STUDIES UPON THE CHEMICAL DETERMINATION AND METABOLISM OF PROGESTERONE

by

WILLIAM PATRICK COLLINS

A Thesis submitted for the Degree of
Doctor of Philosophy in the
University of London

Endocrine Laboratory Unit,
Institute of Obstetrics and Gynaecology,
Chelsea Hospital for Women,
Dovehouse Street,
London, S.W.3.

ABSTRACT

A brief history is presented of the isolation and characterisation of the principal steroid hormones affecting reproductive processes with particular reference to progesterone.

Section I is concerned with information on the presence and concentration of progesterone and related Δ^4-3-ketosteroids in human fluids and tissues, and contains a review of the development of chemical methods for the extraction, purification, separation and determination of these compounds.

A description is given of spectrophotometric and gas-liquid radiochromatographic methods developed during the present study.

The results from the application of these methods to human subjects during pregnancy and the menstrual cycle are presented and discussed.

In Section II an investigation is reported, into the capacity of blood to metabolise the hormone. This aspect is followed by *in vitro* studies upon the biosynthesis and role of progesterone as an intermediary compound in the formation of androgens and oestrogens in the human ovary. The results are related to ovarian morphology and a comparison is made

between the production and metabolism of progesterone in developing follicles, corpora lutea and postmenopausal tissue. Examples of abnormal ovarian tissue are studied in the same manner and the results compared with healthy tissue.

A preliminary investigation into the peripheral metabolism of progesterone in human myometrium is reported as an introduction to studies on transformations which may be related to the mechanism of action, or degradation of the hormone in a target tissue.

Finally some related aspects of progesterone metabolism are presented in a brief review.

These studies were undertaken in an attempt to contribute information essential for rational therapy in various forms of placental and ovarian dysfunction.

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PREFACE

The isolation and characterisation of the principal steroid hormones affecting reproductive function.

The rate of progress in any scientific field is dependent upon the development of new techniques for the verification and amplification of theories suggested, but inadequately supported, by the results of older methods. The isolation of the steroid hormones and the elucidation of their metabolism and role in reproductive function is a good example of this phenomenon, and furthermore illustrates the gradual adaptation of chemical and physical techniques to the study of biological problems.

Any account of the isolation and characterisation of the principal steroid hormones must include the establishment of both physiological and chemical criteria. The substances must be formed in an endocrine gland, released into the blood stream, and produce a discernible effect in another tissue. They must also be shown to possess the perhydrocyclopentenophenanthrene nucleus. This dual definition has confused the history of the steroid hormones, for although the perhydrocyclopentenophenanthrene nucleus was demonstrated in many biologically active substances isolated from the urine of pregnant women, the hormonal nature of these compounds was not established until much later. Further confusion arises from the experimental limitations which resulted in only

partial establishment of these criteria, in a variety of species, at widely different times.

The history of reproductive endocrinology has been traced to the empirical observations of ancient civilisations (Selye, 1950). However, perhaps the first significant advance was the anatomical description of the female reproductive system by Regner de Graaf in 1672, although indications as to the function of these organs had to await the refinement of experimental surgery, some two centuries later. These studies on the ablation and transplantation of organs and tissues, demonstrated that biochemically active substances, later called hormones, were released from ductless glands into the bloodstream, and transported to peripheral tissues where a biological response could be related to various phases of reproductive cycles.

However the isolation from glands of specific compounds responsible for these biological effects was more difficult to achieve, and towards 1927 attention was turned to the urine of pregnant women, which was found to be a rich source of some of the active principles and their metabolites.

During this period considerable progress was being made on the chemical characterisation of sterols and bile acids, and this proved of immense value in the subsequent isolation and identification of the active compounds found in

the urine of pregnant women. The pioneer work on the molecular structure of sterols, depended largely on the study of degradation products by such methods as dichromate oxidation, pyrolysis, and selenium dehydrogenation, and on reflection it is remarkable that so much progress was made with the limited techniques available at that time. These results indicated that a possible formula for sterols was as shown in Fig. I.

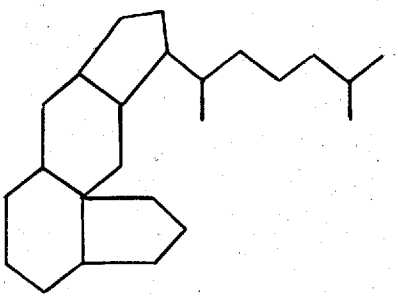


Fig.I. Windaus-Wieland formula for sterols.

However the development and application of X-ray crystallography to the study of single crystals of ergosterol (Bernal, 1932) indicated that the molecules were long and thin (7.2 x 5 x 17-20Å), while the Windaus-Wieland formula should have the dimensions (8.5 x 7 x 18Å). These dimensions were similar in implication to those inferred by Adam and Rosenheim (1929) from the measurement of surface films of sterols. These studies were reviewed by Rosenheim and King

in 1932, and it was concluded that the formula shown in Fig.2 would best account for the established facts.

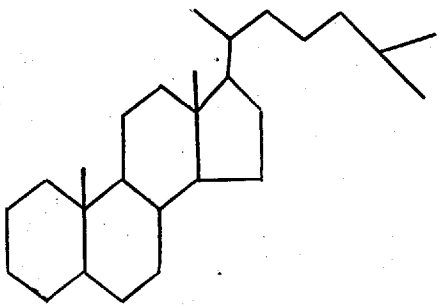


Fig.2. Rosenheim and King formula for sterols.

The first application of standard chemical techniques to reproductive endocrinology followed the demonstration by Aschheim and Zondek,(1927) that the urine of pregnant women contained relatively large amounts of a compound which induced oestrus in rats and mice. The pharmacological potentialities of this compound stimulated intensive research into its isolation, and this was finally accomplished independently by Doisy et al., (1929) and Butenandt, (1929). Further studies by Butenandt et al., (1933) demonstrated that the substance, later named oestrone, was chemically related to the sterols, and contained the perhydrocyclopentenophenanthrene nucleus. A similar structure was attributed to a related compound 'oestriol' which had been isolated from pregnancy urine by Marrian in 1930. Further evidence for the structure of these oestrogens was reported by Cook and Girard in 1934.

As the present work is largely concerned with the rôle of progesterone as an intermediary in the biosynthesis of androgens and oestrogens, the early work upon these compounds will be briefly considered. Furthermore, a brief summary of the technical difficulties involved in their identification, is relevant to subsequent progress in the development of physico-chemical methods for the determination of these compounds in body fluids and tissues.

The isolation of the first oestrogenic hormone from the ovary was reported in 1935 when Doisy and his coworkers (MacCorquodale et al., 1935) isolated a substance from the liquor folliculi of sow ovaries, which was identical with a compound previously obtained by the reduction of oestrone (Schwenk and Hildebrandt, 1933). So small is the amount of oestrogen stored in the ovary that about four hundred litres of liquor folliculi was processed to obtain a few milligrams of crystalline material. It was found that the substance which was named oestradiol accounted for most of the oestrogenic activity of the sow ovary, the remainder was attributed to oestrone.

Oestradiol and oestrone were tentatively identified in human ovarian tissue by Smith and Smith in 1952, and subsequently by Zander et al.,(1959), and Kecskes et al.,(1962). Evidence for the presence of these compounds in human placental

tissue was reported by Mitchell and Davis, (1954) and Diczfalusy and Linkvuist, (1956).

The chemical determination of oestrone and oestradiol-17β in human peripheral plasma during late pregnancy was reported by Aitken et al., (1958), and Preedy and Aitken, (1961). More positive identification was reported by Oertel et al. in 1959, who isolated oestrone, oestradiol-17β, and oestriol from late pregnancy plasma by two countercurrent separations (160-200 transfers), and two paper chromatographic steps. The final products were identified by infra-red spectroscopy.

Progress in the isolation of testicular hormones was first reported by McGee, (1927), who demonstrated the presence of an androgenic principle in bull testis. However, the first pure crystalline androgen was obtained from urine. In 1928 Loewe et al. reported the presence of an androgenic principle in male urine, and in 1931 Butenandt, (1931a) isolated 'androsterone' from this source. The technical difficulties involved are reflected in the fact that in the original isolation only approximately 15 mg. of the crystalline substance were obtained from 15,000 litres of urine. Preliminary chemical analysis indicated that androsterone might be related to the oestrogens, and a formula was proposed which was finally proved correct by Ruzicka et al., (1934), by synthesis from cholesterol.

The most potent androgen from bull testis was isolated by David et al., (1935) and named testosterone. The structure of this compound was conclusively proved by Butenandt and Hanish, (1935), and Ruzicka et al., (1935) by synthesis from cholesterol and dehydroepiandrosterone.

Testosterone was subsequently isolated from *in vitro* incubations of human testis by Brady in 1951, and found in testicular perfusates by Savard et al., (1952). However, direct evidence for the presence of testosterone in human peripheral venous blood has only recently been obtained (Finkelstein et al., 1961; Riondel et al., 1963; Brownie et al., 1964).

Progesterone and related progestational compounds were not found in urine, and so had to be extracted from the endocrine glands. The history of their isolation may be traced to the first description of the corpus luteum by Regner de Graaf in 1672, and the subsequent investigations into the function of this structure. After some unproven hypotheses, Fraenkel, (1903) and (1910), investigated experimentally a theory, that the corpus luteum was necessary for the implantation and protection of the embryo in the uterus. In these studies the corpus luteum was surgically removed from rabbits after ovulation and it was conclusively shown that the corpus luteum was vital for implantation of

the fertilised ovum. During the same period confirmatory evidence was obtained by histologists who observed microscopic changes in the uterine glands of the rabbit during pregnancy, and demonstrated that these changes were also associated with the presence of a corpus luteum.

As a result of these and similar studies it was concluded that the corpus luteum secreted a compound which had the property of maintaining pregnancy in certain ovariectomised animals, and was responsible for transformations in secretory glands of the uterine mucosa. In 1929, Corner and Allen utilised this transformation to develop a biological assay method, which proved of immense value in the subsequent isolation of the active principle, since it provided a qualitative and quantitative assay for material obtained by chemical extraction and purification.

By 1932 there were reports of progestationally active ovarian extracts from three different laboratories, and in 1934 the isolation of crystalline progesterone from sow ovaries was reported independently and almost simultaneously by four groups of workers (Butenandt and Westphal, 1934a; Slotta et al., 1934a; Allen and Wintersteiner, 1934; Hartmann and Wettstein, 1934a,b). In general, approximately one hundred kilograms of ovarian tissue had to be processed to obtain fifty milligrams of crystalline progesterone. The

prodigious amount of work involved in this achievement formed the basis for many subsequent techniques used in micro methods for the biological and chemical determination of progesterone in human tissues and fluids. However, these were not fully exploited for many years until the development of sensitive physicochemical methods of measurement.

The preliminary characterisation of progesterone had indicated that it was related to the androgens and oestrogens, and some confusion was resolved on demonstrating that the substance was dimorphous forming prisms M.P. 128°C and needles M.P. 121°C. Combustion analyses were in agreement with an empirical formula of C_{21} H_{30} O_2, and the substance exhibited an ultra violet spectrum λ max. 240 mμ., indicative of an α/β unsaturated ketone. These studies led Slotta et al., (1934b) to suggest a structure for progesterone, which was subsequently proved correct by Butenandt and Westphal, (1934b) and Fernholz, (1934a,b). In these studies progesterone was synthesised from stigmasterol - the structure of which was known. Confirmatory evidence was also obtained by the synthesis of progesterone from the physiologically inactive urinary metabolite, pregnanediol, (Butenandt and Schmidt, 1934).

Progesterone was subsequently isolated from bovine adrenal cortical extracts by Beal and Reichstein, (1938), but the isolation from human placentae was not achieved until 1952,

when Salhanick et al., (1952); Pearlman and Cerceo, (1952); and Diczfalusy, (1952), independently announced its separation. Conclusive evidence, based on infra-red spectroscopy, for the presence of progesterone in human peripheral blood during the latter stages of pregnancy was presented by Zander, (1954), who in 1958 also isolated the hormone from human liquor folliculi and corpora lutea.

Stages in the isolation and identification of progesterone in body fluids and tissues of the human subject are summarised in Fig.3.

It is remarkable that the interest of both academic and industrial investigators was so intense that within ten years from the isolation of the first steroid affecting reproductive processes, the three most potent steroids as indicated by biological assay were isolated, identified, characterised and made available as partially synthetic substances. However, it proved more difficult to demonstrate that these compounds were actually secreted by glands in human subjects. In the case of progesterone, progress was impeded by the low concentration of the substance in blood and tissues, and the biological inactivity of the urinary metabolites.

Advances in the chemical determination of progesterone and studies into its metabolism, coincided with the development of chromatography and the application of radioactive

1963 - 1965 - chemical determination in peripheral venous blood of non-pregnant women.

1963 - chemical determination in human ovarian venous blood.

1962 - chemical determination in human pregnant myometrium.

1960 - chemical determination in human adrenal venous blood after ACTH stimulation.

1958 - chemical determination in human follicular tissue, corpora lutea; isolation of 20α and 20β hydroxypregn-4-en-3-one

1954 - isolation and chemical determination in human peripheral plasma and uterine venous plasma during late pregnancy.

1952 - isolation from human placental tissue.

1951 - total synthesis of progesterone

1934 - crystallisation, elucidation of structure and partial synthesis of progesterone.

1933 - isolation of active progestational extracts from ovarian tissue.

- the perhydrocyclopentenophenanthrene formula for sterols established.

1929 - biological assay method developed for progestational activity.

Fig.3. Progress in the isolation, identification and chemical determination of progesterone.

isotopes to biochemical problems. These techniques gave insight into the large number of steroid compounds present in the human body and urine, but also indicated a common basic sequence of biosynthesis in all steroid producing glands. The difference between the glands appeared to be only in the later steps of biosynthesis, where the relative activity of enzyme systems determined the ultimate products of the gland.

Improved methods of analysis also indicated the presence of other steroids possessing biological activity. Thus in 1958, Zander et al. working on the marked discrepancy of results between biological and chemical assay methods for progesterone, isolated two other progestogens shown to be 20α and 20β hydroxypregn-4-en-3-one. Similar advances with regard to the androgens and oestrogens suggests that the earlier concept of a few important active principles is an oversimplification. Furthermore, one steroid may exert several biological effects either as a function of its unique structure, or because it is readily converted to another compound.

Recent work on the analysis of human ovarian venous blood (Mikhail et al., 1963; Mahesh and Greenblatt, 1963), has indicated that this gland secretes more than one steroid, and it is tempting to speculate that the regulation of metabolic processes depends upon the ratio of these compounds

as well as the quantity of the main hormone. However, for the practical purpose of obtaining information upon levels in peripheral blood and tissues, the classical hormones are in general the only ones in sufficient concentration to be measured with the techniques available at the present time. In the future, the simultaneous study of related compounds and their metabolites, may well afford better insight into the regulatory mechanisms of the steroid hormones.

SECTION I

The development of chemical methods for the quantitative determination of progesterone.

Apart from technical problems, previous methods designed to provide information about the endogenous secretion of progesterone are open to criticism in that they constitute a somewhat indirect approach. Thus for two decades following the isolation of the hormone from sow ovarian tissue, attempts to obtain information upon the concentration of progesterone in human peripheral blood depended upon the extension of biological assay techniques for progestogens (McGinty et al., 1939; Hooker and Forbes, 1947). These techniques indicated that the progestational activity was mainly confined to the plasma, and this observation has been confirmed by chemical techniques (Zander, 1954; Sandberg and Slaunwhite, 1958).

However, widely different quantitative results were obtained by the various methods for bioassay. These anomalies may be explained by the fact that the end point of any bioassay is the product of a number of interrelated factors and is not necessarily due to the activity of any one compound. This limitation does not affect the usefulness of these techniques as in the pharmacological testing of new drugs but it does mean that the methods are of limited specificity and influenced by a number of unknown parameters.

Another indirect approach to the investigation of

progesterone metabolism in human subjects was the quantitative determination of urinary pregnanediol. This compound had been isolated from the urine of pregnant women by Marrian in 1929, and subsequently identified by Butenandt, (1931b). In 1937, Venning and Browne demonstrated the metabolic conversion of progesterone to sodium pregnanediol glucuronidate (NaPG) prior to excretion in human urine, and later many methods were developed for the quantitative determination of the glucuronide or the free compound after hydrolysis.

Subsequent investigations however have shown that there are many limitations in the interpretation of progesterone metabolism from urinary pregnanediol estimations. These studies have shown that pregnanediol is only one of at least twelve metabolites of progesterone; furthermore the proportion excreted in the urine is low, and varies from 10-25% in different individuals (Klopper and Mitchie, 1956). It has also been shown that desoxycorticosterone (Horwitt et al., 1944), Δ^5 pregnenolone and Δ^5 pregnenolone sulphate (Arcos et al., 1964) are probable precursors of pregnanediol.

The urinary pregnanediol levels therefore do not provide accurate information on progesterone secretion, or allow for alternate pathways in progesterone metabolism. They are also the result of total metabolism for a number of hours, and do not show possible changes in secretion and metabolism which may occur

over short periods of time - as during labour or at abortion.

Some of the limitations of the previous two methods are overcome by the specific determination of progesterone in blood plasma by chemical techniques. However the development of satisfactory methods for this purpose first necessitates the separation of microgram amounts of progesterone from other lipophilic substances and closely related steroids, followed by the adaptation of sensitive physicochemical techniques for measurement.

The first attempt to measure progesterone in human serum using a chemical technique was reported by Reynolds and Ginsburg in 1942. They extracted the serum with ethanol and after solvent partition isolated a non-volatile neutral fraction containing the progesterone. The extract was dissolved in ethanol and the optical density at 240 mμ was compared with that of authentic progesterone prepared in a similar manner. The results were disappointing for although it was possible to detect 0.1 - 5.0 mg of progesterone added to 10 ml of serum, which was within the concentration suggested by biological assay, no endogenous progesterone could be found.

Further work was not published for another eight years until Haskins, (1950) introduced a simplified ether extraction of plasma, which, without further purification, was dissolved in ethanol and the optical density determined at 240 mμ. Once

again the method was unsuccessful when applied to peripheral plasma from pregnant and non-pregnant women, however results were obtained following the intravenous administration of progesterone, which demonstrated a rapid removal from the blood.

In the same year Haskins et al., (1950) reported the application of a paper chromatographic technique for the isolation of progesterone from oil in commercial preparations. A monophasic system of 80% methanol was used in which progesterone had an Rf value of 0.84 and was effectively separated from the oil which remained at the origin.

However further progress towards introducing greater specificity into methods for the determination of plasma progesterone was reported by Butt et al., (1951) who devised a procedure which involved simplifications of the extraction and preliminary purification, and also included a step for the more specific isolation of progesterone. This important development was achieved by partition of the extract between hexane and 70% methanol on a Hyflosupercel column, followed by a polarographic measurement of the progesterone fraction as its Girard complex. This method indicated the presence of progesterone in human placental blood, and in the peripheral blood of one pregnant subject with hepatitis, but was unable to detect its presence in the peripheral plasma of normal pregnant and non-pregnant women. This suggested to the authors that the level must lie

below 0.1 µg/ml plasma.

In 1952 the techniques of column chromatography and countercurrent distribution based on the principles of Craig, (1944) were successfully applied by Salhanick et al., (1952); Pearlman and Cerceo, (1952); and Diczfalusy, (1952) for the isolation and determination of progesterone from human placentae. However it was the work of Zaffaroni and coworkers (1950) and Bush, (1952) on the development of liquid partition paper chromatographic systems for the separation of steroids which had the most impact on further advances in the isolation and measurement of plasma progesterone.

Edgar, (1953a,b) using one of the volatile liquid partition systems of Bush detected progesterone and obtained evidence for its identity in the ovarian venous blood of various domestic animals; while in 1954 Zander, using a similar paper chromatographic system, reported the isolation of progesterone from human peripheral blood. The identification was confirmed in pooled samples by infra-red spectroscopy. The levels found during the latter half of pregnancy ranged between 0.04 and 0.27µg/ml plasma, some ten times lower than indicated by biological assay.

Once the range of progesterone levels had been indicated other successful chemical methods were developed. For the next eight years these were generally similar in principle and depended on UV spectroscopy for the final measurement. This limited their

application to plasma samples of not less than 10 ml volume from pregnant subjects, although increased sensitivity was obtained by the use of micro-cells which contained as little as 0.6 ml of reagent.

These methods have been reviewed by Short, (1961) and Zander, (1962) in terms of sensitivity, specificity and accuracy as defined by Borth, (1952; 1957). A variety of techniques for extraction, preliminary purification, chromatographic separation and spectrophotometric determination were developed and these were summarised by Zander, (1962) and the essential procedures as applied to human subjects are tabulated in Fig.4.

In general it is unfortunate that increased specificity was often accomplished by the introduction of additional steps for purification, as this resulted in diminished accuracy or sensitivity. Conversely, however, more rapid procedures were open to criticism on the grounds of lack of specificity.

Furthermore, because of the complexity of these methods vital information was frequently not available for statistical analysis and there was an urgent need for less laborious and time-consuming procedures which would also be less expensive in materials, space and the working time of highly qualified personnel. For this reason the introduction of thin-layer chromatography and gas-liquid chromatography were of especial interest. At the same time, the value of methods based upon the principle of double isotope dilution became apparent although these methods, as will subsequently be discussed, did not have the advantages of speed and simplicity.

AUTHORS	EXTRACTION	PURIFICATION	ISOLATION	MEASUREMENT
Reynolds & Ginsberg, (1942)	ethanol	partition in organic solvents, removal of lipids in cold 70% methanol	-	ultra-violet absorption at 240 mμ
Haskins, (1950)	ether	-	-	ultra-violet absorption at 240 mμ
Butt et. al., (1951)	ethanol-ether 3:1	partition in organic solvents, removal of lipids in cold 70% methanol	column partition chromat-ography	polarographic, as Girard complex
Zander & Simmer, (1954)	ethanol-ether 3:1	partition in organic solvents, removal of lipids in cold 70% methanol	liquid partition paper chromat-ography	u.v. abs. at 240 mμ
Hinsberg et al., (1956)	ether	chromatography on aluminium oxide	chromat-ography of 2,4 dinitro phenylhydrazone der. on Al_2O_3	u.v. abs. of deriv. at 380 mμ
Short, (1958)	ether after pretreatment with alkali	partition in organic solvents	liquid partition paper chromatography	u.v. abs. at 240 mμ
Sommerville & Deshpande, (1958)	ether-methylene chloride 4:1 after treatment with alkali	chromatography on aluminium oxide		u.v. abs. of isonicotinic acid hydrazide deriv. at 380 mμ
Oertel, et al., (1959)	ethanol	partition in organic solvents, removal of lipids in cold 70% methanol	liquid partition paper chromatography	u.v. abs. of sulphuric acid/ ethanol chrom. 290 mμ

Fig. 4. The development of chemical methods for the determination of progesterone in human plasma prior to 1962.

A modified spectrophotometric method for the determination of progesterone in human plasma.

In 1958 Deshpande and Sommerville described procedures for the determination of progesterone and pregnanediol in human plasma, and later presented results from the application of these methods to ten ml serial plasma samples from patients in labour (Deshpande et al., 1960). Initially an attempt was made to devise a method for the determination of both compounds in the same plasma sample, but at the time this proved impracticable for it was necessary to incubate the plasma for at least twentyfour hours with β-glucuronidase to hydrolyse the conjugated pregnanediol and the hydrolysate contained material which interfered with the spectrophotometric determination of the progesterone. However, after initial division of the plasma, the two methods involved similar extraction, chromatography and measurement.

In this study the plasma was treated with sodium hydroxide prior to extraction with ether:methylene chloride (4:1v.v). This procedure gave a clean extract which was chromatographed on a column of aluminium oxide. The progesterone was eluted with 20% chloroform in hexane, after a preliminary washing of the column with hexane and 5% chloroform in hexane. The final measurement was made spectrophotometrically as progesterone bisisonicotinic acid hydrazone, by a modification of the method of Umberger, (1955). Formation of the more sensitive thiosemicarbazone derivative (Pearlman and Cerceo, 1953) had been

reluctantly abandoned owing to the persistence of trace impurities in the final extract.

In a subsequent modification of the method (Deshpande et al., 1960) specificity was increased by introducing a liquid partition paper chromatogram after the alumina column, but to obtain a sufficiently pure extract for spectrophotometry this additional step was followed by a second alumina chromatogram. A flow sheet of the method is shown in Fig. 5.

In this form the method gave satisfactory results which were shown to be in good agreement (Table 1) with those obtained by Short on the same plasma samples (Sommerville and Pickett, 1962).

TABLE I.

PLASMA PROGESTERONE
µG per 20 ml

	A	B	C	D	Pregnancy Plasma
AMOUNT ADDED(µg)	1.5	1.5	2.5	4.35	-
Short, 1958	1.7	1.7	3.2	4.2	3.2
Sommerville and Deshpande, 1958	1.8	1.9	2.2	3.3	3.0

However, the progesterone technique had several disadvantages. In the first place the use of three chromatographic steps - paper chromatography preceded and followed by alumina chromatography, is time-consuming and laborious and the alternatives of split fractions or pooled eluates reduce respectively the sensitivity and specificity of the method. Secondly the formation of

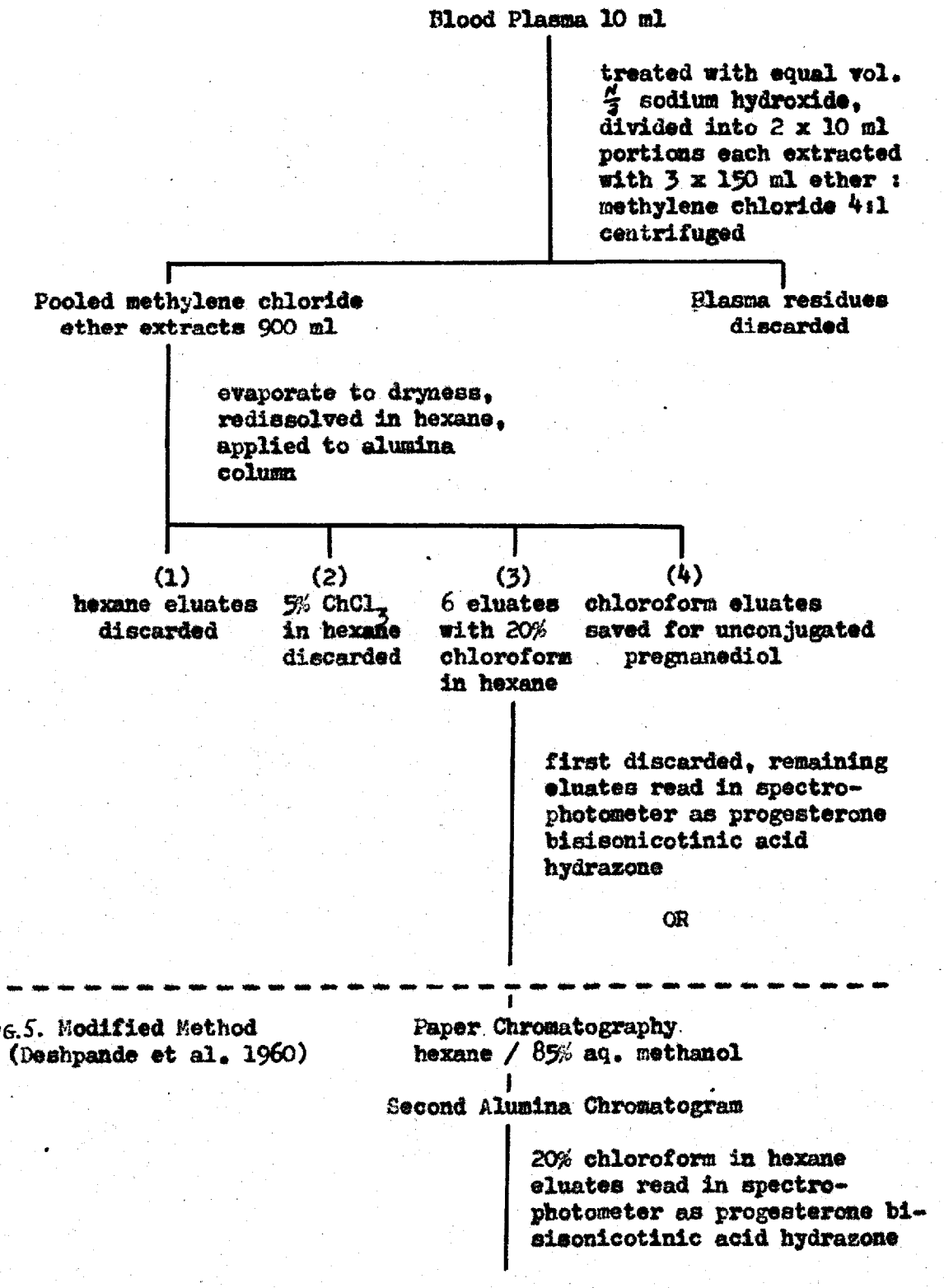


FIG.5. Modified Method (Deshpande et al. 1960)

progesterone bisisonicotinic acid hydrazone is not ideal in view of its low molar extinction coefficient (ε max. circa 11,800).

To overcome these disadvantages the first alumina chromatogram was replaced by a silica gel defatting column followed by paper chromatography and formation of the more sensitive thiosemicarbazone (ε max. circa 39,000). The advantages derived from formation of the thiosemicarbazone derivatives are illustrated in the absorption spectra and calibration curves shown in Fig. 6. The higher degree of purity following silica gel chromatography minimises interference in the final reaction, allowing a tenfold increase in sensitivity over the original procedure. At the same time the method is less complicated. To obtain reproducible results all reagents and materials were carefully standardised.

MATERIALS.

1. Solvents and reagents.

   a) Ether, peroxide free (May & Baker Ltd., England), washed once with volume of saturated aqueous solution of ferrous sulphate and twice with distilled water; then distilled slowly on a steam bath immediately before use.

   b) Methylene chloride laboratory reagent (British Drug Houses Ltd.), redistilled immediately before use.

   c) n-Hexane, low in aromatic hydrocarbons (Hopkin & Williams Ltd., England), redistilled before use.

   d) Heparin B.P., pure, (technical grade not satisfactory for this purpose), 1,000 units per ml distilled water.

   e) Ethanol, analar quality, spectrosol grade (James Burrough Ltd., England).

   f) Methanol, analar reagent (James Burrough Ltd.), redistilled and stored.

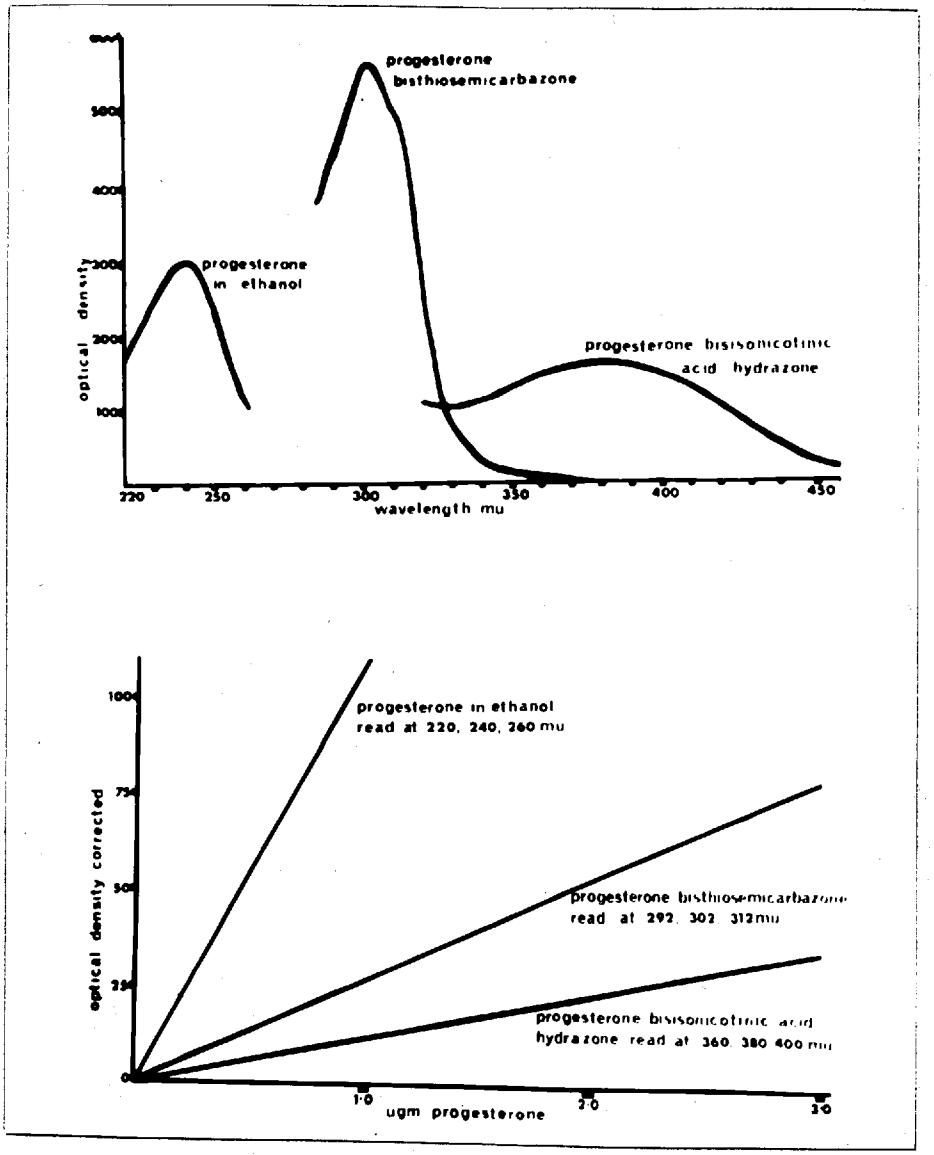


Fig.6. Absorption spectra and calibration curves of progesterone in ethanol, progesterone bisthiosemicarbazone, and progesterone bisisonicotinic acid hydrazone.

g) Ethyl acetate, analar reagent, (Hopkin & Williams Ltd.), redistilled immediately before use.

h) Thiosemicarbazide reagent (L. Light & Co. Ltd.). Thiosemicarbazide re-crystallised from methanol, 180 mg. dissolved in 20 ml of N hydrochloric acid (previously filtered) 100 ml absolute alcohol (spectrosol grade).

i) Silica gel, 60-200 mesh and 200-300 mesh (re-sieved in the laboratory) for chromatography (L. Light & Co. Ltd.). Washed with large volumes cold distilled water; hot distilled water; hot methanol (redistilled); ethyl acetate (redistilled); ethyl acetate : hexane (1:4) mixture. Dried at 120°C overnight and stored in airtight containers.

2. Glassware and other materials.

a) Centrifuge bottles, 250 ml, with ground glass necks (made to specification by Scientific Supplies Ltd., England) to fit all-glass siphon. The latter has a hooked stem so that only the extracting solvent is withdrawn.

b) Chromatography columns: straight, diameter 10 mm, length approx. 20 cm, with bulb reservoir of 50 ml and precision bore oblique stopcock, (Jencons Scientific Ltd., England).

c) Paper chromatogram spotter, (A.& H. Hall (Engineers) Ltd.).

d) Elution apparatus as described by Sander & Simmer, 1954 (Camlab Ltd., England).

e) Filter paper. Fluted paper (Greens Hyduro hardened paper 18.5 cm diameter), discs (10 mm Whatman No.1) and cotton wool used in chromatographic columns are all cleaned with methanol for 48-hours in a Soxhlet apparatus and dried in an oven. Paper for chromatography (Whatman No.2), is washed in a chromatography tank with 70% methanol for 48 hours, and then dried in an oven overnight.

f) Ultra violet spectrophotometer with microcell attachment.

Cleaning:- With the exception of the microcuvettes, all glassware is soaked in chromic-acid solution after use for at least 24 hours, and rinsed thoroughly first with water and then distilled water. The microcuvettes are rinsed with spectrosol alcohol after use. In addition, the tubes for the collection of the paper eluates and final reaction are washed 5/with ethanol and dried before use.

A flow sheet of the method is shown in Fig. 7. The peripheral venous blood is withdrawn by a non-greased syringe and carefully transferred to a centrifuge tube containing Heparin B.P. (10 units/ml blood). The plasma layer is removed after centrifugation, the volume measured, and transferred to a 250 ml centrifuge bottle or stoppered in a deep freeze (-15°C) until it is convenient to assay. Before the thirtieth week of pregnancy, and in the luteal phase of the menstrual cycle 10 ml plasma is required for the assay. In late pregnancy and during labour 5 ml plasma is adequate, and is extracted with half volumes of solvent. When the volume of plasma obtained falls short of 5 ml or 10 ml, it is diluted to the appropriate volume by the addition of distilled water. The use of the smaller volume of plasma in late pregnancy is preferred where a large number of serial analysis are required and since, as will be shown, it is more convenient to assay concentrations within the range 0.3 - 3.0 µg/100 ml plasma. When higher concentrations are encountered (e.g. in umbilical venous blood) the final reagent may also be diluted by the addition of a further 0.6 ml thiosemicarbazide reagent. Tissues are finely chopped, extracted with hot acetone, filtered and evaporated to dryness. The extract is then pre-treated with alkali and re-extracted in the same manner as plasma.

Alkali pre-treatment and extraction:-

This procedure is designed to obtain a complete extraction of progesterone, with the minimum of interfering material. For this

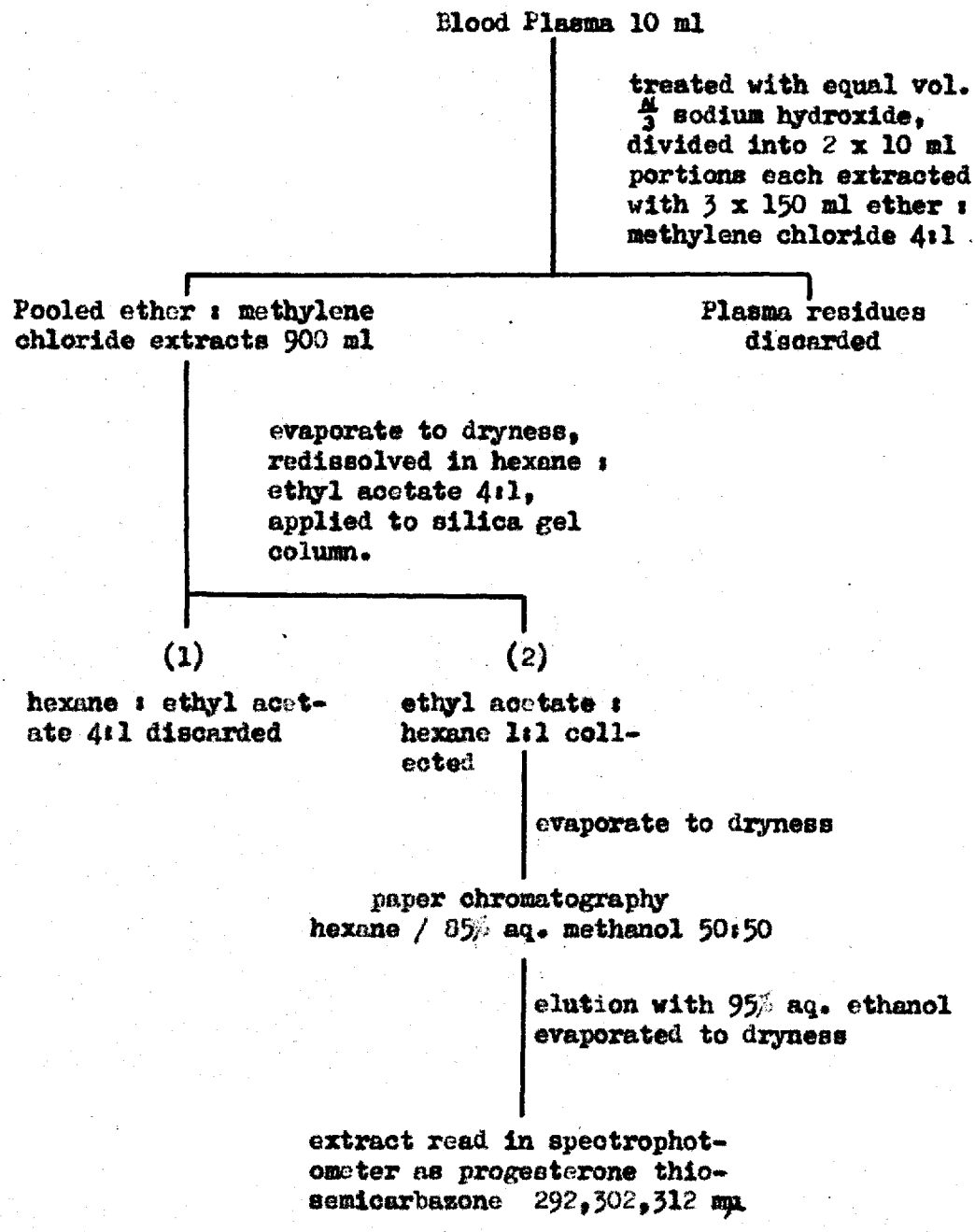


Fig. 7. Method of Sommerville et al., (1963).

purpose an equal volume of $\frac{4}{3}$ sodium hydroxide is added to the plasma, which is hydrolysed by gently shaking the bottle for five minutes. If 10 ml of plasma is used one half of the diluted alkaline sample is transferred to a second centrifuge bottle, the pipette being washed with solvent mixture which consists of ether : methylene chloride (4:1 v/v). To each centrifuge bottle is added 150 ml solvent mixture and extraction is effected by stirring rapidly for two minutes with a mechanical glass stirrer which is washed down each time. Centrifugation is not employed routinely but could be used if there is emulsion formation. The extracts from each bottle are siphoned through a fluted filter paper into a 500 ml long necked round bottomed flask, and evaporated to a small volume.

This extraction process is repeated twice and the solvent reduced to a small volume after each extraction. In this manner, 10 ml of plasma sample is extracted with 900 ml of solvent mixture. The residue from the third distillation (approx. 10 ml) is allowed to cool, and is then transferred into a 100 ml short-necked round bottomed flask. The larger flask is rinsed three times with approximately 15 ml solvent, each rinsing being added to the 100 ml flask. The solvent is then evaporated under reduced pressure.

Silica gel chromatography:-

This step is introduced to further purify the extract before liquid partition paper chromatography.

A small plug of cotton wool is pressed into position at the foot of the column which is then filled with a mixture of ethyl acetate / hexane (1:4). A few grains of silica gel (60 - 200 mesh) are poured on to the cotton wool to prevent the silica gel of finer mesh from passing through into the collecting vessel. The stem is then allowed to fill with solvent. Two gm of silica gel 200 - 300 mesh measured in a calibrated bottle is slowly poured into the column which is tapped continuously to ensure even packing. The column is then allowed to settle and a small filter paper disc (1 cm diameter) is gently placed onto the top. The 1:4 mixture is then allowed to pass through the column until the meniscus is just above the paper disc.

During the preparation of the column, 2.0 ml ethyl acetate is added down the sides of the round bottomed flask (containing the plasma extract) which is stoppered and left to stand for five minutes. 8.0 ml hexane is then added to the flask in the same manner and the contents of the flask (10 ml of 1:4 ethyl acetate / hexane) poured on to the column through a small filter funnel. The flask is rinsed with a further 5 ml of the 1:4 mixture which is also added to the column. When the 15 ml has passed through, a further 15 ml rinsing is also added. These eluates are discarded. Progesterone is eluted from the column by the addition of 15 - 20 ml (depending on the activity of the silica gel) of 1:1 ethyl acetate / hexane and this is

collected in a 25 ml conical test tube. The eluate is taken to dryness under nitrogen in a water bath at 60°C. After evaporation the sides of the tube are washed down with a small volume of ethyl acetate; this is also removed under nitrogen and the tube may be stored in a desiccator.

Paper chromatography:-

In this step the progesterone is adequately separated from other known Δ^4-3-ketosteroids which, if present, would otherwise be determined by the final measurement.

The solvent system consists of 85% aqueous methanol / hexane (50 : 50) and this is allowed to equilibrate in a separating funnel for at least one hour at 35°C.

The paper chromatogram is cut into strips each 1 cm wide and extending 30 cm from the starting line. The extract is applied with a long capillary tube. The solvent is evaporated by a Hall chromatogram spotter which applies a stream of nitrogen under the starting line. The content of the conical tube is transferred with 3 x 0.1 ml absolute ethanol. In addition to the plasma extracts, a blank strip of paper is run and the eluate from this is later used to correct for interfering material eluted from the paper only. At this stage two progesterone standards of appropriate concentration (e.g. 0.7 µg) are prepared - one to run on paper and the other to be evaporated to dryness and stored in a desiccator to be read at the end of the method. Sudan red is

applied to the starting line of the outer strips in order to locate the solvent front when the chromatogram is running and on these strips a line is drawn in ink at a distance of 20.5 cm from the origin. If the chromatogram is allowed to run to this line the progesterone besides separating from the other Δ^4-3-ketosteroids is adequately separated from interfering material running near the solvent front.

The chromatography tank is prepared with both phases in the usual fashion; the paper placed in position; the lid sealed with glycerine / water (2:1) and the tank allowed to equilibrate for three hours at 35°C. At the end of this time, sufficient mobile phase is added. When the sudan red reaches the 20.5 cm line, the chromatogram is removed from the tank, dried in air and inspected in ultra violet light. The progesterone should be approximately 13 cm from the origin and an area extending slightly above and below the shadow (approximately 4 cm in all) is marked off in pencil on the standard and unknown strips. The solvent front is also marked; the area of the paper strip containing the progesterone standard is cut out and an identical section equidistant from the solvent front is taken from all strips including the blank.

Elution:-

Each 4 cm section is shaped to a blunt point and a cut is made at the tip into which a wedge of filter paper is inserted (Bush, 1961). The paper is suspended in an elution apparatus as

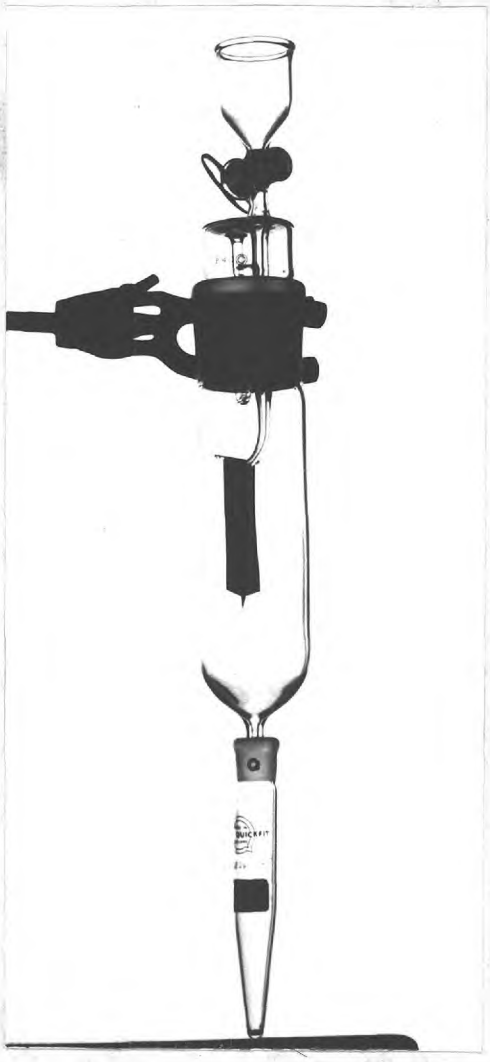


Fig.8. Elution apparatus for paper chromatograms.

shown in Fig. 8, and slowly eluted with 15 ml 95% ethanol. The elute is collected into the developing tubes which are taken to dryness under a stream of nitrogen at 60°C and may be stored in a desiccator.

Spectrophotometry:-

0.6 ml thiosemicarbazide reagent is added down the sides of each tube, and a set of tubes comprising the unknowns, the paper standard, paper blank, reference standard (0.75 µg) and reagent are stoppered and maintained at 25°C in the dark for 1.5 hours. At the end of this time each solution is read in a silica micro-cuvette at 292, 302 and 312 mµ. The reference standard is read against the reagent and the unknowns and paper standard against the paper blank. The progesterone content of the unknowns is calculated from a calibration curve based upon the Allen correction factor (Allen, 1950) for pure progesterone bis-thiosemicarbazone. By comparison of the Allen factor (D corr.) of the paper standard read in this way, with that of the standard read against the reagent a check can be made upon the straight line absorption of the paper background and the efficacy of the elution step.

Time required:-

The extraction of 4 x 10 ml samples and silica gel chromatography can be carried out on the first day of the procedure. Application to the paper, subsequent chromatography and elution

occupy the second day and the final reaction and spectrophotometry is usually performed on the following morning.

Accuracy:-

The accuracy of the method can be assessed from the recovery experiments shown in Table 2.

TABLE 2

QUANTITATIVE DETERMINATION OF PROGESTERONE

RECOVERY FROM MALE PLASMA
(pooled for replicate determination in 10 ml samples)

Progesterone added (µg)	Progesterone recovered (µg)	Recovery (%)
0.375	0.38, 0.31, 0.36, 0.26	88.5
0.75	0.69, 0.72, 0.62, 0.78	93.5
1.50	1.35, 1.35, 1.29, 1.39	89.7
3.00	2.79, 3.03, 2.76, 2.35	91.1
4.50	3.45, 3.52, 3.79, 3.43	80.5
6.00	4.64, 4.65, 5.19, 4.83	79.0

Further evidence for the accuracy of the method has been obtained by recoveries of progesterone from the silica gel column (Table 3) and from the paper chromatograms (Table 4).

TABLE 3

RECOVERY FROM SILICA GEL

Progesterone added (ug)				Progesterone recovered (%)			
1.5,	1.5,	1.5,	1.5	102,	90.5,	95.3,	95.0
3.0,	3.0,	3.0,	3.0	98.5,	98.0,	100.0,	96.2

TABLE 4

RECOVERY FROM PAPER CHROMATOGRAMS

Progesterone added (ug)					Progesterone recovered (%)				
1.5,	1.5,	1.5,	1.5	1.5	100.0,	95.3,	90.0,	100.0,	100.0
1.5,	1.5,	1.5,	1.5	1.5	108.0,	89.5,	97.8	95.3,	92.6
3.0,	3.0,	3.0,	3.0		100.0,	96.0,	98.2,	97.6	

It may be seen that the silica gel and paper chromatography involve negligible losses and in view of the level of recovery attained by the total procedure at concentrations of 0.375 - 3.0µg/ 10 ml plasma, no correction is applied. The recovery from male plasma at higher concentrations (4.0 ug and 6.0 µg / 10 ml) is somewhat lower approx. 80% but such concentrations have not been encountered in the determination of progesterone of endogenous origin since smaller volumes of plasma are used for the analysis of umbilical venous blood.

Precision:-

The precision of the method may be assessed from the reproducibility of recovery experiments from male plasma (Table 2), and from replicate determinations in pooled pregnancy plasma (Table 5).

TABLE 5

REPLICATE DETERMINATIONS IN PREGNANCY PLASMA

Week of pregnancy	Progesterone (µg per 100 ml)
9th - 11th (pooled)	2.1, 1.9, 2.1, 2.1
22nd - 30th (pooled)	7.3, 8.7, 8.7, 8.1

Specificity:-

The specificity of the method depends upon the adequate preliminary purification by silica gel chromatography, and the separation of progesterone from other known Δ^4-3-ketosteroids by paper chromatography (hexane / 85% aqueous methanol) as shown in Table 6.

TABLE 6.

STEROID	rF VALUE	U.V. ABS. AT 240 mµ
20 α hydroxypregn-4-en-3-one	0.23	+
androst-4-ene 3,17 dione	0.28	+
20 β hydroxypregn-4-en-3-one	0.45	+
3 β hydroxypregn-5-en-20-one	0.55	
progesterone	0.60	+
5 α and 5 β androstanedione	0.63	
5 α and 5 β pregnanedione	0.69	

5α and 5β pregnanedione, Δ^5 pregnenolone, 5 α and 5β androstanedione, and allopregnanolone have rF values close to that of progesterone, and if present in the plasma would form mono or bisthiosemicarbazones (λ max. 270 mμ) which would tend to increase the reading at 292 mμ, and thereby lower the Allen correction factor. However subsequent work with gas-liquid chromatography has not demonstrated the presence of signficant amounts of these compounds.

Further evidence for specificity at the sensitivity of the spectrophotometer is afforded by the linearity of the male plasma 'blank', and the fact that the bisthiosemicarbazone of the endogenous progesterone has an absorption maximum at 302 mμ.

Sensitivity:-

The limit of sensitivity of the method is 0.1 μg progesterone, i.e. this concentration yields a bisthiosemicarbazone with an Allen factor significantly different from that of the male plasma 'blank'. This allows the determination of progesterone in 10 ml aliquots of peripheral venous plasma in early pregnancy and during the luteal phase of the menstrual cycle.

Application:-

The method has been applied to the determination of progesterone in serial samples of peripheral plasma during pregnancy, labour and postpartum, and six samples of myometrium (Section 1d).

A gas-liquid radiochromatographic method for the determination of progesterone in human plasma.

Since the development of the preceding spectrophotometric method, attempts have been made to use the more sensitive techniques of fluorimetry, double isotope dilution, and gas-liquid chromatography for the determination of progesterone in human plasma.

In 1960 Touchstone and Murawec described a fluorimetric reaction which consisted of heating progesterone at 60°C for 30 minutes in 2N potassium hydroxide in methanol. When this mixture was dissolved in sulphuric acid at room temperature a maximal fluorescence was obtained at 490 mμ with excitation at 390 mμ.

This reaction was adapted by Short and Levitt, (1962) and applied to the paper eluate obtained from the extraction and purification procedures of Short, (1958). The increased sensitivity suggested the presence of progesterone in both follicular and luteal phases of the menstrual cycle, and the results implied that the progesterone concentration was generally higher in the luteal than in the follicular phase of the cycle. The values in the luteal phase were in the same region as those previously determined spectrophotometrically by Sommerville et al., (1963) and were some hundred times less than reported by the biological assay method of Forbes, (1951). However fluoroscopy at this sensitivity is liable to interference by non-specific material, which may produce high and erratic blank values. The results from the determination of endogenous progesterone at low concentrations must therefore be

interpreted with caution.

Another more sensitive approach is that of double isotope dilution. The principles of this technique were first described by Keston et al., (1950) for the determination of amino acids. The procedure involved formation of radioactive derivatives with ^{131}I p-iodobenzenesulphonyl chloride. From the amount of ^{131}I in the isolated derivative the amount of each endogenous amino acid could be determined, while the recovery throughout the method was checked by the addition of a known quantity of the appropriate ^{35}S derivative.

Similar procedures were subsequently applied to the determination of cortisol and corticosterone (Bojeson and Degn, 1956) and to the determination of plasma and urinary aldosterone using ^{14}C acetic anhydride (Kliman and Peterson, 1960).

The technique was applied to the determination of plasma progesterone by Woolever and Goldfien in 1963. In this study progesterone-4-^{14}C was added to the plasma sample as an internal control on recovery and the total progesterone was extracted and subsequently isolated by paper chromatography. A tritium labelled derivative was prepared by reduction with sodium borotritide followed by partial re-oxidation with manganese dioxide. The derivative was then isolated and purified by paper chromatography. The carbon 14 and tritium content of the samples were determined by liquid scintillation counting and the relative amount of each isotope calculated by the method of Okita et al., (1957).

The technique of double isotope dilution is theoretically the most sensitive, accurate and precise method available, as an internal standard is added to each sample, and the sensitivity is only limited by the specific activity of the reagent. However in practice the accuracy and precision are limited by error in pipetting and counting, whilst the sensitivity is limited by the amount of background radioactivity in the final measurement.

The criterion of specificity is the most difficult to satisfy as any compound in the solvents or reagents containing the reactive group (in the case of progesterone the carbonyl group) will interfere with the final measurement. Therefore the purity of the derivative must be rigorously tested, and adequate specificity is usually only achieved after many chromatographic steps. This limitation has resulted in the development of methods, which at the present are time consuming, laborious and expensive, but the principle offers great potential for development possibly in the form of gas-liquid radiochromatography.

The principles of gas-liquid chromatography were developed by James and Martin, (1952 a,b) for the analysis of readily volatile fatty acids. In these studies a stream of inert gas (the mobile phase) was led through a glass column containing a polar liquid (the stationary phase) on an inert support (10 - 12% w/w), which was maintained at a suitable temperature to render the fatty acids volatile in the gas. Separation depends upon the relative partition coefficients of the fatty acids in the gas and stationary

phase. The acids leaving the column were measured by titration against a base.

The vast potentialities of the technique were soon appreciated and adaptations devised for the separation and measurement of other compounds.

The first application to sterol chemistry was reported in 1959 by Eglinton et al. These authors found that cholestanone could be chromatographed on a column of Apieson L as developed for the separation of fatty acids, but even at 220°C the retention time was about four hours. Shorter retention times were reported by Beerthuis and Recourt, (1960) who demonstrated the separation of oestrone, oestradiol, oestriol and testosterone as their acetates on a 10% column of silicone grease.

Further improvements were introduced by Vanden-Heuvel et al. in 1960. Shorter retention times and finer separations were achieved by the development of stationary phases which were more stable at higher temperatures, and by the application of 1 - 3% of these stationary phases to the supporting material. These advances allowed the separation of many pure sterols and steroids.

This work with pure solutions offered the hope of more rapid, sensitive and specific methods for the determination of steroids in biological extracts. There were however two main problems - to effect adequate preliminary separation without resort to laborious techniques and to ensure that the methods were quantitative.

In the present study upon the determination of plasma progesterone the first difficulty has been solved by the use of two dimensional thin layer chromatography and the second problem has been overcome by the simultaneous determination of an isotopically labelled internal standard in terms of integrated radio-activity (Collins and Sommerville, 1964).

These procedures together with a simplified extraction of the plasma give a high degree of specificity, accuracy and precision. The sensitivity is adequate for the determination of progesterone in 5 ml samples of pregnancy plasma and 10 ml samples collected throughout the menstrual cycle. The time required for the estimation is three hours plus the duration of the gas-liquid radiochromatography, which may last from 20-80 minutes depending on the choice of stationary phase and temperature. This time factor represents a considerable advantage over other procedures.

While this work was in progress there were preliminary reports on the application of gas liquid chromatography to the determination of progesterone in extracts of human myometrium, (Kumer et al., 1962) and to the identification of the hormone in pregnancy plasma, (Futterweit et al., 1963).

MATERIALS

All solvents were purified by standard techniques and redistilled prior to use. The stationary phase for thin layer chromatography was 'Kieselgel' with calcium phosphate binder

and fluorescent indicator from Camag Ltd. (obtained from Griffin and George Ltd., London). Gas-liquid radiochromatography was performed on a Pye panchromatograph with a macro-argon ionization detector; the radioactivity being measured simultaneously by a gas proportional counter coupled to an Echo ratemeter (type N701A) which incorporates an integration unit. The instrument was modified so that all the effluent gas from the column passes through the argon detector before combustion and detection in the gas proportional counter. A block diagram of the apparatus is shown in Fig. 9. The columns are five feet in length and for routine use the stationary phase preferred is 1% cyclohexane-dimethanol succinate (CDMS) on Gas Chrom. P. 60-80 mesh (W. G. Pye and Co. Ltd., Cambridge). Progesterone-4-^{14}C (21.7 mc/mM) was obtained from the Radiochemical Centre, Amersham, Bucks.

METHOD

The peripheral venous blood is withdrawn and the plasma removed as previously described. An internal standard (0.1 - 0.5 µg) of progesterone-4-^{14}C is added to each sample. After the addition of $\frac{n}{3}$ sodium hydroxide, the mixture is shaken for five minutes; extracted with 2 x 100 ml ether; the extract washed with 20 ml distilled water and evaporated to dryness in vacuo. The extract is then transferred with 3 x 5 ml methanol:chloroform (1:1) to a small conical test tube and evaporated to dryness under nitrogen at 60°C (a small volume of the same mixture is added to wash the

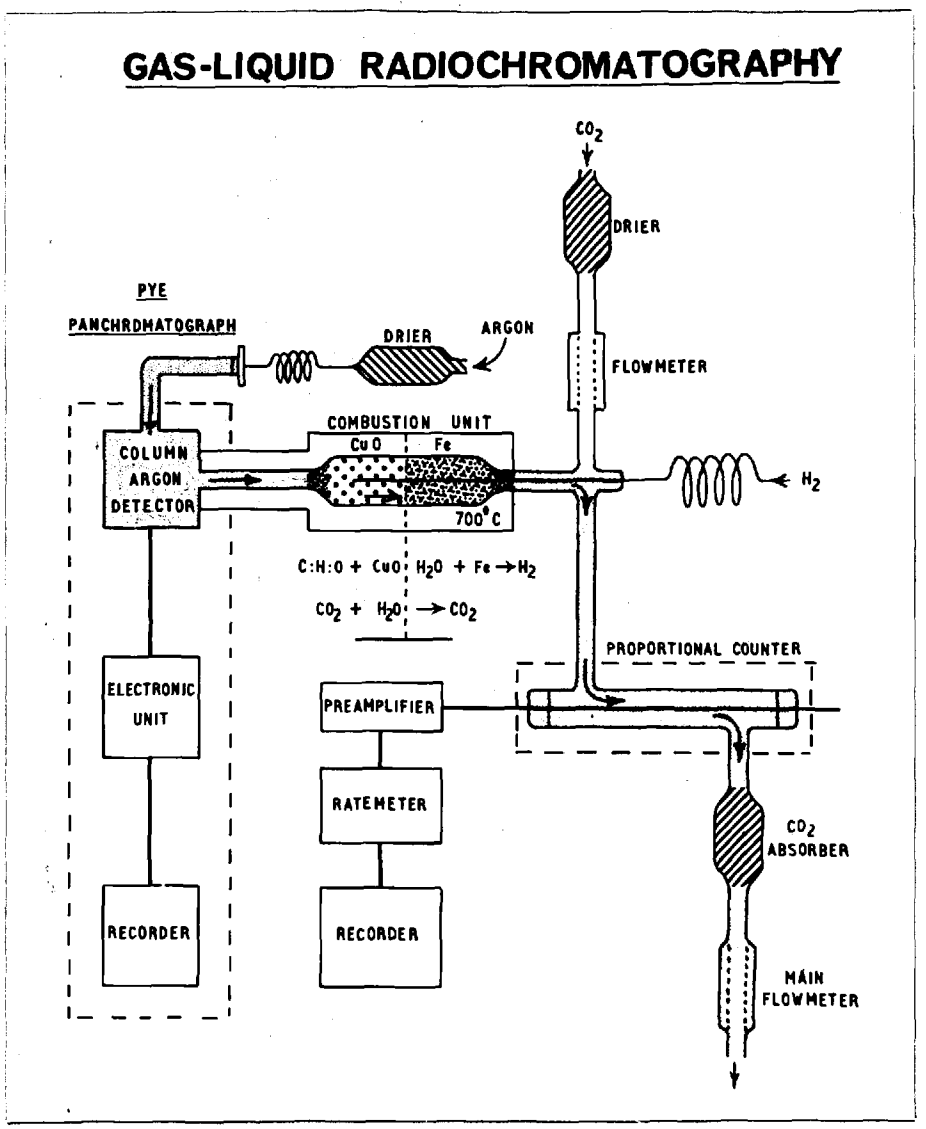


Fig.9. Block diagram of Pye Panchromatograph for gas-liquid radiochromatography.

extract into the tip of the tube and after re-evaporation the tube may be stored in a desiccator.

Glass plates 20 cm x 20 cm are coated with Kieselgel and stored in a desiccator before use. The extracts are applied to separate plates (using a glass capillary and 3 x 0.05 ml methanol:chloroform (1:1) on a spot 2 cm in from either side of the right hand corner. The first system used is benzene / ethyl acetate (3:2), the solvent being allowed to travel 15 cm. The plates are then removed, thoroughly dried, the compounds are located under ultra-violet light (2537 Å) and demarcated with a fine needle. As an additional safeguard, the position of the steroid may be located either by extrapolation from two standards applied to the contralateral corners of the plate at the start of each chromatogram, or by autoradiography over night on Kodirex X-ray film. The silica gel containing the compound is loosened with a microspatula; aspirated on to a filter disc (1 cm diameter No.3 grade); eluted with 3 x 0.5 ml ethanol under positive pressure and the eluate evaporated under nitrogen at 60°C. The elution apparatus was devised in the department by Dr. M. I. Stern and is shown in Fig. 10.

Fig. 11 is a photograph of the ultra-violet absorption of four authentic steroid standards, and Fig. 12 shows the result obtained in an extract of pregnancy plasma. In some instances (e.g. post mature plasma samples) examination of the plate in ultra-violet light reveals other compounds which are under investigation.

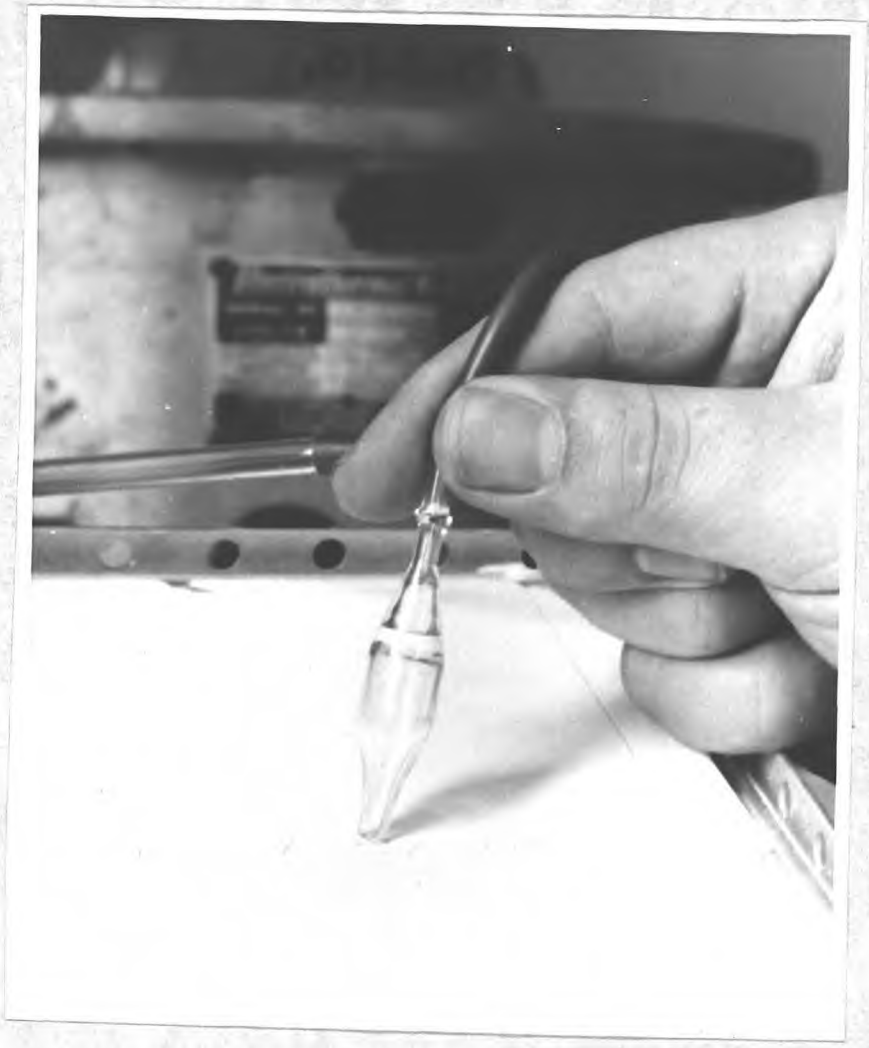


Fig.10. Elution apparatus for thin-layer chromatograms.

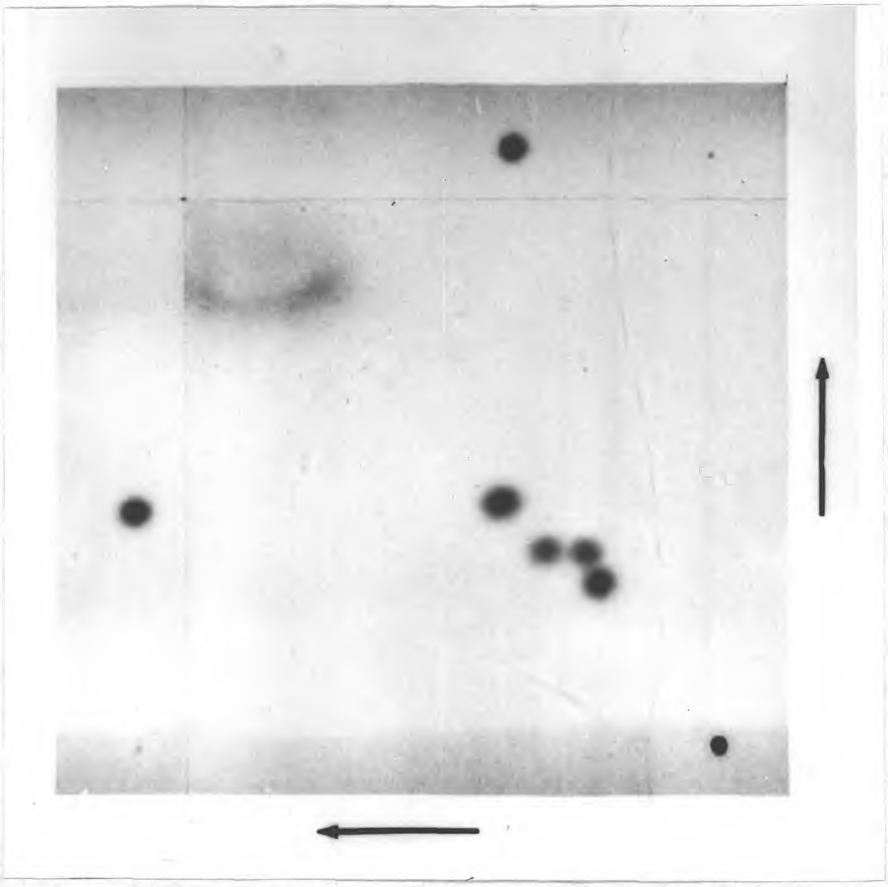


Fig.11. Two-dimensional thin-layer chromatogram showing 20 α -hydroxypregn-4-en-3-one (right); androst-4-ene-3,20-dione (left); 20β hydroxy-pregn-4-en-3-one (left); progesterone.

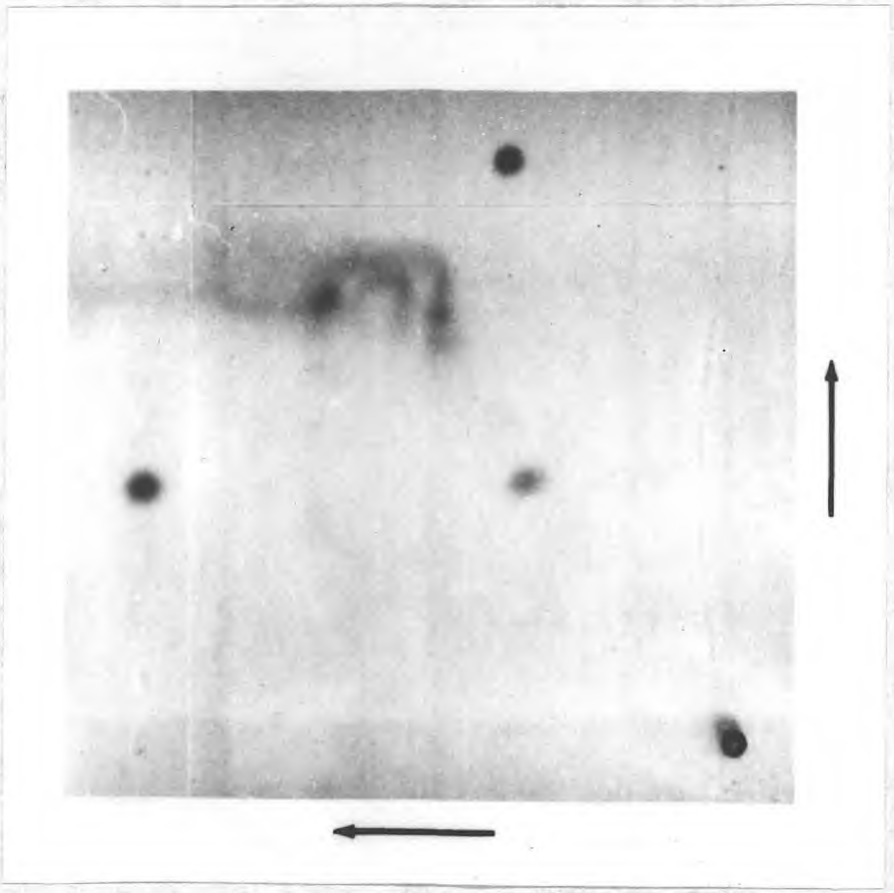


Fig.12. Two-dimensional thin-layer chromatogram showing progesterone (centre of plate) from extract of 8.0 ml. pregnancy plasma (spot includes internal standard).

Where gas-liquid chromatography is not available, the progesterone in the eluate (if more than 0.1 µg) may be determined spectrophotometrically after formation of the bisthiosemicarbazone, and the recovery determined by liquid scintillation counting.

For gas-liquid radiochromatography, the eluate is transferred with 3 x 20 µl methanol / chloroform (1:1) to the centre of a rectangle of folded stainless steel gauze (previously flamed and held over a warm surface) which is dropped on to the top of a GLMS column after cessation of the gas flow. All conditions are carefully standardised and the chromatography carried out at 210°C. The retention time of progesterone is 72 minutes and the relative retention time of other steroids can be expressed in terms of cholestane or using progesterone itself as a marker. For routine work the retention time of progesterone may be shortened by raising the temperature. The amount of progesterone on the differential trace from the argon detector is calculated from the peak area mm^2 and reference to a standard curve. From this, the amount of internal standard recovered (calculated from integrator units on the radiochromatography recorder) is subtracted and the results corrected for loss.

Table 7 shows the relationship between progesterone, peak height, area and integrator units at one of the concentrations used for calibration. Peak height is not satisfactory at relevant concentrations whereas satisfactory calibration curves are obtained from peak area and integrator units. The sensitivity of the argon

TABLE 7

QUANTITATIVE GAS-LIQUID RADIOCHROMATOGRAPHY

0.5 µg progesterone - 4 - ^{14}C

Argon (differential trace) 210°C : 10^{-9} amp : 1,000 V : 5/hr		Integrated radioactivity FSD 1,000 p.p.s. recorder sensitivity
Peak height (mm)	Peak area (mm^2)	Integrator units
128	694	330
110	710	320
125	705	330
141	709	335
123	721	330
147	730	335
Mean S.D. 129 ± 13.3	711 ± 12.5	330 ± 5.5
Coeff. of variation 10.3	1.8	1.7

0.10 µg progesterone - 4 - ^{14}C

225°C : 10^{-9} amp : 1,000 V : 10/hr	FSD 1,000 p.p.s.
101	1,111	205
101	1,313	210
102.5	1,308	202
99	1,138	218
92.5	1,156	215
98.5	1,231	208
Mean S.D. 99.1 ± 3.5	1,258 ± 80.2	209 ± 6.1
Coeff. of variation 3.6	6.4	2.9

detector is tested daily by calculating the peak area and recovery of a sample of pure progesterone-4-^{14}C. In this connection, it was found that a small proportion (2.5 per cent) of the standard consists of an isotopically labelled component (possibly the 17α-epimer) which appears immediately before the main peak.

For the determination of progesterone during the luteal phase of the menstrual cycle and pregnancy, adequate sensitivity is obtained on the panchromatograph with a current range of 10^{-9} amp; the ratemeter at minimum sensitivity (full-scale deflection 1,000 p.p.s. ½ recorder sensitivity) and a chart speed of 5"/hr. This permits the determination of 0.01 µg. However as shown in Table 6 higher sensitivity can be obtained by decreasing the attenuation or amplifying the signal from the argon detector through a second integrator and by increasing the chart speed.

Figs. 13 and 14 are photographs of differential traces at 10^{-9} amp. It will be seen that there is adequate separation of a variety of steroids which in the thin layer system have Rf values close to that of progesterone. Androst-4-ene-dione is not shown in Fig. 13, but it has a retention time between that of 5β-pregnane-3,20 dione and Δ^5 pregen-3β-ol-20-one. At this sensitivity progesterone has been the only steroid present in the progesterone' eluate from 80% of the plasma samples collected during the luteal phase of the cycle or pregnancy, however in a

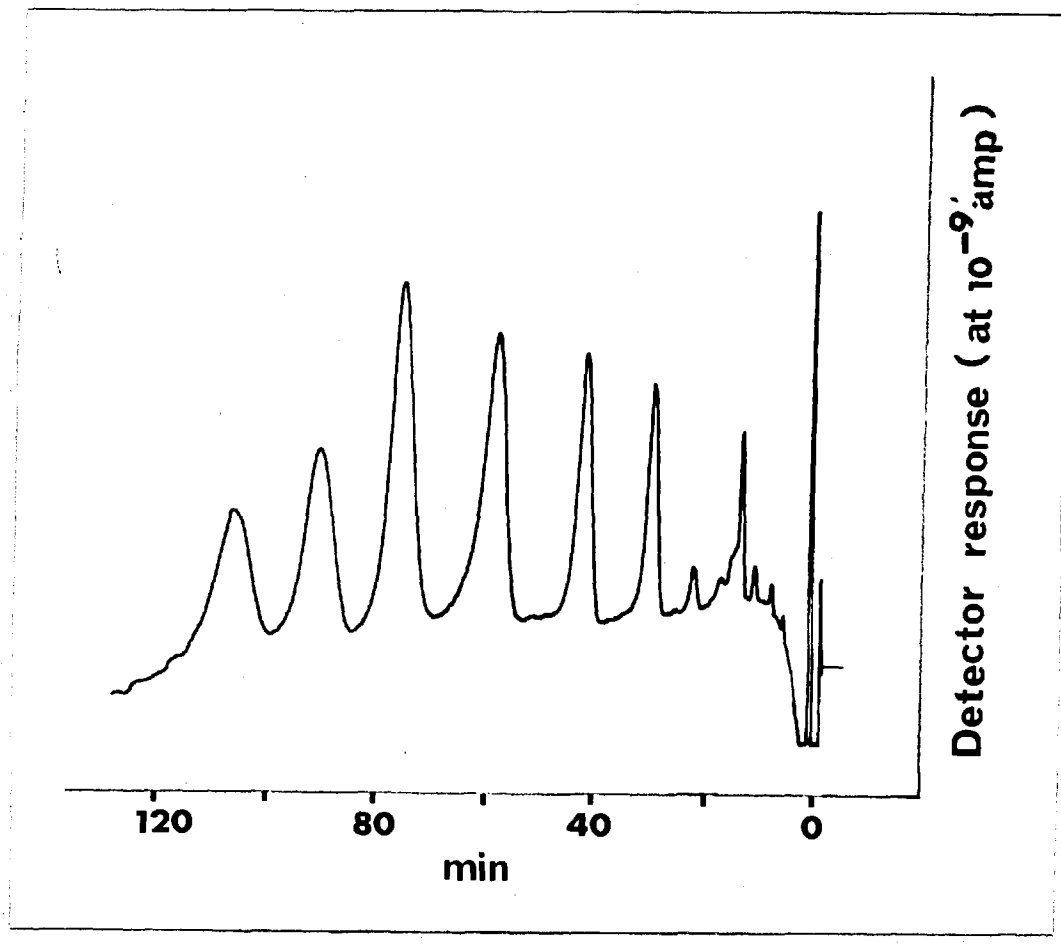


Fig.13. Differential trace from macro-argon detector (1% CDMS: 210°C, 10^{-9} amp; 1,000 v; chart speed 5"/hr). Peaks represent from right to left: solvent; 5β-androstane-3, 20-dione, 5β-pregnane-3,20-dione; Δ^5-pregnen-3β-ol-20-one; progesterone, 20β-hydroxy-pregn-4-en-3-one; 20α-hydroxy-pregn-4-en-3-one.

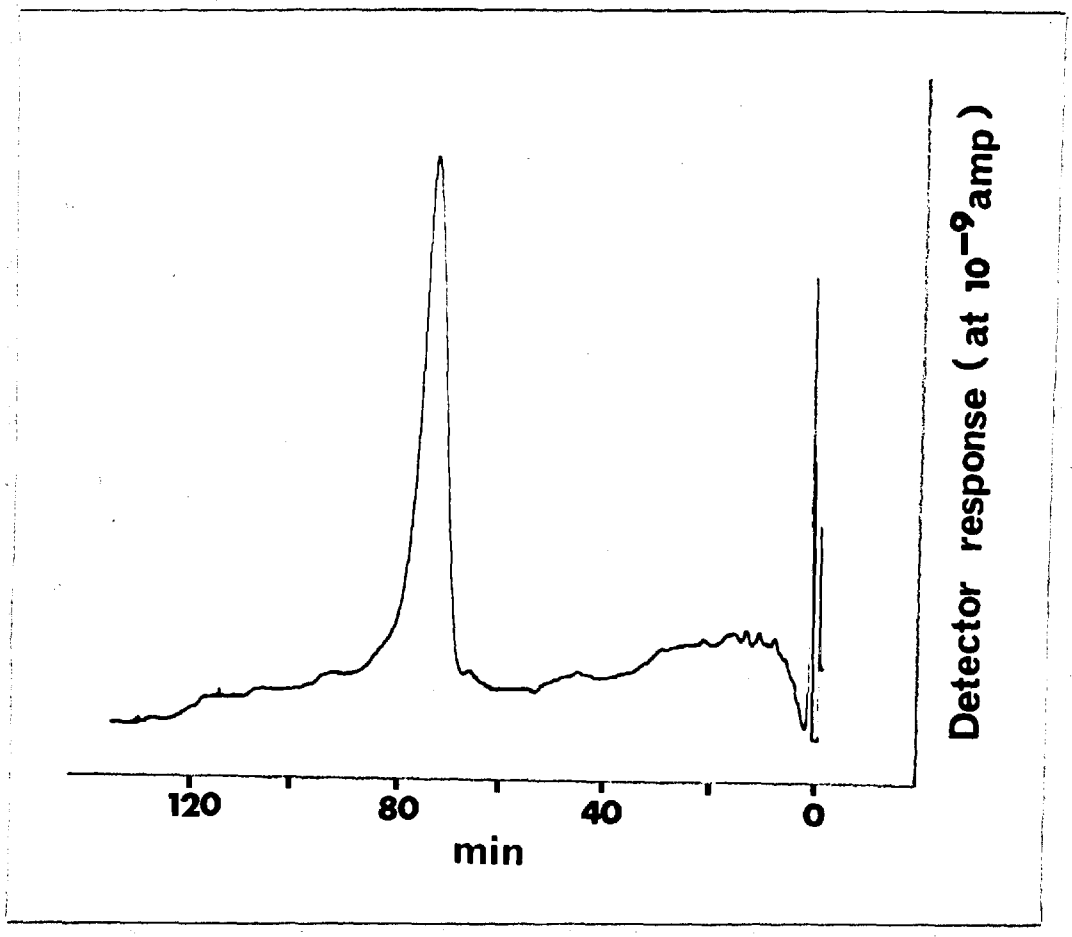


Fig.14. Same conditions as Fig.13.: progesterone (endogenous plus internal standard) eluted from plate shown in Fig.12.

few cases another peak well separated from progesterone has been visible on the gas-chromatogram.

The specificity is achieved by the two-dimensional chromatogram and by the 1% CDMS column. The simultaneous recording of radioactivity confirms the identity of the progesterone peak at each analysis. If other Δ^4-3-ketosteroids are detected on the thin layer plate, these can be eluted separately or as a group and then subjected to gas-liquid chromatography. Further evidence on specificity has been obtained by the use of other stationary phases - 1% neopentyl glycol adipate (NPGA) and the silicone polymers, 3% 'QF-1' and 3% 'SE-30'. In addition, the 1:1 dimethylhydrazone and the bisethylene ketal derivatives of endogenous progesterone from pregnancy plasma have been chromographed on 1% CDMS and 3% 'QF-1'.

It will be noted from Fig.14 that the minimum of non-steroid material is applied to the column and this obviates contamination of the macro-argon detector.

The results from the application of the method together with the recoveries of the internal standard are presented in the next section. The validity of the recoveries and hence the precision of the method was investigated by a series of replicate determinations on a pool of early pregnancy plasma. The results from a series in which the recoveries were both poor and variable are shown in Table 8.

TABLE 8

Early pregnancy (replicates on pool)			
Recovery of internal standard		progesterone/5ml	
84	100	0.22	0.21
20	85	0.22	0.19

However, the recoveries are usually higher than 80%, and the loss appears to be mainly due to the technique of transferring the eluate to the metal gause (since a similar loss occurs in a proportion of the standards).

More recent refinements of this technique include the introduction of a stream of hydrogen into the combustion chamber to clear tritium produced and so enable the use of tritiated progesterone S.A.750 mc/mM as an internal standard. This modification decreases the ratio of exogenous/endogenous progesterone by a factor of ten, and is now employed routinely for the determination of peripheral progesterone during the menstrual cycle. Using progesterone-^{3}H of high specific activity a significant number of integrator units is obtained with as little as 0.005 µg., although for the present, 0.1 µg is used as carrier to facilitate the calculation of specific activity. A series of standards at this level (and using minimum sensitivity; 1,000 p.p.s./full-scale deflection) yields 1775 integrator units $\pm$ 6.3 (mean $\pm$ S.D.) with a coefficient of variation of 0.04%.

It should be emphasised that the gas-liquid chromatography of steroids presents special problems with regard to column efficiency and the fact that the temperature for work under isothermal conditions is relatively high. Above all, attempts to increase the sensitivity of the method for the determination of nanogram amounts of steroids is obviously essential for the microanalysis of the quantities present in plasma samples of reasonable volume.

In view of the limited quantity of steroids available in serial analyses in human subjects it may be necessary to transfer the total final product of the analysis to the column and some form of technique for solid injection is essential, because it would not be possible to transfer the final product with the small volumes of solvents which can be used for liquid injection at high sensitivities. Accordingly, throughout the present work solid injection has been employed and the top of the column was modified so that the metal gauze to which the extracts have been transferred could be dropped on to the column and removed without the necessity to disconnect the base of the column. This modification is based upon a more elaborate system designed by Dr. Menini. In the present work the gauzes are suspended upon a cross wire in an oven at 60°C so that the transferring solvent is evaporated. Quantitative transfer, e.g. 3 x 10 µl can than be conveniently performed. The gauzes (obtainable from Griffin and George, Ltd.) are placed in a glass

sidearm (up to six gauzes at a time) and moved along the sidearm on to the column by a magnet without interruption of the gas flow. The elimination of the majority of the solvent front which would have resulted from liquid injection yields chromatograms in which the signal from the steroid rises from a satisfactory base line.

Developments in instrumentation are actively in progress and their application to the present problem continues, but the results obtained by the technique as described are presented in the following section.

RESULTS

1) Studies upon the chemical determination of progesterone during pregnancy.

   a) The individual variation in progesterone levels in peripheral venous plasma.

   b) The serial determination of progesterone in peripheral venous plasma during normal labour.

   c) The serial determination of progesterone in peripheral venous plasma post partum.

   d) The determination of progesterone in uterine venous plasma.

   e) The determination of progesterone in umbilical arterial and venous blood.

   f) The determination of progesterone in amniotic fluid.

2) Studies upon the chemical determination of progesterone during the menstrual cycle.

   a) The range and variation of progesterone levels during normal menstrual cycles, and the serial determination from the same subject.

   b) The determination of progesterone in ovarian venous blood.

   c) The determination of progesterone in adrenal venous blood.

3) Studies upon the chemical determination of progesterone in tissues.

   a) The determination of progesterone in myometrium.

1) Studies upon the chemical determination of progesterone during pregnancy.

a) The individual variation in progesterone levels in peripheral venous plasma.

The results obtained by biological assay before the chemical isolation and determination of progesterone in human peripheral plasma were very variable. These results are shown in Table 9.

TABLE 9

Authors	Range of progesterone levels	
	menstrual cycle	pregnancy
Haskins, (1941)	-	13.0µg / 100ml serum
Hoffman and V.Lam,(1949)	0.3 - 0.4 µg / 100 ml blood luteal phase	up to 9µg / 100ml blood
Forbes, (1950, 1951)	170 - 520 µg / 100 ml plasma luteal phase	up to 200µg/100ml plasma
Schultz, (1953)	-	38 > 25µg / 100ml plasma 8 up to 200µg/100ml plasma 2 above 200µg/100ml plasma

As already mentioned (Section 1a), the first attempts at chemical measurement by Reynolds and Ginsburg, (1944), Haskins, (1950)

Butt et al., (1951) failed to detect progesterone in the peripheral blood of normal women, but indicated that, if present, the level was below 0.1 µg / ml plasma. The chemical isolation, identification and measurement of progesterone in peripheral plasma was reported by Zander in 1954, followed by a more detailed account in 1955. He studied 9 women in the second half of pregnancy and found that the concentration of progesterone ranged from 4 - 27 µg / 100 ml plasma.

These levels were confirmed in a preliminary report by Sommerville, (1957) upon plasma samples taken from 4 women at the 36th week of pregnancy. Progesterone was demonstrated in all cases in amounts ranging from 9.8 - 12.3 µg / 100 ml plasma.

In 1958 Short published a few preliminary results of progesterone levels in peripheral plasma during pregnancy, followed by a more extensive paper on both progesterone and oestrogen levels in the peripheral blood of women undergoing Caesarian sections and in women in labour. In all these cases the progesterone values fell within the range previously reported by Zander, (1955).

However early in 1959 Oertel et al. published the results of 7 determinations on progesterone in peripheral plasma from the nineth to the fortyfirst week of pregnancy. These values ranged from 11.9 - 44.9 µg / 100 ml plasma and appeared to be slightly higher than those previously reported.

In 1959 Zander published a further 35 observations from

the sixteenth week of pregnancy until parturition. These values showed a marked scatter and no obvious rise in progesterone level as pregnancy progressed. In contrast to these results Short and Eton, (1959) published figures based on 47 individual determinations on different women, which indicated a steady increase in progesterone concentration from the eleventh to the thirtyfifth week. In the same study it was also observed that the plasma progesterone levels were considerably higher in twin pregnancies than in singles, moreover the levels in two women with triplets appeared even higher.

These discrepancies prompted a preliminary investigation into the individual variation of peripheral plasma progesterone concentrations at the same week of gestation. These results are shown in Fig. 15 which illustrates the variation between 22 subjects at the thirtysixth week of pregnancy.

b) The serial determination of progesterone in peripheral venous plasma during normal labour

Since the demonstration by Corner in 1928 that progesterone could maintain pregnancy in certain ovariectomised animals, there has been much stimulating controversy on the function of this hormone in human pregnancy. One hypothesis implicates a major role in the maintenance of pregnancy and associates the initiation of labour with a decrease in the production of progesterone by the placenta.

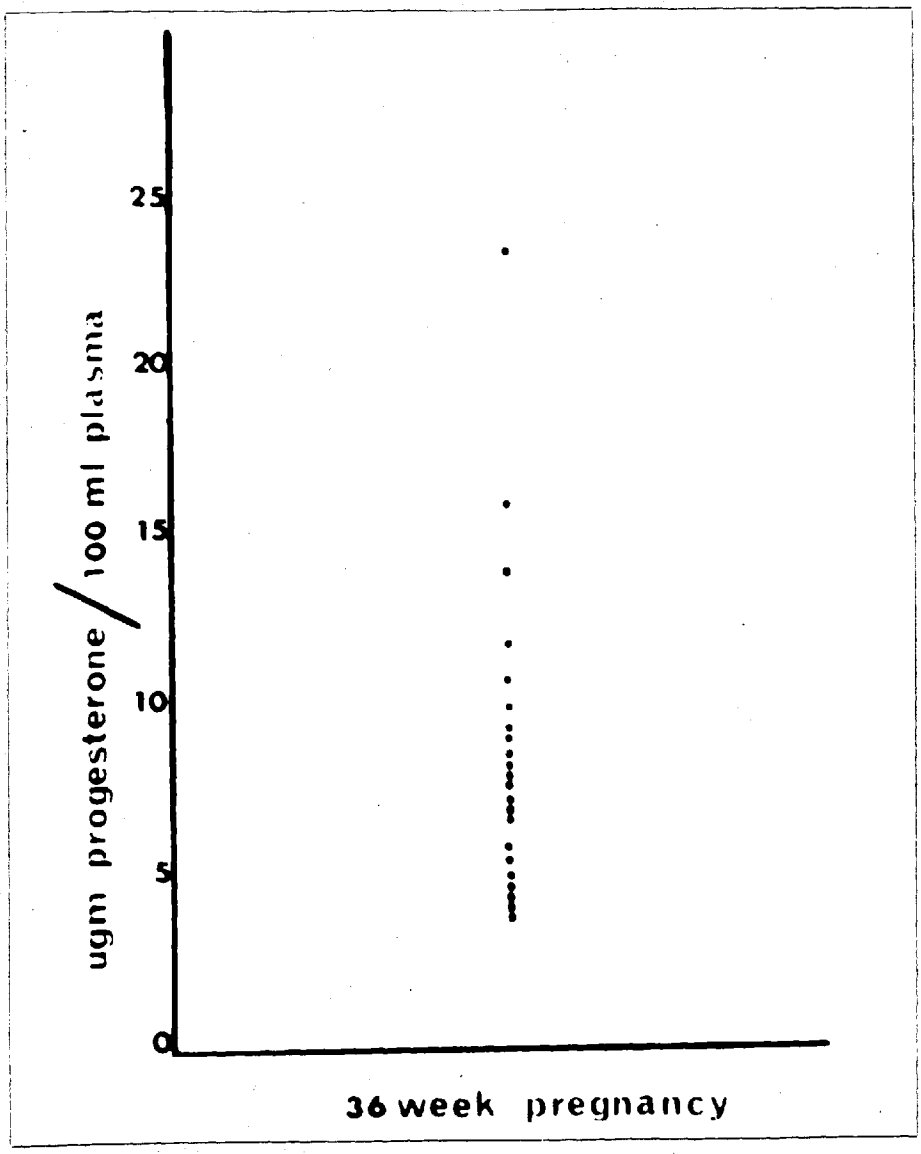


Fig. 15. The variation in progesterone levels from 22 subjects at the 36th week of pregnancy.

In 1961 Zander investigated this possibility by measuring progesterone levels in 11 women 1 - 5 days before parturition and again during labour when the cervix was fully dilated. In every case a fall in the level of progesterone was observed. These results were in apparent contradiction with those of Aitken et al., (1958), who found that there was no significant differences between either the maternal or foetal levels of progesterone (or oestrogens) when Caesarian sections and normal labour groups were compared, and concluded that parturition in women is not associated with any major changes in peripheral progesterone (or oestrogen).

This hypothesis was also investigated by Deshpande et al., (1960) who carried out serial assays of peripheral venous progesterone and pregnandiol upon a series of 13 women before and during labour. In these cases there was no evidence of a fall in plasma progesterone concentration in late pregnancy or labour. On the contrary there was a rise in 4 out of 8 subjects in whom relevant data was obtained. Similarly the plasma pregnanediol concentration did not show a marked fall until after delivery of the placenta and, in addition, it was observed that elevated values occurred before or during labour. It was also noted that the rise in pregnanediol was frequently unaccompanied by a proportionate rise in the concentration of progesterone. Thus whereas the mean progesterone : pregnanediol ratio between the twentyninth and thirtyfifth weeks was of the

order of 1 : 4, ratios of 1 : 8 or even 1 : 14 were obtained in later samples.

This experimental approach was continued in the present study in another patient. The plasma progesterone was measured by the modified method involving thiosemicarbazide formation, the plasma pregnanediol by the procedure of Sommerville and Deshpande, (1958) and in addition eight hourly urinary pregnanediol determinations were performed according to the method of Klopper et al., (1958). The results are shown in Fig. 16.

c) The serial determination of progesterone in peripheral venous plasma post-partum.

Although the peripheral progesterone levels do not appear to fall either before or during parturition, or during therapeutic abortion (Short et al., 1965), it has been shown that they do fall rapidly after removal of the placenta (Short and Eton, 1959). This fact, together with the assumption that the contribution to progesterone secretion by the ovaries and adrenals was negligible at this time, has been used by Short and Eton, (1959) to investigate the half life of progesterone in women undergoing Caesarian sections. Using this method an average half life of approximately 5 minutes was calculated, which was in good agreement with that obtained by another method, (Pearlman, 1957b). In the present study, plasma progesterone was determined in peripheral venous plasma collected from five women after normal delivery. The results are shown with respect

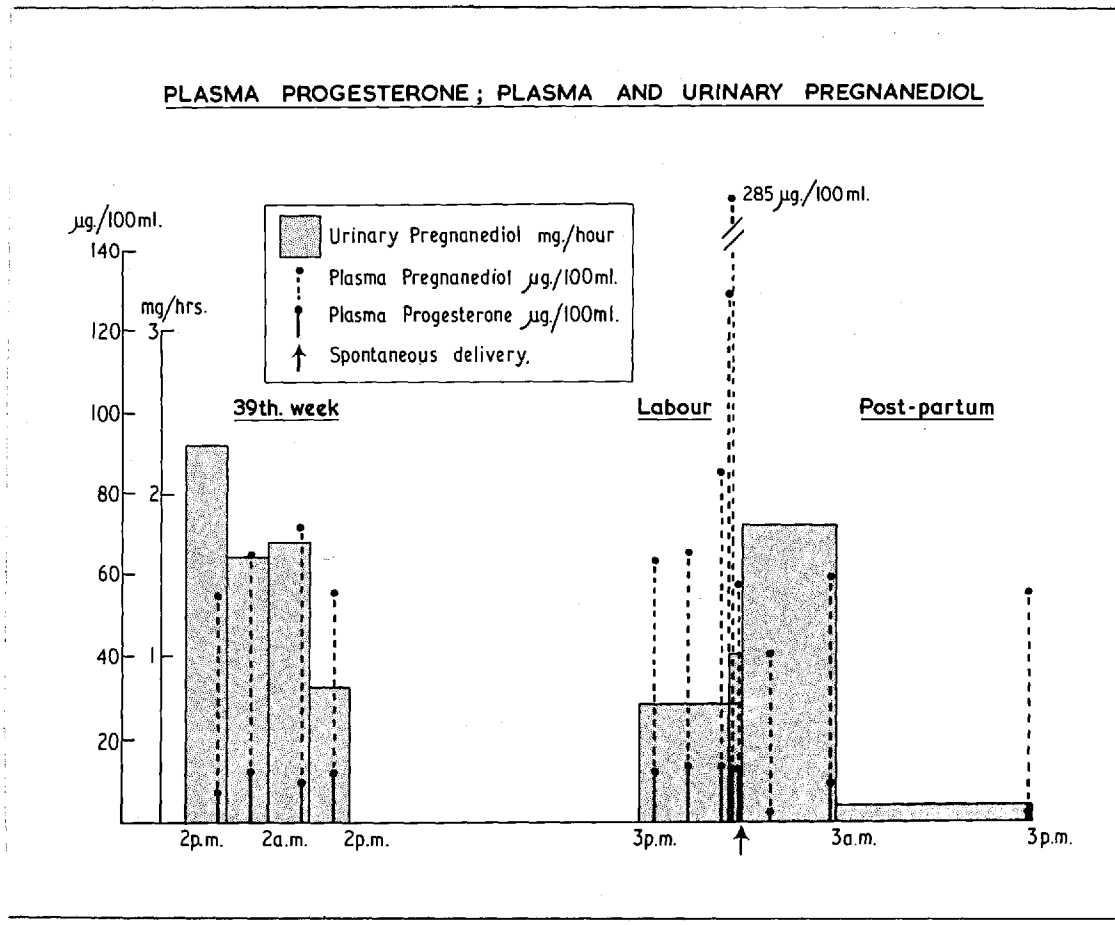


Fig. 16. Plasma progesterone; plasma and urinary pregnanediol levels during normal labour.

to time after delivery in Fig. 17.

d) The determination of progesterone in uterine venous blood.

Zander and von Münstermann, (1956) determined the concentration of progesterone in 5 samples of uterine venous blood, and found that the levels were higher than in the peripheral venous blood. During the third month of pregnancy the concentrations were below 10 µg / 100 ml blood; by the fifth month they had risen to 14 µg / 100 ml blood, and the mean of three determinations carried out during the tenth month was 26 µg / 100 ml blood.

A more elaborate study upon the first half of pregnancy was presented by Fuchs et al., (1963). They determined progesterone levels in 28 samples of uterine venous blood collected from women between the ninth and twentysecond weeks of pregnancy. In these cases the mean concentration was 35.4 µg/ 100 ml plasma (range 8 - 97), which is some ten fold higher than the concentration in the peripheral blood in early pregnancy.

e) The determination of progesterone in umbilical arterial and venous blood

Progesterone has been measured in umbilical venous blood by numerous workers Butt et al., (1951); Pearlman and Thomas, (1953); Zander and von Münstermann, (1954); Sommerville et al., (1958) and Aitken et al., (1958). From the results

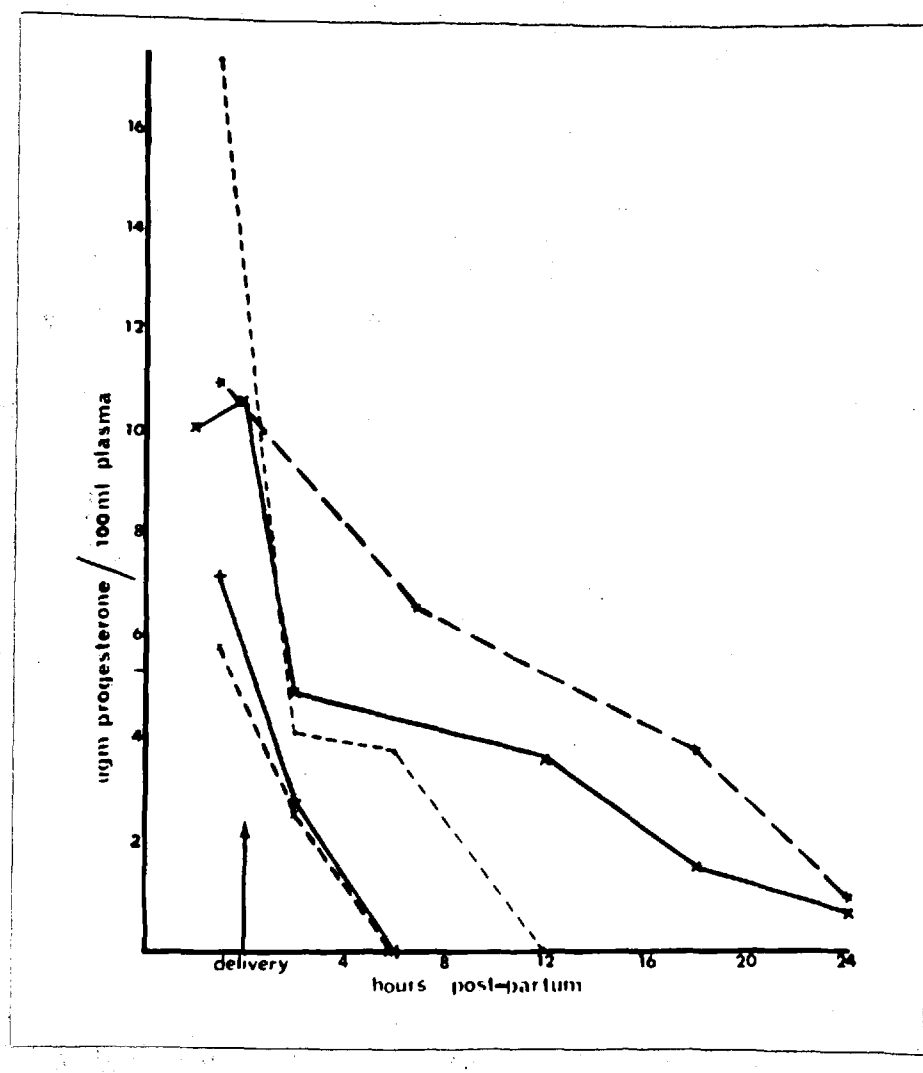


Fig. 17. Plasma progesterone levels in five subjects after normal deliveries.

presented it would appear that the progesterone concentration in umbilical venous blood is three to four times higher than in the peripheral blood of the mother.

It appears therefore that a considerable amount of the progesterone produced by the placenta enters the umbilical vein, and considerable interest has been focused on the metabolism of this compound by the foetus. In 1962 Runnebaum and Zander published results comparing the levels of progesterone 20α and 20β -hydroxypregn-4-en-3-one, and 17α -hydroxyprogesterone in the plasma of the umbilical vein and artery. It was found that the average concentration of progesterone in the plasma of the umbilical vein (37.2 $\pm$ 0.6 μg / 100 ml) was higher than the concentration in the umbilical artery (14.0 $\pm$ 1.8 μg / 100 ml). However, in contrast, the concentration of 20α and 20β hydroxypregn-4-en-3-one and 17α hydroxyprogesterone were higher in the artery than in the vein.

f) The determination of progesterone in amniotic fluid.

In 1961 Zander reported the isolation of small amounts of radioactivity in the amniotic fluid after the intravenous injection of progesterone-4-^{14}C into the maternal blood stream. However, no progesterone has hitherto been identified in amniotic fluid. In the present work $\frac{1}{10}$ of a 280 ml sample of amniotic fluid was treated with alkali and extracted according to the method of Collins and Sommerville, (1964). The extract was then purified on silica gel according to the method of Goldzieher et

al., (1961) and the eluate containing the steroids placed on a thin layer plate and chromatographed in two dimensions.

The spot corresponding to progesterone was eluted and one third of the extract applied to gas chromatography using 3% S.E.30 as stationary phase. The peak area was compared with that of authentic progesterone.

In this case the total concentration of progesterone in the 280 ml sample was 38 µg. The ultra-violet absorbing area immediately below that of progesterone was eluted and one third of the extract analysed by gas chromatography. Two peaks were obtained from the S.E.30 column with retention times identical with those of Δ^4 androstenedione and 20αhydroxypregen-4-en-3-one. The concentrations of these two constituents were 14 and 19 µg respectively.

2) <u>Studies upon the chemical determination of progesterone during the menstrual cycle.</u>

a) The range and variation of progesterone levels between different individuals during normal cycles.

The biological assay method of Forbes, (1951) indicated the presence of large amounts of progesterone in peripheral plasma during the menstrual cycle. However, the work of Zander, (1955) failed to detect the hormone which suggested that if present at all the level was below 5.0 µg / 100 ml plasma. The first figures from chemical analyses were reported by Oertel et al. in 1959. They analysed 5 samples from the 7 - 21 day after the last

menstrual period and found values between 2.5 and 5.0 µg / 100 ml plasma.

In 1963 Sommerville et al. using a spectrophotometric method published figures on plasma samples from 6 subjects during the luteal phase of the cycle as shown in Table 10.

TABLE 10

DAY	DURATION	PROGESTERONE (µg / 100 ml plasma)
18	25	1.5
20	28	3.5
21	*	3.9
22	26	1.3
23	25	1.1
27	31	0.9

* conception occurred during this cycle

The level of progesterone was below 0.1 µg / 10 ml of plasma in both the follicular phase of the cycle and in male plasma.

In 1962 Short and Levitt used a fluorimetric method for the determination of progesterone in 17 samples of peripheral blood from women during the menstrual cycle. The concentrations found during the follicular phase (1.1 - 2.3 µg / 100 ml plasma) were lower than those found during the luteal phase (1.8 - 2.3 µg / 100 ml plasma). In one woman from whom serial samples were taken during the course of the cycle, a well defined rise in the level of peripheral progesterone after the presumed date of ovulation was

reported.

In 1963 Woolever and Goldfien described a double isotope dilution method for the serial determination of progesterone in the menstrual cycle and applied the method to 5 subjects, (Woolever, 1963). Daily samples of 10 - 15 ml blood were removed and analysed. The preovulatory values ranged from 0.0 - 0.53 ug/100 ml plasma, with a mean value of 0.17 µg/100 ml. Values for the second half of the cycle ranged from 0.6 - 2.1 µg/100 ml plasma, with a mean value of 1.08 µg. A male plasma pool averaged 0.66 µg/100 ml plasma.

In 1964 Collins and Sommerville published a gas-liquid radiochromatographic method, which permitted serial analysis of progesterone during the course of the menstrual cycle. The application of this method to random plasma samples from different individuals at various stages of the cycle is shown in Table 11.

TABLE 11.

Subject No.	Age (yr)	Day of cycle	Duration (days)	Recovery of internal standard(%)	Progesterone (µg/100 ml)
1	28	4	32	24	0.52
2	23	9	29	95	0.75
3	24	10	30	76	0.97
4	18	13	32	96	0.90
5	17	17	29	12	3.90
6	34	19	28	101	1.40
7	35	19	28	90	2.30
8	38	21	27	99	0.60
8	38	23	27	100	0.53
4	18	24	30	100	1.10
3	24	26	30	94	1.10
9	25	28	35	80	1.10

Following the introduction of H^3 progesterone as an internal standard, serial assays were performed on one subject during a complete cycle. The results from this study are shown in Fig. 18 and compared with those obtained by Riondel et al., (1965) using a recently published sensitive and specific double isotope dilution technique. The results from the analysis of a 200 ml plasma sample taken on the twentysecond day of another cycle are also illustrated. In this instance the ether extract was subjected to a preliminary purification on silica gel according to the method of Goldzieher et al., (1963). The extract was then chromatographed as in the method of Collins and Sommerville, (1964). Progesterone and 20∝hydroxypregn-4-en-3-one were identified after running the free compounds and various derivatives on several thin-layer and gas-liquid chromatographic systems. The concentration of 20∝hydroxypregn-4-en-3-one was found to be approximately twenty times lower than progesterone.

b) The determination of progesterone in ovarian venous blood.

Mikhail et al., (1963) determined the concentration of progesterone in ovarian venous blood from three patients 5, 10 and 11 days after ovulation as determined from temperature curves. The levels found were 110.0, 48.2, and 28.6 µg/100 ml blood respectively. In blood obtained during an anovulatory cycle no progesterone was detected, the level being below 2.0 µg/100 ml. The analyses of pooled samples also revealed the presence of 9.1 µg/100 ml blood 17∝hydroxyprogesterone, 2.2 µg/100 ml blood

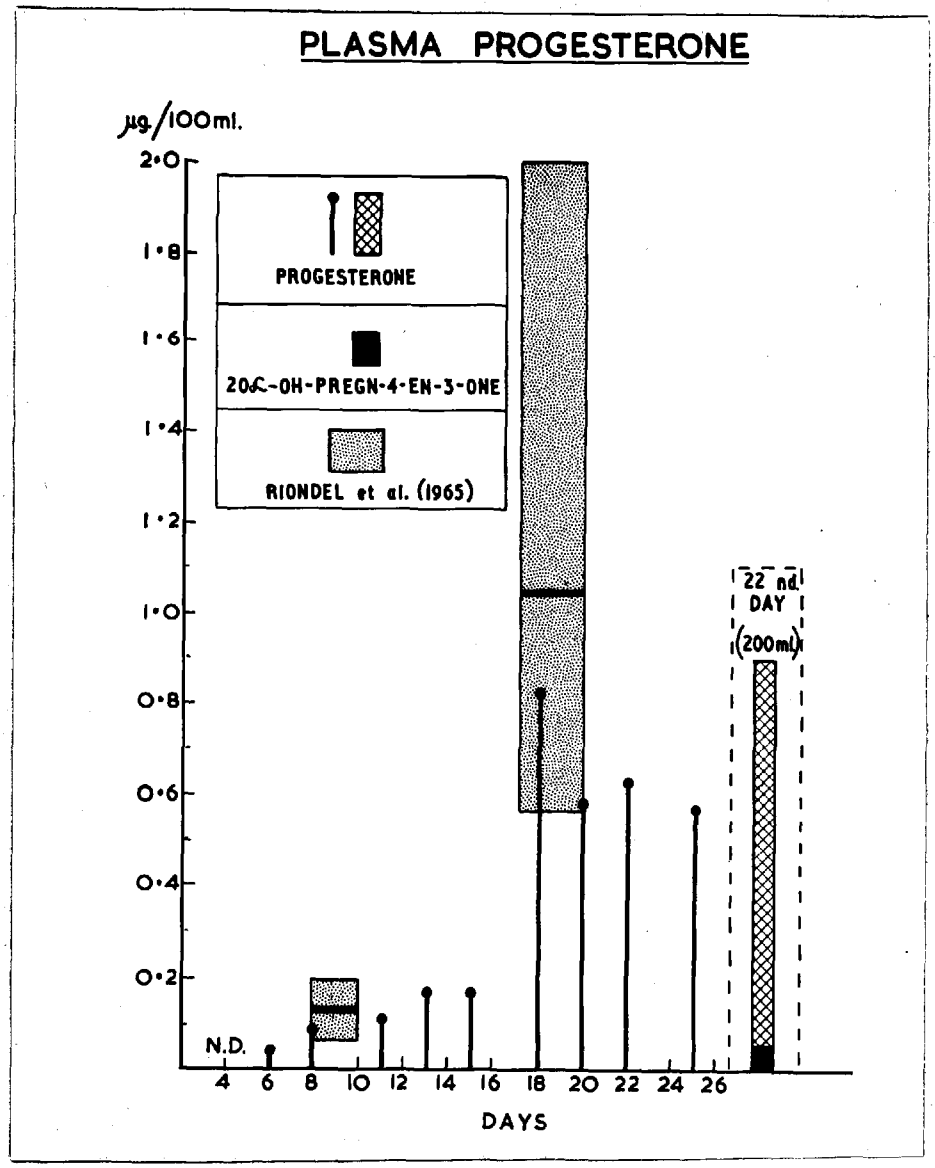


Fig. 18. Quantitative determination of plasma progesterone in peripheral venous blood throughout the menstrual cycle of a healthy woman and in 200 ml. plasma collected on 22nd day. The range of values reported by Riondel et al. (1965) for samples collected between 8th - 10th and 17th - 20th days is shown.

20 α hydroxypregnen-4-en-3-one, and 3.9 µg/100 ml blood of Δ^4 androstenedione. The minimum volume of blood used for these analyses was 40 ml.

c) The determination of progesterone in adrenal venous blood.

Short, (1960 detected progesterone in two out of three samples of adrenal venous blood from post-menopausal women after excessive stimulation with adrenocorticotrophic hormone (ACTH). The concentrations found were 5.0 - 6.0 µg/100 ml blood, and it was estimated that the output of the two adrenals was of the order of 25 µg / hour.

3) Studies upon the chemical determination of progesterone in tissues.

a) The determination of progesterone in myometrium.

In 1962 Kumer et al. reported the isolation of progesterone from 40 - 45 gm of myometrial tissue composed of pooled biopsies, and in three cases from women undergoing Caesarian hysterectomy. Gas-liquid chromatography was used after preliminary digestion of the tissue with 5% sodium hydroxide, extraction with ether and preliminary purification on silica gel. The level of progesterone ranged from 0.08 - 0.63 µg/gm wet weight of tissue. In the few cases studied the concentration at the placental site appeared to be higher than at the anti-placental site, thus supporting the concept of endocrine asymetry proposed theoretically by Csapo, (1961). In view of the limitations imposed by the use of such large quantities

of tissue, it was decided to study further with more sensitive methods 1.0 - 2.0 gm myometrial tissue obtained at biopsy. In all cases the spectrophotometry was used for the final measurement, however in a few instances the results were confirmed by gas-liquid chromatography. The results are shown in Table 12.

TABLE 12.

Week of pregnancy	Placental site	Anti-placental site
	µg progesterone/gm. wet weight of tissue	
14	0.294	0.294
14	0.120	0.105
35	0.158	0.004
36	0.500	N.D.
36	0.120	0.260
36	0.104	0.215

Three extracts of myometrium of similar weight from hysterectomised postmenopausal subjects gave zero readings at all three wavelengths.

Discussion.

The technical difficulties involved in the development of a practical method for the determination of progesterone in human peripheral plasma are reflected in the many different procedures which have been advocated for this purpose. In fact there have been more reports on the development of adequate techniques than on their application to biological problems. The situation has been complicated by two factors. First, the preliminary results obtained from large

plasma samples indicated a wide range of levels from different individuals at the same week of pregnancy and this was confirmed in the present study (Section 1d). This implied that if only one sample could be taken from each individual, large groups would have to be assayed to follow the course of progesterone levels. To overcome this difficulty it was considered desirable to develop a more sensitive method which would allow the serial determination of smaller plasma samples from the same individual.

Secondly it was demonstrated that the levels of progesterone in peripheral plasma during the menstrual cycle were below 5.0 µg/100 ml plasma, but could rise up to 40.0 µg/100 ml during late pregnancy. Hence to be applicable to all conditions the method must be extremely sensitive and valid over a wide range of concentrations.

In the first methods to be developed, progesterone was measured either as the free compound or as a derivative by comparison with a standard solution in a spectrophotometer. In general these methods tended to be laborious because the final measurement required the preparation of very pure extracts, and accurate and reproducible results depended to a large degree on the skill of the investigator. The spectrophotometric measurement also limited the sensitivity to 0.1 µg of progesterone, so that these methods were best suited for studies on plasma samples from late pregnancy.

Greater accuracy and precision in the methods followed

the introduction of isotopically labelled progesterone as an internal standard, and later the formation of labelled derivatives offered the possibility of greater sensitivity. However both of these advantages in the form of double isotope dilution could only be obtained after numerous chromatographic steps, and the time involved for the assay was lengthened accordingly. This situation was improved by the consecutive application of thin-layer chromatography and gas-liquid radiochromatography to the problem of determining micro amounts of steroids in blood and tissues. These techniques allowed the advantages from the use of isotopes to be obtained with quicker methods of purification and separation.

The first successful application of chemical assay methods demonstrated low levels of progesterone in peripheral plasma. Biological assay methods had given variable results but indicated that the levels were some ten times higher in late pregnancy and some hundred times higher in early pregnancy. These discrepancies have only been partly resolved by the isolation of 20α and 20β hydroxypregnen-4-en-3-one. It also appears likely that the biological assay methods are complicated by the antagonistic activity of non 'progestational' substances such as oestrogen (Olsen et al., 1952).

The methods developed during the present study were first applied to the serial analyses of progesterone in peripheral plasma during normal labour, for the mechanisms which initiate

parturition are not understood although explanations involving either the hypophysis, oestrogen, or progesterone metabolism have been suggested.

However the following facts have been fairly well established:

1) Total hypophysectomy does not interfere with the normal course of parturition in women (Little et al.,1958).
2) In cases of twin pregnancies in women with a double uterus the twins may be born several weeks apart (Csapo, 1961).
3) The maternal ovaries are not required to maintain pregnancy after approximately three months (Tulsky and Koff, 1957).

These findings suggest that the 'uterus-placenta-foetus unit' is an independent self-regulating structure (Diczfalusy, 1961) and mainly as a result of studies on the rabbit a concept has evolved that progesterone produced in the placenta plays a dominant role in the maintenance of pregnancy by a local effect on the myometrial cells. A decrease in the production of hormone might then initiate parturition.

However it has also been shown that:

a) The levels of pregnanediol in 24 hour specimens of urine do not show a fall before parturition (Russell et al., 1957).
b) The concentration of progesterone in the placenta does

not fall before parturition (Zander and von Münstermann, 1956)

c) The level of progesterone in cord blood does not fall before parturition (Aitken et al., 1958).

d) Peripheral injections of progesterone will not delay the onset of labour (Pose and Fielitz, 1960).

Most of the findings listed above have been derived from studies on isolated fluids or tissues from different subjects and may not therefore represent a true picture as to events occurring in one subject. Accordingly it was decided that as a preliminary study, information on changes occurring over short periods in the 'uterus-placenta-foetus unit', might be obtained from the serial analysis of maternal peripheral venous plasma. From the results of the six cases presented by Deshpande et al., (1960) and in the present studies there was no indication of a fall in progesterone or pregnanediol, in fact there was usually a rise in both substances but in particular pregnanediol. In studies upon isolated blood samples it might be more valid to express the results as µg progesterone/100 ml blood rather than /100 ml plasma, although by measuring the ratio of progesterone to its principal metabolite this criticism is overcome to a certain extent.

The increased concentration of plasma pregnanediol may reflect either:

a) An increased secretion of progesterone followed by a rapid conversion to pregnanediol.

b) A change in progesterone metabolism during late pregnancy (Bengtson, 1959).

c) A decreased excretion of pregnanediol.

Further information on the initiation of labour may be obtained from cases of therapeutic abortion. In this connection it is interesting that replacement of the amniotic fluid by a hypertonic solution usually precipitates an abortion within forty-eight hours. This phenomenon may be explained either by an adverse effect on the enzymes responsible for progesterone production in the placenta, or by a local ionic effect resulting in the depolarisation of myometrial cells.

In the present work the possible effect on progesterone production was studied by measurement of the level of progesterone in serial samples of peripheral plasma taken before and after replacement of the amniotic fluid with 20% saline solution. In two cases investigated there was no evidence for a fall in the level of plasma progesterone, in fact the concentration tended to remain steady, or slightly increase. Similar conclusions were reached by Short et al., (1965) from the measurement of progesterone in both peripheral and uterine venous plasma during therapeutic abortion.

It may be concluded, that the evidence regarding the role of progesterone in the maintenance of pregnancy in human subjects is somewhat conflicting and inconsistent. However a rise in the level of peripheral progesterone has been observed

during labour and therapeutic abortion and this may suggest either:

a) An increased production and secretion of progesterone.

b) A decrease in the metabolism of progesterone.

c) A general release of bound progesterone by the tissues.

After delivery of the placenta the level of circulating progesterone falls quite rapidly. In the five cases studied two had undetectable levels after two hours, one after twelve hours, while the remaining two had detectable amounts after twentyfour hours. These results are in slight contradiction with those of Short and Eton, (1959) on women undergoing Caesarian section and may represent activity from incomplete removal of the placenta, or the slow release of 'buffered' progesterone from the tissues.

The analysis of umbilical venous blood may give better indication as to endocrine events occurring in the 'uterus-placenta-foetus unit'. These studies have indicated that the level of progesterone is some ten times higher than in peripheral venous blood, but owing to the inaccessibility of the material no serial studied have been performed.

The analysis of umbilical arterial and venous blood have yielded interesting results. Runnebaum and Zander, (1962) have shown a higher concentration of progesterone in the vein than in the artery which suggests production in the placenta and subsequent transport to the foetus, where the progesterone appears to be reduced at carbon 17 and carbon 20, for the

concentrations of 20α and 20β hydroxypregn-4-en-3-one, and 17α hydroxyprogesterone are higher in the artery than in the vein. It is tempting to speculate that modification of the hormone by the foetus, which may be difficult to detect in the mother, might contribute to the initiation of labour. In this connection Runnebaum and Zander suggest that the production of 17α hydroxyprogesterone by the foetus might be necessary as an intermediary in the formation of oestrogens by the placenta. Further studies in this direction are likely to be rewarding.

The results from the determination of peripheral progesterone during pregnancy although still in a preliminary stage have been somewhat disappointing as an index of progesterone production. This may be explained by a number of factors. First the half life of progesterone in blood is very short and has been calculated by different methods to be in the order of five minutes. This fact explains the low level of progesterone in plasma despite the very high production, and means that the analysis of plasma levels is but a small part of a very large dynamic process. The results during pregnancy are also complicated by the hormone being metabolised by both mother and foetus, so that the level in the maternal peripheral blood reflects both of these processes. It also appears likely that the body may use compensatory mechanisms to maintain a relatively constant blood level of hormone.

The concentration of progesterone in myometrial tissue

during pregnancy has been shown to be very small (0.08 - 0.63 µg / gm wet weight of tissue), although Kumer et al., (1962) presented results which indicated that these levels were much higher than could be accounted for by plasma progesterone within the tissue. The large production and rapid metabolism of progesterone may be necessary to provide a constant and steady stimulus at these and other receptor cells.

The agreement between the results obtained during the menstrual cycle in the present study (1963, 1964) and those of double isotope derivative methods (Woolever et al., 1963; Riondel et al., 1965) is encouraging in view of the dissimilar procedures. Also the interpretation of the results is not complicated by the progesterone being metabolised in two separate compartments as is the case during pregnancy. As the changes in blood volume during the cycle are not pronounced, peripheral analysis should provide useful information on ovarian function and may indicate whether or not ovulation has occurred.

The analysis of adrenal venous blood by Short, (1960) in three postmenopausal subjects whose adrenals were stimulated with ACTH indicated that under normal conditions these glands contribute little to the level of plasma progesterone, although Klopper et al., (1957) have shown that there is still a substantial level of urinary pregnanediol in the absence of the gonads.

The analysis of ovarian arterial and venous blood is

the best method to determine exactly what this organ is secreting, and the presence of progesterone and other steroids in the effluent blood during the luteal phase suggests that the regulation of metabolic processes and hypophyseal function may depend upon the secretion and ratio of all these compounds (Mikhail et al.,1963).

In the future, development of more sensitive methods for the simultaneous determination of these related steroids and their metabolites (free or conjugated) in peripheral plasma, may provide more information on the regulation of reproductive function, and suggest a more rational basis for adequate substitution therapy. The use of sensitive and specific methods of measurement may also be used for the more valid determination of secretion rates, and also to elucidate the important problem of the extent to which endogenous or exogenous hormone enters the cells of the target organ. The measurement of steroid concentrations in tissues is also an essential preliminary for the study of the intracellular action of the hormones on metabolic processes.

SECTION II.

The metabolism of progesterone in human peripheral blood.

The first attempt to establish a quantitative relationship between the amount of progesterone secreted by the various glands with the amount present in peripheral blood was reported by Pearlman in 1957. He collected together data on blood volume and blood progesterone concentration, and deduced that the turnover time, which may be defined as the time taken for the endocrine glands to secrete as much hormone as is present in the circulating blood at one instant, may be calculated by multiplying the blood hormone concentration, C, by the total blood volume, V, and dividing the product by the endogenous rate of hormone production, r, thus:

$$T = \frac{CV}{r}$$

The blood hormone concentration, C, may be determined by methods as described in Section I, and the total blood volume by the use of dyes, or isotopic labelling of the cells or plasma. An estimate of the endogenous rate of progesterone production may be obtained by a variety of methods:

a) A known amount of hormone is injected and the percentage of the dose recovered as urinary pregnanediol determined. From the average of pre-injection levels of pregnanediol in the urine, it is possible to estimate the average rate of progesterone production per 24 hours.

b) Method (b) involves the same principle as method (a) but much smaller amounts of isotopically labelled progesterone are injected, and the specific activity of urinary pregnanediol determined.

c) By the determination of the progesterone concentration in blood entering and leaving the endocrine gland, and by the simultaneous determination of the blood flow through the organ.

d) By infusing a trace amount of isotopically labelled hormone into the blood stream until equilibrium is reached with respect to the endogenous production, and then measuring the rate in decline of the specific activity of the hormone in serial blood samples.

The validity of methods a, b and d depend upon the exogenous progesterone being metabolised in an identical manner to the endogenous. Furthermore, methods a and b are complicated by the formation of urinary pregnanediol from other precursors, while method d involves the measurement of small amounts of radioactivity and progesterone. Method c is limited by the availability of the material. Methods a and b give values for 24 hours, while methods c and d give values for the time of assay.

The determination of production rates during pregnancy is complicated by the fact that the progesterone is

metabolised by the mother and foetus, and it is unlikely that progesterone injected into the maternal blood stream is treated in the same manner. However, Pearlman (1957a) estimated the production of progesterone during late pregnancy to be approximately 250 mg./24 hours. This figure was obtained by calculating the specific activity of urinary pregnanediol after the injection of tritiated progesterone, and calculating the secretion rate from the following formula:

$$\text{Secretion rate} = \frac{R}{a \times t}$$

where R = the amount of radioactivity injected

a = the specific activity of pregnanediol in pooled urine

t = the time during which the urine was collected

Assuming a blood volume of 7 litres, a turnover time of 3.3 minutes was calculated.

The validity of method (b) for the determination of progesterone secretion rates has been investigated by Arcos et al., (1964). These authors injected progesterone - 4 - ^{14}C and tritiated preg-5-enolone and determined the ratio of carbon 14 to tritium in the urinary pregnanediol. Analysis of the results indicated that preg-5-enolone (or possibly preg-5-enolone sulphate) contributed in a significant amount to the level of urinary pregnanediol.

However the results of Pearlman were similar to

those calculated by Zander (1959) who determined the secretion rate of progesterone from the concentration of progesterone in the uterine venous blood and the rate of blood flow through the organ.

A more specific method than those involving urinalysis for determination of the secretion rate of progesterone, is by the intravenous administration of a small dose of labelled progesterone (method d) until blood analyses show the exogenous hormone to be in equilibrium with the endogenous. It is then possible to stop infusion and measure the removal rate of labelled progesterone from the blood. This method has been successfully applied to male subjects by Romanoff et al., (1963).

The early work of Hasking, (1950), on the development of a chemical method for the determination of plasma progesterone indicated that the rate of removal of intravenously administered progesterone from the blood depended on its concentration. This observation has been confirmed by subsequent workers and furthermore it has been shown that the removal approximates to a first order reaction. This work has led to the concept of a half life of progesterone in blood, rather than to a turnover time. The relationship between the two is:

$$\text{Half-life} = \frac{T}{2}$$

The half-life may be defined as the time taken for the total blood hormone level to fall by half should the

endogenous secretion cease. This situation may occur at the end of pregnancy after removal of the placenta. Short and Eton, (1959), assumed that the ovarian and adrenal secretion was negligible at this time, and calculated the half-life of progesterone in women undergoing Caesarian section. In these subjects the removal of the placenta could be accurately timed in relation to the collection of blood samples. Using this method they arrived at a half-life of five minutes, which is longer than predicted by the turnover time of Pearlman, (1957b). Future work with more sensitive and specific methods may obviate these anomalies and illustrate some relationship between the secretion of progesterone and the level in circulating blood.

The administration of labelled progesterone, besides contributing valuable information on the secretion rate of endogenous progesterone, has also solved many problems regarding its metabolism. Numerous experiments using this technique have shown that probably the most important factors accounting for the rapid disappearance of progesterone from the blood are the 'buffering action' of the subcutaneous tissues, and the rapid change to biologically inactive metabolites.

The first 'in vivo' experiments on blood levels with progesterone - 4 - ^{14}C were reported by Davis et al., (1956). They injected the hormone intramuscularly to a woman during

the eleventh week of pregnancy and found a peak of plasma radioactivity twenty-five hours later, with radioactivity still detectable up to thirteen days after the injection. These results showed a slow release of radioactivity from the site of injection but gave no indication as to the identity of the radioactive compounds.

A study upon the intravenous injection of labelled progesterone was reported by Plotz and Davis in 1957, followed by a more detailed account, using both progesterone - 4 - ^{14}C and progesterone - 21 - ^{14}C, in 1961 (Plotz). They injected the labelled compounds into women destined for therapeutic abortion and found that the radioactivity in both free and conjugated fractions extracted from the plasma declined rapidly during the first two hours, and that only small amounts of radioactivity were present in both fractions thirty-seven hours after the injection.

It was calculated that approximately 11% of the radioactivity was removed from the blood by the kidneys, liver and lungs within three hours after administration, while only 5% was present in the total plasma volume at that time. From these observations it was concluded that the majority of the radioactivity had rapidly disappeared from the blood into the tissues, from which it was released more slowly into the circulation and subsequently excreted as conjugated metabolites

through the urinary and gastrointestinal tracts.

Another study following the intravenous administration of progesterone - 4 - ^{14}C was reported by Sandberg and Slaunwhite in 1958. They extracted the plasma samples for total free and conjugated steroids, and demonstrated that the total free steroid was cleared from the plasma at two different rates with half-lives of twenty and ninety minutes respectively. The half-life of conjugated steroid was considerably longer.

A more detailed study was conducted by Zander in 1959. He injected progesterone - 4 - ^{14}C intravenously into a postmenopausal woman, and found that nine minutes after the injection most of the plasma radioactivity was already in the form of conjugates. Chromatographic analysis of this fraction after hydrolysis revealed the presence of pregnanediol and some 5/3pregnan-3-ol-20-one. The greatest proportion of the free radioactivity was unmetabolised progesterone, but there were also small amounts of free pregnanolone, 20α-hydroxypregn-4-en-3-one and pregnanediol.

It has been shown that the excretion of metabolites probably starts immediately after intravenous injection. In the studies by Zander 1% of the dose had accumulated in the urine by thirty minutes, and 9% by four hours. A delayed appearance in the faeces was shown by Davis and Plotz, (1956), and Zander, (1959).

Metabolic transformation of progesterone in blood:

Notwithstanding the limitations in the methods employed and minor differences in results, the half-life of progesterone in blood appears to be very short, and many explanations have been put forward to explain this phenomenon. However, little attention has been paid to possible metabolic transformations of progesterone by the blood and such evidence as has been obtained is conflicting. In 1949 Hooker and Forbes reported that the biological activity of progesterone declined rapidly from plasma maintained at body temperature, while Edgar, (1953), using a chemical method for measurement, demonstrated that there was no loss of progesterone after incubation with whole blood for one hour at 37°C.

An experiment with ox blood was reported by Short in 1958. He incubated whole blood with progesterone at 37°C and withdrew samples every hour. Analyses indicated the presence of a new compound tentatively identified as 20β hydroxy-pregn - 4 - en - 3 - one. The half-life of progesterone in vitro was calculated to be approximately seven hours. In 1958 Axelrod and Wertheesen in a preliminary report upon the metabolism of hormones in human and other mammalian blood under aseptic conditions indicated that the conversion was towards biologically inactive forms, and the main conversion of progesterone was to 5β pregnan - 3α ol-20-one.

As an extension to these studies, and to investigate the possible metabolic contribution of blood to 'in vitro' studies with tissues, blood from two women in the luteal phase of the menstrual cycle and from two during late pregnancy, was independently incubated with progesterone.

METHOD.

Ten micrograms of progesterone - 4 - ^{14}C in benzene were added to each fifty ml. incubation flask, and evaporated to dryness under nitrogen. Five thousand units of heparin and 20,000 units of penicillin were added to each flask, followed by twenty ml. of blood and ten ml. of Krebs Ringer phosphate buffer pH 7.4. The flasks were vibrated at 60 vib./min. at 37°C. for four hours. The plasma was then separated from the cells by centrifugation and removed by Pasteur pipette. The cells were then washed with ten ml. saline which was retained after centrifugation.

The plasma was hydrolysed and extracted as in the method for plasma progesterone, one hundreth was removed for liquid scintillation counting and the remainder chromatographed on paper in the system hexane / 85% aq. methanol.

The cell washings were extracted directly with 2 x 100 ml. ether and the total number of counts determined.

The cells were lysed and hydrolysed by the addition of 25 ml. N/6 sodium hydroxide, extracted with 2 x 100 ml.

ether, and the total amount of radioactivity determined. Small amounts of quenching were corrected by the addition of a ^{14}C - hexadecane standard.

It was found that after this procedure the percentage distribution of radioactivity was as shown in Table 13.

TABLE 13.

	MENSTRUAL CYCLE		PREGNANCY	
	I	2	I	2
PLASMA	89.9	90.2	90.6	92.3
CELL WASHING	4.5	5.1	4.7	4.3
CELLS	5.6	4.7	4.7	3.4

The paper chromatogram of the plasma fractions was inspected in ultraviolet light and scanned for radioactivity. It was found that 4.9 - 6.8 per cent (Table 14) of the radioactivity was transformed to a compound less polar than progesterone with an Rf of 0.25 similar to that of authentic 20 α hydroxypregn - 4-en-3-one and 5 β pregnan-3α-ol-20-one.

TABLE 14.

	MENSTRUAL CYCLE		PREGNANCY	
	I	2	I	2
% conversion to more polar compound	6.1	6.8	5.1	4.9

Table 14 - percentage conversion of progesterone 4 ^{14}C to more polar compound.

No material other than progesterone was visible on inspection under ultraviolet light; after elution of the unknown radioactive area with 15 ml. Ethyl acetate/methanol 3:1 and formation of the thiosemicarbazone derivative (case 2) there was no peak at 392 mμ, however a positive Allen correction factor was obtained - corresponding to 0.2 μg.

The unknown compound from case 1 was eluted in the same manner and subjected to gas liquid radiochromatography on 3% S.E. 30. The radioactivity was found to be distributed in the following manner:

Steroid	% of Initial Radio-activity in unknown compound.
20α-hydroxypregnen-4-en-3-one	23.0%
Progesterone	49.0%
5β pregnan-3α-ol-20-one	28.0%

The presence of progesterone in this fraction was probably due to overloading of the paper chromatogram.

The biosynthesis and role of progesterone as an intermediary compound in human ovarian tissue.

The possibility that progesterone may function as an intermediary compound in the biosynthesis of adrenocortical steroids, androgens and oestrogens (Samuels, 1955), arose from analytical studies on the hormone producing glands, and from 'in vivo', perfusion, and 'in vitro' studies involving both labelled and non-labelled compounds. These studies showed that progesterone was synthesized by all steroid producing glands, and indicated that a common pathway involving acetate --- cholesterol --- pregnenolone --- progesterone was of major importance in these organs, although the presence of alternative schemes were not excluded.

The present study is concerned with the biosynthesis of progesterone, and its importance as an intermediary compound in the formation of androgens and oestrogens in the human ovary. It is essential that any study of this kind be correlated with ovarian morphology, because the tissue components are in a continual state of change.

The nature of the ovarian secretion is best evaluated by a comparison of ovarian arterial and venous blood. However, greater insight into which ovarian structures are contributing to this secretion and the mechanisms involved in the elaboration of the hormones may be obtained by 'in vitro' studies with isotopically labelled substrates. In recent years with the

increased availability of labelled substrates, much information has been obtained upon the mode of steroid transformations in the human ovary. A summary of this information is given in Table 15.

TABLE 15.

PRECURSOR : ACETATE

AUTHORS	OVARIAN TISSUE	TREATMENT	STEROIDS PRODUCED
Wotis et al., (1956)	Normal	chorionic gonadotrophin	oestrone oestradiol
O'Donnel et al. (1959)	polcystic	human menopausal gonadotrophin	oestrone oestradiol
Sweat et al., (1960)	stroma or corpora lutea	-	progesterone
Ryan & Smith, (1961a)	folliole cyst linings	sheep follicle stimulating hormone	cholesterol oestrone oestradiol progesterone 17α hydroxyprogest. Δ^4 androstenedione Δ^5 pregnenolone 17α OH pregnenolone dehydroepiandrosterone
Noall et al., (1962)	wedge resections Stein-Leventhal syndrome	human pituitary luteinising hormone	dehydroepiandrosterone
Hammerstein et al. (1964)	corpora lutea	-	Δ^5 pregnenolone progesterone 20α olpregn-4-en-3-one 17α OH progesterone Δ^4 androstenedione oestrone, oestradiol

AUTHORS	OVARIAN TISSUE	TREATMENT	STEROIDS PRODUCED
Huang & Pearlman, (1963)	corpus luteum	-	progesterone 17α OH progesterone 20α OH pregn-4-en-3-one
Rice et al., (1964b)	normal stromal tissue	human chorionic gonadotrophin	testosterone
	PRECURSOR : PREGNENOLONE		
Lanthier & Sandor, (1960)	normal	human chorionic gonadotrophin	progesterone 17α OH progesterone androstenedione
Lanthier & Sandor, (1960)	polycystic	human chorionic gonadotrophin	Δ^4 androstenedione
Axelrod & Goldzieher, (1962)	polycystic	-	17α OH pregnenolone 17α OH progesterone Δ^4 androstenedione testosterone dehydroepiandrosterone 19 OH androstenedione 17α20α diOH progesterone 17α20β diOH progesterone
Ryan, (1963)	corpus luteum	-	progesterone 17α OH progesterone 17α OH pregnenolone oestradiol

AUTHORS	OVARIAN TISSUE	TREATMENT	STEROIDS PRODUCED
	PRECURSOR : PROGESTERONE		
Sweat et al., (1960)	stroma or corpora lutea	-	20α OH pregn-4-en-3-one 20β OH pregn-4-en-3-one 17α OH progesterone 17α : 20ε diOH pregn-4-en-3-one Δ^4 androstenedione oestrone oestradiol pregnanedione allopregnanedione 6β OH progesterone
Ryan & Smith (1961b)	follicular cyst linings	sheep follicle stimulating hormone	oestrone oestradiol
Kase et al., (1961)	polycystic with corpora lutea	-	testosterone Δ^4 androstenedione
Warren & Salhanick (1961a)	normal and post-menopausal		17α-OH progesterone Δ^4 androstenedione 20α-OH-pregn-4-en-3-one progesterone
Warren & Salhanick, (1961b)	normal		16α OH progesterone
Axelrod & Goldzieher, (1962)	normal	-	17α OH progesterone 20α OH pregn-4-en-3-one 20β OH pregn-4-en-3-one 17α 20α diOH pregn-4-en-3-one 17α 20β diOH pregn-4-en-3-one Δ^4androstenedione testosterone 19 OH testosterone 19 OH androstenedione 19 aldo-androstenedione

AUTHORS	OVARIAN TISSUE	TREATMENT	STEROIDS PRODUCED
			6β OH androstenedione oestradiol 17α
Huang & Pearlman, (1963)	corpus luteum	-	17α OH progesterone 20α OH pregnen-4-en-3-one
	PRECURSOR : ANDROSTENEDIONE		
Sweat et al., (1960)	stromal or corpus luteum	-	oestrone oestradiol-17β
Smith & Ryan, (1961)	polycystic	-	oestrone oestradiol
Huang & Pearlman, (1963)	corpus luteum	-	oestrone oestradiol
	PRECURSOR : TESTOSTERONE		
Wotiz et al., (1956)	postmenopausal	-	oestrone oestradiol-17β oestriol
Baggett et al., (1956)	normal	equine follicle stimulating hormone	oestrone oestradiol-17β Δ^4 androstenedione

These studies have identified numerous intermediary compounds and it appears that several interelated pathways are involved in the biosynthesis of androgens and oestrogens. A tentative scheme based on probable transformations is shown in Fig.19.

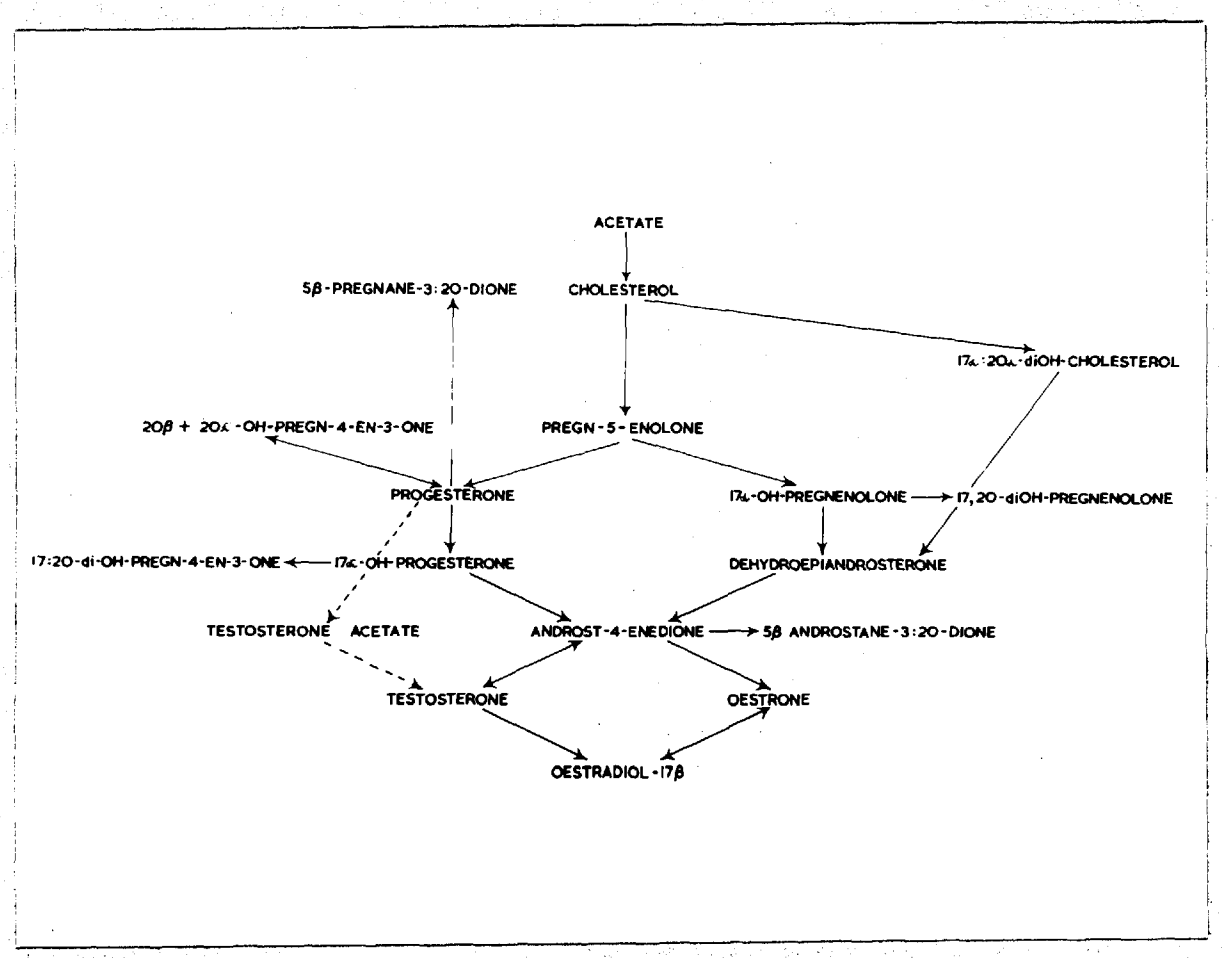


Fig.19. Scheme of probable steroid transformations in human ovarian tissue.

The pathway from progesterone to oestradiol, involving 17 hydroxylation, followed by the formation of Δ^4 androstenedione and testosterone was the first to be established (Sweat et al., 1960; Ryan and Smith, 1961; Axelrod and Goldzieher, 1962; Lanthier and Sandor, 1962). On the other hand there is now convincing evidence for the alternative pathway from Δ^5 pregnenolone to Δ^4 androstenedione with dehydroepiandrosterone as an important intermediary (Axelrod and Goldzieher, 1961; Ryan and Smith, 1961d; Noall et al., 1962). Other possible transformations are the formation of dehydroepiandrosterone directly from cholesterol with 17, 20 dihydroxycholesterol as an important intermediary, as has been demonstrated in an adrenal adenoma (Gual et al., 1962), and the formation of testosterone from progesterone without prior formation of Δ^4 androstenedione, testosterone acetate being postulated as an intermediary (Dorfman et al., 1963).

In view of the many possible pathways of steroid transformations it was decided to investigate 'in vitro' which were quantitatively the most important, in the various components of the human ovary in health and disease.

MATERIALS AND METHODS

a) Solvents and reagents:

All solvents were purified by standard techniques and redistilled prior to use. The silica gel was recieved and

and purified as previously described. Alumina was prepared as in the method of Brown, (1955), for the determination of urinary oestrogens. Silica gel G for thin-layer chromatography was as previously described.

b) Reference steroids; radioactive standards; and coenzymes.

Reference steroids were obtained from the MRC reference collection (by courtesy of Professor W. Klyne). Co-enzymes from Courtin & Warner Ltd., Lewes, Sussex. Sheep FSH (NIH-S-I ovine standard) was prepared and distributed by the Endocrine Study Section, N.I.H., Bethesda, Maryland, U.S.A. Human pituitary F.S.H. containing approximately 1% L.H. was kindly donated by Dr. Crooke, Department of Clinical Endocrinology, Birmingham. ^{14}C and ^{3}H hexadecane reference standards from The Radiochemical Centre, Amersham, Bucks.

c) Substrates:

The following steroids were obtained from the Radiochemical Centre, Amersham, Bucks: pregnenolone-4-^{14}C 19.8 mc/mM, progesterone-4-^{14}C 21.7 mc/mM, androstenedione-4-^{14}C 4.2 mc/mM, testosterone-4-^{14}C 21.5 mc/mM, oestradiol-16-^{14}C 12.0 mc/mM, oestrone-16-^{14}C 12.0 mc/mM. The first four steroids were tested for chemical and radiochemical purity by gas-liquid radio-chromatography in the Pye Panchromatograph using 1% NPGA as the stationary phase; the latter two for radiochemical purity on paper using the system Petroleum ether

(40° - 60°B.P.) / benzene 50:50 / 70% aq. methanol (1:1). All were 97 - 100% pure.

Fig.20 is a flow sheet of the method.

Tissue:

Human ovaries were obtained directly from the operating theatre, and microdissected under an illuminated magnifying lens. Part of the structures isolated were taken for histological examination and the remainder chopped into small pieces (approx. 0.5 mm) with an instrument consisting of parallel razor blades 1 mm. apart, held at one end in a metal holder (donated by Dr. Max Sweat, Utah, U.S.A.). For purposes of comparison 0.5 g. of tissue was taken, but in some cases only 0.2 g. of tissue was available.

Incubation:

All incubations were started within thirty minutes after interruption of the blood supply, the tissues being at room temperature during weighing and mincing. The radioactive substrates prepared in benzene were added to each 25 ml. incubation flask and dried under a stream of nitrogen. The weighed tissue was then washed into the flask with Krebs Ringer phosphate buffer, and the coenzymes and gonadotrophins added to give a final ratio of tissue to buffer of 1:5 w/v.

The final incubation medium contained:

Krebs Ringer Phosphate Buffer pH 7.4, plus glucose 200 mg./100 ml.

NAD 1 μM Nicotinamide-adenine dinucleotide (DPN).

NADP 1 μM Nicotinamide-adenine dinucleotide phosphate (TPN)

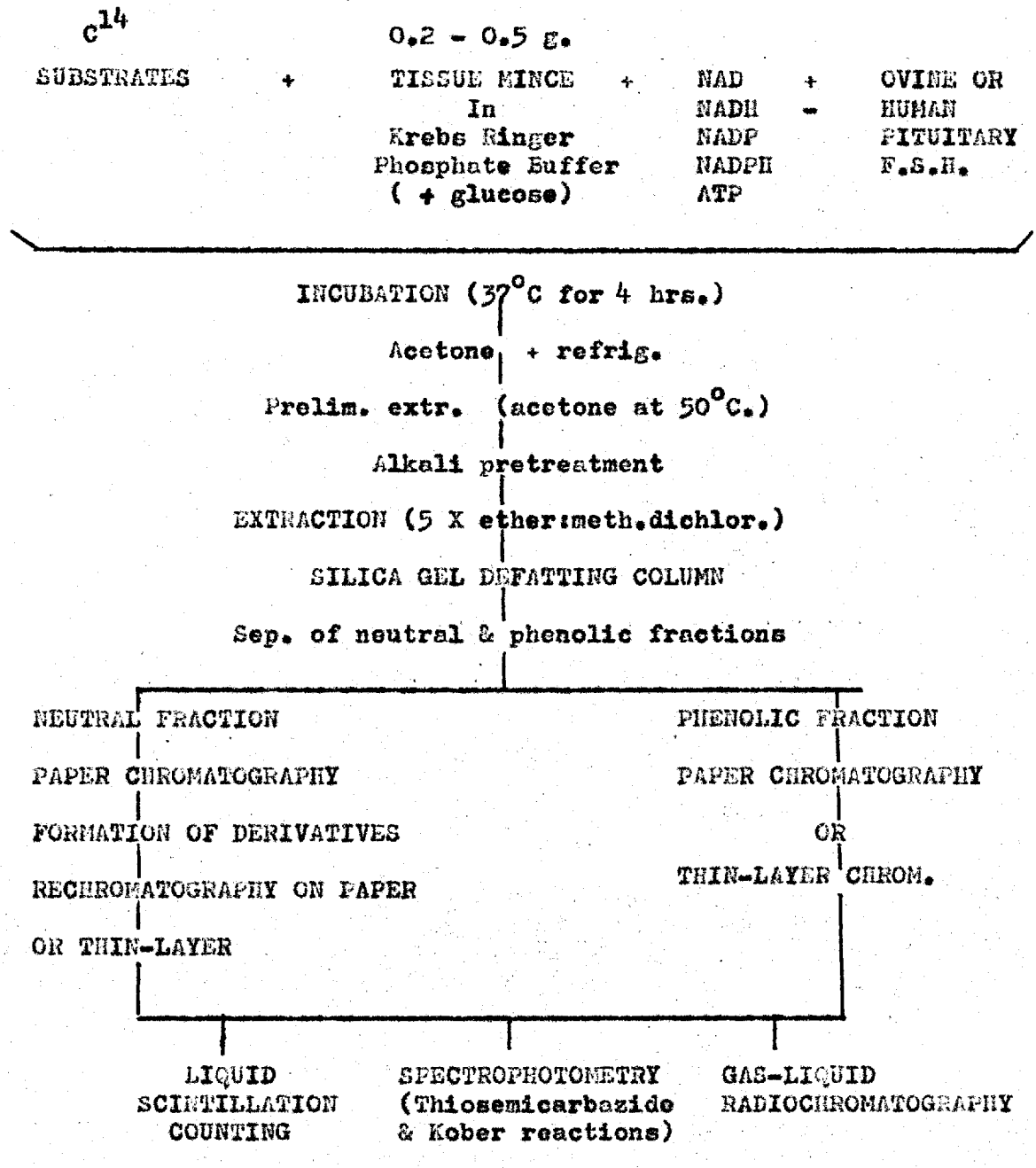


Fig.20. - The 'in vitro' transformation and analyses of isotopically labelled steroids by human ovarian tissue.

NADH 1 µM reduced Nicotinamide-adenine dinucleotide (DPNH)

NADPH 1 µM reduced Nicotinamide-adenine dinucleotide phosphate (TPNH)

ATP 1 mg. Adenosine triphosphate

FSH 2 mg. Follicle stimulating hormone

In the cases where Δ^5 pregnenolone, progesterone or testosterone was the substrate, 10 µg. of steroid was added, whereas to obtain a similar number of counts it was necessary to use 25 µg. Δ^4 androstenedione. The flasks were then placed in a mechanical shaker at 37°C., and incubated for four hours at 120 vib./min.

Extraction:

The reaction was terminated by the addition of 20 ml. acetone to each flask to precipitate the protein and extract the steroids. Each flask was agitated by hand, and left in the refrigerator overnight at 4°C. The flasks were then re-extracted with 230 ml. hot acetone (50°C.), and filtered into a 250 ml. centrifuge bottle. The recovery of radioactivity at this stage varied between 95 - 100%. The acetone/buffer mixture was then evaporated until reappearance of the buffer under vacuum.

Alkali pre-treatment:

Twenty ml. of sodium hydroxide (pH 9.0) was added to each bottle and shaken by hand for ten minutes. The steroids were then re-extracted five times with 150 ml. ether/methylene

dichloride 80:20, the extract siphoned into a round-bottomed flask and evaporated to dryness under vacuum. The recovery was 90 - 100% of the previous step.

De-fatting column:

This procedure was only employed if the extract from the ether/methylene dichloride extraction was so impure that emulsion formation during the subsequent separation of neutral and phenolic fractions was anticipated.

A slight modification of the method of Goldzieher et al., (1961) was used.

A plug of cotton wool was placed in the base of the column which was filled with hexane. The column was then packed with two gm silica gel 200/300 mesh. The dried lipid extract was dissolved in 1.5 ml. hexane, and left overnight or for at least one hour, and then applied to the column. The vessel was then rinsed with another 5 ml. hexane, which was also applied to the column, followed by 35 ml. of 5% ether in benzene (v/v). Finally, 50 ml. of ethyl acetate was added to the column and the eluate collected. The first portion of this solvent was added when about 3 cm. of the ether/benzene mixture was above the silica gel; further portions were added until the transition from one solvent to the other was complete. The ethyl acetate fraction was collected in a 100 ml. round-bottomed flask and evaporated to dryness under reduced pressure.

1.0 - 3.5% of the preceding radioactivity was found on pooling the hexane and ether/benzene fractions and from 4 - 7% on stripping the column with 90% ethanol.

Separation of neutral and phenolic fractions:

The neutral and phenolic fractions were separated according to the method of Brown, (1955), for the determination of urinary oestrogens. 1.0 ml. of ethanol was added to the ethyl acetate extract and the sample transferred with 25 ml. benzene to a separating funnel containing 25 ml. 1.6% sodium hydroxide and 25 ml. petroleum ether (40° - 60°C.). The mixture was extracted twice with 25 ml. 1.6% sodium hydroxide, and the extracts run into another separating funnel. The petroleum ether/benzene fraction was washed twice with 15 ml. distilled water, and the washings transferred to the phenolic fraction.

The neutral fraction was evaporated under reduced pressure, transferred to a pointed tube and dried under nitrogen at 60°C.

The phenolic fraction was adjusted to pH 7.0 with conc. hydrochloric acid, re-extracted with 2 x 150 ml. ether, washed with 25 ml. distilled water, and evaporated under reduced pressure, transferred to a pointed tube and re-evaporated under nitrogen at 60°C.

Analysis of neutral fraction:

The neutral extracts were dissolved in 0.1 ml.

chloroform/methanol 1:1 and transferred with glass capillaries to Whatman No. 2. chromatography paper previously washed for 48 hours with 70% methanol and cut into strips each 1 cm. width. The extract was applied at the starting line above a stream of nitrogen delivered by a Ball chromatogram spotter. Every vessel was re-washed twice with 0.05 ml. chloroform/methanol mixture and applied to the paper in the same manner. Radioactive standards or Δ^4-3-ketosteroids (visible in U.V. light) were applied to parallel strips. The initial chromatographic system was: hexane/85% aqueous methanol.

This system effectively separated the neutral fraction into four regions which were designated α, β, γ, and Δ (Fig.21). The subsequent treatment depended upon the substrate used.

If Δ^5 pregnenolone was the substrate the α fraction was eluted in the Camlab apparatus with 20 ml. ethyl acetate/methanol 2:1 and re-run in the system petroleum ether (40 - 60°C.): benzene 50:50 / methanol:water 70:30.

Testosterone was subsequently separated from 17α hydroxyprogesterone and 17α hydroxypregnenolone by acetylating the mixture and re-running in the hexane/85% methanol system. After reduction with sodium borohydride and subsequent oxidation with sodium bismuthate, the two 17 hydroxy compounds were separated as Δ^4 androstenedione and dehydroepiandrosterone in the system hexane/85% methanol.

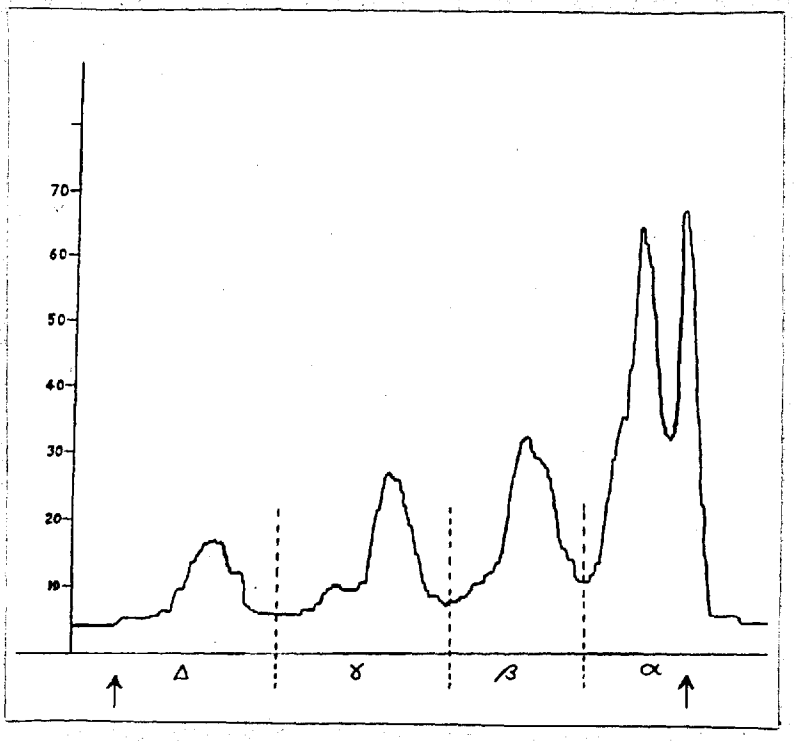


Fig.21. Incubation of gonadal tissue (from patient with testicular feminisation) with Δ^5 -pregnenolone-4-^{14}C: paper chromatogram of neutral fraction scanned for radioactivity.

17α : 20ξ dihydroxyprogesterone and 17α : 20ξdihydroxypregnenolone (pregnenetriol) were also separated and measured as Δ^4androstenedione and dehydroepiandrosterone after oxidation with sodium bismuthate.

The β fraction was eluted in the same manner and run on thin-layer chromatography using silica gel G and the system ether : chloroform 9:1. This system separates Δ^4androstenedione, dehydroepiandrosterone and 20α hydroxypregn[illegible]-4-en-3-one. The two Δ^4-3-ketosteroids may be located under ultraviolet light, and the Δ^5-3β-hydroxy compounds with 10% phosphomolybdic acid in ethanol. Alternatively the steroids may be located by autoradiographic techniques.

The γ fraction was acetylated and re-run in the system hexane/85% methanol. The steroids were located by viewing under ultraviolet light and by scanning the strip for radioactivity.

The Δ fraction was only obtained when Δ^5 pregnenolone was the substrate. Gas-liquid radiochromatography indicated that the compounds were relatively volatile and suggested members of the Δ^{16} series (Brooksbank and Gower, 1964). Further separation on thin-layer plates (before and after acetylation) according to the methods of Gower, (1964), followed by gas-liquid chromatography on 3% C.D.M.S. at 190°C. suggested the presence of androsta-5,16 dien-3β-ol and at least two other steroids of the same series. The formation of this fraction

from various substrates by both human ovarian and testicular tissue has been the subject of a special study not relevant to the present thesis.

The total number of counts in each fraction was determined by removing the paper containing the compound from the chromatogram and counting directly in a toluene based scintillating fluid. If thin-layer chromatography was used the silica gel containing the compound was removed by vacuum as previously described and eluted into a counting vial, thoroughly dried, and scintillating fluid added. Small amounts of quenching were corrected by the addition of C^{14} hexadecane standard.

Analysis of the phenolic fraction:

The extracts were transferred to paper as previously described and run in the system petroleum ether (40° - 60°C.): benzene 50:50/methanol water 70:30, for one hour at 37°C. The paper was then scanned for radioactivity. Five areas of radioactivity were often recorded, three of which corresponded to standard oestriol (at origin), oestradiol and oestrone, the other two have not yet been identified.

Initially the areas corresponding to oestriol, oestradiol and oestrone were eluted and, after the addition of respective non-labelled carriers, were taken through the methylation and alumina chromatography according to the method of Brown, (1955), for the determination of urinary oestrogens.

Aliquots were taken for the Kober reaction to determine the recovery of the carrier, and a fraction counted in a liquid scintillation counter. When the number of counts were sufficient for detection on paper, the methyl ethers were chromatographed in a Bush A system, eluted, and subjected to gas-liquid radiochromatography using 1% CDMS as stationary phase.

In these studies, after extraction of the methyl ethers with petroleum ether and benzene, the sodium hydroxide fraction was adjusted with conc. hydrochloric acid to pH 7.0 and re-extracted with ether. The ether extract from the 'oestriol' fraction contained from 80 - 100% of the starting radioactivity prior to methylation. After acetylation, the compound had the same Rf as standard 19 - hydroxy-androst-4-endione acetate in a Bush B1 system.

In the latter experiments the eluates of the three oestrogens from the first chromatogram were re-run on thin-layer in the system chloroform: acetone 7:3. The compounds were located by autoradiography, and the respective eluates acetylated and run on gas-liquid radiochromatography using 3% S.E. 30 as stationary phase.

IDENTIFICATION OF COMPOUNDS

a) Formation of derivates:

1. Acetylation.

The extract was dissolved in 0.1 ml. pyridine and

an equal volume of acetic anhydride added. The mixture was left overnight at room temperature, or heated for 1 hour at 60°C. The reagents were removed by evaporation under a stream of nitrogen at 60°C., 1.0 ml. of methanol was added and re-evaporated under nitrogen.

2. Oxidation.

The extract was dissolved in a small volume of aqueous acetic acid (50% v/v 0.5 ml.), and an aqueous solution of sodium metaperiodate (1% w/v 1.0 ml.) added. The tube was gently shaken stoppered and left in the dark for 1½ hours at room temperature. The solution was diluted with 5 - 10 volumes of distilled water, extracted with ethyl acetate and washed with saturated sodium bicarbonate (5 ml.) and water (5 ml.). The ethyl acetate extract was dried over anhydrous sodium sulphate and evaporated under vacuum.

3. Reduction.

The extract was dissolved in 2 ml. methanol and 10 mg. sodium borohydride was added. After 90 minutes at 0°C., excess glacial acetic acid was added to decompose the reagent. The methanol was removed under vacuum, water added and the steroids extracted with ethyl acetate.

4. Hydrolysis of acetate esters.

The extract was dissolved in phosphate buffer pH 7.4 and incubated for 4 hours at 37°C. with acylase from

L. Light & Co. Ltd., England. The products were extracted with ethyl acetate.

5. Methylation.

The extract was dissolved in 25 ml. of 1.6% sodium hydroxide, to which 0.9 g. of boric acid was added. Dimethyl sulphate (1.0 ml.) was added using a safety pipette. The flask was shaken until the boric acid and dimethyl sulphate had dissolved and was then placed in an oven at 37°C. for 20 minutes. The procedure was repeated by adding more dimethyl sulphate (1.0 ml.) together with 20% sodium hydroxide (2 ml.). The flask was shaken and maintained at 37°C. for a further 30 minutes or allowed to stand at room temperature overnight. The flasks containing the oestradiol and oestrone fractions were extracted with petroleum ether (40° - 60°C.), while the oestriol fraction was extracted with benzene.

6. Thiosemicarbazone formation.

Thiosemicarbazide (L. Light & Co. Ltd., England) re-crystallised from methanol, was dissolved in 20 ml. 1/10 N HCL (previously filtered) and 100 ml. absolute ethanol (spectrosol grade) added. The extract was dissolved in 1.2 ml. reagent, maintained at 25°C. for 1½ hours, and read in spectrophotometer against a paper blank.

IDENTIFICATION OF STEROIDS.

Δ^5 Pregnenolone:

Same Rf as standard on paper in system hexane/85%

methanol, forms acetate with same Rf as standard on paper in the system methylcyclohexane/acetic acid : water 9:1. Same retention time as standard Δ^5 pregnenolone and after acetylation same retention time as Δ^5 pregnenolone acetate on gas-liquid radiochromatography using 1% NPGA as stationary phase.

Progesterone:

Abs. spectrum (max. abs. 240 mμ) forms bisthiosemicarbazone with max. abs. at 302 mμ, same Rf as standard on paper in the system hexane/85% methanol, same Rf on thin-layer chromatography in the system benzene : ethyl acetate 3:2, resists acetylation, same retention time as standard in gas-liquid radiochromatography using 1% NPGA, 1% CDMS and 3% Q.F.I. as stationary phases.

17α hydroxyprogesterone:

Same Rf as standard on paper in system hexane/85% methanol, resists acetylation, same Rf as standard on thin-layer chromatography in the system chloroform : acetone 7:3. On oxidation yields Δ^4 androstenedione.

17α hydroxypregnenolone:

Same Rf as standard on paper in the system petroleum ether (40° - 60°C.) : benzene 1:1 / 70% methanol 50:50. Separated from 17α hydroxyprogesterone on thin-layer chromatography using the system chloroform : methanol 99:1. On oxidation yields dehydroepiandrosterone.

Dehydroepiandrosterone:

Same Rf as standard on paper in the system hexane/85% methanol, separated from 20α hydroxypregn...-4-en-3-one on thin-layer chromatography in the system chloroform : ether 9:1. Same retention time as free steroid and as acetate standard on gas-liquid radiochromatography using 1% CDMS as stationary phase.

Δ^4 androstenedione:

Abs. spectrum (max. abs. 240 mu), same Rf as standard on paper in the system hexane/85% methanol resists acetylation, on reduction yields testosterone. Same retention time as standard on gas-liquid radiochromatography.

20α hydroxypregn...-4-en-3-one:

Abs. spectrum (max. abs. 240 mu) forms an acetate which has the same Rf as standard on paper in the system methylcyclo-hexane / acetic acid water 9:1. On oxidation yields progesterone.

Testosterone:

Abs. spectrum (max. abs. 240 mu), same Rf as standard in system petroleum ether (40° - 60°C.) : benzene / methanol water 70:30, forms acetate same Rf as standard in the system hexane/85% methanol, yields Δ^4 androstenedione on oxidation. Separated from 17α hydroxyprogesterone on paper after acetylation, or as the free compounds on thin-layer chromatography in the system chloroform : acetone 7:3. Free steroid and acetate

has same retention time as standards on gas-liquid radiochromatography using 1% CDMS as stationary phase.

Oestrone:

Same Rf as standard on paper in the system petroleum ether (40° - 60°C.) : benzene /methanol water 70:30. Same Rf on thin-layer chromatography in the system chloroform : acetone 7:3. Forms acetate with same Rf as standard on GLRC using 3% S.E.30. as stationary phase. Forms methyl ether same Rf as standard on paper and in GLRC.

Oestradiol-17β :

Same Rf as standard on paper in the system petroleum ether : benzene /70% methanol. Same Rf on thin-layer chromatography in the system chloroform : acetone 7:3. Forms acetate and methyl ether with same retention times as standards on gas-liquid radiochromatography using 1% CDMS as stationary phase.

RESULTS.

The ovarian cells which have been shown by transplantation and histochemical studies to have a possible role in hormone synthesis include the granulosa and theca cells of the follicle and corpus luteum, the ovarian stromal cells and their luteinised derivatives, and the hilar Leydig type cells (Morris and Sculley, 1958). In ovarian tumours other types of active cells may also be encountered.

In an attempt to evaluate the function of these

cells, and the structures they comprise, ovarian tissues obtained from the operating theatre were divided into their main constituents and incubated with a variety of labelled substrates.

The total number of counts (corr. for quenching and counting efficiency) was determined at each successive step in the isolation of the radio metabolites. At each step, the recovery of steroid was corrected to 100% in terms of loss of total counts. The results are expressed as percentage conversion of substrate:

$$\frac{\text{Steroid recovered (d.p.s. corr.)}}{\text{Substrate added (d.p.s.)}}$$

The recovery in the total neutral and total phenolic fraction includes unidentified products.

1. Follicular Tissue:

a) Incubation with Δ^5 pregnenolone-4-C^{14}.

500 mg. of ovarian tissue obtained ten days after the last menstrual period was incubated with 10 µg. Δ^5 pregnenolone. Histological examination of a portion of the same tissue indicated the presence of developing follicles surrounded by stromal tissue. After extraction, 88% of the total radioactivity was recovered in the neutral fraction and 12% in the phenolic fraction.

The percentage of total radioactivity recovered in the identified metabolites is shown in Fig. 22.

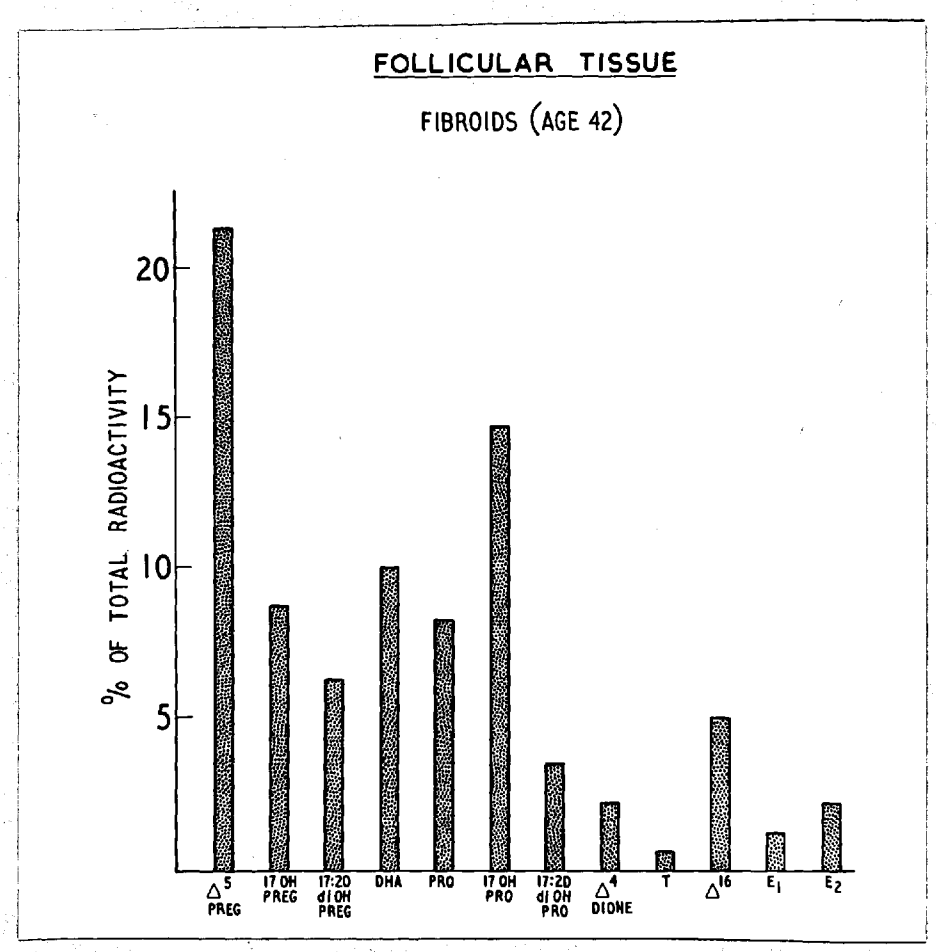


Fig.22. Distribution of radioactivity after incubation of follicular tissue with Δ^5-pregnenolone-4-^{14}C.

Δ^5 Preg = Δ^5-pregnenolone

17 OH Preg = 17αOH pregnenolone

17:20diOH Preg = 17:20 diOH pregnenolone

DHA = dehydroepiandrosterone

pro = progesterone

17 OHpro = 17αOH progesterone

17:20diOHpro = 17:20 dihydroxy progesterone

Δ^4 dione = Δ^4 androstenodione

T = testosterone

E_1 = oestrone

E_2 = oestradiol

It may be seen that there was a high conversion of substrate with a good yield of testosterone, oestrone and oestradiol. More radioactivity had accumulated in the Δ^5 3β-hydroxysteroids prior to the formation of Δ^4 androstenedione than in the C^{21}-Δ^4-3-ketosteroids. There was characteris able amounts of Δ^4 androstenedione and a small accumulation of 17:20 ξ diOH pregnenolone, but an undetectable amount of 20α-hydroxypregn[illegible]-4-en-3-one and low concentrations of 17:20ξ-diOH pregn[illegible]-4-en-3-one.

b) Incubation with progesterone-4-^{14}C.

Two samples of ovarian tissue containing normal developing follicles and stromal tissue were incubated with progesterone. The percentage transformations of substrates are shown in Table 16.

TABLE 16.

AGE	37	43
DAY OF CYCLE	post FSH and HCG	24
% neutral fraction	92.9	92.3
progesterone	4.3	12.9
20α OH pregn-4-en-3-one	1.2	0.2
17:20 ξ diOH pregn-4-en-3-one	37.2	28.8
17α OH progesterone	34.2	31.7
Δ^4 androstenedione	6.7	11.4
testosterone	7.6	6.8
% phenolic fraction	7.1	7.7
oestriol	-	-
oestradiol	1.5	4.2
oestrone	0.1	0.05

It may be seen that on incubation of follicular tissue with progesterone there was a high transformation with good evidence of 17 hydroxylation and desmolase activity. In both cases there was only a small yield of 20αhydroxypregnen-4-en-3-one, but in both a high conversion to 17α : 20ξ diOH pregnen-4-en-3-one.

c) Incubation with Δ^4androstenedione-4-^{14}C.

Two pieces of ovarian tissue containing developing follicles were incubated with androstenedione. The percentage of radioactivity in the metabolites is shown in Table 17.

TABLE 17.

AGE	37	38
DAY OF CYCLE	post FSH and HCG	24
% neutral fraction	96.4	97.8
Δ^4androstenedione	55.0	72.3
testosterone	2.1	9.2
% phenolic fraction	5.4	2.2
oestriol	0.08	-
oestrone	0.2	0.06
oestradiol	0.7	0.8

Testosterone was the main radiometabolite identified; from these two experiments there was no indication that the yield of oestrogen was greater than when progesterone or Δ^5 pregnenolone was substrate. When the 'androstenedione fraction' was acetylated and re-run in the system hexane/85% methanol a small compound tentatively identified as androsterone acetate appeared near the solvent front.

d) Incubation with testosterone-4-^{14}C.

Only one cortex containing some developing follicles was incubated with testosterone. The percentage of radioactivity associated with the identified metabolites is shown in Table 18.

TABLE 18.

AGE	37
day of cycle	22
% neutral fraction	95.9
Δ^4 androstenedione	15.5
testosterone	78.7
% phenolic fraction	4.1
oestriol	-
oestrone	0.01
oestradiol	1.3

It may be seen that Δ^4 androstenedione and oestradiol were identified as the principal radiometabolites.

2. Micropolycystic ovaries associated with the Stein-Leventhal Syndrome:

In 1935 Stein and Leventhal reported a series of cases in which amenorrhea or oligomenorrhea and sterility were associated with enlarged polycystic ovaries. Hirsutism, virilism, or obesity were also present in a number of cases. In the present study ovarian wedge resections containing microcysts from patients labelled Stein-Leventhal syndrome were examined as in the previous study. The incubations were limited to those ovaries which were shiny pearly white; moderately or grossly enlarged with irregular surfaces due to the presence of numerous small subcapsular cysts.

a) Incubation with Δ^5 pregnenolone-4-^{14}C.

500 mg. of ovarian tissue containing numerous microcysts, obtained at wedge resection, was incubated with pregnenolone. The patient was 18 years old and designated as Stein-Leventhal syndrome after careful investigation.

The percentage distribution of radioactivity in the identified metabolites is shown in Fig.23.

It may be seen that the radio-metabolites containing the Δ^5-3β hydroxy group appear to contain most of the radioactivity prior to the formation of Δ^4 androstenedione. In keeping with similar investigations in this syndrome (Axelrod

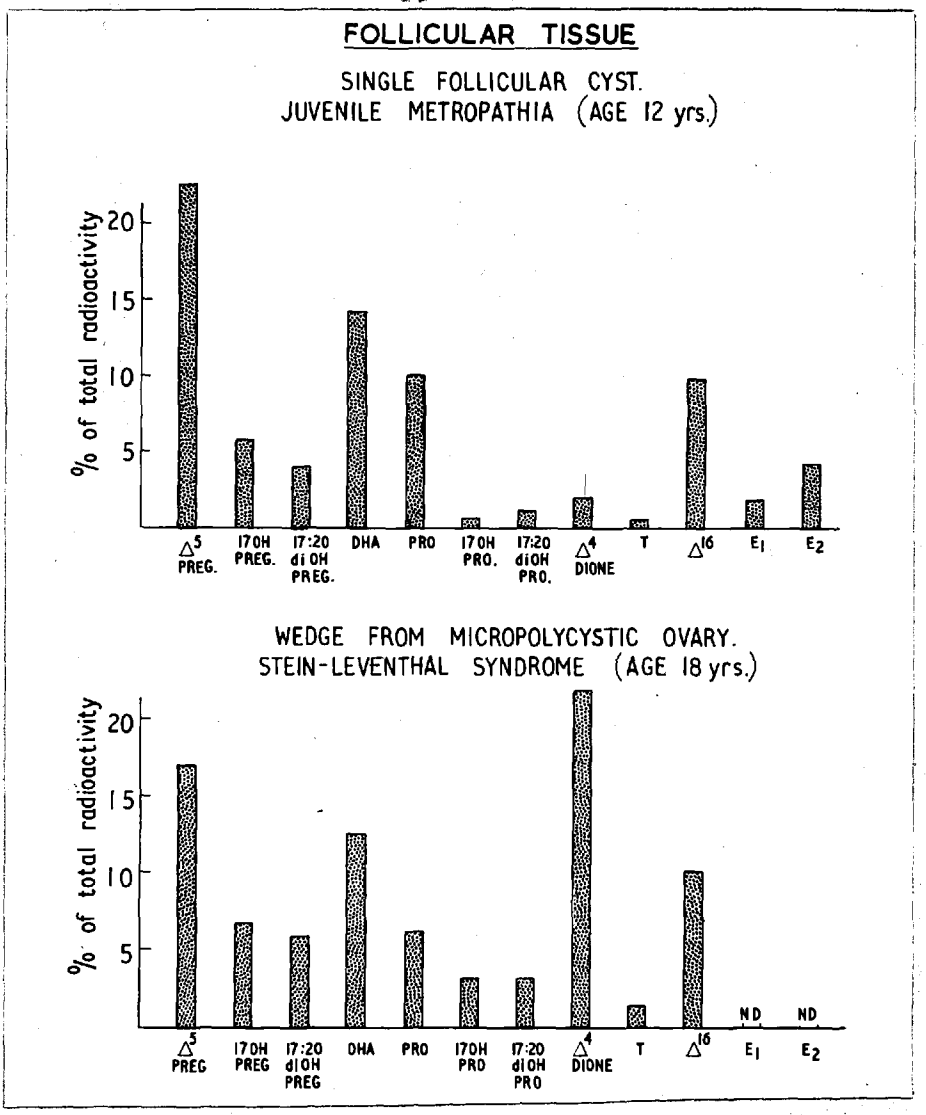


Fig.23. Distribution of radioactivity after incubation of a single follicular cyst (upper case) and a wedge from a micropolycystic ovary (lower case) with Δ^5-pregnenolone-4-^{14}C.

and Goldzieher, 1963), there was a large accumulation of Δ^4 androstenedione, with little evidence for the production of free oestrogens.

b) Incubation with progesterone-4-^{14}C.

500 mg. of tissue obtained at wedge resection from a second patient (age 22 years) and containing multiple micro-cysts was incubated with progesterone. The percentage of radioactivity in the identified metabolites is shown in Table 19.

TABLE 19.

Progesterone	23.9
17αOH progesterone	22.1
20αOH pregn-4-en-3-one	N.D.
17: 20 diOH pregn-4-en-3-one	2.1
Δ^4 androstenedione	25.8
testosterone	1.8
oestriol	N.D.
oestrone	N.D.
oestradiol	N.D.

It may be seen that there was a large accumulation of Δ^4 androstenedione with little evidence for the aromatisation to oestrogens.

c) Incubation with Δ^4 androstenedione-4-^{14}C.

500 mg. of wedge resection tissue from a third subject (age 19 years) was incubated with Δ^4 androstenedione. The distribution of radioactivity in the identified radio-metabolites is shown in Table 20.

TABLE 20.

% neutral fraction	94.8
Δ^4 androstenedione	78.2
testosterone	7.3
% phenolic fraction	5.2
oestrone	N.D.
oestradiol	N.D.

It may be seen that there was a good transformation to testosterone but no oestrogenswere characterisable. There was tentative evidence for the production of 5ξ androstanedione, androsterone and aetiocholanolone or dehydroepiandrosterone.

3. A Single Follicular Cyst:

A single follicular cyst was removed from a 12 year old girl with juvenile metropathia which was suspected to be associated with ovarian hyperfunction. Histological examination revealed marked hyperplasia of the theca cells with only slight retention of the granulosa layer. 200 mg. of the cyst was

homogenised with a glass hand homogeniser in 5 ml. Krebs Ringer Phosphate buffer, and separate portions of the same tissue were incubated with Δ^5pregnenolone, progesterone, Δ^4 androstenedione and testosterone.

a) Incubation with pregnenolone-4-^{14}C.

After extraction, 20% of the radioactivity was found in the phenolic fraction. The percentage of radioactivity recovered in the identified metabolites is shown in Fig.23.

It may be seen that there was a good transformation to oestradiol and oestrone from only 40 mg. of tissue. Again more radioactivity had accumulated in the Δ^5-3β-hydroxysteroids prior to the formation of Δ^4androstenedione than in the Δ^4-3-ketosteroids related to progesterone.

b) Incubation with progesterone-4-^{14}C.

After extraction 19.7% of the radioactivity was recovered in the phenolic fraction. The distribution of radioactivity is shown in Table 21.

TABLE 21.

progesterone	40.0
17α OH progesterone	22.7
20α OH pregn-4-en-3-one	5.6
17:20 diOH pregn-4-en-3-one	15.3
Δ^4 androstenedione	0.7
testosterone	0.1
oestriol	N.D.
oestradiol	4.2
oestrone	1.4

In this tissue a much smaller amount of exogenous progesterone was metabolised, but there was evidence of 17 hydroxylation and desmolase activity with the formation of oestradiol. There was little accumulation of Δ^4 androstenedione. A compound less polar than progesterone was identified as 5β pregnane-3:20-dione.

c) Incubation with Δ^4 androstenedione-4-^{14}C.

The distribution of radioactivity in the identified metabolites was as follows:

androstenedione	80%	testosterone	2.0
oestrone	2.8	oestradiol	4.6

20.3% of the radioactivity was recovered in the phenolic fraction.

d) Incubation with testosterone-4-^{14}C.

After extraction 22.6% of the radioactivity was recovered in the phenolic fraction. The percentage distribution of radioactivity is shown below:-

testosterone	71.0%	androstenedione	10.2
oestradiol	3.6	oestrone	1.6

It may be seen that when Δ^4 androstenedione and testosterone were the substrates there was a good transformation to oestrone and oestradiol, although 70 - 80% of the radioactivity was recovered as substrate. When Δ^4 androstenedione was substrate there was more oestrone formed than oestradiol.

There was also less Δ^4 androstenedione formed when

testosterone was substrate, than testosterone when androstenedione was substrate.

4. Corpus Luteum:

Two corpora lutea removed on day 22 and day 13 after the last menstrual period, were incubated with 10 µg. Δ^5 pregnenolone.

It may be seen (Fig.24) that there is more radioactivity associated with the Δ^4-3-ketosteroids prior to the formation of androstenedione than in the Δ^4-3β-hydroxysteroids. There was a similar yield of oestrone and oestradiol to follicular tissue.

a) Incubation with progesterone-4-^{14}C.

Five corpora lutea were incubated with progesterone. The results are shown in Table 22.

TABLE 22.

	(1)	(2)	(3)	(4)	(5)
AGE	37	38	37	48	51
DAY OF CYCLE	Post FSH & HCG	24	22	7	?
% neutral fraction	93.0	84.2	95.9	94.6	86.4
progesterone	41.7	35.0	5.8	51.9	46.4
17αOH progesterone	25.8	16.8	65.9	23.4	21.7
20αOH pregn-4-en-3-one	3.6	5.2	4.6	4.2	4.1
17:20 diOH pregn-4-en-3-one	7.3	17.5	7.6	9.3	8.7
Δ^4androstenedione	2.5	2.0	1.1	6.4	5.9
testosterone	1.7	-	6.6	5.4	1.8
oestradiol	2.3	8.2	0.8	0.8	1.3
oestrone	2.8	0.09	0.06	0.1	0.6
oestriol	0.2	0.7	N.D.	N.D.	N.D.

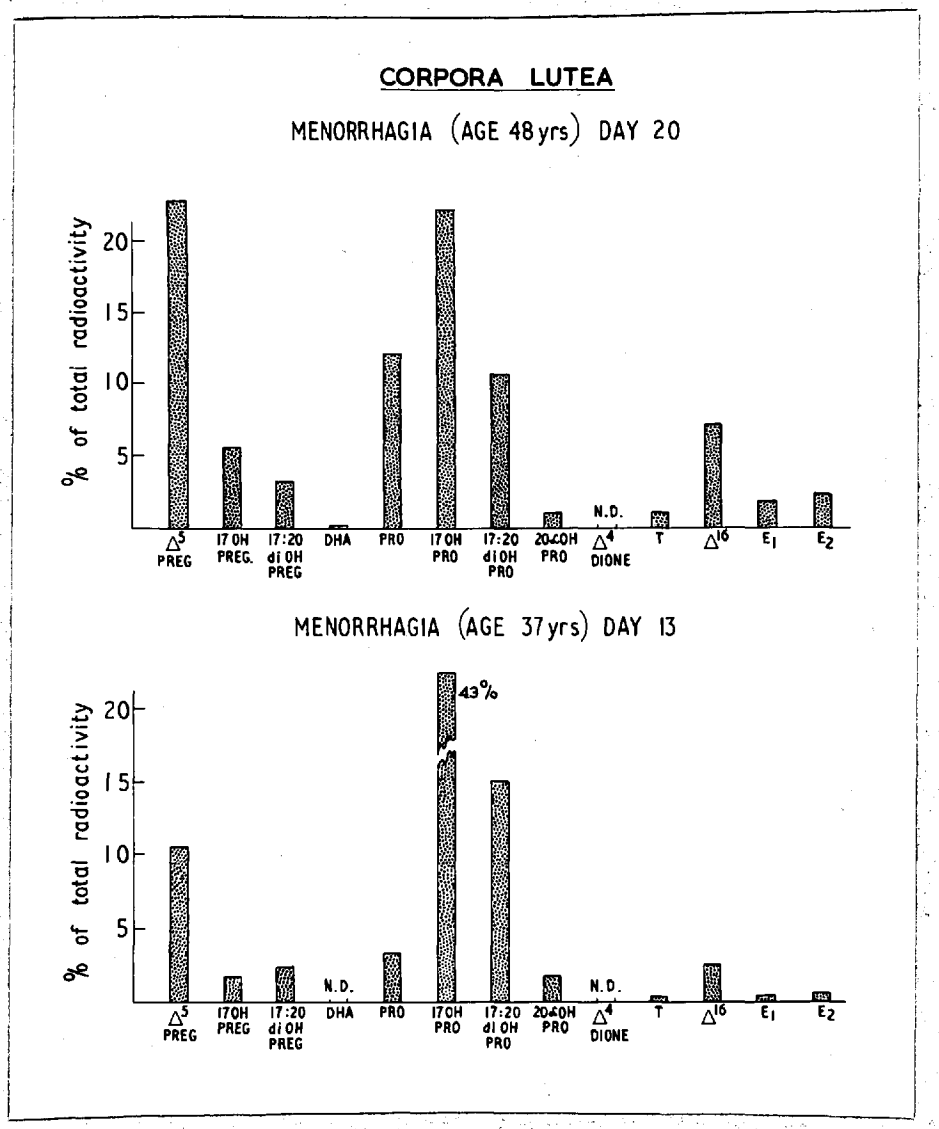


Fig.24. Distribution of radioactivity after the incubation of corpora lutea with Δ^5-pregnenolone-4-^{14}C.

In all cases there was a high transformation of substrate with good evidence of 17-hydroxylation and 20-hydrogenation. However, there was only a small accumulation of Δ^4 androstenedione. There was a similar production of oestrogens as in follicular tissue.

b) and c) Incubation with Δ^4 androstenedione-4-^{14}C and testosterone-4-^{14}C.

Four aliquots of corpora lutea were incubated with androstenedione, and two aliquots with testosterone. The results were as follows:-

With androstenedione as substrate.

	1.	2.	3.	4.
AGE	37	38	48	51
DAY OF CYCLE	Post FSH & HCG	24	7	?
% neutral fraction	97.7	91.1	96.4	89.1
Δ^4 androstenedione	84.3	56.6	89.1	90.1
testosterone	3.0	30.9	4.0	2.3
5β androstanedione	-	-	1.2	N.D.
% phenolic fraction	2.3	8.9	3.6	0.9
oestriol	-	1.2	-	-
oestrone	0.1	N.D.	0.2	0.8
oestradiol	0.7	3.5	1.1	1.4

It may be seen that testosterone was formed in all cases, with a high yield in case 2. A high percentage of the radioactivity was recovered as substrate as was the case with follicular tissue.

The results were as follows:-

With testosterone as substrate.

	1.	2.
AGE	48	51
DAY OF CYCLE	7	?
% neutral fraction	91.4	90.6
testosterone	81.2	86.1
Δ^4 androstenedione	10.3	1.8
5β androstanedione	1.6	0.6
% phenolic fraction	8.6	9.4
oestriol	N.D.	N.D.
oestrone	0.09	0.07
oestradiol	0.91	1.1

It may be seen that less Δ^4 androstenedione is formed when testosterone is substrate, than testosterone when Δ^4 androstenedione is substrate.

5. Postmenopausal Stromal Tissue:

Two gm. of ovarian tissue, from a 62 year old subject, was minced in the usual manner and separate portions of the same tissue incubated with Δ^5 pregnenolone, progesterone, androstenedione and testosterone.

a) Incubation with Δ^5 pregnenolone-4-^{14}C.

In this incubation (and another incubation in a 51

year old woman) two compounds (other than the substrate) were separated on the first chromatogram. One of these was tentatively identified as dehydroepiandrosterone, and the other as a mixture of 17αOH pregnenolone, 17: 20ξ dihydroxypregnenolone and 20αhydroxypregnenolone. However, these only accounted for approximately 6% - 8% of the total radioactivity, the remainder being accounted for as unmetabolised substrate. There was no evidence for the production of significant amounts of progesterone, testosterone or oestrone or oestradiol.

b) Incubation with progesterone-4-^{14}C.

There was marked evidence of 20-hydrogenation with the production of approximately 12% 20αhydroxypregn[illegible]-4-en-3-one. Analyses of the incubation products from 6 other post-menopausal ovaries indicated that this conversion varied from 3 - 12%. In 4 cases a small yield (approx. 3%) of 5β pregnane-3:20-dione was obtained. There was no significant yield of oestradiol or oestrone.

c) Incubation with Δ^4androstenedione-4-^{14}C.

Four postmenopausal ovaries were incubated with androstenedione and the results shown in Table 23.

Small amounts of testosterone were produced in cases 1 and 2. In cases 1,2 and 3 small amounts of oestrone, and oestradiol were characterised.

TABLE 23.

	(1)	(2)	(3)	(4)
AGE	52	52	57	64
L.M.P.	2 yrs.	5 mths.	9 yrs.	10 yrs.
% neutral fraction	96.7	95.7	91.5	98.2
Δ^4 androstenedione	74.6	78.7	81.9	98.2
testosterone	16.3	2.5	-	-
% phenolic fraction	3.3	4.3	8.5	1.8
oestriol	-	-	-	-
oestrone	0.06	0.01	0.003	-
oestradiol	0.28	0.02	0.005	-

d) Incubation with testosterone-4-^{14}C.

Two aliquots of postmenopausal ovarian tissue were incubated with testosterone. The percentage of radioactivity in the identified metabolites was as follows:-

	(1)	(2)
AGE	52	52
L.M.P.	1½ yrs.	2 yrs.
% neutral fraction	92.9	92.2
testosterone	71.9	72.4
Δ^4 androstenedione	17.3	10.9
% phenolic fraction	7.1	7.8
oestriol	-	-
oestrone	0.6	0.3
oestradiol	1.3	1.84

It may be seen that, with this substrate, oestrone and oestradiol were obtained in comparable yield to pre-menopausal tissue.

6. Papillary Adenocarcinoma:

1.0 gm. of ovarian tissue from a 63 year old subject with cystic glandular hyperplasia of the endometrium was divided in half and separate portions incubated with Δ^5 pregnenolone and progesterone. Histological examination indicated a papillary adenocarcinoma.

a) Incubation with Δ^5-pregnenolone-4-^{14}C.

The radioactivity was associated with the metabolites shown in Fig.25.

It will be seen that there was transformation to oestrogens by the abnormal ovarian tissue of this postmenopausal subject, but no progesterone was detectable.

b) Incubation with progesterone-4-^{14}C.

The radioactivity was associated with the following metabolites (Table 24).

TABLE 24.

progesterone	64.9%
17αOH progesterone	11.6%
20αOH pregn-4-en-3-one	6.5%
17:20 diOH pregn-4-en-3-one	3.4%
Δ^4 androstenedione	1.4%
5β pregnane 3:20 dione	9.9%
oestrone	N.D.
oestradiol	N.D.

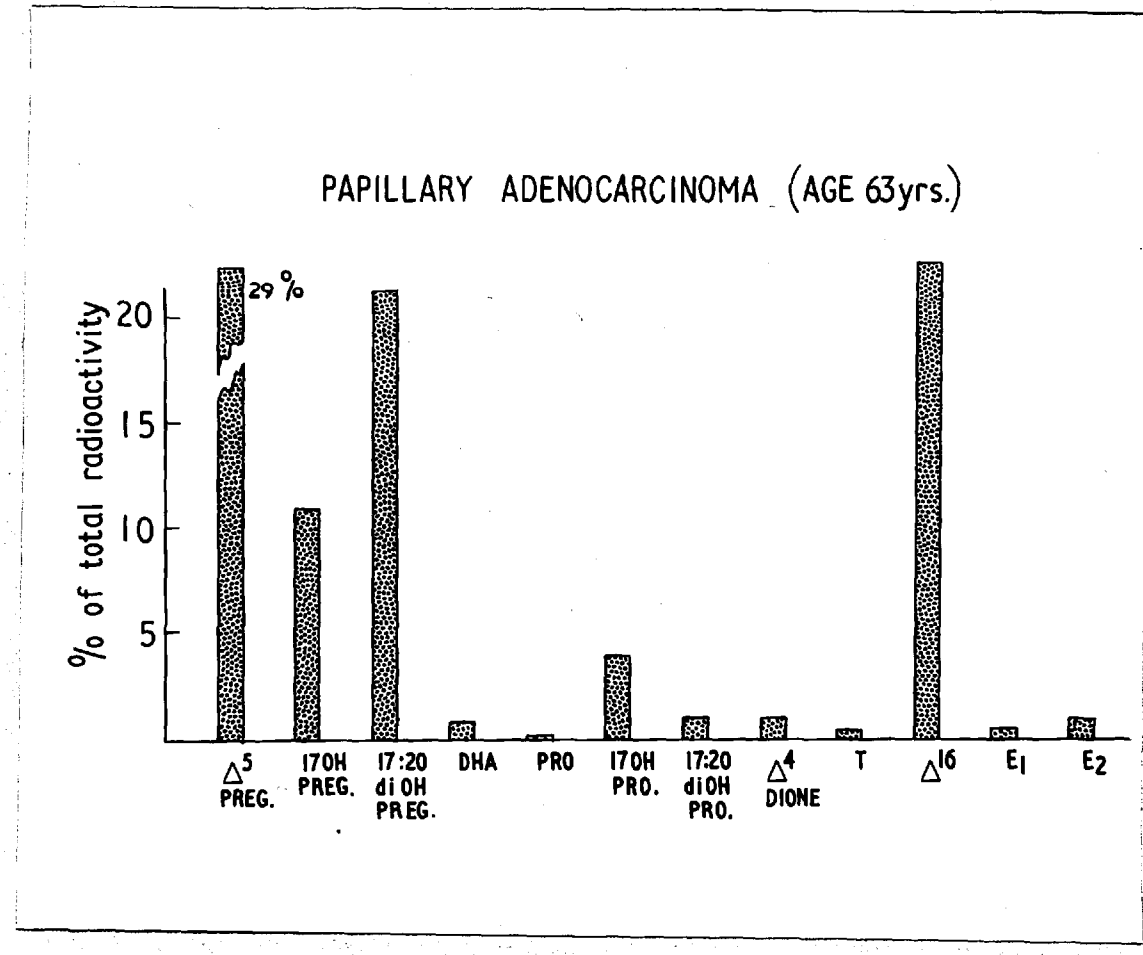


Fig.25. Distribution of radiometabolites after the incubation of a papillary adenocarcinoma of the ovary with Δ^5-pregnenolone-4-^{14}C.

It may be seen that there is an increased yield of 5β-pregnane-3:20 dione and a good yield of 20α-hydroxypregn-4-en-3-one indicative of increased stromal cell activity, but for the first time in postmenopausal ovarian tissue there was good evidence for 17 hydroxylation. There was only a small yield of Δ^4androstenedione, oestrone and oestradiol.

The metabolism of progesterone in human myometrium and myometrial fibroid tissue.

Notwithstanding the large production of progesterone during the menstrual cycle and pregnancy, the myometrium does not appear to concentrate the hormone to any great extent. The experiments of Zander, (1961), and Plotz, (1961), involving the injection of C^{14} progesterone indicated that the concentration was of the order of 0.6 - 0.7 mμg./gm. of tissue.

The first chemical measurement of progesterone in myometrium during late pregnancy was intimated by Kumer et al., and reported in more detail by Barnes et al., (1962). They analysed four samples and found levels between 0.1 - 0.6 μg./gm. wet weight of tissue. Similar concentrations to these were found during the present study.

There has also been little investigation into the metabolism of progesterone by uterine muscle. In the 'in vivo' experiments of Zander, (1961), with progesterone-4-^{14}C, most of the free radioactive steroids recovered from the myometrium could be accounted for as progesterone.

Some 'in vitro' studies with cultured human uterine fibroblasts were reported by Sweat et al., (1958). These authors incubated the fibroblasts with C^{14} progesterone overnight at 37°C. in a stationary position, and then in a roller apparatus for an additional four days. The reaction mixture was then extracted four times with warm acetone and filtered.

The acetone was removed under vacuum in the presence of a stream of nitrogen. The aqueous residue was re-extracted with chloroform, evaporated, redissolved in methanol:chloroform 1:1 and chromatographed on paper in the system hexane / formamide.

This procedure resolved the radiometabolites into six peaks, from which 20α hydroxypregn-4-en-3-one, 20β hydroxypregn-4-en-3-one and allopregnanedione, and unmetabolised progesterone were identified. Evidence was also presented for presence of 5α or 5β pregnan-20α ol-3-one and / or their epi-isomers of 5α or 5β pregnan-3αol -20-one and / or their epi-isomers.

These results prompted further studies into the metabolism of progesterone 'in vitro' by myometrial tissue from non-pregnant subjects.

METHOD

The tissue obtained directly from the operating theatre was processed and incubated with progesterone-4-^{14}C as previously described for the experiments with ovarian tissue. The products were again extracted with hot acetone followed by a re-extraction of the alkaline aqueous extract with ether / methylene dichloride 4:1. The extract was then transferred directly to paper with methanol / chloroform 1:1 and chromatographed in the system hexane / 85% aq. methanol 1:1.

The chromatogram was subdivided as shown in Fig.26.

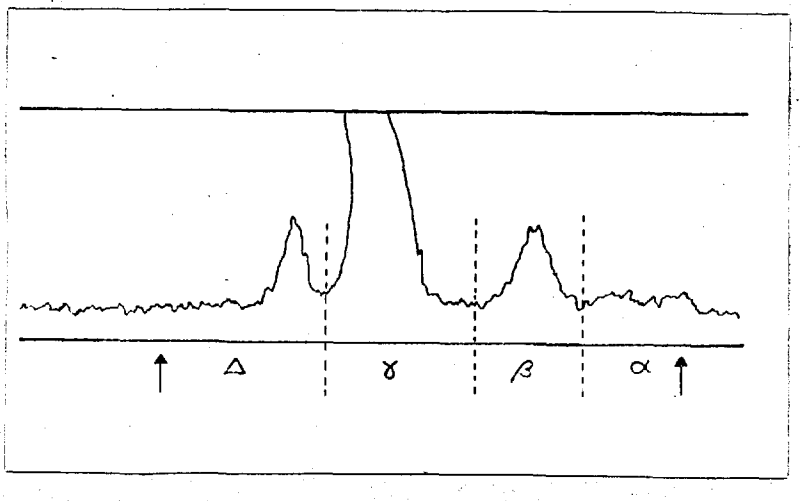


Fig.26. Incubation of myometrial tissue with progesterone-4-^{14}C: paper chromatogram of neutral fraction scanned for radioactivity.

The α fraction was eluted and re-run in the system petroleum ether (40° - 60°C.) / benzene 50:50 / 70% aq. methanol 1:1, acetylated and re-run in hexane /85% aq. methanol.

The β fraction was eluted and subjected to gas-liquid radiochromatography using 1% CDMS as stationary phase.

The γ fraction was eluted, acetylated and re-run in the same system.

The total number of counts in each fraction was calculated as in the experiments with ovarian tissue.

RESULTS

1. Time Study:

Five flasks each containing 500 mg. of myometrium and 10 µg. of progesterone-4-^{14}C were incubated at 37°C. One flask was removed every hour and the reaction termined by the addition of 20 ml. acetone. On analysis a radioactive spot was found with Rf 0.25 in the system hexane / 85% methanol.

Time (Hrs.)	d.ps. (Corr.)	% of substrate transformed
1	74	2.9
2	156	6.1
3	199	7.8
4	260	10.2
5	330	12.0

The unkhown compound was eluted from each strip with 15 ml. ethyl acetate / methhanol 3:1, pooled and subjected to

gas-liquid radiochromatography on 1% CDMS. The radioactivity was associated with the following compounds:

progesterone	21%
5β pregnan-3β-ol-20-one	40%
20α OH pregn-4-en-3-one	38%

2. Normal myometrium after 5 hours incubation:

Patient	1.	2.
Age	47	39
Progesterone	88.4	90.1
5β pregnane 3:20 dione	N.D.	1.2
20α OH pregn-4-en-3-one) 5β pregnanolone)	6.3	5.1

3. Fibroid Tissue:

Patient	1.	2.
Age	51	47
Progesterone	67.4	82.4
5β pregnane 3:20 dione	5.4	3.0
20α OH pregn-4-en-3-one) 5β pregnanolone)	15.1	9.2

As a result of these studies it was decided to study the exogenous metabolism of progesterone by normal myometrium and fibroid tissue from the same uterus.

Patient	1.		2.		3.	
Age	42		46		48	
	M.	F	M	F	M	F
Progesterone	81.7	78.3	89.4	86.0	87.4	81.4
5β pregnane 3:20 dione	3.7	7.9	0.3	1.0	-	-
20αOH pregn-4-en-3-one) 5β pregnanolone)	14.5	13.8	6.9	7.5	9.2	11.2

The possible presence of Δ^5 - 3β ol dehydrogenase activity in human uterine tissue was investigated by incubating one gm. of fibroid tissue with pregnenolone -4 - ^{14}C. In this experiment all the radioactivity was recovered as Δ^5 - pregnenolone.

DISCUSSION

Although 'in vitro' techniques may suggest many possible transformations of steroid substrates in various tissues, the results must be interpreted with caution, for there are many possible limitations inherent in this approach to the study of metabolism.

The first essential requirement is a stable substrate of adequate purity. The ^{14}C progesterone used was tested for purity in a liquid-liquid partition paper chromatographic system, on thin-layer chromatography, and gas-liquid radiochromatography. In the first two systems only one spot was visible under ultraviolet light, and only one peak obtained on scanning the paper strip for radioactivity. However, when

subjected to gas-liquid radiochromatography using 1% NPGA and 1% CDMS as stationary phases, a small peak, tentatively identified as the 17α epimer, and constituting 2.5% of the total radioactivity, was observed immediately preceding the main peak.

When progesterone was incubated with buffer and coenzymes alone at 37°C. for 4 hours, extracted with ether, and chromatographed on paper in the system hexane / 85% aq. methanol, a small unidentified peak (approx. 3% of total radioactivity) with a Rf of 0.19 was observed on scanning the strip for radioactivity.

The preliminary studies on the incubation of progesterone with blood afforded evidence on the presence or absence of enzymes in the circulating fluid under physiological conditions, and at the same time served as a control for the 'in vitro' experiments with tissue. In this respect the metabolic activity of the peripheral plasma appears to be low as only 4 - 7% of the progesterone was reduced at either C = 20, or at C = 3 and C = 5. Since the largest amount of tissue used was 500 mg. it would appear that any blood present would not significantly contribute to the metabolic activity attributed to the tissue 'in vitro'.

Similar precautions with regard to purity, and control incubations with buffer and coenzymes, were applied to all substrates used. All were found to be 97 - 100% pure

initially, and after incubation without tissue 96 - 100% of the radioactivity was recovered as the original substrate.

Implicit in the interpretation of results involving minced tissue is the assumption that all substrates can enter the cells and all at comparable rates. Since in most cases up to 75% of the substrate may be metabolised the first part of the assumption would appear to be valid, and since similar results are obtained with homogenised tissue the rate of uptake would not appear to affect the result.

During the study on the relative importance and inter-relationship of various pathways in the biosynthesis of steroid hormones in the human ovary, it was apparent that the results obtained would be influenced by the concentration of substrate available for the respective enzymes. Consequently whenever sufficient material was available, the tissue was minced, subdivided and separate portions incubated with different substrates in order to evaluate the relative enzyme activities more effectively. However, due to the unpredictable availability of human material this approach was not always possible, for in various pathological conditions (e.g. Stein-Leventhal syndrome) only portions of the ovaries were obtained.

The results from ovarian tissue, although in a preliminary stage, do appear to suggest marked differences in enzyme activity within the various ovarian components. However,

this assumes an adequate supply of Δ^5 pregnenolone to all structures 'in vivo', and the presence of alternative pathways not involving pregnenolone should not be precluded.

The developing follicles showed a preference for retaining the Δ^5 - 3β-hydroxy group prior to the formation of Δ^4 androstenedione, rather than an immediate production of progesterone and related Δ^4 - 3 - ketosteroids. All the enzymes necessary for the formation of testosterone, oestrone and oestradiol from Δ^5 -pregnenolone were shown to be present.

When progesterone was used as a substrate there was a high transformation to 17αOH progesterone, Δ^4 - androstenedione, testosterone, oestrone and oestradiol.

Incubation with Δ^4 - androstenedione gave a small yield of testosterone and no consistent increase in the yields of oestrone and oestradiol. These results and similar observations with corpora lutea tend to indicate that the process of aromatisation is a rate limiting step in the production of oestrogens.

Incubation of follicular tissue with testosterone gave slightly larger yields of Δ^4 - androstenedione, than testosterone when Δ^4 - androstenedione was substrate. This indicates that under these conditions the reaction equilibrium tends to favour the formation of Δ^4 - androstenedione.

From these results it would appear that the normal developing follicle is capable of producing progesterone,

testosterone, oestrone and oestradiol. However, it would appear that in this structure progesterone is not an obligatory intermediary in the biosynthesis of both androgens and oestrogens.

Similar results to these have recently been presented by Gospodarowicz, (1964a,b), for steroid transformations in the rabbit follicle.

The multiple microcysts associated with the Stein-Leventhal syndrome appeared to have a similar pattern with regard to the production of other Δ^5 -3β hydroxycompounds from Δ^5 pregnenolone, but differed from normal follicles in the accumulation of Δ^4-androstenedione and the complete or almost complete blockage in the biosynthesis of free oestrogens.

When progesterone is added as substrate there is again a rapid metabolism with an accumulation of Δ^4-androstenedione, and, as with the incubations with Δ^5 - pregnenolone, no significant amounts of oestrogens are produced.

Incubation of this tissue from ovarian wedge resections with Δ^4- androstenedione results in the production of testosterone and small amounts of various reduction products tentatively identified as androstanedione, androsterone and aetiocholanolone or dehydroepiandrosterone. A similar equilibrium between testosterone and Δ^4- androstenedione is observed in these structures as in normal follicles. It would appear that in these polycystic ovaries there is a metabolic block, probably in 19-hydroxylation which impedes the formation of oestrogens.

and probably results in the secretion of androgenic precursors which would account for the virilism and masculinisation associated with this syndrome.

In the case of juvenile metropathia in the twelve year old subject, there was a very high excretion of oestrogens in the urine. At laparotomy a small single follicular cyst was removed for histological and 'in vitro' metabolic investigation. In this instance 200 mg. of tissue was homogenised in Krebs Ringer phosphate buffer and separate aliquots incubated with the precursors previously mentioned. Analysis of all the radiometabolites indicated that the tissue was potentially capable of producing large amounts of oestrogen, and was in fact the most active tissue studied in the present series, when compared to constant weight.

Incubation with Δ^5 - pregnenolone gave only a small yield of progesterone, and when progesterone was used as substrate only a small percentage was metabolised. Also more androstenedione was formed when Δ^5 - pregnenolone was substrate than when progesterone was substrate. Incubatim with androstenedione gave results essentially similar to those recorded for normal follicular tissue.

From these results it would appear that biosynthetic pathways other than those involving progesterone are relatively more important in many forms of follicular tissue.

After ovulation, followed by luteinisation of the

granulosa cell layer and the possible migration of 'K' cells from the theca into the granulosa layer, the resultant corpus luteum exhibits changes in the metabolism of exogenous substrates.

In corpora lutea incubation with Δ^5 - pregnenolone results in the formation of larger quantities of progesterone and related Δ^4 - ketosteroids. However, there is still a good yield of testosterone, oestrone and oestradiol as in the developing follicle. The increase in hormone secretion in this phase of the cycle as demonstrated by both blood and urinary assays is probably the result of the continued activity of both these structure.

Incubation of corpora lutea with progesterone demonstrates 20-hydrogenation with increased yields of 20α-hydroxy-pregn-4-en-3-one and 17:20 ξ dihydroxyprogesterone when compared with follicular tissue. The latter compound was formerly considered to be an important intermediary in the formation of Δ^4-androstenedione (Sweat et al., 1960). However, more recent studies by Sweat et al. (Personal Communication), Ball and Kadis, (1964) and Axelrod, (1965) have shown that it is not an intermediary compound and may represent an artefact of the 'in vitro' system due to the unphysiological level of substrate available. The studies of Ball and Kadis, (1964), have also shown that in the sow ovary 17-hydroxylation must precede 20-hydrogenation.

In all studies with corpora lutea there was a much

lower yield of Δ^4-androstenedione than with follicular tissue, but on using Δ^4-androstenedione as substrate comparable yields of oestrone and oestradiol were obtained.

The function and possible endocrine activity of ovarian stroma cells has been the subject of a great deal of indirect study since hyperplasia of these cells has been associated with cancer of the breast and various postmenopausal gynæcological disorders. Preliminary experiments with labelled sodium acetate (Lemon et al., 1958) indicated that these cells could synthesize oestrogens although the yields were extremely small and no intermediary compounds were identified. Warren and Dalhamick, (1961), reported that incubation of postmenopausal stromal tissue with progesterone-4-^{14}C resulted in the formation of 20αhydroxypregn-4-en-3-one, while, in another study, Notis et al., (1956), reported that testosterone could be transformed to oestrogens by this tissue.

In the present work, experiments with Δ^5 pregnenolone show that only a small percentage of the substrate was metabolised to a small yield of dehydroepiandrosterone and that a more polar compound, tentatively identified as 20α-dihydroxypregnenolone was produced. There was no evidence for the synthesis of progesterone. Small amounts of radioactivity were associated with non-labelled oestrone and oestradiol, but were insufficient for complete identification.

Incubation with progesterone resulted in its reduction to 20α hydroxy-pregn-4-en-3-one in amounts varying from 3 - 12% as previously found by Warren, although the presence of 5β pregnanolone in this fraction was not excluded. One ovary produced a 9% yield of a compound tentatively identified as 20β-hydroxypregn -4-en-3-one. 17-hydroxylation appeared to be completely absent.

Incubation with Δ^4 androstenedione gave a small yield of testosterone and slightly increased amounts of oestrogens. In contrast with the previous results, incubation with testosterone gave oestrogens in yields comparable to premenopausal tissue. This indicates that testosterone can be converted to oestrogen without the formation of androstenedione as an intermediary compound.

It would appear that if these stromal cells are active 'in situ', and ovariectomy was limited to those post-menopausal ovaries which were thought to be active, only small amounts of oestrogen are produced compared with premenopausal tissue and progesterone does not appear to be an important intermediary compound. These studies do not preclude the possibility that larger amounts of oestrogenic material are produced by completely different routes to those studied.

The effects of FSH, CG and HMG on progesterone metabolism by postmenopausal ovaries was investigated by sub-dividing tissue from the same patient; adding progesterone as

substrate and comparing results after the addition of various gonadotrophic preparations. No increase in metabolism was observed with any of the preparations.

Studies on the tissue from the case of papillary adenocarcinoma of the ovary was an extension to these investigations. Separate portions of the tissue were incubated with Δ^5 pregnenolone and progesterone. The incubation with Δ^5 pregnenolone gave characterisable amounts of oestrogens but no progesterone was detectable and most radioactivity in the intermediaries prior to the formation of Δ^4 androstenedione resided in the Δ^5 -3β - hydroxysteroids. On incubation with progesterone there were increased yields of 5β pregnanedione and 20α hydroxypregn -4-en-3-one indicative of increased stromal cell activity, and for the first time in ovarian tissue of this age there was evidence for the production of 17α hydroxy-progesterone. However, the yields of Δ^4 androstenedione, oestrone and oestradiol were very low.

The metabolism of progesterone by ovarian fibroid tissue was investigated in a 23 year old patient, and once again there was only a 12% reduction at carbon 20.

Recent 'in vitro' studies by Rice et al., (1964b) on stromal tissue from normal premenopausal ovaries have indicated that these cells produce testosterone from acetate, the conversion being enhanced by preparations of trophic hormones with luteinising action.

In one case in the present study where hilar tissue was obtained, incubation with progesterone again yielded a small percentage of 20α hydroxypregn-4-en-3-one, and if androgens are produced by these cells as has been suggested due to their histological similarity to Leydig cells in the testis, progesterone does not appear to be an important intermediary in the process.

From these studies it would appear that progesterone is not an obligatory intermediary compound in the biosynthesis of androgens and oestrogens in the human ovary, and indeed in many of the ovarian tissues it does not appear to be of major importance as an intermediary. However, it must be stressed that far more must be learned about the relationship between production and secretion and factors controlling secretion before the true significance of these results can be ascertained.

The 'in vitro' experiments with myometrial tissue illustrate that, although progesterone has an undoubted biological effect on this tissue, it is comparatively slowly metabolised. The focal points for enzymatic change appear to be reduction of the double bond at C 5, and reduction of the ketone groups at carbon atoms 3 and 20. The order of these metabolic reductions is unknown. Analyses of the results did not reveal any consistant pattern in these changes, or between normal myometrial tissue and myometrial fibroids. No pregnanediol was identified in these studies and there was also

no evidence for the formation of either 20α or 20β-hydroxypregnane-3-one.

If the steroid metabolism observed in the same 'in situ' where there is far less substrate, the question remains as to whether the conversions are associated with the mechanism of hormone action or represent a process employed by the cell to remove excess hormonal stimulation.

ADDENDUM

ADDENDUM :- Related aspects of progesterone metabolism.

The preceding sections have been particularly concerned with two approaches to the study of progesterone metabolism. These have been the development of a new micro method for the quantitative determination of progesterone in body fluids and tissues and the application of 'in vitro' techniques to the investigation of ovarian steroidogenesis.

Generally speaking, because of the necessity for the development and application of specialised techniques, individual research workers can only cover a few aspects of any biochemical problem, and for this reason it is vitally important to constantly relate and integrate the results obtained by other experimental approaches. Furthermore, the constant introduction of new techniques in all disciplines has generally exposed far more problems than the techniques were originally intended to resolve. This has necessitated the repeated reappraisal and restatement of the 'established' facts.

With these premises in mind the following brief comments are intended to place the work reported in the present study in a more general context with regard to the collection of essential information for rational therapy in placental and ovarian dysfunction. The situation at the present time may be summarised as follows:-

1. Biosynthesis of progesterone and effect of gonadotrophins.

a) Non-pregnant women.

During a normal cycle it would appear that up to 30 mg. of progesterone are secreted daily by the combined production of the follicles, corpus luteum and to a lesser extent the adrenal cortex. The secretion rates during the follicular phase and the variation between individuals in health and disease has yet to be determined. The resolution of this problem necessitates the development of more specific methods which may be conveniently performed for the determination of secretion rates.

Tentative evidence has been presented that the largest concentration of enzymes associated with the biosynthesis of progesterone are present in the luteinised granulosa cells of the corpus luteum, but the inter-relationship of these cells with others within the corpus luteum and surrounding tissue is unknown. The subcellular localisation of enzymes responsible for progesterone biosynthesis in ovarian tissue is also as yet unknown.

Little is known with regard to the effect of FSH, LH and luteotrophic hormone on progesterone production. 'In vitro' studies with corpora lutea have suggested that LH promotes the incorporation of labelled acetate into a variety of intermediary compounds (Rice et al., 1964a), but these observations have still to be confirmed 'in vivo'. The differences between the two approaches could be explained by

the differences in production and secretion (Short, 1964).

The mechanism of progesterone secretion has not yet been explored, and the regulatory mechanisms are not clearly defined. However, if all the progesterone produced is immediately secreted then the activity of the rate limiting enzymes in its production could also control secretion. The function of the gonadotrophins is important in this connection, for if they regulate the formation of Δ^5 pregnenolone from cholesterol as is postulated for ACTH in the adrenal cortex, then the gonadotrophins could control the secretion of progesterone. However, in the ovary where the biosynthetically active structures are under constant morphological change, the action of the gonadotrophins may occur in the regulation of the growth of structures which produce the hormones and the actual quantity produced and secreted may be subject to additional factors.

b) Pregnant women.

During pregnancy there is good evidence that the placenta also secretes progesterone and the total production increases until at the end of gestation at least 200 - 300 mg./ 24 hours are secreted. The situation is complicated by the secretion and metabolism of steroids by the developing foetus. As with the ovary the cells involved and the biosynthetic pathways and subcellular localisation of enzymes for progesterone

biosynthesis is still under investigation. The problem as to whether the initiation of labour is the result of a decrease in progesterone production by the placenta is still unresolved.

2. Distribution and concentration in peripheral blood.

After release into the blood stream the progesterone rapidly disappears from the circulation. This appears to be due in the main to rapid reduction and conjugation in the liver (Atherden, 1959), and also to a rapid diffusion into body tissues, particularly into the subcutaneous fat. Furthermore, it has been shown by both Plotz (1961) and Zander (1961) that although the excretion of metabolites starts almost immediately after intravenous injection of the C^{14} labelled hormone, this fact does not significantly account for the disappearance of more than 90% of the dose from the plasma within 25 minutes.

It is apparent that the production, reduction, conjugation, diffusion and rediffusion, determine to a great extent the level of the substance in the blood under both physiological and pathological conditions.

Characteristic changes in the level of plasma progesterone have been shown in the menstrual cycle, and these probably reflect the development, maturation and regression of the corpus luteum. With regard to the progesterone

concentration in peripheral venous plasma of pregnant women, most authors agree that there appears to be a rise especially during labour, but it remains to be determined what factors are responsible for the rise. At the moment it is impossible to draw any definite conclusions with regard to the physiological significance of these changes.

It has been established that progesterone is transported in the blood in association with the albumin fraction of the plasma proteins, but the physiological significance of this phenomenon, apart from increasing the solubility of progesterone is unknown.

3. Concentration at target tissues.

The concentration of progesterone at target tissues is very low and the high rate of production and metabolism may provide the target cells with a slow sustained stimulus.

The myometrial cells do not appear to metabolise progesterone very rapidly, but over a period of time effect small reductions (in vitro) at C 5, C 3 and C 20. The order and significance of these changes is unknown, but it has been postulated that they may represent changes in the molecule during the regulation of certain aspects of intermediary metabolism, or represent an effort by the cells to remove excessive stimulation.

4. Mechanism of action at the molecular level.

Just as the current trend is for chemical determination

rather than biological assay, so chemical explanations are being sought for the diverse biological effects of progesterone. The pure compound has been shown to affect various isolated enzyme systems and has also been observed to decrease P/O ratios in rat liver (Wade and Jones, 1956a,b). This basic effect on energy production could account for many of the observed effects 'in vivo' but remains to be explored in far greater detail.

Another method by which the progesterone molecule might affect metabolic processes is in the provision of oxidised coenzymes during extensive reduction in the peripheral tissues.

Other possibilities include a direct effect on protein synthesis and since all enzymes are proteins this would have far reaching effects on cellular biochemistry. It is probably that far more emphasis will be placed on this important line of research in the future.

Finally, with regard to the basic problem that many forms of therapy are based upon unsupportable hypothesis of absolute progesterone deficiency or relative deficiency associated with defects in biosynthesis or metabolism, it would appear that one of the most difficult criteria to investigate is whether or not the target cells are receiving an adequate supply of hormone in the right form. Furthermore,

since the analysis of gonadal and peripheral venous blood indicates that the regulation of metabolic processes associated with reproduction may depend upon a close balance of numerous related compounds, more precise information about this balance and the factors responsible for its regulation, should lead to important advances in therapy.

ACKNOWLEDGMENTS

I should like to thank Dr. Ian F. Sommerville for his constant help and encouragement, and Professor S. G. Clayton and other surgeons at Queen Charlotte's and Chelsea Hospitals for providing much of the experimental material. I am also grateful to my friends and colleagues in the Endocrine Unit for useful discussion and advice, especially Mr. M. J. Sharples who also prepared the specialised glassware and photographs shown in this thesis.

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Nomenclature.

The trivial names of the principal steroid hormones and their metabolites are well established, and to facilitate reading have been used in the present thesis. However for reference purposes the systematic names are listed below:

Trivial Name.	Systematic Name.
progesterone	pregn-4-ene-3:20-dione
17 OH progesterone	17αhydroxypregn-4-en-3-one
pregnanedione	5β-pregnane-3:20-dione
pregnanolone	5β-pregnan-3α-ol-20-one
pregnanediol	5β-pregnan-3α,20α-diol
pregnenolone	3β-hydroxypregn-5-en-20-one
dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
17 OH pregnenolone	17α-hydroxypregn-5-en-20-one
androstenedione	androst-4-ene-3:20-dione
androstanedione	5β-androstane-3:17-dione
testosterone	17β-hydroxyandrost-4-ene-3-one
oestrone	3-hydroxyoestra-1:3:5(10)-triene-17-one
oestradiol-17	oestra-1:3:5(10)-triene-3:17β-diol
oestriol	oestra-1:3:5(10)-triene-3&6α:17β-triol