

Endocrine Unit, Chelsea Hospital for Women
& Institute of Obstetrics and Gynaecology, University of London,
London, S.W.3.

STEROIDS IN THE CYST FLUID FROM HUMAN OVARIES

by Eleonora Giorgi, M.D.

P R E F A C E

The development of new micro-analytical techniques for the isolation and quantitative determination of steroids in human tissues and body fluids has made possible a more direct approach to many problems in reproductive physiology. This type of research is still in a preliminary phase and the main experimental work embodied in this thesis describes an attempt to study ovarian function in women with normal and abnormal menstrual cycles by means of steroid determinations in ovarian fluid and ovarian venous blood. First, however, it was considered that it was desirable to bring together relevant observations upon the relationship between ovarian morphology and function in the normal cycle and in some gynaecological conditions. Finally, the results of the present work are discussed in the context of previous work and current hypotheses.

Chapter I - ANATOMY OF THE OVARY, WITH ESPECIAL REFERENCE TO CYSTIC FORMATIONS.

1 - Embryological derivation of ovarian tissues.

The three major components of the ovary, germ cells, cortex and medulla, derive embryologically from different tissues (Witschi, 1963). The germ cells separate from stem cells during cleavage stages and migrate during the 5th week of embryonic development from the dorsal yolk sac epithelium to the site of the gonadal primordial^{um}. They are the only source of ova, according to the view most widely accepted today.

The cortex is a specialised strip of coelomic epithelium that arises as a thickening along the median edge of the mesonephric bodies. It furnishes the primitive gonad with follicle cells which become granulosa cells in advanced stages of ovogenesis. The medulla originates from the medial mesonephric blastema; it furnishes interstitial or stromal cells and later contributes to the formation of the follicular theca. A condensation of the mesenchyme just beneath the coelomic epithelium forms the tunica albuginea.

By the ninth month of pregnancy the medulla is very reduced, in size. In the cortex, the primary ovarian follicles, which comprise the oocyte and a layer of follicular cells, enlarge with accumulation of fluid; the follicular granulosa cells proliferate and constitute a multilayered coat around the oocyte. This is centrally located and separated from the granulosa layer by an antrum. The theca layer,

consisting of differentiating stromal cells, develops at this stage, and a Graafian follicle is formed.

2 - The ovary of the sexually mature woman.

The ovaries of the sexually mature woman are paired, bean-shaped bodies, attached to the back of the broad ligament by a double fold of peritoneum - the mesovarium. The longitudinal diameter is approximately 1 to 2 inches; the transverse diameter is 1 to 1 1/2 inch and the thickness is approximately 1/2 inch. The surface is smooth and glistening.

A cross section reveals a cortical zone - the cortex - containing follicles and stromal cells; an intermediate zone - the medulla - which consists of loose connective tissue, numerous blood vessels, nerves and bundles of smooth muscle fibres; and a connective tissue adjacent to the mesovarium - the hilum - which contains chiefly numerous large arteries and veins, but also lymphatics, nerves and ganglia. The hilum also contains clusters of plump polyhedral cells closely connected with the nerve trunks; in their behaviour towards fixation fluids and stains these cells are identical with the Leydig cells of the testicles. Through the hilum the ovarian artery penetrates into the ovary and gives rise to primary, secondary and tertiary branches. These branches exhibit spiralling in different degree - minimal in the primary branches which are undulated; more evident in the secondary branches and maximal in the tertiary branches. The veins have a similar distribution and converge towards the hilum into

the ovarian vein. This forms a rich network called the pampiniform plexus between the layers of the broad ligament. The lymphatic system of the ovary has a particular abundance of labyrinthine channels which become lacunae around the follicles.

The cortex comprises more than half the thickness of the mature ovary. It is covered by a single layer of 'coelomic' or serosal epithelium. Infolding of the serosal epithelium, with invaginations into the cortical stroma, may form small cysts known as germinal inclusion cysts. Underlying the epithelium there is a slight condensation of the stroma - tunica albuginea -, but this is sometimes absent. The albuginea merges with the cortical zone without sharp demarcation. Thickening of the albuginea, due to an increase in the number and diameter of the collagenous fibres, occurs in some cases of chronic pelvic inflammation and endometriosis. This is considered to be a constant feature of polycystic ovaries as seen in the Stein-Leventhal syndrome (Stein, Cohen & Elson, 1949; Leventhal, 1958; Goldzieher & Green, 1962).

The ovarian stroma has scanty blood vessels and is formed mostly by spindle shaped cells enmeshed in reticular fibres. Hyperplasia of the stromal cells, with increased density of their nuclei, and stromal fibrosis have been described in the Stein-Leventhal syndrome as a constant finding (Stein et al., 1949), although doubts have been expressed as to whether this histological feature is entirely characteristic of the ovary in this syndrome (Morris & Scully, 1958; Roberts & Haines, 1960).

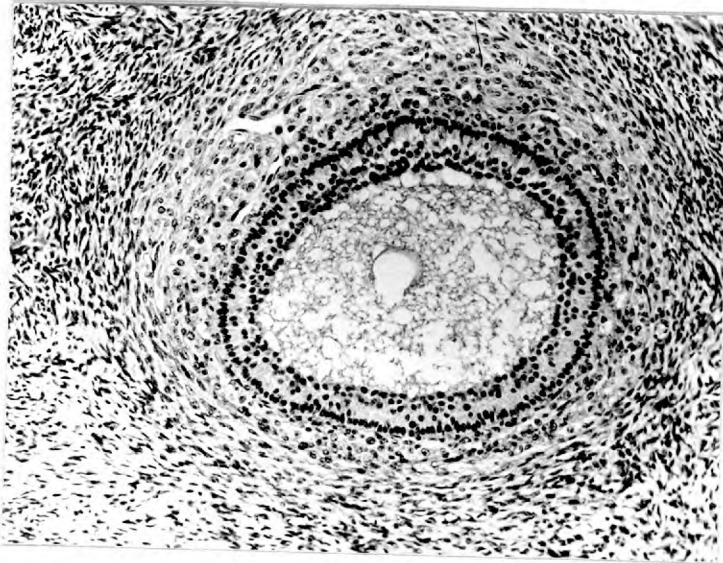
Maturation of the follicle.

At the onset of each menstrual cycle a few of the many thousands of the primordial follicles in the cortex begin to increase in size and to show some degree of maturation. Only one follicle, or rarely more than one, reaches full maturity, ruptures to release the ovum and is finally transformed into a corpus luteum (Fig. 1). In the other follicles the process of maturation stops at different stages and is followed by involution - atresia folliculi.

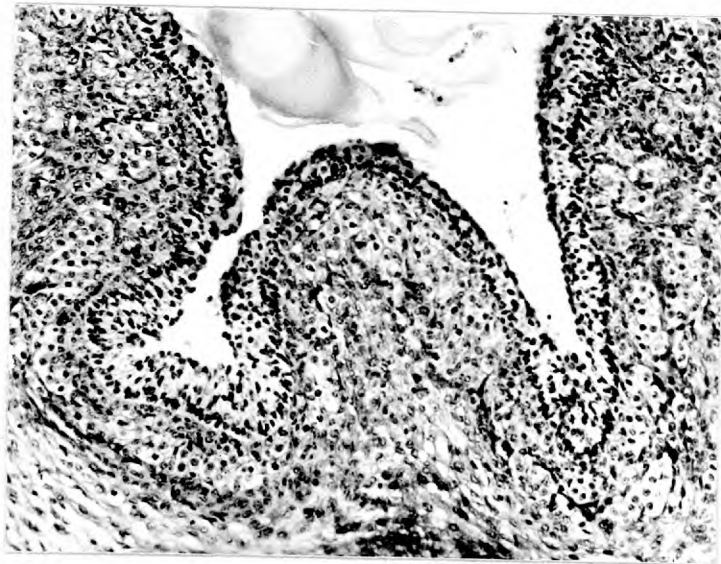
Three stages can be recognized in the process of maturation (Rubin & Novak, 1956). 1) Growth of primary and vesicular follicles : - Primary follicles and follicles at the antrum stage undergo enlargement through proliferation of the granulosa cells and accumulation of fluid. The ovum is displaced to a polar position and becomes embedded in a well-marked peninsula of granulosa cells - the cumulus oophorus - and surrounded by cells arranged in radial fashion - corona radiata.

2) Theca differentiation : - While proliferation of the granulosa cells and accumulation of fluid continue, the ovarian stroma surrounding the follicle differentiates into an investing cellular layer- the theca, which soon comes to exhibit two concentric layers : a layer of large polyhedral cells with abundant lipid content - the theca interna - and the theca externa in which the cells are fusiform. The theca is separated from the granulosa by a basement membrane. At this stage, a special blood supply for the follicle has developed. Two systems of capillaries can be demonstrated, one for each of the two theca layers. The vessels in the theca externa run parallel to the outer surface

Fig.1- The ovarian structures.



a) Maturing Graafian follicle.



b) Early corpus luteum.

of the follicle and have a thick connective wall. In the theca interna the capillaries are disposed radially and consist only of a basal membrane lined by endothelium. The capillaries do not penetrate into the granulosa layer until after rupture of the follicle and formation of the corpus luteum.

3) Preovulatory swelling : - An acceleration of follicular growth follows the two preceding stages, and the follicle reaches its maximal size, mainly due to a rapid increase in the accumulation of fluid. A special type of theca cells, the "K cells", which become an important feature in the formation of the corpus luteum, can be first seen at the time of preovulatory swelling (White, Hertig, Rook & Adams, 1951). The ovum itself participates in the growth and maturation of the follicle and its diameter increases to 150-200 μ m. It dehisces from the follicular wall and floats free in the antrum in a covering of cumulus cells.

Ovulation.

Ovulation is the rupture of a ripe follicle with release of a mature ovum. The mechanism of ovulation is not completely understood. During the stage of maturation the follicle ascends towards the surface of the ovary. Formation of a "theca cone" at the peripheral hemisphere of the follicle near the cumulus oophorus has been described in human ovaries and in ovaries of other mammals (Strassman, 1938). This cone may prepare the path for the follicle to the surface of the ovary, acting in a purely mechanical way. When the follicle reaches the

surface, the theca and granulosa layers become thinner and avascular in an area corresponding to the protruding pole - stigma folliculi. The stigma finally tears, probably due to the effect of intrafollicular pressure or because of diminished elasticity of the follicular wall (Rondell, 1964). The ovum is extruded and the follicle collapses with the escape of the fluid.

Corpus luteum formation.

Four principal stages are recognised in the formation of the corpus luteum (Meyer, 1911). 1) Stage of proliferation and hyperaemia : -The wall is thin and folded. The granulosa layer shows evidence of early transformation into large polyhedral cells laden with lipoid material - granulosa lutein cells.

2) Stage of vascularisation : - The layer of lutein cells is invaded by blood vessels from the theca. The vessels extend to the very lumen. Haemorrhage is a normal feature of this phase. At times, however, the cavity may be distended with blood-corpus luteum haematoma.

3) Stage of maturity : - The broad lutein zone becomes festooned and subdivided by wedgelike septa of theca cells into folds and alveoli. The theca cells appear shrunken through disappearance of their rich lipoid content. They often show some degree of luteinisation, constituting the theca lutein cells, which are smaller than the granulosa lutein cells. The K cells retain their identifying characteristics during luteinisation and are conspicuous by their attenuated shape, small dark nuclei and highly eosinophilic cytoplasm. The cavity

always contains a small amount of fluid, shut off from the lutein zone by a layer of fibroblastic tissue.

4) Stage of regression : - This stage is marked by fatty degeneration, fibrosis and later hyalinization of the lutein zone, with increase of the cicatricial tissue within the cavity. The end product is the corpus albicans, a whitish, hyalinised, convoluted structure which slowly decreases in size and usually disappears.

Atresia folliculi.

According to a classical description (Rubin & Novak, 1965), atresia folliculi consists of death of the ovum and disintegration of the granulosa layer, followed by degeneration of the theca cells. Frequently the cavity becomes distended with fluid. The cystic stage is followed by cicatricial obliteration, proceeding slowly from the periphery toward the centre. The end result of this process is the corpus fibrosum a condensed zone of hyalinised tissue surrounding a small central cicatrix.

Recently, it has been maintained (Mossman, Koering & Ferry, 1964) that the theca cells of atretic follicles do not degenerate after disintegration of the granulosa. On the contrary, the theca cells would differentiate into rounded, polyhedral cells, arranged in rows or groups around the disappearing cavity of the follicle. These formations are considered to be an endocrine tissue - the interstitial gland. The interstitial gland tissue can be recognized by the disposition of the cells and by their characteristics. The cells are not as large as the theca cells of mature follicles and have a relatively scarce cytoplasm, which contains large rounded vacuoles filled with

lipoid material.

The mass of the interstitial gland seems to vary in relation to the reproductive age and cycles.

3 - Cellular origin of the ovarian hormones.

The type of ovarian cells responsible for the production of individual steroid hormones (progestogens, oestrogens and androgens) has not yet been clearly established. This is partly due to the low specificity of the histochemical reactions, which can only indicate whether there is secretory activity in the cells or whether the cells contain steroid precursors. The application of various histochemical techniques has demonstrated a considerable degree of metabolic activity in the theca interna of maturing follicles. On the contrary, granulosa cells show minimal activity and this also applies to follicular fluid (McKay & Robinson, 1947). In the corpus luteum both the granulosa lutein cells and the theca lutein cells appear to be actively secreting; they contain lipid droplets rich in materials which are sudanophilic, birefringent, autofluorescent and Schultz-positive.

On the basis of histochemical studies, it has been proposed that the theca cells produce progesterone and oestrogens in the developing follicle. In the corpus luteum, the granulosa and lutein cells would be responsible for progesterone production and either type of cells for the production of oestrogens (McKay & Robinson, 1947). Some authors attribute great importance to the K cells in progesterone formation (White et al., 1951). Some evidence has been presented for oestrogen

production by the interstitial cells (Corner, 1938; McKay & Robinson, 1947; Pinkerton, 1959). Production of androgens has been attributed to the Leydig cells of the hilum (Allen & Woolf, 1959). It has also been reported that the concentration of androgens in the hilum is much higher than in the cortex or medulla (Simmer & Voss, 1960). However, the definition of 'interstitial' cells may have varied from one study to another. Recently the morphology and derivation of the interstitial cells supposedly producing steroids has been defined, but their function still awaits clarification (Mossman et al. 1964).

The demonstration of 3β -hydroxy-steroid dehydrogenase by means of a very specific reaction (Wattenberg, 1958) in both layers of the follicle and in larger quantities in the corpus luteum (Deane, Lobel & Romney, 1962), has confirmed that the follicular and lutein cells can actually produce steroids, as suggested by the above mentioned histochemical investigations.

A different experimental approach has thrown new light upon the activity of the ovarian cell types. Autotransplantation of cells from the follicle or corpus luteum of the rat into the anterior chamber of the eye has shown that granulosa cells are indispensable for oestrogen production. Under these experimental conditions, oestrogens are formed only if theca interna or interstitial cells are transplanted together with granulosa or granulosa lutein cells. Since the combination of a progesterone implant and theca interna cells also results in oestrogen formation, it would appear that granulosa cells are the source of progesterone and that the theca interna cells transform the

latter into oestrogens (Falck, 1959).

In agreement with this hypothesis, it has been demonstrated that the corpus luteum of the mare, which consists predominantly of granulosa lutein cells, produces progesterone but not oestrogens. Granulosa cells, in contrast to theca cells, would appear to be deficient in the enzyme systems involved in oestrogen synthesis (Short, 1962a).

4 - Cystic formations of the ovary.

An abnormal development of follicles or corpora lutea may give rise in the ovary to cystic formations. These have a similar histological structure to normal follicles or corpora lutea, but are larger in size and contain more fluid. They are further characterized by their life span, which can extend over the length of two or more cycles.

The definition of a 'pathological' cyst formation is still a matter of debate. Accumulation of fluid can occur in maturing follicles, in corpora lutea and in atretic follicles, which are otherwise normal. However, a follicle with a diameter of more than 1.5 cm or a corpus luteum with a diameter of more than 2.5 cm is generally regarded as pathological and is termed a follicular or corpus luteum cyst (Hertig & Mansell, 1957).

The non-neoplastic cysts of the ovary can be classified according to their number - single or multiple - and to their derivation - from the unruptured or from the ruptured follicle.

a) - Single cysts from the unruptured follicle.

Cystic follicles : - Their diameter does not exceed 1.5 cm. More than one at a time may be present. Cystic follicles are not usually accompanied by any appreciable endocrine disturbance, although a particular type has been described in women of premenopausal age with hyperplastic changes in the endometrium and irregular bleeding. In this type of follicle both the granulosa and theca layer appear intact : the ovum is mature.

Follicle or follicular cysts : - This is the most common variety of single cyst of the ovary. The cyst is unilocular, translucent and rarely measures more than 10 cm in diameter. It has a smooth outer surface and thin, membranous wall. The cavity contains a clear yellow fluid, free of mucus. Microscopically the cyst is composed of flattened granulosa and theca cell layers, but often the lining is only of theca cells. Both cellular layers may show varying degrees of luteinisation (Hertig & Mansell, 1957). These cysts are considered persisting follicles which do not ovulate (Rubin & Novak, 1956).

In the majority of cases, these cysts do not produce clinical symptoms. In infancy, they may be associated with precocious puberty and irregular bleeding. During the reproductive age and at the time of the menopause, follicular cysts are sometimes accompanied by menstrual abnormalities, such as irregular uterine bleeding alternating with periods of amenorrhoea (Schroeder, 1954; Zondek, 1954). It has been suggested that the presence of follicular cysts may be associated

with ovulatory failure and may account for atrophy of the contralateral ovary (Morris & Scully, 1958). It is generally believed that follicular cysts have a limited life span and regress completely within weeks or months.

b) - Single cysts of the ruptured follicle.

Corpus luteum cysts : - This type of cyst, usually larger in size than follicular cysts, is derived from a corpus luteum into which there has been an abnormal amount of haemorrhage (corpus luteum haematoma). If reabsorption of the haematoma does not follow, organisation of the coagulum results in an inner zone of hyaline, lined by flattened cells and enclosing a cavity in which fluid may accumulate. The wall of the corpus luteum cyst so formed is of variable thickness and consists of a layer of granulosa lutein cells. The cyst may exhibit all the phases of development or regression of a corpus luteum. Corpus luteum cysts can sometimes be accompanied by periods of amenorrhoea lasting a few weeks and followed by prolonged but not heavy bleeding. More often they do not provoke any clinical features.

c) - Multiple cysts.

Multiple cysts of the ovary derive from unruptured follicles.

a) Some ovaries are studded with tiny cysts of not more than 3-5 mm in diameter. These are formed by follicles blighted at various stages of development. Such change is not pathological and precedes follicular atresia. The other ovarian constituents appear normal and ovulation occurs regularly. This type of cystic ovaries does not cause endocrine disturbances, although they may be associated with irregular endometrial

hyperplasia and bleeding. This occurs mainly during puberty and adolescence (Rubin & Novak, 1956).

b) Additional changes in ovarian morphology may occur in cystic ovaries in which ovulation does not seem to be impaired. Chronic pelvic inflammation may produce thickening of the albuginea, which, in turn, hinders rupture of the follicles and leads to formation of cysts. Also in endometriosis, the albuginea may be thickened as a reaction to haemorrhage from ectopic endometrium, and the ovary contains many cysts of the follicular type, in addition to typical 'chocolate' cysts. Fibroids of the uterus are frequently associated with cystic changes and some enlargement of one or both ovaries, which may be distorted, oedematous and hyperaemic. The cysts are small and occasionally show some degree of luteinisation and haemorrhage. Whether the association of cystic ovaries with fibroids is coincidental or whether the ovarian changes are due to interference with ovarian circulation by the tumour mass, is difficult to resolve (Haines & Taylor, 1962).

c) Vesicular mole and chorionic epithelioma are characteristically accompanied by cystic ovaries. The cysts - theca lutein cysts - are histologically similar to follicles, but have a striking hyperplasia of the theca interna cells. The size of the cysts and the thickness of the lining varies from case to case and in different cysts in the same ovary. The cyst lining may be formed by a layer of granulosa cells, which rarely shows signs of luteinisation, but in some instances this is substituted by a zone of connective tissue. Numerous plump theca cells with eosinophilic cytoplasm are arranged in ribbons, clusters

or columns in the cyst wall and they may form bands of yellow tissue in the ovarian stroma (Novak, 1952).

d) Ovaries containing numerous small cysts - from 2 to 15 mm in diameter - may be considerably larger than the normal size. Some authors reserve the term 'polycystic' ovaries for this type of cystic ovary. The appearance is characteristic, due to the smooth, white surface of the glands and the presence of numerous cysts in the cortex.

Microscopically the cysts represent follicles at various stages of maturation and atresia. Atretic follicles are particularly abundant, while no follicles in the pre-ovulatory stage are observed. The cysts are lined by granulosa and theca cells. Sometimes there is marked hyperplasia and luteinisation of the theca interna. There may be also hyperplasia of the ovarian stroma, with scattered nests of large, rounded, lipoid - laden cells. This microscopic picture of theca and stroma cell hyperplasia has been designated as 'theca hyperplasia', 'thecomatosis' and 'hyperthecosis'. The tunica albuginea is thick and sclerotic in most cases and the stroma swollen and oedematous. Although corpora lutea are more commonly absent, they are encountered in some cases.

Ovaries with these macro - and microscopical features are present in patients with the Stein-Leventhal syndrome, but also in patients who are not example of the syndrome, e.g. metropathia haemorrhagica, metrorrhagia and virilism. In metropathia haemorrhagica, as an additional finding, one ovary may contain a large follicular cyst (Schroeder, 1954; Zondek, 1954).

In the few cases of virilism associated with polycystic ovaries reported in the literature, no difference in the histological appearance of the ovaries has been noted with respect to the classical 'polycystic' ovaries (Geist & Gaines, 1942; Morris & Scully, 1957). Finally, it is important to note that enlarged cystic ovaries have also been found in women with regular menstrual cycles. Accordingly, as already mentioned in connection with stromal fibrosis, the original opinion that the histological appearance of the ovaries in the Stein-Leventhal syndrome is characteristic and pathognomonic has been questioned (Roberts & Haines, 1960; Haines & Taylor, 1962; Goldsicher & Green, 1962).

d) - Artificially induced cysts

Formation of ovarian cysts has been observed after administration of gonadotropins. The cystic change is reversible and regression occurs shortly after treatment is discontinued.

The presence of germinal tissue is essential for the response of the ovaries to the administered gonadotropins (Gemsell, Dickfalusi & Tillinger, 1960). The administration of extracts with prevalent follicle stimulating effect provokes enlargement of the ovaries and formation of numerous large cysts; subsequent administration of human chorionic gonadotropin induces further enlargement and ovulation in a high proportion of cases (Gemsell et al., 1960; Buxton & Herrmann, 1961; Crooke, Butt, Palmer, Morris, Logan-Edward, Taylor & Short, 1963a). Macroscopically many haemorrhagic areas are observed on the surface of the ovaries with prominent cysts of varying size. The ovarian stroma is oedematous and congested.

Microscopically, the cyst lining shows active proliferation of both granulosa and theca layer, with numerous mitotic figures (Geist, Gaines & Salmon, 1941). Luteinisation is present to a variable degree in the cells of the theca interna and is observed in the granulosa cells when administration of FSH is followed by HCG. Two or more corpora lutea at different stages of development are often seen after combined treatment with FSH and luteinising hormone (Gemzell et al., 1960).

The ovaries can undergo similar macro- and microscopic changes after administration of some non-steroidal synthetic compounds, analogues of chlorothianisene (Clomiphene citrate and derivatives). These compounds appear to interfere with oestrogen metabolism, and through this would mediate release of pituitary gonadotropins (Charles, 1962; Smith, Smith & Kistner, 1963; Mahesh & Greenblatt, 1964). Some authors have reported that these drugs can induce ovulation in amenorrhoeic women and in women with functional uterine bleeding (Roy, Greenblatt, Mahesh & Jungok, 1963; Puebla & Greenblatt, 1964).

5 - Origin of liquor folliculi and cyst fluid.

Liquor folliculi and cyst fluid are probably formed in the ovary by a similar process, but it is not clear why, in the case of cysts, fluid is produced in excess. An understanding of the mechanism of formation of fluid in follicles should help to discover the causes of pathological fluid accumulation.

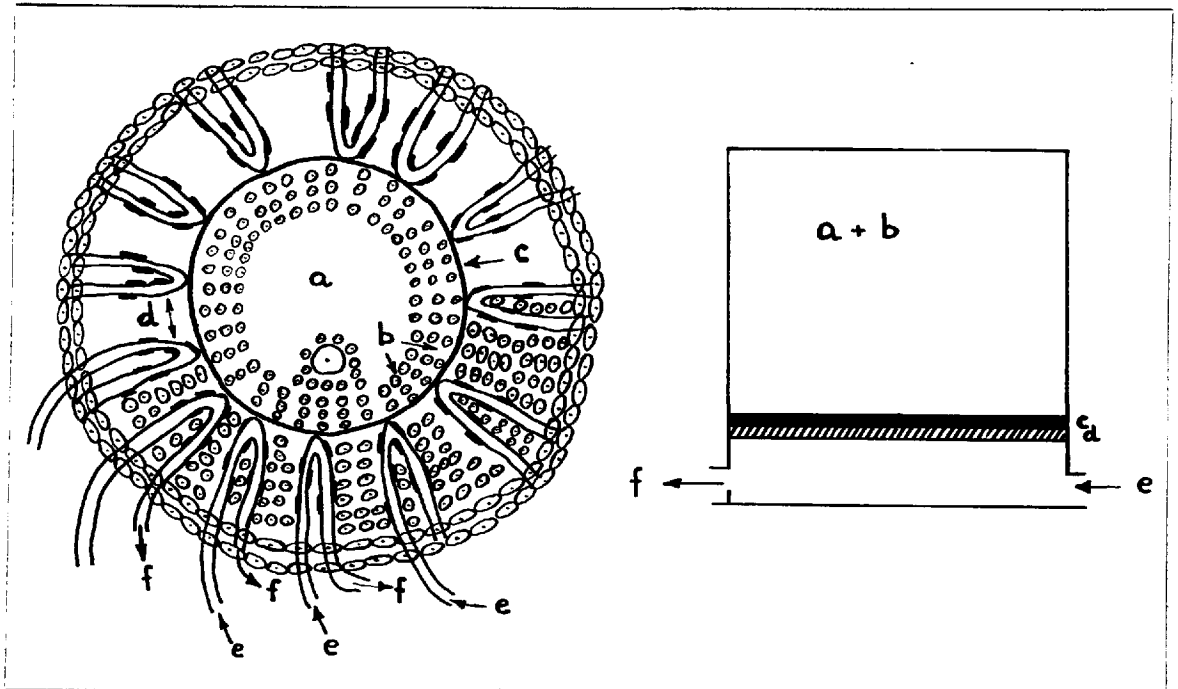
Earlier authors maintained that liquor folliculi is primarily a secretory product of the follicle lining. The hypothesis that it

originates from theca cells was based upon the concept that these were the hormone-secreting cells; on the other hand, since fluid does not accumulate after destruction of the granulosa cells by X-ray irradiation, it was suggested that it is secreted by the granulosa cells (Selye, 1947).

Later, it was recognised that filtration from the capillaries of the theca interna could be important. It has been noted that in most species the liquor folliculi appears at a time when the capillary net in the theca interna is developing. In this net, some large capillaries of a 'filtration' system can be distinguished from smaller capillaries of a 'nutritional' system (V. Thielen, 1953). Interposed between the 'filtration' capillaries and the fluid there is the basement membrane, which divides the theca from the granulosa and also the avascular layer of the granulosa. Intercellular spaces in the latter are in continuity with the follicular cavity.

Biological structures can be represented as physical models, although the fact that the tissues may play an active rôle - in this particular instance the transport of fluid and solutes - cannot be excluded. According to the description given above, the follicle can be regarded as a two compartment system - the fluid in the cavity and the blood in the capillary vessels of the theca - separated by a semi-permeable membrane, the basement membrane. (Fig. 2) The exchange of water and water soluble compounds between two compartments separated by a membrane is regulated, as is well known, by a complex of factors : permeability of the membrane and hydrostatic , osmotic and

Fig.2- The follicle as a two-compartment system.



a)Follicle cavity.-b)Intercellular spaces of granulosa layer.-
c)Membrana propria.- d)Capillary endothelium.- e)Tertiary arteriolar
branches.- f)Venous end of capillaries.

colloidosmotic pressures in the two compartments.

The permeability of the blood-follicle barrier to water and small molecules from the capillaries has been demonstrated in the human ovary. After intravenous infusion the concentration of tritiated water and ^{131}I , reaches equilibrium between blood and follicular fluid in a very short time. Since, in these experiments, no increase in the volume of the follicles was observed, the mechanism of passage must have been one of simple diffusion, without net transfer of fluid (V. Kaulla, Aikawa & Pettigrow, 1958; Peckham & Kiekhofers, 1959). The basement membrane is also permeable to proteins and ^{131}I -labelled globulin is found in follicular fluid after systemic administration, although the equilibrium is attained after a longer interval than with free iodine (V. Kaulla et al., 1958). In fact, in experiments with intact and hypothyroid rats pretreated with gonadotropins to induce ovarian cysts, it has been shown that the rate of diffusion of individual proteins into the follicular cavity depends upon the molecular weight (Mancini, Vilar, Heinrich, Davidson & Alvarez, 1963; Yatvin & Leatham, 1964).

Labelled proteins are found in higher concentration in the fluid than in blood for a considerable time following intravenous injection. (V. Kaulla et al., 1958; Mancini et al., 1963; Yatvin & Leatham, 1964). This phenomenon of "retention" in follicular fluid has been noted also for ^{131}I , some drugs and for steroid hormones, as will be discussed later. It has been suggested that proteins cannot be released back into the blood and that they remain in the follicle until they are completely metabolised (Mancini et al., 1963). Another explanation,

based on the geometry of the follicle, has been proposed (Yatvin & Leatham, 1964). The follicle is spherical and when fluid accumulates in a sphere, the volume increases proportionally more than the surface, so that the area of exchange between fluid and capillaries is reduced. This might retard the passage of proteins from the follicle into the blood (Yatvin & Leatham, 1964). On the other hand, it is probable that proteins can cross the blood-follicle barrier in both directions. The total protein content of follicular fluid from human ovaries is similar to that of blood, and only slight differences in the albumin and globulin percentage have been reported (Perloff, Schultz, Farris & Balin, 1955; Caravaglios & Cilotti, 1957; Zachariae and Jensen, 1958).

^tElectrolyte concentration in liquor folliculi from human ovaries has not been ^othoroughly studied. In the cow, ovarian venous blood and fluid are in equilibrium with respect to Na, Ca, K and Cl (Lutwak - Mann, 1954; Olds & van Denmark, 1957); therefore the gradient in osmotic and colloid osmotic pressure between the two compartments is very small (Zachariae & Jensen, 1958). At the stage of pre-ovulatory swelling, the colloid osmotic pressure suddenly increases in the liquor folliculi because of depolymerization of mucopolysaccharides. This would provoke accumulation of fluid in the follicle and lead to its rupture (Zachariae & Jensen, 1958), but, as already indicated, other factors may be involved. Direct measurement in vivo in the rat has shown that intrafollicular pressure is not significantly higher than capillary pressure in pre-ovulatory follicles (Blandau & Rumery, 1963). However, even a small increase in intrafollicular pressure would cause rupture of the follicle

owing to the reduced distensibility of the wall of the pre-ovulatory follicle (Rendell, 1964). It would appear therefore that hydrostatic, osmotic and colloidosmotic pressure play only a minor rôle in the formation of fluid in follicles.

The situation could be very different in large cysts of the human ovary. In fluid drawn from these cysts, the electrolyte concentration and the total protein content range within wide limits, but are generally lower than in blood, so that the fluid is hypotonic with respect to blood (Watts & Adair, 1944):

The concentration in the fluid of ^{131}I administered systemically is also lower than in blood, so that the 'retention' phenomenon is no longer apparent (v. Kaulla, Aikawa, Bruns & Wilke, 1957). Under these circumstances, accumulation of fluid cannot be due to simple diffusion and another mechanism of passage may be considered. In this connection it should be noted that any sudden change in blood pressure in the ovarian arterioles is prevented by their spiral configuration. The pressure of a fluid is reduced when this flows in a curved system. Should the ovarian arteries become less convoluted, the blood pressure could rise and the amount of water filtered through the capillaries into the tissues could increase. Follicles, and corpora lutea, are the only structures in the compact ovarian stroma which can accommodate excess fluid and any increase in blood pressure would therefore result in accumulation of fluid in them.

The hypothesis that vascular alterations may be the origin of accumulation of fluid in cysts has been supported by the finding of

distortion of the spiral arteries supplying some cysts (Delson, 1949). Furthermore, cysts have been obtained experimentally in rabbits by clamping the efferent ovarian vein, a manoeuvre which affects the blood flow in the arteries (Burr & Davies, 1951).

The occurrence of steroids in ovarian fluid has been known for a long time, and their significance will be extensively discussed in later sections of this thesis. In the present context, it is important to emphasize that, in general, the fluid contains higher steroid concentration than the peripheral blood (see Table I). It would seem reasonable to assume that any substance which is present in cyst fluid in higher concentration than in peripheral venous blood is a secretory product of the cyst lining.

The high concentration of steroids in ovarian cyst fluid is to be attributed either to the fact that the filtrate, from which follicular fluid is derived, diffuses past steroid-producing cells (Short, 1961) or to active transport by the cells. Theca interna and granulosa cells appear to be actively involved in the transport of compounds which are metabolised by the cells of the two layers. Autoradiographic investigations of the ovary after injection of labelled compounds have confirmed earlier results obtained by vital staining. Thus, Na_2SO_4 and some amino-acids are at first concentrated in the theca interna; pass slowly into the granulosa and finally, after 10-48 hours, reach the fluid (Moricard & Gothie, 1956; Zachariae, 1957; Yatvin & Leatham, 1964). A similar process could be postulated for the steroid hormones and their precursors.

Table I - CONCENTRATION OF PROGESTERONE AND FREE OESTRADIOL-17 β IN PERIPHERAL BLOOD, OVARIAN FLUID AND OVARIAN VENOUS BLOOD OF WOMEN.

$\mu\text{g}/100 \text{ ml}$			
	Peripheral blood	Ovarian fluid	Ovarian venous blood
Progesterone			
(follicular phase)	0.00-0.53 (1)	95.0 (7)	
	0.05-0.20 (2)	(G. Follicle)	
	0.05-0.20 (3)	15.0-100.0 (7) (Foll.cysts)	
(luteal phase)	0.60-2.10 (1)		
	0.58-2.00 (2)		28.6-110.6 (9)
	0.50-2.00 (3)		
Oestradiol-17β			
(follicular phase)	0.00-0.20 (4)	32.5 (8) (pool)	0.4-5.6 (10)
	0.00-1.03 (5)	70.0 (7)	
	0.01 (6)	(G. Follicle)	
		22.0-156.0 (7) (Foll.cysts)	
(luteal phase)	0.01-0.05 (4)	10.4 (8) (pool)	
	0.00-0.34 (5)		
	0.03 (6)		

(1) Woolever, 1963.

(2) Sommerville & Collins, 1964.

(3) Riendel, Tait, Tait, Gut & Little, 1965.

(4) Ichii, Forachielli & Dorfman, 1963.

(5) Svendsen & Sørensen, 1964.

(6) Roy, Harkness & Kerr, 1965.

(7) Short & London, 1961.

(8) Smith, 1960.

(9) Mikhail, Zander & Allen, 1963.

(10) Mahesh & Greenblatt, 1964.

The cyst fluid does not seem to possess the enzymes necessary to metabolise steroids (Short, 1961; Tomaaki & Pincus, 1961), but if steroids are introduced into the follicular cavity, conversion to new compounds takes place (Short, 1964). Probably, the fluid carries the compounds to the cells where they are further transformed. Thus, it has been suggested that progesterone can be converted to oestrogens during the passage, from the fluid to the blood, through the granulosa layer of the follicle (Ryan, 1963a).

It is apparent that much remains to be learned about the mechanism by which steroids are ultimately released from cyst fluid into the blood stream. This subject will be considered in relation to the present work in the Discussion.

In summary, follicular fluid is formed by diffusion of water and solutes from the blood, and is in continuity with interstitial fluid. It has been considered in fact as a "sequestration" of lymph (Short, 1961). It contains specific secretory products of the cells lining the follicle or the cyst, and probably acts as a medium for the transport of steroids from one layer to the other, so that some metabolic transformations are made possible.

The causes of excessive fluid accumulation in cysts are not completely understood, but vascular factors might be responsible in some cases.

It is especially relevant to the present work to note that the volume of fluid depends mainly upon physical factors, while the amount of steroids present in the fluid is related to the secretory activity

of the cyst lining. In follicles and medium size cysts, accumulation of fluid is associated with cellular proliferation and fluid volume and metabolic activity probably vary in the same direction. In large cysts, where the increase in size is mainly due to accumulation of fluid rather than to cellular growth (Geist, Gaines & Salmon, 1941) there is unlikely to be a correlation between the amount of fluid and its steroid content.

Chapter II - OVARIAN FUNCTION IN RELATION TO THE MENSTRUAL CYCLE

The rôle of the ovary in puberty, implantation of the conceptus and pregnancy is outside the scope of the present work and the present Chapter is confined to the subject of ovarian function in relation to the menstrual cycle. It is divided in three sections; the regulatory mechanism of ovarian function; ovarian steroid production and secretion; functional and morphological changes depending upon ovarian secretion.

1 - The regulatory mechanism of ovarian function.

a) - Hypothalamic control of the hypophysis.

The neurohormonal concept (Green & Harris, 1947)°affirms that exteroceptive stimuli impinge upon the hypothalamus as a primary focus in the neurovascular reflex arc. Here, what may be termed gonadotropic impulses are integrated with local blood levels of circulating hormones. From the hypothalamic centres, impulses travel by nerve conduction to the median eminence and affect the liberation of neurohormones. The latter enter the primary plexus in the median eminence and are distributed to the cells of the anterior hypophysis through the hypophyseal portal system. Recently it has been possible to isolate and purify one of these neurohormones, the luteinising releasing factor-LRF- from the median eminence of beef hypothalamus (Schally & Bowers, 1964).

In women, a rôle of the hypothalamus in reproductive function is indicated by derangements of menstrual cycles, such as 'environmental amenorrhoea' or the menstrual irregularities which follow from emotional disturbances. In these cases it would appear that impulses from higher nervous centres act on the hypothalamus. In addition, hypothalamic lesions, such as occur in diabetes insipidus, the Hand-Schüller-Christian syndrome and tumours, or following resection of the pituitary stalk, can also lead to amenorrhoea (Bishop, 1962).

b) - Pituitary secretion and its effect on the ovary.

The gonadotropic principles of the mammalian pituitary have not yet been isolated in a pure form. However, it is known (Chester-Jones & Ball, 1962) that the anterior hypophysis of mammals secretes at least three gonadotropic principles: these have been named follicle stimulating hormone-FSH; luteinising hormone-LH or interstitial cell stimulating hormone-ICSH, and lactogenic hormone which has luteotropic properties as well and has also been termed luteotropin-LTH.

The presence of FSH and LH activity in human pituitaries has been established (Bahn, Lorenz, Bennet & Albert, 1953), but it has not been possible to achieve complete separation of the two gonadotropic principles.

In animals, it would appear (Greep, 1961; Chester-Jones & Ball, 1962) that FSH is not active on primordial or small vesicular follicles, which grow to the antrum stage independently of pituitary stimulation. At the antrum stage the follicles become 'competent' to respond to pituitary stimulation. The processes involved in the more advanced

development of follicles, i.e. the formation of liquor folliculi, the mitotic proliferation of the granulosa cells and the differentiation of the theca cells, are considered to be dependent on FSH. The luteinising hormone alone causes stimulation of interstitial cells and enlargement of theca cells without follicular stimulation. A combination of FSH and LH can cause a twofold increase in ovarian weight and maturation of follicles associated with stimulation of thecal and interstitial cells and secretion of oestrogens. Even minute quantities of LH can act synergistically with FSH to produce secretion of oestrogens without obvious histological changes in the interstitial cells. Ovulation, transformation of the ruptured follicle into a corpus luteum and the production of progesterone can be attributed to the action of LH (Greep, 1961). It has not been proved unequivocally that the luteotropic hormone is essential for the maintenance of luteal function, except in the rat (Rothchild, 1960).

In women, considerable information concerning pituitary function has been derived from assays of urinary gonadotropin excretion and from studies involving the administration of pituitary extracts.

Urinary excretion of gonadotropins.

Many biological tests have been proposed for the detection and quantitation of gonadotropins in urine. The most generally used 'end points' are the increase in weight of the uterus of the immature mouse (Klinefelter, Albright & Griswold, 1943) and of the ventral prostate of the hypophysectomized rat (Greep, VanDyke & Cow, 1942). The mouse uterus test measures the 'total' gonadotropic activity, while the

rat ventral prostate test is more specific for LH. Recently many attempts have been made to develop an immunological assay for gonadotropins (Wide, Roos & Gemzell, 1961; Wide, 1964).

The bioassay of urinary gonadotropins during the normal menstrual cycle has failed to show a sharp increase of excretion at the time of ovulation (Buchholz, 1956; Borth, Lunenfeld & DeWatteville, 1957; McArthur, Ingersoll & Worcester, 1958; Albert, 1959). In other studies, higher values of excretion of LH or both gonadotropins have been reported, sometimes in coincidence and sometimes just after the increase in oestrogen excretion characteristic of ovulation (Ingersoll & McArthur, 1950; Brown, Klopfer & Loraine, 1958; Loraine & Bell, 1963; Fukushima, Stevens, Gantt & Vorys, 1964). A marked increase in LH excretion immediately preceding ovulation has been demonstrated by immunological assay (Wide, 1964). The ratio of potency of the two gonadotropins approximates to unity at all stages of the menstrual cycle. This fact may indicate that the two gonadotropins are excreted in the same proportions or that there is a single gonadotropin with two activities (Albert, 1959).

Little information is available regarding gonadotropin excretion in patients with menstrual abnormalities. Although some studies were performed in the past, the methods of extraction and bioassay available at that time did not afford a quantitative determination of the amounts of gonadotropins excreted. However, gonadotropin assays in cases of amenorrhoea provide information which is of value in differentiating amenorrhoea resulting from pituitary failure from amenorrhoea due to

ovarian deficiency (Klinefelter et al. 1943; Bishop, 1962). In pituitary amenorrhoea the excretion of gonadotropins is generally very low and often no activity can be detected even by the most sensitive methods; on the other hand ovarian amenorrhoea is usually associated with a greatly increased output of urinary gonadotropins (Klinefelter et al. 1943; Bishop, 1962).

In an extensive study of gonadotropin excretion in 222 patients with various types of menstrual anomaly other than amenorrhoea, abnormal values were reported in 34% of patients with hypo- or oligo menorrhoea and in only 19% of patients with irregular bleeding (Pedersen - Bjergaard & Tønnessen, 1951). In a few cases of the Stein-Leventhal syndrome, serial determinations of LH and FSH excretion have shown marked fluctuations and no definite trend (Ingersoll & McDermott, 1950; Evans & Riley, 1960), although it has been claimed that the composite excretion value for LH for the period observed was higher than that of normal patients (Ingersoll & McArthur, 1959; Taymor & Barnard, 1962). This finding could not be confirmed by immunological assay (Genzell, 1964).

Administration of pituitary extracts.

The administration of extracts obtained from the pituitary of various animals gave variable results in the hands of earlier authors. This might be due to species difference or to the fact that the extracts almost certainly contained more than one gonadotropin, so that the effects observed could not be considered specific for any individual pituitary hormone.

A series of studies employing pituitary extracts has recently been reported. Although considerable work has been performed upon the isolation of pituitary gonadotropins, it is doubtful if any preparations are entirely free from contamination by other pituitary factors.

Human pituitary follicle stimulating hormone - HP-FSH - has been generally used in these studies, in association with human chorionic gonadotropin - HCG -, which, although chemically dissimilar to LH, has many of its biological properties (Gemzell et al., 1960; Burton & Herrmann, 1961; Crooke et al., 1963a; Crooke, Butt, Palmer, Morris, Logan-Edward & Anson, 1963b; Burton, Kase & vanOrden, 1963; Diczfalusy, Johannisson, Tillinger & Bettendorf, 1964).

It is interesting to note that in normally menstruating women, ovine pituitary extracts with a very high FSH activity induce an increase in oestrogen excretion in women in the luteal phase of the cycle as well as in those who start treatment in the follicular phase (Maner, Saffen & Preedy, 1962). No alteration of the menstrual rhythm is evident after treatment in most cases.

In amenorrhoeic women, the administration of HP-FSH does not elicit any response unless urinary gonadotropin excretion is within normal limits and some germinal tissue capable of endogenous production of oestrogens is present in the ovaries (Gemzell et al., 1960). The ovarian response consists of bilateral enlargement, formation of multiple cysts and increased production of steroids. The urinary excretion of oestrogens is definitely increased, whereas pregnanediol

excretion is only slightly elevated in the majority of cases. The administration of HCG after FSH enhances the ovarian response and appears necessary to induce ovulation. Pregnenediol excretion becomes high in all cases and oestrogen excretion may rise further. All these effects regress after discontinuance of treatment. Menstrual bleeding sometimes occurs within a few days of the last HCG injection (Gemzell et al., 1960; Buxton & Hermann, 1961; Crooke et al., 1963b; Buxton et al., 1963; Dicsfalussy et al., 1964).

Similar results have been obtained in patients with the Stein-Leventhal syndrome, although all the patients do not respond in the same manner. Ovarian enlargement, ovulation and menstrual bleeding is observed in only a few cases (Mahesh & Greenblatt, 1964; Crooke et al., 1963a.). It would appear that in patients with the syndrome treatment with FSH alone can induce ovulation (Gemzell et al., 1960). The gonadotropin administration does not seem to stimulate ovarian steroid production to the extent noted in normal women. Increased excretion of 17-ketosteroids and Δ^5 - pregnenetriol has been reported as a characteristic response to HP-FSH treatment in patients with the Stein-Leventhal syndrome (Gemzell et al., 1960; Mahesh & Greenblatt, 1961).

c) - The 'feed-back' effect of the ovary on the hypophysis.

The body of evidence derived from animal experiments on the effects of steroids on pituitary function has been reviewed recently (Greep, 1961; Chester - Jones & Ball, 1962; Everett, 1964), and clinical trials with steroid preparations in women have been critically examined (Kotz & Hermann, 1961a,b). In animals it has been established that

the ovarian hormones all exert, in variable degree, an action on the function of the hypophysis. This action has been termed a 'feed-back' effect. The steroids seem to influence either the secretion or the production of gonadotropins, and from recent studies it would appear that the hormonal action is exercised at the hypothalamic level, by interference with neurosecretion of releasing factors.

Among the known compounds having an inhibitory action on gonadotropin secretion, the oestrogens are the most powerful, if administered in sufficiently large amounts. On the other hand, discordant results have been obtained with dosages around the postulated physiological levels. In animals, the same low doses which would stimulate FSH secretion if administered for a short term, suppress FSH secretion and stimulate LH release if administered for a longer period. The action of oestrogens on the median eminence would result in inhibition of LH production by the Hypophysis, while a direct action on the hypophysis would induce release of LH (Kanematsu & Sawyer, 1964).

In women, oestrogens administered in high dosage inhibit the secretion of pituitary gonadotropins as suggested by a sharp decrease in urinary excretion (Loraine, 1958). On the other hand, there are indications that oestrogens can release LH from the pituitary also in women, and such a mechanism has been invoked to explain induction of ovulation after administration of oestrogens in a single injection (Kupfermann, 1963). However, the general experience has been that administration of oestrogens early in the cycle suppresses ovulation (Kots & Herrmann, 1961b).

With regard to the rôle of progesterone in the 'feed-back' effect on the hypophysis, in animals progesterone may either inhibit or hasten ovulation, depending upon the time of administration during the oestrous cycle. The action of progesterone may be related to the level of circulating oestrogens. Thus, progesterone could be responsible for induction of ovulation at mid-cycle, when oestrogen levels are high, probably by facilitating oestrogen action on the hypophysis (Everett, 1964). Progesterone secretion by follicles immediately prior to ovulation could be important in the triggering mechanism of ovulation. Once ovulation has taken place, the action of progesterone changes to inhibition of gonadotropin release (Rothchild, 1962).

The fall of the luteal activity in the absence of the conceptus may be related either to this negative 'feed-back' effect of progesterone on the hypophysis or to the production by the endometrium of a luteolytic substance (Rothchild, 1960; du Mesnil du Boisson, 1961). In species in which the corpus luteum secretes oestrogens as well as progesterone, the rôle of oestrogens in the termination of the oestrous cycle should also be considered.

Although there have been equivocal reports of progesterone - induced ovulation in women, the suppression of ovulation by administration of progesterone or progestational synthetic compounds is well established (Pincus, 1960).

The third group of steroids involved in the 'feed-back' mechanism is the androgens. Testosterone, the most potent androgen, is a weak inhibitor of pituitary secretion. In animals, prolonged treatment with testosterone

results in deprivation of pituitary LH, while the storage of FSH increases (Greep & Chester - Jones, 1950). As a consequence, testosterone provokes both cessation of the cycles and regression of corpora lutea in intact animals. Less potent androgens - Δ^4 - androstenedione and DHA - appear to exert an effect on the hypophysis, either by suppressing FSH secretion or by causing a shift in the FSH/LH ratio as to inhibit ovulation (Roy, Mahesh & Greenblatt, 1962).

In women, androgens are relatively weak inhibitors of pituitary gonadotropin secretion. Thus, testosterone, in a dosage sufficient to cause virilisation, fails to decrease urinary gonadotropin excretion (Loraine, 1958). Δ^4 - androstenedione and DHA are much less active than testosterone in this respect (Dorfman, 1963).

In conclusion, many theoretical possibilities of interaction between ovarian and pituitary hormones have been advanced in an attempt to explain the cyclic nature of ovarian function (Chester - Jones & Ball, 1962). A continuous production of oestrogens from developing follicles or from interstitial cells has been postulated as the stimulus responsible for the growth of primary and vesicular follicles to the 'competent' stage and for the release of FSH from the hypophysis. FSH action stimulates the growth and maturation of the follicles until the stage of pre-ovulatory swelling. The larger amounts of oestrogens produced at this point, and/or the secretion of progesterone by the pre-ovulatory follicle, release LH and provoke ovulation. The formation of the corpus luteum seems to be dependent on LH, but it is not known which factors are

responsible for the maintenance of the corpus luteum in women. Finally, the pituitary-ovarian equilibrium of the luteal phase is interrupted either by inhibition of LH progesterone and/or oestrogens or by the presence of a luteolytic substance in the endometrium.

2 - Ovarian steroid production and secretion.

a) - Ovarian steroidogenesis.

It need not be pointed out that any information about the biosynthesis of ovarian steroids is extremely relevant to the present work. While it is recognized that phenomena observed 'in vitro' depend upon the choice of experimental conditions, the demonstration that ovarian tissue has the potentiality of forming the individual steroids is useful for the interpretation of 'in vivo' observations. The numerous studies involving incubation 'in vitro' of ovarian tissue reported in the last few years have been extensively reviewed (Axelrod & Goldzieher, 1962a; Dorfman, 1963; Savard, Marsh & Rice, 1965). The following comments will be confined to results obtained by the incubation of human ovarian tissue.

Normal ovary.

The term 'normal' ovary in this section should be accepted with reservations and many authors omit details of the clinical state of the patients (although in general there was no evidence of ovarian dysfunction) and of the histological composition of the specimen incubated .

The ovary, in common with other steroid-producing glands, forms pregnenolone and progesterone from acetate via cholesterol (Sweat, Berliner,

Bryson; Nabors, Haskell & Holmstrom, 1960; Ryan & Smith, 1961a; Hammerstein Rice & Savard, 1964). Progesterone is subsequently transformed to oestrogens via androgens (Baggett, Engel, Savard & Dorfman, 1956; Solomon, Vandewiele & Lieberman; 1956; Lanthier & Sandor, 1960; Ryan & Smith, 1961b; Kase, Forchielli & Dorfman, 1961). (Fig.3). This conversion of progesterone to oestrogens is not specific to the ovary, but is also performed by the adrenal cortex and the testis. In the ovary, however, progesterone has a double rôle; it acts both as a biosynthetic intermediary and as a hormonal principle. It is one of the focal points in a variety of metabolic transformations. The suggested pathway via pregnenolone $\rightarrow$ 17 α - hydroxy - pregnenolone and DHA provides an alternate route which does not involve progesterone as an intermediary.

Attempts to express in quantitative terms the incorporation of radioactivity from a substrate into various products are open to criticism, but the approximate inter-relationships are summarized in Table II. There is an increasing yield of oestrogens with substrates closer to the end products in the biosynthetic scheme, which confirms the proposed sequence of reactions.

In addition to the steroids depicted in Fig. 3, other compounds have been isolated as incubation products. Derivatives of progesterone hydroxylated in the C20-position have been shown to be formed with acetate or progesterone as precursors, mainly in the presence of stromal tissue. These hydroxylated derivatives of progesterone are considered to be degradation products (Sweat et al., 1956; Warren & Salhanick, 1961a). 16 α - hydroxy - progesterone is also formed

Fig.3- Steroid biosynthesis in human ovaries.

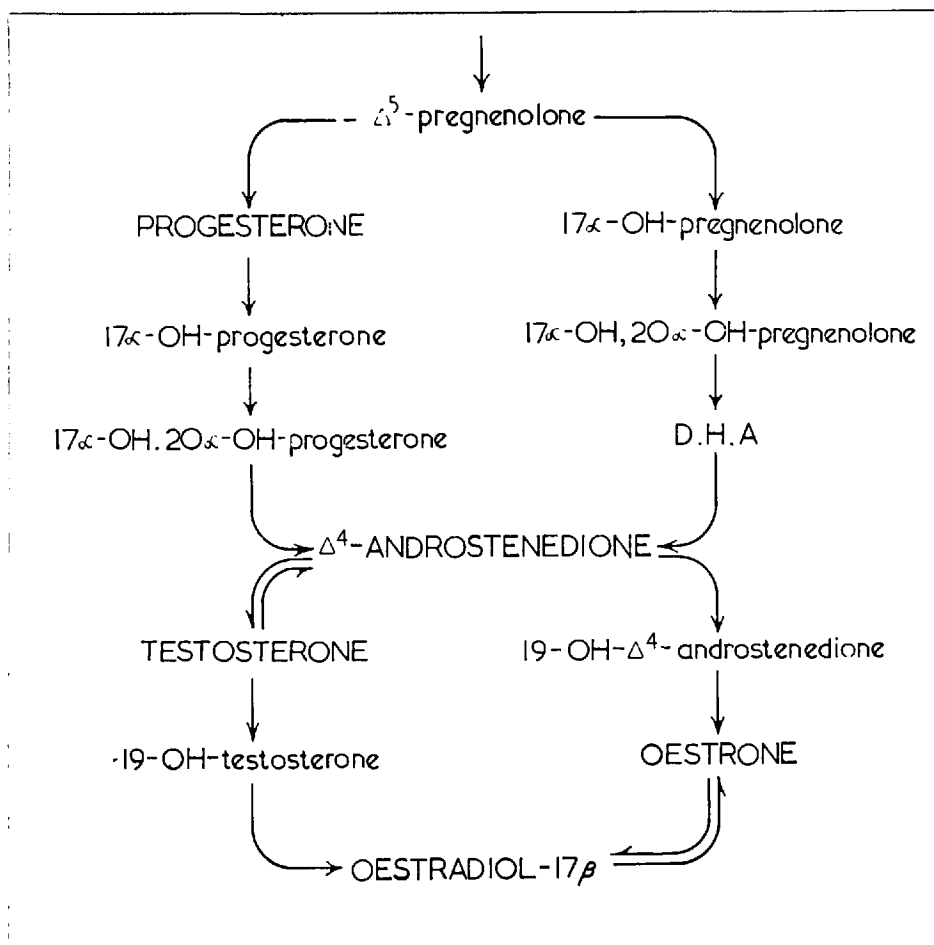


Table II - COMPARATIVE YIELDS OF METABOLITES AS RELATED TO SUBSTRATE
(from Smith & Ryan, 1961)

Radioactive metabolite	Substrate			
	Acetate-	Cholesterol	Progesterone	Δ^4 -androstened.
Cholesterol	0.34%			
Pregnenolone	0.0025%			
17 α -hydroxy-pregnenolone	0.0006%	★		
DHA	0.0046%	★		
Progesterone	0.0002%			
17 α -hydroxy-progesterone	0.0013%	★	14.8%	
Δ^4 -androstenedione	0.0047%		3.7%	
Testosterone	★		0.2%	2.9%
Oestrone	0.012%	0.09%	3.5%	10.9%
Oestradiol-17 β	0.016%		2.1	4.4%

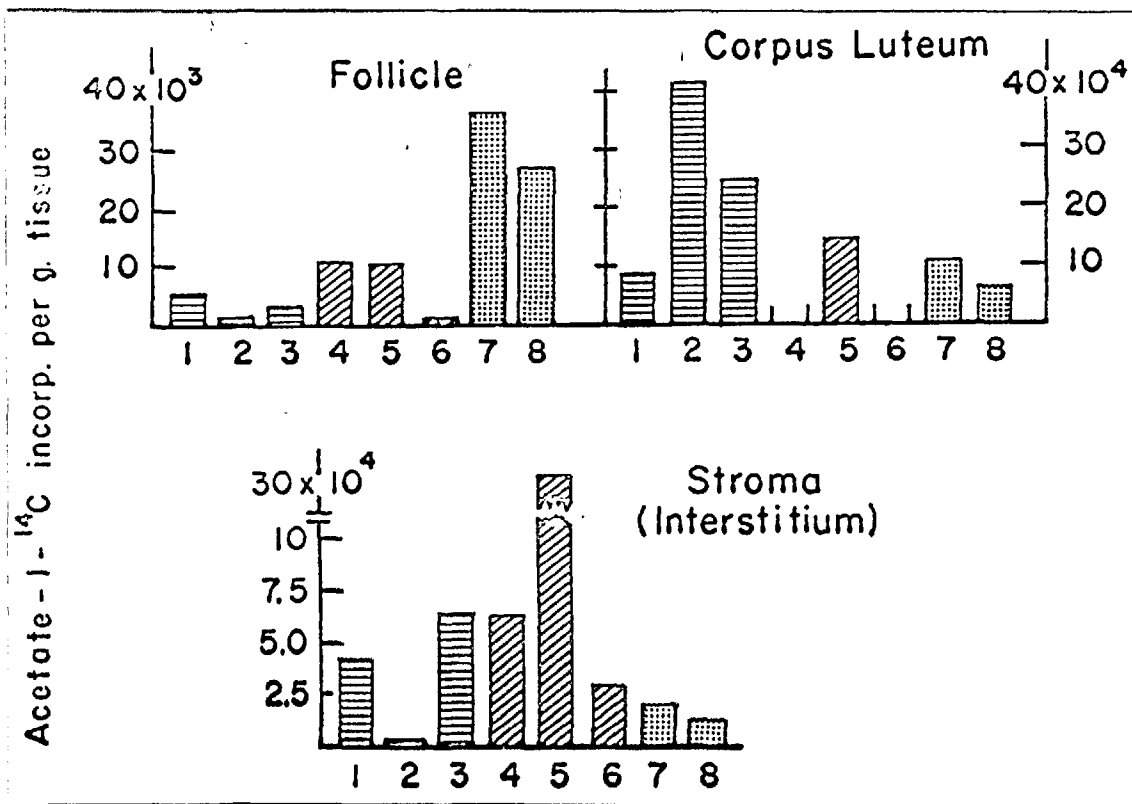
★ possibly a trace- not identified.

(Warren & Salhanick, 1961b) - a compound which can act as an oestrogen precursor in the human placenta (Little, Shaw & Purdy, 1963).

19 - hydroxy - or aldo - derivatives of androstenedione and testosterone proved to be efficient precursors of oestrogens in the ovary. The sequence has been suggested : Δ^4 -androstenedione $\rightarrow$ 19 -hydroxy-androstenedione $\rightarrow$ $\rightarrow$ 19 -oxo-androstenedione $\rightarrow$ oestrone, or, starting from testosterone, 19-hydroxy-testosterone $\rightarrow$ 19-oxo-testosterone $\rightarrow$ oestrone (Axelrod & Goldzieher, 1962b).

A few quantitative studies have been performed with dissected Graafian follicles, corpora lutea and ovarian stroma, in an attempt to correlate morphology with synthetic pattern. The results have varied according to the precursors used in the incubations. Thus, with acetate as precursor, a clear-cut difference is evident in the biosynthetic activity of follicles corpora lutea and ovarian stroma. Follicles form predominantly oestrogens - oestradiol and oestrone - (Smith & Ryan, 1961); the major synthetic product of corpora lutea is progesterone (Hammerstein et al., 1964), while stroma synthesises larger amounts of androgens - Δ^4 - androstenedione and DHA - than of progesterone and oestrogens (Savard et al., 1965). The pattern of steroids formed in these three structures of the ovary is illustrated in Fig.4. Using progesterone as precursor, the conversion to 17 α -hydroxy-progesterone and androstenedione is very high in luteinised tissue as compared with stromal or medullar tissue (Warren & Salhanick, 1961a). The yield of oestrogens from progesterone, androstenedione and testosterone is of the same order - between 0.1 and 4.2% - in follicles and corpora lutea, but the rate of conversion of progesterone to androstenedione in corpora lutea is lower than in follicles (Forleo &

Fig.4-Radioactive steroids formed from 1-¹⁴C-acetate 'in vitro' from follicles, corpora lutea and stroma of normal human ovary.



1=Δ⁵-pregnenolone; 2=progesterone; 3=17β-hydroxy-progesterone; 4=DHA; 5=Δ⁴-androstenedione; 6=testosterone; 7=oestradiol-17β; 8=oestrone.

Data on follicle in cpm (from Smith&Ryan, 1961); data on corpus luteum and stroma in dpm (from Savard, Marsh & Rice, 1965).

Collins, 1964).

Many hypotheses have been advanced to explain the change in steroidogenesis in the two phases of the cycle. Gonadotropins augment the steroidogenic activity of the ovary (Savard et al., 1965). The capacity of follicles to form steroids from acetate is increased under FSH stimulation (Smith & Ryan, 1961), while corpora lutea are stimulated only by homologous LH or HCG (Rice, Hammerstein & Savard, 1964). It has been suggested that gonadotropins could induce an increased conversion of sterol precursors to progesterone and this would result in progesterone accumulation for secretion and still allow a portion for further transformation to oestrogens (Ryan, 1963a). The choice of one or the other of the alternative pathways might be the important factor. The pathway pregnenolone $\rightarrow$ 17 α -hydroxy-pregnenolone $\rightarrow$ DHA $\rightarrow$ androstenedione could be operative in the follicular phase, with exclusion of the C19- Δ^4 -3-keto-steroids (Kase, 1964); oestrogens may also arise through 19-hydroxylation, successive demethylation and aromatisation of DHA sulphate (Goldberg, Seegar - Jones & Woodruff, 1963). In the luteal phase, on the contrary, synthesis of oestrogens through the Δ^5 -3 β -hydroxy-steroids appears to be less important (Ryan, 1963b; Hammerstein et al., 1964). However, it cannot be overemphasised that the results of 'in vitro' incubation experiments in human ovarian tissue must be interpreted with caution. Many factors require further investigation before we can speak of the relative importance of different pathways or individual transformations.

Polycystic ovaries.

A comparison of results obtained by means of experiments of incubation 'in vitro' of normal and polycystic ovarian tissue is given in Table III. The results agree on the point that in polycystic ovaries there is a more marked transformation of substrates to Δ^4 -androstenedione than in normal follicular tissue and a somewhat reduced formation of oestrogens. An accumulation of Δ^4 -androstenedione is noted when specimens from polycystic ovaries are incubated with pregnenolone, progesterone and 17 β -hydroxy-progesterone (Lanthier & Sandor, 1960; Warren & Salhanick, 1961a; Axelrod & Goldzieher, 1962a; Kase, Kowal & Soffer, 1963). The rate of conversion of progesterone and 17 β -hydroxy-progesterone to testosterone is also very much increased in polycystic ovaries, in contrast to normal ovaries (Axelrod & Goldzieher, 1962a; Kase et al., 1963). In other polycystic ovaries, accumulation of DHA instead of Δ^4 -androstenedione has been noted (Axelrod & Goldzieher, 1962a; Kase, 1964). A very interesting comparison between 'in vitro' and 'in vivo' hormone production has indicated that polycystic ovaries containing large amounts of androstenedione do not convert this compound to oestrogens; on the contrary, ovaries in which the endogenous content of DHA is high, can transform Δ^4 -androstenedione to oestrogens, but when they are incubated with DHA as substrate oestrogen synthesis is impaired (Mahesh & Greenblatt, 1964). Decreased ability to transform androgens to oestrogens in polycystic ovaries has been observed by some authors (Axelrod & Goldzieher, 1962a; Mahesh & Greenblatt, 1964) and not by others (Chapdelaine, Sandor & Lanthier, 1963; Kase, 1964). On the basis of such

Table III - STEROID BIOSYNTHESIS IN NORMAL AND POLYCYSTIC OVARIES,
(Data from Dorfman, 1963).

Metabolite	Substrate	Percentage conversion . substrate	
		Normal ovaries	Polycystic ovaries
Δ^4 -androstenedione	Progesterone	1.3-1.7 (1)	2.6-29.3 (1)
		1.1 (2)	0.8-58.6 (2)
Testosterone		0.1-0.2 (1)	0.4-2.1 (1)
		0.4 (2)	0.0-3.4 (2)
Δ^4 -androstenedione	17 α -hydroxy- progesterone	0.9-2.2 (1)	0.4-55.8 (1)
		0.1-0.3 (1)	0.4-2.7 (1)
Testosterone	Δ^4 -androstened.	0.3 (3)	1.1 (1)
19-oxygenated- C19 steroids	Δ^5 -pregnenolone	3.9 (2)	1.2-67.8 (2)
Oestradiol-17 β		4.8 (2)	0.0- 5.9 (2)

(1) Kase, Kowal & Soffer, 1963.

(2) Axelrod & Goldzieher, 1962a.

(3) Lanthier & Sandor., 1960.

results, the hypothesis of one or more biosynthetic defects in the Stein-Leventhal has been proposed.

However, it has been shown recently that stromal tissue synthesises Δ^4 -androstenedione as a major product, and there is a striking resemblance of steroidogenic pattern in stromal tissue and tissue from polycystic ovaries (Savard et al., 1965). The difference between steroid synthesis in normal and polycystic ovaries, which is quite obvious in Table III, could be explained by the fact that the normal ovaries had a different cellular composition from the of the polycystic ovaries with which they were compared.

b) - Steroids determined in the ovary.

Normal ovary.

Many steroids, in addition to those considered as the classical hormones, have been determined in human ovarian tissue or fluid. They are listed in Table IV. Most of the Δ^4 -3-keto-steroids intermediaries in ovarian steroid biosynthesis have been isolated from human ovaries, although testosterone, its 19-oxygenated derivatives and the oxygenated derivatives of Δ^4 -androstenedione have not as yet been detected. Of the alternative pathway through the Δ^5 -3 β -hydroxy-steroids, DHA has been found in normal ovaries (Simmer & Voss, 1960), while pregnenolone and 17 α -hydroxy-pregnenolone have been detected only in ovaries of women treated with FSH (Crooke et al., 1963a; Mahesh & Greenblatt, 1964).

Oestradiol and oestrone have been isolated from ovarian tissue and fluid. Oestriol has not been detected in most instances, apart from one

Table IV - STEROIDS DETERMINED IN NORMAL OVARIAN TISSUE OR FLUID OF WOMEN.

A. Neutral steroids.

Steroid	Source	Quantity	
Progesterone	Corpora lutea	15.5	µg/gm (1)
	" "	4.4-44.6	µg/gm (2)
	Graafian follicles	1.3-17.1	µg/gm (2)
	Follicular fluid	0.9	µg/ml (3)
20α-hydroxy-pregn-4-en-3-one + 20β-hydroxy-pregn.	Corpora lutea	2.4	µg/gm (1)
16α-hydroxy-prog.	Corpora lutea	0.3	µg/gm (4)
17α-hydroxy-prog.	Corpora lutea & follicles	1.7	µg/gm (1)
	Follicular fluid	0.8	µg/ml (3)
	Ovarian tissue	1.5- 5.0	µg/gm (5)
Δ ⁴ -androstenedione	Corpora lutea & follicles	0.6	µg/gm (1)
	Corpora lutea	0.3- 0.7	µg/gm (6)
	Ovarian tissue	0.8-15.0	µg/gm (5)
	Cortex & medulla	0.05-0.1	µg/gm (6)
	Hilus	0.04	µg/gm (6)
	Follicular fluid	0.78	µg/ml (3)
DHA	Corpora lutea	0.04	µg/gm (6)
	Cortex & medulla	0.01	µg/gm (6)

Table IV - Steroids determined in normal ovarian tissue or fluid of women.

B. Phenolic Steroids,

Steroid	Source	Quantity	
Oestradiol-17 β	Corpora lutea	0.07	$\mu\text{g/gm}$ (7)
	Graafian follicles	0.10-0.90	$\mu\text{g/gm}$ (7)
	Ovarian tissue	0.50-0.69	$\mu\text{g/gm}$ (8)
	Fluid(follicular phase)	0.32	$\mu\text{g/ml}$ (9)
	Fluid(luteal phase)	0.10	$\mu\text{g/ml}$ (9)
	Follicular fluid	0.70	$\mu\text{g/ml}$ (3)
	Follicular cysts	0.01-0.25	$\mu\text{g/ml}$ (10)
	id.+FSH + HCG	0.60-1.88	$\mu\text{g/ml}$ (11)
Oestrone	Corpora lutea	0.27	$\mu\text{g/gm}$ (7)
	Graafian follicles	1.98	$\mu\text{g/gm}$ (7)
	Ovarian tissue	0.26-0.94	$\mu\text{g/gm}$ (8)
	Fluid (follicular phase)	0.06	$\mu\text{g/ml}$ (9)
	Fluid (luteal phase) ,	0.14	$\mu\text{g/ml}$ (9)
	Follicular fluid	0.68	$\mu\text{g/ml}$ (3)
	Follicular cysts	0.01-1.10	$\mu\text{g/ml}$ (10)
	id. + FSH + HCG	0.07-0.24	$\mu\text{g/ml}$ (11)
Oestriol	Corpora lutea	0.02	$\mu\text{g/gm}$ (7)
	Graafian follicles	0.05	$\mu\text{g/gm}$ (7)
	Ovarian tissue	0.00-0.61	$\mu\text{g/gm}$ (8)
	Fluid (luteal phase)	0.24	$\mu\text{g/ml}$ (9)
Conjugated oestrogens	Corpora lutea	0.01	$\mu\text{g/gm}$ (7)
	Fluid (follicular phase)	0.02	$\mu\text{g/ml}$ (9)
	Fluid (luteal phase)	0.04	$\mu\text{g/ml}$ (9)
	Ovarian tissue	0.08	$\mu\text{g/gm}$ (12)

References to Table IV, page 50

- (1) Zander, 1958.
- (2) Zander, Forbes, v. Muenstermann & Neher, 1958.
- (3) Short & London, 1961.
- (4) Zander, 1962.
- (5) Mahesh & Greenblatt, 1964.
- (6) Simmer & Voss, 1960.
- (7) Zander, Brendle, v. Muenstermann, Diczfalusy, Martinsen & Tillinger, 1959.
- (8) Kéckés, Mutschler, Thàn & Farkas, 1962.
- (9) Smith, 1960.
- (10) Granjon, Yannotti & Cédard, 1958.
- (11) Smith & Ryan, 1962.
- (12) Schild, 1961.

study, in which this was the predominant oestrogen in cyst fluid obtained in the luteal phase. In this fluid, the presence of large amounts of oestrogenic substances other than the "classical" oestrogens has also been revealed by bioassay (Smith, 1960).

As far as conjugated steroids are concerned, conjugated oestrogens have been demonstrated in human ovary (Zander, Brendle, V.Muenstermann, Diczfalussy, Martinsen & Tillinger, 1959; Smith, 1960; Schild, 1961), but no attempt has been reported so far to measure conjugated neutral steroids- namely conjugated testosterone, pregnenolone, 17 α -hydroxy-pregnenolone and DHA.

It should be added that most of the results reported in Table IV have been obtained by means of methods of rather low specificity. Identification of the compounds has proved possible only in pooled samples of tissue or fluid, while in analyses of single samples there has been, at best, strong evidence of identity for the various compounds. Accordingly, the quantitation of the compounds cannot be considered satisfactory. With these reservations, it would appear from the data reported in Table IV that the concentrations of steroids, when expressed as $\mu\text{g/gm}$ of tissue or $\mu\text{g/ml}$ of fluid is of a similar order in tissue and fluid. In addition, progesterone, oestradiol and oestrone are quantitatively the most important steroids in the ovary. Oestrogens are found in larger amounts in follicles than in corpora lutea, while progesterone concentration is much higher in corpora lutea than in follicles.

Polycystic ovaries.

Quantitative or semi-quantitative results reported by various authors in the Stein-Leventhal syndrome are shown in Table V. The presence of a high concentration of Δ^4 -androstenedione in cyst fluid or in ovarian tissue of patients with this syndrome seems to confirm the existence of a biosynthetic defect, in agreement with some studies 'in vitro'. The accumulation of androstenedione is accentuated after gonadotropin stimulation. It would appear that there is at least one variant of the Stein-Leventhal syndrome, in which Δ^5 - β -hydroxy-steroids instead of androstenedione accumulate. Testosterone has never been detected in significant amounts.

Determination of total neutral 17-ketosteroids in ovarian tissue from 19 patients with the syndrome has shown a mean concentration of 4.5 $\mu\text{g/gm}$ of tissue (1.2 to 8.6 $\mu\text{g/gm}$), against a mean value of 4.0 $\mu\text{g/gm}$ (1.9 to 7.8 $\mu\text{g/gm}$) in 5 normal ovaries (Trace, Keaty & McCale, 1960). However, the biological significance of total 17-ketosteroids in the ovary is still not clear.

While the results of steroid determinations in ovarian tissue of patients with the Stein-Leventhal syndrome agree on the presence of abnormally high concentrations of androgens, they are at variance in regard to the oestrogen content. Oestrogens were not detectable in the cyst fluid of 3 out of 5 patients treated with FSH, and in almost all the untreated patients (Short & London, 1961; Crooke et al., 1963b). On the other hand, oestrone was present in pooled cyst fluid from polycystic ovaries and in specimens of ovarian tissue from 6 patients with the syndrome

Table V - STEROIDS ISOLATED IN POLYCYSTIC OVARIES.

Steroid	Source		Quantity
Progesterone	Cyst fluid	49-13	µg/100 ml (1)-(2)
	" "	179	µg/100 ml (3)
	id.+FSH+HCG	45-55	µg/100 ml (4)
17α-hydroxy-progesterone	Cyst fluid	23-29	µg/100 ml (1)-(2)
	id+FSH+HCG	12-124	µg/100 ml (4)
	Ovarian tissue	2	µg/gm (5)
	id+FSH+HCG+β-dexamethas	2	µg/gm (5)
17α-hydroxy-pregnenolone	Ovarian tissue	41	µg/gm (5)
	id+FSH+HCG+β-dexamethas	.4	µg/gm (5)
DHA	Cyst fluid	10	µg/100 ml (1)-(2)
	id+FSH+HCG	0.5	µg/100 ml (4)
	Ovarian tissue	41-64	µg/gm (5)
	Ovarian tissue	0.5	µg/gm (6)
	id+FSH+HCG+β-dexamethas	41-13	µg/gm (5)
Δ ⁴ -androstenedione	Cyst fluid	88-196	µg/100 ml (1)-(2)
	Cyst fluid	323	µg/100 ml (3)
	id+FSH+HCG	12-63	µg/100 ml (4)
	Ovarian tissue	41-12	µg/gm (5)
	Ovarian tissue	0.5	µg/gm (6)
	id+FSH+HCG+β-dexamethas	41-4	µg/gm (5)
Oestrone	Cyst fluid	42	µg/100 ml (1)
	Cyst fluid	79	µg/100 ml (3)
	id+FSH+HCG	42-34	µg/100 ml (4)
	Ovarian tissue	0-0.1	µg/gm (5)
	Ovarian tissue	0.7	µg/gm (7) *
Oestradiol-17β	Cyst fluid	42	µg/100 ml (1)-(2)
	id+FSH+HCG	41-2	µg/100 ml (4)
	Ovarian tissue	0-0.2	µg/gm (5)
	Ovarian tissue	1.6	µg/gm (7) *
Oestriol	Cyst fluid	42	µg/100 ml (1)
	id+FSH+HCG	40.3	µg/100 ml (4)
	Ovarian tissue	0.1	µg/gm (7) *

References to Table V of page 53.

- (1) Short & London, 1961
- (2) Short, 1962b.
- (3) Mahajan, Shah & Eik-Nes, 1963.
- (4) Crooke, Butt, Palmer, Morris, Logan Edward, Taylor & Short, 1963a.
- (5) Mahesh & Greenblatt, 1964.
- (6) Baulieu, Mauvais-Jarvis & Coperchot, 1963
- (7) Kéckés, Mutschler, Thàn & Farkas, 1962.

* Total oestrogens.

(Mahajan, Shah & Eik-Nes, 1963; Kèckès, Mutschler, Thàn & Farkas, 1962). Conjugated oestrogens were not detected in the pooled fluid, while they might have been present in the ovarian tissue, which had been subjected to acid hydrolysis prior to extraction. It is difficult to establish whether polycystic ovaries are really unable to produce oestrogens, or whether the different results are related to the degree of sensitivity or specificity of the methods employed.

No other gynaecological condition has been studied so thoroughly as the Stein-Leventhal syndrome by means of direct determination of steroids in ovarian tissue or fluid. A few biological assays of oestrogens in fluid from follicular cysts associated with irregular bleeding have been reported by earlier authors (Zondek, 1954; Perloff et al., 1955). Oestradiol and oestrone have been measured by chemical methods in follicular cysts (Granjon, Yannotti & Cédard, 1958).

c) - Steroids secreted by the ovary.

The presence of a steroid in ovarian tissue or fluid does not necessarily mean that it will be secreted into the effluent blood. There are many methods for the investigation of steroid secretion from the ovary, but the only direct one is determination of steroids in ovarian venous blood.

The compounds so far isolated from ovarian venous blood of normally menstruating women and of patients with the Stein-Leventhal syndrome are shown in Table VI. The determination ~~of~~ of progesterone in ovarian venous blood during the follicular phase has not yet been reported. Progesterone is present in very high concentrations in ovarian venous blood during the luteal phase but undetectable in anovulatory cycles.

Table VI - STEROIDS DETERMINED IN OVARIAN VENOUS BLOOD OF WOMEN.

Steroid	Phase of the cycle or clinical condition	Concentration µg/100 ml of plasma	
Progesterone	Pool from luteal phase	56.1	(1)
17α-hydroxy-progesterone	Pool from luteal phase	10.5	(1)
20α-hydroxy-pregn-4-en-3-one	Pool from luteal phase	2.5	(1)
Δ ⁴ -androstenedione	Pool from luteal phase	4.3	(1)
	Stein-Leventhal syndrome	13.1-20.9	(2)
DHA	Stein-Leventhal syndrome	0.0-15.4	(2)
Oestradiol-17β	Follicular phase	1.7-5.6(total)	(2)
	Follicular phase	0.0-3.6 "	(3)
	Luteal phase	0.3-0.6 "	(3)
Oestrone	Follicular phase	0.9-3.0 (total)	(3)
	Luteal phase	0.3-0.7 "	(3)
Oestriol	Follicular phase	0.0-10.4(total)	(3)
	Luteal phase	0.0-4.5 "	(3)

(1) Mikhail, Zander & Allen, 1963.

(2) Mahesh & Greenblatt, 1964.

(3) Varangot & Cédard, 1959.

20 α -hydroxy-pregn-4-en-3-one, 17 α -hydroxy-progesterone and Δ^4 -androstenedione appear to be secreted in the luteal phase.

Androstenedione and DHA have been detected in high concentration in the ovarian venous blood of patients with the Stein-Leventhal syndrome, mainly after FSH administration (Mahesh & Greenblatt, 1964). The concentration of total neutral 17-ketosteroids in ovarian venous blood from polycystic ovaries has been reported as 40 to 475 μ g/100ml (Seeman & Saracino, 1961). Considering the values reported by other authors for DHA and androstenedione, which are the only androgenic compounds found in human beings with a 17-keto structure apart from androsterone (Dorman & Shipley, 1956), it is probable that most of the 17-ketosteroids measured in blood were of a non-androgenic nature.

It would appear that oestradiol and oestrone are secreted by the ovary in either phase of the cycle and also in amenorrhoea. Estrogens secretion increases after FSH administration (Varangot & Cédard, 1959; Mahesh & Greenblatt, 1964).

It must be noted that the values reported in Table VI cannot give any indication upon the secretion rate of the individual steroids, since this depends on the blood flow through the ovary as well as on the amount of steroids secreted. Measurement of ovarian blood flow in the woman has not been attempted, and it appears to be a very difficult task indeed.

While information about the steroid concentrations in ovarian venous blood has been limited due to the difficulty in obtaining adequate samples, a considerable amount of work has been directed towards the

chemical determination of steroids in peripheral venous blood. The results are summarized in Table VII. It is interesting to note that as techniques improve, especially with regard to specificity progressively lower values are obtained. In the more recent methods, colorimetric and fluorimetric determination has been replaced by double isotope analysis and gas-liquid-chromatography.

Some steroids are present in blood partially in a conjugated form (Antoniades, Pennell, Slaunwhite & Sandberg, 1957; Migeon, Wall & Bertrand, 1959; O'Donnel & Preedy, 1961). The sulphates of DHA, androsterone and oestrone and the glucuronidates of oestrogens and testosterone have been identified (Twombly & Levitz, 1960; Purdy, Engle & Oncley, 1961; Treen, Nilsson, Wiquist & Diczfalusy, 1961; Touchstone, Greene, McElroy & Murawec, 1963; Dignam, Pion, Lamb & Simmer, 1964; Burger, Kent & Kellie, 1964).

Progesterone is present through the cycle and the concentration in the luteal phase is consistently higher than in the follicular phase. With regard to the considerable variations in oestrogens values obtained by different methods, it should be noted that hydrolysis of conjugated oestrogens was not employed in all instances. In addition, the determination of such low concentrations as appear to be present in a blood sample of reasonable volume constitutes a formidable problem which has not been fully resolved. A definite increase in oestradiol concentration is observed at mid-cycle (Svendsen & Sprensen, 1964). The peripheral blood level of testosterone, DHA and androstenedione in normal women is lower than in men, and probably the greatest contribution comes from

Table VII - STEROIDS DETERMINED IN PERIPHERAL VENOUS BLOOD OF WOMEN.
A. Neutral steroids.

Steroid	Phase of cycle or clinical condition	Concentration µg/100 ml of plasma	
Progesterone	Follicular phase	2.5-6.00	(1)
	" "	1.1-2.30	(2)
	" "	0.0-0.53	(3)
	" "	0.5-0.97	(4)
	" "	20.1-1.60	(5)
	" "	0.013	(6)
	" "	0.05-0.20	(7)
	Luteal phase	3.2-4.70	(1)
	" "	1.8-2.30	(2)
	" "	0.6-2.10	(3)
	" "	0.5-3.90	(4)
	" "	0.4-1.25	(5)
	" "	1.04	(6)
	" "	0.05-2.00	(7)
	" "	0.09-3.90	(8)
DHA	Whole cycle	3-116	(9)
	" "	84-127	(10)
	" "	25.51 (sulphate)	(11)
	Stein-Leventhal syndrome	50.90 (sulphate)	(12)
	Hirsutism	42.90 (sulphate)	(12)
Testosterone	Whole cycle	0.11	(11)
	" "	0.02-0.26	(13)
	" "	0.12	(14)
	" "	0.04	(15)
	" "	0.05-0.26	(16)
	" "	0.05	(17)
	Stein-Leventhal syndrome	0.45	(12)
	" " "	0.25-0.42	(13)
	" " "	0.08-0.62	(18)
	Hirsutism	0.11-0.32	(13)
	"	0.03-0.64	(15)
	"	0.08-0.12	(18)
	Virilism	0.30-0.74	(13)

Table VII - Steroids determined in peripheral venous blood of women.
B. Phenolic steroids.

Steroid	Phase of cycle or clinical condition	concentration µg/100 ml of plasma	
Oestradiol -17β	Follicular phase	0.0-0.20 (total)	(19)
	" "	0.01-0.05	(20)
	" "	0.0 -0.10	(21)
	" "	0.013 (total)	(22)
	Luteal phase	0.0-0.40 (total)	(19)
	" "	0.08-0.02	(20)
	" "	0.030	(21)
	" "	0.028 (total)	(22)
	Whole cycle	0.07 (total)	(23)
	" "	0.5-1.10 (total)	(24)
	" "	0.3-0.80 (total)	(25)
	Stein-leventhal syndrome	0.01-0.04	(20)
Oestrone	Follicular phase	0.7-0.30 (total)	(19)
	" "	0.008-0.10	(20)
	" "	0.0-0.10	(21)
	" "	0.020 (total)	(22)
	Luteal phase	0.7-3.00 (total)	(19)
	" "	0.03-0.10	(20)
	" "	0.01-0.03 (total)	(21)
	" "	0.07 (total)	(22)
	Whole cycle	0.06-0.20 (total)	(23)
	" "	0.6-1.70 (total)	(24)
	" "	0.5-1.50 (total)	(25)
	Stein-Leventhal syndrome	0.0-0.13	(20)

References ~~to~~ Table VII of page 58 ~~▲~~

- (1) Oertel, Weiss & Eik-Nes, 1959.
- (2) Short & Levett, 1962.
- (3) Woolever, 1963.
- (4) Collins & Sommerville, 1964.
- (5) vanderMolen, Runnebaum, Nishizawa, Kristensen, Kirschbaum, Wiest & Eik-Nes, 1965.
- (6) Riandel, Tait, Tait, Gut & Little, 1965.
- (7) Sommerville, Sheerin, Collins & Wyman, 1965.
- (8) Sommerville, Pickett, Collins & Denyer, 1963.
- (9) Saier, Campbell, Sticker & Grauer, 1959.
- (10) Mahesh & Greenblatt, 1962.
- (11) Lamb, Dignam, Pion & Simmer, 1964.
- (12) Dignam, Pion, Lamb & Simmer, 1964.
- (13) Forchielli, Sorcini, Nightingale, Burst, Dorfman, Perloff & Jacobson, 1962.
- (14) Hudson, Coghlan, Dulmanis, Wentour & Ekkel, 1963.
- (15) Riandel, Tait, Gut, Tait, Joachim & Little, 1963.
- (16) Burger, Kent & Kellie, 1964.
- (17) Lobotsky, Wyss, Segre & Lloyd, 1964.
- (18) Kirschner, Lipsett & Collins, 1965.
- (19) Varangot & Cédard, 1959.
- (20) Ichii, Forchielli & Dorfman, 1963.
- (21) Svendsen & Sørensen, 1964.
- (22) Roy, Harkness & Kerr, 1965.
- (23) Preedy & Aitken, 1957.
- (24) Ittrich, 1960.
- (25) Oertel, 1961.

the adrenals. In hirsute women or in women with polycystic ovaries, the reported values overlap with those of men. A decrease of plasma testosterone levels has been noted in hirsute women after ovariectomy and wedge resection (Forchielli, Sorcini, Nightingale, Burton, Dorfman, Perloff & Jacobson, 1962). This would indicate that at least a portion of testosterone is secreted by the ovaries. Also the plasma level of total 17-ketosteroids is increased in the Stein-Leventhal syndrome, and decreased after ovariectomy (Seeman & Saracino, 1961).

d) - Urinary excretion of metabolites of ovarian hormones.

Several steroid precursors may contribute to the urinary concentration of one steroid. Furthermore, chemical methods for the determination of urinary steroids are far from comprehensive. To take an example, urinary pregnanediol is only one of the reduction products of progesterone and the urinary excretion represents only a proportion of the hormone - between 10 and 20% (Sommerville & Marrian, 1950; Klopper & Michie, 1956). Other precursors of pregnanediol include pregnenolone, pregnenolone sulphate, desoxycorticosterone and 20 α -hydroxy-pregn-4-en-3-one. In addition, most available methods measure a mixture of the C5-stereoisomers of pregnane-3 α -20 α -diol. The same applies to oestrogen metabolites; the three 'classical' oestrogens-oestrone, oestradiol -17 β and oestriol -account for less than 20% of the administered oestrone and oestradiol (Brown, 1957). Seven other metabolites have been isolated from human urine, and two of these -16-epioestriol and 16 α -hydroxy-oestrone-are usually present in higher concentration than oestradiol and oestrone in the urine of normally menstruating women (Levitz, Spitzer & Twombly, 1956).

Accordingly, it will be appreciated that considerable assumptions are involved in attempts to interpret the results of the determination of any urinary metabolite in terms of endogenous secretion of a precursor.

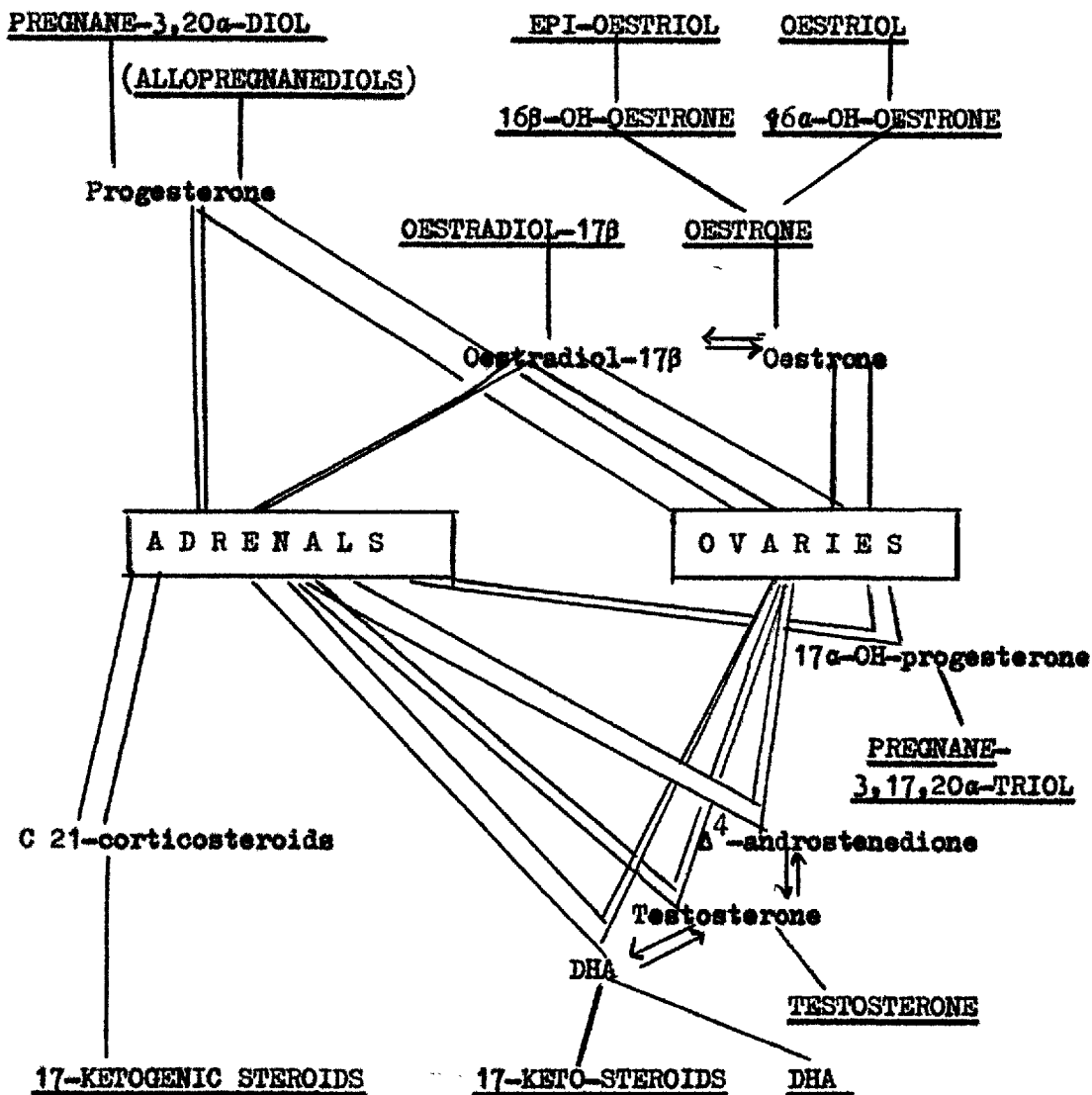
These problems have been the subject of intensive study and details of the relevant contributions to our knowledge of the degradation of ovarian hormones is outside the scope of the thesis. However, Fig.5 summarizes the relationships between ovarian hormones and urinary metabolites, with particular regard to steroids for which methods of assay are available and routinely used. With these reservations in mind, the following comments refer to the pattern of urinary steroid excretion in normal and abnormal cycles.

Normal cycle.

This has been investigated systematically with regard to urinary excretion of pregnanediol, pregnanetriol, 17-ketosteroids, 17-ketogenic steroids and oestrogens (Brown et al., 1958; Pickett & Sommerville, 1962).

The level of urinary pregnanediol tends to decrease in the first days of the cycle and reaches a steady excretion level at around 1 mg/24 hr; there is some evidence for a pre-ovulatory rise, but the main feature is a sustained rise in the luteal phase up to levels of 3-5 mg/24 hr. The onset of bleeding is preceded by a sharp fall in pregnanediol output. This pattern is interpreted as indicating a slight residual activity of the corpus luteum of the previous cycle at the beginning of the new cycle, followed by a low output which represents pregnanediol mainly derived from adrenal precursors; upon this is superimposed the contribution from the progesterone of the corpus luteum.

Fig.5-Urinary metabolites of steroid hormones.



underlined urinary metabolites.

The three 'classical' oestrogens exhibit two peaks of excretion, one at about the time of ovulation, the other during the luteal phase. The amounts of the three oestrogens excreted usually rise and fall together but oestriol lags behind during the ovulatory rise (Brown, 1955). Usually oestradiol is excreted in smaller quantities than oestrone (about one half), while there is no constant relationship between oestradiol or oestrone and oestriol. Immediately prior to menstruation the excretion of all three oestrogens falls, although the lowest levels are frequently reached several days after the onset of the bleeding. Table VIII shows the excretion values found in 10 normal menstrual cycles (Brown, 1955). The steady increase in oestrogen excretion prior to ovulation derives from the follicle, while the second rise is related to the activity of the corpus luteum.

The ratio of excretion pregnanediol/total oestrogens is similar in the follicular (1 mg/13 μ g) and in the luteal phase (5 mg/42 μ g), pregnanediol being excreted in quantities which are approximately 80 - 120 times greater than the oestrogens.

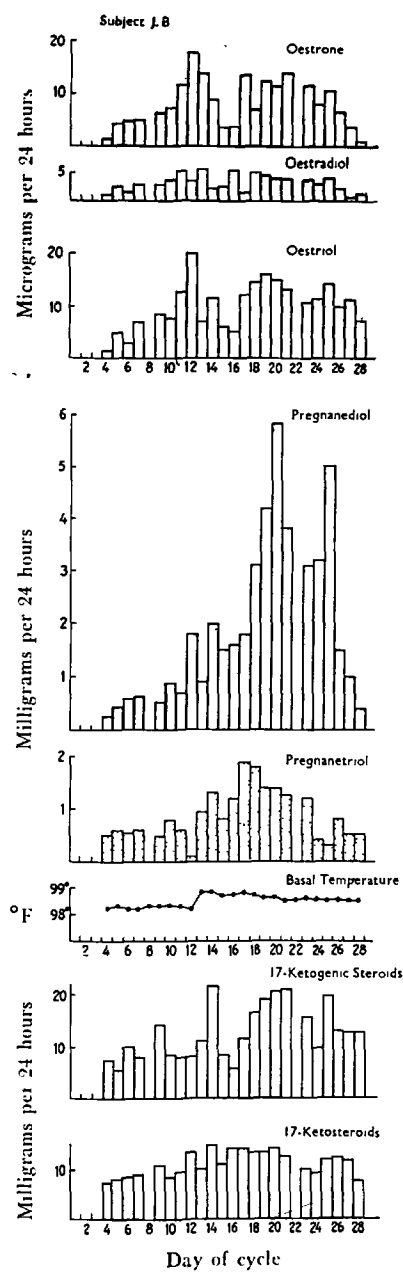
The excretion of pregnanetriol also undergoes cyclical variations with a mid-cycle peak (Pickett, Kyriakides, Stern & Sommerville, 1959; Pickett & Sommerville, 1962; Fotherby, 1962). 17-ketosteroids and 17-ketogenic steroids have not been shown to bear any constant relationship to the time of the cycle (Pickett & Sommerville, 1962). A typical pattern of urinary steroid excretion in the normal menstrual cycle is shown in Fig. 6.

Table VIII - EXCRETION OF OESTROGENS DURING THE NORMAL MENSTRUAL CYCLE.

(from Brown, 1955)

Phase of cycle	Average excretion $\mu\text{g}/24\text{hrs.}$			Range of excretion, $\mu\text{g}/24\text{hrs.}$		
	Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol
Onset of menstruation	6	5	2	0-15	4-7	0-3
Ovulation peak	27	20	9	13-54	11-31	4-14
Luteal maximum	22	14	7	8-72	10-23	4-10

Fig.6- Steroid excretion in the normal menstrual cycle.



Irregular Bleeding.

The pattern of oestrogen excretion in anovulatory cycles and in patients with functional haemorrhage is different from that observed during the normal menstrual cycle. There is no rhythmic fluctuation in oestrogen output and no ovulation peak can be demonstrated. In some cases with hyperplasia of the endometrium, oestrogen excretion is maintained at high levels -around 30 $\mu\text{g}/24$ hrs.- for many days. Bleeding occurs when oestrogen excretion levels are constant, rising or falling. Pregnanediol might be excreted in low or increased amounts, but no definite trend can be discerned and no change appears to precede the onset of the bleeding (Brown & Matthew, 1962; Fotherby & Brown, 1964).

Stein-Leventhal syndrome.

Critical reviews of all the reported studies have been published (Zander & Henning, 1961; Goldzieher & Axelrod, 1962).

Pregnanediol levels are low, comparable to those found in normal women during the proliferative phase of the cycle (Evans & Riley, 1960; Lanthier & Sandor, 1964). Also the urinary oestrogen excretion values are within the normal range for the follicular phase, but the characteristic fluctuations of the normal cycle are absent (Evans & Riley, 1960; Jayle, Scholler, Mauvais-Jarvis & Metay, 1961). 17-ketosteroids have been found elevated in 23% of a total of 358 patients collected from the literature. Fractionation of urinary 17-ketosteroids has revealed an increased excretion of androsterone and aetiocholanolone, in slightly more than half of 62 cases, and of DHA in 10 out of 60 patients. Pregnanetriolone, whose precursor is still unknown, has been found in

the urine of patients with the syndrome, and a tendency for high values of excretion of Δ^5 -pregnenetriol - arising from Δ^5 -17~~α~~-hydroxy-pregnenolone- has been reported (Cox & Shearman, 1961; Stern & Barwell, 1963). Suppression tests of adrenal secretion gave a significant response in only 50% of the cases examined and, in general, normal excretion values for 17-hydroxy-corticoids have been reported (Goldzieher & Axelrod, 1962). These findings indicate that in some cases of Stein-Leventhal syndrome there is adrenocortical dysfunction; but, since cortisone metabolites are excreted in normal or only slightly increased amounts, the adrenal secretion of glucocorticoids appears to be normal.

It would appear possible to differentiate cases in which adrenal dysfunction is the aetiological factor from cases in which ovarian production of androgens is increased. In the latter, adrenal suppression with dexamethasone does not decrease significantly the urinary excretion of 17-ketosteroids, pregnanetriol or Δ^5 -pregnenetriol, while FSH administration leads to higher levels of excretion (Mahesh & Greenblatt, 1961; Jayle et al., 1961).

e) - Production rates of progesterone,
oestrogens and androgens.

Tentative estimates of production rates of progesterone and oestrogens from the data of urinary metabolite excretion and the observed conversion of administered precursor to metabolites, indicates that the daily secretion of progesterone during the luteal phase is in the range 20-30 mg and that the daily secretion of oestradiol is 0.1 to 0.4 mg according to the phase of the cycle (Zander & Henning, 1961).

In the last few years, the rate of secretion of ovarian steroids has been studied by means of determination of the specific activity of urinary ~~metabolites~~ metabolites after administration of isotopically labelled compounds. The secretion rate of progesterone has been calculated as 2.5-5.4 mg/day in the pre-ovulatory phase, rising to 22-43 mg/day during the post-ovulatory phase (Dominguez, François, Watanabe & Solomon, 1962). Oestradiol secretion rate in normal women at the time of ovulation ranges between 0.2 and 0.5 mg /day (Gurpide, Angers, Vandewiele & Lieberman, 1962). By this method of calculating production rates, healthy women are estimated to produce 1.2 mg of DHA and up to 1.6 mg of testosterone plus androstenedione per day. In hirsute women and in women with polycystic ovaries under adrenal suppression, the secretory rate of DHA is increased to 16 mg/day and that of testosterone plus androstenedione to 5.6 mg (McDonald, Vandewiele & Lieberman, 1963). On the other hand, production of oestrogens seems reduced, since secretion rates of 0.16 and 0.29 mg per day have been obtained (Mahesh & Greenblatt, 1964).

It is interesting to note that the results obtained by these more sophisticated methods agree with the rough estimates made on the basis of the daily urinary excretion of oestrogens and pregnanediol.

More recently, secretion rates of certain steroids - for example testosterone - have been calculated in terms of the metabolic clearance rate of intravenously administered hormone and the mean plasma concentration. This obviates errors which arise in the calculations based on urinary studies - where the product may not be a unique metabolite.

By this type of method the testosterone production rate is found to be 0.34 mg/day in healthy women and of this 0.2 mg is derived from Δ^4 - androstenedione (Horton & Tait, 1966).

Summarizing the evidence which has been described in this section - 'ovarian steroid production and secretion' - the following conclusions may be reached: 1) From 'in vitro' studies it appears that both follicles and corpora lutea have the capacity to form progesterone, oestrogens and other intermediaries in oestrogen synthesis, but that there is a difference in biosynthetic pattern between follicles and corpora lutea. 'In vivo' determinations have proved that these steroids are indeed formed, and that follicles produce more oestrogens, while ~~the~~ corpora lutea produce more progesterone than oestrogens. The production of hormones by the ovary is therefore cyclic in nature.

2) From methods of investigation which indicate the actual output of steroids from the ovaries - determination in ovarian venous blood or in peripheral blood, measurement of the metabolites in urine and secretion rate studies - it would also appear that the steroids are released in a cyclic fashion, but the information at present available is not adequate for a definite conclusion to be reached on whether there is a free interchange of all the steroids formed between the ovary and the effluent blood.

3) - Functional and morphological changes dependent upon ovarian hormone secretion.

In this final section, reference will be made to some of the functional

and morphological changes induced in the target organs of women by ovarian secretion in the normal cycle and in a few abnormal conditions. It has been possible to establish definitely that these changes--duration and features of the cycle, variation in the endometrium and vaginal epithelium-- depend upon the action of ovarian hormones, since they can be reproduced in ovariectomized or post-menopausal women by cyclic administration of oestrogens and progesterone (Peel, 1955; Rubin & Novak, 1956; Buxton & Harrmann, 1961a; Eekstein, 1962). Information upon the effect of other hormones - for example the androgens- has been obtained by this type of experiment (Dorfman, 1963). These studies have emphasised that the responsivity of the target organs conditions the hormonal effect and that there is great variability in the minimal effective doses in different individuals.

a) - The normal menstrual cycle.

The menstrual cycle is classically divided into two phases. The follicular phase starts the first day of the menstrual flow and terminates, after 12-14 days, with ovulation; during this phase, as mentioned in the previous section, oestrogens are secreted by the maturing follicle in increasing amounts. The luteal phase begins with the day of ovulation and terminates with the onset of the bleeding; usually it lasts 12-16 days. Progesterone and oestrogens are secreted in increasing amounts from the corpus luteum during this phase until a few days before the onset of the bleeding.

The length of the cycle, that is the interval between the appearance of successive menstruations, is therefore considered normal when it falls

between 26 and 28 days. The bleeding should last 3-4 days, be moderate and not painful. A characteristic of the ovulatory cycle is a biphasic curve of basal body temperature, due to the thermogenic effect of progesterone produced by the corpus luteum (Buxton & Engle, 1950).

In the follicular phase, under the influence of oestrogens, the endometrium proliferates and increases in thickness. The narrow and widely scattered endometrial glands become much more numerous and elongated. The lining cells become columnar and show presence of numerous mitotic figures. The whole endometrium is richly vascularised and a great many intercellular lymph spaces are formed. The spiral arteries in the stratum basalis appear more coiled (Rubin & Novak, 1956). After corpus luteum formation, progesterone induces further increase in the thickness of the endometrium, while the stroma becomes more abundant and oedematous. The stromal cells are hypertrophied, resembling decidual cells and tend to accumulate near the surface of the mucosa. The glands continue to grow, they become very tortuous and distended with secretion. Three cellular layers can be recognized at this stage : the stratum compactum, formed by the mass of swollen stromal cells; the stratum spongiosum, where the glands are more obvious; the stratum basalis. The spiral arterioles become more and more coiled and numerous thin-walled sinuses develop near the surface of the endometrium (Rubin & Novak, 1956).

When the hormone secretion by the corpus luteum declines, the endometrium becomes necrotic and is shed. A fall in progesterone secretion would appear to be of greater significance than that of oestrogen secretion in determining the bleeding (Peel, 1955). The mechanism of endometrial

breakdown at the end of the cycle appears to be a sudden temporary ischaemia caused by vaso-constriction of the spiral arteriales which end abruptly under the surface epithelium in tufts of numerous capillaries (Markee, 1940). Autolytic disintegration of the endometrium by tryptic enzymes contributes to the destruction of the stratum compactum and spongiosum, while the stratum basalis is retained and proliferates at the beginning of the next cycle (Rubin & Novak, 1956).

The vaginal epithelium becomes taller and stratified during the follicular phase, while the cells present keratinisation and nuclear pyknosis. Three cell layers can be recognised : an intraepithelial cornified layer, a deep basal layer and a superficial functional layer. From the epithelium many keratinised pyknotic cells desquamate and are found in the vaginal secretion (deAllende & Orias, 1950; Papanicolaou, 1954). In the vaginal epithelium 'primed' with oestrogens, progesterone increases the thickness, mainly of the intermediate layer, and produces cellular deposition of glycogen. These changes are reflected in vaginal smears by a reduction of the number of cornified cells, which are replaced by cells with much larger nuclei and irregularly pleated. These cells show a characteristic tendency to cluster formation (Papanicolaou, 1954).

b) - Irregular bleeding.

There is some disagreement about the classification and terminology of the many types of irregular uterine bleeding occurring in women during the reproductive age and around the menopause, so that it is sometimes difficult to compare results obtained in different clinical studies. In this thesis, a classification based upon endocrinological

rather than clinical criteria (Peel, 1955) has been adopted, and all the terms will be clearly defined in this section.

Irregular uterine bleeding is frequently due to anatomical causes without any apparent relation to endocrine dysfunction, but may be related to disorders of the endocrine glands and this is the type which will be considered here. When no anatomical cause is demonstrable, irregular bleeding is somewhat empirically classified as "functional" and the term "functional uterine haemorrhage" has been adopted in this thesis (F.U.H.). Functional uterine haemorrhage can be divided into two main groups, cyclic and acyclic, according to whether the rhythm of the menstrual cycle is maintained or is completely lost.

In cyclic functional bleeding ovulation usually takes place, and the main symptom may be either shortening of the cycle of bleeding for a longer period than the duration of the patient's normal period. This symptom will be referred to as menorrhagia.

Acyclic functional bleeding is more common at the extremes of reproductive life, at puberty and before the onset of the menopause. Ovulation occurs at irregular intervals or is completely absent. Prolonged bleeding alternates with phases of amenorrhoea. The bleeding may vary from a few days at a time to continuous haemorrhage for a month or longer, and the loss can be very heavy. The periods of amenorrhoea are of very variable duration, from six weeks to a few months.

On the basis of the histological appearance of the endometrium, some of these cases of acyclic functional bleeding are termed metropathia haemorrhagica (Schroeder, 1954); the cases which do not present the

the typical endometrial features of metropathia haemorrhagica will be referred to as metrorrhagia.

As mentioned in the previous section, very little is known about ovarian secretion in these conditions, apart from the presence of variable amounts of oestrogens in follicular cysts associated with irregular bleeding (Granjon et/ al., 1958). It would appear that the mechanism of bleeding is different than ^{that of} in the normal cycle, since the bleeding is not necessarily preceded by a fall in the excretion of pregnanediol and/or oestrogens.

In metropathia haemorrhagica a characteristic feature of the endometrium is that it does not correspond to any of the stages of the cycle. The thickness of the mucosa varies to a high degree in different areas, and therefore the surface is uneven. It is impossible to differentiate between basal and functional layers. All the glands are proliferative in character, many of them dilated to form small cysts. The stroma is homogeneous and dense, and many mitotic figures can be seen in the cells. There are numerous straight vessels throughout the endometrium, which form large sinusoids. According to some authors, haemorrhage could occur from these sinusoids, without loss of tissue (Hertig & Mansell, 1957).

In metrorrhagia, the endometrium is most commonly proliferative in character or hypertrophic, without reaching the degree of hyperplasia seen in metropathia haemorrhagica. In contrast, it can be atrophic. Occasionally secretory changes are seen at biopsy.

These changes in the endometrium have been attributed to prolonged secretion of oestrogens, unopposed by progesterone secretion (Schepder, 1954).

Uninterrupted administration of oestrogens can cause hyperplasia of the endometrium, but this cannot be maintained in a proliferative or hyperplastic state indefinitely, since a 'break-through' bleeding intervenes. Bleeding intervenes also after abrupt discontinuance of treatment - 'withdrawal' bleeding. The mechanism of 'break-through' and 'withdrawal' bleeding is fundamentally the same, and is related to the tropic effect of the oestrogens on the endometrium and particularly on its vascular system. In 'break-through' bleeding, the constant levels of oestrogens may not suffice to maintain the viability of an augmented mass of endometrium, while in 'withdrawal' bleeding the sudden cessation of the supporting stimulus would produce vascular destruction. The bleeding in F.U.H. is very similar to a 'break-through' bleeding, since it can take place while excretion of oestrogens is constant or even increasing; or it could be considered as a 'withdrawal' bleeding, in the cases in which haemorrhage follows a sharp fall in oestrogen excretion. Another similarity between the bleeding induced by continuous oestrogen administration and the haemorrhage of F.U.H. is that this is protracted for many days and the loss is very heavy. This is probably due to the incomplete shedding of the endometrium (Kuppersmann, 1963).

'Break-through' and 'withdrawal' bleeding is also observed during progesterone administration, but the 'withdrawal' bleeding is not prolonged and excessive as with oestrogens, and the endometrial shedding is complete (Kuppersmann, 1963).

Vaginal cytology has shown that there is a marked oestrogenic influence in most cases, but a fluctuating progestational effect may

also be observed (Osmond-Clarke & Murray, 1964). Since fluctuations of the pregnanediol excretion, similar although less marked to those of oestrogens, have been noted (Brown & Matthew, 1962; Fotherby & Brown, 1964), it might be that the rôle of progesterone in irregular bleeding is more important than is generally accepted.

c)- The Stein-Leventhal syndrome.

The secondary amenorrhoea which is a feature of the Stein-Leventhal syndrome is generally due to ovulatory failure. However, neither amenorrhoea nor anovulation are constant findings. About 21% of the patients experience episodes of irregular, prolonged bleeding and a functioning corpus luteum is found at operation in 19% (Goldzieher & Green, 1962). Amenorrhoea often follows a long period of oligomenorrhoea, with periods becoming more and more delayed. As a consequence of ovulatory failure, sterility is a very common complaint.

In the majority of cases, the endometrium is proliferative in character, and in a few cases it is hyperplastic (Evans & Riley, 1960); (Goldzieher & Green, 1962). This would seem to be in contrast with the finding of diminished oestrogen production reported by some authors (Short & London, 1961; Mahesh & Greenblatt, 1964). On the other hand, it has been claimed that androgens alone could maintain a proliferative endometrium (Leventhal, 1958), and androgens appear to be secreted in increased amounts in the syndrome, as already mentioned.

The vaginal epithelium shows evidence of oestrogenic effect (Evans & Riley, 1960) and in vaginal smears, together with pyknotic cells, there are numerous round cells of the stratum basalis, which would

indicate an androgenic effect (Osmond - Clarke & Murray, 1964).

The high level of circulating androgens is also responsible, according to some authors, for the failure of ovulation, through a negative 'feed-back' effect on the hypophysis (Roy, Mahesh & Greenblatt, 1962; Goldzieher & Axelrod, 1963) and for the hirsutism observed in some patients with the syndrome (Short & London, 1961; Goldzieher & Axelrod, 1962; Mahesh & Greenblatt, 1964).

Chapter III - SCOPE OF THE PRESENT RESEARCH

Different biochemical approaches to the study of ovarian function have been outlined in the previous Chapter. Indirect methods, like the determination of steroid levels in peripheral blood and urine or the study of secretion rates, indicate the actual steroid output of the ovary together with the output of other steroid-producing glands.

Steroid determination in ovarian fluid or tissue and 'in vitro' experiments are of a more direct nature, but the information is restricted to the functional state of the ovary at the moment of operation and in most instances to one part of the ovary only. This is a serious limitation for the understanding of ovarian physiology and pathology, since ovarian function is typically cyclic and the ovary is constituted of tissues with differing functional capacities. In recent years, the 'in vitro' approach has, in general, been the method of choice for the more direct investigation of ovarian function. However, it cannot be assumed that reactions which are demonstrated in these incubation experiments actually occur 'in vivo', and the results may not indicate more than interesting possibilities. Accordingly, the 'in vitro' studies must be supplemented by the determination of the steroid content of the ovary and, when possible, of the effluent blood.

In fact, as has been mentioned, there is some evidence to suggest that the production of steroids as determined by assays in ovarian tissue or fluid does not necessarily reflect steroid secretion into the ovarian vein, and concurrent determination in the ovary and effluent

blood appear to be indispensable in order to establish which steroids are actually secreted. With regard to ovarian venous blood, it will be understood that samples are not readily available and that interpretation is complicated by lack of information about ovarian blood flow.

Owing to the small amounts of steroids present in ovarian tissue or fluid, it is usually impossible to identify the compounds in individual samples by classical methods (re-crystallization, determination of the melting point, infrared spectrum). Despite these limitations, the determination of ovarian steroids has already contributed to the knowledge of ovarian function in normal and pathological conditions. Particularly valuable have been investigations where either identification of the compounds has been achieved or a quantitative method of adequate specificity has been employed. Until recently (Short, 1965), very little work has been reported upon the concurrent measurement of progesterone, neutral intermediaries and oestrogens in human follicles or corpora lutea.

Comprehensive information upon steroid production and secretion by the normal ovary is required as a valid control for comparison of results obtained in abnormal conditions. For instance, steroid biosynthesis and production has been studied in atretic and cystic follicles from the ovaries of patients with the Stein-Leventhal syndrome and not from the ovaries of women with regular menstrual cycles.

On the other hand, apart from the studies of this relatively rare syndrome, ovarian steroid production in women with menstrual disorders has not been examined extensively and the whole question of the relationship between ovarian morphology and steroid production requires further investigation.

In the belief that progress in the treatment of ovarian dysfunction awaits clarification of the problems to which reference has been made, it was decided to study the ovarian steroid production of normally menstruating women at various stages in the cycle; of patients receiving gonadotropins and of carefully selected patients with menstrual disorders.

It was first necessary to develop a sensitive and specific technique for the quantitative determination of Δ^4 -3-keto-steroids, Δ^5 -3 β -hydroxy-steroids, oestradiol, oestrone and oestriol and conjugated oestradiol and oestrone in small volumes of cyst fluid. Cyst fluid, which, as already mentioned, contains specific secretory products of the cyst lining, was chosen in preference to ovarian tissue as it could be obtained without untoward trauma to the gland. In some instances analysis of cyst fluid was supplemented by determinations in ovarian venous blood. In a few cases, 'in vitro' experiments were performed by departmental colleagues, and through their courtesy it has been possible to correlate ovarian steroid production 'in vivo' and 'in vitro'.

The present work concerns the results of steroid determination in 62 ovarian fluids and 10 ovarian venous blood samples from 59 patients, of whom 22 were women with normal menstruation (13 of whom had polycystic ovaries); 5 were under gonadotropin stimulation; 11 patients complained of acyclic uterine bleeding and 19 were examples of Stein-Leventhal syndrome.

Chapter IV - METHOD FOR STEROID DETERMINATION IN OVARIAN FLUID

Many difficulties are encountered in the determination of steroids in biological fluids. The quantity of steroids present in most samples is usually of the order of a few micrograms and the amount of interfering material is frequently large. The organic solvents, which are commonly used, extract the steroids together with the interfering material and the steroids have to be separated from this before they can be separated from each other. Spectrophotometric methods for the detection and quantitative estimation of steroids are in general non specific and adequate preliminary purification is essential to ensure that the procedure measures only the steroids in question.

Steroids present in ovarian fluid can be divided into three groups : neutral, phenolic and conjugated steroids. The group of neutral steroids comprises the C21 and C19 steroid intermediaries in the synthesis of oestrogens; the group of phenolic steroids comprises the C18 oestrogens so far isolated from the human ovary. Free neutral and phenolic steroids can be extracted by organic solvents from biological fluids. By shaking this extract with aqueous alkaline solution, the phenolic steroids pass quantitatively from the organic solvent into the aqueous phase, whereas the neutral steroids remain in the organic phase (Baggett et al., 1956). Conjugated steroids, being hydrophilic, are not extracted by most organic solvents and can be separated from free steroids by means of solvent partition between an aqueous phase and a very polar organic solvent (Diczfalusy & Magnusson, 1958; Migeon et al., 1959).

The only procedure published up to date for the concurrent determination

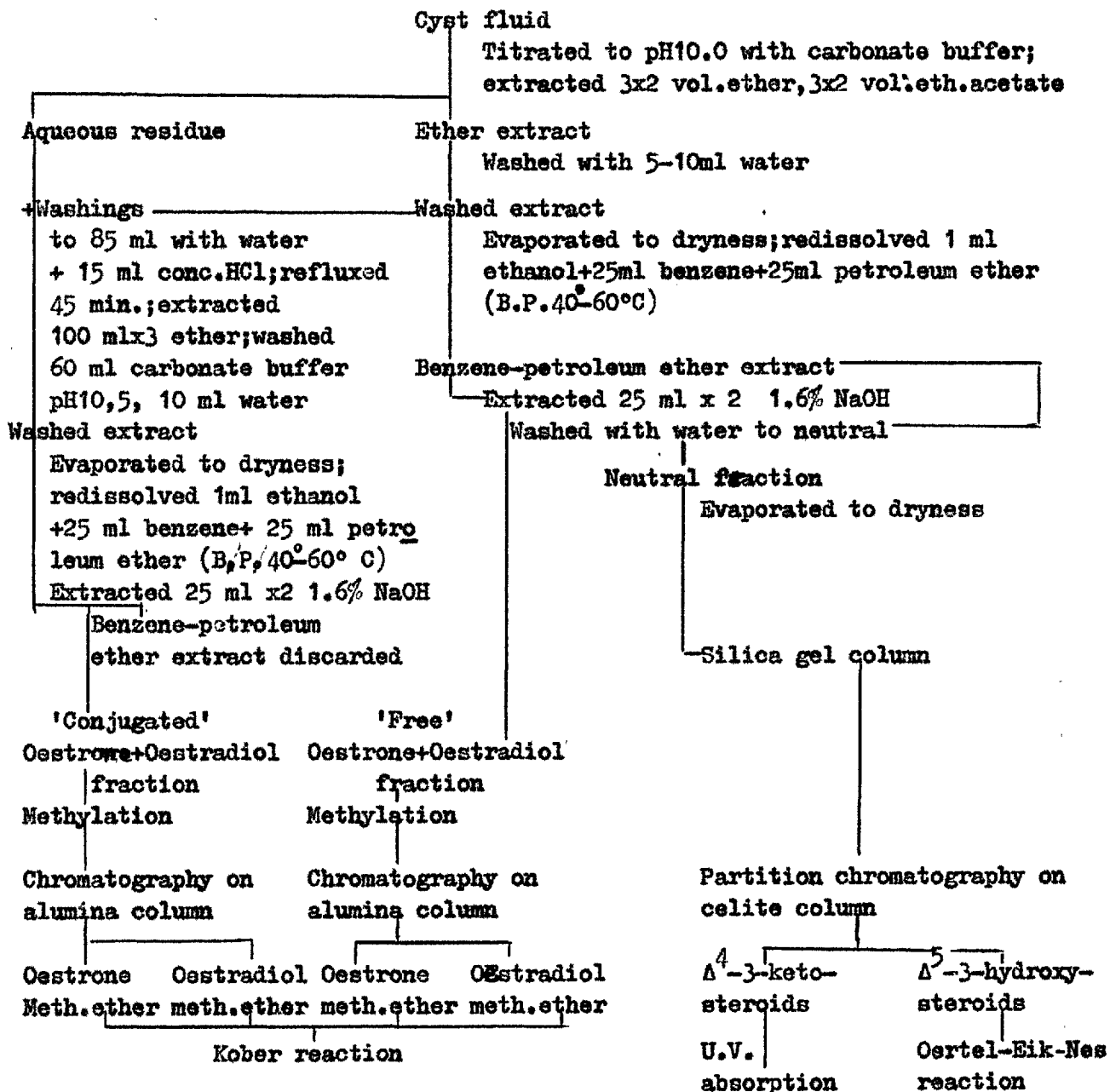
of free neutral and phenolic steroids in small volumes of ovarian fluid is the method of Short (1961), and this has been applied to steroid determination in fluid from human ovaries (Short & London, 1961; Short, 1962b; 1965; Crooke et al., 1963a,b) and to follicular fluid, ovarian venous blood and lymph of different species of domestic animals (Short, 1961; 1962a, 1964; Lindner, Sass & Morris, 1964). By this method individual steroids can be determined in concentrations above 2.0 $\mu\text{g}/100\text{ ml}$. The method involves extraction with ether and ethyl acetate followed by solvent partition of neutral and phenolic steroids. Neutral steroids are chromatographed and paper in Bush systems and measured by means of U.V. absorption. Phenolic steroids are separated by chromatography on paper in Zaffaroni systems and determined by the Kober reaction.

A similar method for extraction and partition into neutral and phenolic fractions was employed in the present research. Subsequently the neutral fraction was subjected to partition chromatography on a celite column and the oestrogens were methylated and their methyl ethers separated by adsorption chromatography on an alumina column according to the method of Roy & Brown (1960). As will be seen, the application of isotope dilution was an essential feature of the method.

a) - The method in detail.

Cyst fluid was withdrawn at operation and stored at -15°C pending analysis. A flow sheet of the method is shown in Fig. 7. In analyses performed over the past two years, $16\text{-}^{14}\text{C}$ -oestrone, $16\text{-}^{14}\text{C}$ -oestradiol- 17β , $4\text{-}^{14}\text{C}$ -progesterone, $4\text{-}^{14}\text{C}$ -androstenedione and $4\text{-}^{14}\text{C}$ -testosterone were added in trace amounts (not more than 0.1 μg) and used as internal standards for correction of losses.

Fig. 7 - Flow sheet of the method for steroid determination.



Extraction. The fluid was adjusted to pH 9.5; extracted successively with ether and ethyl acetate and the pooled ether-ethyl acetate extracts were washed with a small volume of water. The aqueous residue was saved in some instances and analysed for the presence of conjugated oestrogens. This was effected by dilution of the aqueous residue to 85 ml with water and hydrolysis by addition of concentrated HCl with refluxing for 45 minutes. After addition of trace amounts of ^{14}C -oestrone and ^{14}C -oestradiol the hydrolysate was extracted with ether, washed with saturated NaHCO_3 and water and evaporated to dryness.

Solvent partition of neutral and phenolic fractions. The dry residues of the non-hydrolysed and of the hydrolysed extracts were dissolved in 1 ml warm ethanol, 25 ml benzene was added and transferred to a separating funnel containing 25 ml of petroleum ether. The 'oestriol' fraction was extracted from the benzene-petroleum ether mixture with water and the 'oestrone-oestradiol' fraction with 0.4 N NaOH. The benzene-petroleum ether mixture corresponding to the extract of the non-hydrolysed fluid, containing the neutral free steroids, was washed with water until neutral, and evaporated to dryness. The benzene-petroleum ether mixture from the hydrolysed fluid was discarded.

Phenolic fractions. The phenolic fractions were methylated and the methyl ethers chromatographed on alumina according to Roy & Brown (1960). In the analyses where isotopically labelled oestrogens had been added, one tenth of the eluate containing oestrone and oestradiol was taken for scintillation counting using a toluene-based scintillator. Radioactivity measurements were carried out with a standard error of 1%.

The Kober reaction was performed on the remainder, adding 0.6 ml of the appropriate reagent and 0.2, 0.1 and 0.04 ml distilled water to the oestriol, oestrone and oestradiol fractions respectively after boiling. Optical densities were read in a spectrophotometer (Unicam SP500) with a micro-attachment at 480,516 and 552 m μ for oestriol and oestrone and 480,518 and 556 m μ for oestradiol. The Allen correction (Allen, 1950) was applied to the spectrophotometric readings and the amount of each steroid was calculated from calibration curves based upon the appropriate standards. The spectrophotometric readings were corrected for the contribution from the labelled internal standards.

Neutral fraction. The neutral fraction, which retained most of the impurities, was defatted on a silica gel column in the solvent system n-hexane/ethyl acetate (Sommerville, Pickett, Collins & Denyer, 1963). The defatted residue of the neutral fraction was transferred to a column of 2.5 gm celite in n-hexane and chromatographed in the system MeOH:H₂O/n-hexane 80:2/100. Fifty 2 ml fractions were collected by means of an automatic fraction cutter. The fifty fractions were evaporated to dryness under N₂ and 0.6 ml of ethanol, spectrosol grade, was added to each vial. The U.V. absorption was read at 220, 240 and 260 m μ for the determination of Δ^4 -3-keto-steroids. The ethanol from vials 5-7 and 11-14 was evaporated to dryness, 1 ml of the sulphuric acid-95% ethanol (2:1) reagent of Oertel and Eik-Nes (1959) was added and the optical densities read at 380,405 and 430 m μ for the determination of Δ^5 -pregnenolone and at 375,400 and 425 m μ for the determination of DHA.

The eluates which contained material absorbing in the U.V. at 240 m μ were pooled and chromatographed either on paper or on a second celite

column. A Bush A system was used for progesterone and Δ^4 -androstenedione, and a Bush B system for testosterone and 17 α -hydroxy-progesterone. The compounds were eluted quantitatively from the paper, the eluates evaporated to dryness and U.V. spectroscopy performed as above, with the exception that the eluates were read against a 'blank' eluted from paper. The solvent system for the second chromatography on celite was MeOH:H₂O/n-hexane 90:10/100.25 fractions of 2 ml each were collected. Each eluate was subjected to U.V. absorption spectroscopy and scintillation counting in order to perform an isotopic dilution analysis as proposed by Preedy and Aitken (1961).

b)- Assessment of the method.

The accepted criteria to assess the validity of quantitative methods are accuracy, precision, sensitivity and specificity. The definitions of these criteria are as follows : accuracy, the extent to which the appropriate substance can be recovered; precision, the degree of agreement between replicate estimations; sensitivity, the least quantity of substance which can be measured; specificity, the extent to which the procedure measures only the substance in question (Borth, 1952).

Accuracy. There were several difficulties involved in attempts to assess the accuracy of the method. First, the volumes of cyst fluid were variable and the fluids contained differing amounts of blood pigment, so that it was not possible to obtain a relevant 'blank' for comparison. Secondly, the volumes were usually small and insufficient for use in recovery experiments. For these reasons, the majority of recovery experiments with unlabelled standards were carried out in pure solution. The recoveries,

employing 0.5 or 1.0 μg of each steroid, ranged from 60 to 85% for neutral steroids, free and conjugated oestrogens. Some of the recovery experiments of unlabelled and labelled steroids, the latter from blood or cyst fluid, are reported in Table IX. It will be seen that the recovery of isotopically labelled steroids from cyst fluid or blood was of the order of 62-72%. Since it was clear that inaccuracies were introduced in the method because of lack of uniformity in the recovery of the single steroids, the use of isotopically labelled compounds as internal standards was adopted for all the analyses.

Sensitivity. The sensitivity of the reactions employed was as follows : the micro-Kober reaction permitted the determination of 0.05 μg oestrone and oestradiol. By U.V. absorption 0.1-0.2 μg of Δ^4 -3-ketosteroids could be detected, provided this amount was distributed between not more than three eluates from the celite column. The isotopically labelled steroids added as internal standards contributed 0.02 μg of oestrogens and 0.05 μg of Δ^4 -3-keto-steroids to the spectrophotometric readings. Therefore as little as 0.03 μg of endogenous oestrogens and 0.05 μg of Δ^4 -3-keto-steroids could be measured. The Oertel-Eik-Nes reaction for Δ^5 -3 β -hydroxy-steroids was slightly less sensitive than U.V. absorption.

Precision. It was possible to perform duplicate analyses on 10 cyst fluids containing detectable amounts of steroids; the average deviation from the mean was as follows : progesterone $\pm 11\%$; Δ^4 -androstenedione $\pm 12\%$; 17 α -hydroxy-progesterone $\pm 1\%$; oestrone $\pm 20\%$; oestradiol $\pm 14\%$.

Specificity. Partition chromatography on celite and the use of U.V. spectroscopy for Δ^4 -3-keto-steroids and the Oertel-Eik-Nes reaction for

Table IX -- RECOVERY EXPERIMENTS FROM PURE SOLUTION, PLASMA OR CYST FLUID.

Steroid added μ g		recovered	Labelled steroid added μ g		recovered
Progesterone	1.0	68-72%	0.5		48-62%
Pregnenolone	1.0	55-65%			
Δ^4 -androstenedione	1.0	62-93%	0.5		66-72%
DHA	1.0	56-78%			
20 α -hydroxy-pregn. 4-en-3-one	1.0	80-94%			
Testosterone	1.0	57-88%	0.5		63-70%
17 α -hydroxy-proges- terone	1.0	56-76%			
Oestradiol-17 β	0.5	71-88%	0.056		63-70%
Oestrone	0.5	66-83%	0.11		66-72%
Oestradiol glucur.	0.9	70-75%			
Oestrone sulphate	1.0	65-66%			

Δ^5 - 3β -hydroxy-steroids effected adequate separation of progesterone, Δ^5 -pregnenolone, Δ^4 -androstenedione, DHA, 20α -hydroxy-pregn-4-en-3-one, testosterone and 17α -hydroxy-progesterone (Fig.8). Thus the partial overlapping of progesterone and pregnenolone, DHA and androstenedione was resolved by the use of the two reactions. The steroids were identified by the position of the 'peaks' from the eluates and additional evidence of identity was afforded by paper chromatography or chromatography on a second celite column (Fig. 9). The results of the second celite chromatography of a cyst fluid are illustrated in Fig. 10. The distribution of radioactivity and U.V. absorption in the eluates from the column was almost superimposable; in consequence all of the 'peak' eluates had similar specific activities, indicating that the compounds were radiochemically pure. The results in Table X supports this conclusion.

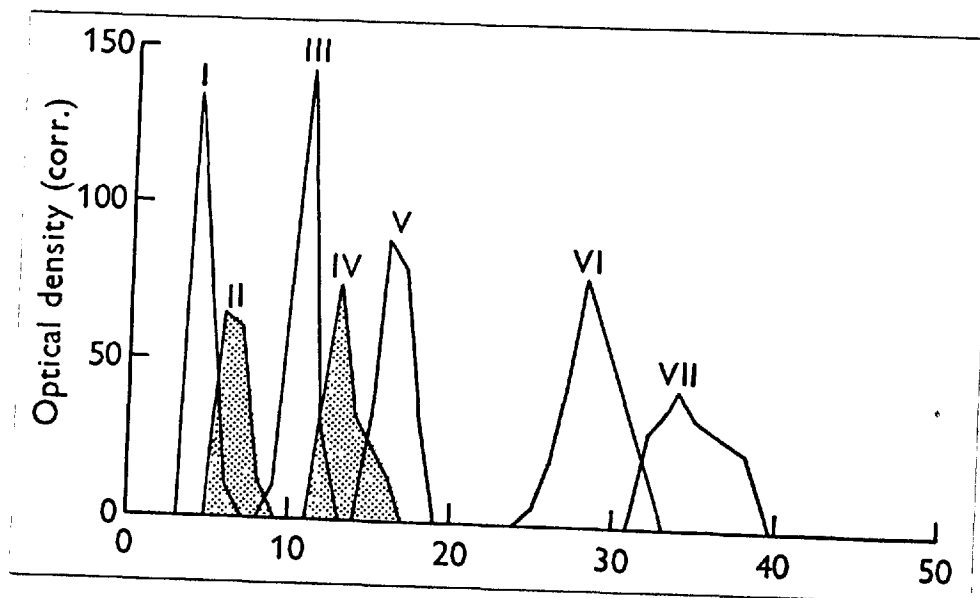
The specificity of the Roy-Brown method for the separation and quantitative estimation of oestrogens was controlled by recovery experiments of labelled oestrogens from plasma or cyst fluid, in which the eluates from the alumina column were measured both colorimetrically and by liquid scintillation counting. The specific activity of the recovered compounds was similar to that of the added standards (Table X).

c) - Investigations on the 'conjugated oestrogen' fraction.

-Indirect evidence that oestrogens in the 'conjugated' fraction were originally present as chemical conjugates :

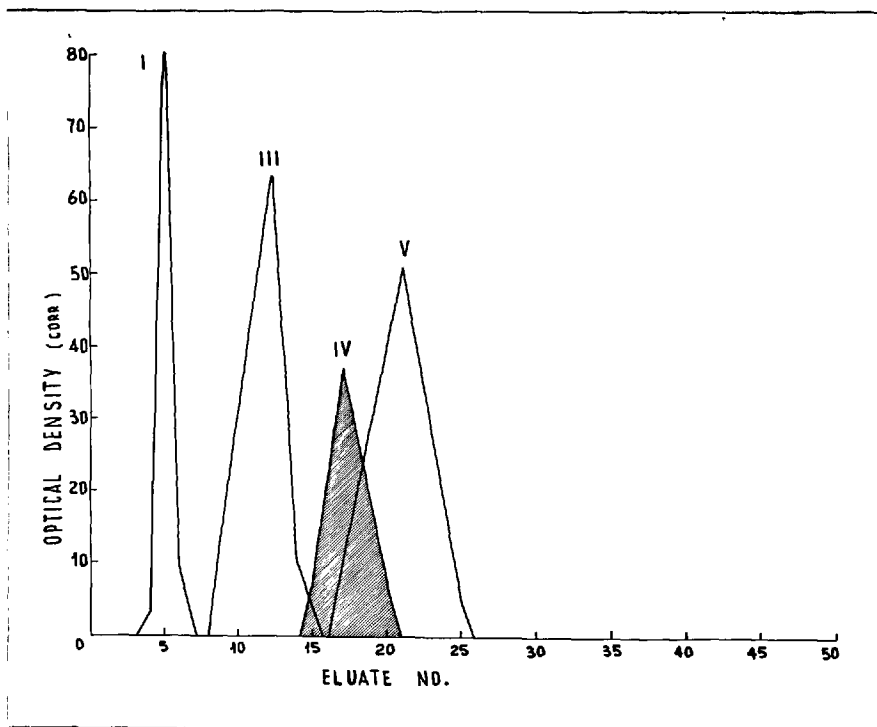
In a series of experiments, unlabelled oestradiol (11.5 μ g) and oestrone (13.5 μ g) were added to plasma which was extracted with ether; the aqueous residue of the plasma was subjected to acid hydrolysis and ether

Fig.8- Elution pattern of steroids from a partition celite column
in 80% aq.methanol/n-hexane.



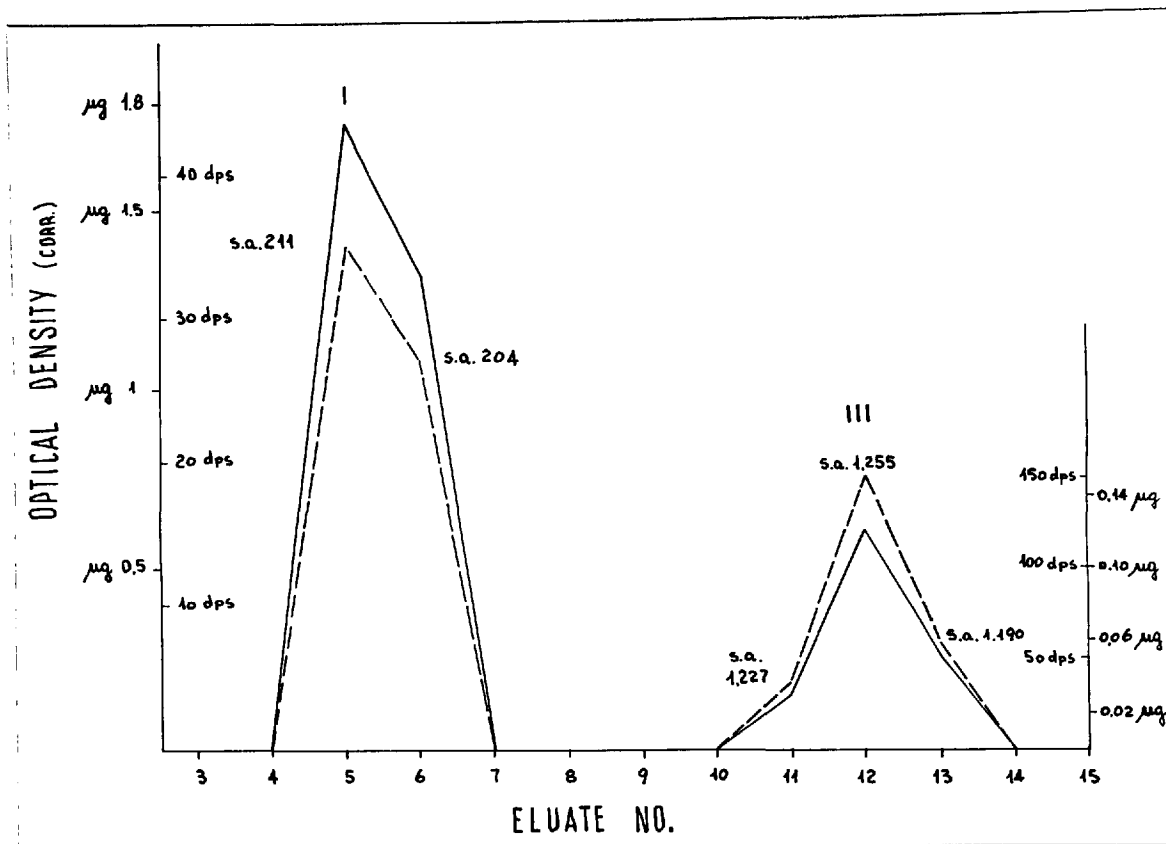
Mixture of 2 μ g each of authentic steroids: U.V. absorption and Cortel-Eik-Nes reaction (shaded areas). I, Progesterone; II, Δ^5 -pregnenolone; III, Δ^4 -androstenedione; IV, DHA; V, 20 α -pregn-4-en-3-one; VI, Testosterone; VII, 17 α -hydroxy-progesterone.

Fig.9- Elution pattern of steroids from a partition celite column
in 90% aq.methanol/n-hexane.



Mixture of 2 µg each of authentic steroids: U.V. absorption and Cortel-Eik-Nes reaction (shaded areas). I, Progesterone; III, Δ^4 -androstenedione; IV, DHA; V, 20 α -pregn-1-en-3-one.

Fig.10- Isotopic dilution analysis on cyst fluid.



Cyst fluid No.11. Celite column in 90% aq. methanol/n-hexane.

Continuous line: μg in vials; broken line: dps in vial. s.a.=specific activity, dps/μg. I, Progesterone, 10.6μg; III, Δ^4 -androstenedione, 0.3μg.

Table X - EXPERIMENTS TO TEST THE SPECIFICITY OF THE METHOD.

Isotopically labelled compounds.

Steroid added	µg	specific activity dps / µg	measured µg ②	specific activity dps/µg
Progesterone	0.5	2,325	0.44-0.44	2,642
Δ ⁴ -androstenedione	0.5	2,780	0.49-0.54	2,879-2,574
Testosterone	0.5	2,780	0.51-0.54	2,737-2,574
Oestradiol-17β	0.056	1,600	0.038-0.038	2,357
	0.039	4,325	0.031-0.032	5,341-5,270
Oestrone	0.11	1,600	0.11-0.13	1,600-1,323
	0.037	4,635	0.039-0.040	4,656-4,287
Na-oestradiol-glucuronidate *	0.60	67	0.55-0.55	74 - 74

② Measured by U.V. spectroscopy or Kober reaction and corrected for losses according to recovery of radioactivity.

* Na-oestradiol-17β-glucuronidate, 6,7-H³ diluted with unlabelled Na-oestradiol-glucuronidate.

extraction, followed by methylation of the extract; chromatography on alumina and Kober reaction on the alumina eluates. The amount of residual oestrogens in the 'conjugated' fraction was never more than 0.6% of the total added. In another series of experiments, tritiated oestradiol glucuronidate and ^{14}C -labelled free oestradiol were added to plasma and this dialysed for 18 hours against 0.9% NaCl. Ether extraction of the dialysed plasma yielded between 3.5 and 3.9% of the ^3H and 89.5 to 93.8% of the ^{14}C . After refluxing with hot HCl, ether extraction of the hydrolysate yielded 91-95% of the tritium and 3.8-4.4% of the ^{14}C . These experiments indicated that the separation of free and conjugated oestrogens was satisfactory. In addition, they showed that the bond of free oestradiol to proteins was resolved completely by ether.

Another series of experiments was performed on three cyst fluids which were available in larger volumes. The results are reported in Table XI. Part a) of this Table shows that there was satisfactory agreement between the values for the total oestrogens as determined by direct hydrolysis of the fluid or by summation of the amounts obtained before and after acid hydrolysis. Part b) indicates that oestradiol was liberated by enzyme hydrolysis in amounts similar to those obtained by acid hydrolysis.

-Effect of acid hydrolysis on the oestrogen moiety of the conjugates :

A mixture of unlabelled oestradiol glucuronidate and ^3H -oestradiol glucuronidate of specific activity 64 dps ,per μg was added to plasma and this subjected to acid hydrolysis followed by methylation and chromatography on alumina of the hydrolysed extract; the specific activity

Table XI - INVESTIGATIONS ON THE 'CONJUGATED' OESTROGEN FRACTION.

a) Replicate determinations upon 5 ml aliquots of a cyst fluid.
(μg / per 100 ml)

Procedure	A	B	C	A + B
Oestrone	2.0	3.4	6.2	5.4
	3.2	1.6		4.8
Oestradiol	4.0	12.8	20.0	16.8
	5.0	15.2		20.2
	"Free"	"Conjugated"	Total	Total

A. Ether-ethyl acetate extract.

B. Hydrolysate of aqueous residue from A.

C. Hydrolysate of cyst fluid without previous extraction.

b) Acid and enzyme hydrolysis of aqueous residue of cyst fluid.

		Oestradiol-17 β $\mu\text{g}/100\text{ ml}$	
		acid hydrolysis	enzyme hydrolysis *
Fluid	a	3.2	4.2
Fluid	b	3.3	3.5-4.1 +

* Helix pomatia, 10.000 U.; acetate buffer pH5.4; 18 hrs at 37°C.

+ Replicate determinations.

of the oestradiol methyl ether was 74 dps/ μ g. When the same procedure was applied to plasma containing either oestrone sulphate or oestradiol glucuronidate, no transformation of oestrone to oestradiol or viceversa was noted. This would seem to exclude that the chemical configuration of the oestrogen moiety was altered by acid hydrolysis.

In order to investigate whether acid hydrolysis destroys part of the conjugates, two other series of experiments were performed. Unlabelled oestradiol glucuronidate (36.6 μ g) and oestrone sulphate (10.0 μ g) were added separately to plasma, and this treated as the 'conjugated' fraction of cyst fluid, with addition of 14 C-labelled oestradiol or oestrone after acid hydrolysis. After correction for losses, 100% of the initial amount of oestrone sulphate and oestradiol glucuronidate was accounted for. Therefore acid hydrolysis in the conditions adopted does not destroy a significant part of oestrogen conjugates. In recovery experiments of oestradiol glucuronidate added to plasma in amounts from 2.9 to 8.7 μ g, using 3 H-labelled glucuronidate as internal standard, the recoveries ranged between 70 and 75%; recoveries after enzyme hydrolysis varied between 65 and 75%.

In conclusion, although direct evidence that conjugated oestrogens were present in cyst fluid was lacking, the indirect evidence is convincing. Furthermore, it could be assumed that the specificity, sensitivity, accuracy and precision of the method were identical for free oestrogens and oestrogens in the 'conjugated' fraction.

Chapter V - CLINICAL MATERIAL AND RESULTS OF STEROID DETERMINATION
IN CYST FLUID AND OVARIAN VENOUS BLOOD

1 - Determination of neutral and phenolic steroids
in cyst fluid.

a) - Normally menstruating women.

Ovarian fluid was obtained at operation from seven patients with cycles normal for rhythm, duration and menstrual loss, who underwent surgery for various gynaecological conditions. The main clinical features and histological findings in these patients are reported in Table XII. It will be seen that, at operation, endometriosis was found in two patients, a dermoid cyst in one and tuberculous pyosalpinx in another. The indication for operation in the other three patients were infertility, dysmenorrhoea and sterilization.

The cystic formations found in the ovaries of five of these patients were follicles or corpora lutea containing more fluid than usual. In one patient (L.E.), the ovaries presented several small cysts which proved on biopsy to be inclusion cysts and it is probable that fluid was aspirated from a Graafian follicle not recognizable by the naked eye. In the seventh patient there was a cyst of medium size of the appearance of a corpus luteum cyst. Evidence of ovulation was obtained in two patients (I.O.;D.O.) by direct inspection of the ovaries.

As the follicles and corpora lutea were a chance finding at operation it was not possible in all cases to examine them histologically, nor was it possible to study ovarian function pre-operatively. Therefore it is not claimed that the ovarian function was normal in these patients, but only that the menstrual cycles were not altered in any appreciable

DETAILS OF NORMALLY MENSTRUATING WOMEN.

Table XII

Name	Age yrs.	Symptoms and duration	Menstrual periods	Operation	Findings at operation	
					Ovaries	Others
S.Y.	40	Pain in abdomen, 6 months	8/28	Laparotomy	(L) sclerotic; (R) endometrial cyst; Graafian follicle (2.7 ml) ^o	Uterus: normal Endometriosis
H.I.	29	Prolonged periods, P.C.B. for 6 months post-partum	5/26-30	(R)salpingo- oophorectomy	(L) Graafian follicle(2.7ml) ^o (R) dermoid cyst	-
G.A.	35	(Sterilization)	2-5/28	Bilateral salpingec tomy	(R) Graafian follicle(1.6ml) ^o	-
L.E.	28	Sterility, 4 years	4-5/27	Tubal insufflation	(L)(R) inclusion cyst (2.0 ml) ^o	Tubes patent
I/O.	28	2nd sterility, 9 years	3/28	(L)salpingo- oophorectomy	(L)cystic follicles; abscesses; corpus luteu ^u m haematoma (2.8 ml) ^o	(L) Tb pyosalpinx
D.O.	43	Dysmenorrhoea & premen- tension for many years	8/26	Hysterectomy, bilat eral wedge resection ovaries	(L)corpus luteum haematoma (0.7 ml) ^o (R)active corpus luteum	Uterus:normal
S.R.	21	Vaginal discharge, dyspareunia, 2 years	5-7/28	Laparotomy	(L) single cyst (6ml) ^o	Pelvic endometriosis

^o = volume of fluid aspirated at operation.

way up to the day of operation, with the exception of patient S.R., in whom menstruation was overdue.

The results of steroid determination in fluids from normally menstruating women were tabulated in chronological order from the onset of the last period (Table XIII).

The method of expression of the results presented certain difficulties. Thus, to calculate the concentration per 100 ml of fluid does not take into account dilution of the steroids by fluid, whereas to calculate the amount of steroids per sample is open to the criticism that fluid may be lost at operation or that it may be impossible to obtain the total volume, as in the case of polycystic ovaries. For these reasons it is not possible to compare quantitatively the steroid content of different fluids, but in the case of fluids aspirated from single cysts the results were expressed as μg steroid per sample, in the belief that this is more representative of the secretory activity of the cyst lining. On the other hand, a comparison of the relative proportions of different steroids within individual samples is valid.

With these reservations, it may be stated that the steroid content of individual samples of fluid from normally menstruating women was of a similar order : five fluids contained from 3.5 to 8.0 μg neutral plus phenolic steroids, while in one fluid obtained at the end of the cycle the steroid content was very low.

Progesterone was present both in the follicular and luteal phase of the cycle; the amounts at mid-cycle and in the early luteal phase were larger than at the end or the beginning of the cycle. In fluid No.7,

STEROID DETERMINATION IN CYST FLUID FROM NORMALLY MENSTRUATING WOMEN.

Table XIII

Fluid No.	Name	Day of cycle	Endometrium	Steroids in cyst fluid µg per sample								Ratio OD/progest
				Volume	Progest.	17α-hydroxy-progesterone	Δ ⁴ -andro- stenedione	DHA	Testos- terone	Oestra- diol	Oestre- ne	
1	S.Y.	6	—	2.7 ml	0.93	0.54	<0.10	—	<0.10	5.72	1.24	6.15
2	H.I.	11	—	2.7 ml	0.30	0.40	0.47	<0.10	<0.10	2.30	0.06	7.29
°°4	L.E.	12	—	2.0 ml	<0.10	0.10	0.33	—	<0.10	3.12	0.22	>31.20
3	G.A.	11	proliferative	1.6 ml	5.61	1.11	<0.10	<0.10	<0.10	3.30	0.21	0.59
5	I.O.	20	vag.cyt.luteal	2.8 ml	<u>2.50</u>	1.19	<0.05	<0.10	<0.10	4.29	0.05	1.71
6	D.O.	24	secretory	0.7 ml	0.10	0.20	<0.10	—	<0.10	<0.05	<0.05	<0.005
7	S.R.	34	—	6.0 ml	<u>7.87</u>	<0.30	<0.15	—	<0.30	<u>0.31</u> *	<u>0.48</u> *	0.39

underlined = values corrected for losses

* = total oestrogens

°° = Fluid No. 4 precedes No.3 for convenience

Values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

collected at the 34th day of an abnormally prolonged cycle, progesterone was still present in a large amount. It may be relevant that the cyst found in the ovary of this patient was larger than those found in the other ~~menstrual~~ patients. Of the intermediaries in oestrogen biosynthesis, 17 α -hydroxy-progesterone was increased in mid and late cycle, generally paralleling progesterone. Δ^4 -androstenedione was detectable only in two fluids obtained in the follicular phase. In three fluids, attempts were made to detect DHA, but this steroid was not found. Testosterone and 20 α -hydroxy-pregn-4-en-3-one were not detectable in any fluid. Oestradiol was the predominant steroid from the early phase of the cycle to the 20th day, and was absent in a corpus luteum at the 24th day. Oestrone was present in much smaller amounts than oestradiol in all fluids, with the exception of fluid No. 7. Oestradiol was not detected.

The ratio oestradiol/progesterone increased from the 6th day to the 12th day of the cycle and decreased thereafter, varying between 0.6 and 31.5 in the follicular and between <0.005 and 1.7 in the luteal phase. These ratios do not agree with that of the urinary metabolites of oestradiol and progesterone, which, as reported earlier, is around 0.01 in both phases of the cycle.

It may be of interest that in patient G.A. the endometrium showed a proliferative pattern, while the fluid contained a large amount of progesterone.

b) - Patients under gonadotropin stimulus.

Four patients of this group had, in common, an increased level of circulating gonadotropins at the time of operation, as a consequence either of treatment with human gonadotropins or of increased endogenous

production of chorionic gonadotropin. A fifth patient had been treated pre-operatively with Clomiphene.

Patient M.K., aged 37 years, had regular cycles; was to be sterilized for psychological reasons and volunteered to be treated with human pituitary FSH, in a dose corresponding to 50 HMG/I.U./day on day 10, 6 and 3 prior to operation and with HCG-6,000/I.U./day, on day 2 and 1 prior to operation. The urinary excretion of steroid metabolites in this patient was very high following treatment. At operation, both ovaries were enlarged; the left ovary presented four cysts, the largest 2 cm in diameter; the cysts proved on biopsy to be cystic atretic follicles or follicular cysts; the right ovary contained three active corpora lutea, the largest one 1 cm in diameter. 10 ml of fluid was collected from the left ovary. Patient D.R., aged 18 years, had primary amenorrhoea and was treated with a preparation of urinary menopausal gonadotropins (Pergonal, Serono), 70 I.U. of FSH and 70 I.U. of LH per day for 12 days. At operation, the ovaries were found to be hypotrophic, but in the left ovary a large corpus luteum cyst had developed. 12 ml of fluid was obtained from the cyst. Patient S.B., aged 22, had an ovarian cystectomy at the 10th week of pregnancy. A simple cyst, 8 cm in diameter, and a corpus luteum were present in the right ovary. 20 ml of fluid was aspirated from the cyst. Patient E.E. was affected by chorionic epithelioma of the uterus. The urinary excretion of HCG in this patient was 65,000 I.U./24 hrs, as measured by immunoassay. At surgery the ovaries were bilaterally enlarged to three times the normal size and presented numerous large cysts, many containing blood-stained fluid. 26.3ml of fluid was aspirated

from the right ovary.

Patient S.H., aged 26, designated as a case of Stein-Leventhal syndrome, had been amenorrhoeic for 7 years; she was given Clomiphene citrate (Merrell) 100 mg/day for 10 days pre-operatively. At operation, the left ovary appeared enlarged to about 3 times the normal size; contained one large cyst, from which 8.6 ml of fluid was obtained, and several small cysts; the right ovary was also enlarged and contained numerous cysts.

The results of steroid determination in the cyst fluid of the five patients under gonadotropin stimulus are reported in Table XIV. The total amount of steroids varied in the fluids from 15.4 to 119.4 μg . These values were much higher than those detected in fluid from normally menstruating women. The amount of steroids present in the fluid of the two amenorrhoeic patients, one treated with postmenopausal gonadotropin (D.R.) and the other with Clomiphene (S.H.) was much lower than in the remainder.

The steroid pattern in fluids No. 8,9,10 and 11 was almost identical. Progesterone was the predominant steroid, reaching the highest value, 90.2 μg in fluid No. 11; 17 α -hydroxy-progesterone was present in large amounts and Δ^4 -androstenedione in relatively small amounts, apart from fluid No. 9. Oestrogen level was much lower than that of neutral steroids, but the difference was less marked in fluid No. 9; the ratio oestradiol/progesterone varied between 0.003 and 0.1, values below those generally observed in the luteal phase of the normal cycle. Oestrone and oestradiol were present in similar amounts, instead of there being a predominance of oestradiol as in fluid from normally menstruating women. In fact, fluid No. 10 contained more oestrone than oestradiol.

STEROID DETERMINATION IN CYST FLUID FROM PATIENTS UNDER GONADOTROPIN STIMULUS.

Table XIV

Fluid No.	Name	Diagnosis and treatment	Steroids in cyst fluid, µg per sample								
			Volume	Progest.	17-hydroxy- progesterone	Δ ⁴ -andro- stenedione	DHA	Testos- terone	Oestra- diol	Oest- one	Ratio OD/progest.
8	M.K.	Normal cycles +HP-FSH+HCG	10.0 ml	<u>77.50</u>	<u>10.22</u>	<u>5.21</u>	<0.25	<0.25	<u>4.63</u> *	<u>3.25</u> *	0.06
9	D.R.	Primary amenorr. + Pergonal	12.0 ml	<u>8.26</u>	—	<u>5.05</u>	<0.60	<0.60	<u>1.41</u> *	<u>0.65</u> *	0.17
10	S.B.	10th week of pregnancy	20.0 ml	<u>23.08</u>	<u>32.00</u>	<u>3.98</u>	—	<0.23	<u>2.26</u> *	<u>7.46</u> *	0.10
11	E.E.	Chorionic epi- thelioma	26.3 ml	<u>90.27</u>	<u>21.91</u>	<u>2.90</u>	<0.88	<0.88	<u>3.36</u> *	<u>0.97</u> *	0.003
12	S.H.	Stein-Leventhal + Clomiphene	8.6 ml	<u>0.94</u>	<0.19	<u>1.42</u>	<0.19	<0.19	<u>18.31</u> *	<u>5.06</u> *	1.95

Underlined = values corrected for losses

* = total oestrogens

— = not assayed

Values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

In fluid No. 12, there was 18.3 μg oestradiol, the second highest amount in the present series of cyst fluids. Only 0.94 μg progesterone and 1.42 μg Δ^4 -androstenedione was detected, while 17 α -hydroxy-progesterone was undetectable. The relative proportions of steroids in this fluid were therefore the same as in fluid from Graafian follicles- although the steroid production was higher- and quite dissimilar from those observed in fluid from polycystic ovaries associated with the Stein-Leventhal syndrome (Table V and Table XVIII).

It will be noted that in this fluid from a follicular cyst, the steroid content differed from that of fluid No. 8, which was also aspirated from follicular cysts.

Despite the evident effect of gonadotropin administration upon the production of other steroids by these ovaries, DHA, testosterone and 20 α -hydroxy-pregn-4-en-3-one were not present in detectable amounts in any of the 5 fluids.

c) - Patients with functional uterine haemorrhage.

Clinical details and histological findings at operation in the 11 patients with functional uterine haemorrhage (F.U.H.) are reported in Table XV. Of these patients, five were approaching the menopause and presented the classical symptomatology and pathology of metropathia haemorrhagica; heavy, prolonged bleedings alternated with periods of amenorrhoea; the uterus was symmetrically enlarged and there was cystic glandular hyperplasia of the endometrium. Of the six patients with heavy bleedings at irregular intervals (metrorrhagia), only three were of menopausal age, and in all, with the exception of patient H.E., uterine fibroids or endometriosis were found at operation. It should be noted

DETAILS OF PATIENTS WITH F.U.H.

Table XV

Name	Age yrs	Duration of symptoms (months)	Menstrual periods	Operation	Findings at operation Ovaries	Others
S.T.	30	12	Prolonged, heavy, every 21-28 days	Hysterectomy, (L) salpingo-oophorectomy	(R) single cyst (8.0ml) ^o	Pelvic inflamm. Uterus: normal
R.O.	51	24	Heavy, lasting 5-21 to 60 days	Hysterectomy	(R) single cyst (3.7ml) ^o	Bulky cervix. C.g.h. ++
S.U.	50	6	Amenorrhoea 6/12, then 3 heavy, prolonged bleedings	Hysterectomy, bilateral salpingo-oophorectomy	(L)&(R) slightly enlarged, several cystic follicles (2.8 ml) ^o	Seedling fibroids
S.N.	48	12	Heavy for 14-21 days, every 28-35 days	Hysterectomy, bilateral salpingo-oophorectomy	(L) corpus luteum cyst (8.0 ml) ^o	Intramural fibroids.
H.E.	43	5	Irregular, flooding at times	Hysterectomy	(R) small cyst (2.5 ml) ^o	Uterus: normal
C.O.	43	24	Periods of amenorrhoea and bleeding for 35-60 days	Hysterectomy	(L)&(R) several small cysts; (L) cyst (6.6 ml) ^o	Uterus: increased in size. C.g.h. ++
G.R.	43	36	Heavy, prolonged, every 14-28 days	Hysterectomy, (L) salpingo-oophorectomy	(L) 2 follicular cysts (6.0 ml) ^o ; regr. corpus luteum; (R) sclerotic	Uterus: increased in size. C.g.h. ++
T.R.	35	7	Heavy, prolonged, every 30-32 days	Hysterectomy, (L) salpingo-oophorectomy	(L) corpus luteum haematoma; (R) cyst (1.3 ml) ^o ; (L)&(R) en- larged, several cystic follicles.	Uterus: fibroids, Pelvic endometriosis
S.M.	23	24	Heavy, for 15 days, every 28-42 days	Bilateral wedge resec- tion of ovaries	(L)&(R) enlarged x2, sev- eral cystic follicles; (L) regressing corpus luteum (9.2ml) ^o (R) follicular cyst (4.2 ml) ^o	Uterus: normal, Endometriosis
C.R.	52	20	Prolonged heavy bleeding & periods of amenorrhoea	Hysterectomy, (L) cystectomy	(L) large cyst (31.6 ml) ^o	Uterus; increased in size. C.g.h. ++
F.I.	45	20	Almost continuous bleeding for 6 months & periods of amenorrhoea	Hysterectomy, (L) ovary biopsy	(L) follicle cyst (1.2ml) ^o (R) regr. corpus luteum; single cyst (8.0 ml) ^o	Uterus: increased in size. C.g.h. ++

^o = volume of fluid aspirated at operation

++ = cystic glandular hyperplasia

that uncomplicated fibroids and endometriosis are not usually associated with metrorrhagia and that in the six patients there was no evidence that the bleeding was due to local uterine lesions. The cysts found in the ovaries of this group of patients were all, with the exception of that found in patient H.E., of medium to large size. In four patients there were enlarged polycystic ovaries, accompanied, in three instances, by a cyst of medium size. In the ovaries of four patients (S.R.; T.R.; S.M. and F.I.) there was either an active or a regressing corpus luteum and in patient S.T. endometrial biopsy indicated that ovulation had occurred in the previous cycle. A corpus luteum cyst was present in the ovary of patient S.N.

Table XVI, which shows the results of steroid determinations in the cyst fluid from patient with F.U.H. is tabulated in chronological order from the onset of the last bleeding. The results are expressed in μg per sample. Bearing in mind the reservations already advanced about the validity of comparison between results in different fluids, it would appear that in the cyst fluid from patients with F.U.H. the total amount of steroids varied within large limits : 0.28 to 55.03 μg . In eight patients, this total was within the range found in fluid from normally menstruating women.

It will be noted that there was no regular sequence in the production of various steroids in fluids taken at progressive intervals from a bleeding. Progesterone was present in ten cyst fluids obtained from 2 to 60 days after the onset of a bleeding and irrespective of the presence in the ovaries of a corpus luteum. 17 α -hydroxy-progesterone content was higher than that of progesterone in cyst fluids No. 17, ~~18~~ and 18, in both

STEROID DETERMINATION IN CYST FLUID FROM PATIENTS WITH F.U.H.

Table XVI

Fluid No.	Name	LMP (days)	Endometrium	Steroids in cyst fluid μ g per sample							Ratio OD/progest.
				volume	Progest.	17-hydroxy- progesterone	Δ^4 -andro- sterone	Testos- sterone	Oestradiol	Oestrone	
13	S.T.	2	ovular bleed ing	8.0ml	<u>2.40</u>	<0.26	<0.13	<0.26	<u>0.32</u>	<u>0.22</u>	0.13
14	R.O.	7	c.g.h. ++	3.7ml	4.39	2.70	0.43	<0.10	0.58	0.05	0.13
15	S.U.	9	postmenstrual	2.8ml	1.10	0.10	0.50	<0.10	2.58	0.20	2.34
16	S.N.	11	proliferative	8.0ml	<u>11.71</u>	<0.40	<0.20	<0.40	<u>15.50</u> *	<u>1.46</u> *	1.32
17	H.E.	11	proliferative	2.5ml	0.44	1.10	<0.10	<0.10	4.85	0.17	11.00
18	G.O.	11	c.g.h. ++	6.6ml	<u>0.88</u>	<u>1.52</u>	<u>1.79</u>	<0.20	<u>3.57</u> *	<u>0.45</u> *	4.00
19	G.R.	13	c.g.h. ++	6.0ml	27.54	10.61	0.40	<0.10	14.69	1.79	0.53
20	T.R.	16	proliferative	1.3ml	0.94	0.37	<0.10	<0.10	<0.05	<0.05	
21	S.M.	16	—	(L) 9.2ml	4.21	0.60	1.00	<0.10	1.31	0.05	0.31
22				(R) 4.2ml	<0.10	<0.10	<0.10	<0.10	—	—	
23	C.R.	60	c.g.h.++	31.6ml	<u>4.55</u>	<1.00	<0.50	<1.00	<u>6.00</u> *	<u>2.16</u> *	1.32
24	F.I.	90	c.g.h.++	(L) 1.2ml	<0.10	<0.10	<0.10	<0.10	0.28	<0.05	
25				(R) 8.0ml	<0.13	<0.26	<0.13	<0.26	<u>0.45</u> *	<u>0.34</u> *	

Underlined = values corrected for losses

* = total oestrogens ++ = cystic glandular hyperplasia

Values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

of which oestradiol was the predominant steroid. Δ^4 -androstenedione was present in 6 cyst fluids, two obtained from polycystic ovaries, (but was absent in fluids No. 20 and 22, which were also obtained from polycystic ovaries. Oestradiol was present in all cyst fluids, apart from No. 20, and even in fluids No. 24 and 25 obtained three months after a bleeding. In two cases, oestradiol concentration was much above the highest value found in fluids from normally menstruating women. The oestradiol content was generally higher than that of oestrone.

The progesterone content was higher than that of oestradiol in cyst fluids No. 13, 14, 19, 20 and 21, all collected from patients in whom bleeding was occurring at short intervals. The ratio oestradiol/progesterone showed no definite trend and it was less than unity both immediately after and at a long interval from a bleeding. Testosterone and 20α -hydroxy-pregn-4-en-3-one were not detected in any cyst fluid.

There was no apparent correlation between the steroid content of the cyst fluids and the histology of the cysts. Results obtained in patients with metrorrhagia did not differ in any way from results in patients with metropathia haemorrhagica. In only one patient (G.R.) the latter condition was associated with a cyst producing large amounts of steroids.

d) - Patients with the Stein-Leventhal syndrome.

Nineteen patients who were considered to have the Stein-Leventhal syndrome because of the clinical features and the presence of bilateral polycystic ovaries, were studied, (Table XVII). All had a history of menstrual irregularities ending in oligomenorrhoea or amenorrhoea and sterility. Hirsutism was present in fourteen patients, obesity in eight.

Table XVII - DETAILS OF THE PATIENTS WITH THE STEIN-LEVENTHAL SYNDROME.

Name	Age yrs.	Symptoms and duration	Hirsutism	Ovarian histopathology	Thickened albuginea	Theca cell hyperplasia
H.U.	20	Oligomenorrhoea (3-6/12) 4 years	Face, body ++ clitoris enlarg.	Polycystic, enlarged; (L)serous cyst	+	-
H.C.	25	Oligomenorrhoea (2-3/12) 12 years; sterility	Face, body, legs ++	Polycystic, enlarged	-	-
K.D.	19	Oligomenorrhoea (28d-6/12) 2 years;	Face, body, legs ++ male escutcheon	Polycystic, enlarged x3; atretic corpora lutea	-	-
S.D.	38	Oligomenorrhoea (1-2/12) 5 years; obesity, sterility	Face ++, chest ++ legs +	Polycystic, enlarged x2	+	+
W.A.	23	Oligomenorrhoea (28d-2/12) 4 years;	Face, body, legs ++	Polycystic, enlarged; (L)corpus luteum	-	-
R.I.	24	Oligomenorrhoea (3-4/12) 11 years; sterility	Face, chest +	Polycystic, normal size	+	+
M.O.	17	Oligomenorrhoea (5-8/52) 3 years; obesity	Face, body, legs ++	Polycystic, enlarged	+	-
B.A.	22	Oligomenorrhoea (4-8/12) 8 years; obesity	Face, legs +	Polycystic, enlarged	-	-
P.E.	27	Oligomenorrhoea (2-9/12) 10 years; sterility	Face, legs ++	Polycystic, normal size	++	+
McC.	21	Oligomenorrhoea (2-6/12) 7 years	Face, legs ++	Polycystic, enlarged	+	-
C.U.	23	Second. amenorrhoea, 6 years; obesity	Face, legs ++	Polycystic, enlarged x2	+	+
V.I.	30	Second. amenorrhoea, 15 years; sterility	Face, chest +	Polycystic, enlarged; atretic corpora lutea	-	-
G.B.	23	Second. amenorrhoea, 2 years; obesity	Face ++	Polycystic, enlarged x2; atretic corpora lutea	++	+
F.O.	28	Second. amenorrhoea, 2 years; sterility, obesity	Face ++	Polycystic, enlarged; several early corpora lutea	-	-
H.O.	26	Oligomenorrhoea (1-2/12) 7 years; sterility	—	Polycystic, enlarged	+	-
G.O.	24	Second. amenorrhoea, 2 years; obesity	—	Polycystic, enlarged; (R) (L); (R) corpus luteum	-	-
P.L.	27	Second. amenorrhoea 2 years; obesity, sterility	—	Polycystic, slightly enlarg. (R) regressing corpus luteum, (L) corpus luteum haematoma	+	-
W.I.	24	Second. amenorrhoea, 3 years	—	Polycystic, normal size; (R) regressing corpus luteum	-	-
S.L.	30	Second. amenorrhoea 14 years; sterility, obesity	—	Polycystic, enlarged	+	-

Breast development was normal in all patients. In patients H.U. and K.D., hirsutism was accompanied by slight signs of virilisation. After bilateral wedge resection regular periods were restored in all patients, and one became pregnant. There was little or no change in the hirsutism.

The ovaries were enlarged from two to three times the normal size in fifteen patients and were of normal size in the remainder. Histological examination of ovarian tissue revealed thickening of the albuginea in eleven patients and theca cell hyperplasia in four. Neither of these features was evident in the wedges from the remaining eight patients. No mature Graafian follicles were seen. A recent corpus luteum was present in the wedges from five patients, but, in general, corpora lutea and corpora albicantia were fewer than in ovaries of normally menstruating women of comparable age.

The results of the determinations of steroids in the cyst fluid from patients with the Stein-Leventhal syndrome are shown in Table XVIII. For the reasons already given, they are expressed as $\mu\text{g}/100\text{ ml}$ of fluid. The volume of fluid available for analysis was smaller in this group of patients than in the three preceding groups. It appears evident from Table XVIII that in dealing with volumes of fluid around 1.0 ml it might have been impossible to detect significant concentrations of steroids.

The steroid content of the cyst fluid obtained from patients with the Stein-Leventhal syndrome showed a definite pattern. Δ^4 -androstenedione was present in sixteen out of twenty fluids and was the predominant steroid in 15 fluids. The concentration of Δ^4 -androstenedione ranged from 9.2 to 161.2 $\mu\text{g}/100\text{ ml}$; it was higher in fluids obtained at long

Table XVIII - STEROID DETERMINATION IN CYST FLUID FROM PATIENTS WITH THE STEIN-LEVENTHAL SYNDROME.

Fluid No.	Name	LMP	Endometrium	Steroids in cyst fluid, µg/100 ml							
				Volume	Progest.	17-hydroxy- progesterone	Δ ⁴ -andro- sterone	Testosterone	DHA	Oestradiol	Oestrone

26	H.U.	9days	Proliferative	3.5 ml	<2.8	<2.8	<2.8	<2.8	—	<1.5	<1.5
27	H.C.	18days	—	3.3 ml	<3.0	36.0	18.0	<3.0	—	39.0	8.1
28	K.D.	30days	proliferative	1.4 ml	<7.1	<7.1	16.4	<7.1	<7.1	<3.5	<3.5
29	S.D.	31days	proliferative	1.3 ml	<7.7	<7.7	<u>38.5</u>	<7.7	<7.7	<3.8	<u>9.5</u> *
30	W.A.	32days	—	2.4 ml	7.1	8.3	25.4	—	<4.0	<2.0	<2.0
31	R.I.	51days	—	4.3 ml	<2.3	2.3	9.2	<2.3	—	1.6	<1.5
32	M.O.	53days	—	0.6 ml	<16.6	<u>20.0</u>	<u>53.5</u>	<16.6	<16.6	<8.3	<8.3
33	B.A.	3months	—	2.8 ml	<3.5	<3.5	99.7	<3.5	—	—	—
34	P.E.	3months	c.g.h. ++	1.1 ml	<8.3	<8.3	31.8	<8.3	—	<4.0	<4.0
35	McC.	5months	—	3.2 ml	<3.1	<6.2	<u>161.0</u>	<6.2	<6.2	<1.2	<3.1
36	C.U.	6months	c.g.h. ++	4.1 ml	<2.5	<2.5	70.7	<2.5	<2.5	<1.2	<1.2
37	V.I.	15months	—	3.0 ml	30.0	<3.3	26.7	<3.3	—	<1.6	<1.6
38	G.B.	18months	proliferat.	0.6 ml	<8.8	<16.6	<u>23.3</u>	<16.6	<16.6	<4.2	<4.2
39	F.O.	2years	—	0.5 ml	<u>32.0</u>	—	<u>36.0</u>	<20.0	<20.0	<u>8.0</u> *	<10.0
40	H.O.	50days	—	2.0 ml	<2.5	<5.0	<u>37.5</u>	<5.0	<5.0	<u>3.2</u> *	<2.5
41			—	0.7 ml	<u>85.8</u>	<14.3	<u>35.8</u>	<14.3	<14.3	<u>42.8</u> *	<2.8
42	G.O.	3months	—	0.6 ml	<8.3	<16.6	<8.3	<8.3	<u>66.8</u>	<u>8.3</u> *	<u>23.2</u> *
43	P.L.	11months	—	4.3 ml	<2.3	<2.3	60.3	<2.3	—	—	—
44	W.I.	1year	—	1.8 ml	<u>8.2</u>	<u>33.1</u>	<u>49.8</u>	<11.1	<11.1	<2.8	<2.8
45	S.L.	2years	—	3.6 ml	<2.7	<2.7	36.0	<2.7	<2.7	2.5	<1.4

underlined = values corrected for losses ++ = cystic glandular hyperplasia

* = total oestrogens

Values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

intervals from the last menstrual period, and the level was consistently high in patients with amenorrhoea. The concentration in the cyst fluid from the five non-hirsute patients (Nos. 40 to 45) was well within the range of that found in hirsute women, varying between 35.8 and 60.3 $\mu\text{g}/100\text{ ml}$. However, all the non-hirsute patients had not had a menstrual period for at least 50 days, and, as just mentioned, Δ^4 -androstenedione concentration in the fluid seemed to increase with the duration of amenorrhoea. In the cyst fluid from one non-hirsute patient (G.O.), DHA- and not androstenedione- was present, in a concentration of 66.8 $\mu\text{g}/100\text{ ml}$. This was the only fluid in which DHA was present in detectable amounts. It is of interest that the most potent androgen, testosterone, was undetectable in all cyst fluids.

Progesterone, 17 α -hydroxy-progesterone and 20 α -hydroxy-pregn-4-en-3-one were either undetectable or present in low concentration, with few exceptions. Thus, progesterone was present in 5 fluids only, in concentration varying from 7.1 to 85.8 $\mu\text{g}/100\text{ ml}$. Some of these fluids had been aspirated from ovaries containing either old or recent corpora lutea; but, on the other hand, other fluids also aspirated from ovaries presenting corpora lutea did not contain progesterone in detectable amounts. It must be noted that progesterone was found in fluid No. 40, aspirated after operation from the ovarian wedges of patient H.O., and not in fluid No. 41, aspirated at operation from the ovaries of the same patient. This stresses the difficulties inherent to the collection of fluid representative of the activity of the whole ovary. 17 α -hydroxy-progesterone was present in 5 fluids in concentrations ranging from 2.3 to 36.0 $\mu\text{g}/$

100 ml; its presence was not always associated with that of progesterone.

Oestradiol was present in higher concentration than Δ^4 -androstenedione in fluid No. 27 and ^{in No.} 41, which contained a substantial concentration of progesterone. In fluids No. 31, 39, 40, 41 and 45, oestradiol concentration was low, between 1.6 and 8.3 $\mu\text{g}/100\text{ ml}$. Oestradiol was undetectable in the remaining 13 fluids. Oestrone was present in only three fluids. It should be pointed out that oestrogens were found in 55% of the fluids examined with the more sensitive method and in only 33% of those examined with the less sensitive method. This might have been a coincidence, but it cannot be excluded that amounts of oestrogens below the sensitivity of the latter method were present in some fluids.

The results obtained in cyst fluid from enlarged ovaries, or ovaries with markedly thickened albuginea and/or hyperplasia of the theca cells did not differ from those in cyst fluid taken from ovaries which did not show these features. Therefore a relative increase in the proportion of Δ^4 -androstenedione in the cyst fluid accompanied by a decrease in the proportion of oestradiol is not correlated to any of the above mentioned histological abnormalities. It appeared to be a more constant finding in the Stein-Leventhal syndrome than the histological changes which have been considered until recently classical of the syndrome.

e) - Patients with polycystic ovaries not associated
with the Stein-Leventhal syndrome.

The type of ovary considered in this sub-section is, as already described in Chapter I, section 3, usually not enlarged and contains numerous atretic follicles. These follicles fail to undergo rapid

cicatrization, as in the case of the atretic follicles in the ovary of the Stein-Leventhal syndrome (Leventhal, 1958). Results of steroid determination in fluid from such ovaries could therefore be used as controls for the results obtained in the syndrome.

The details of the patients with polycystic ovaries are reported in Table XIX. Patients S.U. and S.M. have already been considered in the group of patients with F.U.H., but are included in the present group in order to compare steroid production in polycystic ovaries associated with metrorrhagia with that of polycystic ovaries associated with other gynaecological conditions. In the remaining eleven patients, the indications for surgery were uterine fibroids, sometimes accompanied by heavy menstrual loss (7), sterility (3) and dysmenorrhoea (1). The average age of this group was higher than that of the Stein-Leventhal patients. Six patients had always had regular menstrual periods; two complained of scanty but regular periods (hypomenorrhoea) and one of oligomenorrhoea. This last patient (C.K.), presented diffuse pelvic inflammation. The hair distribution was normal in all patients, except in patient C.I. who had slight facial hirsutism and patient A.X. who was hirsute on the face and on the body.

The ovaries of five patients were of normal size and contained several small cysts bilaterally. The ovaries were slightly enlarged in eight patients and had numerous cysts of various size. Histological examination was performed and revealed cystic follicles in all patients. Corpora lutea were present in the ovary of the last seven patients in the Table. The left ovary of patient J.O. contained a small corpus luteum cyst.

Table XIX - DETAILS OF PATIENTS WITH POLYCYSTIC OVARIES.

Name	Age yrs	Symptoms and duration	Menstrual periods	Operation	Findings at operation Ovaries	Others
J.O.	41	Abdominal pain, 6 months	6/28	Hysterectomy, L.S.O.	Polycystic, normal size; (L) small corpus luteum cyst (1.1ml)°	Fibromyoma
S.I.	41	Abdominal pain, 19 months	6/28	Hysterectomy	Polycystic, normal size (1.6ml)°	Fibromyoma
M.L.	24	Dysmenorrhoea, 11 years	7-11/28	Bilateral di- vision ovarico- pelvic ligaments	Polycystic, slightly enlarged, (0.6 ml)°	Uterus: normal
S.U.	50	Metrorrhagia, 3 months	irregul.	Hysterectomy B.S.O.	Polycystic, slightly en- larged, (2.8 ml)°	Uterus: seedling fibroids
C.L.	37	Abdominal pain, 5 years	4-5/28	Hysterectomy	Polycystic, slightly en- larged, (2.4ml)°; endometriosis	Fibromyoma
C.I.	36	Abdominal pain, 18 months; hirsutes of face	5-6/28	Hysterectomy	Polycystic, normal size (1.6 ml)°	Fibromyoma
M.S.	36	Mass in abdomen, 3 months	6/24	Hysterectomy	Polycystic, normal size (0.6ml)°; (L) corpus luteum	Fibromyoma
S.M.	23	Metrorrhagia, 15 months	5-7/28-42 heavy loss	Bil. wedge res.	(R) enlarged x2, cystic (4.2ml)° (L) follicular cyst, corp. luteum	Pelvic endometriosis
C.H.	43	Menorrhagia, 6 months heavy loss	7/28	Hysterectomy	Polycystic, enlarged (1.1ml)° (R) corpus luteum	Fibromyoma
A.X.	32	Hypomenorrhoea, 4 years; sterility, hirsute	2-3/28 scanty	Bil. wedge res.	Polycystic, enlarged; (R) > (L), (1.0ml)°; (L) corp. luteum	Uterus: normal
A.B.	39	Menorrhagia, 7 years	8/25 Heavy loss	Hysterectomy, L.S.O.	Polycystic, enlarged; (L) corp. luteum, corp. luteum haematoma (2.2 ml)°	Uterus: small fibroids
B.E.	26	Sterility, 5 years	1-2/25 scanty	(R) wedge res.	Polycystic, normal size, (0.9ml)° tubal insuffl. capsule slightly thickened, corp. luteum	Chronic salpingitis
C.K.	34	Oligomenorrhoea, 11 years; Sterility	5/42-120	Bil. wedge res.	Polycystic, enlarged (0.9ml)°; (R) corp. luteum, corp. luteum haematoma.	Retroverted uterus.

° = volume of fluid aspirated

L.S.O. = Left salpingo-oophorectomy,

B.S.O. = Bilateral salpingo-oophorectomy.

Endometrial biopsy in eight patients showed a pattern corresponding to the phase of the menstrual cycle.

Results obtained in cyst fluid from patients with polycystic ovaries are reported in Table XX. The results are expressed in $\mu\text{g}/100\text{ ml}$.

The steroid content in cyst fluid from this group of patients varied considerably. In fluids No. 22, 51 and 52, obtained in the luteal phase, no steroid could be detected and fluid no. 46, aspirated from a corpus luteum cyst at the 4th day of a cycle, did not contain any steroid in detectable amounts. The volume of some of these fluids was small and significant concentrations below the limit of sensitivity of the method might have been present.

Fluid No. 49 should be considered apart, since the steroid pattern was very similar to that observed in Graafian follicles (see Table XXI); probably it was aspirated from a pre-ovulatory follicle not recognized at operation.

In the remaining eight fluids, Δ^4 -androstenedione was present in concentrations from 12.3 to 100.0 $\mu\text{g}/100\text{ ml}$ and was the predominant steroid in 5 fluids. These values are well within the range observed in the fluid of patients with the Stein-Leventhal syndrome. Progesterone was detected in 4 fluids in concentrations from 6.2 to 39.2 $\mu\text{g}/100\text{ ml}$, 17 α -hydroxy-progesterone was present in detectable amounts in only two fluids. Oestrogens were detected in 3 fluids; oestradiol in concentrations from 10 and 92.8 $\mu\text{g}/100\text{ ml}$ and oestrone from 4.1 to 7.1 $\mu\text{g}/100\text{ ml}$. The concentration of oestrogens was generally lower in the luteal phase, while no significant variation in the concentration of the

Table XX - STEROID DETERMINATION IN CYST FLUID FROM POLYCYSTIC OVARIES.

Fluid No.	Name	LMP	Endometrium	Steroids in cyst fluid, µg/100 ml						
				Volume	Progest.	17-hydroxy-progesterone	Δ ⁴ -andro-stenedione	Testosterone	Oestradiol	Oestrone

46	J.O.	4th	proliferative	1.1 ml	<9.1	<9.1	<9.1	<9.1	<4.5	<4.5
47	S.I.	4th	proliferative	1.6 ml	<6.2	<6.2	37.5	<6.2	5.0	<3.1
48	M.L.	4th	—	0.6 ml	<8.1	<16.3	16.3	<16.3	<3.2	<3.2
15	S.U.	9th	post-menstrual	2.8 ml	39.2	3.6	17.8	<3.5	92.8	7.1
49	C.L.	12th	proliferative	2.4 ml	95.7	82.2	<4.1	<4.1	249.6	25.3
50	C.I.	13th	proliferative	1.6 ml	6.2	13.0	56.2	<6.2	58.7	3.1
51	M.S.	15th	proliferative	0.6 ml	<16.6	<16.6	<16.6	<16.6	<8.3	<8.3
22	S.M.	16th	—	(R) 4.2 ml	<2.3	<2.3	<2.3	<2.3	—	—
52	C.H.	19th	early secretory	1.1 ml	<9.1	<9.1	<9.1	<9.1	<4.5	<4.5
53	A.X.	19th	—	7.0 ml	<u>17.6</u>	<3.3	<u>36.0</u>	<3.3	<u>1.0</u>	<1.5
54	A.B.	21st	early secretory	2.2 ml	<u>27.8</u>	<4.5	<u>100.0</u>	<4.5	<0.9	<u>4.1</u>
55	B.E.	26th	—	0.9 ml	<11.0	<11.0	12.3	<11.0	5.5	<5.5
56	C.K.	30th	—	0.9 ml	<5.5	<11.0	<u>44.4</u>	<11.0	<u>9.9</u> *	<u>6.6</u> *

* = total oestrogens

underlined = values corrected for losses

Values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

other steroids was noted in relation to the phase of the cycle.

There was no correlation between androstenedione concentration in the fluid and the presence of hirsutism; nor between steroid pattern in the fluid and the type of menstrual disorder.

Since results of steroid determination in Graafian follicles and follicular cysts have been reported in the previous Tables in μg per sample, they are again shown in Table XXI in $\mu\text{g}/100\text{ ml}$. By comparing the data in Table XVIII, XX and XXI, it appears evident that Δ^4 - androstenedione is the predominant steroid in fluids from polycystic ovaries whether or not these are associated with the Stein-Leventhal syndrome, and that androstenedione concentration in these fluids is higher than in Graafian follicles or follicular cysts. On the contrary, progesterone and oestrogen concentration is much lower in fluids from polycystic ovaries than in fluid from follicles or cysts derived from follicles.

2 - Determination of neutral and phenolic steroids in ovarian venous blood.

Neutral steroids and free oestrogens were determined in 8 samples of ovarian venous blood by means of the same method employed for cyst fluid. Either plasma or whole blood was analysed. Steroid determination in cyst fluid from the same ovary was performed concurrently. Results in 5 fluids have already been described.

Clinical details of patients L.E., J.O., M.K., T.R. and F.I. have been given in the previous section. Patient M.A., 33 years old, had

Table XXI - STEROID CONCENTRATION IN GRAAFIAN FOLLICLES AND SMALL FOLLICULAR CYSTS.

Fluid No.	volume	STEROIDS			µg/ 100 ml	
		Progesterone	17 α -hydroxy- progesterone	Δ^4 -andro- stenedione	Oestradiol * °	Oestrone * °
1	2.7 ml	34.5	22.2	43.7	204.1	41.8
2	2.7 ml	11.1	14.8	17.5	89.1	2.2
3	1.6 ml	363.5	69.7	46.2	206.2	13.2
4	2.0 ml	45.0	5.0	16.5	156.0	11.0
5	2.8 ml	89.3	43.2	41.7	153.0	1.7
14	3.7 ml	118.6	72.9	11.4	5.6	1.3
17	2.5 ml	17.6	44.0	44.0	234.2	26.8

* ° Free oestradiol and oestrone.

The values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

regular cycles but complained of sterility; tubal insufflation was carried out at the 25th day of a cycle. The right ovary contained a cystic corpus luteum from which 0.5 ml of blood-stained fluid was aspirated. Patient T.O., a girl aged 12 years, had profuse bleeding for 5 months; the left ovary contained a theca lutein cyst of medium size; 5.4 ml of fluid was collected from this cyst at operation.

In Table XXII results of steroid determination in cyst fluid and in ovarian venous blood are reported in $\mu\text{g}/100$ ml of fluid or plasma.

* Isotopically labelled internal standards were used for all the analyses in blood and for certain fluids, as indicated in the Table.

The volume of plasma was generally larger than that of fluid, and therefore it should have been possible to detect lower concentrations, as little as 1.0 to 3.1 $\mu\text{g}/100$ of neutral steroids and 0.5 to 1.0 $\mu\text{g}/100$ ml of oestrogens. However, steroids were detected in 5 cyst fluids and in only 3 blood samples, indicating that the concentration in blood was lower than in fluid.

In the case of patient M.K., who had been treated pre-operatively with FSH and HCG, blood samples from the two ovarian veins were examined. The blood from the right ovarian vein was draining three corpora lutea. In the left ovary there were follicular cysts and atretic follicles but no active * corpus luteum. Both samples of blood contained progesterone, free oestradiol and oestrone, but no detectable 17 α -hydroxy-progesterone or androstenedione. The steroid concentration, especially of progesterone, was higher in the effluent blood from the right ovary. The relative proportions of the steroids were different in cyst fluid and blood

Table XXII - DETERMINATION OF NEUTRAL STEROIDS AND FREE OESTROGENS IN CYST FLUID AND OVARIAN VENOUS BLOOD.

Name	Fluid No.	Blood volume	Steroids $\mu\text{g}/100$ ml of fluid or plasma									
			Progesterone		17 α -progesterone		A ⁴ -androstenedione		Oestradiol ^{oo}		Oestrone ^{oo}	
			fluid	blood	fluid	blood	fluid	blood	fluid	blood	fluid	blood
I L.E.	4	4.2 ml	45.0	42.4	45.0	42.4	16.5	42.4	156.0	41.2	11.0	41.2
II J.O.	46	9.0 ml	49.1	41.0	49.1	41.0	49.1	41.0	44.5	40.5	44.5	40.5
III M.A.	57	3.3 ml	410.0	43.1	420.0	43.1	420.0	43.1	410.0	41.5	410.0	41.5
IV M.K.	8 (L)	1.6 ml	<u>435.0</u>	<u>27.4</u>	<u>75.0</u>	43.1	<u>32.0</u>	43.1	<u>26.5</u>	<u>6.2</u>	<u>18.7</u>	<u>5.0</u>
	(R)	1.6 ml		<u>125.0</u>		43.1		43.1		<u>10.6</u>		<u>8.5</u>
V T.R.	20	2.7 ml	72.2	<u>12.2</u>	15.4	42.5	47.7	42.5	43.8	41.2	43.8	41.2
VI F.I.	24 (L)	5.6 ml	^(L) 28.3	41.8	48.3	41.8	48.3	41.8	23.3	40.9	44.2	40.9
VII T.O.	58	3.3 ml	<u>34.8</u>	41.5	<u>30.0</u>	—	<u>35.5</u>	41.5	<u>156.6</u>	40.6	<u>23.8</u>	40.6

^{oo} = free oestradiol and oestrone
underlined = values corrected for losses
 — = not assayed

Values preceded by the sign < were calculated from the volume of the samples and the limit of sensitivity of the method.

from the left ovary. The ratio oestradiol/progesterone was 0.06 in the fluid and 0.22 in the blood; the ratio progesterone/17 α -hydroxy-progesterone was 5.7 in the fluid and >9.9 (27.4/43.1) in blood.

In case V, the ovary contained a corpus luteum haematoma in addition to a small cyst. Progesterone was present in the fluid in a concentration of 72.2 μ g/100 ml and in the blood in a concentration of 12.2 μ g/100 ml.

In cases I and VII, free oestradiol concentration was very high in the fluid and undetectable in blood. It may be relevant that fluid No.4 was aspirated from a Graafian follicle and fluid No. 58 from a theca lutein cyst in the absence of ovulation. In case VI, the fluid obtained from a follicular cyst during the luteal phase contained 23.3 μ g/100 ml oestradiol but the concentration of oestradiol in the blood was less than 0.9 μ g/100ml.

It is evident that oestradiol concentration in the blood was not in the same relationship to concentration in the fluid in the different cases. Thus, in case IV, oestradiol concentration was about 1/4 of that in the fluid and in cases I, VI and VII it was less than 1/130, 1/26 and 1/280 respectively. The same applies to progesterone. Progesterone concentration in the blood was 1/60 of that in fluid in case IV, 1/6 in case V and less than 1/20 in case VII. 17 α -hydroxy-progesterone and/or Δ^4 -androstenedione were present in substantial amounts in fluids No.4, 8, 20 and 58, but were undetectable in the blood samples from the same ovary.

3 - Determination of free and conjugated oestrogens
in cyst fluid.

Separate determination of free and conjugated oestrogens was performed on 23 cyst fluids. The results are shown in Table XXIII. The fluids were divided into four groups : those obtained from women with regular ovulatory cycles, from patients under gonadotropin stimulation, from patients with functional uterine haemorrhage and from patients with the Stein-Leventhal syndrome. Clinical details of 19 patients and the results of oestrogen determination as 'total' oestradiol and oestrone have been reported in the previous section.

Results in four additional cyst fluids are shown in Table XXIII. In these, neutral steroids were not assayed. Fluid No. 59, 2.8 ml in volume, and No. 60, 2.4 ml, were aspirated at the 10th and 13th day of a cycle respectively from cystic follicles in the ovaries of patients undergoing hysterectomy for fibromyoma. Patient C.J., fluid No. 61, had menorrhagia and was operated upon at the 14th day of the cycle; a cystic corpus luteum was found in the left ovary and 0.7 ml of fluid aspirated. Patient P.M., fluid No. 62, complained of profuse, irregular bleeding for many years; the last bleeding had occurred 16 days before operation. A follicular cyst was present in the right ovary; 5.4 ml of Fluid was collected.

In order to include the results obtained in polycystic ovaries in an overall comparison, the results in Table XXIII are expressed as $\mu\text{g}/100\text{ ml}$. In addition to results expressed in this way and the ratios, the 'total' oestrogens is expressed as μg per sample, to permit evaluation of

Table XXIII - DETERMINATION OF FREE AND CONJUGATED OESTROGENS IN CYST FLUID.

Fluid No.	Name	'Total' oestrogens in sample	OESTRADIOL-17 β		OESTRONE		Ratio free/conjug. oestradiol	Ratio free/conjug. oestrone
			μ g/100 ml free	μ g/100 ml conjug.	μ g/100 ml free	μ g/100 ml conjug.		
<u>Patients with ovulatory cycles</u>								
48	M.L.	<0.1 μ g	<3.2	<3.2	<3.2	<3.2		
59	N.W.	2.9 μ g	89.8	5.3	6.2	1.2	16.8	5.1
60	B.P.	3.6 μ g	98.4	18.7	31.8	2.6	5.2	12.2
61	C.J.	2.0 μ g	198.8	30.6	23.3	39.4	6.4	0.6
53	A.X.	0.2 μ g	2.0	<0.6	<1.6	<1.6		
7	S.R.	0.8 μ g	2.5	3.0	5.0	3.0	0.7	1.6
56	C.K.	0.2 μ g	5.5	9.4	—	9.4	0.6	
<u>Patients under gonadotropin stimulation</u>								
8	M.K.	7.9 μ g	26.5	7.0	18.7	4.5	3.8	4.1
9	D.R.	2.1 μ g	2.8	8.9	3.2	2.2	0.3	1.5
10	S.B.	9.7 μ g	6.0	5.2	29.7	7.6	1.1	3.9
11	E.E.	4.3 μ g	11.0	1.9	2.0	1.3	5.8	1.5
<u>Patients with F.U.H.</u>								
58	T.O.	11.1 μ g	166.6	24.4	23.8	10.3	6.8	2.3
13	S.T.	0.5 μ g	2.2	2.2	2.9	<0.6	1.0	
18	C.O.	4.0 μ g	32.5	26.5	4.0	3.3	1.2	1.2
62	P.M.	4.8 μ g	50.4	30.1	4.5	3.5	1.6	1.3
23	C.R.	8.2 μ g	10.0	9.0	2.5	4.3	1.1	0.5
25	F.I.	0.8 μ g	<0.6	6.3	<0.6	4.3		
<u>Patients with the Stein Leventhal syndrome</u>								
29	S.D.	0.1 μ g	<3.8	<3.8	9.5	<3.8		
35	McC.	<0.1 μ g	<1.2	<3.1	<1.2	<3.1		
39	F.O.	0.1 μ g	8.0	<10.0	<5.0	<10.0		
40	H.O.	0.3 μ g	31.4	11.4	<2.5	<2.5	2.8	
42	G.O.	0.2 μ g	<5.5	8.3	<11.5	23.2		
12	S.H.	22.4 μ g	191.7	7.5	45.8	9.1	25.5	5.0

— not assayed.

All the values were corrected for losses. Values preceded by the sign < were calculated from the volume of fluid assayed and the limit of sensitivity of the method.

oestrogen production. All results were corrected for losses.

It will be seen that, with the exception of No. 48 and 35, all the fluids contained free and/or conjugated oestrogens in detectable amounts. With the reservations already advanced on the comparison of quantitative results in different fluids, it would appear that the amount of 'total' oestrogens differed in the four groups of fluids; it ranged from undetectable to 3.6 μg in group I, from 2.1 to 7.9 μg in group II; from 0.5 to 11.1 μg in group III and from undetectable to 0.3 μg in the last group (excluding fluid No. 12, which was obtained after treatment with Clomiphene).

In the fluids of the first group, the concentration of free oestradiol varied according to the phase of the cycle, and higher concentrations were found in fluids aspirated from cystic follicles (No. 59 and 60) or a cystic corpus luteum at the 14th day of a cycle (No. 61) than in fluids collected late in the luteal phase (No. 7, 53 and 56). The concentration of conjugated oestradiol and oestrone was low relative to that of the free compounds in fluids collected during the follicular phase. On the contrary, in the luteal phase, conjugated oestrogen concentration was about the same or even higher than that of free oestrogens. This is clearly illustrated by a comparison of the ratios free/conjugated reported in the last two columns of Table XXIII.

Two other fluids, No. 12 and 58, exhibited relative proportions of the oestrogens similar to those observed in fluids obtained from cystic follicles. They were collected from an ovarian cyst in a patient with the Stein-Leventhal syndrome treated pre-operatively with Clomiphene

(fluid No. 12) and from the ovary of a girl with prolonged bleeding (fluid No. 58) . Both the cysts were of the follicular type. Ovulation had not occurred at the time of operation in these two patients.

In group II, fluid No. 8 and 11 were aspirated from follicular and theca lutein cysts and the ratio free/conjugated oestradiol was similar to that observed in fluids of group I obtained in the follicular phase of the cycle. Fluid No. 9 and 10, collected from a corpus luteum cyst and a large cyst in pregnancy, had ratios free/conjugated near or below unity as in the late luteal fluids in group I.

Considering the values for the two oestrogens in each fluid, it will be noted that the ratio free/conjugated oestradiol differed from that free/conjugated oestrone in all fluids of group I and II.

Four fluids of group III, obtained from patients with irregular bleeding, were characterized by a concentration of conjugated oestrogens almost equal to that of the free compounds. In a fifth fluid, No. 25, only conjugated oestrogens were detected. The ratio free/conjugated oestradiol- as well as that free/conjugated oestrone- approximated unity, as in the luteal phase of the cycle.

In fluids from polycystic ovaries of patients with the Stein-Leventhal syndrome the amount of oestrogens per sample was small or undetectable. However, it would appear that polycystic ovaries associated with the Stein-Leventhal syndrome are still capable of producing oestrogens when adequately stimulated, since fluid No. 12 contained a very large amount of free oestrogens.

Summarizing the main findings, it would appear that in fluids obtained from follicles, follicular cysts and early corpora lutea, the concentration of free oestrogens was much higher than that of conjugated oestrogens. In fluids obtained from cystic corpora lutea, there was a similar concentration of free and conjugated oestrogens. In all the fluids obtained from patients with F.U.H., irrespective of the histological structure of the cysts or the presence of corpora lutea, almost as much conjugates as free steroids were detected. Fluids from polycystic ovaries associated with the Stein-Leventhal syndrome, contained very little or undetectable free oestrogens, whereas conjugated oestrogens could be detected in only two out of seven fluids.

4 - Determination of free and conjugated oestrogens in ovarian venous blood.

Determination of free and conjugated oestrogens was performed in 3 samples of ovarian venous blood and the results are shown in Table XXIV. Clinical details of the first patient have been given in the previous section. Patient W.O., aged 49 years, was one year post-menopausal but the endometrium still showed proliferative changes; the indication for surgery was fibromyoma. Patients F.L., 30 years old, also underwent hysterectomy for fibromyoma; her cycles were regular and the operation was performed at the 22nd day of a cycle.

It will be seen that all the samples contained conjugated oestradiol or oestrone in detectable amounts. In addition, sample VIII contained free oestradiol. Conjugated oestrogen concentration varied between 0.7 and 3.2 $\mu\text{g}/100\text{ ml}$ of plasma and was much lower than that found in the

Table XXIV - DETERMINATION OF FREE AND CONJUGATED OESTROGENS IN OVARIAN
VENOUS BLOOD.

Sample No.	Name	Plasma volume	Oestradiol free	µg/100 ml conjugated	Oestrone free	µg/100 ml conjugated
VII	T.O.	3.3 ml	<0.6 (166.6)	1.8 (24.4)	<0.6 (23.8)	4.7 (10.3)
VIII	W.O.	3.2 ml	1.5	0.7	<0.6	<0.6
IX	F.L.	2.4 ml	<0.8	<0.8	<0.8	3.2

Values preceded by the sign < were calculated from the volume of plasma assayed and the limit of sensitivity of the method.

In parentheses, oestrogen concentration in the fluid, µg/100 ml.

majority of cyst fluids (Table XXIII). In case VII the fluid contained a high concentration of free oestradiol and low concentration of the conjugates, whereas in blood conjugates were present in larger amounts than the free oestrogens. Accordingly, in this preliminary series, the relative proportions of free to conjugated oestrogens were different in fluid and blood.

5 - Comparison between results in cyst fluid and upon incubation of the cyst wall.

In a few cases, the capacity of the cyst lining to transform isotopically labelled steroid precursors 'in vitro' was studied by departmental colleagues (Forleo & Collins, 1964; Collins & Sommerville, 1965; Collins, 1965). Reference has already been made to the problems involved in the interpretation of the results obtained 'in vitro', but it was of interest to attempt to correlate this information with the results of analyses of cyst fluid.

For each experiment 'in vitro', 0.2-0.5 gm of tissue was minced with Kreb's Ringer phosphate buffer; appropriate coenzymes (DPN, DPNH, TPN, TPNH, ATP and sheep FSH) were added to the medium. The incubation time was 5 hours. Identification of the various metabolites was achieved by means of chromatography on paper or thin layer, formation of derivatives, paper or thin layer chromatography of the derivatives and finally gas-liquid radio-chromatography. The results of the incubation experiments with 4-¹⁴C-progesterone and 4-¹⁴C- Δ^5 -pregnenolone are reported in Table XXV. They are expressed as percentage conversion of the

Table XXV - COMPARISON OF 'IN VIVO' and 'IN VITRO' RESULTS.

Name Steroids	Cyst fluid	µg/sample	Incubation 'in vitro' percentage conversion substrate	
D.O.	No. 6		precursor : progesterone	
Progesterone		0.10	25.2	
17α-progesterone		0.20	15.5	
20α-pregn-4-en.		< 0.10	3.8	
17α-20 ξ-progesterone		—	33.5	
Δ ⁴ -androstenedione		< 0.10	1.1	
Testosterone		< 0.10	1.9	
Oestradiol-17β *		< 0.05	0.7	
Oestrone *		< 0.05	0.003	
M.K.	No. 8		precursor : progesterone	
			follicle	atretic follicle
Progesterone		77.50	4.3	25.5
17α-progesterone		10.22	34.2	21.7
20α-pregn-4-en		< 0.25	1.2	nil
17α-20 ξ-progesterone		—	27.2	2.3
Δ ⁴ -androstenedione		5.21	6.7	26.4
Testosterone		< 0.25	77.6	1.7
Oestradiol-17β *		3.65	1.5	0.09
Oestrone *		2.62	0.15	0.03

* Free oestrogens

Table XXV - Comparison of 'in vivo' and in 'in vitro' results.

Name Steroid	Cyst fluid	µg/sample	Incubation 'in vitro' percentage conversion substrate	
S.H.	No. 12		precursors : progesterone	pregnenolone
Pregnenolone		—		4.5
17α-pregnenolone		—		11.1
17α-20ξ -pregnenolone		—		8.6
DHA		40.19		4.9
Progesterone		0.94	8.3	5.6
17α-progesterone		40.19	31.9	8.0
17α-20 ξ -progesterone		—	12.4	2.1
Δ ⁴ -androstenedione		1.42	22.6	19.8
Testosterone		40.19	6.1	—
Oestradiol-17β *		17.62	4.1	6.8
Oestrone *		4.22	0.8	2.9
T.O.	No. 58		precursors : progesterone	pregnenolone
Pregnenolone		—		23.0
17α-pregnenolone		—		6.0
17α-20 ξ -pregnenolone		—		4.0
DHA		40.27		14.0
Progesterone		1.48	40.0	10.0
17α-progesterone		1.62	22.7	0.8
20α-pregn-4-en.		40.27	5.6	—
17α-20 ξ -progesterone		—	15.3	1.2
Δ ⁴ -androstenedione		2.10	0.7	2.2
Testosterone		40.27	0.1	0.5
Oestradiol-17β *		8.40	4.2	4.0
Oestrone *		1.18	1.4	2.2

substrate. The results of steroid determination in the 4 cyst fluids are also reported in the Table, as μg per sample.

Progesterone, 17 α -hydroxy-progesterone, Δ^4 -androstenedione, oestradiol and oestrone were present in the fluids in detectable amounts; 20 α -hydroxy-pregn-4-en-3-one, testosterone and DHA were undetectable. In incubation experiments with progesterone as substrate, all the above steroids were isolated and in addition 17 α -20 ξ -dihydroxy-pregn-4-en-3-one was formed in good yield. On incubation of the tissue with pregnenolone, 17 α -hydroxy-pregnenolone, 17 α -20 ξ -dihydroxy-pregnenolone and DHA were isolated in large amounts.

Considering the results in cyst fluid and those 'in vitro', it would appear that neutral steroids which were present in the fluid in detectable amounts were formed 'in vitro' at a rate of conversion of not less than 10%. On the other hand, the conversion of the substrates to oestrogens was much lower, despite the finding of significant levels of oestrogens in most fluids.

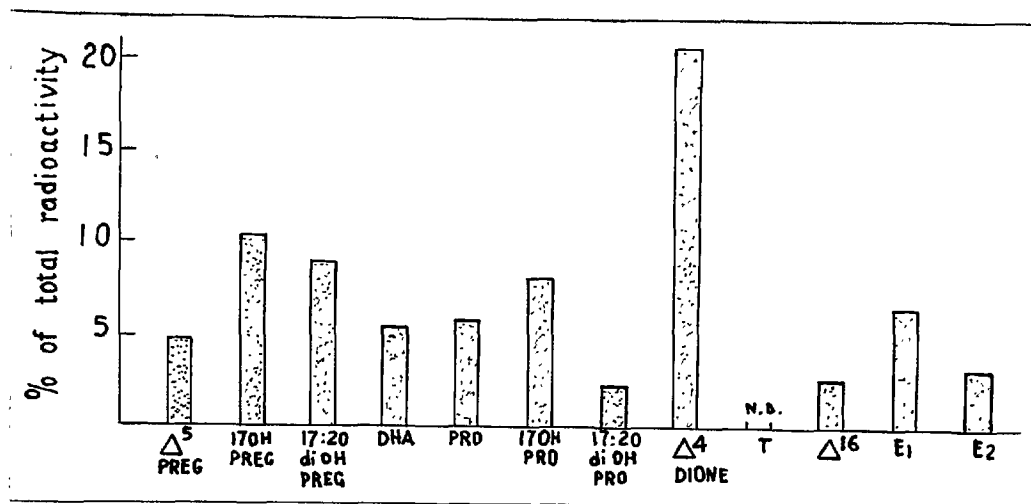
Interesting analogies were noted in the results of the two types of investigation in each of the four cases. Thus, in the fluid of patient D.O., obtained from a corpus luteum at the 24th day of a cycle, there were small amounts of neutral steroids while oestrogens were undetectable. 'In vitro', there was active metabolism of progesterone by the corpus luteum, but the percentage conversion to oestrogens was very low.

The fluid from follicular cysts and cystic follicles in the left ovary of patient M.K. contained a very large amount of progesterone and comparatively little 17 α -hydroxy-progesterone, Δ^4 -androstenedione and

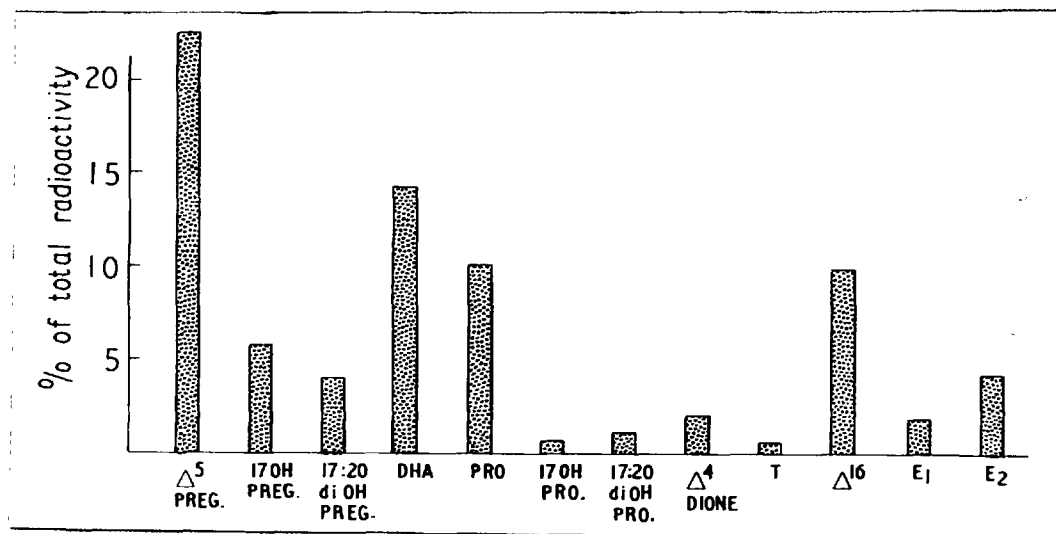
oestrogens. On incubation of progesterone with tissue from the cysts, the precursor was metabolized almost completely, with significant yields of Δ^4 -androstenedione (especially in the atretic follicle), testosterone and oestradiol. Progesterone was therefore actively metabolised 'in vitro' by the lining of cysts which contained a high level of the hormone. On the other hand, on incubation of a corpus luteum from the right ovary, 41.7% of progesterone was not transformed and conversion to 17 α -hydroxy-progesterone and Δ^4 -androstenedione was lower than on incubation of the follicle wall. In this case, follicles and corpora lutea exhibited 'in vitro' a different metabolism of the precursor; 'in vivo' cystic formations derived from follicles contained steroids in relative proportions similar to those observed in corpora lutea.

In the last two patients, S.H. and T.O., ovulation had not occurred at the time of operation. In these two cases, the findings 'in vivo' and 'in vitro' were similar, apart from the discrepancy between the fluid content of Δ^4 -androstenedione and the capacity to form this compound 'in vitro' (Fig. 11). The cyst fluid contained an exceptionally large amount of intermediaries in oestrogen synthesis. Androstenedione was present in larger amounts than 17 α -hydroxy-progesterone and pregnenolone. On incubation of the cyst wall with progesterone and pregnenolone, there was active metabolism of the two precursors and a good yield of oestrogens. Incubation with pregnenolone showed that there was a preferential retention of the Δ^5 - β -hydroxyl group prior to the formation of androstenedione, whereas progesterone and 17 α -hydroxy-progesterone were not important intermediaries in this transformation.

Fig.11-Radioactive steroids formed 'in vitro' from 4-¹⁴C- Δ^5 -pregnenolone by follicular cysts.



a) Patient S.H. (Data from Collins, 1965).



b) Patient T.O. (Data from Collins & Sommerville, 1965).

Finally, it would appear of interest to note that although the findings were similar in the two cases, the cyst from patient T.O. was luteinized, whereas that from patient S.H. was not.

Chapter VI - DISCUSSION

1 - Introduction.

In this Chapter an attempt will be made to examine whether the results presented in this thesis can contribute some information to the knowledge of ovarian function in the human, both in physiological and pathological conditions. It is hoped that the following discussion will justify the assumption that cyst fluid is a suitable material for the study of ovarian function. However, it is necessary to stress again the limitations of this type of investigation; namely that the results refer to the function of the ovary at the time of operation and to a portion of the ovary only.

During the course of the investigation, the importance of another factor became more evident. According to the data presented, some steroids are secreted from the ovary more rapidly than others, and it cannot be excluded that under some circumstances steroids present in the fluid are not secreted at all. Presence of steroids in ovarian fluid cannot therefore be equated to secretion. Furthermore, it is impossible to deduce the amount of steroids produced by the cyst lining from the concentration in the fluid. Evidently, the concentration depends upon the equilibrium between production and secretion. However, comparison of the steroid content in different fluids can give some indications as to whether steroid production is increased or not.

An understanding of the mechanism of steroid secretion from the ovary into the effluent blood appears to be essential for the interpretation of the results of steroid determinations in ovarian fluid.

Results in ovarian venous blood will therefore be discussed first. The results in cyst fluid will then be examined in relation to 'in vivo' biosynthesis; to the histological features of the cysts, and finally the results for each group of patients will be considered in detail.

2 - Steroid secretion from ovarian cystic formations.

The mechanism by which steroids present in cyst fluid are ultimately liberated into the blood is not known. It has been reported that steroids, like proteins and some other compounds of smaller molecular weight, can still be found in the follicle of the mare some time after local injection (Short, 1964). According to some authors, binding to protein is a form of steroid storage in peripheral blood (O'Donnel & Preedy, 1957) and it may well be that protein binding is also important in the storage of steroids within the fluid. On the other hand, the possibility that the formation of a steroid - protein complex is necessary for the transport of steroids from the fluid to the blood cannot be excluded, at least for water-insoluble compounds, such as free steroids.

In the ewe, the secretion of unconjugated steroids is increased in the luteal phase, when the vessels and the basement membrane allow the passage of a greater quantity of protein (Lindner et al. 1964), due to a direct effect of the luteinising hormone on these ovarian structures (Yatvin & Leathem, 1964). Thus, it would appear that steroid secretion can vary in relation to the permeability to protein of the basement membrane and the ovarian vessels. With regard to conjugated oestrogens, which are soluble in water, they could be transported by the water,

although it is known that binding to protein takes place (Purdy et al., 1961). Recent work upon the binding 'in vitro' in human plasma has shown that there is a lower affinity for oestradiol glucuronidate, but that the resultant complex is more stable than that formed with free oestradiol (Giorgi & Crosignani, 1966). This point will be considered in more detail in the section of the Discussion devoted to conjugated oestrogens.

The passage of free and conjugated oestrogens into the blood should be favoured by the contiguity between fluid and vessels. In follicles and cysts derived from them, the cavity is separated from the vessels by the granulosa layer and the membrana propria; in corpora lutea, the vessels reach the cavity through the inner cellular layer. It may be therefore that in cystic corpora lutea, a greater proportion of the specific products passes directly into the effluent blood and less escapes into the fluid than in follicles or follicular cysts.

While nothing more than interesting possibilities can be postulated with regard to the mechanism of passage of steroids from the fluid into the blood, there is no doubt that the concentration of steroids in the blood depends upon the blood flow through the ovary as well as upon the rate of steroid production. The relationship between blood flow and steroid concentration is not a simple one. Under certain circumstances there appears to be a positive correlation between the two parameters, whereas it would be anticipated that an increase in blood flow would lower the concentration. A concomitant increase in blood flow and steroid levels is probably due to the fact that the blood carries to the steroid-producing glands precursors and energy supplies, so that more steroids are produced and secreted (Romanoff, Deshpande & Pincus, 1962).

As already mentioned, the blood flow through the ovary was not measured in the patients studied, and there is no relevant information in the literature. In domestic animals, the blood flow is known to vary within narrow limits; the highest values, which are found in the luteal phase and after acute gonadotropin stimulus, being just about twice the lowest levels of the follicular phase (Romanoff et al., 1962; Hayward, Hilliard & Sawyer, 1963; Short, McDonald & Rowson, 1963; Lindner et al., 1964). In women, it is probable that variations of blood flow cover a similar range and are not responsible for major changes of steroid concentration in the ovarian venous blood.

Obviously, steroid concentration in the effluent blood represents the contribution of the whole ovary, and not only of the cysts present in it. With this limitation, a comparison between concentration in fluid and in blood was considered to be of especial interest.

In the present work, considerable discrepancies were noted in the relationship between the concentration of steroids in cyst fluid and in venous blood in the various cases. The concentration of individual steroids in blood was from $1/280$ to $1/4$ that in fluid. According to the preceding comments, it seems unlikely that these discrepancies were related to variations in blood flow. In addition, the relative proportions of the steroids differed in the fluid and venous blood of the same subject, as if steroids were secreted at different rates. A similar observation has been reported in the mare (Short, 1964) and these findings suggest the existence of a selective retention of steroids within the ovary.

It would be premature to attribute a physiological significance to a

selective retention of steroids; but an effect on steroidogenesis within the cysts--through accumulation of compounds which could influence some transformations -- or a homeostatic action on steroid secretion could be envisaged.

This mechanism of selective retention appears to be more efficient in the follicular than in the luteal phase, since only a small portion of the free oestrogens contained in follicles or follicular cysts was secreted into the effluent blood (Table XXII), whereas oestrogen conjugates were secreted in larger proportions (Table XXIV). Progesterone was apparently retained in follicles, and this would be in agreement with the low secretion rate of progesterone and the constant low excretion of its main metabolite reported during the follicular phase of the cycle (Dominguez et al., 1962; Brown et al., 1958; Pickett & Sommerville, 1962). It should be noted, however, that there is some evidence in monkeys for progesterone secretion prior to ovulation (Hayward et al., 1963) and this may depend upon the stage of maturation -- starting at a time when physico-chemical changes occur in the follicular wall. It may be significant that in the present work, progesterone was found in venous blood draining follicular cysts persisting after ovulation. Neutral intermediaries on the pathway from progesterone to oestrogens did not seem to be secreted in quantity into the ovarian venous blood during the follicular phase of the cycle.

In the luteal phase, progesterone and free and conjugated oestrogens were detected in the ovarian blood and the steroid concentration was not much lower than in fluid. Since blood flow is increased in the luteal phase (Short et al., 1963; Lindner et al., 1964), the rate of steroid

production should be increased as well. This is in keeping with the secretion rates of progesterone and oestrogens which have been reported (Dominguez et al., 1962; Gurpide et al., 1962). Neutral intermediaries were not detected in the samples examined from the luteal phase, although large amounts were found in fluid. Other authors have isolated 17 α -hydroxyprogesterone, 20 α -hydroxy-pregn-4-en-3-one and androstenedione in pooled ovarian blood collected during the luteal phase, in concentrations definitely lower than that of progesterone (Mikhail, Zander & Allen, 1963).

In conclusion, the data strongly suggest the existence of a mechanism of selective retention of steroids within cyst fluid, which varies according to the phase of the cycle. The high concentration of oestrogens in follicular fluid might be the expression of a high degree of retention in the fluid. Conjugated oestrogens seem to escape more freely from the fluid than the free oestrogens. The secretion of progesterone from follicles appears to be correlated with the stage of follicular maturation. In the luteal phase, secretion of both progesterone and oestrogens is increased.

3 - Biosynthesis 'in vivo'.

a) - Free neutral and phenolic steroids.

Steroids determined in the cyst fluids of the present series—progesterone, 17 α -hydroxyprogesterone, Δ^4 -androstenedione, DHA, oestradiol and oestrone — have already been isolated from ovarian fluid or tissue (Table IV). They were present in the fluid in higher concentrations than

in peripheral blood, and therefore were most probably produced by the cyst lining. Unequivocal proof of this was obtained in a few cases, when the same steroids were isolated 'in vivo' and upon incubation of the cyst wall with isotopically labelled progesterone and pregnenolone. The amount of steroids present in the fluid is correlated to the extent to which they accumulate in the various steps of biosynthesis, as could be directly demonstrated in these few cases. However, oestrogen yield 'in vivo' was apparently much higher than 'in vitro'. Apart from limitations inherent in incubation experiments, this might be due to two factors : a) oestrogens, as just discussed, may be retained in the fluid; b) precursors and energy supply is continuous 'in vivo' so that final products may be formed in higher yield.

In all the other fluids assayed, the relationship between the intermediaries in oestrogen synthesis varied very much, so that it was impossible to reach any general conclusion as to the relative importance of the various biosynthetic reactions 'in vivo'. However, it is clear from the present work, that in follicles the transformation to oestrogens from precursors goes to completion, whereas in corpora lutea progesterone accumulates, although a part may be further metabolized and contribute to the formation of oestrogens.

The presence of progesterone, 17 α -hydroxy-progesterone and Δ^4 -androstenedione in most cyst fluids indicates that the pathway through the Δ^4 -3-keto-steroids is active in both phases of the cycle; in atretic follicles; in pregnancy and in some abnormal conditions. As already mentioned in the section on ovarian steroidogenesis (Chapter II), follicles can also

synthesise oestrogens from acetate through Δ^5 - β -hydroxy-steroids (Ryan & Smith, 1961a). Failure to demonstrate the presence of DHA in detectable amounts in all but one of the fluids examined in the present study cannot be considered as evidence that this compound is not an important intermediary 'in vivo'. The rate of metabolism of DHA could be higher 'in vivo' than 'in vitro', so that the compound does not accumulate in the fluid. The same may apply to testosterone, which has been shown to be an intermediary 'in vitro', but has not so far been isolated from normal ovarian tissue. Δ^4 -androstenedione, which arises from conversion of both Δ^4 - β -keto- and Δ^5 - β -hydroxy-steroids, was present in substantial quantities in fluid from follicles, cystic and atretic follicles and some follicular cysts. On the other hand, aromatization of androstenedione appears to be a rate limiting step in many tissues (Collins, 1965), and this might condition its high concentration in the fluid.

The 'shift' of biosynthesis in the luteal phase of the cycle, from a predominant formation of oestrogens to the production of both progesterone and oestrogens (Chapter II), has been attributed to many factors. These include the effect of gonadotropins (Savard et al., 1965); changes in the enzymes of the granulosa lutein cells (Short, 1962a); increased metabolic activity and alternate pathways of hormone formation (Ryan, 1963a).

It has also been suggested that the different anatomical relationship of cells to vessels in the follicle and in the corpus luteum may be important (Ryan, 1963a). In connection with such a hypothesis, the rôle of a selective retention of steroids in the ovary should again be considered. In follicles, it is probable that progesterone, pregnenolone and other intermediaries are retained in the fluid, in contact with the

metabolically active cells. They could then be transformed into oestrogens in high yield. From the corpus luteum, on the other hand, progesterone passes in quantity into the effluent blood, but oestrogens are still formed. The presence of large amounts of 17 α -hydroxy-progesterone and of smaller amounts of Δ^4 -androstenedione in cyst fluid indicates that progesterone is actively metabolized, although the two compounds could also be derived from the Δ^5 -3 β -hydroxy-steroids pathway. The relative importance of different pathways of oestrogen biosynthesis in the luteal phase is by no means established, especially for the human ovary.

Accumulation of Δ^4 -androstenedione has been considered by some authors (Short & London, 1961; Mahesh & Greenblatt, 1964) to reflect a biosynthetic block in the ovaries of patients with the Stein-Leventhal syndrome. The results reported in this thesis on cyst fluids obtained from polycystic ovaries of women with regular cycles suggest that high conversion to androstenedione is a characteristic feature of cystic or atretic follicles, and is not necessarily correlated with menstrual disorders. According to a recent hypothesis (Mossman et al., 1964), the cells lining these types of follicles form part of the interstitial gland tissue, interspersed in the stroma (Chapter I). It is therefore very interesting that incubation 'in vitro' of stroma from the ovaries of pre-menopausal women with 1- 14 C-acetate has demonstrated formation of Δ^4 -androstenedione in high yield (Savard et al., 1965).

Finally, the results obtained in the cyst fluid of ovaries stimulated by exogenous or endogenous gonadotropin suggest that this approach may be useful for the elucidation of gonadotropic action. Thus, stimulation of ovarian steroidogenesis was clearly reflected in the high concentration

of steroids in the cyst fluid and the steroid pattern in the fluid was different according to the predominant type of gonadotropin.

b) - Conjugated oestrogens.

Little is known about the formation of oestrogen conjugates in the human ovary. The results of the present study afford new information on this subject and will therefore be discussed at some length.

In the 23 fluids assayed, acid hydrolysis was effective in liberating additional oestrogens. This and other indirect evidence suggests that some oestrogens are originally present as conjugates. The nature of conjugation is not known. Different forms of oestrogen sulphates and glucuronidates have been identified in human blood (Twombly & Levitz, 1960; Purdy et al., 1961; Troen et al. 1961; Touchstone et al. 1963), and it would appear probable that the complex designated as 'conjugated' oestradiol and oestrone in cyst fluid contains more than one form of conjugate.

The concentration of conjugated oestradiol and oestrone determined in cyst fluid in the present investigation was above that reported for 'total' oestradiol and oestrone in either ovarian venous or peripheral blood of non-pregnant women (see Table VI and VII). This can be regarded as evidence that conjugated oestrogens are synthesised in the ovary.

There are two tentative explanations for the presence of conjugated oestrogens in the ovary : a) they are conversion products of conjugated precursors; b) they are formed by conjugation of free oestrogens. The possibility that conjugated precursors may participate in ovarian steroidogenesis has been suggested (Goldberg et al., 1963; Savard et al., 1965). Studies 'in vivo' have demonstrated that pregnenolone sulphate is

converted to DHA sulphate without cleavage of the sulphate ester group (Calvin, Vandewiele & Lieberman, 1964), but it would seem that in non-pregnant women DHA sulphate is not transformed into oestrogens in significant amounts (Mancuso, Mancuso, Tillinger & Diczfalussy, 1965).

The human ovary can conjugate steroids with sulphuric acid 'in vitro' (Wallace & Silberman, 1964), but the enzyme systems responsible for the synthesis of steroid conjugates in the ovary have not yet been isolated. Sulphatases (Burstain & Dorfman, 1963; Warren & French, 1965) as well as β -glucuronidase (Fishman & Baker, 1956) have been demonstrated in follicles and corpora lutea and may have a synthetic rôle in the formation of conjugates. On the other hand, the hydrolytic action of β -glucuronidase seems to prevail, at least in mouse liver and kidney (Karunairatnam, Kerr & Levy, 1949). Another enzyme, glucuronyl transferase, is involved in glucuronidate formation in these tissues (Dutton, 1959).

This lack of knowledge about the biological reactions leading to the formation of conjugated oestrogens in the ovary makes more difficult the interpretation of results of separate determinations of free and conjugated oestrogens in cyst fluid. Furthermore, consideration must be given to the evidence obtained in the present work that, of the two types of compounds, free oestrogens seem to be retained to a larger extent in the fluid. The ratio free/conjugated oestrogens was high in follicles and follicular cysts, and low in corpora lutea. Many factors could influence this ratio, but there is information about only one of these factors-protein binding. As already mentioned, a smaller portion of conjugated than free oestrogens is bound to protein 'in vitro', especially when the two forms

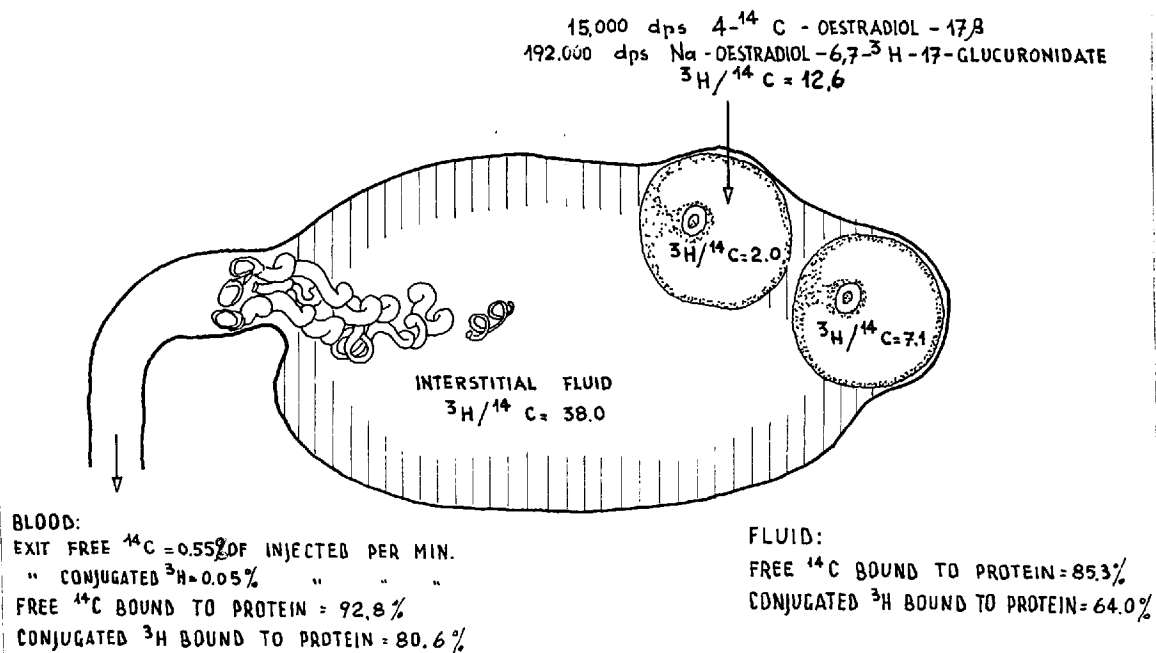
of oestrogens are mixed together, since there is competition for the protein (Giorgi & Crosignani, 1966). If this applies under physiological conditions, protein-bound conjugated oestrogens are retained in the fluid together with free oestrogens, while the major part of the conjugates, not bound to protein, exchange freely with the interstitial fluid outside the cyst (Fig. 12). When the exit of protein is restricted, as in follicles, the unbound conjugated leave the fluid and the bound free oestrogens are retained in the fluid. A high free/conjugated ratio would result. When permeability to protein increases and the vessels come into close contact with the fluid, as in corpora lutea, oestrogens may be secreted at a similar rate, whether bound or not, and the ratio free/conjugated would be close to unity.

In fluids from patients with F.U.H., the concentration of oestrogen conjugates was characteristically high and, conversely, was low in fluid from polycystic ovaries of patients with the Stein-Leventhal syndrome. It would be premature, owing to the small number of patients studied and in the absence of the determination of free and conjugated oestrogens in ovarian venous blood, to assume that these variations in conjugate concentration have an effect on 'total' oestrogen secretion from the ovary in these two conditions.

Another point arising from the results presented here merits discussion. The ratio free/conjugated oestradiol differed from that of free/conjugated oestrone in all fluids, with the exception of those from patients with F.U.H. This may imply the presence of two conjugating enzymes with very high substrate specificity, one for oestradiol and one for oestrone. In this connection it may be pertinent that studies 'in vitro'

Fig.12-Distribution of Na-oestradiol-6,7-³H-17-glucuronidate and 4-¹⁴C-oestradiol-17 β injected into a follicle of a mare.

EXPERIMENT ON A MARE



The ratios $^3\text{H}/^{14}\text{C}$ and the percentages of steroids bound to protein in the fluid refer to the 36th minute after injection. The percentages of protein-bound steroids in the blood are mean values of 4 samples collected at 10 min. interval.

(Data from Giorgi, Addis, Colombo & Polvani, unpublished).

with a preparation of microsomal glucuronyl transferase of rabbit liver have indicated that probably more than one transferase is involved in oestrogen glucuronidate synthesis (Smith & Breuer, 1963; Wessendorf, 1965). On the other hand, it is known that interconversion of oestrone to oestradiol takes place in the liver mainly in the conjugated form (Schwers, Eriksson & Diczfalussy, 1965) and the rate of interconversion of the two conjugates in the ovary could be different from the rate of transformation of the free compounds.

Neutral conjugates were not assayed in the present study, but it is possible that future investigation of this problem would afford interesting information upon ovarian function.

To summarise this section upon biosynthesis 'in vivo', it may be concluded that steroidogenesis is different in the two phases of the cycle and that gonadotropins induce quantitative as well as qualitative changes upon hormone production by the ovary. Oestrogens are formed in high yield in follicles, both progesterone and oestrogens are synthesised in large amounts in corpora lutea. Atretic follicles or follicle cysts produce mainly androstenedione and minimal amounts of progesterone and oestrogens.

Thus, despite considerable variations of the relative proportions of steroids within individual fluids—which cannot readily be interpreted at the present stage of knowledge—certain patterns of steroid content in cyst fluid emerge from the present study.

In the following section, a possible relationship between these findings and ovarian morphology will be discussed.

4 - Cyst morphology and steroid content in the fluid.

Histological examination of the cysts was performed in those cases in which all or part of the ovary was removed. The report of the pathologist (Dr. Magnus Haines, Chelsea Hospital for Women) was available for 41 cysts. The classification of the various types of ovarian cysts and their histological features have already been described.

In the fluid from seven follicular cysts, the steroid content varied according to whether or not ovulation had occurred. Thus, in the fluid from two patients who had not ovulated at the time of operation, there was a large amount of oestradiol and comparatively little progesterone and neutral intermediaries on the pathway to oestrogen synthesis. One of these two cysts presented extensive luteinisation of the theca layer. In the other fluids, obtained from follicular cysts during the luteal phase, more progesterone than oestradiol was found, and in some instances 17 α -hydroxy-progesterone was present in high concentration. These observations may indicate that the 'shift' of biosynthesis in the luteal phase is independent of luteinisation and the other morphological changes characteristic of corpus luteum formation.

As already pointed out, the ratio free/conjugated oestrogens was high in all fluids from follicular cysts, with the exception of fluids from patients with F.U.H.

Fluid from follicle cysts and atretic follicles showed a characteristic accumulation of Δ^4 -androstenedione in both phases of the cycle and in amenorrhoeic women. On the basis of 'in vitro' studies (Forleo & Collins, 1964; Savard et al., 1965), it would appear that this is correlated with

increased formation of the steroid. Furthermore, a retention effect does not seem likely since progesterone, 17 α -hydroxy-progesterone and free oestrogens, which are retained within follicles, were present in very low or undetectable concentrations. The inability of this type of follicles to produce increased amounts of progesterone in the luteal phase may indicate that the steroidogenic process is different ^{from} ~~than~~ in maturing follicles. It must be noted, however, that, according to some reports, cystic and atretic follicles exhibit an abnormally high metabolic activity 'in vitro' (Warren & Salhanick, 1961a).

Theca lutein cysts were found in a case of chorionic epithelioma. The cysts were producing much more progesterone than oestrogens, and since, as mentioned above, luteinisation 'per se' does not imply production of progesterone in large quantities, it is most ^r ~~p~~robable that in this case the steroid pattern reflected stimulation by endogenous HCG.

The relative proportions of steroids in two fluids from corpus luteum cysts were not very different from those observed in normally developed corpora lutea, although the total steroid content was higher. In a third fluid, aspirated from a corpus luteum cyst at the 4th day of a cycle, no steroid could be detected.

The conjugate oestrogen concentration in corpus luteum cysts was similar to that of the free compounds.

In conclusion, there is no apparent correlation between cyst lining morphology and steroid content in the fluid. Cysts with similar histological features contain steroids in different relative proportions and these seem to vary in relation to the phases of the cycle or, in

other words, to the type of gonadotropin acting on the ovary. Follicular, theca lutein and corpus luteum cysts contain large amounts of steroids in most cases. Follicle cysts and atretic follicles exhibit a characteristic accumulation of Δ^4 -androstenedione; low concentration of the other neutral steroids and of oestrogens.

In contrast to the comparison of free steroid content with ovarian morphology, there appear to be a good correlation between the anatomical configuration of the cysts and the ratio free/conjugates oestrogens-high in follicles and approximating unity in corpora lutea.

5 - Consideration of the results in selected groups of patients.

a). - Women with regular cycles.

In agreement with the concept of ovarian function indicated by other methods of investigation, determination of steroids in normally developing follicles and corpora lutea confirmed the cyclic character of hormone production by the ovary. In the follicular phase of the cycle, oestradiol appears to be the principal product, but high concentrations of progesterone are found around the time of ovulation; production of oestrogens and progesterone in large quantities persists throughout the luteal phase until shortly before the onset of menstruation.

The 'shift' in ovarian biosynthesis towards production of the two hormonal principles seems therefore to precede ovulation. This may indicate that luteinising hormone is released from the hypophysis ^{some time} before ovulation takes place. However, the rise in progesterone concentration in the mature follicles is not apparently followed by secretion, since

progesterone was not found in the few samples of ovarian venous blood. Similarly there is no appreciable rise in the excretion of its main urinary metabolite, pregnanediol, at this time (Brown et al., 1957 ; Pickett & Sommerville, 1962). If progesterone is not secreted in substantial amounts before corpus luteum formation, it could not influence the release of FSH, or LH, from the hypophysis; at the same time it could not induce progestational changes in the endometrium or vaginal epithelium.

Secretion of oestrogens during the follicular phase is proved by the oestrogenic effect on the target organs, as well as by the raised excretion of oestradiol, oestrone and oestriol in the urine. However, since follicles retain free oestrogens, it is probable that the oestrogens secreted during the follicular phase are mainly represented by conjugated oestrogens, as the results in ovarian venous blood would seem to indicate. (Table XXIV).

As already mentioned, the urinary excretion of total oestrogens and pregnanediol represents roughly the same percentage - 20% - of the amount of parent hormones produced. The contribution of the adrenals to urinary oestrogens and pregnanediol is approximately constant throughout the cycle. If progesterone and oestradiol were secreted from the ovarian fluid at the same rate, the ratio oestradiol/progesterone in the fluid and total oestrogens/pregnanediol in the urine should be similar. This appears not to be the case. The ratio oestradiol/progesterone is much higher - especially in the follicular phase - than the ratio of the metabolites in the urine. This could be deduced by previous data upon the determination of the two hormones separately in different fluids

(Table IV), but the concurrent determination in the same fluid, as in this thesis (Table XIII) and in recent work by another author (Short, 1965), affords direct evidence of this phenomenon.

The different rate of secretion from the ovary of progesterone and oestradiol may be the result of a selective retention within the follicle and the corpus luteum, which may affect oestrogens more than progesterone.

It is interesting to note that, in contrast to some pathological conditions or in the presence of excessive ovarian stimulation by gonadotropins, the total steroid content in fluid from normally menstruating women varied within narrow limits in the individual fluids and in the two phases of the cycle, apart from the period preceding menstruation. A well-balanced steroid production by the ovary may be indispensable to maintain regular menstrual cycles. The steroid content in the fluid dropped towards the end of the cycle, in accord with the hypothesis that bleeding in ovulatory cycles is provoked by a fall in the levels of circulating hormones.

Determination of steroids in fluid from polycystic ovaries of normally menstruating women gave an opportunity to explore hormone production in ovarian structures other than Graafian follicles or corpora lutea- that is the interstitial gland tissue. It appears that this endocrine tissue produces Δ^4 -androstenedione as the predominant steroid in both phases of the cycle, and that also the production of the other steroids is not changed. The physiological significance of the interstitial gland tissue and the reasons why its follicular components do not respond to gonadotropin stimulation like developing follicles, is not yet clear.

In conclusion, the direct study of steroid production by the ovary has confirmed the cyclic nature of steroid production, and secretion, in the menstrual cycle. It has also confirmed the hypothesis of a selective retention of steroids within follicles and corpora lutea. The amounts of steroids produced are relatively consistent from one subject to another. Steroid production by follicle cysts and atretic follicles seems to be independent of the phase of the cycle.

b) - Ovarian response to gonadotropin stimulation.

The administration of human FSH in association with LH or HCG has the effect of raising steroid production by normal and abnormal ovaries to many times the values found in women with regular cycles. In one case, steroid determination in ovarian venous blood afforded direct evidence that steroid secretion from the ovaries was increased. Similarly elevated amounts of steroids were found in fluid from a patient at the third month of pregnancy and from a patient with chorionic epithelioma, whose urinary excretion of HCG was within the limits found in pregnancy. It is evident that the ovary has a biosynthetic capacity which is not fully exploited under normal conditions.

It cannot be excluded that in the pregnant patient and in the patient with chorionic epithelioma there was some endogenous production of FSH and LH, either from the hypophysis or from placental tissue. The human pituitary FSH administered to a third patient was contaminated with LH. In these three cases, therefore, there was probably a combined effect by FSH, LH and HCG, although HCG was predominant. In agreement with many reports in the literature, the effect of such combined stimulation

consisted in a great increase of progesterone production, while production of other steroids, particularly of oestrogens, was not so elevated.

Progesterone production was also increased after administration of human post-menopausal gonadotropins (Pergonal), which contained FSH and LH, but oestrogen formation was also much increased. The results may be considered as an indication that the two luteinising gonadotropins have a different action on the ovary, and that LH induces a steroid pattern which is more similar to the 'luteal' type than that induced by HCG.

The increase in metabolic activity in comparison with untreated cases was less easily appreciated in the fluid from a patient with the Stein-Leventhal syndrome given Clomiphene pre-operatively, because, as discussed in Chapter V, it is impossible to calculate the total amount of steroids present in fluid from polycystic ovaries. On the contrary, the difference in the steroid pattern in the fluid from that observed in unstimulated polycystic ovaries was quite evident. After treatment, the fluid contained a large amount of free oestradiol and no accumulation of Δ^4 -androstenedione was noted, in striking contrast to the results in untreated patients. Clomiphene administration did not induce ovulation in this patient, but may have enhanced the release of FSH from the hypophysis, with consequent increase in oestrogen formation.

c) - Functional uterine haemorrhage.

This term has already been defined and the ^{an}alogy between the haemorrhage in F.U.H. and bleeding intervening during ovarian hormone administration has been noted (Chapter II). It has been postulated that continuous oestrogen secretion from functioning ovarian cysts, unopposed by progester-

one secretion, is the main aetiological factor (Schroeder, 1954; Rubin & Novak, 1958; Kuppermann, 1963). While this hypothesis has not been substantiated by extensive study of ovarian steroid production, it appears probable that an endocrine disorder underlies this condition, since cyclic hormonal therapy can control the haemorrhage (Peel, 1955). Furthermore, the excretion of urinary metabolites of progesterone and oestradiol differs from that of the normal cycle (Brown & Matthew, 1962; Fotherby & Brown, 1964).

It must be noted, however, that follicular cysts are not a constant finding in F.U.H., which may be associated with polycystic ovaries and, in many cases, with no macro - or microscopical abnormality. In addition, the syndrome can last for many years, while it seems unlikely that follicular cysts persist for longer than a few months (Hertig & Mansell, 1957).

The possibility of pituitary involvement has not been explored so far, although the absence of ovulation in F.U.H. may imply an altered ^{hypothalamo} pituitary-ovarian function and it may be significant that irregular bleeding is more frequently observed at the extremes of reproductive life. The recent observation that Clomiphene, probably by releasing gonadotropins from the hypophysis, can induce ovulation and regular cycles in patients with F.U.H. ^{hypothalamo} ^{complex} also implicates the pituitary in the aetiology.

All the cases examined in the present investigation were associated with functioning ovarian cysts, either of the follicular or corpus luteum type, or with polycystic ovaries. More than one third of the patients had ovulated shortly before operation. This high incidence of ovulatory cycles is in contrast with the classical description of this form of

menstrual disorder (Schroeder, 1954; Peel, 1955). This discrepancy could be explained by the adoption of different criteria of classification of the patients, and by the fact that the ovaries were available for examination in the present series.

The direct study of ovarian function, by means of steroid determination in cyst fluid, has lent little support to the hypothesis of a predominant rôle of oestrogens in F.U.H. Progesterone in varying amounts was present in most fluids, at any interval from a bleeding and irrespective of the presence of a corpus luteum. Free oestrogens were detected in all fluids, but generally not in abnormally high amounts. The distinctive feature of this series of fluids was a greater variation of total steroid amounts and the absence of a regular sequence of oestradiol-progesterone secretion as seen in normal cycles.

In a previous section, it has been suggested that steroid production in follicular cysts varies according to the stimuli impinging on the ovary. The presence of progesterone and oestrogens in a 'luteal' pattern in most fluids may indicate stimulation by luteinising hormone even in cases in which a corpus luteum was not seen at operation. The response of follicular cysts to this stimulus, however, would seem to be abnormal, since they fail to evolve into corpora lutea. The persistence of corpora lutea, in the form of cysts, for a period longer than the normal life span, could also be the expression of an abnormal response to gonadotropin stimulation.

In order to attribute to the cysts a pathogenetic rôle, it would be necessary - as has been emphasised in this thesis - to demonstrate that the steroids contained in the fluid are secreted into the effluent blood,

Progesterone was detected in the ovarian venous blood from one patient with F.U.H., while free oestrogens were undetectable. On the basis of the observations on steroid secretion from cystic formations reported in section 2 of this Chapter, it appears probable that the follicular cysts found in the patients studied were secreting only a small portion of the progesterone and free oestrogens present in the fluid, and a large portion of conjugated oestrogens. Since conjugated oestrogen concentration in the fluid was high and there was no evidence for retention, it may be justifiable to assume an elevated secretion of conjugates. Thus the 'total' oestrogen output from the ovary may be above the level observed in normally menstruating women, although the free oestrogen content in the fluid is not above this level.

With regard to the action of secreted steroids upon target organs, it is probable that progesterone as well as oestrogens is responsible for the abnormal endometrial development, in contrast to the current opinion that this is mainly an oestrogenic effect. Progesterone was present in large amounts in fluid from patients with frequent bleedings and it may be that progesterone secretion influences the shedding of the endometrium.

In conclusion, determination of steroids in cyst fluid from patients with F.U.H. has afforded new information on steroid production by cysts associated with this syndrome. In particular, progesterone seems to be as important as oestrogens in the induction of irregular bleeding. The results strongly suggest the existence of an abnormal response of the ovary to gonadotropin stimulation.

d) - The Stein-Leventhal syndrome.

Apart from reference to recent work upon steroid biosynthesis 'in vitro' and 'in vivo' by ovarian tissue obtained at wedge resection and the description of the present findings, the results in cyst fluid have not been discussed in relation to the pathogenesis of the syndrome.

It is generally agreed that there is no characteristic histological change in the ovaries of patients with the Stein-Leventhal syndrome and that pituitary function is abnormal, either as a primary factor or secondary to ovarian dysfunction. The latter could consist of an enzymatic defect in ovarian tissue leading to increased formation of androgens and to a partial block in the synthesis of oestrogens. However, the concept of pituitary dysfunction is largely speculative and awaits improved techniques for gonadotropin assay (Chapter II), while the nature of the biosynthetic defect, mainly the ability to form oestrogens, is still under discussion (Chapdelaine et al., 1963, Kase, 1964).

In considering this situation, the importance of the choice of relevant controls cannot be overemphasised and this was evident from the present study when results of steroid analysis in fluid from patients with the syndrome were compared with those obtained in fluid from follicle cysts and atretic follicles-histologically similar to those observed in the syndrome. Although these were in the ovaries of normally menstruating women, the accumulation of androstenedione was demonstrated to a comparable extent (Table XVIII and XX). Accordingly, this finding appears to reflect a peculiarity of steroid production in follicle cysts and atretic follicles rather than an enzymatic defect characteristic of the Stein-Leventhal syndrome.

Reference has already been made to the fact that the cells lining this type of cyst are the first stage in the evolution of the interstitial gland tissue (Mossman et al., 1964). If accumulation of androstenedione is a peculiarity of an endocrine tissue present in all ovaries - whether or not polycystic - this raises two questions : a) - is the presence of a great number of follicles at different stage of development and regression the expression of same abnormality; and if so, is the abnormality primarily ovarian or extra-ovarian in origin? b) - why increased production of androgens, and probably diminished oestrogen synthesis, is compatible with normal follicular maturation and ovulation in some cases and not in others?

With regard to a), it must be noted that ovarian cystic changes can be induced in animals by various manipulations. Continuous exposure of hypophysectomized rats to FS# or of hypothyroid rats to PMS and HCG causes formation of follicle cysts and some ovarian enlargement (Evans & Riley, 1960; Yatwin & Leatham, 1964). Injection of normal rats with testosterone and other steroids immediately after birth or of adult rats with DHA and androstenedione also produces enlarged polycystic ovaries (Barraclough & Gorski, 1961; Roy et al., 1962; Goldzieher & Axelrod, 1963).

In women, polycystic ovaries, very often dissociated from menstrual irregularities, have been reported, on one hand, in conjunction with conditions which could affect pituitary function, such as acromegaly and increased intracranial pressure; on the other hand, in case of androgenic adrenal hyperfunction. (Morris & Scully, 1958; Goldzieher & Green, 1962).

In experimental animals, the effect of androgens and other steroids

on the ovary is mediated through the hypothalamo-pituitary system (Barraclough & Gorski, 1961). This could apply to women, but attention has been drawn to the fact that the androgens secreted by the ovary -DHA and Δ^4 -androstenedione- exert a weak inhibitory effect on the hypophysis (Dorfman, 1963). It has been suggested that the two androgens are metabolised outside the ovary to testosterone, a more potent inhibitor of gonadotropin secretion (Mahesh & Greenblatt, 1964). Accordingly, the increased concentration of testosterone which has been observed in the peripheral blood of patients with the syndrome could be attributed either to the peripheral transformation of the ovarian androgens, or to testosterone secretion by the adrenal cortex.

A diminution in oestrogen formation has been considered an important factor in the pathogenesis of the syndrome (Goldzieher & Axelrod, 1963); but, as already indicated, there is conflicting evidence as to whether oestrogen production is defective. There are no clinical symptoms of *gross* oestrogen deficiency and urinary oestrogen excretion is higher than in ovariectomized women. Oestradiol and oestrone have been detected in the ovarian venous blood of patients with the syndrome, although in slightly lower levels than in normally menstruating women (Mahesh & Greenblatt, 1964). In the present series oestradiol was undetectable in the majority of the fluids (13 out of 19). This is a larger series than has hitherto been studied and it is concluded that a relative decrease in the proportion of oestrogens in cyst fluid is characteristic of the Stein-Leventhal syndrome. However, the overall production of oestrogens must depend upon the total mass of steroid-producing tissue.

With regard to b), an explanation for the persistence of ovulation and regular cycles in some cases of polycystic ovaries could be that the production of androgens has to reach a certain level to become pathogenic. In fact, in the present series, the concentration of Δ^4 -androstenedione in fluid from patients with the syndrome seemed to increase with the duration of amenorrhoea. Furthermore, as discussed for oestrogens, the total amount of androgens produced is related to the number of actively secreting follicles or the quantity of interstitial tissue, and this, together with the concentration of steroids in the fluid, has to be taken into account.

The hypothesis has been put forward by some authors that, since the reduction of the mass of ovarian tissue after wedge resection allows restoration of ovulatory cycles (Zander, Wiest & Ober, 1962; Kase, 1964), the increased size of the ovary, rather than a biosynthetic defect may be responsible for the insurgence of the syndrome. However, since ovarian enlargement is sometimes absent in patients with the syndrome, while the number of follicles is definitely very large in all cases, the above hypothesis should probably be modified in the sense that the mass of interstitial tissue - either atretic follicles or stromal - is the determining factor, and not the size of the ovary.

The observation, reported in previous sections of this Chapter, that steroid production in follicle cysts and atretic follicles does not vary with the phases of the cycles, may help to explain the pathogenesis of the syndrome. It may be that these follicles do not mature because physiological amounts of gonadotropin are insufficient to stimulate

follicle maturation on such a large scale. This is supported by the fact that after excessive dosage of gonadotropins or administration of Clomiphene the follicles in the ovaries of the Stein-Leventhal patients produce androgens and oestrogens in a physiological ratio and some reach full maturity and ovulate. The efficacy of wedge resection can be regarded as a corollary to this, in that endogenous gonadotropins, after surgery, would be acting upon a small number of responsive follicles. At the same time, the reduction in the overall production of androgens by the resected ovary, could modify the 'feed-back' effect upon the hypophysis and a situation similar to that found in normally menstruating women with non enlarged polycystic ovaries would become established.

While it is hoped that the present work has added information about ovarian function in the Stein-Levanthal syndrome, it is felt that a full comprehension of the very complex pathogenesis of the Stein-Leventhal syndrome is far from achieved, and that further investigation should be directed mainly towards the study of ovarian secretion and of the interaction between androgens and the hypothalamo-hypophyseal axis.

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