107 (0.056 - 0.152)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Thecal, pool 2+</td>
<td>0.149 (0.027 - 0.426)</td>
<td>0.099 (0.064 - 0.147)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Zona parenchymatosa++</td>
<td>0.015 (0.011 - 0.130)</td>
<td>0.031 (0.026 - 0.050)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 3.12 Conversion of [4-\(^{14}\)C]progesterone to testosterone (nmol/20 mg wet tissue per hour) by ovarian homogenates at two different periods of the ovulatory cycle. Median values are given with the range in parenthesis (see table 3.9 for explanatory footnotes).

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>1 - 2 hours</th>
<th>15 hours</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thecal, F2*</td>
<td>0.025 (0.018 - 0.032)</td>
<td>0.019 (0.015 - 0.022)</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Thecal, pool 1**</td>
<td>0.079 (0.049 - 0.099)</td>
<td>0.070 (0.061 - 0.087)</td>
<td>NS</td>
</tr>
<tr>
<td>Thecal, pool 2†</td>
<td>0.043 (0.037 - 0.061)</td>
<td>0.052 (0.045 - 0.056)</td>
<td>NS</td>
</tr>
<tr>
<td>Zona parenchymatosa***</td>
<td>0.006 (0.005 - 0.007)</td>
<td>0.005 (0.004 - 0.005)</td>
<td>NP</td>
</tr>
</tbody>
</table>

NP - comparison not possible due to the low conversion rate obtained
the cycle. This result should be regarded with caution, since the percentage conversions involved are less than 1%. Thecal homogenates produced approximately 10 times more testosterone than did homogenates of zona par
chymatosa. This was similar to the data observed for androstenedione production (see table 3.11). Thecal homogenates from small follicles (pools 1 and 2), however, produced more testosterone than did thecal homogenates of large (F2) follicles.

f) Identification of metabolites

After the initial development in solvent system 1, the metabolites were separated into 5 zones. The first 3 zones I, II and III corresponded to the carrier steroids progesterone, androstenedione and 20β-hydroxy-4-pregnen-3-one respectively. Zone IV contained small amounts of an unknown metabolite not previously detected as a product of [4-14C]progesterone metabolism (section 3:1:3). Zone V contained the more polar metabolites and in addition, testosterone and 17α-hydroxyprogesterone (previously Zone IV).

ZONE I, the area corresponding to progesterone, was run in solvent system 13. Most of the activity on the chromatogram, i.e. 93%, 87%, 83% and 71% for F2, 1, 2 and Z.P. respectively, was associated with carrier progesterone (and also 5β-androstenedione which has the same Rf as progesterone in this solvent system). In addition, there were three smaller peaks with Rf's similar to 5α-pregnanedione, 5β-pregnanedione and
5α-androstanedione. No further attempt to identify these steroids was made. The radioactive areas corresponding to progesterone were eluted separately and chromatographed in solvent system 10 in order to separate progesterone from 5β-androstanedione. Two radioactive peaks were obtained, the first corresponding to progesterone and accounting for 73%, 43%, 28% and 30% of the total activity on the chromatogram for F, 1, 2 and Z.P. respectively. The second peak, which contained the remainder of the activity, corresponded to 5β-androstanedione.

**ZONE II**, which was coincident with carrier androstenedione, was chromatographed in solvent system 13. All of the activity from F2 and 1, and 90% and 59% of the activity from 2 and Z.P. respectively, remained associated with androstenedione. The other peak of activity present in Zone II from 2 and Z.P. remained unidentified. The activity corresponding to androstenedione from F2, 1, 2 and Z.P. was eluted and aliquots from each were taken for oxidation and acetylation. The remaining activity was pooled and recrystallised with authentic androstenedione (see table 3.13). Upon oxidation 97%, 94%, 95% and 44% of the activity from F2, 1, 2 and Z.P. respectively, was still associated with androstenedione, the rest of the activity corresponding to 5β-pregnane[...](solvent system 13). Upon acetylation, similar results were obtained, with most of the activity from F2, 1 and 2, and slightly less than half the activity from Z.P., remaining coincident with androstenedione. Two other peaks of
Table 3.13 Recrystallisation data for the radiometabolite isolated from Zone II with androstenedione, radiometabolite from acetylated Zone V with testosterone acetate and radiometabolite from Zone V (after acetylation) with 17α-hydroxyprogesterone.

<table>
<thead>
<tr>
<th>Recrys. No.</th>
<th>Solvent Pair</th>
<th>Specific Activity (dpm/mg)</th>
<th>17α-hydroxyprogesterone</th>
<th>androstenedione**</th>
<th>testosterone acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crystals</td>
<td>ML*</td>
<td>Crystals</td>
<td>ML*</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>24,191</td>
<td>-</td>
<td>27,101</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
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<td>24,604</td>
<td>28,245</td>
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<td>93,980</td>
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<td>21,933</td>
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<td>Acetone/ethyl acetate</td>
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<tr>
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<td>Acetone/hexane</td>
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<td>8,261</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>Acetone/cyclohexane</td>
<td>1,081</td>
<td>11,521</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mother liquor
+ Recrystallisation number

** Following rechromatography of Zone II after acetylation, the specific activity of the crystals was initially 943. Values of 654, 531, 498, 488, 478 and 445 were obtained for 6 successive recrystallisations.
<table>
<thead>
<tr>
<th>Recrys. No.</th>
<th>Solvent Pair</th>
<th>Specific Activity (dpm/mg)</th>
<th>20β-hydroxy-4-pregnen-3-one</th>
<th>5β-pregnan-3α-ol-20-one</th>
<th>5β-pregnanedione</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crystals</td>
<td>ML*</td>
<td>Crystals</td>
<td>ML*</td>
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<tr>
<td>0</td>
<td></td>
<td>8,987</td>
<td>-</td>
<td>3,446</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Acetone/hexane</td>
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<td>21,821</td>
<td>3,448</td>
<td>6,465</td>
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<td>Acetone/cyclohexane</td>
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<td>3,091</td>
<td>2,834</td>
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<td>Acetone/hexane</td>
<td>8,498</td>
<td>10,797</td>
<td>2,675</td>
<td>1,391</td>
</tr>
</tbody>
</table>

* Mother liquor
+ Recrystallisation number
** zona parenchymatosa
activity were obtained after acetylation, one of which was similar in \( R_F \) but not identical to \( 5\beta -\text{pregnan-3}\beta -\text{ol-20-one} \) monoacetate (solvent system 4 and 6). The radioactivity corresponding to androstenedione after acetylation from F2, 1, 2 and Z.P. was eluted, pooled and recrystallised to constant specific activity with authentic androstenedione (see footnote to table 3.13).

ZONE III from F2, 1 and 2 was coincident with \( 20\beta -\text{hydroxy-4-pregnen-3-one} \) in solvent system 1. Upon subsequent chromatography in solvent system 13, two peaks of activity were obtained. The first corresponded to \( 20\beta -\text{hydroxy-4-pregnen-3-one} \) and represented 87%, 54% and 52% of the total activity on the chromatogram for F2, 1 and 2 respectively. This peak gave progesterone upon oxidation (solvent system 13), \( 20\beta -\text{hydroxy-4-pregnen-3-one} \) monoacetate upon acetylation (solvent system 4), and was subsequently recrystallised to constant specific activity with authentic \( 20\beta -\text{hydroxy-4-pregnen-3-one} \) (see table 3.14). The second peak of activity gave \( 5\beta -\text{androstanedione/progesterone} \) (solvent system 13) and upon acetylation, two acetates were obtained; one with the same \( R_F \) as \( 5\beta -\text{androstan-3}\beta -\text{ol-17-one} \) monoacetate, and the other with an \( R_F \) similar to \( 5\beta -\text{androstan-17\beta -ol-3-one} \) monoacetate in solvent systems 4 and 6.

Zone III from Z.P. which accounted for approximately 20% of the total activity on the chromatogram after running in the initial solvent system (1) was not coincident with \( 20\beta -\text{hydroxy-4-pregnen-3-one} \) after
chromatography in solvent system 1. Oxidation gave pre-
dominately $5\beta$-pregnanedione accompanied by a small amount
of $5\beta$-androstanedione and another trace of activity which
was not identified (solvent system 13). The oxidised peak
corresponding to $5\beta$-pregnanedione was recrystallised to
constant specific activity with authentic $5\beta$-pregnan-
dione (see table 3.14). Zone III from Z.P. was then
recrystallised to constant specific activity with auth-
entic $5\beta$-pregnan-3$\alpha$-ol-20-one. (See table 3.13).

ZONE IV had the same $R_F$ as 20$\alpha$-hydroxy-4-pregnen-
3-one and $5\beta$-androstan-3$\alpha$-ol-17-one after the initial
chromatographic development in solvent system 1. After
elution from the chromatogram this zone was purified by
re-chromatographing in the same solvent system in order
to remove small amounts of 20$\beta$-hydroxy-4-pregnen-3-one
present. For F2, 1, 2 and Z.P., 81%, 92%, 94% and 93%
respectively, of the total activity present was still
associated with 20$\alpha$-hydroxy-4-pregnen-3-one and/or
$5\beta$-androstan-3$\alpha$-ol-17-one after development of the
chromatogram. Due to the small amounts of activity
present, Zone IV from F2, 1, 2 and Z.P. was pooled and
aliquots taken for oxidation and acetylation. Oxidation
gave one radioactive peak with the same $R_F$ as progest-
erone and $5\beta$-androstanedione in solvent system 13.
Upon acetylation, one radioactive peak with exactly the
same $R_F$ as $5\beta$-androstan-3$\alpha$-ol-17-one monoacetate was
obtained (solvent system 4) which was clearly separated
from 20$\alpha$-hydroxy-4-pregnen-3-one monoacetate. The
Table 3.15  Partition of Zone V between 1M NaOH (phenolic fraction) and toluene/petroleum spirit, 60 - 80°C b.p. (1:1, v/v) (neutral fraction) using a counter current distribution procedure as described in 2:5:2. Results are expressed as a percentage of the total activity in each tube.

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Neutral Fraction</th>
<th>Phenolic Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thecal, F2*</td>
<td>92.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Thecal, pool 1*</td>
<td>78.6</td>
<td>14.9</td>
</tr>
<tr>
<td>Thecal, pool 2**</td>
<td>84.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Zona parenchymatosa**</td>
<td>88.9</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*second largest follicle
*thecae pooled from follicles > 4 < 10 mm in diameter
**theca pooled from follicles > 2 < 4 mm in diameter
++contains follicles < 2 mm in diameter
steroid present in Zone IV was therefore tentatively identified as $5\beta$-androstan-3$\alpha$-ol-17-one.

**ZONE V** was partitioned between 1M NaOH and toluene: light petroleum, b.p. 60 to 80°C (1:1, v/v) as described in materials and methods (section 2:5:2). The distribution of radioactivity after this partition procedure is shown in table 3.15. The phenolic fractions from 1 and 2 contained the highest amounts of activity, indicating that thecal homogenates from small follicles may have a greater ability to convert progesterone to oestrogens than thecal homogenate from large F2 follicles and homogenates of zona parenchymatosa (Z.P.). The phenolic fractions were run in solvent system 7 which separates oestrone, oestradiol and oestriol from each other. In all the phenolic fractions, the majority of the activity remained at the baseline and was associated with oestriol. In 1 and 2 however, this activity was streaked upwards and included both the oestriol and oestradiol standards. In F2 and Z.P. a spot of activity similar in $R_F$ to oestradiol, and in each case accounting for about 3.0% of the total activity present on the chromatogram, was detected. Small amounts of activity corresponding to oestrone were also present, accounting for 1%, 5%, 6% and 3% of the total activity on the chromatogram for F2, 1, 2 and Z.P. respectively. Two additional spots of activity, with an $R_F$ higher than oestrone, were also detected, notably in the phenolic fractions from 1 and 2. These metabolites were not identified.
The neutral fractions were run in solvent system 8 which separated a zone containing the carrier steroids testosterone and $17\alpha$-hydroxyprogesterone from the remaining steroids (probably androstane/pregnane diols and triols) which were not identified further. 10%, 31%, 36% and 21% of the activity from F2, 1, 2 and Z.P. respectively, were associated with testosterone and $17\alpha$-hydroxyprogesterone. This area of the chromatogram was cut out and eluted. After acetylation, carrier $17\alpha$-hydroxyprogesterone and testosterone acetate were separated by chromatography in solvent system 2. 20%, 21%, 12% and 2% of this activity from F2, 1, 2 and Z.P. respectively, was associated with testosterone acetate, the rest corresponded to $17\alpha$-hydroxyprogesterone. The activity corresponding to testosterone acetate was eluted, pooled and recrystallised to constant specific activity with authentic testosterone acetate (see table 3.14). The activity corresponding to $17\alpha$-hydroxyprogesterone was also eluted, pooled and recrystallised with authentic $17\alpha$-hydroxyprogesterone but did not yield crystals of constant specific activity (see table 3.13). It is possible that a small amount of $17\alpha$-hydroxyprogesterone was present in addition to another unidentified metabolite.
Discussion

a) Conversion of $[4^{14}C]$progesterone to androstenedione, testosterone and $20\beta$-hydroxy-4-pregnen-3-one

Figure 3.5 summarizes the metabolism of $[4^{14}C]$-progesterone by the different homogenates. From these results it is evident that thecal tissue homogenates from follicles of 2 mm in diameter and over have a greater capacity to convert progesterone to androstenedione than the zona parenchymatosa homogenates, which comprised follicles less than 2 mm in diameter together with some interfollicular stromal tissue. It could be argued that the zona parenchymatosa homogenates contained a lower proportion of interstitial cells compared to the thecal homogenates, due to the inclusion of yolk and a small amount of stromal tissue. If this is the case, their ability to convert progesterone to androstenedione may be slightly greater than it appears in figure 3.5. Even so, the present results indicate that thecal homogenates from follicles of $>2$ mm in diameter possess a greater $17\alpha$-hydroxylase and $C-17,20\alpha$-lyase activity than the zona parenchymatosa. In addition, the present results demonstrate that thecal homogenates obtained from small follicles of 2 to 10 mm in diameter converted significantly more $[4^{14}C]$progesterone to androstenedione at 1 to 2 hours before ovulation compared with earlier in the cycle (15 hours before ovulation). It is possible that these follicles, unlike the
**Legend to Figure 3.5**

To illustrate the metabolism of [4-\(^{14}\)C]progesterone by the various homogenates 1 to 2 hours before ovulation. Values are given as means.

The following abbreviations have been used:

- \(P_4\) - progesterone
- \(A_4\) - androstenedione
- \(T\) - testosterone
- \(20\beta P\) - \(20\beta\)-hydroxy-4-pregnen-3-one
- \(5\beta P3\alpha\) - \(5\beta\)-pregnan-3\(\alpha\)-ol-20-one
- \(5\beta A3\alpha\) - \(5\beta\)-androstan-3\(\alpha\)-ol-17-one
- \(5\beta A\) - \(5\beta\)-androstenedione
- ? - remaining unidentified (\(5\beta\)-reduced) metabolites at baseline
1.0
0.8
0.6
0.4
0.2
0

nmol/20mg fresh tissue per h

F₂ follicle ≥ 30mm i.d.
follicles ≥ 4mm < 10mm i.d.
follicles ≥ 2mm < 4mm i.d.
zona parenchymatosa

P₄
A₄
T
20βP

5βP₃α
5βA₃α
5βA

?
large preovulatory follicles (as exemplified by F2), respond to the plasma surge of LH with an increase in thecal 17α-hydroxylase and/or C-17,20lyase activity. Thus LH may play an important regulatory role during follicular development by stimulating thecal 17α-hydroxylase and/or C-17,20lyase activity in the small (2 to 10 mm in diameter) follicles, as has been proposed in the rat (Bogovich and Richards, 1980).

Conversion of [4-14C]progesterone to testosterone reflected the conversion of [4-14C]progesterone to androstenedione in that the thecal homogenates produced more testosterone than did the zona pellucida homogenates (see figure 3.5). With the exception of the second largest follicle (F2), testosterone production also appeared to be related to follicular development, being of the order: follicles 4 to 10 mm in diameter > follicles 2 to 4 mm in diameter > zona pellucida. The lack of conversion of [4-14C]progesterone to testosterone by thecal homogenates from the second largest follicle is puzzling since the same homogenates were able to convert large amounts of [4-14C]progesterone to androstenedione and subsequent studies (see section 3:4) were able to show that they also possess an active 17β-hydroxysteroid dehydrogenase enzyme using either [4-14C]androstenedione or [4-14C]testosterone as a substrate. Perhaps in the present experiment, the incubation conditions favoured androstenedione formation in the F2 thecal homogenates.
Thecal homogenates from the second largest follicle converted greater amounts of [4-\(^{14}\)C]progesterone to 20\(\beta\) -hydroxy-4-pregnen-3-one than did thecal homogenates from the smaller follicles (2 to 10 mm in diameter). In conjunction with the previous experiments (3:1 and 3:2), it would appear that large preovulatory follicles contain a higher thecal 20\(\beta\) -hydroxysteroid dehydrogenase activity than the smaller (2 to 10 mm in diameter) follicles.

Wyburn and Baillie (1966) also obtained a positive histochemical reaction for this enzyme in the theca interna of developing follicles (2 mm, 7 mm, 15 mm and 35 mm in diameter). 20\(\beta\) -Hydroxy-4-pregnen-3-one and its 20\(\alpha\) -epimer have been identified previously in ovarian extracts and in the plasma of the laying hen (Furr, 1970; O'Malley et al, 1968). Taken together with the present results, these findings suggest that 20\(\beta\) -hydroxy-4-pregnen-3-one, synthesized by the thecal tissue of developing follicles, may be a secretory product of the ovary in vivo. The thecal homogenates used in this study did not convert [4-\(^{14}\)C]progesterone to 20\(\alpha\) -hydroxy-4-pregnen-3-one. Nevertheless, Nakamura et al (1974) have shown that ovarian homogenates are capable of converting [4-\(^{14}\)C]-17\(\alpha\) -hydroxyprogesterone to 17\(\alpha\),20\(\alpha\) -dihydroxy-4-pregnen-3-one in addition to its 20\(\beta\) -epimer. Galli and Wasserman (1972; 1973) also found that ovarian slices from 7, 10 and 15 day old chick embryos were capable of converting [4-\(^{14}\)C]progesterone to both 20\(\beta\) -hydroxy-4-pregnen-3-one and 20\(\alpha\) -hydroxy-4-pregnen-3-one, although
Figure 3.6 Proposed metabolic pathway of progesterone metabolism by thecal and zona parenchymatosa homogenates in vitro.

5β-pregnanedione \rightarrow progesterone \rightarrow 20β-hydroxy-4-pregnen-3-one

5β-pregnan-3α-ol-20-one

5β-pregnan-3α,20β-diol

5β-androstanedione \rightarrow androstenedione \rightarrow 17α-hydroxyprogesterone

5β-androstan-3α-ol-17-one

5β-androstane-3α,17β-diol

Testosterone
only small amounts of the latter were formed.

The failure of zona parenchymatosa homogenates to convert \( [4^{-14}\text{C}]\)progesterone to \( 20\beta\)-hydroxy-4-pregnen-3-one does not necessarily imply that they lack the enzyme \( 20\beta\)-hydroxysteroid dehydrogenase. These homogenates produced large amounts of \( 5\beta\)-pregnen-3\(\alpha\)-ol-20-one. \( 5\beta\)-reduction of \( [4^{-14}\text{C}]\)progesterone followed by reduction of the keto groups at the 3 and 20 position, would result in the formation of \( 5\beta\)-pregnane-3\(\alpha\),20\(\beta\)-diol rather than \( 20\beta\)-hydroxy-4-pregnen-3-one.

b) Conversion of \( [4^{-14}\text{C}]\)progesterone to other metabolites

The other metabolites which were formed from \( [4^{-14}\text{C}]\)progesterone by the homogenates included \( 5\beta\)-androstane-3-one, \( 5\beta\)-pregnan-3\(\alpha\)-ol-20-one and \( 5\beta\)-androstane-3\(\alpha\)-ol-17-one. Small amounts of \( 5\beta\)-pregnane-3\(\beta\)-ol-20-one and \( 5\beta\)-androstane-3\(\beta\)-ol-17-one may also have been produced. With the exception of \( 5\beta\)-pregnane-3\(\alpha\)-ol-20-one which was crystallised to constant specific activity, these metabolites were only tentatively identified i.e. by isopolarity with authentic reference compounds before and after derivative formation. The remaining group of metabolites was thought to consist of a mixture of \( 5\beta\)-pregnane and \( 5\beta\)-androstane derivatives, and probably included \( 5\beta\)-pregnane-3\(\alpha\),20\(\beta\)-diol and \( 5\beta\)-androstane-3\(\alpha\),17\(\beta\)-diol. Figure 3.6 summarizes the metabolism of \( [4^{-14}\text{C}]\)progesterone by thecal and zona parenchymatosa homogenates from the
ovary of the laying hen.

Nakamura et al (1974) found pregnanedione to be the major metabolite after incubation of whole ovarian homogenates for one hour with [4-14C]progesterone in the presence of NADPH as cofactor. This was presumably 5β-pregnanedione because when [4-14C]17α-hydroxyprogesterone was used as a substrate under the same conditions, 17α-hydroxy-5β-pregnanedione was identified as the major metabolite produced. In this study, the zona parenchymatosa homogenates produced large amounts of 5β-pregnan-3α-ol-20-one. The further hydroxylation of 5β-pregnanedione at the 3α-position may reflect differences in the incubating conditions used. The preferential utilization of progesterone for 5β-pregnan-3α-ol-20-one formation by the zona parenchymatosa homogenates could explain the low conversion of progesterone to androstenedione by the zona parenchymatosa homogenates as compared to the thecal homogenates.

Although thecal homogenates from follicles of 2 mm in diameter and upwards did not convert [4-14C]progesterone to 5β-pregnan-3α-ol-20-one, they still exhibited 5β-reductase activity; producing 5β-androstenedione and 5β-androstan-3α-ol-17-one. Nakamura et al (1974) were unable to demonstrate the conversion of [4-14C]progesterone to these 5β-reduced androgens by whole ovarian homogenates, although the same homogenates did convert [4-14C]androstenedione and [4-14C]testosterone-ol to both 5β-androstenedione and 5β-androstan-3α-ol-17-one.
in the presence of NADPH. 5β-androstan-17β-ol-3-one and 5β-androstane-3α,17β-diol were also produced using either [4-14C]androstenedione or [4-14C]testosterone as a substrate. These metabolites may have been produced from [4-14C]progesterone by the thecal or zona pannicular homogenates used in this study (see section 3:3:3 (e). Colombo and Colombo (1978a, 1978b) using similar homogenates from the quail ovary have reported conversion of [4-14C]progesterone to 5β-androstanedione, 5β-androstane-3α-ol-17-one and 5β-androstane-3α,17β-diol. 11-Ketotestosterone, 11-ketoandrostenedione, 11β-hydroxysterone and 11β-hydroxyandrostenedione were also produced by the quail homogenates. Although Wyburn and Baillie (1966) have reported a weak histochemical reaction for 11β-hydroxysteroid dehydrogenase in both the theca interna and granulosa of developing follicles (2 mm, 7 mm, 15 mm and 35 mm in diameter), there is no evidence to suggest that these steroids are products of the fowl ovary in vivo.

Thecal homogenates of the small follicles also produced small amounts of a steroid similar in Rf to oestrone. Unfortunately, the amounts produced were too small to permit positive identification. Similarly, Nakamura et al (1974) were unable to demonstrate conversion of either [4-14C]progesterone or [4-14C]pregnenolone to oestrogens using whole ovarian homogenates. It is likely that in the ovary of the hen, the rate of aromatization of androgens to oestrogens is so low that using either progesterone
or pregnenolone as substrates conversion to oestrogens is barely detectable. This has been found in a variety of other non-mammalian vertebrates i.e. fish, amphibians and reptiles (Callard and Leathem, 1965, 1966; Redshaw and Nicholls, 1971).

3:4 [4\textsuperscript{-14}C]testosterone metabolism by thecal tissue from the three largest follicles, the small follicles and by the zona parenchymatosa

3:4:1 Introduction

Thecal tissue from both the small follicles and the zona parenchymatosa have been shown to possess interstitial cells with an ultra-structure characteristic of steroid synthesis (Dahl, 1970a; 1971a) and have been postulated as a site of oestrogen synthesis (Dahl, 1970a; Senior and Furr, 1975). To date however, the only evidence that these tissues possess aromatase activity was provided by Nakamura et al (1974) and Skrivanek (1975) who used homogenates of the whole ovary after removal of the large yellow-yolky follicles. Huang, Kao and Nalbandov (1979) and Wang and Bahr (1983) have shown that thecal cells isolated from the second and third largest follicles (F2 and F3 respectively) are also able to convert exogenous testosterone to oestradiol in a dose-dependant manner. These authors have also obtained evidence that aromatase activity in thecal cells from F3 follicles was higher than that of cells isolated from F2 follicles, whilst the largest follicle possessed negligible amounts of aromatase activity. These findings
suggest that thecal aromatase activity may decline during the final phase of follicular maturation (Huang, Kao and Nalbandov, 1979; Wang and Bahr, 1983). Measurements of thecal oestrogen content support this view and indicate that the smaller follicles and/or the zona perehymatosa may possess a greater aromatase activity (Senior and Furr, 1975; Shahabi et al., 1975a; Imai and Nalbandov, 1978; Nakamura et al., 1979).

Thecal tissue homogenates from both the large and small ovarian follicles were previously shown to be capable of converting [4-14C]progesterone to androstenedione and testosterone (see section 3:1 and 3:3) as were homogenates of the zona perehymatosa, albeit to a much lower extent (see section 3:3). The aim of the work presented in this section was to see whether these homogenates also possess aromatase in vitro, and if so, to find out whether this alters during the course of follicular development.

3:4:2 Experimental procedures

a) Experiment 1

Thecal homogenates were prepared from the three largest follicles (F1, F2 and F3) of a hen (Nick Chick) killed 1 to 2 hours before ovulation. After centrifugation, aliquots of the supernatant (0.1 ml) were incubated in duplicate with either [4-14C]testosterone (9.5 nmol) or [4-14C]androstenedione (9.5 nmol) as described in section 2:4, except that the incubation period was 2 hours. At the end of the incubation period, 20 µg of androstenedione, testosterone, oestrone, oestradiol
and oestriol were added as carriers before extraction of the metabolites with dichloromethane. Each extract was partitioned as described in section 2.5.2 to obtain phenolic and neutral fractions. The phenolic and neutral fractions were chromatographed in solvent systems 12 and 1 respectively.

b) **Experiment 2**

Six hens (1 Nick Chick and 5 Shavers) were killed 1 to 2 hours before ovulation. Follicles between 4 to 10 mm in diameter were removed from each ovary and following expulsion of the yolk contents, were combined and homogenized (pool 1). Whole follicles between 2 to 4 mm in diameter were also combined and homogenized without prior removal of the yolk (pool 2). Duplicate aliquots (0.2 ml) of each homogenate were incubated with $[^{14}C]_{[4-]}$testosterone (9.5 nmol) for 3 hours. Thecal homogenates from the second largest follicle ($F_2$) were also obtained from each ovary and a single aliquot (0.2 ml) incubated with $[^{14}C]_{[4-]}$testosterone as described above, for comparison of thecal aromatase activity with that of the two groups of small follicles. For each hen, a control incubation was also carried out in which phosphate buffer (0.2 ml) was added to the incubation medium in place of the homogenate. These incubations were used to assess the amount of neutral steroids carried over into the phenolic fraction during the partition procedure. The incubation procedure was as described for Experiment 1 except for the following modifications. Firstly, the incubation period was
increased from 2 hours to 3 hours and oxygen:carbon dioxide (95%:5%, v/v) replaced air as the gas phase. Secondly, centrifugation of the homogenate prior to incubation was omitted, in case any aromatase activity was lost in the nuclear membrane fraction (Skrivanek, 1975). At the end of the incubation period, 50 μg each of oestrone, oestradiol and oestriol were added as carriers and the glass vials frozen at -20°C until extraction of the metabolites with dichloromethane. The recoveries obtained were routinely about 80%.

Each extract was partitioned as described previously. The neutral extracts from each homogenate type (i.e. F2, pool 1, pool 2 and the control) were combined and run in solvent system 1. The phenolic extracts were similarly combined. One-tenth of each phenolic extract was run in solvent system 12, with a 30 minute overrun to give a better separation of oestrone, oestradiol and oestriol (see plate 3.2). The remaining nine-tenths of each phenolic fraction were chromatographed in solvent system 7, using a separate t.l.c. plate for each phenolic fraction (control, F2, pool 1 and pool 2). Each extract was applied along the baseline as a streak in order to avoid overloading the chromatogram with carrier oestrogens (see plate 3.3 and 3.4).

c) Experiment 3

Eight hens (Ross Brown's) were killed 1 to 2 hours before an expected ovulation and six hens were killed
approximately 15 hours before an expected ovulation. Thecal homogenates were prepared from the second largest follicle (F2), follicles of between 4 to 10 mm in diameter (pool 1) and from follicles between 2 to 4 mm in diameter (pool 2). The zona pellucida (Z.P.) was also collected and homogenized. Duplicate aliquots of each homogenate were incubated with [4-\textsuperscript{14}C]testosterone (8.9 nmol) for 3 hours as in Experiment 2. In addition, six control incubations were carried out. At the end of the incubation 1 µg each of testosterone, androstenedione, oestrone, oestradiol and oestriol were added as carriers and the glass vials frozen at -20°C until extraction.

The incubates from each tissue type (F2, pool 1, pool 2 and Z.P.) were combined before extraction of the metabolites, keeping incubates from each period of the ovulatory cycle separate. The replicate sets were likewise combined, as were the six control incubations.

The metabolites were extracted by shaking for 5 minutes with dichloromethane (10 volumes). After allowing to stand for 30 minutes, the aqueous layer was discarded and the extract washed three times with distilled water (1 volume) to remove water-soluble impurities. Following this extraction procedure, recoveries of radioactivity ranged from 74% to 87%. Each extract (F2, pool 1, pool 2, Z.P. and the control) was then partitioned to separate the neutral and phenolic steroids.

The phenolic fractions were chromatographed in solvent system 7 (see plates 3.5 to 3.9). The neutral
fractions from both periods of the ovulatory cycle were combined, keeping the neutral fractions from each tissue type separate (F2, pool 1, pool 2, Z.P. and the control). To remove the large amount of lipid present, each combined fraction was purified on a Florisil column before i.t.l.c. chromatography. Columns (width 1 cm, height 2 cm) of Florisil, prewashed with redistilled deionized water, were prepared in glass pipettes and washed with diethyl ether. The neutral fraction was applied to the column in 0.5 ml dichloromethane. Diethyl ether (6 ml) was percolated through the column to remove lipid residues which were discarded. The steroids were eluted with 30% methanol in diethyl ether (10 ml). Approximately 95% of the radioactivity added to the column was recovered in this eluate. Each steroid fraction was evaporated to dryness, transferred to an i.t.l.c. strip and chromatographed in solvent system 1. The metabolites were separated into 4 major zones designated A, B, C and D respectively. Each zone was eluted and an aliquot counted to determine the percentage of radioactivity in each zone of the chromatogram.

3:4:3 Conversion of [4-^14C]testosterone to phenolic steroids

The distribution of radioactivity following the partition procedure is shown in tables 3.16, 3.17 and 3.18. In Experiment 1 (table 3.16), conversion of either [4-^14C]testosterone or [4-^14C]androstenedione to phenolic steroids was less than 0.5% after subtraction
Table 3.16  Partition of the extracts obtained after incubation of \([4-^{14}C]\)androstenedione or \([4-^{14}C]\)testosterone with thecal homogenates from the 3 largest follicles (F1, F2 and F3) 1 to 2 hours before ovulation. The extracts were partitioned as described in section 2:5:2. The results are given as a percentage of the total activity per tube and are the mean of two determinations.

<table>
<thead>
<tr>
<th></th>
<th>Neutral Fraction</th>
<th>Phenolic Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>[(4-^{14}C)]androstenedione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>96.3</td>
<td>3.2</td>
</tr>
<tr>
<td>F2</td>
<td>95.4</td>
<td>3.7</td>
</tr>
<tr>
<td>F3</td>
<td>94.2</td>
<td>5.1</td>
</tr>
<tr>
<td>[(4-^{14}C)]testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>93.9</td>
<td>5.3</td>
</tr>
<tr>
<td>F2</td>
<td>95.1</td>
<td>3.4</td>
</tr>
<tr>
<td>F3</td>
<td>95.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Control</td>
<td>96.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>
Table 3.17  Partition of the extracts obtained after incubation of [4-^{14}C]testosterone with thecal homogenates from either the second largest follicle or from two groups of small follicles collected 1 to 2 hours before ovulation. The extracts were partitioned as described in section 2:5:2. The results are given as a percentage of the total activity per tube and are the means of 6 determinations ± standard error of the mean.

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Neutral Fraction</th>
<th>Phenolic Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control+</td>
<td>95.2 ± 0.5</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Thecal, F2</td>
<td>91.4 ± 1.8</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>Thecal, pool 1**</td>
<td>84.0 ± 2.9</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>Follicular, pool 2**</td>
<td>91.0 ± 0.8</td>
<td>5.3 ± 0.7</td>
</tr>
</tbody>
</table>

+ phosphate buffer
** second largest follicle (approximately 30 mm in diameter)
++ pooled thecae from follicles > 4 mm < 10 mm in diameter
++ pooled follicles > 2 mm < 4 mm in diameter
Table 3.18  Partition of the extracts obtained after incubation of [4-14C]testosterone with zona parenchymatosa homogenates or with thecal homogenates from either the second largest follicles or from two groups of small follicles collected either 1 to 2 hours or 15 hours before ovulation. The extracts were partitioned as described in section 2:5:2. Results are expressed as a percentage of the total activity.

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Neutral Fraction</th>
<th>Phenolic Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Thecal, F2+</td>
<td>93.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Thecal, pool 1*</td>
<td>78.5</td>
<td>7.9</td>
</tr>
<tr>
<td>Thecal, pool 2**</td>
<td>82.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Z.P.+*</td>
<td>94.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Thecal, F2</td>
<td>92.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Thecal, pool 1</td>
<td>83.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Thecal, pool 2</td>
<td>86.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Z.P.</td>
<td>93.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Control*</td>
<td>94.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

+ second largest follicle (approximately 30 mm in diameter)

* pooled thecae from follicles > 4 mm < 10 mm in diameter

** pooled thecae from follicles > 2 mm < 4 mm in diameter

++ zona parenchymatosa (follicles < 2 mm in diameter)

# phosphate buffer
of the control values. It was considered that the failure to demonstrate conversion of either [4-\(^{14}\)C]testosterone or [4-\(^{14}\)C]androstenedione to oestrogens may have been due to the incubations used. For example, lack of molecular oxygen which, together with NADPH, is required as a co-factor for the aromatase complex (Thompson and Siiteri, 1974a) may have limited aromatase activity since air was used as the gas phase in Experiment 1. The possibility that aromatase activity had been lost during centrifugation of the homogenate to spin down broken cells and nuclear debris was also considered. In one previous report, as yet unsubstantiated, it was claimed that the aromatase complex of the hen ovary is associated with the nuclear membrane fraction (Srivanek, 1975). However, following the omission of the centrifugation step during preparation of the homogenate, and the replacement of air by oxygen:carbon dioxide (95\%:5\%, v/v) as the gas phase, thecal homogenates from the second largest follicle still exhibited negligible aromatase activity (Experiment 2 and 3). The latter converted approximately 1\% of [4-\(^{14}\)C]-testosterone to phenolic steroids after subtraction of the control value (see table 3.17 and 3.18). The corresponding value obtained for the zona parenchymatosa homogenates was also low, averaging less than 1\% (Experiment 3).

Thecal homogenates from the small follicles however, converted the greatest amounts of [4-\(^{14}\)C]testosterone to phenolic steroids. Values ranged from 6.6\% (Experiment 2)
to 11.9% (Experiment 3) for the group of small follicles between 4 mm and 10 mm in diameter. No differences were observed according to the period of the ovulatory cycle (table 3.18). Conversion of $[4^{-14}C]$testosterone to phenolic steroids by thecal homogenates from follicles of 2 mm to 4 mm in diameter averaged 7.8% (Experiment 3). The same group of follicles homogenized without removal of the yolk (Experiment 2) gave a value of 2.3%. Measurement of the protein concentration of the latter homogenate showed that it contained approximately half as much protein compared to thecal homogenates from the same follicles. Allowing for this, a value of approximately 4.6% would be obtained for the conversion of $[4^{-14}C]$testosterone to phenolic steroids by thecal homogenates from follicles of 2 mm to 4 mm in diameter in Experiment 2.

The consistently higher values obtained in Experiment 3 compared with Experiment 2, may reflect genetic differences between the strains of hen used (Shavers versus Ross Brown's), since the same incubation conditions were used in both these experiments.

3:4:4 Identification of phenolic steroids

a) Experiment One

In chromatograms of the phenolic fraction obtained from incubations of thecal homogenates from the three largest follicles with either $[4^{-14}C]$testosterone or $[4^{-14}C]$androstenedione, most of the radioactivity remained at the baseline, associated with oestriol (solvent system 12). There was no activity in the area of the
chromatogram corresponding to oestradiol. In phenolic fractions from F2 and F3 (but not F1) a trace of radioactivity corresponding to oestrone was also present. Traces of androstenedione and testosterone were also present but were clearly separated from the oestrone, oestradiol and oestriol standards. Further identification of the metabolites present in the phenolic fraction was not possible due to the low yield of radioactivity.

b) **Experiment Two**

Plate 3.2 shows the autoradiograph obtained after running one-tenth of the phenolic fraction from each homogenate type (control, F2, pool 1 and pool 2) in solvent system 12. The control fraction contained a small amount of radioactivity most of which remained at the baseline; no activity was detected in the areas corresponding to oestrone or oestradiol. Most of the radioactivity in the phenolic fraction from F2 incubations was streaked upwards from the baseline, covering an area which included and extended slightly beyond the oestriol standard. No oestradiol was detected, although there was a peak of radioactivity coincident with carrier oestrone which represented approximately 16% of the total activity present on the chromatogram. Other traces of activity were also present on the chromatogram. The phenolic fraction from pool 1 incubations (thecal homogenates from follicles > 4 mm < 10 mm in diameter) gave one major radioactive peak coinciding with oestrone which accounted for 50% of the radioactivity present on the
Legend to Plate 3.2

Chromatography of the phenolic fractions in solvent system 12.

C - control

F2 - thecal homogenates from second largest follicle

1 - thecal homogenates from follicles $> 4 \text{ mm} < 10 \text{ mm}$ in diameter

2 - homogenized follicles $> 2 \text{ mm} < 4 \text{ mm}$ in diameter

The following abbreviations have been used to denote the positions of authentic steroids:

$E_1$ - oestrone

$E_2$ - oestradiol

$E_3$ - oestriol

$T$ - testosterone

$A_4$ - androstenedione
Legend to Plate 3.3

Chromatography of the phenolic fraction in solvent system 7.

C - control

F2 - thecal homogenates from the second largest follicle

The following abbreviations have been used to denote the positions of authentic steroids:

E₁ - oestrone
E₂ - oestradiol
E₃ - oestriol
T - testosterone
Legend to Plate 3.4

Chromatography of the phenolic fraction in solvent system 7.

Pool 1 - thecal homogenates from follicles

\( \geq 4 \text{ mm} < 10 \text{ mm in diameter} \)

Pool 2 - homogenized follicles \( \geq 2 \text{ mm} < 4 \text{ mm in diameter} \)

The following abbreviations have been used to denote the positions of authentic steroids:

- \( E_1 \) - oestrone
- \( E_2 \) - oestradiol
- \( E_3 \) - oestriol
- \( T \) - testosterone
t.l.c. plate. Most of the remaining activity on the chromatogram was associated with oestriol at the baseline. A similar distribution of radioactivity was obtained for the phenolic fraction from pool 2 follicles (2 mm < 4 mm in diameter).

Plates 3.3 and 3.4 show the autoradiographs obtained after chromatography of the remainder of each phenolic extract in solvent system 7. Again, there was no radioactivity in the area of the chromatogram corresponding to oestradiol. Phenolic fractions from all incubations except the control, contained radioactivity coincident with oestrone. Most of the remaining radioactivity remained at the baseline as did oestriol. Two other areas of radioactivity were also present in all the phenolic fractions except those from control incubations, plus a trace of unknown activity of higher $R_F$ than oestrone.

The radioactive areas corresponding to oestrone were eluted, combined and acetylated. A radioactive product with the same $R_F$ as oestrone acetate was obtained after chromatography in solvent system 5. No further identification was attempted due to the small quantity of radioactivity recovered.

c) Experiment Three

One set of phenolic fractions from each period of the ovulatory cycle were run in solvent system 7, and the duplicate set kept aside. Plate 3.5 shows an autoradiograph of the phenolic fractions from hens killed
Legend to Plate 3.5

Autoradiograph of the phenolic fraction after chromatography in solvent system 7.
Abbreviations of carrier steroids were:-

E₁ - oestrone
E₂ - oestradiol
E₃ - oestriol
A₄ - androstenedione
T - testosterone
C - control
F₂ - second largest follicle
1 - follicles > 4 mm < 10 mm in diameter
2 - follicles > 2 mm < 4 mm in diameter
ZP - zona parenchymatosa
15 hours before ovulation; the autoradiograph (not shown) of phenolic fractions from hens killed 1 to 2 hours before ovulation was similar. A radioactive spot similar in $R_F$ to oestradiol was detected in the phenolic fractions from the zona perechermatosa homogenates only. However, in the phenolic fractions from pool 1 (follicles $> 2$ mm $< 10$ mm in diameter) and pool 2 (follicles $> 2$ mm $< 4$ mm in diameter) the radioactivity at the baseline was streaked upwards as far as the oestradiol standard. In all the fractions except the control, there was a radioactive spot corresponding to oestrone. This was most intense in the pool 1 fractions which contained, in addition, an unknown metabolite of higher $R_F$ than oestrone.

The radioactive areas corresponding to oestrone were combined, and after the addition of authentic oestrone, recrystallised 6 times. The recrystallisation data (see table 3.19(i)) shows clearly that this metabolite was not oestrone. It was noted however, that the solution obtained after elution of the radioactivity from the chromatogram was yellow rather than colourless as expected, and that the first crop of mother liquor, which contained most of the radioactivity, yielded yellowish-white coloured crystals. Solutions of oestrone kept in the laboratory at room temperature for some time also develop a yellow colouration, therefore it is possible that the "oestrone" isolated from the phenolic fraction had undergone chemical decomposition. It was thought that the most likely stage during which degradation of
oestrone may have occurred was during autoradiography when, due to the low amounts of activity present, the chromatograms were left in contact with the "X" ray film for about 2 weeks. It was, therefore, decided to repeat the whole procedure using the duplicate set of phenolic fractions, but locating the metabolites straight away using the Panax scanner and eluting immediately in order to avoid keeping the metabolites on the chromatogram for any length of time. Sufficient radioactivity for detection of the metabolites using the Panax scanner was obtained by pooling the remaining set of phenolic fractions from both periods of the ovulatory cycle, keeping the tissue types (F2, pool 1, pool 2 and Z.P.) separate. Plates 3.6 to 3.9 show the radioactive peaks as located by the Panax scanner. After chromatography in solvent system 7, all the phenolic fractions contained a peak of activity corresponding to carrier oestrone. In addition to oestrone, there was a peak of activity which was not completely separated from the remaining peak of activity at the baseline (coincident with oestriol). The phenolic fraction from zona parenchymatosa homogenates also contained a peak of activity with the same $R_F$ as oestradiol. The phenolic fractions from pool 1 (follicles $\geq 4$ mm $< 10$ mm in diameter), pool 2 (follicles $\geq 2$ mm $< 4$ mm in diameter) but not those from the second largest follicles, also contained some activity in this region of the chromatogram, but covering an area which extended beyond the oestriol standard. The radioactive
Scan showing phenolic fraction from F2 (second largest follicle) thecal homogenates. The direction of run is from the baseline at 0 cm to the solvent front at 14 cm. Instrument settings were as follows:-

Time Constant 3 sec., Range 30 cps,
Paper Speed 600 mm/h

The following abbreviations have been used:-

E₁ - oestrone
E₂ - oestradiol
E₃ - oestriol
A  - androstenedione
T  - testosterone
Legend to Plate 3.7

Scan showing phenolic fraction from group 1 (follicles > 4 mm < 10 mm in diameter) thecal homogenates. The baseline is 0 cm and the solvent front is at 14 cm. Instrument settings were as follows:-

Time Constant 10 sec., Range 100 cps,
Paper Speed 600 mm/h

The following abbreviations have been used:-

$E_1$ - oestrone
$E_2$ - oestradiol
$E_3$ - oestriol
$A$ - androstenedione
$T$ - testosterone
Scan showing phenolic fraction from group 2 (follicles > 2 mm < 4 mm in diameter) thecal homogenates. The baseline is at 0 cm and the solvent front is at 14 cm. Instrument settings were as follows:-

Time Constant 10 sec., Range 100 cps,
Paper speed 600 mm/h

The following abbreviations have been used:-

E₁ - oestrone
E₂ - oestradiol
E₃ - oestriol
A - androstenedione
T - testosterone
Legend to Plate 3.9

Scan showing phenolic fraction from ZP (zona parenchymatosa) homogenates. The baseline is at 0 cm and the solvent front is at 14 cm.

Instrument settings were as follows:-
- Time Constant 10 sec., Range 30 cps,
- Paper speed 600 mm/h

The following abbreviations have been used:-
- $E_1$ - oestrone
- $E_2$ - oestradiol
- $E_3$ - oestriol
- $A$ - androstenedione
- $T$ - testosterone
Table 3.19 Recrystallisation data for the pooled radiometabolite from the phenolic fractions with oestrone i) first recrystallisation, ii) second recrystallisation.

<table>
<thead>
<tr>
<th>Recrys. No.</th>
<th>Solvent Pair</th>
<th>Specific Activity (dpm/mg)</th>
<th>Solvent Pair</th>
<th>Specific Activity (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>i) Crystals</td>
<td>Mother Liquor</td>
<td>ii) Crystals</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>11,224</td>
<td>-</td>
<td>4,807</td>
</tr>
<tr>
<td>1</td>
<td>Acetone/hexane</td>
<td>2,264</td>
<td>47,207</td>
<td>736</td>
</tr>
<tr>
<td>2</td>
<td>Acetone/cyclohexane</td>
<td>375</td>
<td>6,316</td>
<td>266</td>
</tr>
<tr>
<td>3</td>
<td>Acetone/ethyl acetate</td>
<td>184</td>
<td>787</td>
<td>135</td>
</tr>
<tr>
<td>4</td>
<td>Acetone/ethanol</td>
<td>116</td>
<td>387</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>Acetone/hexane</td>
<td>103</td>
<td>100</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>Acetone/cyclohexane</td>
<td>93</td>
<td>24</td>
<td>68</td>
</tr>
</tbody>
</table>

* Recrystallisation number
areas corresponding to oestrone, oestradiol and oestriol standards were eluted from each chromatogram and pooled separately. Once again, the solution of oestrone obtained was yellow-coloured and after the addition of authentic oestrone, constant specific activity was not obtained after 6 successive crystallisations (see table 3.19(ii)).

The radioactive areas corresponding to oestradiol and oestriol were re-chromatographed in solvent system 12, allowing the solvent to run off the plate. The radioactive area thought to be oestradiol gave one radioactive peak with the same $R_F$ as oestradiol in this solvent system. There was not sufficient radioactivity present however for recrystallisation with authentic oestradiol. In addition, small amounts of "oestrone" and "oestriol" were obtained together with traces of androstenedione and testosterone.

Re-chromatography of the pooled oestriol in solvent system 12 gave an area of radioactivity streaked upwards from the baseline and which appeared to contain several steroids. This area included the $16\alpha,17\beta$ and $16\beta,17\alpha$ epimers of oestriol. In addition, there was some activity of slightly higher $R_F$ which was streaked upwards, as was also the $16\beta,17\beta$ epimer of oestriol. There was no activity coincident with the $16\alpha,17\alpha$ epimer of oestriol which had a higher $R_F$ than the other epimers and was completely separated from them. Further identification was not carried out.
Conversion of [4-\(^{14}\)C]testosterone to neutral steroids

Following incubation of the homogenates with [4-\(^{14}\)C]testosterone, four major metabolites corresponding to androstenedione, 5\(\beta\)-androstan-3\(\alpha\)-ol-17-one and 5\(\beta\)-androstane-3\(\alpha\),17\(\beta\)-diol were obtained. Table 3.20 shows the distribution of radioactivity on the chromatogram after initial chromatography in solvent system 1 (Experiment Three). Control incubations (Experiments Two and Three) gave one radioactive peak only with an \(R_F\) identical to that of carrier testosterone (Zone C).

Androstenedione was found to be the major metabolite produced by thecal homogenates of the 3 largest follicles after incubation with [4-\(^{14}\)C]testosterone. Similarly, when [4-\(^{14}\)C]androstenedione was used as the substrate, testosterone was the major metabolite produced (Experiment One). Thecal homogenates from pool 1 follicles (> 4 mm < 10 mm in diameter) exhibited a similar pattern of [4-\(^{14}\)C]testosterone metabolism. In contrast, the zona parenchymatosa homogenates converted [4-\(^{14}\)C]testosterone preferentially to 5\(\beta\)-reduced metabolites. Thecal homogenates from pool 2 follicles (> 2 mm < 4 mm in diameter) exhibited an intermediate pattern of [4-\(^{14}\)C]testosterone metabolism (see Table 3.20).

Identification of neutral steroids

ZONE A (\(R_F\) 0.91 - 0.57) corresponded to androstenedione in solvent system 1. Upon oxidation, most of the radioactivity remained coincident with androstenedione.
Table 3.20  Radioactivity associated with each Zone (A, B, C and D) after chromatography of the neutral fractions in solvent system 1. The results are expressed as a percentage of the total activity present on the chromatogram. The major steroid present in each Zone is indicated in parenthesis.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Homogenate</th>
<th>Thecal</th>
<th></th>
<th>Z.P.</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone A</td>
<td>F2+</td>
<td>Pool 1*</td>
<td>Pool 2**</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>(Androstenedione)</td>
<td>50</td>
<td>51</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone B</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>(5β-androstane-3α-ol-17-one)</td>
<td>2</td>
<td>6</td>
<td>14</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Zone C</td>
<td></td>
<td></td>
<td></td>
<td>18</td>
<td>86</td>
</tr>
<tr>
<td>(testosterone)</td>
<td>33</td>
<td>18</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone D</td>
<td></td>
<td></td>
<td></td>
<td>59</td>
<td>9</td>
</tr>
<tr>
<td>(5β-androstane-3α-17β-diol)</td>
<td>13</td>
<td>21</td>
<td>32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* second largest follicle (approximately 30 mm in diameter)
* pooled theca from follicles > 4 mm < 10 mm in diameter
** pooled theca from follicles > 2 mm < 4 mm in diameter
+++ zona parenchymatosa (follicles < 2 mm in diameter)
after chromatography in solvent system 13. A small amount of activity corresponding to 5β-androstane-3-one was also present. Upon acetylation, 98% of the radioactivity present in Zone A remained coincident with androstenedione after chromatography in solvent system 9. The remaining activity had an \( R_F \) identical to 5β-androstane-3β-ol-17-one-monoacetate (but not 5β-androstane-3α-ol-17-one-monoacetate). On re-chromatography in solvent system 4, however, the \( R_F \) of this acetate was similar to but not exactly coincident with that of 5β-androstane-3β-ol-17-one-monoacetate.

ZONE B (\( R_F 0.57 - 0.32 \)) corresponded to 5β-androstane-3α-ol-17-one in solvent system 1. Oxidation of Zone B gave 5β-androstane-3-one in solvent system 13. Following acetylation of Zone B, 75% of the radioactivity corresponded to 5β-androstane-3α-ol-17-one-monoacetate in solvent systems 4 and 5. In addition, two smaller peaks of activity were obtained, one with an \( R_F \) corresponding to 5β-androstane-17β-ol-3-one-monoacetate and the other with an \( R_F \) identical to that of testosterone acetate (solvent system 4).

ZONE C (\( R_F 0.32 - 0.18 \)) contained unreacted testosterone. This gave testosterone acetate upon acetylation (solvent system 4), and following oxidation, only one peak of radioactivity with an \( R_F \) identical to androstenedione (Solvent system 13).

ZONE D (\( R_F 0.18 - 0.13 \)) gave 5β-androstane-3-one upon oxidation in solvent system 13. Acetylation of
zone D gave one radioactive peak in solvent system 2 which had an $R_F$ coincident with that of $5\beta$-androstane-3$\alpha$, 17$\beta$-diol diacetate. Although this peak was clearly separated from $5\beta$-androstane-3$\beta$, 17$\beta$-ol-diol diacetate, the presence of a large tail on one side (sloping towards the baseline) indicated that other metabolites may have been present.

3:4:7 **Discussion**

a) **Conversion of $[4-^{14}C]$testosterone to androstenedione and to $5\beta$-reduced metabolites**

Both thecal and zona parenchymatosa homogenates were shown to convert $[4-^{14}C]$testosterone to androstenedione, confirming the previous histochemical demonstrations of the enzyme 17$\beta$-hydroxysteroid dehydrogenase within these tissues (Wyburn and Baillie, 1966; Boucek and Savard, 1970). When thecal homogenates from the 3 largest follicles were incubated with $[4-^{14}C]$androstenedione as the substrate, similar values were obtained for the percentage conversion of androstenedione to testosterone, indicating that this enzyme is readily reversible under the incubation conditions used. Nakamura et al (1974) also reported the inter-conversion of these substrates by hen ovarian homogenates as did Colombo and Colombo (1978a; 1978b) using homogenates from the quail ovary. Furthermore, these results of the present study, show that thecal 17$\beta$-hydroxysteroid dehydrogenase activity increases during follicular growth, the highest activity being associated
with follicles over 4 mm in diameter (see figure 3.6). Zona parenchymatosa homogenates, which contained follicles of up to 2 mm in diameter, exhibited much lower levels of 17β-hydroxysteroid dehydrogenase activity in vitro.

Unlike 17β-hydroxysteroid dehydrogenase activity which showed an increase during follicular growth, 5β-reductase activity tended to decrease with increasing follicle size and was maximal in the zona parenchymatosa homogenates (see figure 3.6). The major 5β-reduced metabolites produced by the homogenates were tentatively identified as 5β-androstan-3α-ol-17-one and 5β-androstane-3α,17β-diol. Small amounts of 5β-androstan-3β-ol-17-one and 5β-androstan-17β-ol-3-one may also have been produced. These metabolites were also identified by Nakamura et al. (1973) after incubating whole ovarian homogenates from laying hens with either [4-14C]testosterone or [4-14C]androstenedione in the presence of NADPH as cofactor. A high 5β-reductase activity is also seen in ovarian homogenates from the laying quail (Colombo and Colombo, 1978a; 1978b). Ovarian minces prepared from the frog, Rana pipiens (Callard and Leathem, 1966) and the sea-bass, Centropristes striatus (Reinboth, Callard and Leathem, 1966) also produced predominately 5β-reduced metabolites in preference to 5α-reduced metabolites when incubated with [4-14C]testosterone in vitro. This is not the case in all non-mammalian vertebrates. In other amphibian species such as Necturus maculosus, Rana temporaria and Xenopus laevis, similar ovarian preparations
Legend to Figure 3.6

To illustrate the metabolism of [4-\(^{14}\)C]testosterone by the various homogenates. Values given are means (1 to 2 hours and 15 hours before ovulation). The following abbreviations have been used:

- **T**: testosterone
- **A\(_{4}\)**: androstenedione
- **5\(\beta\) 3\(\alpha\)**: 5\(\beta\)-androstan-3\(\alpha\)-ol-17-one
- **5\(\beta\)A3\(\alpha\)17\(\beta\)**: 5\(\beta\)-androstan-3\(\alpha\)-ol-17-one
M control

Follicle ~ 30 mm in diameter

Follicles ≥ 4 ≤ 10 mm

Follicles ≥ 2 ≤ 4 mm

Zona parenchymatosa

nmol/20 mg fresh tissue per h

T

A4

5β3α

5βA3α, 17β
were found to reduce testosterone preferentially to products with the 5\(\alpha\)-configuration (Ozon, Breuer and Lisboa, 1964; Callard and Leathem, 1966; Redshaw and Nicholls, 1971). Whether the high 5\(\beta\)-reductase activity found in the hen ovary in this study is of any biological significance is not clear. The high activity found in the zona parenchymatosa homogenates may explain both the low conversion of [4-\(^{14}\)C]progesterone to androstenedione and testosterone and of [4-\(^{14}\)C]testosterone to androstenedione and to phenolic steroids (see tables 3.18 and 3.20) by these homogenates. The possibility that some of the 5\(\beta\)-reduced metabolites produced act as inhibitors of steroidogenic enzymes cannot be excluded, although these metabolites are generally believed to be inactive (Dorfman and Ungar, 1965). A high 5\(\beta\)-reductase activity has also been observed in the neuroendocrine tissues of the laying hen (Nakamura and Tanabe, 1974; Sharp and Massa, 1980).

It was suggested that 5\(\beta\)-reduced metabolites may play a role in modulating neural activity (Sharp and Massa, 1980).

b) Conversion of [4-\(^{14}\)C]testosterone to phenolic steroids

Thecal homogenates from follicles between 2 and 10 mm in diameter exhibited the highest aromatase activity, as judged by the percentage conversion of [4-\(^{14}\)C]testosterone to phenolic steroids. Within this size range of follicles, thecal aromatase activity was consistently higher in the 4 to 10 mm group. A much lower aromatase activity was found in the zona parenchymatosa homogenates,
which comprised numerous small follicles of up to 2 mm in diameter. These results confirm and extend a preliminary study by Senior and Furr (1975) in which it was reported that 60 to 70% of the oestrogen content of the ovary was associated with the small follicles and ovarian stroma. No distinction was made by these workers however, between the small follicles (i.e. all follicles remaining after removal of the 5 largest follicles) and the rest of the ovary (termed ovarian stroma). The present results imply that developing follicles do not acquire significant amounts of thecal aromatase activity until they have reached a diameter of about 2 mm, and that by the time the follicles have reached ovulatory size, aromatase activity has declined to low levels.

This interpretation of the results is based on the assumption that the radioactivity in the phenolic fraction consisted entirely of oestrogens and thus provided a true reflection of the aromatase activity of the homogenates. A recent study (Armstrong, 1983) has shown that similar homogenates from the ovary of the laying hen can convert testosterone to oestrogens. Aromatase activity was determined by the method of Thompson and Siiteri (1974a) in which the stereospecific release of tritiated water from [1 -2-3H]testosterone is measured. Values of 0.64, 3.41, 0.73 and 0.25 pmol/minute per mg protein, were obtained for thecal homogenates from F2, yellow-yolky follicles (as pool 1 in this study), white-yolky follicles (as pool 2) and the zona parenchymatosa
respectively. The corresponding average values obtained for the conversion of [4-\(^{14}\)C]testosterone to a steroid with a chromatographic mobility identical to oestrone as calculated from Experiment Two and Experiment Three (regardless of the period of the ovulatory cycle) were as follows: 0.11, 2.45, 1.95 and 0.13 pmol/minute per mg protein (for F2, pool 1, pool 2 and Z.P.). The good agreement between these two methods is encouraging.

Attempts to positively identify the metabolites present in the phenolic fraction however, met with some difficulty, since the relatively low aromatization rate obtained in the hen ovary meant that even after pooling the homogenates from several incubations, there was only sufficient radioactivity available for one or occasionally two further chromatographic runs. This problem has been encountered by other workers attempting to demonstrate aromatase activity in the ovaries of other non-mammalian vertebrates. In experiments using ovarian homogenates or tissue slices incubated with labelled testosterone or androstenedione, aromatization rates were either very low, ranging from 0.2% to 0.4% (Ozon and Breuer, 1964; Oxon, 1967; Simpson, Wright and Hunt, 1968; Redshaw and Nicholls, 1971; Xavier and Ozon, 1971) or were undetectable (Callard and Leatham, 1966; Ozon, Breuer and Lisboa, 1964). In most of these studies the very low yields of oestrogens have allowed only a tentative identification of oestrone and oestradiol to be made; usually by isopolarity with the authentic steroid in one or more thin-layer chromatographic
systems, both before and after derivative formation. Indeed, in this study these criteria were met by a steroid present in all the phenolic fractions (except the control) and which was initially believed to be oestrone. In addition, a steroid which was isopolar with oestradiol was isolated from the phenolic fractions from the small follicles and the zona parenchymatosa after incubation of the homogenates with [4-\(^{14}\)C]testosterone.

The phenolic steroid which co-migrated with oestrone was the major constituent of the phenolic fraction obtained after incubating thecal homogenates from follicles of 4 to 10 mm in diameter with [4-\(^{14}\)C]testosterone. Recrystallisation with authentic oestrone however, provided conclusive evidence that this steroid was not oestrone. Most of the radioactivity which corresponded to oestrone was lost to the first crop of mother liquor. Nevertheless, during the last 3 recrystallisations, the crystals approached constant specific activity (see table 3.19), indicating that small amounts of [4-\(^{14}\)C]oestrone may have been present together with large amounts of an unknown [4-\(^{14}\)C]-radiometabolite. It was considered that the latter could be an oxidation product of [4-\(^{14}\)C]oestrone since the first crop of mother liquor formed yellow-coloured crystals. The presence of Fe\(^{++}\) in the silica gel may have catalysed autoxidation of oestrone during i.t.l.c. chromatography and storage on the plate.

In order to test the possibility that autoxidation of oestrone had occurred during the isolation procedure,
[\textsuperscript{3}H]oestrone (1.5 KBq) was added to a thecal homogenate (second largest follicle) and subjected to the same procedures (see 3:6:2). The specific activity after addition of 20 mg of authentic oestrone was 2,843 dpm/mg. Following recrystallisation using acetone/hexane as the solvent pair, the specific activity of the crystals was 2,483 dpm/mg and that of the mother liquor 4,540 dpm/mg (see table 3.19 for comparison). It would appear that [\textsuperscript{3}H]oestrone is not extensively oxidized by the separation procedures used in the present study. It does not seem likely therefore that the unidentified phenolic steroid, produced in large amounts by thecal homogenates of small follicles incubated with [4-\textsuperscript{14}C]testosterone, is an oxidation product of [4-\textsuperscript{14}C]oestrone. In addition, other workers using similar techniques for the isolation of oestrogens have been able to demonstrate conversion of 4-\textsuperscript{14}C-labelled precursors to oestrone and oestradiol using either ovarian explants from chick embryos (Haffen and Cedard, 1968; Weniger and Zeiss, 1971; Galli and Wasserman, 1972), or ovarian homogenates from the laying hen (Nakamura et al., 1974). In these experiments, oestrone or oestradiol (or their methyl ether derivatives) were recrystallised to constant isotopic ratio following the addition of the corresponding tritiated steroid.

Vernon, Diershke, Sholl and Wolf (1983) have also reported the production of a polar radioactive steroid corresponding to oestrone after incubating thecal wedges from preovulatory follicles of the Rhesus monkey for 3
hours, in the presence of \([4-^{14}C]\)progesterone and \([^{3}H]\)-androstenedione. A large amount of radioactivity was lost to the first crop of mother liquor, as in this study, following recrystallisation with authentic oestrone. The specific activity of \([^{3}H]\)oestrone subsequently remained constant through successive recrystallisations, whilst that of "\([4-^{14}C]\)oestrone" did not, indicating the presence of a small amount of oestrone in addition to the unknown metabolite. It was also found that the same polar metabolite was produced (from both \([4-^{14}C]\)progesterone and \([^{3}H]\)androstenedione) by control incubations in the absence of thecal tissue, albeit in much smaller quantities. The identity of this metabolite was not established.

Clearly further work is needed to establish the identity of the phenolic steroid produced in the present study by thecal and zona pannychmatosa homogenates, before an assessment of the significance of these findings can be made. Oestrone and oestradiol may have been produced early on during the 3 hour incubation period and then further metabolized to hydroxylated derivatives. It is of interest to note here that Brown, Long, Bacon and Braselton (1979) have obtained evidence for the presence of 15-hydroxylated oestrogens in the plasma of the laying turkey. Preliminary work based on h.p.l.c. chromatography and mass spectral analysis indicated that in addition to oestrone and oestradiol, 15\(\alpha\)-hydroxyoestrone, 15\(\beta\)-hydroxyoestosterone, 15\(\alpha\)-hydroxyoestradiol and 15\(\beta\)-hydroxyoestradiol are present in plasma. It was postulated
that these steroids may be of physiological significance since they are apparently present in greater quantities in the plasma of laying as opposed to broody or immature turkeys. These authors have also reported that 15-hydroxylated oestrogens comprised 40% of the oestrogens present in the ovary of the laying turkey. It is conceivable therefore that the unidentified oestrogen produced by the thecal homogenates in this study was a 15-hydroxylated oestrogen, possibly either 15α-hydroxyoestrone or 15β-hydroxyoestrone. As pure preparations of 15-hydroxylated oestrogens are not readily available, it was not possible to determine their \( R_F \) values in the solvent systems used. It should be stated here, that 15-hydroxyoestrone would not be expected to migrate as far as oestrone in the solvent systems used. Again, it is possible that the radioactivity corresponding to oestrone which was isolated from the chromatogram was an oxidation product of 15-hydroxyoestrone, especially since Brown et al. (1979) showed that use of a phenolic partition procedure during the isolation of 15-hydroxyoestrone resulted in the disappearance of part of the 15-hydroxyoestrone peak and the appearance of a new peak with the same retention time as oestrone following Sephadex LH-20 chromatography.
4. CONCLUDING DISCUSSION
CONCLUDING DISCUSSION

The results presented in this thesis support the hypothesis that the interstitial tissue of the ovary is a site of androgen and oestrogen synthesis in the laying hen (Gallus domesticus). Both thecal and zona parenchymatosa homogenates converted $[4-^{14}C]$progesterone to androstenediolone and testosterone, and $[4-^{14}C]$testosterone to phenolic steroids. These results have been attributed to the presence of interstitial cells in these tissues. Although granulosa cells were also present in thecal homogenates from the small follicles (2 to 10 mm in diameter) and in the zona parenchymatosa homogenates, it is unlikely that they will have contributed to the metabolism of $[4-^{14}C]$progesterone or $[4-^{14}C]$testosterone for several reasons. Firstly, they do not start to become steroidogenically active until the follicle has reached a diameter of 5 to 8 mm (Davidson et al., 1979; Gilbert et al., 1983), and secondly, the granulosa cells do not possess the enzymes necessary for conversion of progesterone to oestrogens (Huang, Kao and Nalbandov, 1979; Wells et al., 1980; Armstrong, 1983).

Furthermore, the results of the present study imply that during the course of follicular development, the activity of steroidogenic enzymes within the interstitial cells may change. Thus during growth of the follicle from less than 2 mm in diameter to 10 mm in diameter, there was an increase in $^{17\alpha}$-hydroxylase/C-17,20lyase, $^{17\beta}$-hydroxysteroid dehydrogenase and possibly aromatase
activities. The latter finding was recently confirmed by Armstrong (1983). During the subsequent phase of rapid follicle growth (when the follicle increases in size to approximately 35 mm in diameter), it has been shown that thecal $3\beta$-hydroxysteroid dehydrogenase (Armstrong, 1982b) and aromatase (Huang, Kao and Nalbandov, 1979; Wang and Bahr, 1983; Armstrong, 1983) activity decline progressively. In contrast, the present results have shown that thecal $17\alpha$-hydroxylase/C-17,20\text{lyase} activity is maintained until shortly before ovulation.

These changes in thecal enzyme activities during follicle growth may reflect alterations in the responsiveness of interstitial cells to gonadotrophic stimulation. For instance, the acquisition of LH receptors by the thecal interstitial cells during growth of the follicle from 2 to 10 mm in diameter may explain the increase in thecal $17\alpha$-hydroxylase/C-17,20\text{lyase} and $17\beta$-hydroxysteroid dehydrogenase activities. At present however, there is no information regarding thecal LH receptors during follicle growth in the hen and, so far, attempts to demonstrate binding of either bovine or ovine LH to hen thecal tissue have proved unsuccessful (Etches and Cheng, 1981; Ritzhaupt, 1983). Nevertheless, in the present study it was shown that thecal homogenates from the small follicles (2 to 10 mm in diameter) converted significantly more [4-\text{\textsuperscript{14}}C]-progesterone to androstenedione when collected after exposure to the endogenous surge of LH (section 3:3).
In view of the well-known stimulatory effect of LH on thecal androgen production in other species (rabbit: Erickson and Ryan, 1976; rat; Fortune and Armstrong, 1977; human; McNatty, Makris, Ostanondh and Ryan, 1980) these results indicate the presence of functional thecal LH receptors in the small follicles of the hen. The failure of thecal homogenates from the larger preovulatory follicles (F2 and F3) to exhibit an increase in conversion of [4-\(^{14}\)C]progesterone to androstenedione following the LH surge may be due to the fact that their 17\(\alpha\)-hydroxylase/C-17,20\(\beta\)lyase activities are already maximally stimulated. The dramatic fall in thecal 17\(\alpha\)-hydroxylase/C-17,20\(\beta\)lyase activity shown by the theca layer of the largest, preovulatory follicle during the interval between the LH surge and ovulation may be associated with a loss of thecal LH receptors, as in the sheep (Webb and England, 1982).

Whereas LH is important in controlling thecal androgen production, it is possible that FSH is responsible for stimulating thecal aromatase activity in the developing hen follicles. A preliminary study by Etches and Cheng (1981) has shown that ovarian homogenates are capable of binding bovine FSH. The greatest binding was exhibited by homogenates of the ovarian stroma, which included follicles of up to 4 mm in diameter. The ability of thecal homogenates from follicles larger than 4 mm in diameter to bind FSH was negatively correlated with follicle size. A decrease in the number of
thecal FSH receptors throughout the final period of follicle growth may account for the accompanying decrease in thecal 3β-hydroxysteroid dehydrogenase (Armstrong, 1982b) and aromatase activities (Huang, Kao and Nalbandov, 1979; Wang and Bahr, 1983; Armstrong, 1983).

According to Armstrong (1983), the thecal aromatase activity increases during the early period of follicular development, reaching a maximum in the fifth largest follicle (approximately 20 mm in diameter) before subsequently declining. The mechanism whereby thecal aromatase activity was initially increased is not clear. The results of Etches and Cheng (1981) rule out the possibility that this is due to an increase in the number of thecal FSH receptors. In addition to acting as substrates for aromatization, androgens produced by the theca layer of developing follicles may act synergistically with FSH to enhance aromatase activity as in the granulosa cells of developing rat follicles (Daniel and Armstrong, 1980; Hillier and De Zwart, 1981). It is also possible that oestrogens themselves may exert a positive feedback action on aromatase activity by enhancing the induction of adenyl cyclase in response to FSH, as in the rat (Richards, Jonassen, Rolfes, Kersey and Reichart, 1979; Zhuang, Adashi and Hsueh, 1982; Adashi and Hsueh, 1982).

The present results suggest that LH, by controlling the availability of androgens for aromatization, may play an important role in regulating follicular oestrogen
synthesis. Whilst the plasma surge of LH resulted in a significant increase in conversion of [4-^{14}C]progesterone to androstenedione by the small ovarian follicles, aromatase activity was apparently unchanged (section 3:4). Thus the rise in plasma oestrogens which accompanies the LH surge (Peterson and Common, 1972; Senior, 1974b; Senior and Cunningham, 1974; Johnson and van Tienhoven, 1980) may be due to an increased production of oestrogens by the small follicles, following an increase in LH-stimulated androgen production.

It is tempting to speculate that by increasing follicular oestrogen production, the plasma surge of LH may be responsible for recruiting a follicle to the final phase of rapid follicle growth culminating in ovulation. Whereas previous workers have emphasised the importance of FSH in the recruitment of preovulatory follicles both in the hen (see section 1:1), and in other species (Hirshfield, 1979), several workers are of the opinion that a small rise in basal LH secretion may be responsible for stimulating the growth of preovulatory follicles (rat: Richards, Jonassen and Kersey, 1980; Bogovich and Richards, 1982; sheep: Webb and England, 1982). In the ovary of the hen it appears that once the follicle has reached a diameter of 8 mm, its fate as an ovulatory follicle is secured (Gilbert et al, 1983). At this stage of development the granulosa cells are starting to synthesise progesterone (Davidson et al, 1979) and are beginning to undergo proliferation (Gilbert et al,
1980). The results of the present study and that of Armstrong (1983) indicate that at this stage of follicular development, the theca layer is capable of producing large amounts of androgens and oestrogens. Perhaps in the fowl, as in the rat, oestrogens act on the granulosa cells to stimulate mitosis (Bradbury, 1961; Goldenberg, Vaitukaitus and Ross, 1972) and to enhance the induction of LH receptors in response to FSH (Richards, Ireland, Rao, Bernath, Midgley and Reichart, 1976).

Oestrogens, produced by the theca layer, might also play a role in modulating progesterone synthesis by the granulosa cells, as has been proposed in mammals (Haney and Schomberg, 1978). Although conflicting results have been reported in the literature concerning the effect of oestrogen on granulosa cell steroidogenesis, these discrepancies appear to be due largely to differences in incubation times. In small porcine follicles, oestradiol inhibited FSH-stimulated progesterone synthesis (Thanki and Channing, 1978) by granulosa cells in vitro. Oestradiol also suppressed both basal and LH-stimulated progesterone synthesis by granulosa cells from porcine preovulatory follicles in vitro (Haney and Schomberg, 1978; Fortune and Hansel, 1979; May and Schomberg, 1980; Veldhuis, Klase and Hammond, 1981a; 1981b). These inhibitory effects of oestradiol were seen in granulosa cell cultures maintained for 2 to 3 days. In longer incubation, oestradiol elicited an increase in both basal and LH-stimulated progesterone synthesis by
granulosa cells from rat (Bernard, 1975), bovine (Fortune and Hansel, 1979) and porcine (Veldhuis et al., 1981a, 1981b) preovulatory follicles. Welsh, Zhuang and Hsueh (1983) have recently reported a stimulatory effect of diethylstilboestrol on gonadotrophin-induced progesterone synthesis by granulosa cells from oestrogen-primed hypophysectomised immature rats. These stimulatory effects of oestrogens on progesterone synthesis could be maintained for 2 to 10 days and could not be attributed to an increase in mitosis (Veldhuis et al., 1981a, 1981b).

It was proposed that oestrogens may augment the production of cAMP in response to gonadotrophic stimulation (Welsh et al., 1983). It is conceivable that in the hen follicle, oestrogens produced by the thecal layer may also regulate granulosa cell function.

Androgens, produced by the theca layer of the hen follicle, might also be responsible for stimulating progesterone production by the granulosa cells throughout follicular development. In the rat, androgens elicited an increase in both basal (Lucky, Schreiber, Hillier, Schulman and Ross, 1977; Hillier, Knazek and Ross, 1977) and FSH-stimulated progesterone synthesis (Armstrong and Dorrington, 1976; Nimrod and Lindner, 1976) by granulosa cells from small antral follicles in vitro. Granulosa cells from both medium and large porcine follicles also responded to exogenous dihydrotestosterone, a non-aromatizable androgen, with an increased progesterone synthesis in vitro (Schomberg,
Stouffer and Tyrey, 1976; Haney and Schomberg, 1978; Veldhuis et al., 1981a). In addition Schomberg, Williams, Tyrey and Ulberg (1978) have demonstrated a reduction in progesterone secretion by granulosa cells from large, porcine follicles following prior treatment with ovarian implants of the anti-androgen flutamide in vivo. Another anti-androgen, cyproterone acetate, also inhibited progesterone production by granulosa cells from small rat follicles (Hillier, Knazek and Ross, 1977), and from large bovine preovulatory follicles in vitro (Henderson and Franchimont, 1983). The present results have shown that in the hen preovulatory follicle, the theca layer maintains the ability to synthesise androgens until shortly before ovulation. The possibility that androgens produced by the theca layer are responsible for enhancing the synthesis of progesterone by the granulosa cells awaits further investigation.
5. **SUMMARY**
SUMMARY

Homogenates of interstitial tissue from the ovary of the laying hen (Gallus domesticus) converted [4-\(^{14}\)C]-progesterone to androstenedione and testosterone, and [4-\(^{14}\)C]testosterone to phenolic steroids. These findings support the hypothesis that the interstitial cells of the ovary are a site of androgen and oestrogen synthesis.

During growth of the follicle from < 2 mm in diameter to 10 mm in diameter, thecal 17\(\alpha\)-hydroxylase/C-17,20lyase activity increased. Thecal homogenates from follicles within this size range converted significantly more [4-\(^{14}\)C]progesterone to androstenedione when collected after exposure to the endogenous surge of LH in vivo. These results suggest that LH acts on these follicles to stimulate thecal 17\(\alpha\)-hydroxylase/C-17,20lyase activity.

Thecal 17\(\alpha\)-hydroxylase/C-17,20lyase activity remained high throughout the final period of follicle development. Thecal homogenates from the largest follicle (approximately 35 mm in diameter) however, converted significantly less [4-\(^{14}\)C]progesterone to androstenedione when collected after the endogenous surge of LH. In contrast, conversion of [4-\(^{14}\)C]-progesterone to 20\(\beta\)-hydroxy-4-pregnen-3-one was significantly increased. These changes, which were not seen in the second largest (approximately 30 mm in diameter) and third largest (approximately 28 mm in diameter) follicles, due to ovulate 24 hours and 48 hours later respectively, may be related to the ovulatory process.
6. REFERENCES


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7. APPENDIX

LLEWELYN, C.A. (1981). Conversion of progesterone to 20β-hydroxy-4-pregnene-3-one by the preovulatory follicle in the hen (Gallus domesticus). IRCS Medical Science, 9, 859.
CONVERSION OF [4-14C]PROGESTERONE TO ANDROSTENEDIONE IN VITRO BY THECAL TISSUE FROM THE OVARY OF THE DOMESTIC FOWL (GALLUS DOMESTICUS)

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SUMMARY

The conversion of [4-14C]progesterone to androstenedione by thecal tissue homogenates from the large yellow follicles of the hen ovary was measured at two periods of the ovulatory cycle after incubation for 1 h in the presence of cofactors. Production of androstenedione by the largest follicle, F1, was reduced significantly 1–2 h before ovulation compared with 15 h before ovulation, whereas production of an unidentified androgen increased. These differences were not observed in the next largest follicles, F2 and F3. Thecal tissue homogenates from all the follicles converted [4-14C]progesterone to 17α-hydroxyprogesterone, androstenedione and testosterone.

INTRODUCTION

Hens, unlike mammals, have only one functional ovary containing many small white follicles, and a hierarchy of follicles filled with yellow yolk which range from the follicle due to ovulate which is about 40 mm, to the smallest of about 7 mm.

It has been shown that thecal tissue of the hen follicle contains cells with an ultrastructural appearance characteristic of steroid-secreting cells (Dahl, 1970; Perry, Gilbert & Evans, 1978). Biochemical and histochemical techniques have also demonstrated the presence in these cells of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase, both key enzymes involved in steroidogenesis (Wyburn & Baillie, 1966; Boucek & Savard, 1970). The presence of progesterone, testosterone and oestrogen in the follicle walls of the three largest follicles, F1, F2 and F3, also supports the hypothesis that thecal tissue is a site of steroid synthesis (Shahabi, Norton & Nalbandov, 1975; Imai & Nalbandov, 1978).

There is also some evidence that thecal and granulosa cells interact within the follicle because although isolated thecal cells from F3 produce small amounts of oestrogen, a fivefold increase is seen when the two cell types are incubated together (Huang, Kao & Nalbandov, 1979). A similar interaction between thecal and granulosa cells for the biosynthesis of oestradiol is thought to occur in mammals (Falck, 1959; Fortune & Armstrong, 1978) where granulosa cells may aromatize androgens, derived from the theca, to oestrogens. In birds, however, no aromatase activity has been detected in the granulosa layer; the major product of these cells being progesterone (Huang et al. 1979; Wells, Gilbert & Culbert, 1980) produced mainly under the influence of luteinizing hormone (LH) (Culbert, Hardie, Wells & Gilbert, 1980). However, isolated thecal cells from F2 and F3 can aromatize exogenous testosterone to oestrogen (Huang et al. 1979). It is possible that progesterone produced by the granulosa layer may be metabolized by the thecal layer to androgens and oestrogens. This paper reports on the conversion of progesterone to androstenedione by thecal homogenates obtained from the follicles of the ovarian hierarchy at two periods of the ovulatory cycle.
Laying hens (Shaver 288) aged about 8 months and in good laying condition, were killed with an overdose of sodium pentobarbitone (Expiral, Abbott Laboratories, Kent). Five to seven of the largest yellow follicles were obtained from the ovary of each of six hens killed 1–2 h before an ovulation was expected. The three largest follicles were collected from a further five hens killed 15 h before ovulation was expected. The time of ovulation was estimated as described by Gilbert, Davidson & Wells (1978). The follicles were slit and the yolk and surrounding granulosa layer were removed (Gilbert, Evans, Perry & Davidson, 1977). The thecal tissue (i.e. the remainder of the follicle exterior to the basal lamina) was weighed and then homogenized at 4°C, using a Polytron homogenizer (several bursts of 2 s at approximately 12 000 rev./min), in 10 vol. 0.25 M sucrose containing MgCl₂ (20 mmol/l), KCl (20 mmol/l) and EDTA-tetrasodium salt (5 mmol/l). Cell debris and nuclei were removed by centrifugation at 800 g for 10 min and the resulting supernatant fraction was used immediately.

Steroids and other chemicals

[4-¹⁴C]Progesterone (specific activity 2.18 GBq/mmol) was obtained from The Radiochemical Centre, Amersham. Nonradioactive steroids, glucose-6-phosphate, NAD⁺ and NADP⁺ were supplied by Sigma Chemical Co., Poole, Dorset. Organic solvents (Analar grade) were redistilled before use.

Incubation, extraction, isolation and measurement of metabolites

Duplicate tubes with a total volume of 0.5 ml phosphate buffer (pH 7.4) containing 1.7 nmol [4-¹⁴C]progesterone, 0.5 μmol NAD⁺, 2.5 μmol glucose-6-phosphate, 2.5 μmol NADP⁺, 0.5 μmol EDTA-tetrasodium salt and 2.0 μmol MgCl₂ plus the supernatant fraction corresponding to 10 mg fresh tissue (0.1 ml) were incubated for 1 h at 37°C in air, using a shaking water bath. These conditions were similar to those reported by Kremers (1976). At the end of the incubation, the tubes were placed on ice to stop the reaction and nonradioactive carrier steroids (20 μg each of progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone and oestradiol in 20 μl acetone) were added. The reaction mixture was extracted twice with 2.5 ml dichloromethane; the extract was then washed with water to remove aqueous impurities before evaporating to dryness. The radioactive extract was redissolved in acetone (1 ml) and 0.1 ml was used for counting to determine the recovery. Recoveries were usually about 70%.

A sample of each extract was applied to an instant thin-layer chromatographic strip (i.t.l.c.—SAF, Gelman Hawksley, Northampton) and run in a partition system with propylene glycol as the stationary phase (applied in acetone, 20% by vol.) and carbon tetrachloride: light petroleum, b.p. 40–60°C (9:1, v/v) as the mobile phase (Goldman, 1973). The areas corresponding to carrier progesterone and androstenedione were located by u.v. light (250 nm), cut out and placed in vials containing 5 ml toluene scintillator (0.4% PPO, 0.032% POPP) which were then counted in a Philips liquid scintillation counter (efficiency approximately 90%). After correction for recovery, the results were expressed as nmol/10 mg fresh tissue per h (Tables 1 and 2). The remaining radioactive areas of the chromatogram were located using a thin-layer radiochromatogram scanner (Panax Equipment Ltd, Redhill, Surrey) or by autoradiography and eluted with methanol.

A further sample of the extract from three birds at each period of the cycle was run in the same solvent system and the whole chromatogram cut into 2.5 mm strips which were counted. The yield of an unknown metabolite which was similar but not identical to 5α-dihydrotestosterone (5α-DHT) was calculated from these data (Table 3).
Identification of metabolites

The following mixtures of solvents were used to identify radiometabolites. These are referred to later as solvent systems 1–6. (a) Gelman i.t.l.c.—SAF strips: 1, hexane: butyl acetate (5: 2, v/v); 2, diethyl ether. (b) Merck F254 silica gel plates (Darmstadt, Germany): 3, chloroform: ethyl acetate (4:1, v/v); 4, cyclohexane: ethyl acetate (1:1, v/v); 5, chloroform: cyclohexane: butyl acetate (1:1:1, by vol.); 6, toluene: acetone (4:1, v/v). Carrier steroids which did not absorb in the u.v. region were located with an anisaldehyde–sulphuric–acetic acid spray (Neher, 1964). Oxidation and acetylation were carried out according to the method of Bush (1961).

Statistical treatment of results

For each follicle, Student's t-test was used to compare the mean values at 1–2 h before ovulation with those obtained 15 h before ovulation.

RESULTS

Identification of metabolites

The area on the initial chromatogram run corresponding to progesterone contained one 14C-labelled peak coincident with carrier progesterone in solvent systems 4, 5 and 6. The area corresponding to androstenedione had the same relative front (RF) value as carrier androstenedione in solvent systems 3, 4 and 6 and was recrystallized to constant specific activity, e.g. after four successive recrystallizations the percentage change in radioactivity per mg was 1·2±1·6 (mean±s.e.m.). A single 14C-labelled peak with an RF value similar to 5β-DHT in solvent systems 2, 3 and 5 was present in incubations of F1, F2 and F3. This yielded 5β-androstane-3,17-dione upon oxidation, but acetylation did not give a 14C-labelled peak coincident with 5β-DHT acetate. Also, repeated recrystallization with authentic 5β-DHT failed to produce crystals with a constant specific activity. A tentative suggestion is that this metabolite is the 17α-epimer of 5β-DHT.

Other metabolites formed included 17α-hydroxyprogesterone and small amounts of testosterone which were separated in solvent system 1 after acetylation. The remaining more polar metabolites were subjected to a partition procedure (Dick, Culbert, Wells, Gilbert & Davidson, 1979) and a neutral and phenolic fraction obtained. The amount of activity in the phenolic fraction was low (less than 1% of the total activity on the chromatogram) and when run in solvent system 4 neither oestrone nor oestradiol was detected, although a 14C-labelled peak with an RF value similar to oestradiol was present.

Progesterone metabolism

There was no significant difference in progesterone metabolism by thecal tissue homogenates of F1, F2 or F3 at 1–2 h before ovulation compared with 15 h before ovulation (Table 1). However, it appeared that less progesterone was metabolized by F1 compared with F2 and F3 at both periods of the cycle and that this reduction was greater 1–2 h before ovulation.

Conversion of progesterone to androstenedione

Thecal tissue homogenates from F1 produced significantly less androstenedione at 1–2 h before ovulation compared with 15 h before ovulation (Table 2). However, F2 and F3 produced similar amounts of androstenedione at both periods of the cycle.

Conversion of progesterone to an unidentified androgen

These results are given in Table 3 and were worked out from the chromatograms cut into strips (see Materials and Methods). There was a significant increase in production of an unidentified androgen by F1 1–2 h before ovulation compared with 15 h before ovulation.
Table 1. Metabolism of \([4-\text{\textsuperscript{14}C}]\)progesterone (nmol/10 mg wet tissue per h) by thecal homogenates 1-2 and 15 h before the expected time of ovulation of the domestic fowl. Values are means ± S.E.M.; no. of follicles are shown in parentheses

<table>
<thead>
<tr>
<th>Follicle</th>
<th>1-2 h before ovulation</th>
<th>15 h before ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.360 ± 0.051 (5)</td>
<td>1.289 ± 0.147 (5)</td>
</tr>
<tr>
<td>F2</td>
<td>1.507 ± 0.023 (5)</td>
<td>1.398 ± 0.107 (5)</td>
</tr>
<tr>
<td>F3</td>
<td>1.472 ± 0.093 (5)</td>
<td>1.394 ± 0.082 (5)</td>
</tr>
<tr>
<td>F4</td>
<td>1.600 ± 0.014 (5)</td>
<td>1.289 ± 0.032 (5)</td>
</tr>
<tr>
<td>F5</td>
<td>1.562 ± 0.032 (5)</td>
<td>1.372 ± 0.037 (5)</td>
</tr>
<tr>
<td>F6</td>
<td>1.519 ± 0.057 (3)</td>
<td>1.394 ± 0.082 (5)</td>
</tr>
</tbody>
</table>

Table 2. Conversion of \([4-\text{\textsuperscript{14}C}]\)progesterone to androstenedione (nmol/10 mg wet tissue per h) by thecal homogenates 1-2 and 15 h before the expected time of ovulation of the domestic fowl. Values are means ± S.E.M.; no. of follicles are shown in parentheses

<table>
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<th>1-2 h before ovulation</th>
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</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.095 ± 0.018 (6)*</td>
<td>0.236 ± 0.026 (5)</td>
</tr>
<tr>
<td>F2</td>
<td>0.265 ± 0.024 (6)</td>
<td>0.231 ± 0.023 (5)</td>
</tr>
<tr>
<td>F3</td>
<td>0.287 ± 0.019 (6)</td>
<td>0.221 ± 0.025 (5)</td>
</tr>
<tr>
<td>F4</td>
<td>0.281 ± 0.041 (6)</td>
<td>0.217 ± 0.030 (3)</td>
</tr>
<tr>
<td>F5</td>
<td>0.279 ± 0.038 (6)</td>
<td>0.217 ± 0.030 (3)</td>
</tr>
<tr>
<td>F6</td>
<td>0.237 ± 0.028 (4)</td>
<td>0.217 ± 0.030 (3)</td>
</tr>
<tr>
<td>F7</td>
<td>0.370 ± 0.076 (3)</td>
<td>0.217 ± 0.030 (3)</td>
</tr>
</tbody>
</table>

*P<0.01 compared with results at 15 h (t-test).

Table 3. Conversion of \([4-\text{\textsuperscript{14}C}]\)progesterone to an unidentified androgen (nmol/10 mg wet tissue per h) by thecal tissue homogenates 1-2 and 15 h before the expected time of ovulation of the domestic fowl. Values are means ± S.E.M.; no. of follicles are shown in parentheses

<table>
<thead>
<tr>
<th>Follicle</th>
<th>1-2 h before ovulation</th>
<th>15 h before ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.836 ± 0.056 (3)*</td>
<td>0.401 ± 0.105 (3)</td>
</tr>
<tr>
<td>F2</td>
<td>0.316 ± 0.048 (3)</td>
<td>0.344 ± 0.078 (3)</td>
</tr>
<tr>
<td>F3</td>
<td>0.329 ± 0.190 (3)</td>
<td>0.217 ± 0.030 (3)</td>
</tr>
</tbody>
</table>

*P<0.05 compared with results at 15 h (t-test).

However, F2 and F3 produced similar amounts of this metabolite at both periods of the cycle.

**DISCUSSION**

This study has shown that thecal tissue from the large yolk-filled follicles of the domestic fowl can convert progesterone to 17α-hydroxyprogesterone, androstenedione and testosterone. These results are consistent with the hypothesis that the thecal tissue of the hen follicle metabolizes the progesterone secreted by the granulosa cells (Huang et al. 1979; Wells et al. 1980).

Thecal tissue homogenates of the largest preovulatory follicle (F1) converted significantly less progesterone to androstenedione 1-2 h before ovulation compared with 15 h before ovulation. Nakamura, Tanabe & Hirano (1979) reported a decrease in 17α-hydroxylase...
activity of F1 as ovulation approached. However, in this study the absence of any change in progesterone metabolism by F1 indicates that such a decrease may only partly account for the observed reduction in androstenedione production 1–2 h before ovulation. Perhaps the LH surge, mediated by the action of steroids (Saidapur & Greenwald, 1979), might be responsible for altering enzyme activities within F1. One such steroid could be the unidentified 5β-androstane derivative whose production was significantly increased in F1 but not in the other follicles (F2 and F3) 1–2 h before ovulation. It is unlikely, however, that this androgen could affect enzyme activities since 5β-reduced metabolites are believed to be biologically inactive (Dorffman & Ungar, 1965; Davies, Massa & James, 1980). The increased production of this androgen by F1 as ovulation approaches would limit the amount of substrate available for oestrogen formation and is consistent with the finding of Nakamura et al. (1979) that the concentration of oestradiol is lowest in the largest follicle of the hierarchy.

There was no evidence of any conversion of [4-14C]progesterone to oestrogens by thecal homogenates in this study which supports earlier work by Nakamura, Tanabe & Katukawa (1974) using homogenates of whole ovarian tissue.

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REFERENCES


CONVERSION OF PROGESTERONE TO 20β-HYDROXY-4-PREGNENE-3-ONE BY THE PREOVULATORY FOLLICLE IN THE HEN (GALLUS DOMETICUS)

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The ovary of the hen contains a hierarchy of 5–7 large yolk-filled follicles of which only the largest (F1) is ovulated in response to the plasma surge of luteinizing hormone (LH). The mechanism by which LH brings about follicular rupture is uncertain. Tojo and Hustin (1) have suggested that progesterone may be directly involved in the hen, as it is in mammals (2). The granulosa cells of all the large follicles produce substantial amounts of progesterone in response to LH (3, 4) but the metabolism of [4-14C] progesterone by thecal homogenates from F1 but not F2 and F3 follicles alters before ovulation. Conversion of [4-14C] progesterone to an unidentified metabolite by homogenates of F1 was found to increase significantly 1–2 h before ovulation compared with earlier in the cycle (5). In this paper evidence is presented to show that this metabolite is 20β-hydroxy-4-pregnen-3-one.

Sufficient quantities of this metabolite for identification were obtained from homogenates of several F1 follicles collected 1–2 h before ovulation. The homogenates comprised the thecal layer and its epithelial covering. Aliquots (0.2 ml) were incubated for 100 mins at 37 °C with 8.5 nmol of [4-14C] progesterone (specific activity 2.18 GBq/nmol) in a total volume of 1 ml 20 mM phosphate buffer (pH 7.4) containing 5.0 μmol NADH and an NADPH-generating system comprising 5.0 μmol glucose-6-phosphate and 1.0 μmol NADP. The incubates were gassed with 95% O2–5% CO2 (v/v) and at the end of the incubation period were frozen at −20 °C until extracted. The metabolites were extracted thrice with 5 ml dichloromethane, pooled, and the unknown metabolite, which was the major conversion product of progesterone, isolated by instant thin-layer chromatography (t.t.l.c.) as previously described (5). In addition a time-study showing the production of this and various other metabolites was carried out by sampling incubates at various times (0–100 mins); the metabolites were separated by t.t.l.c. and quantified by liquid scintillation counting as before (5). These results are shown in the figure.

The unknown metabolite had an Rf value which was identical to those of 20β-hydroxy-4-pregnen-3-one and 17α-hydroxyl-17β-ol-3-one in the initial t.t.l.c. system (5) and upon subsequent thin-layer chromatography (silica gel plates, Merck F254) in the solvent system butyl acetate: chloroform: cyclohexane (1:1:1, by vol). After acetylation the unknown metabolite yielded a product whose Rf (0.26) was the same as 20β-hydroxy-4-pregnen-3-one-acetate (Rf 0.30), but different from 17α-hydroxyl-17β-ol-3-one-acetate (Rf 0.43), on t.t.l.c. using the solvent system, light petroleum, b.p. 40–60 °C: isopropyl ether (1:1, v/v) (2 developments). Oxidation of the metabolite with chromic acid gave a product which recrystallised to constant specific activity with progesterone (mean ± SEM, 1913 ± 18 dpm/mg after 6 recrystallisations) but not with 17α-hydroxyl-17β-ol-3-one (specific activity decreased from 8692 to 264 dpm/mg after 6 recrystallisations).

The isolated metabolite was also chromatographed in a high-pressure liquid chromatography system using a ‘Hypersil’ M22 column, 5 × 100 mm with methanol: water (60: 40, v/v) as the solvent. With this system, a single ultra-violet absorbing peak was recorded at 242 nm, confirming the presence of a conjugated system, presumably a Δ3-3-keto structure in the molecule. The retention time of the metabolite (5.2 min) was identical to that of 20β-hydroxy-4-pregnen-3-one, and was clearly separated from the following Δ3-3-keto steroids: androstenedione (2.0 min), 17α-hydroxy-progesterone (2.4 min), testosterone (2.5 min), progesterone (3.7 min) and 20α-hydroxy-4-pregnen-3-one (4.1 min). After passing through the detector, the metabolite was recovered by evaporating the solvent. As a final proof of identity, the metabolite was then recrystallised to constant specific activity with 25 mg of authentic 20β-hydroxy-4-pregnen-3-one (mean ± SEM, 48 650 ± 615 dpm/mg after 6 recrystallisations).

These results show that the metabolite produced in large quantities from progesterone by thecal homogenates of F1 just before ovulation is 20β-hydroxy-4-pregnen-3-one. Homogenates of the F2 and F3 follicles due to ovulate 24 and 48 h later, produce much lower amounts of 20β-hydroxy-4-pregnen-3-one at this time (5). A possible involvement of this metabolite of progesterone with the process of follicular rupture in the hen deserves further investigation. Progesterone has been shown to inhibit collagenase activity in the rat uterus (6) and in the human cervix (7). Hence rapid removal of progesterone in the thecal layer by metabolism to 20β-hydroxy-4-pregnen-3-one might also play a role in the follicular rupture by ensuring maximal collagenase activity.


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