THE DEVELOPMENT OF METHODS OF STEROID ANALYSIS IN REPRODUCTIVE PHYSIOLOGY

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A Thesis presented to
The University of Aston in Birmingham
in application
for the Degree of Master of Science
MAY 1971

Thesis 577.17 MOR

22AUG71 141308

The development of methods of steroid analysis in reproductive physiology

Synopsis

The recent development of methods employing competitive protein binding techniques for the estimation of certain hormones has made possible the determinations of circulating levels in the human that were previously impossible.

This thesis has been concerned with the investigation of these methods and their application to problems in reproductive physiology.

Conventional chemical methods and the newer methods have been compared and used for the estimation of steroids in blood and urine during normal menstrual cycles and in infertile patients receiving gonadotrophins.

The joint effect of follicle stimulating hormone and luteinizing hormone has been examined and a new method of detecting a possible hyperstimulation with these hormones is indicated.

A novel relationship between oestrogens and haematological values has been demonstrated and a new method for the estimation of oestrogens by labelling with radioiodine is proposed.

The difficulties involved in competitive protein binding are discussed together with the future applications of the methods.

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INTRODUCTION

Steroids in infertility.

The measurement of the steroid hormones has wide applications in the field of reproductive physiology. In particular these measurements can provide information about the processes of ovulation and pregnancy in health and disease. This thesis is concerned with the estimation of these hormones and their application to endocrine problems, especially in the field of infertility.

The predominant steroid hormones involved in the female reproductive cycle are oestradiol and progesterone. Formation and secretion of these steroids by the ovary is under the control of the gonadotrophic hormones secreted by the anterior pituitary gland. During each cycle a number of ovarian follicles develop under the influence of follicle-stimulating hormone (FSH). Only one matures into a Graafian follicle which is destined to release an ovum, and the others become atretic and regress. During this stage of the cycle, increasing amounts of oestrogens are secreted by the ovary and they may be measured in the urine (Brown, 1954; Brown & Matthew, 1962). At about day 14 an increased secretion of luteinizing hormone (LH)

takes place by the pituitary, the Graafian follicle ruptures and the ovum is released. The ruptured follicle is converted by LH into a corpus luteum which secretes both oestradiol and progesterone and this phase of the cycle is characterised by the presence of increased amounts of the latter steroid in the blood and its metabolite, pregnanediol, in the urine (Klopper, 1957, Loraine, 1958). The corpus luteum slowly diminishes in its activity unless fertilization of the ovum takes place. Chorionic gonadotrophin (CG) produced by the developing placenta is then able to maintain the corpus luteum for a further 10 to 12 weeks. However, if conception does not occur, the uterine endometrium lacking the hormonal support it needs, degenerates and menstruation ensues.

Hypothalamic centres are considered to be responsible for the secretion of releasing factors which control the pattern of the gonadotrophin secretion (Harris, 1961) and these centres are influenced by the concentration of circulating steroids. Although the factors responsible for the release of gonadotrophins have not been identified with certainty they are probably of small molecular weight and contain polyamine groups.

Infertility of hormonal origin is characterized by failure at the hypothalamic, pituitary or ovarian level. Thus, the production of gonadotrophin may be low or the separate hormones may not be released in a cyclic fashion, or the ovaries may fail to produce oestrogen or progesterone. In the latter case the secretion of gonadotrophins may become greater than normal.

Although these processes have been studied, and the postulated regulation mechanisms accepted for many years, advances in knowledge have been limited by the difficulties encountered in isolating and measuring accurately the various hormones involved. During the last few years methods of purifying human pituitary gonadotrophins (HPG) have been developed and their application in the treatment of infertility has led to an increased interest in the normal ovulation mechanisms. New problems have also arisen since the timing, and dose-level of these preparations is critical and they can only be controlled by careful analyses of steroid excretion patterns.

The first group to examine the effects of a fairly crude human gonadotrophin preparation containing FSH was Gemzell,

Diczfalusy and Tillinger (1958). A course of treatment with this preparation was followed by an injection of human chorionic gonadotrophin (HCG). This material, which is available commercially, is extracted from the urine of pregnant women and is biologically similar to LH in its activity. Ovarian steroid production was stimulated in infertile women and the first pregnancy following this treatment was reported (Gemzell, Diczfalusy and Tillinger, 1959). Subsequently a commercial preparation of FSH extracted from the urine of post-menopausal women was used by the Israeli group, Lunenfeld (1963); Lunenfeld and Rabau (1967). Methods of detecting responses were based on palpation of the ovaries, vaginal cytology, examination of cervical mucus and on random steroid analyses. As well as successful single pregnancies, misfortunes occurred including hyperstimulation with varied degrees of severity, cerebral thrombosis and death (Mozes, Bogokowsky, Antebi, Lunenfeld, Rabau, Serr, David and Salomy, 1965). It was apparent that careful control of treatment with FSH was necessary with each patient to ensure that dangerous

hyperstimulation and possible multipregnancies were avoided.

In Birmingham, following the separation of FSH and LH fractions from human pituitaries by Butt, Crooke and Cunningham (1961) treatment of patients began, controlled entirely by steroid analyses (Crooke, Butt, Palmer and Morris, 1962). Statistical designs were used to examine factors such as variation between patients, variation in treatments with HCG and the effects of joint action of FSH and LH or HCG (Crooke, Butt, Palmer, Morris, Edwards and Anson, 1963; Crooke, Butt, Palmer, Bertrand, Carrington, Edwards and Anson, 1964). The wide variation in sensitivity between patients was noted (Crooke, Butt, Bertrand, 1966) and subsequently Crooke, Butt, Bertrand and Morris (1967, a) investigated the within-patient variability. This was done by constructing a series of probability curves at different doses of FSH based on the concept of the ED 50 - the dose, which if repeated would produce a positive response 50% of the time it was given. If the ED 50 of a subject is known the probability of ovulation, of pregnancy or hyperstimulation could be estimated. A response was defined in terms of the rise of urinary

steroid levels.

Work on the effect of administering FSH in the treatment of infertility has continued during recent years (Crooke, Butt, Bertrand and Morris 1967, b): Crooke, Butt, Bertrand and Morris, (1968); Crooke, Bertrand, Butt and Morris, (1970) and the results of these and other workers have been summarised (Crooke, 1970). One of the interesting findings was that a single injection of FSH followed by HCG seven to nine days later could lead to successful ovulation and pregnancy. This suggests that, once initiated the growth of the Graafian follicle may be autonomous. Using this, and other methods involving FSH treatment in Birmingham, 85 healthy babies have been born to date by previously infertile women.

When the original work on the application of FSH was started in Birmingham the effect of ovarian stimulation was assessed retrospectively by estimations of urinary oestrone, oestradiol-17B and oestriol, by the method of Brown (1955). With the limited laboratory facilities available at the time it became apparent that it would be necessary to choose one oestrogen as an index and Palmer (1964) developed a short modified method for the estimation

of oestriol only, based on the original Brown technique. Later, it was considered that treatment could be used to a greater advantage if the ovarian response in a patient could be assessed before each dose of FSH was given. The ovulating dose of HCG could then be delayed until the oestrogen excretion was at a sufficiently high level. This would ensure that an ovulation (as judged by the steroid excretions) took place in every month of treatment. Alternatively, if the rate of oestrogen secretion appeared to be excessive HCG could be withheld and an undesirable hyperstimulation syndrome avoided. A rapid method for the estimation of total oestrogens (Brown, Macnaughton, Smith and Smyth, 1968) was therefore adopted. Using this method patients bringing a 24-hour collection of urine at 9.00 a.m. would have their oestrogen response assessed by early afternoon and the next dosage of FSH could be calculated accordingly. A system of treatment based on this method has recently been described by Brown, Evans, Adey, Taft and Townsend, (1969).

Many workers have used changes in basal body temperature as an indication of progesterone secretion by the corpus

luteum following ovulation. A more quantitative assessment is obtained by the measurement of the excretion of pregnanediol (Klopper, Michie and Brown 1955) and this has been the index used in Birmingham.

The influence of FSH on the production of other steroids is also being studied. Urinary pregnanetriol has been shown to arise from the ovary as well as the adrenal cortex (Fotherby, 1962; Pickett and Kellie, 1962). It is suggested in a summary by Diczfalusy and Palmer (1967) that the increase in urinary 17-hydroxycorticosteroids (which include pregnanetriol) following the administration of FSH to amenorrhoeic women reflects increased formation of the precursor of pregnanetriol viz. 17α-hydroxyprogesterone by the ovary. An increase in urinary androgens following treatment with FSH while the adrenals are suppressed with B-methasone indicates that these steroids or their precursors are also of ovarian origin in certain conditions (Mahesh and Greenblatt, 1961).

It is likely that the examination of other parameters may enlarge our knowledge of the basic endocrine phenomena involved in the reproductive processes. At the moment we have no certain method of knowing without surgery when, or

indeed whether ovulation has taken place unless pregnancy ensues. It is incompletely understood why at certain stages of pregnancy a deficiency in the endocrine system of the foeto-placental unit may occur placing the life of the foetus in jeopardy. A method of detecting this failure by demonstrating hormone changes at an early stage may be valuable. In the field of contraception further work remains to be done regarding the mode of action of the 'pill' and the intra-uterine devices.

Most of the information which has been assembled hitherto on the effects of FSH has been based on urinary excretion patterns of steroids. The estimation of blood steroids has been limited because of the low levels encountered and the practical difficulties of the methods involved. Most of them are too lengthy to be of value if they are required to assess a rapidly changing clinical situation.

The estimation of blood steroids.

The chemical methods for the estimation of progesterone and oestrogen in blood described prior to about 1960, involved the use of colorimetry or fluorimetry, and they were only sufficiently sensitive to measure the levels found during pregnancy. However, extraction and purification procedures devised earlier found application in the later, more sensitive methods.

The chromatographic systems devised by Butt, Morris, Morris and Williams, (1951) in the determination of blood progesterone (by a polarographic technique), for example, are being used in current steroid assay methods. The application of gas chromatography, particularly with the use of the electron capture detector (Van der Molen and Green, 1965) and radioisotope methods, using the formation of a labelled derivative (Riondel, Tait, Tait, Gut and Little, 1965) permits the estimation of progesterone in the male and the non-pregnant female.

Similarly the method for blood oestrogens (Diczfalusy and Magnusson, 1958) which adapted the colorimetric Kober reaction as described by Brown (1955) or the fluorimetric method of Preedy and Aitken (1961) were only suitable for use in pregnancy. Gas chromatography (Wotiz, Charransol and Smith, 1967) and a double isotope method using ^{35}S-pipsyl chloride (Baird 1968) have extended the sensitivity range as well as increasing specificity.

The extremely low levels of testosterone in blood have necessitated the application of isotope methods. Labelling of the steroid with ^{35}S-thiosemicarbazide (Riondel, Tait, Gut, Tait, Joachim and Little 1963) or ^{3}H-acetic anhydride (Hudson, Coghlan, Dulmanis, Wintour and Ekkel, 1963; Burger, Kent and Kellie, 1964; Casey and Kellie, 1964; New, Gross and Peterson, 1968) have been described but extensive purification of the product is necessary to remove the excess label.

Radioisotopic methods are available for the blood

corticoids but for clinical investigations colorimetric and fluorimetric methods have been found useful and these have been summarised by Braunsberg and James (1961) and DeMoor and Steeno (1963). In particular the simple fluorimetric method of Mattingly (1962), although lacking in specificity, is widely used for the determination of plasma 11-hydroxycorticosteroids. The important mineralocorticoid, aldosterone, can only be measured satisfactorily however by preparing an isotopically labelled derivative followed by purification of the resulting material. The method of Kliman and Peterson (1960) for the acetylation of aldosterone using ^{3}H-acetic anhydride is generally accepted and this has been applied in plasma aldosterone methods by Coghlan and Scoggins (1967) and Brodie, Shimizu, Tait and Tait (1967).

All these methods which provide a high degree of sensitivity and specificity are demanding in time, skill and laboratory facilities. Although they will remain as reference procedures, a new concept in chemical methods, the competitive protein binding technique, may make blood steroid assays available for routine clinical use.

The binding of steroids to plasma proteins.

The first demonstration that steroids may circulate in the blood bound to protein was made by Brunelli (1935) when he showed that oestrogen added to serum did not dialyze through a collodion membrane. By a crude fractionation of the serum proteins Brunelli considered that a globulin fraction was responsible for the binding. The binding property of serum proteins was confirmed by Rakoff , Paschis and Cantarow (1943); Szego and Roberts (1946, 1947, 1953 and 1956); and Bischoff and Stauffer (1957), although the latter authors showed that albumin was the protein bound to oestrogen.

The binding of progesterone and other steroids to protein was studied by Westphal (1955, 1957); Westphal and Ashley (1958), and they considered that the interaction of the steroids with protein involved the Δ^4-3-ketone group. A similar finding was made by Oyakawa and Levedahl (1958) when they examined the binding of testosterone to albumin.

Earlier methods of separating the free and protein bound steroids involved one of the following procedures :-

(1) precipitation of proteins with an organic solvent (2) precipitation of proteins by salting out or by organic protein precipitants (3) equilibrium dialysis (4) ultra-filtration or (5) electrophoresis.

It was shown subsequently that when the protein was separated under conditions that could involve denaturation or if the steroid was adsorbed on to a surface misleading results were obtained. Ultra-filtration and dialysis were satisfactory however, and using these methods Sandberg, Slaunwhite & Antoniades (1957) found that labelled oestradiol, testosterone, progesterone, cortisol, corticosterone, and androstenedione were all bound to plasma proteins shortly after these steroids had been intravenously injected. The plasma proteins were fractioned by the Cohn procedure and it was shown that Fraction IV-1 and Fraction V were the most important in the transport of steroids although binding was distributed throughout the other fractions. Similar results were reported by Antoniades, Pennel, Slaunwhite & Sandberg (1957). Oestrone and oestradiol, were particularly associated with albumin (Slaunwhite and Sandberg 1959). These authors suggested

that most hormones are transported in the blood in association with carrier proteins.

The binding of corticosteroids to proteins was extensively studied by Bush (1957), Slaunwhite et al. (1959) and Daughaday (1958). It was demonstrated that the binding of labelled cortisol and corticosterone was critically dependent on the amount of steroid added in the in vitro protein system. When 0.5 μg. of cortisol was added to 10 ml. plasma virtually all the steroid became bound to the protein. On increasing the added cortisol to 10 μg. the amount of steroid bound was reduced to about 60%. The effect was much less evident with testosterone, progesterone and oestrogens, within the limits examined. The principal binding protein in plasma, an α1 globulin, was designated transcortin by Slaunwhite et al, (1959) and cortisol binding globulin (CBG) by Daughaday (1958). It became apparent that the displacement of bound cortisol with added steroid followed a dose-response pattern and with standardized conditions an assay for the steroid could be devised.

The competitive protein binding assay

The first application of the reversible binding properties of proteins to provide an assay method was in the determination of insulin by Yalow and Berson (1960). The binding protein used was an antibody to insulin. At the same time Ekins (1960) introduced a similar method for the measurement of thyroxine and shortly afterwards for Vitamin B-12 (Barakat and Ekins, 1961).

The first application in a steroid assay was made by Murphy, Engelberg and Pattee (1963) in the estimation of plasma corticosteroids. The binding protein used was a solution of human plasma and the tracer was cortisol-^{14}C. Sufficient tracer was added to saturate the binding sites of the protein and then, when the steroid to be measured was added to the mixture, part of the radioactive cortisol was displaced. The percentage of tracer remaining bound to protein thus fell in proportion to the amount of added steroid. The free and bound steroids were separated by dialysis. Subsequently Murphy and Pattee (1964) used a Sephadex column to separate the

two fractions and later Murphy (1967) introduced a simple method of removing the free steroid by adsorption on to inert materials such as Florisil and Fullers earth. This latter paper included a comprehensive survey of protein binding methodology in the estimation of steroids.

In recent years methods have been devised for measuring by competitive binding assay a number of steroids in blood and urine and papers in which full working methods are presented are tabulated. The methods differ in the extraction and purification processes, in the choice of binding protein and in the method of separating free and bound steroids.

One of the interesting recent developments has been the application of binding proteins extracted from tissue cells. Korenman (1968) has prepared a specific oestrogen binding extract from a pregnant rabbit uterus and this has been applied in sensitive methods for blood oestradiol - 17β (Corker and Exley 1969; Korenman, Perrin & McCallum 1969) Receptor-site proteins in the kidney (Edelman & Fimognari 1968) and in toad bladder (Ausiello & Sharp 1968) have been shown to have aldosterone binding activities. Preparation of this type of material may lead to methods with a high degree of sensitivity and specificity.

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of separating free & Bound Steroid
Cortisol in plasma	Murphy et al. (1963)	Deproteinization by heat or extraction with chloroform	Human Plasma (night specimen) 12.5% Cortisol-4-C^{14}	Dialysis
	Murphy et al. (1964)	Ethanol precipitation	Human plasma 2.5% Cortisol-4-C^{14}	Sephadex column
	Murphy (1967)	Ethanol precipitation	Dog plasma 2.5% Cortisosterone-1, 2-T	Florisil
	Nugent et al. (1966)	Dichloromethane extraction	Human plasma (Dexamethasone treatment) 50% Cortisol-4-C^{14} (or Ethinyl Oestradiol treatment 25%)	Dextran -charcoal
	Bowman et al. (1968)	Dichloromethane extraction	Rabbit a-Globulin Cortisol-1,2-T	Fuller's earth

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of separating free & Bound Steroid
11-Deoxy-corticosteroids	Strott (1969) et al.	Dichloromethane extraction Benzene-water Partition	As for Nugent et al. (1966) 11-Deoxycortisol - 1,2-T.	Dextran-Charcoal
Cortico-sterone in plasma	Grad et al. (1968)	Method as described for Cortisol (Murphy 1967)		
Cortisol in urine	Murphy (1968)	Dichloromethane extraction	Human plasma 5.0% Cortisol-1,2-T	Fuller's Earth
	Beardwell et al. (1968)	Dichloromethane extraction	Human plasma 5.0% Cortisol-1,2-T.	Florisil
Oestrogen in blood	Corker et al. (1969)	Ether extraction TLC (1)	Rabbit uterus Oestradiol-6, 7-T.	Dextran-Charcoal
	Korenman et al. (1969)	Ether extraction Celite column	Rabbit uterus Oestradiol-6,7-T.	Dextran-Charcoal

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of separating free & Bound Steroid
Oestrogen in blood	Shutt (1969)	Ethyl Acetate extraction Paper Chromatography(1)	Uterus from ovariectomised ewe Oestradiol-6,7-T.	Dextran-Charcoal or Sephadex column
	Dufau et al. (1970)	Dichloromethane extraction Sephadex column	Human plasma pregnancy treated with charcoal. Applied to Sephadex column with oestradiol-6,7-T. Diluted to 3.3%	Dextran-Charcoal
Progesterone in plasma	Neill et al. (1967)	Petroleum ether extraction TLC (1)	Dog plasma 2.5% Corticosterone-1, 2-T	Florisil
	Johansson et al. (1968)	Petroleum ether extraction TLC (1) or without TLC (rapid method)	Dog Plasma 2.5% Corticosterone-1,2-T.	Florisil
	Yoshimi et al. (1968)	Ether extraction TLC(1) Silica-Alumina column	Human plasma female ovariectomised receiving oestrogen and dexamethasone 1%. Progesterone-1,2-T.	Sephadex Column

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of Separating free & Bound Steroid
Progesterone in plasma	Johansson (1969)	Petroleum ether extraction	Human plasma female treated with ethinyl oestradiol o.05% Corticosterone-1,2-T.	Florisil
	Martin et al. (1970)	Ethyl acetate extraction Paper chromatogram (1)	Dog plasma oestradiol implant 2.5% Corticosterone-1,2-T.	Florisil
	Reeves et al. (1970)	Petroleum ether extraction TLC (1) Ether: water partition	Human plasma ovariectomised women treated with oestrogen and Dexamethazone o.2% Corticosterone-1,2-T.	Florisil
17-Hydroxy-progesterone in plasma	Strott et al. (1968)	Ether extraction TLC (1) Acetylation.TLC(2)	Human plasma female treated with Dexamethasone and Stilboestrol 0.4% 17α-Hydroxy-progesterone-7α-T.	Florisil

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of separating free & Bound Steroid
Testosterone in plasma	Horton et al. (1967)	Ether extraction	Human plasma late pregnancy female receiving Dexamethazone 30% Testosterone-1,2-T	Dextran-Charcoal
	Mayes et al. (1968)	Dichloromethane extraction.Paper chromatogram (1) Alumina column(1) TLC (1) Alumina column (2)	Human plasma late pregnancy 3.0% Testosterone-1,2-T.	Precipitation with ammonium sulphate
	Kato et al. (1968)	Dichloromethane extraction Paper chromatogram (1)	Human plasma late pregnancy, albumen free 2-5% Testosterone -1,2-T.	Sephadex
	Frick et al. (1969)	Ether extraction TLC (1) TLC (2)	Human plasma orchidectomised men treated with stilboestrol 0.5-1.0% Testosterone-1,2-T.	Florisil

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of separating free & Bound Steroid
Testosterone in plasma	Hallberg et al. (1968)	Dichloromethane extraction TLC(1) Paper chromatogram (1)	Human plasma late pregnancy 0.25% Testosterone-1,2-T.	Florisil
	Rosenfield et al.(1969)	Ethyl Ether extraction TLC(1) TLC (2) - redevelopment of (1)	Human plasma late pregnancy 1% Testosterone-1,2-T.	Dextran - Charcoal
	Maeda et al. (1969)	Dichloromethane extraction Paper chromatogram (1)	Human plasma female treated with Oral contraceptive and Dexamethazone. 1% Testosterone-1,2-T.	Ammonium sulphate precipi-tation.
Testosterone in Urine	Rudd et al. (1969)	Hexane extraction TLC (1) Acetylation TLC(2) Saponification	Human plasma late pregnancy 1% Testosterone-1,2-T.	Dextran-Charcoal

Steroid	Reference	Extraction method	Binding Protein Tracer	Method of separating free & Bound Steroid
Androstenedione in plasma	Rosenfeld (1969)	Ether extraction TLC (1) Acetylation (enzymic) Reduction TLC(2)	Human plasma late pregnancy 0.67% Testosterone-1,2-T.	Dextran-Charcoal
	Moshang et al. (1970)	Hexane extraction TLC (1) TLC(2) Reduction (Borohydride) (3)	Human plasma late pregnancy 0.33% Testosterone-1,2-T.	Dextran-Charcoal

Technical aspects of competitive protein binding.

The term competitive protein binding (cpb) applied to a method of assay was introduced by Murphy (1964). It implies the use of a system containing a protein and a steroid for which it has specific binding sites. In the presence of excess steroid all the binding sites on the protein becomes occupied. If this steroid is isotopically labelled, addition of increasing amounts of unlabelled steroid will progressively displace the labelled material, i.e. there is a competition for the binding sites on the protein. The isotopically labelled steroid may be measured and this provides an assay for the unlabelled steroid added.

Competitive protein binding methods involve the following stages:-(1) Extraction and purification of the steroid to be estimated (the ligand) (2) Addition of the binding-protein solution containing the labelled tracer (3) Equilibration (4) Separation of the free and protein bound steroids (5) Radioactive counting of the bound tracer and calculation of ligand.

The procedures are now examined in detail.

1. (a) Preliminary extraction

It is necessary in every method to remove any binding protein in the specimen to be assayed. This may be achieved by heat denaturation, by precipitation of the proteins with ethanol or by partitioning with an immiscible solvent. The latter method is the most widely used. The solvent is then removed

and the extract is submitted to cpb with or without preliminary purification depending on both the specificity of the initial partition and of the protein binding system. It is usually desirable to include a labelled steroid at the outset for assessment of recovery, especially if thin layer, paper or column chromatography is to be used. If these purification processes are to be applied, scrupulous attention to cleanliness of apparatus and purification of solvents is necessary and blanks must be taken through the complete procedure.

1. (b) Choice of binding protein

Human plasma is the usual source of binding protein. Several of the plasma proteins have binding properties but they differ in their capacity and affinity for the ligand (the hormone bound). Albumen has a high capacity but a low affinity for steroids whereas the binding globulins show the reverse effect.

Daughaday (1958) and Slaunwhite et al. (1959) found that undiluted plasma gave a comparatively flat curve when bound cortisol-^{14}C was displaced with increasing amounts of unlabelled cortisol. Dilution of the plasma 1:5 with saline improved the slope of the curve with respect to its application in an assay for cortisol. Dilution apparently reduced the binding to the albumen and the binding to the higher affinity globulins is then

more apparent. Removal of the albumin fraction by salt precipitation or the use of commercially prepared globulin fractions can also lead to improved dose-response curves. The sensitivity of the process increases with increasing dilution, i.e. there is a steeper fall in the percentage tracer bound, but the effective range of added ligand decreases. There is a limit however, to the sensitivity obtainable since with too dilute a protein it becomes impossible to control the separation of the free and bound ligand adequately.

It is desirable to have a minimal concentration of endogenous ligand and this is achieved by treating the donor with a suppressive drug, e.g. dexamethasone. Alternatively, the sample may be taken at the nadir of a circadian rhythm, or after removal of the hormone-producing gland. During pregnancy the concentration of testosterone-binding globulin in the plasma increases considerably (Pearlman, Crepy and Murphy, 1967) and this is made use of in testosterone assays.

The binding affinity for steroids had been investigated with the plasma of other species (Seal and Doe, 1965; Murphy, 1967). Simian plasma has a higher affinity for corticosterone than that from the human while dog plasma has a higher affinity for most steroids compared with human.

2. Choice of Radioactive tracer

Early methods used ^{14}C labelled steroids but with the availability of tritiated steroids with high specific activity more sensitive assays are possible. It is usual to have the same steroid labelled as the unlabelled steroid to be measured. In methods for cortisol and progesterone however, tritiated corticosterone has been used since it is easier to adsorb this unbound tracer on to a small quantity of Florisil. Beardwell et al. (1968) found tritiated cortisol satisfactory, however, in their method for urinary cortisol.

The quantity of tracer present must not be in excess of the amount required to saturate the binding sites. In practice the minimum count is determined by the precision with which a count may be made in a reasonable time. A gross count of 10,000 is usually selected, i.e. a 1% standard counting error ($100\%/\sqrt{\text{count}}$) at 68% confidence.

The plasma is diluted so that a satisfactory curve (λ of the order of 0.2) over the range required is obtained. It should be borne in mind that the initial count of the solution will be reduced by between 10 and 50 per cent when it is treated with the adsorbent in the absence of added ligand.

The most satisfactory method of preparing the protein-tracer mixture is to evaporate a portion of a solution of the

labelled steroid in ethanol (usually 10uCi/ml) to dryness and then add the diluted plasma.

3. Equilibration

The protein-tracer solution (usual volume 1 ml) is added to the dry, solvent-free extracts, standards and blanks. In order to ensure complete solution of the ligand and to achieve equilibration the tubes are incubated with shaking at 45°.

A 2% ethanol-saline solution is used by Beardwell et al. (1968) to ensure that the ligand is in solution before the addition of the protein-tracer.

After incubation the tubes are transferred to an ice-bath at 4°C. The binding affinity of the globulins decreases with increasing temperature and is greatly increased at 4°C (Daughaday, Holloszy and Mariz, (1961). Unsaturated CBG binds all the available cortisol present at 4°C but only 80% at 20°C (Murphy, 1964).

The time of incubation at 45°C and at 4°C do not appear to be critical and between 5 and 10 minutes at each temperature is sufficient.

4. Separation of free and bound steroids

The methods using dialysis or Sephadex columns were slow and not suitable for multiple analyses in clinical work. The methods now applied in the separation of free and bound steroid usually involve the use of Florisil or dextran-coated charcoal.

A fixed amount of the adsorbent is added to the protein-steroid mixture and shaken for a definite time in order to remove the free tracer. The time in contact with Florisil must be rigidly controlled because while the reaction between steroid and protein is reversible, that between steroid and adsorbent is not (Murphy, 1969). Hence the Florisil will progressively strip off the tracer from the protein with a resulting diminution of the protein-bound count. It is necessary to wash the Florisil carefully before use to remove fines.

Charcoal is a general adsorbent and is able to bind both steroids and polypeptides. The larger molecules are excluded however, by coating the charcoal particles with dextran. Charcoal-dextran is added as a suspension in water or buffer and is easily dispensed reproducibly. A disadvantage is that it increases the total reaction volume and also, when there is poor protein affinity the irreversible adsorption is difficult to control.

Other adsorbents which, so far have had limited application, are ion exchange resins, Füller's earth and Lloyd's reagent. Precipitation of the protein with ammonium sulphate has also been applied.

It would be useful to be able to count both the protein bound and free labelled steroids, but it is usually found convenient to count only the bound portion.

5. Radioactive counting and calculation

A measured portion of the aqueous phase containing the protein-bound tracer is transferred to a counting vial together with a suitable scintillation fluid.

The results may be plotted as counts against amount of standard or as percentage of tracer bound against standard. A convenient method is to plot time to reach a predetermined count against standard concentration. The shape of the curve varies but the latter method usually gives a straight line with a positive slope over the usable portion. Some curves may be linearised by plotting the counts against the logarithm of the standard values.

Steroids that may be assayed using human plasma as the binding protein source.

A table showing the relative binding affinities of steroid to human plasma protein is given by Murphy (1969). Cortisol shows the greatest binding affinity. Absence of the C11, C17 and C21 hydroxyl groups as in progesterone reduces the binding by 50% when the two steroids are compared using 10% plasma. Removal of the side-chain at C17 as in testosterone still further reduces activity and an unsaturated ring as in oestradiol reduces the binding to less than 1% that of cortisol.

MATERIALS AND METHODS

Full details of methods are described when modifications to published procedures have been introduced. Details are also given of the competitive protein binding methods which are relevant to this thesis.

Preparation of Gonadotrophic Hormones

Preparations containing follicle stimulating hormone (FSH) were extracted from human pituitaries preserved in acetone. The initial extraction procedure was that described by Steelman, Segaloff and Mays, (1958) and Steelman, Segaloff and Anderson (1959). Purification was carried out by chromatography on carboxymethyl cellulose. (Butt, Crooke and Cunningham, 1961) and on DEAE cellulose (Butt, 1967).

Methods of assay

The FSH activity of the preparations was measured by the method of Steelman and Pohley (1953) modified by Brown P.S. (1955). The assay depends on the increase in weight of mouse ovaries *in vivo* when preparations containing FSH are administered together with an excess of luteinizing hormone (LH).

The LH activity of the preparations was assayed by the method of Parlow (1961).

The method depends on the depletion of ascorbic

acid in the ovaries of pseudo pregnant rats in vivo when the animals are treated with preparations containing LH.

Standard.

The reference standard (2nd. IRP-HMG) was prepared from Human Menopausal Gonadrotrophin by Serono, Rome (Pergonal 23). One ampoule contains an amount of standard defined as equivalent to 40 iu FSH and 40 iu LH.

Activity of preparations.

Two preparations were used. One contained FSH and LH in the ratio of approximately 1:1 and the other was richer in FSH the ratio being approximately 4:1. The preparations were stored as dry powders at 4°C. When required for injection they were dissolved in a sterile solution containing 0.5% gelatine and 0.01% chlorocresel to give a solution containing 1000 iu FSH/ml.

One human pituitary yielded approximately 100 iu FSH. On average 1500 iu were required to induce ovulation and 1750 iu to achieve pregnancy i.e. 17-18 pituitary glands.

Commercial preparations

A commercial preparation of gonadotrophin was also used. This material, (Humegon) was prepared from human menopausal urine by Organon Laboratories Ltd. and supplied in ampoules containing 75 iu. FSH. The ratio of FSH to LH was about 1:3.

Human chorionic gonadotrophin (HCG) was prepared from pregnancy urine (Paines and Byrne Ltd) and supplied in ampoules containing 5000 i.u.

The subjects

The patients and their husbands were examined in the Endocrine Clinic to assess the cause of their infertility. When it had been established that a patient would benefit from treatment with FSH preparations a course of injections was initiated.

On each visit to the clinic the subject brought a 24 hour sample of urine (last collection about 8.00 a.m. that morning) which was analysed for total oestrogen content by the method of Brown et al. (1968). Blood samples were also taken for the estimation of steroids and for haematological investigations, in all cases between 9.00 a.m. and 10.00 a.m. Attendances were separated by three to four days intervals. On the first two visits of a particular cycle a predetermined injection of human pituitary FSH was given. On subsequent visits the dose was adjusted according to the total oestrogen level of the 24 hour sample brought in that morning, the oestrogen estimation having been completed that afternoon. When an oestrogen level at least 35 µg per 24 hours higher than the initial value was attained an injection of HCG was given two or three days later to induce ovulation. Subsequent urine samples were analysed for pregnanediol by the method of Klopper et al.(1955)

to give evidence whether or not ovulation had been followed by a progesterone-secreting corpus luteum.

A new cycle of treatment was started eighteen days after the HCG injection providing there was evidence that conception had not occurred viz. menstruation, or a return to the basal steroid levels.

The doses of FSH, which varied considerably from patient to patient, were carefully adjusted with the aim of producing as nearly as possible, the steroid excretion pattern seen in the normal cycle. Following conception, urine was collected at intervals in order to assess, from the steroid excretion values, the well-being of the foetus.

Other experimental designs.

When different methods of administering FSH and LH were being investigated the sensitivity of each member of a group of subjects was first established by a method similar to that described above. The total dose of FSH required per month by each subject in order to cause ovulation was found. A factorial design was then made so that several factors e.g. timing of treatment with FSH or HCG or the effect of combining FSH with HCG could be compared independently. The responses to these treatments were usually assessed by determinations of oestriol and pregnanediol

on 24 hour or 48 hour collections of urine.

Estimation of oestriol in urine.

The method of Palmer (1964) was used. The urine (100 ml) was hydrolysed by boiling with hydrochloric acid and then extracted with diethyl ether. The extract was washed with buffer pH10.5 and then the oestrogens were extracted into sodium hydroxide solution. Methylation was carried out by the addition of dimethyl sulphate and the oestrogen methyl ethers were separated chromatographically on an alumina column. The Kober reaction was carried out on the oestriol methyl ether fraction and the colour produced was measured spectrophotometrically after extraction into chloroform.

Total oestrogens in urine.

The method of Brown et al. (1968) was applied. The urine (2 ml) was hydrolysed with acid and extracted with diethyl ether. After purification of the extract by washing with buffer pH 10.5 petroleum ether was added and the oestrogens were extracted from the diethyl ether - petroleum ether mixture with sodium hydroxide solution. The alkaline extract was partially neutralised with sodium bicarbonate and the oestrogens were re-extracted into diethyl ether. The solvent was removed and the Kober reaction was

carried out on the residue. The product was extracted with tetrachloroethane containing p - nitrophenol and measured fluorimetrically.

Pregnanediol in urine.

Pregnanediol was measured by the method of Klopper et al. (1955). One fortieth of the 24 hour specimen of urine was simultaneously hydrolysed with acid and extracted with toluene. The toluene extract was purified by shaking with sodium hydroxide and potassium permanganate solutions and then applied to a column of alumina. The pregnanediol fraction was acetylated with acetyl chloride and then rechromatographed on a second alumina column. The solvent was removed from the fraction containing pregnanediol diacetate and the chromogen formed after the addition of sulphuric was measured colorimetrically.

Cortisol in plasma by fluorimetry.

The method of Mattingly (1962) was used for the estimation of plasma cortisol. Volumes were modified so that smaller quantities of plasma could be used.

Reagents.

Methylene chloride (BDH) was washed with sulphuric acid and water before distillation.

Ethanol-sulphuric acid. To 2.5 volumes of ethanol was added 7.5 volumes of sulphuric acid cooled in ice. The addition of the acid was made slowly to avoid excessive generation of heat. The reagent was stored for up to one week at $4^{\circ}C$.

Cortisol standard. A solution containing 1 mg/100ml. ethanol was prepared from pure cortisol (Koch-Light). This was diluted with water to give a solution containing 20μg/100ml. for plasma determinations.

Fluorimetric readings. An Aminco-Bowman S.P.F. was used with slit sequence No.3. A Wratten (Kodak 2) yellow correction filter was inserted in the light path. A solution of quinine sulphate in dilute sulphuric acid was used to standardise the instrument.

Method.

To 1ml. of plasma in a stoppered tube was added 5 ml. of methylene chloride. The tube was shaken gently for 1 minute and then allowed to stand until the two phases had separated. The supernatant plasma and precipitated protein were removed and 2 ml. of the methylene chloride extract were transferred

to a special fluorimeter tube containing 1ml of ethanol-sulphuric acid. The tube was stoppered, shaken vigorously for 30 seconds, and then allowed to stand for 12 minutes exactly. The fluorescence of the lower layer was read at an activation wave length of 470 mu and an emission wave length of 520 mu. A blank (1 ml. water) and a standard consisting of 0.2ug of cortisol in 1 ml. water were treated identically to the plasma. The reading of the blank was subtracted from those of the standard and test, and the plasma cortisol was then calculated by direct comparison with the standard.

17-Hydroxycortiosteroids in urine-separation into 11-deoxy and 11-oxycorticosteroids.

The method of separation by chromatography on silica gel was described by Morris (1959). The differential solvent extraction procedure was that of Few (1968). Oxidation and reduction procedures were the modifications of Metcalfe (1963).

Reagents

Solvents were A.R. quality and were distilled 15% $^{w}/v$ potassium borohydride (A.R. B.D.H.). solution in 0.1 N-sodium hydroxide was prepared daily. 10% $^{w}/v$ sodium metaperiodate solution was prepared with freshly distilled water.

Standard dehydroepiandrosterone (D.H.A. - B.D.H.) A solution

of 50 mg/100 ml ethanol was stored at 4°C.

Zimmermann reagents(a) A 1.0% w/v m-dinitrobenzene solution in ethanol was prepared weekly.(b) Aqueous benzyl trimethyl ammonium hydroxide (40% v/v) (Hopkin and Williams).

Silica gel 100/200 mesh - Davison (Koch-Light). Water was added to the gel to give a concentration of approximately 7.0%. Standardisation was then carried out with pure aetiocholanolone (Koch-Light). A solution of 50 ug. of this steroid in 20 ml. 25% v/v ethyl acetate in petroleum ether was applied to a 10 x 1 cm. chromatographic column containing 2g. of the gel suspended in the same solvent. Aetiocholanolone was eluted from the column with 20 ml. 35% v/v ethyl acetate in petroleum ether and the Zimmermann reaction was applied to the residue after removal of the solvent. When the gel had been correctly standardised at least 95% of the steroid added to the column was eluted in the 35% ethyl acetate/petroleum ether fraction.

Method

The pH of the urine was adjusted to between 7 and 8 and then 25 ml was transferred to a stoppered tube together with a drop of octanol and 2.5 ml of 15% w/v potassium borohydride in 0.1 N-sodium hydroxide. After 45 minutes at 55°C, 2.5 ml of 4 N-acetic acid was added and incubation was continued for a further 2 minutes.

A 10% $^{w}/v$ solution of sodium metaperiodate (10 ml) was added and incubation at 55°C was continued for 15 minutes after which 2.5 ml. of 5 N-sodium hydroxide was added.

One of the following two separation procedures was then applied:-

(a) Chromatography on silica gel (Morris, 1959)

After the addition of 5 N-sodium hydroxide the urine was extracted with 30 ml of ethylene chloride. The upper layer was discarded and the extract was filtered on a Whatman no. 1 paper. A 20 ml portion of the filtrate was evaporated to dryness, redissolved in 20 ml of 25% $^{v}/v$ ethyl acdate and then applied to a 10 x 1 cm chromatographic column containing 2g silica gel suspended in the same solvent. The extract was allowed to pass through the gel and the eluate was discarded. The 11-deoxy-17-oxosteroids were eluted with 20 ml of 35% $^{v}/v$ ethyl acetate in petroleum ether and the 11-oxy-17-oxosteroids were eluted in 20 ml 65% $^{v}/v$ ethyl acetate in petroleum ether. The eluates were evaporated to dryness and the Zimmermann reaction was applied to the residues.

(b) Differential solvent extraction (Few, 1968).

After the addition of 5 N-sodium hydroxide the urine was transferred to a separating funnel and extracted first with 100 ml isopentane and then with 100 ml diethyl ether. The urine was

discarded and the two separate extracts were washed once with 10 ml of 5% w/v sodium dithionite in 2 N-sodium hydroxide and 3 times with 10 ml portions of water.

The isopentane extract contained the 11-deoxy-17-oxosteroids and the diethyl ether extract contained the 11-oxy-17-oxosteroids. They were evaporated to dryness and the Zimmermann reaction was carried out on the residues.

The 11-deoxy fraction gave a measure of the 11-deoxy-17-hydroxycorticosteroids derived mainly from pregnanetriol and the 11-oxy-fraction represented the 11-oxy-17-hydroxycorticosteroids derived from the metabolites of cortisol.

Zimmermann reaction. The method of James and de Jong (1961) was used with pure D.H.A. (50 ug) as standard. To the dry residues, the standard, and a blank tube was added 0.4 ml of 1% w/v m-dinitrobenzene in ethanol and 0.2 ml of 40% v/v benzyl trimethyl ammonium hydroxide in water. After 1 hour at room temperature 2 ml of 30% v/v ethanol in water was added to each tube followed by 5 ml diethyl ether. The chromogen was extracted into the ether and was read against the blank at 515 mu. No correction was applied for the difference in molecular weights between the extracted 17-oxosteroids and the standard but the 11-oxy-17-oxosteroid fractions were multiplied by 1.33 to correct for the lower chromogenicity of these relative to the standard.

Cortisol in urine by CPB

Free cortisol in urine was measured by the method of Beardwell, Burke & Cope (1968).

Reagents

Cortisol - 1,2-T (S.A. 32 Ci/mM Radiochemical Centre, Amersham) was diluted with ethanol to give a solution containing 10 μC/ml. and stored at 4°C.

Dichloromethane BDH was shaken with sulphuric acid, washed with water and distilled.

2% $^{v}/v$ ethanol in 0.9% saline

Florisil BDH. 60-100 U.S. mesh was washed repeatedly with water until the finer particles had been removed and then dried overnight at 120°C.

Cortisol standard. A stock solution was prepared from cortisol (Koch-Light) 1 mg./100 ml in ethanol.

Serum or plasma for CBG was collected from normal subjects and stored in small aliquots at -20°C.

Preparation of CBG.0.5 ml. of the cortisol-1,2-T solution 10 μC/ml. was evaporated to dryness and 100 ml of water was added followed by 5.0 ml. serum. The mixture was allowed to stand at room temperature 1 hour before use. A fresh preparation was made up prior to each set of analyses.

Scintillation fluid. 2.5 g PPO and 0.05 g dimethyl POPOP were dissolved in 1 litre of toluene.

Radioactive counting was carried out with a Packard Tri-Carb Model 3375 Liquid Scintillation Spectrometer (Efficiency for Tritium = 63%).

Method

To 2ml urine in a stoppered tube was added 10 ml of methylene chloride. The tube was shaken for 1 minute and then allowed to stand to allow the two phases to separate. The aqueous layer was removed and 3 ml of the extract was transferred to a tube for competitive protein binding. The solvent was evaporated in ~~by~~ a current of nitrogen after placing the tube in a water-bath at 35°C and the residue was dissolved in 1 ml. of 2% $^{v}/v$ ethanol in 0.9% $^{w}/v$ sodium chloride solution.

A series of 14 standards were prepared. Tubes 1 to 4 contained 1 ml. of the ethanol-saline solution and tubes 5 to 14 contained 5,10, 20, 30 and 40 ng cortisol in duplicate, dissolved in the same solvent.

To the urine extract and the standards was added 1 ml. of CBG solution and the tubes were placed in a water-bath at 45°C for 5 minutes. The tubes were then transferred to a water-bath at 4°C and allowed to equilibrate for 10 minutes. At the end of this time 80 mg Florisil was added and each tube was shaken for 2 minutes.

The tubes were returned to the cold bath and the Florisil allowed to settle. The procedure was repeated for all the tubes except numbers 1 and 2 of the standard curve. After 10 minutes in the ice bath 0. 5 ml. of the supernatant solution was transferred to a counting vial containing 10 ml. of scintillation fluid. The vials were placed in the Packard TriCarb counter and the time required to attain a total of 10,000 counts was noted.

A curve was drawn showing these times against the amount of cortisol in each standard tube. The amount of cortisol in the urine extracts was then found by reference to this curve.

Cortisol in plasma by CPB.

Reagents. The reagents described in the method for cortisol in urine were used.

Method.

To 1 ml. of plasma in a stoppered tune was added 5 ml.of methylene chloride. The tube was shaken gently for 1 minute and then allowed to stand until the two phases had separated. The supernatant plasma and protein were removed and 0.5 ml. of the extract (equivalent to 0.1 ml. of plasma) was transferred to conical centrifuge tube for competitive protein bimding assay, exactly as described for the assay of cortisol in urine.

Plasma oestradiol-17β by competitive binding

The method of Corker and Exley (1970) was used.

Reagents.

Ethyl Acetate.

Nanograde - Mallinckrodt (M & B).

Diethyl Ether.

Peroxide free (M & B) was shaken with saturated ferrous sulphate and then washed with water before distillation.

Ethyl Alcohol.

(Burroughs) was refluxed either with calcium hydride or 2.4 dinitrophenyl hydrazine and then distilled.

Tris buffer pH 8.0

A solution containing 2.42g (0.01 M) Tris, 170g. (0.25 M) sucrose, and 0.744g EDTA disodium salt was made up to 2 litres with freshly distilled deionised water after adjustment of the pH to 8.0 with N-hydrochloric acid.

Oestradiol-17β, 6, 7-T.

S.A. 30.2 Ci/m M (Radiochemical Centre, Amersham) was diluted with ethanol to give a solution containing 10 uCi/ml.

Alumina.

G.F. 254 Type 'E' (Merck, Darmstatt, Germany)

Thin layer plates were prepared using a mixture 60g of alumina

and 65 ml water. A 0.25 mm.thickness was spread and the plates were activated at 100° for 1 hour before use.

TLC and Solvents.

A mixture of benzene (Nanograde) 200 volumes, and methyl alchohol (AR. B.D.H. Redistilled) 10 volumes were used to develop the plates. Unlined tanks were used.

Dextran-Charcoal.

0.005g Dextran T40 (Pharmacia) and 0.5g Charcoal (Acid washed BDH) were shaken with 200 ml tris buffer. The mixture was stable for several weeks at 4°.

Glassware.

New glassware was soaked in chromic acid and washed in 50% aqueous methanol. After drying the glass was silanized with a solution of 5% dimethyl dichlorosilane (BDH) in toluene. Subseqnently the glassware was soaked in 2% Decon before washing in tap water, distilled water and ethanol.

Binding Protein.

The method of Korenman (1968) was used for preparation of binding protein.

Pseudo-pregnancy was induced in rabbits by injecting 50 units

of chorionic gonadotrophin (Pregnyl, Organon, Oss,) intravenously. After six days the uteri were dissected and homogenized at 4°C in 3 volumes of Tris buffer. After centrifugation for 15 minutes at 5,000g, the supernatants were separated and re-centrifuged at 94,000g for 90 minutes, and then freeze-dried in small aliquots. The protein was stored at -20°C. When required for use it was reconstituted with glass-distilled water.

Extraction

To 1 ml. of plasma was added 50 μl of the solution of oestradiol-17B, 6, 7-T in ethanol (approximately 800 cpm) and 10 ml. of diethyl ether. After shaking the tubes for 5 minutes they were centrifuged. The ether extract was filtered through sodium sulphate supported on cotton wool, and then evaporated to dryness under nitrogen. A blank consisting of 1 ml. of water instead of plasma was treated identically. The residues were applied to a TLC plate and developed in the benzene-methanol (20:1) system. The oestradiol-17B was located and areas corresponding to the marker were scraped off and eluted

by shaking the powder with 3 ml ethyl acetate for 1 to 2 minutes and allowing to stand for 30 minutes, after which 100 μl water was added. After shaking and centrifuging 0.5 ml. ethyl acetate was removed and evaporated to dryness under nitrogen, in a counting vial. To the residue was added 10 ml scintillating fluid and a count was made to assess the recovery. A 2 ml. portion of the ethyl acetate extract was evaporated for protein binding assay.

Standard Curve.

To six standard tubes was added 2 ml. of ethyl acetate and 320 cpm of oestradiol-17B-6,7-T to simulate the average counts in the sample tubes prior to assay.

Tube 1 was blank and tubes 2-6 contained 20, 50, 100, 200 and 300 pg unlabelled oestradiol-17B. The solvents were evaporated and the residues were treated as the samples in the protein binding assay.

Protein binding assay

To all the tubes was added 0.2 ml. of Tris buffer containing approximately 5,500 cpm oestradiol-17B, 6,7-T. The tubes were

shaken, placed in an ice bath until cold and then 50 μl of cold uterine protein was added. After equilibration at 4°C for between 3 and 20 hours, 1 ml of dextran-charcoal suspension was added, and after shaking, the tubes were re-equilibrated at 4°C for 10 minutes. The tubes were centrifuged at 2,500 rpm for 10 minutes and 1 ml of supernatant was transferred to a scintillation vial for counting.

A calibration curve was constructed from the results obtained with the standards. The unknowns were calculated from the curve, corrected for recovery and expressed as ng/100 ml of plasma.

Estimation of oestriol in plasma during pregnancy by competitive protein binding

The method of Corker (1970) was used.

Reagents.

In addition to those described for the estimation of oestradiol-17B the following reagents were used.

Radioactive oestriol

Oestriol - 6, 7-T (S.A. 2.5 curies/mM) (Radiochemical Centre, Amersham), was dissolved in ethanol and stored at 4°C at a concentration of 10 uC/ml.

Recovery Solution.

A 0.8 ml portion of the tritiated oestriol solution (10μCi/ml)

was diluted to 100 ml with ethanol.

Benzene-petroleum ether mixture.

Benzene A.R. (B.D.H.) and petroleum ether A.R. (BDH)b.pt. range 60-80°C were shaken with sulphuric acid, washed with water and distilled. Equal volumes of the two solvents were mixed.

Standard oestriol solution.

Stock oestriol (Koch-Light) contained 1mg/10ml ethanol. A working standard contained 10 ng/ml.

Method.

To 0.2 ml plasma was added 1 ml of 18% hydrochloric acid. The tube was stoppered and placed in boiling water for 1 hour. After cooling 50 μl of the tritiated oestriol recovery solution (approximately 3000 cpm) was added followed by 10 ml. ether. The tube was shaken for 5 minutes and then centrifuged at 2500 r.p.m. for 5 minutes. A 2 ml. portion of the ether extract was evaporated to dryness and 1 ml. of water was added followed by 1 ml. of the benzene-petroleum ether mixture. After shaking and centrifuging the supernatatant solvent mixture was removed. The benzene-petroleum ether wash was repeated and then 0.5 ml. of the aqueous phase was transferred to a counting vial to assess the recovery of oestriol. A 0.1 ml. portion was transferred to a small tube for the protein binding assay.

A blank consisting of 0.2 ml. water instead of plasma was also taken through the procedure.

Standard Curve.

Six tubes were numbered for the standard curve. Tube 1 was blank and tubes 2 - 5 contained 80, 160, 280, 400 and 800 p.g. oestriol. The solvents were evaporated to dryness and 0.1 ml. water was added to each tube.

Protein binding assay.

To all the tubes was added 0.2 ml. of tritiated oestradiol-17B tris buffer and 0.05 ml. of binding protein solution. After 3 hours at 4°C, 1 ml. of dextran-charcoal suspension was added and incubation was continued for a further 10 minutes.

The tubes were centrifuged and 1 ml. of supernatant fluid was transferred to a counting vial. Counting and recovery correction were carried out as in the method for the determination of oestradiol-17B.

Progesterone in Plasma by C.P.B.

The method of Johanssen (1969) was used.

Corticosterone - 1,2-T

(S.A. 39.1.Ci/m M Radiochemical Centre, Amersham) was diluted with ethanol to give a solution containing 10 μ Ci/ml. and stored at 4°C.

Petroleum ether A.R. BDH

Boiling point range 30 - 60°C. was shaken with sulphuric acid, washed with water and distilled.

Florisil.

(60-100 mesh) (BDH) was washed repeatedly until the finer particles were removed. The washed material was dried overnight at 120°C.

Dog plasma

Blood was collected in Heparin from untreated dogs. The plasma was separated and stored in small aliquots at 4°C.

Progesterone standard.

A solution of crystalline progesterone (BDH) 1 mg/10 ml in ethanol was stored at 4°C. A working standard 50 ng/ml was prepared from this.

Protein binding solution

A 0.5 ml. portion of the corticosterone-1,2-T solution (10 μC/ml) was evaporated to dryness under nitrogen and dissolved in 100 ml. of glass distilled water containing 0.5 ml. of dog plasma. The solution was allowed to stand for one hour at room temperature or overnight at 4° before use.

Method

The plasma (0.25 ml) was extracted with 2.5 ml of petroleum ether in a stoppered tube by application to a vortex mixer for 1 minute. After centrifuging 2 ml. of the supernatant extract was transferred to a conical centrifuge tube and evaporated to dryness in a stream of nitrogen.

A set of 12 tubes were numbered for use in the preparation of a standard curve. Tubes 1 to 4 contained no progesterone, tubes 5 to 12 contained 0.5, 1, 2 and 4 ng progesterone in duplicate.

To the dry plasma extract was added 1 ml. of binding protein solution and the tube was incubated for 10 minutes at 40°C. The tube was applied to the mixer for 30 seconds and then transferred to an ice bath for 10 minutes.

Florisil (80 mg) was added, the tube was applied to the mixer for exactly 30 seconds and then returned to the ice-bath.

The Florisil settled out rapidly and within 15 seconds 0.5 ml. of the supernatant fluid was transferred to a counting vial containing 10 ml. scintillation fluid. The standard tubes were treated similarly with CBG and all except tubes 1 and 2 were shaken with Florisil.

A curve was drawn showing the time to reach a total of 10,000 counts for each concentration of standard. The concentration of progesterone in the plasma extract was calculated from the curve.

The procedure was identical when monkey and baboon plasma were examined except that the 0.5 ml. sampled were extracted with 5 ml. petroleum ether and 4 ml. of the extract was evaporated for the protein binding assay.

EXPERIMENTAL AND RESULTS

Examination of methods for the estimation of 11-hydroxycorticosteroids in blood and urine

The method of Mattingly (1962) is widely used for the estimation of plasma 11-hydroxycorticosteroids. The plasma sample is extracted with methylene chloride and this is then re-extracted with a mixture of sulphuric acid and ethanol. The fluorescence of the ethanolic-sulphuric acid is measured after a period of development. Cortisol and corticosterone are the principal fluorogens present but other substances contribute significantly to the total fluorescence. Spencer-Peet, Daly and Vivien Smith (1965) showed that whereas pure cortisol and corticosterone give a constant fluorescence between 8 and 20 min. after extraction into ethanolic sulphuric acid, extracts from plasma show an increase during the same period. They suggested that a measurement of the rate of increase of fluorescence in plasma could be used to measure the stable component and thus increase the specificity with respect to cortisol and corticosterone.

This rate of increase was measured in a series of plasma samples and the results after correction have been compared with those obtained by a single measurement of the fluorescence. The extraction procedure was investigated in order to find the minimum

volume of solvent for the extraction of maximum fluorescence. The effect of varying the composition of the fluorescence reagent was also examined.

A simple competitive protein binding method for the estimation of cortisol in urine has been described by Beardwell et al. (1968). This method was now applied to the estimation of plasma cortisol and the results compared with those obtained by fluorimetry.

It was required to find whether treatment with FSH influenced the level of circulating adrenal steroids. The estimation of cortisol was therefore applied.

Haematological investigations were also carried out on the same blood specimens.

Experimental

Fluorescence was measured in an Aminco-Bowman Spectrofluorimeter (SPF). It was found convenient to use the fluorimetric cells described by Brown et al. (1968) in the estimation of oestrogens since it was possible to extract the fluorogens from a methylene chloride extract into ethanolic-sulphuric acid and measure the fluorescence in the same tube. The fluorimetry readings were increased three-fold by the use of cell mirrors (Aminco-Bowman) inserted in the cell compartment facing the activation and emission beams.

Investigations were carried out on pooled plasma, on samples received routinely for the estimation of 11-hydroxycorticosteroids

and on plasma samples from patients receiving FSH therapy.

The fluorimetric and cpb methods were compared.

1) Investigation of optimum relative volumes of solvent and plasma

All estimations were carried out in duplicate.

To 1 ml. samples of pooled plasma in stoppered tubes were added volumes of methylene chloride between 4ml. and 8ml. The tubes were shaken for 1 min., the supernatant plasma was removed and 2 ml. of the methylene chloride extract was transferred to a fluorimetric tube containing 1 ml. of ethanol-sulphuric acid. The tube was stoppered and shaken vigorously for 15 seconds after which the two phases were allowed to separate. The fluorescence was read after 12 min. at an activation wavelength of 470 mu and an emission wavelength of 530 mu. A standard containing 20 ug. cortisol in 100 ml. water and blank (1 ml. water) were treated identically with each plasma sample and, after subtracting the blank reading from the test and standard the 11-hydroxycorticosteroid concentration of the plasma was calculated.

The results are shown in Table 1 where it is seen that the optimum volume of methylene chloride to extract 1 ml. plasma was 5 ml. No further increase in the amount of fluorogens was observed on increasing the volume of methylene chloride up to 8 ml.

The experiment was repeated using a mixed urine sample from fresh specimens received for the estimation of free 11-hydroxy-corticosteroids. These results are also shown in Table 1.The optimum

volume of methylene chloride for 1 ml. urine was 8 ml.

2) Investigation of composition of fluorescence reagent

Mixtures of ethanol and sulphuric acid were prepared containing between 65% $^{v}/v$ and 80% $^{v}/v$ of the acid. The reaction was carried out as in (1) using 2 ml. samples of a methylene chloride extract of pooled plasma and 1 ml. of the respective fluorescence reagents. Fluorimetric readings were taken at 10 min. and the plasma 11-hydroxycorticosteroid was calculated. The results (Table 2) show that similar values are obtained with all the concentrations of acid examined up to 75%. However, it was noted that the rates of development varied, being particularly slow with the lowest acid concentration and if this was used it would be necessary to take particular account of the time of reading of the fluorescence. The concentration of acid giving the most stable fluorescence between 0 and 20 mins. was 75% and this was chosen for subsequent work.

3) Investigation of time of development of fluorescence.

Specimens of plasma (1 ml.) from individual subjects were extracted with 5 ml. methylene chloride and 2 ml. of the extract was treated with 1 ml. of ethanol-sulphuric acid. The fluorescence of each sample was read after 8 min. and again after 16 min. as recommended by Spencer-Peet et al. (1965). After subtraction of the blank reading from the standard and plasma readings the concentration of 11-hydroxycorticosteroids in μg/100 ml. was given by the formula:-

$$\frac{(\text{F. of test at 8 min.} \times 2) - (\text{F. of test at 16 min.})}{(\text{F. of standard})} \times (\text{Concentration of std. in } \mu g/100 \text{ ml.})$$

(Where F. = Fluorimetric reading).

The formula derives an extrapolated value that would be obtained by a fluorimetric reading at 0 min. and should represent the cortisol and corticosterone fraction only, since the fluorescence of these components does not increase with time.

The results obtained from 150 plasma samples are shown in Table 3. The mean values over 6 ranges between 0 and 70 μg/100 ml. obtained from readings taken at 0 min. and at 16 min. are compared. It may be seen that with the 148 samples in the ranges 0-50 μg/100 ml. there is a difference between the 0 min. and the 8 min. reading of between 0.9 and 1.8 μg/100 ml. (Mean 1.2 μg/100 ml.) irrespective of the level of 11-hydroxycorticosteroids. Between the 0 and 16 min. readings there is a difference of between 1.1 and 3.3 μg/100 ml. (Mean 2.6 μg/100 ml.).

It was decided therefore that, for use in examining possible changes in steroid level (e.g. during FSH treatment) a single reading at 12 min. as suggested by Mattingly (1962) would be satisfactory and the application of the correction formula was not necessary. It was noted however, that values of 5 μg/100 ml. or less would be at least 40% too high if a single reading of fluorescence is accepted.

Results obtained by fluorimetry

The precision of the fluorimetric method adopted was 1.05 μg/100 ml. with a co-efficient of variation of 8.8% in the range 0 - 25 μg/100 ml. (n = 150).

The reagent blank was 1.0 μg /100 ml. and the mean recovery was 94%.

The plasma 11-hydroxycorticosteroid levels were measured fluorimetrically in 12 patients while they were receiving FSH treatment. The total oestrogen content of their urine in the 24 hours immediately prior to the blood collection was also measured and the results are shown in the Table 4.

Part of the blood sample collected for steroid analysis was used for haematological investigations.

All the plasma 11-hydroxycorticosteroid levels with the exception of those from one patient were within the normal range (7-26 μg/100 ml) and they were not correlated ($P>0.2$) to changing total oestrogen levels. There was no correlation ($P>0.2$) between 11-hydroxycorticosteroid levels and the total leukocyte, neutrophil and monocyte counts carried out on the same samples. However, a highly significant ($P<0.001$) negative correlation was demonstrated between plasma 11-hydroxycorticosteroids and the lymphocyte counts. These results have been accepted for publication (Cruickshank, Morris, Butt and Crooke, 1970).

4) Plasma cortisol by competitive protein binding.

Standard curves were prepared by first diluting a cortisol solution containing 1 mg/100 ml. 1 in 100 with 2% ethanol/saline.

Working standards were then prepared by diluting this solution.

1) 0.5 ml. to 10 ml. ethanol-saline (5 ng/ml.)

2) 1.0 ml. to 10 ml. ethanol-saline (10 ng/ml.)

3) 2.0 ml. to 10 ml. ethanol-saline (20 ng/ml.)

4) 3.0 ml. to 10 ml. ethanol-saline (30 ng/ml.)

5) 4.0 ml. to 10 ml. ethanol-saline (40 ng/ml.)

1 ml. of each solution was subjected to competitive protein binding.

A standard curve was obtained using 5% $^{v}/v$ normal male plasma as the binding protein (Fig. 1). In this figure the time to reach a predetermined count is plotted against concentration of steroid. The plasma from a normal female treated with ethinyl-oestradiol was also found to give a satisfactory standard curve. In Fig. 2 the amount of tritiated cortisol bound to protein is plotted against standard and the increase in sensitivity with increasing dilution of plasma is seen.

<u>Results obtained by cpb</u>.

The precision of the cpb method in the range 0-60 ug/100 ml, was 1.6 ug/100 ml with a coefficient of variation of 8.7% (n = 40). The reagent blank was equivalent to less than 0.5 ug/100 ml of cortisol.

The results obtained for plasma cortisol by cpb on a series of samples from hospital in-patients are shown on table 5. The results obtained by fluorimetry on the same specimens are also shown. The cpb method gave a mean result 60% that obtained by fluorimetry (range 30% to 118%).

The correlation between the two methods was highly significant ($p < .001$).

Urine cortisol by cpb

The cpb method for cortisol was applied to a research project carried out under the direction of Prof. P. Eckstein, University of Birmingham. In these experiments the sexual activity of rhesus monkeys after administration of dexamethazone was investigated.

Materials and Method

The cpb method of Beardwell et al (1968) for the estimation of urinary cortisol was applied. Ovariectomised monkeys treated with oestradiol benzoate were used in the experiments.

Results.

The standard curve obtained with pure cortisol shown in fig. 1 was used. After the method had been established it was applied in experiments to investigate the role of the adrenal gland in the sexual receptivity of female monkeys. It was shown that the administration of dexamethazone (0.5 mg/day/kg) suppressed the production of corticoids as indicated by the urinary excretion of cortisol (Fig. 3) and this was accommpanied by a marked lowering of the female monkeys sexuality. Administration of testosterone restored the receptivity while the urinary free cortisol remained low. This suggested that the effect was related to adrenal androgens rather than to other adrenal hormones. These results were published (Everett & Herbert, 1969).

Separation of 17-hydroxycorticosteroids in urine into pregnanetriol and cortisol fractions

It was shown in a previous section (p.61) that there was no correlation between blood cortisol levels and the amount of oestrogen excreted in the urine by subjects receiving FSH treatment. Corticosteroids excreted in the urine, however, include metabolites of steroids secreted by both the adrenal cortex and the ovary and when the latter is stimulated it might be expected that increased amounts of these metabolites would be detected in the urine.

The relationship between the production of progesterone and 17a-hydroxyprogesterone in the ovary, and their metabolites pregnanediol and pregnanetriol (a 17-hydroxycorticosteroid) in the urine is outlined on p. 69.

During the normal cycle the excretion of pregnanetriol starts to increase on the day that the excretion of oestrone and oestradiol-17b reach a maximum in the follicular phase (Fotherby, 1962). Pregnanediol does not rise until later in the cycle when the excretion of pregnanetriol is beginning to fall. Lunenfeld, Eshkol, Insler and Kraiem (1968) reported that increased amount of pregnanetriol were excreted in the urine by infertile women when they were excessively stimulated with gonadotrophins.

Using the method of Morris (1959) and the modification shown on p. 39 it is possible to separate the 17-hydroxycorticosteroids in urine into two fractions. Pregnanetriol may be oxidised to a 11-deoxy-17-oxosteroid (aetiocholanolone) and measured by the Zimmermann reaction after chromatography on silica gel. A second fraction representing the 17-hydroxycorticosteroids derived from the metabolites of cortisol is measured separately.

In current work the chromatographic stage has been replaced by a differential solvent partition method (Few 1968). This method has been compared with the earlier chromatographic procedure and a highly significant correlation was shown between the two techniques (Morris and Leeson 1969).

Pregnanetriol excretion was measured during the menstrual cycle of a normal subject and during treatment of two infertile women with FSH. Pregnanediol and oestriol or total oestrogen estimations were also carried out.

Results.

In fig. 4 the excretion of oestriol and pregnanediol by a normal subject during one cycle are shown. During a second month the total oestrogens excreted were measured and the 17-hydroxycorticosteroids were fractionated (fig. 5).

In both months a peak in oestrogen excretion was seen at day 13 with a small secondary rise between days 17 and 24. The pregnanediol peak was at day 20 in the first month and at day 21 in the second month (4.5 mg/24 hr. - not shown in figure).

There was a small rise in the pregnanetriol excretion just before the oestrogen peak and this was at a maximum at about day 16. The ratio of the pregnanetriol to cortisol fractions (R) fluctuated only slightly and did not rise above 0.6.

In fig. 6 is shown the total oestrogens and 17-hydroxy-corticosteroid fractions excreted by a subject receiving FSH therapy. She was given a total of 750 units of FSH between days 1 and 15 and 5,000 units of FSH on day 17. The oestrogen excretion rose to levels of 320 and 400 ug/24 hrs. after treatment. A subsequent rise supported the evidence that conception has occurred. The pregnanediol excretion was 10.2 mg/24 hr at day 25. The pregnanetriol excretion rose slowly to maximum near day 40. The ratio R did not exceed 0.6. No abnormal symptoms were observed during the treatment.

In fig. 7 is shown the oestriol excretion and 17-hydroxy-corticosteroid fractions excreted by a subject who received a total of 1125 i.u. FSH divided between days 1, 3 and 5 and 4500 i.u. HCG on days 1, 3 and 10. Two days after the last injection the oestriol reached a peak of 400 ug/24 hr. i.e.

about 15 times the normal average peak value. The pregnanetriol excretion was three to four times the normal value at its peak and this coincided with that of oestriol. The ratio R reached a value of 2.0.

It may be seen that the rise in pregnanetriol excretion had become marked after the first injection of FSH and before the ovulating dose of HCG had been given.

Symptoms of hyperstimulation were observed in this subject. She became pregnant in a subsequent course after modification of the treatment scheme.

OVARY		URINE
Acetate		
↓		
Cholesterol		
↓		
Pregnenolone →	Progesterone →	Pregnanediol
↓	↓	
17α OH-Pregnenolone →	17α OH-Progesterone →	17α OH-Pregnanolone
↓	↓	↓
Dehydroepiandrosterone	Androstenedione	Pregnanetriol
↓	↓	
Androstenediol →	Testosterone	
	↓	Oestriol
	Oestradiol →	Oestradiol
		Oestrone

Steroid biosynthesis in the ovary and metabolites excreted in the urine.

Application of urinary oestriol and pregnanediol assays in the study of the joint action of FSH and LH.

The effect of the joint action of FSH and HCG on the excretion of oestriol and pregnanediol by infertile women was examined. The experiment was one of a series in which a number of variables are being examined to find the optimum conditions for the induction of ovulation.

A commercial preparation of gonadotrophin was used in which the ratio of FSH to LH was 1 to 3. Ten patients commenced the trial but three became pregnant during the treatment. Of the seven that completed the trial six received four months of treatment and one was treated for eight months.

The total monthly dose of FSH, which in this group was between 1800 i.u. and 3150 i.u., was calculated for each patient after they had been subjected to a sensitivity test.

Urinary oestriol was measured by the method of Palmer (1964) and pregnanediol by the method of Klopper et al. (1955).

A factorial design was used in order to compare independently the effects of three factors. Factor A was the timing of the treatment with FSH. It was given on days 1, 3 and 5, or on days 1, 4 and 8. The second factor B was the addition of 4500 i.u. HCG to the first injection of FSH

compared with no addition to that injection. The third factor C was the addition of 4500 i.u. HCG to the second injection of FSH compared with no addition. An ovulating dose of 4500 i.u. HCG was given on day 10 in every month of treatment.

Results.

Fig. 8 shows the geometric means of significant effects. In (a) and (b) it is shown that FSH given on days 1, 3 and 5 produced more oestriol between days 5 and 10 than FSH given on days 1, 4 and 8. The peak in excretion of oestriol occurred two days later in the latter scheme.

In (c) and (d) it is shown that HCG given with the first injection of FSH decresaed the excretion of oestriol between days 9 and 10.

The third row (e) and (f) shows that HCG given with the second injection of FSH increased the excretion of oestriol between days 9 and 14 as well as the pregnanediol excretion on days 15 and 18. It also tended to delay the peak of oestriol.

In the fourth row (g) and (h) show the decrease in sensitivity during the second two months of treatment compared with the first two.

These results were published (Crooke, Bertrand, Butt and Morris, 1970).

Estimation of oestradiol-17B in plasma by cpb

The method of Corker et al.(1969) involved the preparation of an extract from the uterus of a pregnant rabbit by the method of Korenman et al. (1969) which was then used as the binding protein.

In the present study pseudo-pregnancy was induced in the rabbit with a single injection of HCG six to eight days before dissection of the uterus. The preparations were not uniformly satisfactory and this may have been due to delay in processing when the temperature of the extract may have risen. In accordance with Korenman et al. (1969) it was found necessary to maintain the temperature of the preparation at 4°C or below.

Stability was improved by lyophilization but sensitivity was lost if reconstituted material was frozen and rethawed.

In the original method (Korenman et al.1969) the uterine preparation was allowed to equilibrate for between 3 and 20 hr in the presence of oestrogen. It was found in the present study and by Corker (1970) that 20 min. at room temperature was equally satisfactory.

Results

The standard curves produced with two different concentrations from a rabbit uterus are shown in figs. 9(i) and 9(ii) and different methods of illustrating the results are indicated.

A plot of the logarithm of oestrogen concentration between 0 and 200 pg against the amount of tracer bound to protein produced a straight line.

The slope of the line produced by plotting the ratio of protein bound tracer to total count varied only with the activity of the uterine preparation and was independent of small variations in the radioactivity of the tracer.

The method was applied in a study involving the examination of blood oestradiol-17B, urinary oestrogens, blood leucocyte counts and haemoglobin levels in subjects receiving FSH therapy. Blood oestrogen levels were also measured during pregnancy.

In view of the difficulties experienced with the uterine preparation other possible oestrogen binding proteins were examined and the results are shown in a subsequent section.

Relationship of urinary oestrogens blood oestradiol-17B and blood leucocyte counts

It has been demonstrated that a fall in leucocyte and neutrophil count occurs in normal women after the menopause

(Allan and Alexander, 1963; Cruickshank and Alexander 1970). The possibility that this is linked with the circulating oestrogen level is supported by the observation that women using oral contraceptive preparations show an increase in neutrophil levels (Cruickshank et al.1970; Pincus 1965; and Morley 1966). An increase in the neutrophil count at the ovulatory peak in the normal cycle has also been noted (Morley 1966; Pepper and Lindsay 1960) although it was suggested that this rise was associated with plasma cortisol changes.

The methods for total oestrogens and blood oestradiol-17 B were applied to investigate whether any relationship occurred between oestrogen excretion and total and differential leucocyte counts in subjects receiving FSH therapy. The blood analyses were carried out on samples taken immediately after the collection of the 24 hr specimen of urine.

Leucocyte counting was carried out in a Model B Coulter Counter at the Group Pathology Laboratory, Warwick. An IBM 1440 computor was used for statistical analysis.

Results.

In the first investigation the relationship between total and differential leucocytes (cells per cmm blood) with urinary total oestrogens (ug/24 hrs) was examined and the result is shown in fig.10. The standard error of the mean and

the numbers in each group are indicated.

The changes in total leucocytes with increasing oestrogen may be seen and analysis showed a highly significant ($P<0.001$) positive correlation between these two parameters.

The principal changes were seen in the neutrophil counts where a progressive increase occurred with increasing oestrogen levels. The marked oestrogen-neutrophil relationship was illustrated in sequential samples taken from four different women (fig.11). These results have been published (Cruickshank, Morris, Butt and Crooke 1970).

In the second investigation blood oestradiol-17B and total urinary oestrogens estimations were carried out on five subjects and the results are shown in Table 6.

Although substantial variation occurred between the individual five subjects there was a significant positive correlation between plasma oestradiol-17B and urinary total oestrogens ($0.01>P>0.001$).

Neutrophil and haemoglobin estimations have also been carried out in this series and an analysis of the correlations between all these parameters will be submitted for publication shortly.

Application of cpb method to the estimation of oestriol in pregnancy.

Estimations of oestriol in plasma were carried out by the cpb method of Corker et al.(1970) on 6 patients during the third trimester of pregnancy. Total urinary oestrogens were also measured in the 24 hr collection of urine preceeding the blood sample.

The results are shown in Table 7. An increase in the daily excretion of urinary oestrogens over the periods examined is seen in 5 of the subjects. The changes in plasma oestriol are less marked. The levels are within the normal ranges given for plasma oestriol measured by a fluorimetric method (Nachtigall et al.1966).

In one subject (5) low urinary and plasma levels were observed and this supported the evidence that the foetus was abnormally small for the expected delivery date.

Application of cpb method to the estimation of free oestrogens in pregnancy.

Korenman et al.(1970) proposed that oestradiol-17B could be measured directly i.e. without a chromatographic step in the plasma of pregnant subjects. No experimental details were given but it is likely that all free and protein-bound oestrogens would be included in the measurement.

It was suggested that such an estimation would provide a rapid and easily carried out assessment of the blood oestrogen status of pregnant subjects. The following procedure was therefore developed:

Plasma (0.05 ml) was shaken with 5 ml of freshly distilled diethyl ether and 1 ml portions of the extract were evaporated to dryness under nitrogen for cpb assay as previously described.

Some preliminary results are shown in Table 8. In this table a comparison is shown of results obtained by cpb and by a method involving labelling with radio-iodine. Details of the latter are given in a later section (page 83).

Examination of other proteins for possible application in oestrogen binding

a) Human plasma as a source of binding protein for oestrogens

A method for the estimation of plasma oestradiol-17β using a partially purified binding protein prepared from plasma collected during pregnancy has been described by Dufau, Dulmanis, Catt and Hudson (1970). Fractionation of the binding plasma was carried out on Sephadex G-200 and isolation of oestradiol from the test plasma was also achieved by gel filtration.

Experiments were carried out in this study to find whether the binding globulin could be separated from pregnancy plasma by a simple salt fractionation. The fraction obtained was used to prepare standard cpb curves with pure oestradiol-17β, oestrone and oestriol.

Materials

1) Binding protein

Plasma obtained from women during the third trimester of pregnancy was shaken with charcoal and then centrifuged. To 2 ml portions was added 2 ml of a saturated solution of ammonium sulphate solution in water. Diethyl ether (approximately 2 ml) was added and the mixture was shaken vigorously, and then centrifuged. The globulin precipitate separated at the interface between the lower phase containing albumen and the upper ether layer.

The precipitate was separated, washed with ammonium sulphate solution, and after suspending in saline was dialysed for 17 hours against running water. The dialysis was carried out in a 5 ml plastic disposable tube, the open end of which was covered with a layer of Visking tubing (Scientific Instrument Co., Ltd.).

After dialysis the protein was dissolved in 80 ml of saline and 4 µC of oestradiol-6, 7-T in 0.4 ml ethanol was added.

2) Charcoal-dextran suspension

A stock suspension contained 250 mg Activated Charcoal (B.D.H.) and 2.5 mg Dextran T 40 (Pharmacia) in 100 ml distilled water. The suspension was diluted 1:5 with water before use.

Cpb assay

A series of oestrone, oestradiol and oestriol standards (0, 0.5, 1, 2 and 4 ng) in 0.05 ml saline were prepared.

One ml of the binding protein - tritiated oestradiol-17β was added to all the tubes which were shaken on a Vortex mixer and then allowed to stand at 4°C for 90 min. To each tube was added 0.2 ml of the charcoal-dextran suspension and, after agitation for 1 minute the tubes were centrifuged for 10 minutes at 3000 r.p.m.

A 1 ml portion of the supernatant solution was transferred to a counting vial, shaken with 10 ml of toluene-based scintillation solution and counted in a Packard Tri-Carb

scintillation counter. The counts obtained were expressed as a percentage of the original total count and plotted against the concentrations of oestrogens.

Results

A typical standard curve obtained with pure oestradiol-17β is shown in fig. 12. together with those obtained for oestrone and oestriol. The curve obtained with oestradiol showed a 35% fall in count over the range 0 to 5 ng. Oestrone gave a less satisfactory curve, while oestriol caused no displacement of tritiated oestradiol over the range examined (0-10 ng).

Although the standard curve for oestradiol was satisfactory the sensitivity obtained was poor compared with that found when uterine preparations were used as a source of binding protein. No improvement was obtained with lower concentrations of the plasma globulin.

The binding protein is easily prepared and readily available but comparatively large volumes of the test plasma would be required for assay.

b) Gonadotrophins as a source of binding protein

A commercial source of gonadotrophin prepared from the serum of pregnant mares (Leo) was examined to find whether it exhibited any binding properties with respect to oestrogens. The reagents used were those used in the previous section.

The material was supplied freeze-dried in ampoules each containing 3000 i.u. FSH. A solution was prepared in saline containing 2000 i.u./ml FSH and this was diluted to give 1000, 500 and 250 i.u./ml.

To tubes containing 0, 2.5 and 5 ng pure oestradiol-17β was added 0.1 ml of tritiated-oestradiol-17β (approximately 18,000 cpm) and 1 ml of each gonadotrophin dilution. After incubation at $4^{\circ}C$ for 1 hour 0.2 ml of charcoal suspension was added and the tubes were maintained at $4^{\circ}C$ for a further 10 min. The tubes were centrifuged and 1.0 ml of the supernatant fluid was transferred to a counting vial containing the toluene based scintillation fluid.

The counts in each supernatant solution was expressed as a percentage of the original total count and plotted against the concentration of gonadotrophin.

Results

The result (fig. 13) shows the binding of tritiated-oestradiol-17β to gonadotrophin in the absence of added unlabelled steroid. It is seen that binding occurs and that 44% of the total tritiated-oestradiol-17β is combined with the protein at a concentration of 2000 i.u./ml.

Addition of up to 5 ng unlabelled oestradiol-17B to displace the tritiated oestradiol-17-B from the protein at any of the concentrations of FSH examined.

Similar results were obtained with a partially purified FSH preparation (CM_4) prepared from pituitaries in this department. It was found that this material was also able to bind tritiated progesterone and it is likely therefore that the effect is a non-specific one.

Work is in progress on the binding of labelled FSH preparations to ovarian receptors and this may provide useful information on the mode of action of the gonadotrophins.

The estimation of oestrogen by radio iodine labelling

Iodine vapour, or a solution of iodine in water or an organic solvent may be used as a colour reagent to detect lipids, steroids or steroid carboxylic acids on thin layer or paper chromatograms. Some compounds react transiently with iodine forming loose addition compounds (Branté, 1949), others, particularly those possessing a phenolic group, react to form comparatively stable compounds. The natural oestrogens, for example, react with avidity, and Bush (1952) was able to detect as little as 0.5μg of oestrone on a paper chromatogram using iodine vapour. The reaction is quite non-specific however, and several nitrogenous compounds react to give colours with iodine on chromatograms under suitable conditions (Barrett, 1962). Although the reaction with iodine has been found useful for the qualitative detection of steroids no work has been reported making use of iodination in the quantitative estimation of those compounds.

Radioactive iodine ^{131}I and ^{125}I have been used to label protein hormones containing tyrosine for use in radio-immunoassay (Greenwood, Hunter and Glover, 1936). A solution of the protein is treated briefly with the isotope in the presence of the oxidising agent chloramine-T. Sodium metabisulphite is added to convert any residual ^{131} iodine to ^{131} iodide and the labelled protein is separated from the reaction mixture by gel filtration.

The phenolic ring A in the oestrogen molecule is analogous to the ring which is iodinated in tyrosine. Experiments were designed in the present work therefore, to investigate whether the isotope ^{125}I could be incorporated into the oestrogen molecule and thus provide a method for the quantitative estimation of the oestrogens in biological fluids.

A simple method of iodination of an oestrogen with ^{131}I or ^{125}I could lead either to a direct measurement of the labelled product or to its application in a sensitive protein bimding method.

Materials and Methods.

Solvents were A.R. quality and were distilled.

Diethyl ether A.R. (Fisons) was washed with 1% $^{w}/v$ chloramine-T solution, 1% sodium metabisulphite and water before distillation.

Petroleum ether Bpt 60°-80° C.A.R. (B.D.H.) was washed with sulphuric acid and water before distillation.

Chloramine-T and sodium metabisulphite solutions were freshly prepared each day in 0.5 M-phosphate buffer pH 7.

Sodium iodide - ^{125}I (Radiochemical Centre, Amersham) was diluted in buffer so that 0.1 ml. gave a count of approximately 10,000 cpm.

Standard oestradiol - 17β (Koch-Light) 1 mg. in 10 ml. ethanol.

Chromatography was carried out on Silica Gel GF 254 (Merck) 0.25 mm thin layer plates.

Visualisation was carried out by immersing the developed plates briefly in a tank containing iodine crystals followed by photography under U.V. illumination. Autoradiography of isotopically labelled steroids was carried out by application of the T.L.C. plate to an Agfa-Gevaert X-ray film for 2 to 3 days before development in Kodak DX-80. Counting was carried out in a Packard Autogamma Counter with the samples in plastic disposable tubes. Petroleum ether and other solvents slowly dissolved the tubes but they remained effective in retaining solvent for at least 24 hours.

Experimental

1) The effect of Iodine vapour on oestradiol -17β.

An experiment was carried out to examine the chromatographic behaviour of the products when oestradiol-17β was exposed to iodine vapour.

A T.L.C. plate coated with Silica Gel GF 254 was divided into five lanes and 10 μg. oestradiol-17β was applied in chloroform to the origin in each lane. Lanes 1 to 4 were exposed to iodine vapour for 20 min.,10 min.,5 min. and 2 min. respectively and lane 5 was left unexposed. The plate was developed in a chloroform-ethyl acetate 9:1 solvent mixture and then allowed to dry. ~~After~~

After exposure of the whole plate to iodine vapour it was photographed using light of wavelength 254 mu. The result is shown in fig. 14. In lanes 2, 3 and 4 three compounds have separated from the original oestradiol-17β, the position of this unchanged steroid can be seen in lane 5. With increasing time of iodination a greater proportion of the least polar compound was produced until at 20 min. (lane 1) the product was almost entirely this material.

It is apparent that the compounds produced depend on the conditions of iodination and that it will be necessary to standardise these conditions carefully to obtain reproducible results. At this stage no attempt was made to isolate the individual products since it was intended to carry out further tests after iodination in solution rather than in vapour form.

2) <u>Iodination of oestradiol-17β in aqueous solution</u>.

Experiment (1) was repeated except that the steroid was iodinated with an aqueous solution of iodine. The oestradiol-17β content of the reaction mixtures (1 ml) was between 6 and 50 μg. and the solutions contained between 100 μg. and 250 μg. iodine.

After iodination for 1 hour excess free iodine was removed with sodium metabisulphite and the mixtures were extracted with chloroform. The extracts were applied to separate lanes of a TLC plate and run in the same system as in experiment (1).

The results (fig. 15) show that three compounds had again separated from the residual oestradiol-17β in each case and traces of others had appeared.

3) Iodination of oestradiol-17β using sodium iodide ^{125}I

A modification of the procedure for the iodination of protein hormones (Greenwood et al. 1963) was applied.

To 0.05 ml. of 0.5M-phosphate buffer pH 7.0 containing 50 ng. oestradiol 17-β was added 0.05 ml. sodium iodide ^{125}I (30,000 cpm) solution and 0.025 ml. 0.4% $^{w}/v$ chloramine-T solution both dissolved in 0.5 M-phosphate buffer. After 1 hour at room temperature 1.0 ml. of petroleum ether was added, the tube was stoppered and shaken on a vertex mixture. A 0.5 ml. portion of the supernatant extract was transferred to a tube for radioactive counting. A blank consisting of 0.05 ml. of the buffer was treated similarly from the outset.

The procedure was repeated but with the addition of between 0.1 ml. and 0.5 ml. of 0.24% $^{w}/v$ sodium metabisulphite in buffer before extraction into petroleum ether.

The results are shown in Table 9 in which the difference in counts between the blanks and the oestradiol-17β extracts are shown. The difference was a maximum after the addition of between 0.1 and 0.3 ml. of 0.24% $^{w}/v$ sodium metabisulphite.

4) Investigation of pH and time of oxidation

Experiment (3) was repeated and the pH of the iodination

mixture and time of oxidation were varied. In Table 10 it is seen that a time of 2 minutes with chloramine-T gave the highest count difference between steroid and blank and the optimum pH was 7.5.

5) Investigation of solvents.

A number of different solvents were examined for the extraction of the iodinated products (Tables 11 and 12). Petroleum ether, hexane and mixtures of petroleum ether and ethyl acetate were among the best. It was noted that the count with 50 ng. oestradiol-17β were almost exactly double those obtained with 25 ng. and it seemed likely that a stoichiometric relationship would be obtained between the amount of steroid and count obtained.

Petroleum ether was used as solvent in subsequent experiments unless otherwise stated.

6) Concentration of chloramine-T and metabisulphite.

In an experiment to find the joint optimum concentration of chloramine-T and metabisulphite the results shown in Table 13 were obtained. Concentrations of 2.5 mg/ml. chloramine-T and 1.25 mg/ml. sodium metabisulphite were the most satisfactory.

7) Specificity.

The specificity of the procedure was examined by iodinating some other steroids with sodium iodide $^{125}1$. Oestrone gave similar results to oestradiol-17β but no iodinated oestriol could be obtained under the conditions of the experiment. It was considered that the oestriol, if iodinated, had remained in the

aqueous solution and had not been extracted by the solvent. Some iodinated material was extracted however, after prior acetylation of oestriol.

None of the following steroids could be extracted as iodinated products when concentrations up to 1 ug were examined: cortisol, progesterone, testosterone, pregnanediol, D.H.A., androsterone and cholesterol. Oestradiol, after methylation, would no longer iodinate and this suggested that a phenolic group is required.

It was found that iodinated oestradiol could be extracted from a petroleum ether extract with sodium hydroxide solution and this indicated that the phenolic group remained intact.

8) Standard Curve for oestradiol 17-B

Using the method described in section 3 and the optimum conditions for oxidation and extraction a standard curve was prepared with concentrations of oestradiol-17B between 0 and 20 ng. The result is shown in Fig. 16.

It may be seen that the curve is almost linear between 2 and 10 ng and this would be a useful range for the estimation of oestradiol-17B in plasma during pregnancy.

9) <u>Application of sodium iodide - ^{131}I.</u>

Iodination of oestradiol-17B was carried out with ^{131}I instead of ^{125}I. An autoradiograph (Fig. 17) showed that the products were similar with regard to their behaviour on a TLC plate to those obtained by iodination in the first two experiments.

Radioactive measurement of the separate products after chromatography was not attempted since a satisfactory standard curve was produced by counting the unfractionated extract. It is likely however that the blank count could be decreased by chromatography.

10). Application to the estimation of oestrogens in plasma.

Several different procedures for the extraction of oestrogens from plasma prior to iodination were examined. A method originally described by Van Baelen, Heynes and De Moor (1967) for the purification of oestrogens from urine using Sephadex gel was found to be one of the most satisfactory. This method has been applied recently to plasma by Jiang, Ryan and Albert (1971). They have shown that oestrogens are retained by the gel when they are applied in an acidified aqueous solution and that they may be eluted in organic solvents.

(a) Preparation of extracts.

Plasma samples collected from subjects during the third trimestre of pregnancy were examined. To prepare the sample for estimation in duplicate 1.1 ml of plasma was diluted with 4.7 ml water. To this was added 3 ml of 0.16 N hydrochloric acid and approximately 5000 cpm of tritiated oestradiol in 0.02 ml water. The mixture was allowed to stand for at least 10 minutes before chromatography.

Sephadex G15 (800 mg), which had been suspended in water for 24 hours, was poured into a chromatography tube to form a column approximately 30 mm by 10 mm and this was washed with 10 ml of water before use.

A 4 ml portion of the diluted plasma was transferred to the

Sephadex column and allowed to pass through. The column was washed with 7.5 of water in order to remove proteins and other interfering substances and was then sucked dry at the pump. Oestradiol and oestrone were eluted in 10 ml benzene and the solvent was evaporated to dryness under a stream of nitrogen in a water bath at $60^{o}C$.

(b) Iodination of Extracts.

All the aqueous reagents were dissolved in $^{M}/15$ phosphate buffer pH 7.5.

The residue, after evaporation of the solvent was shaken with 0.12 ml phosphate buffer and 0.02 ml was transferred to a vial containing 10 ml scintillation fluid in order to assess the radioactive recovery.

To the remainder was added 0.02 ml of sodium iodide-^{125}I (approximately 15,000 cpm) and 0.025 ml of a 2.5 mg/ml chloramine T solution. After 2 minutes 0.1 ml of a 1.25 mg/ml solution of sodium metabisulphite solution was added followed, after a further 5 minutes, by 2 drops of 1% $^{w}/v$ potassium iodide solution to act as an iodine carrier.

The mixture was extracted with 1 ml of petroleum ether by agitation of the tube on a Vortex mixer and a 0.5 ml portion of the extract was transferred to the Autogamma counter for a count of 1 minute.

(c) Calculation of result.

A series of oestradiol standards (0 to 10 ng) in 0.1 ml phosphate buffer were iodinated, extracted and counted under identical conditions to the plasma extracts. The concentration of oestrogen in the extract was found by reference to the standard curve, corrected for losses and expressed as ng/ml plasma.

It was found useful to find the total amount of ^{125}I in cpm added to each tube and then the percentage extracted into petroleum ether could be calculated.

The total petroleum ether extract (1 ml) contained appromimately 8% of the total count when no oestrogen was present and between 24% and 28% in tubes containing 10 ng oestradiol.

Results.

Some preliminary results are shown in Table 8 . The mean recovery of radioactive oestradiol was 72% (n=15) and levels of between 13 and 20 ng/ml were obtained in plasma during the third trimester of pregnancy. These values are similar to those obtained by Korenman, Tulchinsky and Eaton (1970) using a competitive protein binding method.

11). Solvents for the extraction of iodinated oestriol.

Further experiments were carried out to find suitable solvents for oestriol after iodination. A 1.1 mixture of ethyl

acetate and petroleum ether was satisfactory.

The method described for the iodination of oestradiol-17B was followed except that the preliminary petroleum ether extract was discarded. It was necessary to include this petroleum ether wash in order to obtain a satisfactorily low blank. The iodinated oestradiol was then extracted with 1 ml 1:1 ethyl acetate: petroleum ether and 0.5 ml was transferred to a disposable tube for gamma counting. A standard curve similar to that obtained with oestradiol-17B was obtained.

12). Application to the estimation of oestriol in pregnancy.

Oestriol is present in blood, mainly in the form of conjugates with sulphuric and glucuronic acids and it is necessary to carry out a preliminary hydrolysis of the plasma before extraction. Ether extracts of acid-hydrolysed samples contained substances which interferred with the iodination procedure. Although these could be removed by washing the extracts with bicarbonate buffer pH 10.5 it was found that traces of HCO_3-ion also produced, erroneous results.

Preliminary experiments indicated that satisfactory purification of extracts could be carried out by chromatography on Sephadex G15. Oestriol was eluted in ethyl acetate after washing the column with benzene to remove oestrone and oestradiol-17B.

The estimation of progesterone by cpb

Dog plasma and plasma from human females during treatment with ethinyl oestradiol and during pregnancy have been examined as sources of binding protein. The radioactive counting procedure and the method for separating free and bound steroids have been investigated,

1) Investigation of scintillation fluid

Several liquid scintillant mixtures have been described for counting aqueous solutions containing radio-isotopes. Toluene based scintillants have been diluted with more polar solvents such as ethanol, methanol, 1:4-dioxane and 2-ethoxyethanol to improve miscibility. (Turner 1967). The most widely used liquid scintillant for aqueous samples is that of Bray (1960) which consists of naphthalene (60g) PPO (4g) POPOP (0.2g) methanol (100 ml.) ethylene glycol (20 ml.) and 1:4 dioxane to give a final volume of 1 litre.

Recently Triton X-100 was introduced by Patterson & Greene (1965) and this appears to be finding increased application in emulsion counting of aqueous samples. The volume ratio of water to scintillating fluid may be made optimal by using a figure of merit (Kinard 1957). The product of water volume (w) and counting efficiency (E) is found and the values of Ew are plotted against (w)

and the optimum volume of water is obtained.

Addition of any of the polar solvents depresses the counting efficiency to some extent and since many steroids may be extracted from aqueous solution by toluene an experiment was carried outto examine whether this addition was necessary. An aqueous solution of corticosterone -1, 2-T containing 0.5% dog plasma used in cpb was extracted directly into a toluene based medium and counted without separating the two phases. The counts were compared with those obtained from the same amount of isotope in a toluene-ethanol scintillation mixture and in Bray's solution.

The results in Table 14 show that the efficiency measured using an external standard, was satisfactory with the aqueous toluene mixtures and there was no need to separate the aqueous layer.

The efficiency was less satisfactory in the ethanol-toluene mixture and in Bray's solution and after correction the resulting counts remained highest in the 2 phase system. Subsequent counts were carried out therefore in a toluene-water system without the addition of a polar solvent.

2). <u>Conditions for separating free and bound steroids.</u>

In preliminary experiments progesterone standards were treated with 0.5% $^{v}/v$ dog plasma solution containing corticosterone -1,2-T.

The unbound steroids were removed by shaking with Florisil. Wide variations between replicates were obtained and it was found that the time of contact with Florisil was critical.

In Fig. 18 is shown the variation in counts of the tritiated corticosterone bound to the protein when shaking with Florisil was carried out for 10, 20, 30, 40, or 50 secs. In this experiment the solutions contained 1.25 ng/ml progesterone in addition to the radioactive tracer.

When the time was varied between 10 and 30 seconds an increase of 1 second caused a drop in count of over 100cpm in the tracer bound to protein. Between 30 and 50 seconds an equilibrium was apparently reached.

In a further experiment, after shaking with Florisil for 40 seconds, solutions containing protein, tracer and 1.25 ng/ml progesterone were transferred to a bath at 4°C and samples of the supernatant solution were removed for counting immediately and at intervals up to 20 minutes. Fig. 19 shows that there was an 18% fall in count over this period. In an experiment without added progesterone incubation at 4°C was continued up to 50 minutes after the preliminary shaking. In Fig. 20 it is seen that a minimum point was reached after which the radio-active

the count in the supernatant started to increase. A shaking time of 30 secs. followed by 15 secs. at 4°C was therefore adopted. Reproducibility was improved by treating each tube identically in this manner but unacceptable replicates were still obtained in each batch of analyses.

3) Variation of concentration of binding protein.

The percentage of tritiated corticosterone bound to various concentrations of plasma after shaking with Florisil is shown in Fig.21.

In this experiment the source of plasma was a normal human female whose endogenous ovarian steroids had been suppressed with ethinyl oestradiol.

4) Protein binding standard curves.

Standard curves were produced with pure progesterone and, as binding protein, various concentrations of plasma from a human female during treatment with ethinyl oestradiol (fig.22) from a dog (fig.23) and from a human pregnant subject (fig.24).

The change in sensitivity and slope of the curves with different concentrations of the binding protein may be seen.

Initially dog plasma was used as the source of protein but later it was found that a 0.1% solution of plasma from a human female after treatment for about 20 days with 50 ng ethinyl oestradiol was more consistently satisfactory.

This concentration of plasma bound 63% of tritiated corticosterone in the absence of progesterone. (Fig.21.)

5) Choice of solvent for extraction of progesterone.

Petroleum ether obtained from different suppliers was examined with the object of finding a solvent which extracted the maximum amount of progesterone from plasma with minimal extraction of cortisol and the more polar steroids cortisol and corticosterone. A product 'Free from aromatic hydrocarbons', Bpt. 30-40°C, an A.R. grade solvent supplied by British Drug Houses was suitable.

Labelled steroids were added to plasma and then extracted with this solvent as described in the method. Typical recoveries were: Progesterone 87.5%, 17a-hydroxyprogesterone 11.5% cortisol 0.38%, corticosterone 1.32%. Addition of 1 ng unlabelled progesterone to 0.5 ml plasma gave a mean recovery of 0.99 ng (S.D. 0.17, n = 14).

6) Application in the rhesus monkey cycle.

A method was required to demonstrate the time of ovulation and the duration of the luteal phase in the menstrual cycle of normal rhesus monkeys. The work was carried out under the direction of Prof. P. Eckstein and will form part of an investigation into the mode of action of intra-uterine devices.

The results obtained with blood samples taken at intervals during the cycle of one monkey are shown in fig. 25. A peak in the luteal phase at day 26 is seen.

DISCUSSION.

This thesis has examined methods of assay of steroids in blood and urine with particular reference to those employing competitive protein binding. Methods have been compared and applied to investigations in the field of reproductive physiology.

The procedures used for the assay of corticosteroids, oestrogens and progesterone are now discussed.

Corticosteroids.

Comparison of methods.

The lack of specificity of the Mattingly (1962) method for the estimation of 11-hydroxycorticosteroids in plasma was demonstrated by James, Townsend and Fraser (1967) when they showed that 41% of the fluorescent material measured by this technique was not cortisol. They reduced the mean proportion of interference to 34% by applying the correction formula of Spencer-Peet et al.(1965). In the present work, application of the formula to a wide range of 11-hydroxycorticosteroids reduced the levels measured by the Mattingly (1962) method by a small constant amount and the possibility of the presence of a constant amount of fluorogen other than cortisol was suspected. This was confirmed recently by Stenlake, Davidson, Williams and Downie (1970) who showed that cholesterol esters contribute to the total fluorescence.

The cpb method used for the estimation of cortisol in plasma was

a modification of that described by Beardwell et al.(1968) for the estimation of cortisol in urine. It presented only slightly more difficulty than the fluorimetric technique, and the precision of the two methods was similar. A disadvantage is that the extraction solvent must be completely removed prior to the cpb assay and this is not necessary in the fluorimetric procedure.

The concentration of cortisol binding globulin in normal plasma is sufficient to enable it to be used satisfactorily as a source of the protein. The equilibrium in the presence of the adsorbent florisil is a comparatively stable one and the time of shaking is not highly critical.

The cpb method gave a mean value 60% that obtained by fluorimetry. Assuming that cortisol is the predominant steroid measured by this technique, the result is in accordance with the earlier finding of James et al.(1967) which was based on a chromatographic separation.

The estimation of plasma cortisol is frequently applied to demonstrate changing blood levels during ACTH stimulation or suppression tests. Providing that the 11-hydroxycorticosteroids measured contain a constant proportion of cortisol it is doubtful whether a more complex method will replace the fluorimetric procedure in the routine chemical laboratory unless automation is facilitated by the new technique.

Applications.

An investigation was carried out to determine whether any changes in the levels of cortisol in the blood could be detected in infertile women receiving treatment with gonadotrophins. In the series examined, no correlation was found between the levels of oestrogen in the urine and the levels of cortisol in the blood. It is known that the gonadotrophin preparations used had a negligible ACTH content and no direct action on the adrenal cortex would be expected. However, an interrelationship between the secretion of ACTH and gonadotrophins by the anterior pituitary has been suggested since the excretion of FSH may rise when patients are treated with cortisone (Brown, 1956). Similarly, suppression of endogenous ACTH by administering dexamethazone can lead to an increase in secretion of oestrogens by the ovary due to an increased release of FSH. (Butt, Crooke, Cunningham and Palmer,1963). (Crooke, Butt, Palmer, Morris, Edwards, Taylor and Short, 1963).

The lack of association between the level of oestrogen in the urine and cortisol in the plasma was surprising in view of the increase in cortisol observed during pregnancy and during treatment with oral contraceptives. (Burke, 1969, Doe, Dickinson, Dimmeman and Seal, 1969).

The method for the determination of cortisol in urine by cpb was found useful in the demonstration of adrenal function in rhesus monkeys. The control levels were lower than those found in the human. The mean 24 hour excretion was 14 μg compared with 42 μg in the normal human

subjects examined by Beardwell et al.(1968), but the normal range in the human was wide (0 - 145 μg/24 hr.). Similar ranges have been reported by workers using chromatographic methods.

Fractionation of corticosteroids in urine.

The methods described for the fractionation of corticosteroids in urine (Few, 1968, Morris et al.1969), show promise in the early detection of a possible hyperstimulation during FSH therapy. Further work is in progress using these methods and preliminary results suggest that an increase in the pregnanetriol fraction preceeds an excessive ovarian response. (Lipede, 1970).

Cox, (1969) has reported that the excretion of pregnenetriol also increases just before ovulation and an abnormal rise in the amount of this steroid is associated with a subsequent excessive response of the ovary.

The estimation of 17α-hydroxyprogesterone in plasma may also be of value since this is a precursor of pregnanetriol. A cpb method for the estimation of this steroid has been described. (Strott and Lipsett 1968), but the procedure is lengthy and is not yet suitable for routine application. The method for the estimation of pregnanetriol on the other hand may be carried out relatively rapidly and if an abnormal level is shown, the ovulatory dose of HCG may be withheld.

The reason for the association between increased pregnanetriol excretion and hyperstimulation is not clear. It is possible that the enzyme system responsible for the conversion of 17α -hydroxyprogesterone

to androstenedione (page 69) is temporarily inadequate and this results in an increased excretion of 17α-hydroxypregnanolone and pregnanetriol in the urine. It is probable that the capacity of the enzyme system varies from one subject to another and high oestrogen levels are not necessarily indicative of imminent hyperstimulation. Recent work suggests that hyperstimulation can be avoided if the rate of oestrogen secretion is carefully controlled, (Hancock, Scott, Stitch, Level, Oakey and Ellis (1970). This supports the theory that the enzyme system may be overloaded by an excessive rate of stimulation. The hyperstimulation syndrome is characterized by marked fluid retention as well as ovarian enlargement. It is possible therefore that aldosterone assays may also be helpful in elucidating the mechanism of the condition.

The assay of Aldosterone by cpb.

Attempts at developing a method for the assay of aldosterone by cpb have met with limited success. The application of an aldosterone binding protein (ABP) has however been described recently by Robinson and Fanestil (1970). Adrenalectomized rat kidneys were homogenized in 0.25 M sucrose and a 10,000 g. supernatant was used for binding after gel filtration.

The ABP aldosterone complex was found to be heat sensitive and all procedures had to be carried out at $4^{\circ}C$. The capacity of the preparation to bind aldosterone decreased rapidly even at this temperature and approximately 25% was lost in two hours.

The material was not specific for aldosterone and attempts to remove interfering corticosteroids resulted in high blanks. A 10 ml. volume of plasma was required for the assay.

It is apparent therefore that several problems must be overcome before the assay can be made of practical value.

It is likely that failure to obtain any binding protein from rat kidneys in the present work was due to a lack of appreciation of the heat labile nature of the material.

A preliminary report by Mayes, Furuyuma, Kem and Nugent,(1970), described a method for the assay of aldosterone in plasma using antibodies to an aldosterone-protein conjugate in a radioimmunoassay technique. The precision, accuracy and sensitivity of the method are claimed to be satisfactory and the method shows promise for clinical application.

Oestrogens.

Comparison of methods.

The methods used for the estimation of oestrogen in urine depended finally on the product obtained by heating the steroid with sulphuric acid in the presence of quinol - the Kober reaction. The reaction,though lacking in sensitivity,has a high degree of specificity and this may be increased by extracting the colour produced by the reaction into an organic solvent (Ittrich, 1958). This technique was applied by Palmer, (1964) in the estimation of urinary oestriol.

The sensitivity of the reaction may be increased considerably by measuring the fluorescence of the extracted material in a suitable solvent, and Brown et al.(1968), developed a method for the estimation of total oestrogens in small volumes of urine using this technique.

Both the methods of Palmer (1964) and Brown et al.(1968) have been used in this work to demonstrate ovarian activity by urinary oestrogen determination.

The method of Brown et al.(1968) has the advantage of speed using the semi-automated procedure which is possible with the small volumes of urine employed. Difficulties can arise however when low concentrations of steroids are subjected to evaporation and heating with strong acids in this method. Variations are particularly noticeable when the pure steroid used as standard is examined. Fluorimetric measurements are particularly sensitive to quenching agents and these must be excluded. With suitable precautions, the method of Brown et al.(1968) is the one of choice for demonstrating the changes during the human menstrual cycle and following ovarian stimulation, particularly if a rapid result is required to monitor treatment.

Applications.

The estimation of oestrogens in urine has been of paramount importance in the monitoring of gonadotrophin therapy. The estimation has been applied in controlled experiments investigating a number of

variables to find the optimum conditions for administering FSH and HCG in the treatment of infertility, (Crooke, 1970).

During the present work the estimation of oestriol in urine was used to examine the effect of the combined effect of FSH and HCG in the development of the ovarian follicle in infertile women. It was found that the addition of HCG to the second of three injections of FSH delayed the peak in excretion of oestriol and increased the total amount excreted. When the FSH injections were prolonged over 8 days, the addition of HCG had no effect. This suggests that the follicle has a natural life cycle which cannot be extended beyond a certain time. A delay in the peak of oestriol excretion had previously been found to be advantageous in the treatment of infertility since it resulted in more pregnancies per month of ovulation (Crooke, et al,1968). It is possible that endometrium which has had longer to mature under the influence of oestrogen is better prepared for the implantation of the fertilized ovum.

An interesting relationship was demonstrated between the levels of total oestrogens in the urine and the circulating neutrophils in 20 infertile women receiving gonadotrophin therapy (Cruickshank,et al. 1970). In a subsequent paper (Cruickshank 1970a) a significant negative correlation was shown between the total oestrogens in urine and the haemoglobin concentrations of the blood.

A regression equation was applied (Cruickshank 1970b) using

haemoglobin and neutrophil values to investigate whether a prediction could be made of the total oestrogens in the urine. The prediction was correct in 70% of the cases examined. In view of the fact that the urinary oestrogen values reflected oestrogen production during the 24-36 hours previous to the collection of the blood sample, the correlation with the haematological values was remarkable.

Although it is not yet claimed that the method is sufficiently accurate to enable it to replace oestrogen assays, it does make available another parameter to demonstrate ovarian activity. It could provide a useful index in clinics where leucocyte counting is carried out but where there are no facilities for the assays of steroids.

Cpb methods for oestrogens in blood.

It is apparent that using isotopically labelled materials a degree of sensitivity may be obtained that cannot be achieved by colorimetric or fluorimetric methods. The cpb methods have overcome to some extent the difficulties involved in the estimation of the very low levels of oestrogens found in blood.

The methods currently available employing cpb are those of Shutt (1969), Korenman et al.(1969), Corker et al.(1970) and Dufau et al.(1970).

The method of Shutt (1969) requires 4 ml of plasma and lengthy

purification of the extract before assay using ovine material as the binding protein.

Korenman et al.(1969) and Corker et al.(1970) both use a rabbit uterus preparation but they differ in the preliminary purification of the plasma extract. In the Korenman procedure 3 to 5 ml. of plasma is extracted with 90 ml. ether and purification is achieved by means of partition column chromatography on Celite. Corker et al.(1970) is able to carry out the estimation using only 1 ml. of plasma with an ether volume of 10 ml. for extraction. Chromatography on an alumina thin layer plate is used before the cpb assay.

Of these methods that of Corker et al(1970) which was used in the present work has the advantage of simplicity and is economical with regard to plasma. However in both cases a supply of uteri is required together with apparatus for processing and freeze drying.

A method using human pregnancy plasma as a source of binding protein has been developed by Dufau et al.(1970) and this shows promise since the plasma is relatively more easily available. It is shown in the present study that fractionation of the binding globulin may be carried out by a simple procedure. However the preparation lacks the sensitivity shown by the rabbit material and the volume of plasma required by the assay (4 or 8 ml.) restricts the use of the method if a regular assessment of oestrogen levels is required

It is of interest that the circulating oestrogens differ considerably in their relative binding affinity, i.e. in competition with tritiated oestradiol - 17B in vitro. Korenman (1970) has shown the comparative binding affinities for oestradiol -17B, oestrone and oestriol to be in the ratio 100:66:16 but this varies with the species from which the uterine material is obtained.

It is suggested by Korenman (1970) that this ratio varies with temperature and that the system can be made more specific for oestradiol under suitable conditions. Johannson (1970) recommends further purification of the uterine binding protein as a method of improving the specificity for oestradiol. Although Dufau et al.(1970) claim no interference from oestrone or oestriol in their system, the globulin prepared from pregnancy plasma in the present study did show some affinity for oestrone as well as oestradiol, although oestriol was ineffective in displacing tritiated oestradiol at the levels examined (0-5 ug).

Some of the features of the intracellular proteins and plasma globulins which exhibit binding properties are common to those found in the protein hormones, particularly the gonadotrophins. Evidence that steroids may be associated with gonadotrophins was shown by Butt and Kinnear (1956). They were able to detect small quantities of oestrogens in their extracts of gonadotrophins prepared from human urine by a kaolin adsorption method.

Preliminary experiments in the present study suggest that preparations of gonadotrophins are able to bind tritiated oestradiol-17β, but it was not found possible to displace the bound label under the conditions of the experiment. Further work is being carried out to investigate the sensitivity and specificity of this binding property.

The use of radioiodine for the estimation of oestradiol.

A method is proposed making use of labelled iodine ^{125}I for the estimation of oestradiol-17 β. It has been shown that Na ^{125}I may be oxidised by chloramine T to liberate the free iodine which reacts quickly with the phenolic group. Hydrogen peroxide in acid solution was also found useful in bringing about the oxidation. Three products were usually obtained and it is likely that mono and diodinated oestradiol (2.4 positions) were present. The iodination procedure is simple, is rapidly carried out and shows a high degree of specificity for oestrogens. The advantage of a radio iodine over a tritium label is that an isotope of high specific activity may be used. In addition gamma ray spectroscopy counting is a much simpler and more economical procedure than that involving liquid scintillation. The method was applied to the estimation of oestradiol-17β in the plasma of pregnant subjects. Preliminary results indicate that the values obtained are comparable to those produced by a cpb method using a rabbit uterine

extract.

A method is available therefore which does not rely on an extract which may have a variable potency depending on the temperature at which it has been prepared and stored.

Theoretically the iodination method should be capable of a much greater sensitivity than has been achieved in the current work. Only about 20% of oestrogen present in a solution could be labelled under the best conditions examined.

A method involving halogenation of oestrogens was described by Saroff, Keenan, Sandberg and Slaunwhite (1967) in which ^{82}Br was used. Testosterone and other androgens in extracts obtained from plasma were converted to oestradiol by means of a placental enzyme capable of aromatisation and then the radioactive halogen was introduced. The isotope has a short half life and the electrolytic method described for its preparation is tedious. However the aromatisation procedure could lend itself to the transformation to oestrogens of other steroids which could then be iodinated by the procedure described in the present work.

The use of direct iodination as a method of estimating oestrogens has not previously been described. Work is in progress however on the use of iodinated oestradiol in a radioimmunoassay

technique (Abraham and Odell, 1970). Difficulties arise since the physical dimensions of the iodine atom approach those of the complete phenolic ring. This may account for the fact that attempts to use iodinated oestradiol in a cpb procedure were unsuccessful in the present work.

The estimation of progesterone by cpb.

A number of methods have been described for the assay of progesterone in plasma and these vary in the number of preliminary purification stages before cpb (see p. 20.).

The method of Johansson (1969) is attractive since only a simple extraction with petroleum ether is required and no purification is needed. The method is based on the original suggestion of Murphy (1967) that petroleum ether would selectively extract progesterone in the presence of other steroids which compete in the binding system.

Johansson (1970) points out that his method stands or falls with the selection of the petroleum ether which varies between batches and distillers. The petroleum ether used in the present study, B.D.H. A.R. Bpt. 30-40°C, has been consistently satisfactory in the extraction of progesterone. The amounts of cortisol, corticosterone and 17-Hydroxyprogesterone in the extracts were acceptably small.

It is recommended (Johansson 1970) that each batch of petroleum ether is checked by running parallel assays which include a thin layer stage before cpb. This is desirable, although the cpb

system for progesterone is particularly sensitive to interfering substances introduced from absorbents used in TLC.

As sources of the binding protein, dog plasma, chicken plasma and human female plasma during treatment with oestrogens have been used. Variations in the binding properties of dog plasma from one animal to another were found and this has also been reported by Hagerman and Williams (1969). Martin, Cooke and Black (1970) however, obtained reproducible standard curves with the five different dogs they tested.

Human female plasma has been found to be the most reliable source of binding protein and Florisil the most satisfactory reagent for separating the free and bound steroids. It was necessary however to examine a number of different batches of Florisil before a satisfactory grade was found. The need for carefully controlling the time of contact with this reagent was confirmed.

Applications.

The application of this assay in the treatment of infertility has been reported by Gemzell, Carlberg, Johansson and Roos (1970). The first application in the present work is concerned with an investigation into the cycle of rhesus monkeys.

Preliminary results indicate that it is satisfactory in demonstrating a peak of progesterone in the luteal phase of this

animal.

Cpb methods.

Practical difficulties.

Strott et al (1968) have emphasised some of the factors to which particular attention must be made in cpb assays. When working in the picogram range all glassware must be scrupulously clean and reagents must be selected for minimal background interference. Solvent residues must be carefully removed since they can displace label from protein.

It has been shown in the present investigation that the mixture of protein, steroid and adsorbent is not a stable one and quite large variations in the results can be obtained by varying conditions slightly. The binding proteins themselves are not stable and deteriorate with time even if stored at -20°C.

Earlier methods suggested that a high degree of specificity may be obtained by cpb methods. With the exception of the Johansson (1969) method for progesterone most of the current methods involve at least one chromatographic stage before protein binding.

As Korenman (1969)points out: "Investigators setting out to develop a radio-ligand binding (cpb) assay, even one which has worked for others are embarking on a difficult process involving meticulous attention to detail."

Future developments involving cpb assays.

Several cpb methods are now available which provide an opportunity for the estimation of steroids in blood when previously it was only possible to measure metabolites excreted in the urine. It is obvious that cpb work will continue to develop for this purpose. However in several instances it is apparent that results obtained from urine by established methods give equally useful information.

Arguments may be proposed in favour of carrying out estimations on either urine or blood from the point of view of the patient and that of the laboratory. For example, although urine collection is inconvenient, collection of a blood sample usually requires the attendance by the patient at a laboratory or clinic and a competent person must be available to carry out the venepuncture. Blood levels give an indication of a situation at one moment of the day and this may be influenced by diet, fluid intake, posture and the diurnal variation.

However the day to day variation in the urine excretion of certain steroid metabolites is often considerable and unexplicable.

Klopper et al. (1969) has found that the co-efficient of variation of repeated oestriol estimations in the urine of healthy pregnant women was 27%. For 48 hour specimens the variation was 20%. He points out that the oestriol may drop by a third from one day to the next without signifying any pathological change.

It is obvious, therefore, that single urine assays are of limited value.

Estimations of oestrogens in blood carried out on samples taken at regular intervals and under the same circumstances may provide a better indication of the progress of a suspected abnormal pregnancy and the series in the present work will be extended for this purpose.

Another application of cpb methods is in progress in an investigation of the mode of action of synthetic materials capable of inducing ovulation in infertile women. The methods are also being applied in a study of the excretion of oestrogens by post-menopausal women. Preliminary results indicate that an excessive excretion of oestrogen is associated with certain pathological conditions of the ovary. Estimations of oestrogens and progesterone in blood may be of value in early diagnosis and treatment.

The most promising development in the techniques of cpb has been in the field of radio-immunoassay. An antigen is produced by linking a steroid to a protein and the complex is injected into a foreign species to produce a specific antibody. This reacts in the usual way with the original steroid and this is used in a quantitative assay. When antigens are freely available they should provide methods for steroid assay unequalled for speed, specificity and accuracy.

TABLE 1

Values of 11-hydroxycorticosteroid obtained by extracting 1 ml. plasma or 1 ml. urine with different volumes of methylene chloride.

Volume of methylene chloride	11-hydroxycorticosteroid	
	Plasma	Urine
ml.	μg/100 ml.	μg/24 hr.
4	25.3	190
5	31.1	189
6	30.0	202
7	30.5	202
8	29.2	220
9	-	213

TABLE 2

Values of plasma 11-hydroxycorticosteroids obtained using sulphuric acid-ethanol reagents of different composition.

Percentage composition of reagent	11-hydroxycorticosteroid Plasma. ug/100 ml.
65/35 sulphuric acid-ethanol	17.1
70/30 "	18.7
72.5/27.5 "	18.1
75/25 "	18.9
80/20 "	32.4 (Fluorescence increasing rapidly)

TABLE 3

Values of plasma 11-hydroxycorticosteroids obtained by fluorimetric readings at 8, 16 & 0 min (by extrapolation)

Range ug/100 ml	No. of samples	0 min	8 min	16 min	Difference 8-0	Difference 16-0
0-5	33	3.0	4.2	5.2	1.2	2.2
6-10	29	7.7	9.5	11.0	1.8	3.3
11-15	34	12.6	13.5	14.6	0.9	2.0
16-20	25	17.9	19.0	20.5	1.1	2.6
21-30	20	26.4	27.8	28.5	1.4	2.1
31-40	0	-	-	-	-	-
41-50	7	44.9	45.9	46.0	1.0	1.1
51-70	2	58.5	62.0	61.5	3.5	3.0

Table 4 Sequential plasma 11-hydroxycorticosteroids (cortisol-µg/100 ml) and total oestrogens (µg/24 hr) in the urine of subjects receiving FSH therapy.

SUBJECT Coc											
Specimen No.	1	2	3	4	5	6	7	8	9	10	11
Oestrogens	15	14	20	30	30	12	32	128	183	238	205
Cortisol	15.3	16.4	19.8	15.5	14.5	22.0	20.5	20.0	18.1	17.5	19.0

SUBJECT Da											
Specimen No.	1	2	3	4	5	6	7	8	9	10	11
Oestrogens	4	5	10	5	5	6	10	19	46	188	344
Cortisol	22.1	22.7	17.2	20.4	16.4	15.7	15.6	17.0	20.0	16.0	20.0

SUBJECT Wa										
Specimen No.	1	2	3	4	5	6	7	8	9	10
Oestrogens	13	15	28	43	20	90	42	42	57	78
Cortisol	14.9	19.5	12.8	17.1	9.1	11.4	15.6	12.5	12.5	14.4

SUBJECT Co.HM								
Specimen No.	1	2	3	4	5	6	7	8
Oestrogens	34	25	20	73	268	55	72	178
Cortisol	10.8	16.3	11.4	8.5	13.2	19.5	11.9	14.4

SUBJECT Mi										
Specimen No.	1	2	3	4	5	6	7	8	9	10
Oestrogens	17	53	92	19	13	23	49	38	45	72
Cortisol	14.5	13.3	10.2	13.8	11.9	13.8	10.5	13.3	15.0	17.5

SUBJECT Mo						
Specimen No.	1	2	3	4	5	6
Oestrogens	25	38	57	113	139	424
Cortisol	12.5	10.5	9.3	7.8	10.2	22.0

Table 4 continued

SUBJECT Br

Specimen No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Oestrogens	12	27	26	32	180	65	87	20	12	24	24	59	28	277
Cortisol	15.0	10.3	15.1	13.6	12.4	11.2	16.0	14.4	15.0	15.6	11.5	10.9	18.1	16.9

SUBJECT Bo

Specimen No.	1	2	3	4	5	6	7
Oestrogens	84	196	25	49	48	44	24
Cortisol	16.4	16.7	20.0	15.0	17.0	16.9	16.9

SUBJECT Co

Specimen No.	1	2	3	4	5	6	7
Oestrogens	13	20	11	14	41	57	172
Cortisol	16.5	13.6	16.7	13.6	9.5	12.2	17.0

SUBJECT Br

Specimen No.	1	2	3	4	5	6
Oestrogens	23	34	126	59	135	610
Cortisol	19.7	19.1	17.7	20.4	-	22.9

SUBJECT Ev

Specimen No.	1	2	3	4	5	6
Oestrogens	24	53	69	54	33	87
Cortisol	28.6	23.1	17.6	15.5	20.0	25.1

SUBJECT Ho

Specimen No.	1	2	3	4	5	6
Oestrogens	70	26	33	25	245	66
Cortisol	33.0	25.0	24.0	23.0	25.6	27.5

TABLE 5.

Comparisons of plasma 11-hydroxycorticosteroid estimations by the fluorimetric method and cortisol estimations by cpb.

Subject	Diagnosis	Fluor-metric μg/100ml	CPB	CPB X 100 (%) / Fluorimetric
1	2ndry amenorrhoea (am)	21.5	9.4	44
2	?Addisons (pm)	5.4	3.0	56
3	Adrenal Ca.	51.9	34.0	67
4	?Cushings (am)	22.2	13.0	59
5	Myopathy	25.8	9.9	38
6	Cushingoid (am)	30.0	14.0	47
7	Addisons Before synacthen	27.0	17.7	66
7(a)	After synacthen	50.6	31.0	61
8	?Addison (am)	37.1	19.6	54
8(a)	" (pm)	34.0	20.3	60
9	?Cushings (pm)	6.5	2.0	31
9(a)	" (am)	8.1	5.0	31
9(b)	" after dexamethazone (pm)	10.0	6.5	65
9(c)	" " (am)	15.2	7.3	48
10	Oligomenorrhoea (pm)	19.6	8.6	44
10(a)	" (am)	25.1	16.6	66
11	— (am)	33.9	23.1	68
12	— (am)	40.0	24.2	60
13	— (am)	11.6	7.3	63
14	Cushings after dexamethazone	6.7	2.0	30
15	Hypertension	15.6	9.5	61
16	2ndry Amenorrhoea (am)	21.3	17.3	80
17	?Addisons (am)	25.3	22.2	88
17(a)	" after synacthen	12.4	14.7	118
17(b)	" "	47.6	33.5	70
17(c)	" "	78.7	58.6	74
	Mean (n = 26)	26.3	16.6	59.6

TABLE 6

Total urine oestrogens and plasma oestradiol-17β during gonadotrophin therapy

Subject	Day of Treatment	Treatment	Total urinary oestrogens μg/24 hr.	Plasma oestradiol ng/100 ml.
1	1	150 iu FSH	13	8
	5	150 "	20	8
	8	225 "	34	2
	12	300 "	25	2
	15	375 "	20	1
	19	375 "	73	2
	21	10,000 "HCG	134	36
2	1	1,200 iu FSH	8	<2
	4	1,200 "	21	<2
	8	1,650 "	23	3
	11	1,650 "	69	30
	13	1,500 "HCG	102	91
	15	-	186	109
3	1	525 iu FSH	16	2
	4	675 "	10	12
	8	900 "	19	26
	11	900 "	46	67
	13	10,000 "HCG	188	112

TABLE 6 (Con't)

Subject	Day of treatment	Treatment	Total urinary oestrogens µg/24 hr.	Plasma oestradiol ng/100 ml.
4	1	900 iu FSH	25	-
	4	900 "	25	1.2
	8	1,200 "	38	2.3
	11	1,200 "	57	2.0
	15	1,200 "	113	6.3
	17	5,000 "HCG	139	8.8
	36	-	424	29.2
5	1	2,250 iu FSH	12	0.7
	4	2,250 "	32	0.7
	8	2,920 "	37	-
	11	2,920 "	128	8.8
	13	15,000 "HCG	183	-
	15	-	238	33.5
	18	-	205	40.7

TABLE 7

Total urinary oestrogens and plasma oestriol during pregnancy.

Subject.	Weeks of pregnancy	Total urinary oestrogens mg/48 hr.	Plasma oestriol ug/100 ml.
1	34½	18.7	11.6
	35	23.8	8.0
	36½	23.2	11.9
	37	29.3	-
2	34	21.4	13.0
	34	26.7	9.9
	35	19.9	12.3
	36	27.2	12.4
	37	30.6	-
3	37	21.8	10.4
	37½	20.3	13.2
4	32½	23.4	7.4
	33	30.5	9.6
5	32	9.1	3.0
	32½	3.0	1.0
	33	2.1	1.0
6	Term + ½	30.6	14.1
	" + 1	47.2	13.6
	" + 1½	-	15.7
	" + 2	33.1	-

TABLE 8.

Plasma free oestradiol in plasma during pregnancy
Comparison of iodination and cpb methods.

Subject	Plasma Oestradiol by iodination (ng/ml)	Plasma Oestradiol by cpb (ng/ml)
Hc	17.5	20.0
Gr	18.8	15.8
Ro	17.7	22.5
Ya	15.2	21.7
Ch	15.0	15.0
Wi	13.0	15.2
Fo	13.0	10.0
Fo	10.5	9.0

TABLE 9

Iodination of oestradiol 17B.

Variation in amount of sodium metabisulphite.

Results are shown in counts per minute.

0.24% metabisulphite ml.	Oestradiol 0 ng	Oestradiol 50 ng	Difference
0	642	3226	2584
0.1	254	3716	3462
0.2	284	3796	3512
0.3	252	3782	3530
0.4	396	4456	3060
0.5	278	3236	2958

TABLE 10

Iodination of oestradiol-17β.

Investigation of pH and time during oxidation of $Na^{125}I$ with chloramine-T. Results in cpm.

Time (min)	pH	Oestradiol 0	Oestradiol 50ng	Difference
1	6.1	377	3358	2981
2		353	3538	3185
5		322	3202	2980
1	6.5	3533	6600	3067
2		2686	6540	3854
5		1289	5456	4167
1	7.0	2105	5410	4305
2		1714	6464	4750
5		1924	6526	4602
1	7.5	1950	6586	4636
2		1771	7254	5483
5		1479	6700	5221
1	8.0	1504	6646	5142
2		2217	6554	4337
5		2618	6378	3760

TABLE 11

Solvents for the extraction of iodinated oestradiol-17β

Results in cpm.

Solvent	Oestradiol		Difference
	0	50 ng	
$CHCl_3$	514	2426	1912
Pet. ether	578	4790	4212
Diethyl ether	988	4914	3926
Cyclohexane	696	4512	3816
Benzene	770	4432	3662
Ethyl acetate	1256	5310	4054
Ethylene chloride	542	3148	2606

TABLE 12

Solvents for the extraction of iodinated oestradiol-17β

Results in cpm.

Solvent	Oestradiol		Difference
	0	25ng	
$CHCl_3$	476	1423	947
Pet. ether	363	2615	2252
Diethyl ether	722	2831	2109
Cyclohexane	470	2679	2209
Benzene	867	2744	1877
Ethyl acetate	1615	3622	2007
Ethylene chloride	509	1647	1138
Hexane	445	2911	2466
Pet. ether/ethyl acetate 20/80	1288	3908	2615
40/60	1388	3588	2200
60/40	951	3207	2256
80/20	750	3652	2902

TABLE 13

Iodination of oestradiol-17β

Effect of variation of chloramine-T and metabisulphite

Difference in cpm between 0 and 20 ng oestradiol-17β.

Chloramine T concentration (mg/ml)	Metabisulphite concentration (mg/ml)			
	10	5	2.5	1.25
10	5500	5687	4989	2890
5	5617	6876	7129	7420
2.5	5980	5170	7283	8586
1.25	6178	6752	7418	7416
0.625	2641	4345	3760	3589
0.31	2679	2326	2793	1607
0.16	1456	3962	1430	1602

TABLE 14

Counts obtained with a solution of corticosterone -1,2-T + 0.5% plasma in different scintillation media.

Medium	Time at 4° before counting (hours)	Uncorrected count per minute	A.E.S.	Corrected count per minute
10 ml.Toluene	2	5040	0.9248	9000
PPO/POPOP	6	5000	0.9198	8960
0.1 ml.water	12	4976	0.9136	8980
(2 phase)	24	4976	0.9103	9000
10 ml.Toluene	2	5002	0.9270	8900
PPO/POPOP	6	4947	0.9228	8840
0.5 ml.water	12	4889	0.9127	8760
(2 phase)	24	4864	0.9058	8750
10 ml.Toluene	2	2110	0.5734	7500
PPO/POPOP	6	2080	0.5138	9100
0.5 ml.water	12	2059	0.5100	8900
5 ml.Ethanol	24	2061	0.5084	9100
(1 phase)				
10 ml.Brays	2	2332	0.5627	8400
solution	6	2358	0.5633	8550
0.5 ml.water	12	2352	0.5639	8500
(1 phase)	24	2366	0.5581	8640

A.E.S. = Automatic external standardisation
(A lower figure indicates greater quenching)
Blank counts (without corticosterone -1,2-T) were identical in each system

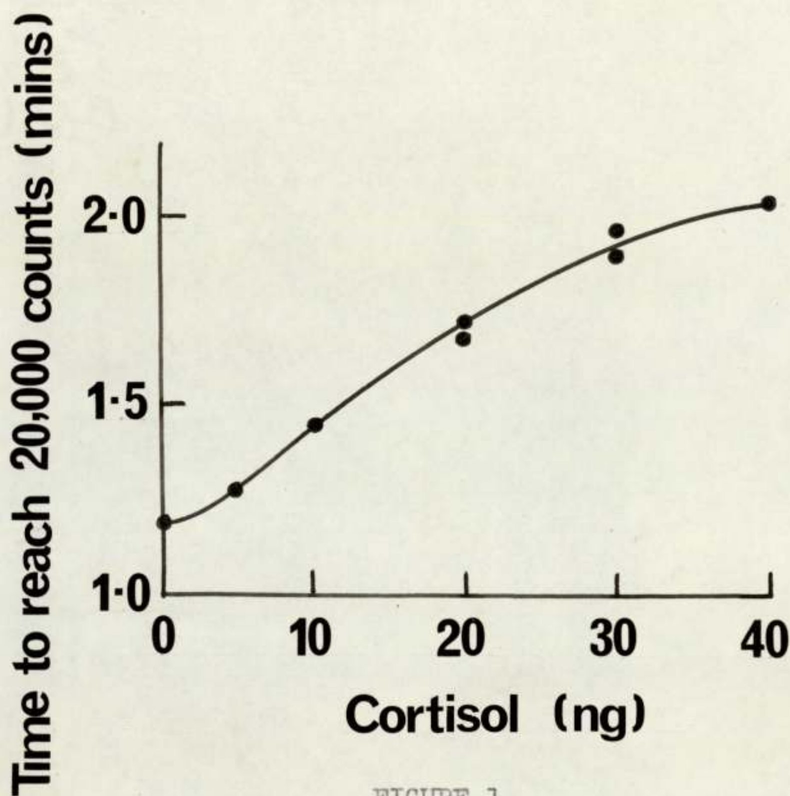


FIGURE 1.

Standard cpb curve for cortisol using 5% v/v human male plasma, tritiated cortisol and Florisil. The amount of tracer bound to protein at each concentration is shown as the time to reach the fixed total amount.

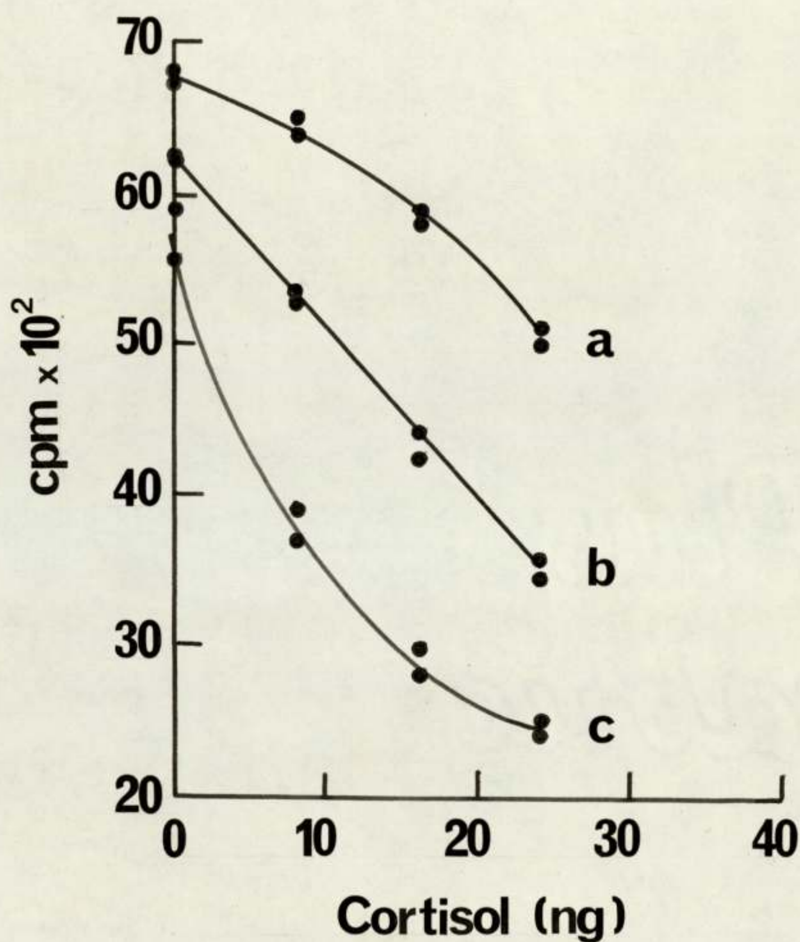


FIGURE 2.

Standard cpb curves for cortisol using plasma from a female treated with ethinyl oestradiol+tritiated cortisol and Florisil. Variation of binding curves with different plasma concentrations in the same total volumes.
Plasma concentrations: (a) 4.0% (b) 2.0% (c) 1.0%

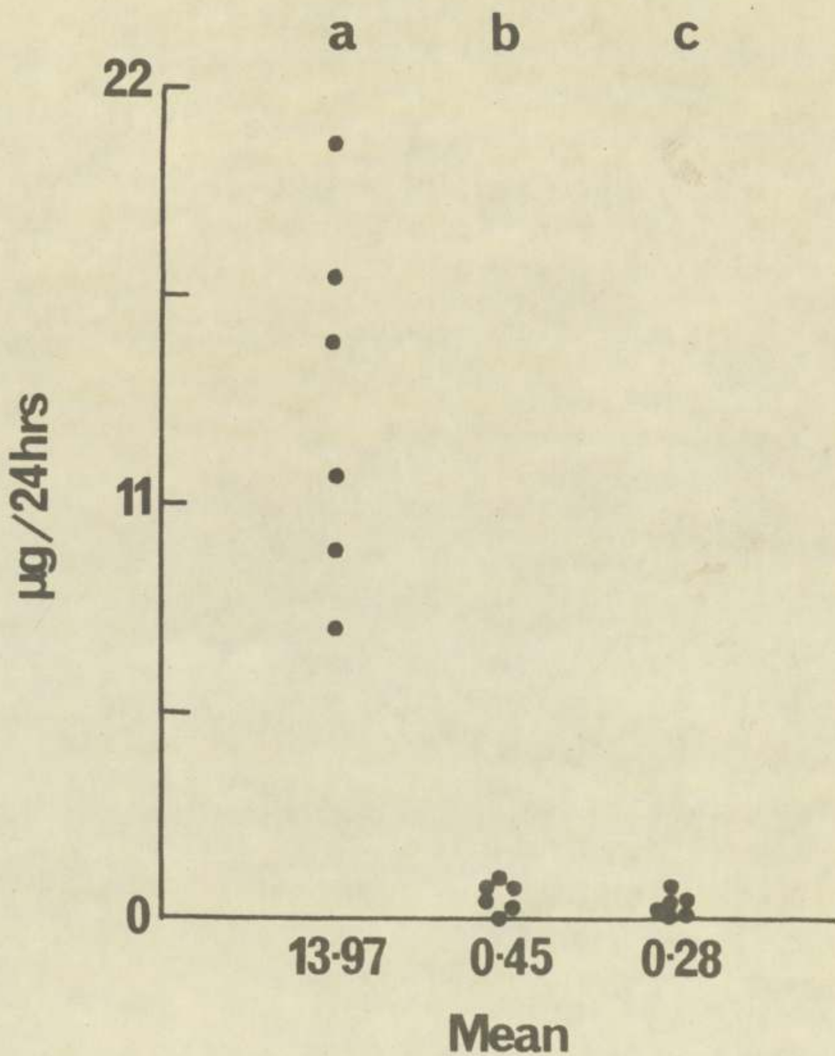


FIGURE 3.

Levels of free cortisol in urine (ug/24 hrs) in six ovariectomised rhesus monkeys. The females treated with oestradiol (a) were given dexamethasone (b) and then testosterone (c)

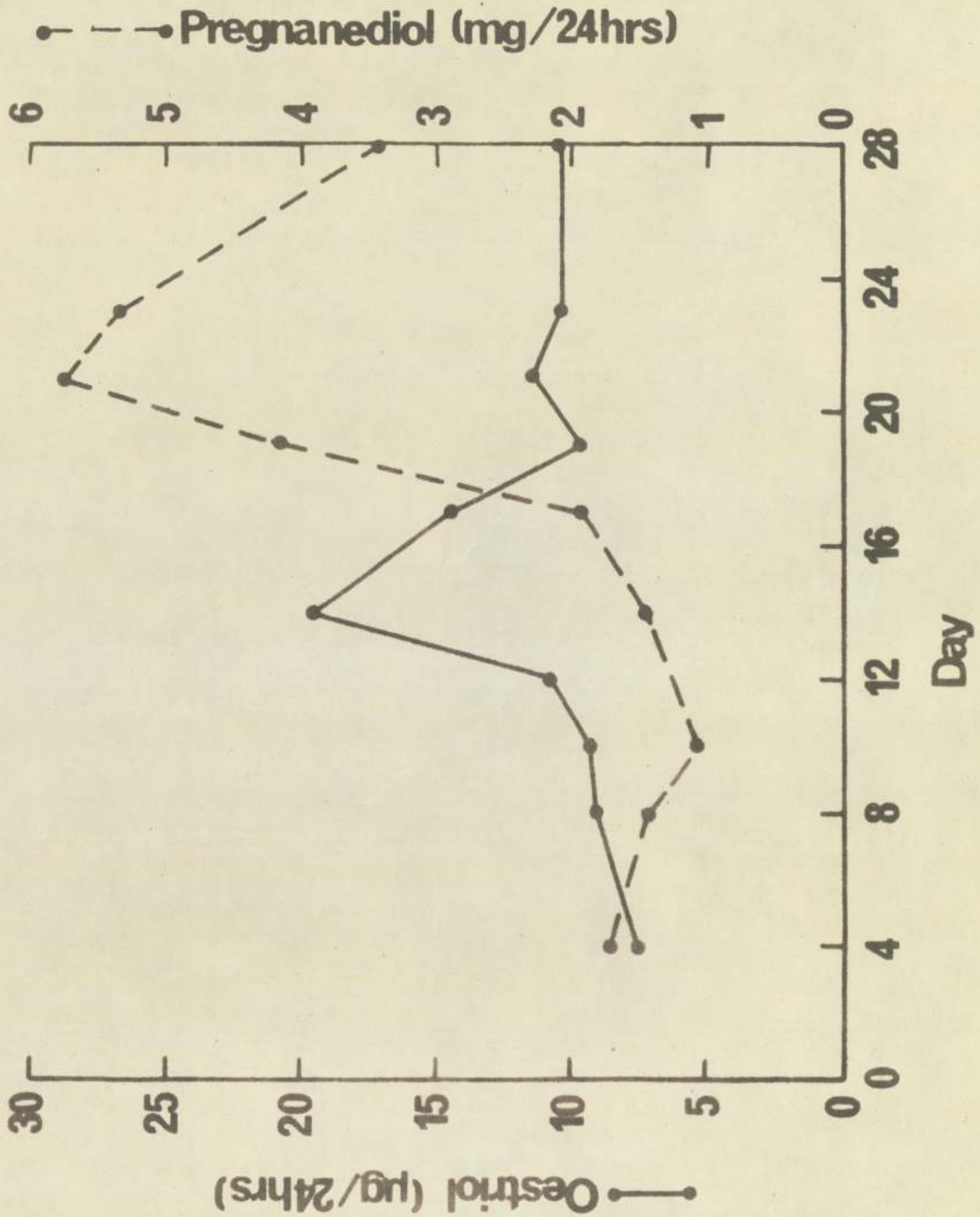


FIGURE 4.

The excretion of oestriol and pregnanediol in urine by a normal woman during one menstrual cycle.

FIGURE 5.

See page 141 .

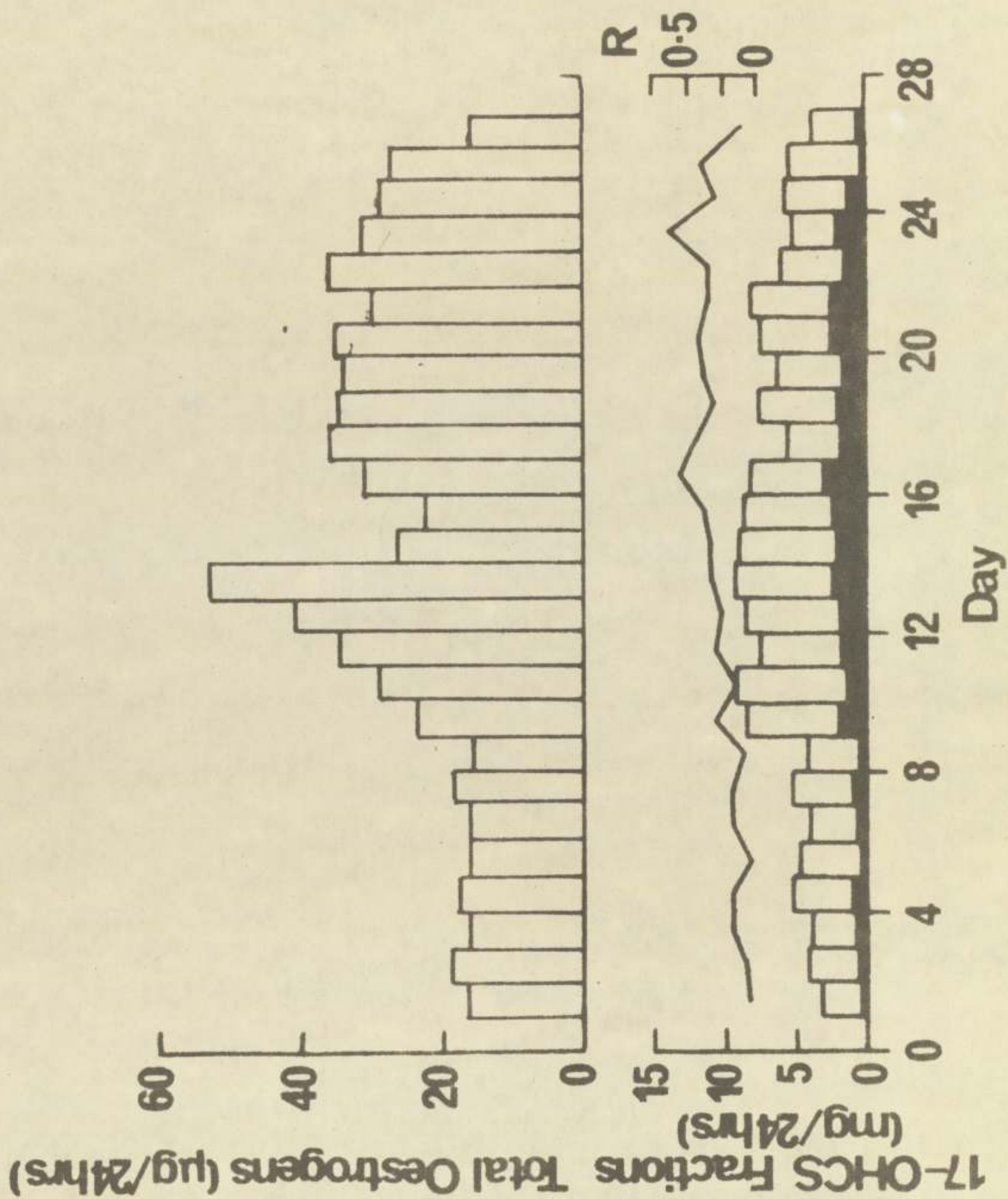
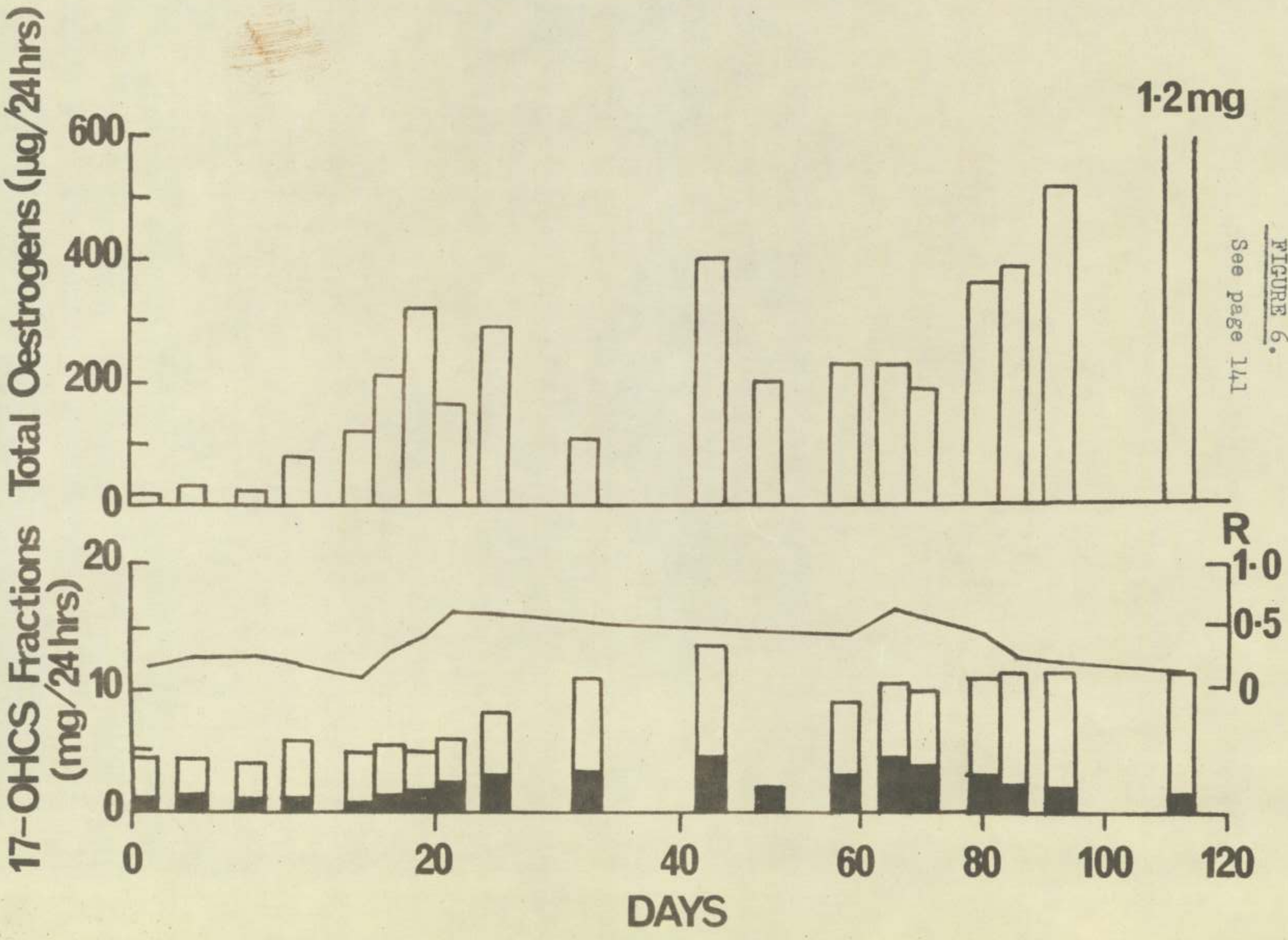


FIGURE 6.

See page 141



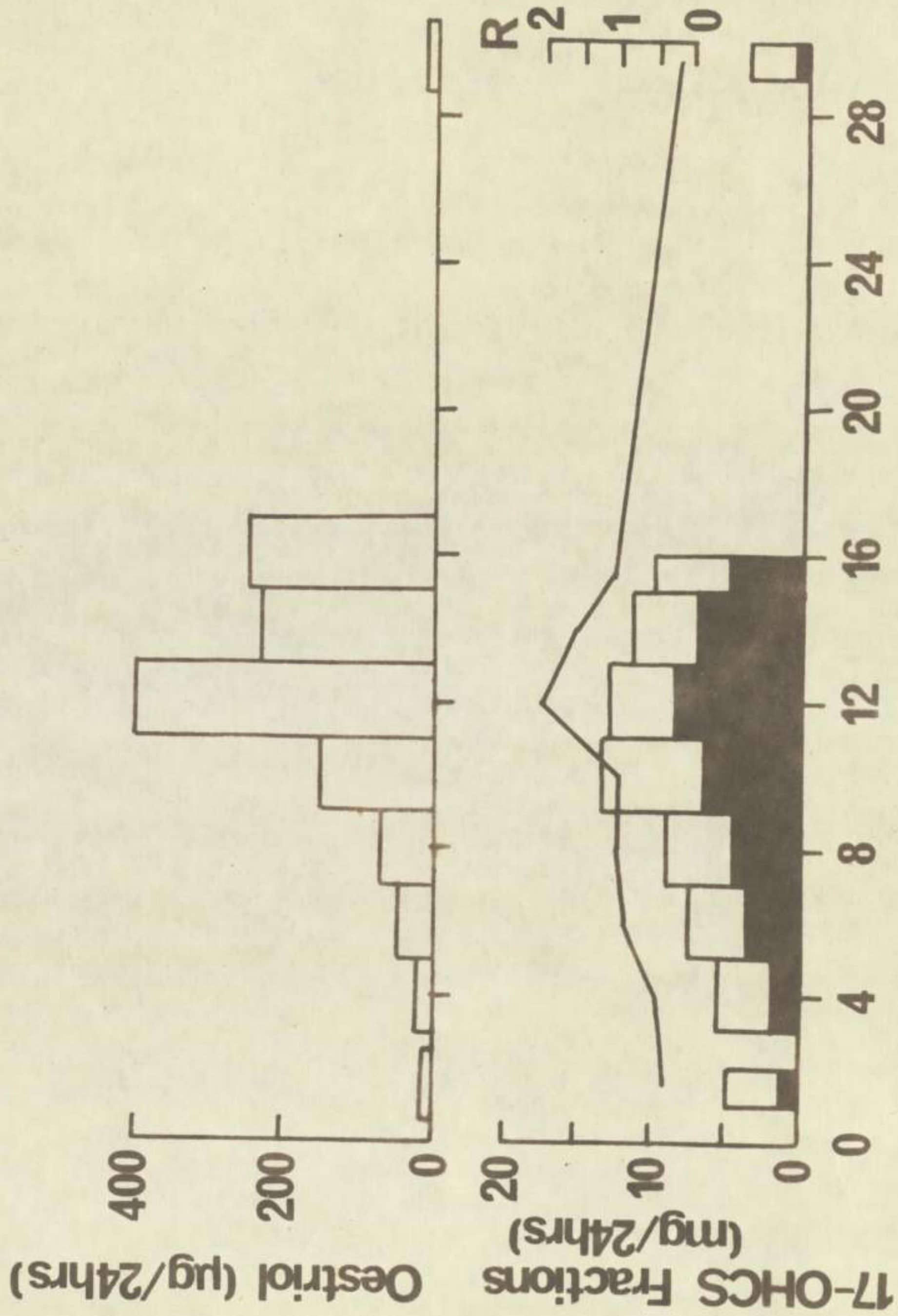


FIGURE 7. See page 141

<u>FIGURE 5</u>. The excretion of total oestrogens and 17-hydroxycorticosteroids (17-OHCS) in urine by a normal woman during one cycle

<u>FIGURE 6</u>. The excretion of total oestrogens and 17-OHCS in urine by a woman during treatment with gonadotrophins. An FSH preparation given on days 1, 4, 8, 11 and 15 (dosage 150, 150, 225, 225 and 150 iu) and HCG (5000 iu) on day 17.

<u>FIGURE 7</u>. The excretion of oestriol and 17-OHCS in urine by a woman receiving gonadotrophin treatment. An FSH preparation (375 iu) was given on each of days 1, 3 and 5 and HCG (4500 iu) on days 1, 3 and 10.

The 11-deoxy-17-hydroxycorticosteroid (pregnanetriol) fraction in represented by ▮, and the 11-oxy-17-hydroxycorticosteroid (cortisol) fraction is represented by ⊓ . R shows the numerical value of the ratio of the pregnanetriol and cortisol fractions indicated by the adjacent line.

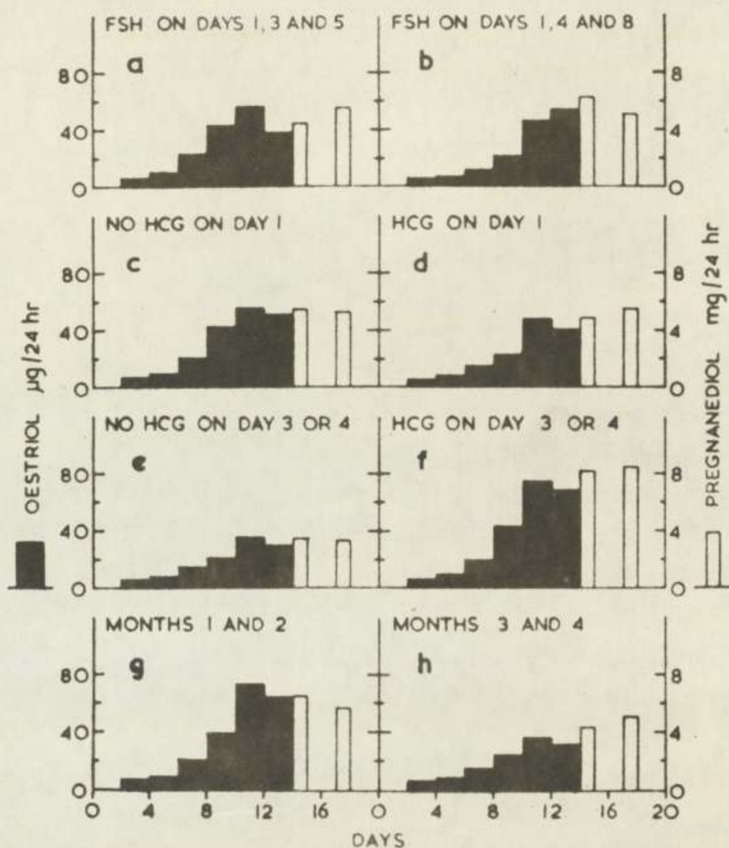


FIGURE 8.

The geometric mean excretion of oestriol and pregnanediol after different treatments with human menopausal gonadotrophins and chorionic gonadotrophin.

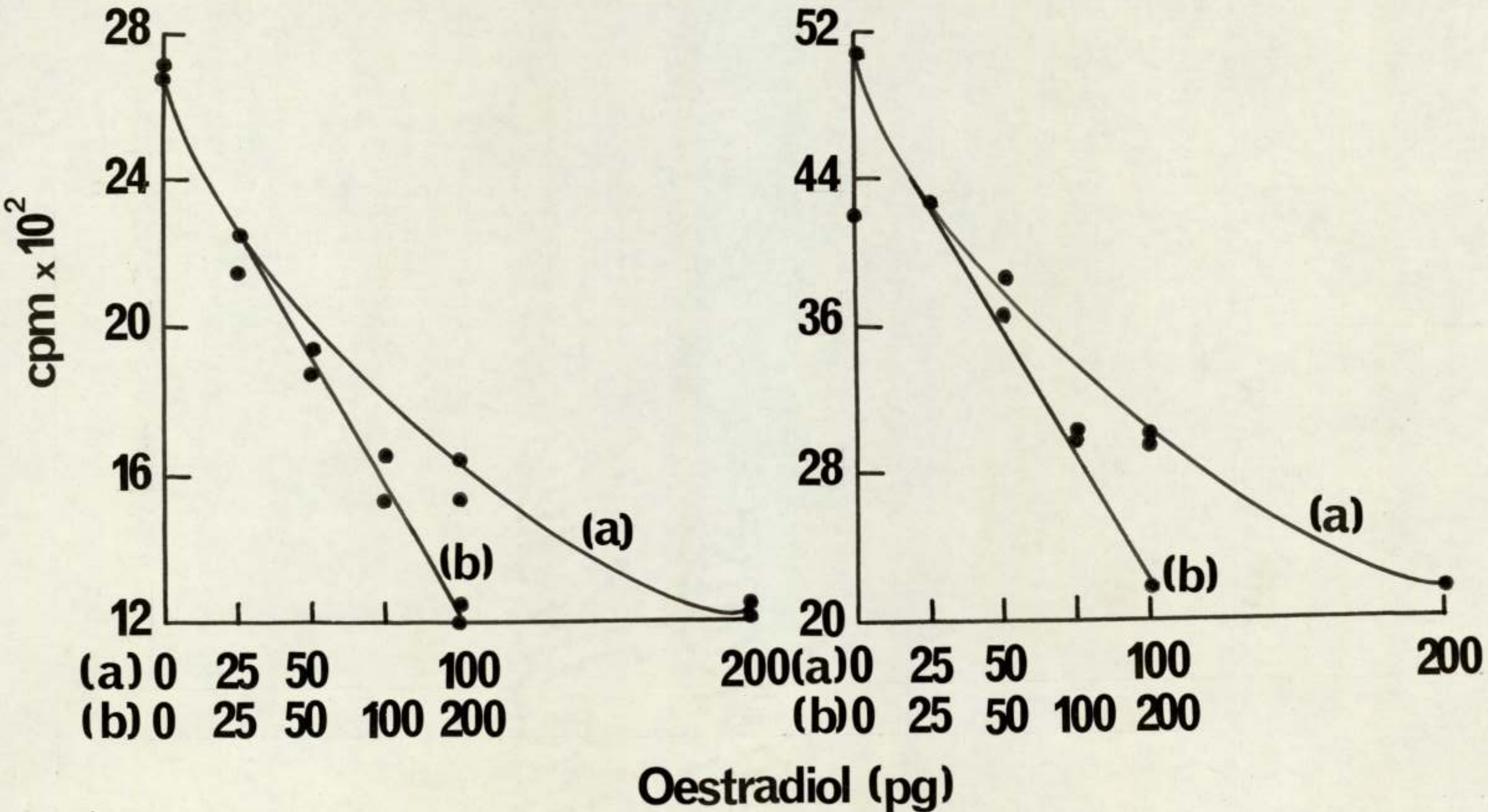


FIGURE 9 (1).

Standard cpb curves for oestradiol using two concentrations of a rabbit uterus preparation
(a) Count of tritiated oestradiol bound at each concentration
(b) Count of tritiated oestradiol plotted against log concentration

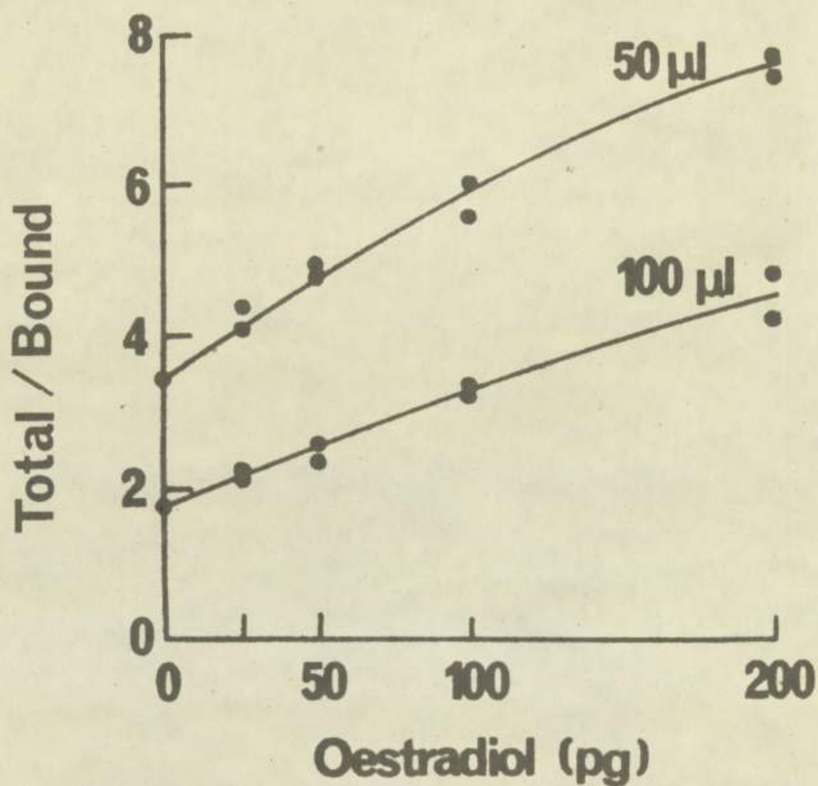


FIGURE 9 (ii).

Standard cpb curves for oestradiol with rabbit preparations as in 9 (i). The ratio of total tritiated oestradiol to bound count is plotted against concentration of oestradiol.

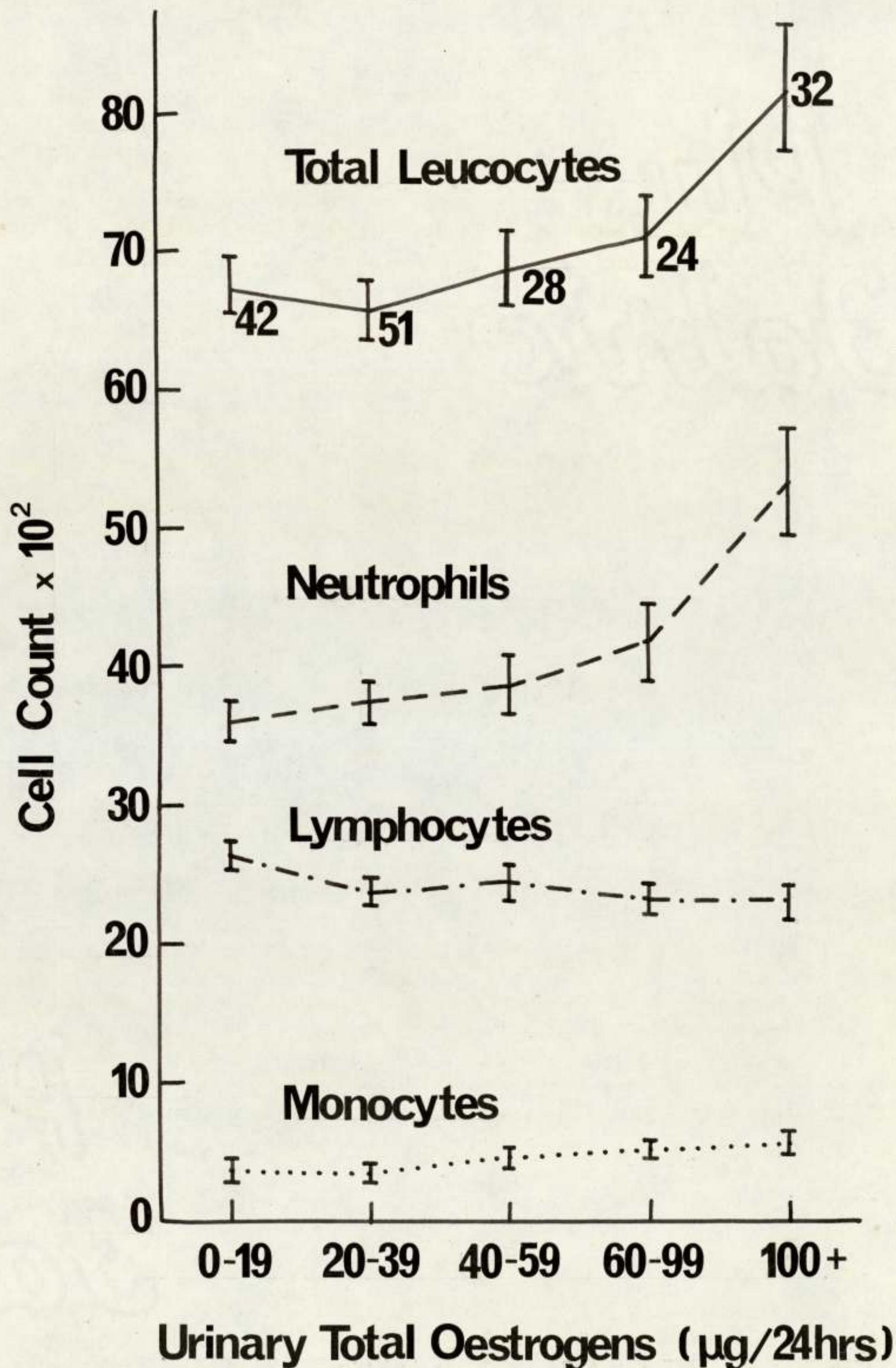


FIGURE 10.

Relationship of total and differential blood leucocyte count (cells per cu.mm.) with urinary total oestrogens (μg/24 hrs). Standard error of mean and numbers in each group are shown.

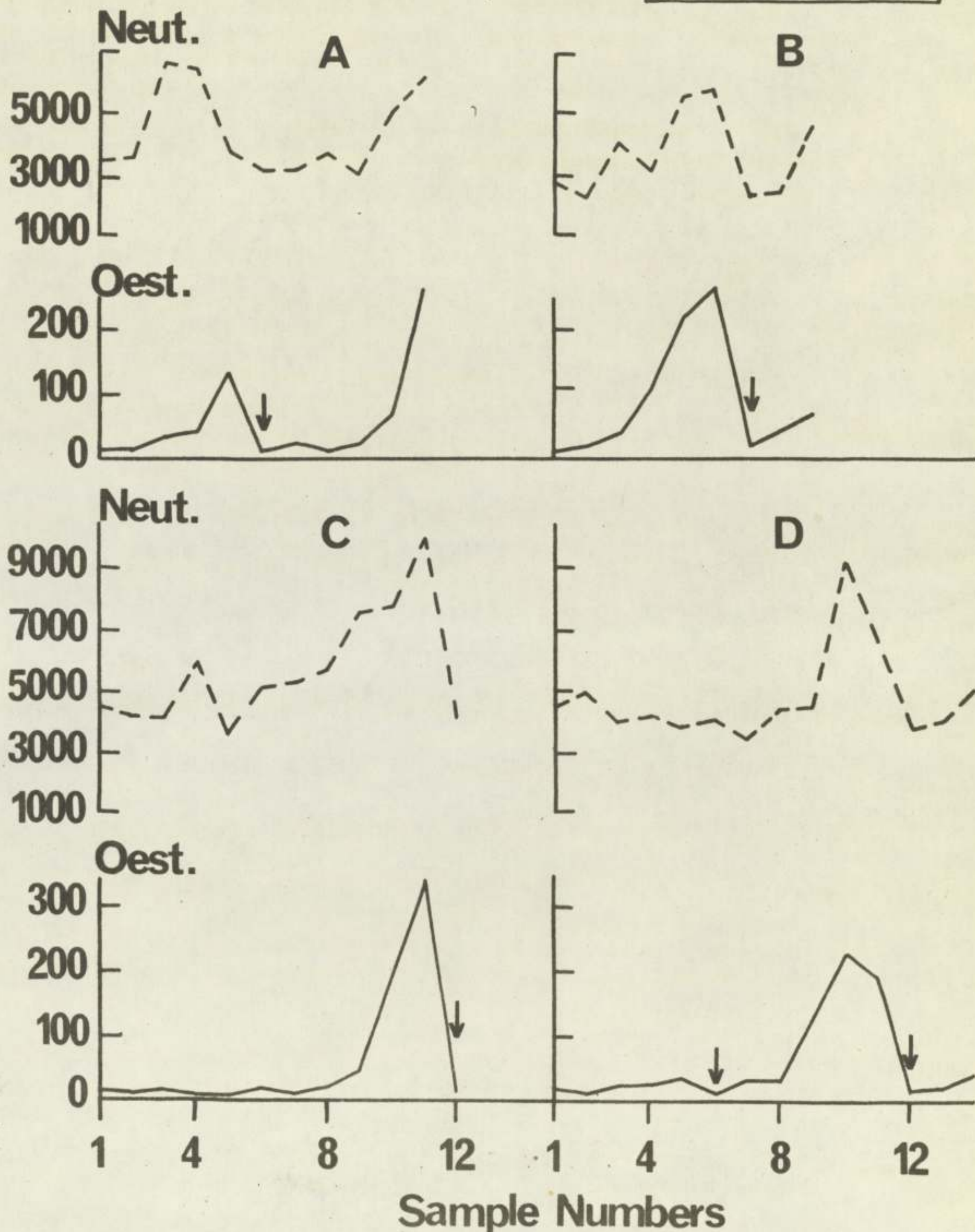


FIGURE 11.

Sequential urinary total oestrogens levels (ug/24 hrs) and blood neutrophil counts (cells per cmm) in four different women.

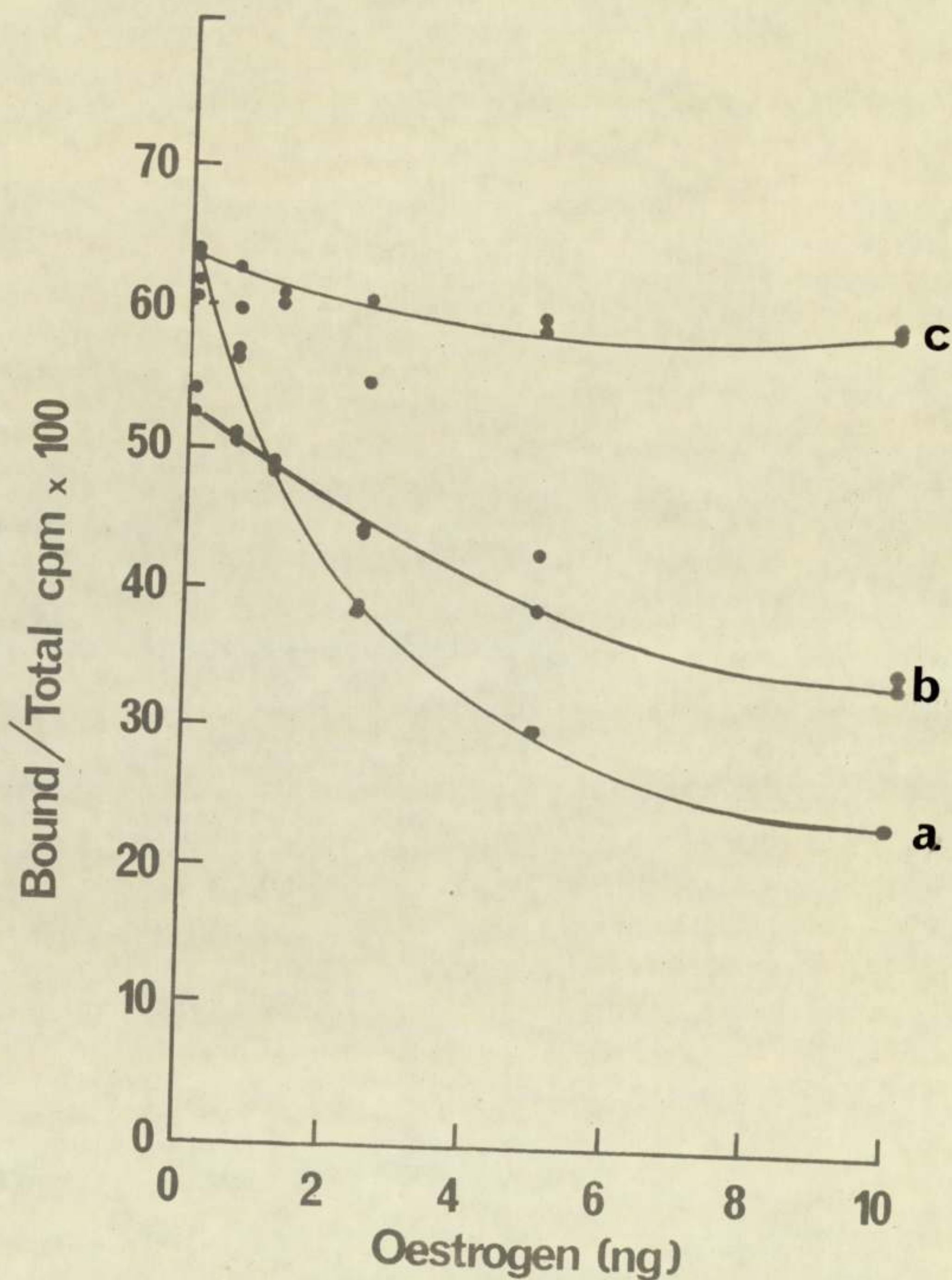


FIGURE 12.

Standard cpb curves for (a) oestradiol-17β, (b) oestrone and (c) oestriol using a globulin fraction from the plasma of a pregnant woman.

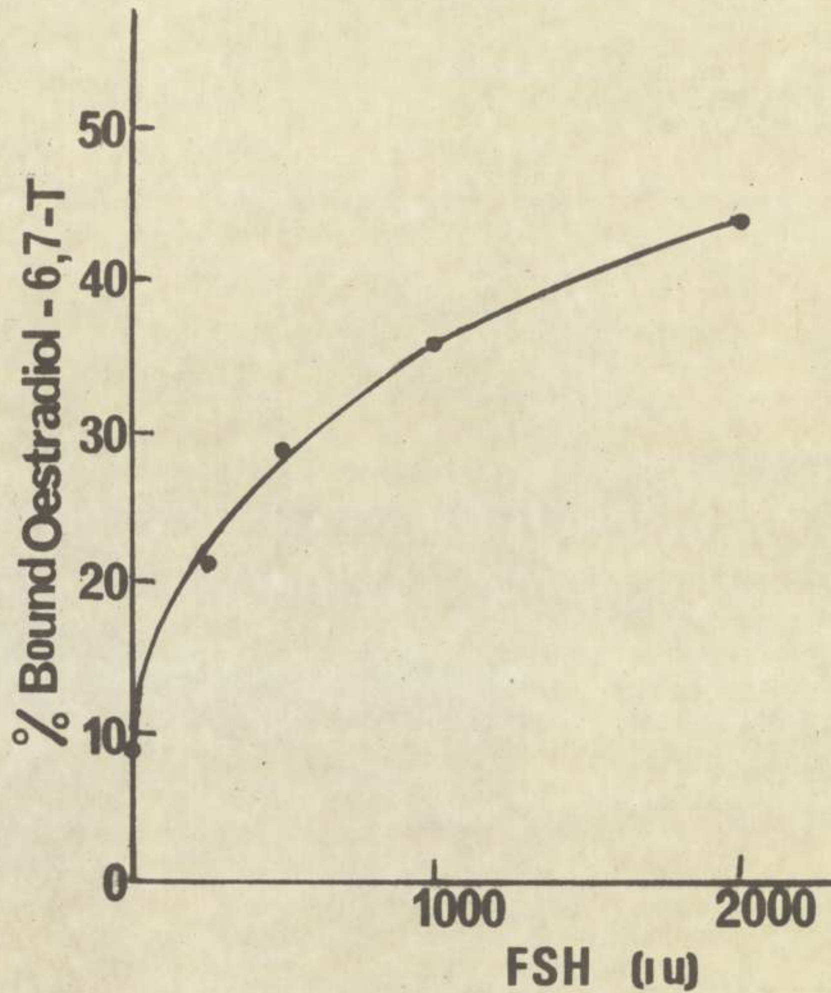


FIGURE 13.

Binding of oestradiol -6, 7-T to Serum gonadotrophin (LEO). Percentage of counts in supernatant (bound) as a function of FSH content of each tube

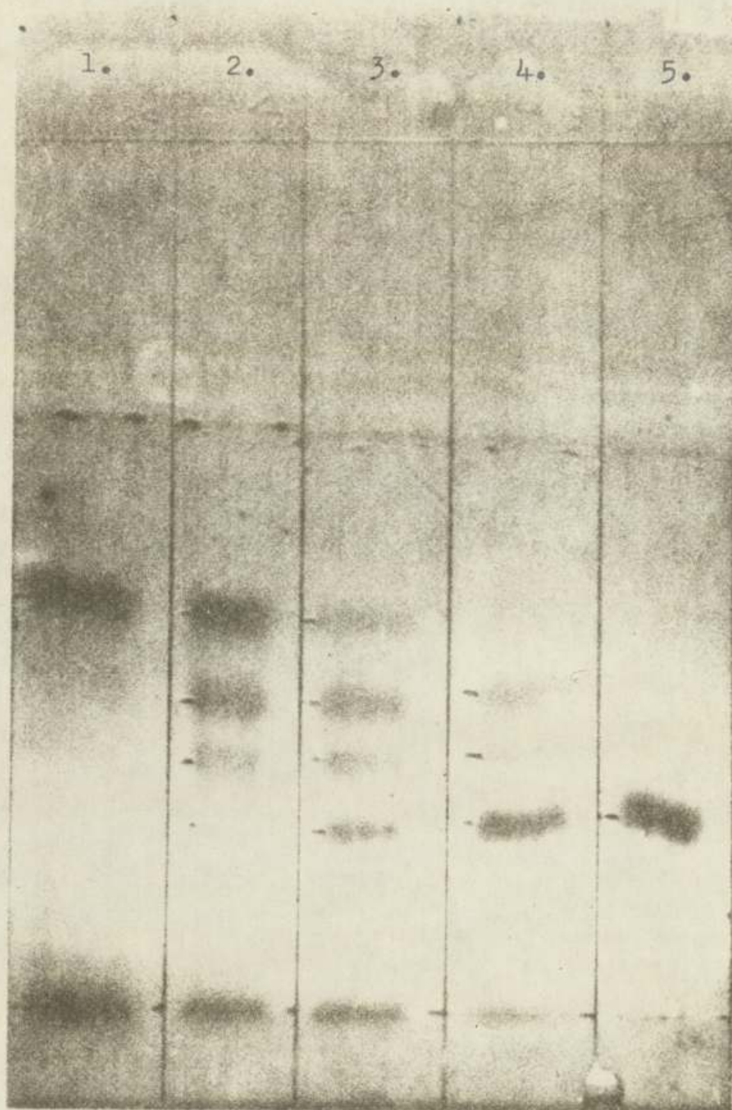


FIGURE 14.

Silica gel TLC plate. Oestradiol was applied to the origin in each lane, exposed to iodine vapour for (1) 20 mins. (2) 10 mins. (3) 5 mins. (4) 2 mins. (5) 0 mins. and then developed in chloroform-ethyl acetate 9:1.

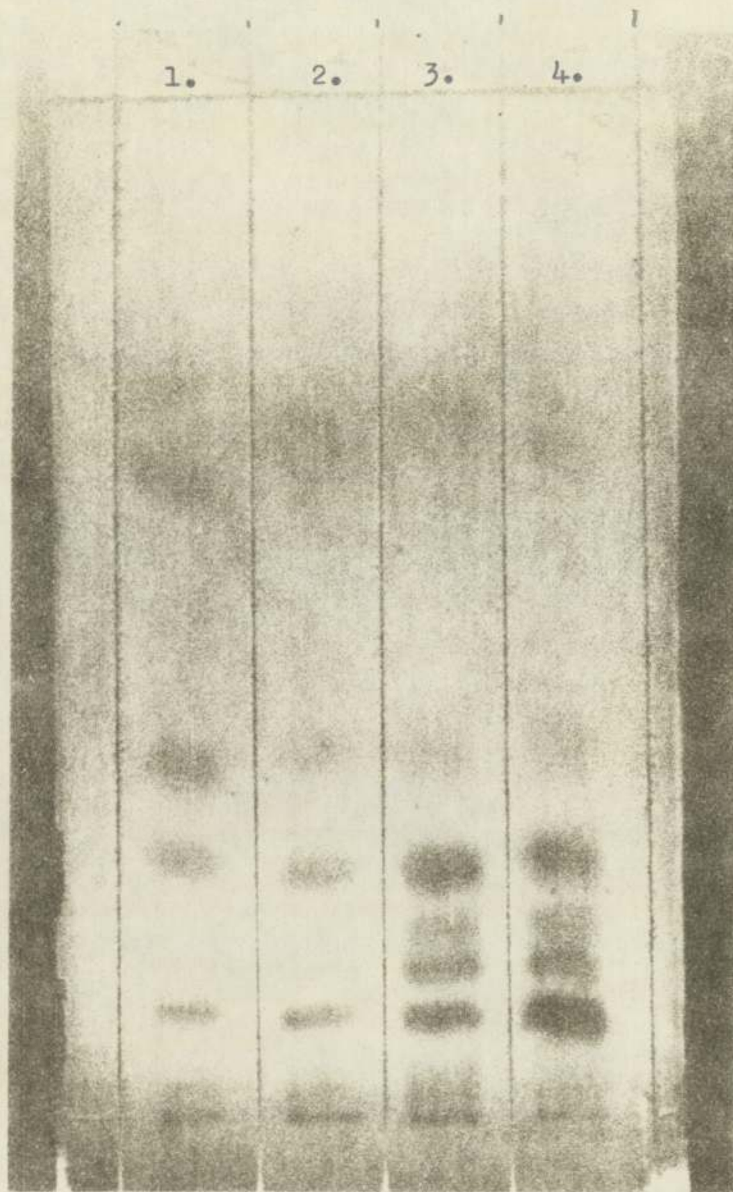


FIGURE 15.

Silica gel TLC plate. Oestradiol was treated with iodine in aqueous solution and then applied to the origin in chloroform-ethyl acetate 9 : 1.

	Lane 1	2	3	4
μg oestradiol	6.25	12.5	25	50.
μg iodine	100	100	100	250

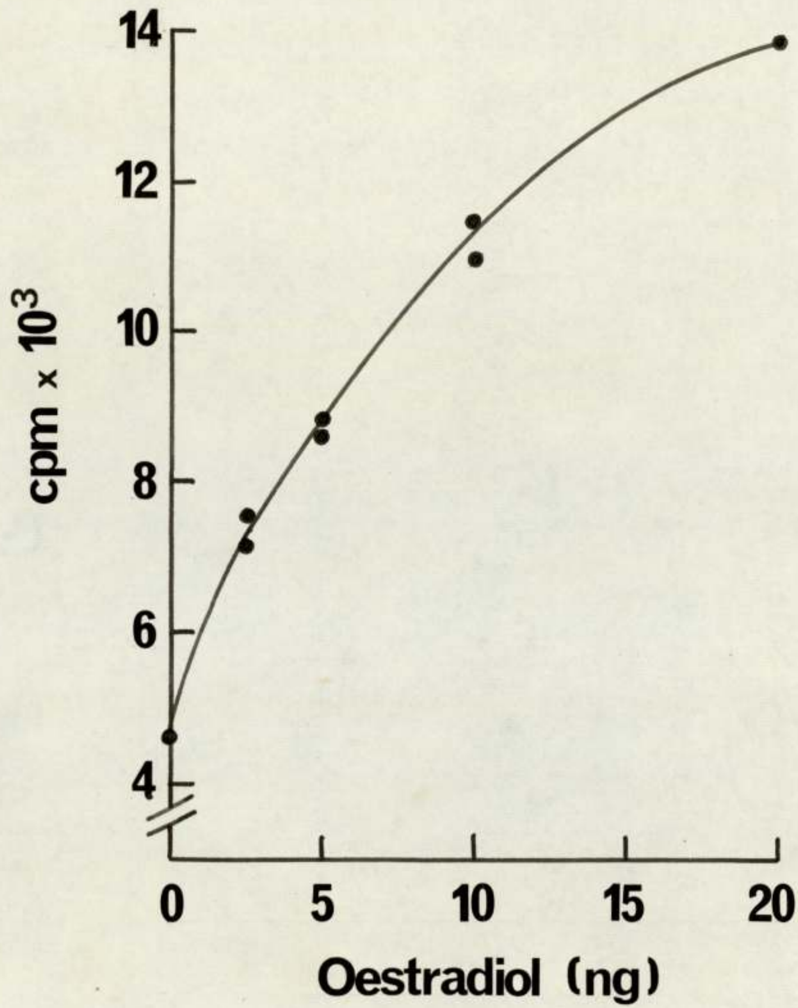


FIGURE 16.

Standard curve for oestradiol after iodination with ^{125}I

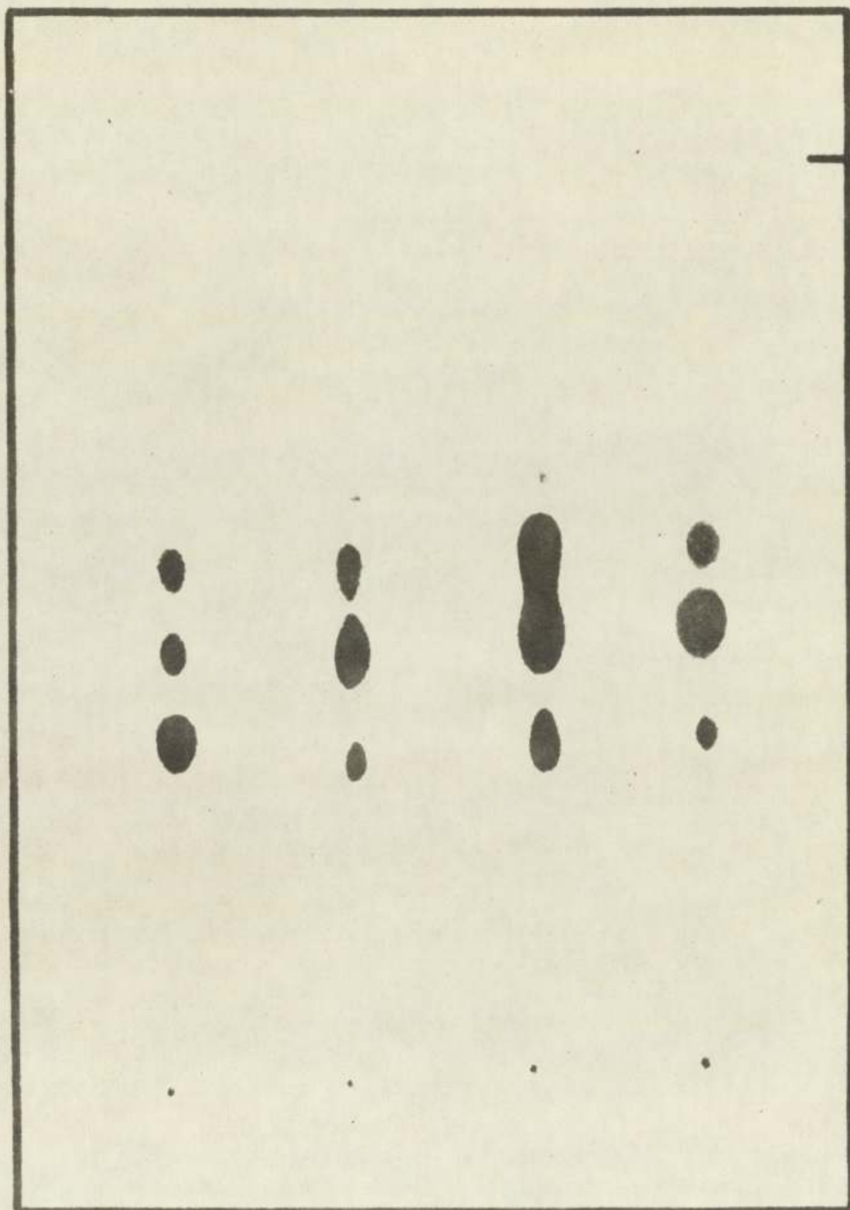


FIGURE 17.

Autoradiograph of silica gel TLC plate. Oestradiol iodinated with ^{131}I applied to the origins and then run in chloroform-ethyl acetate 9:1

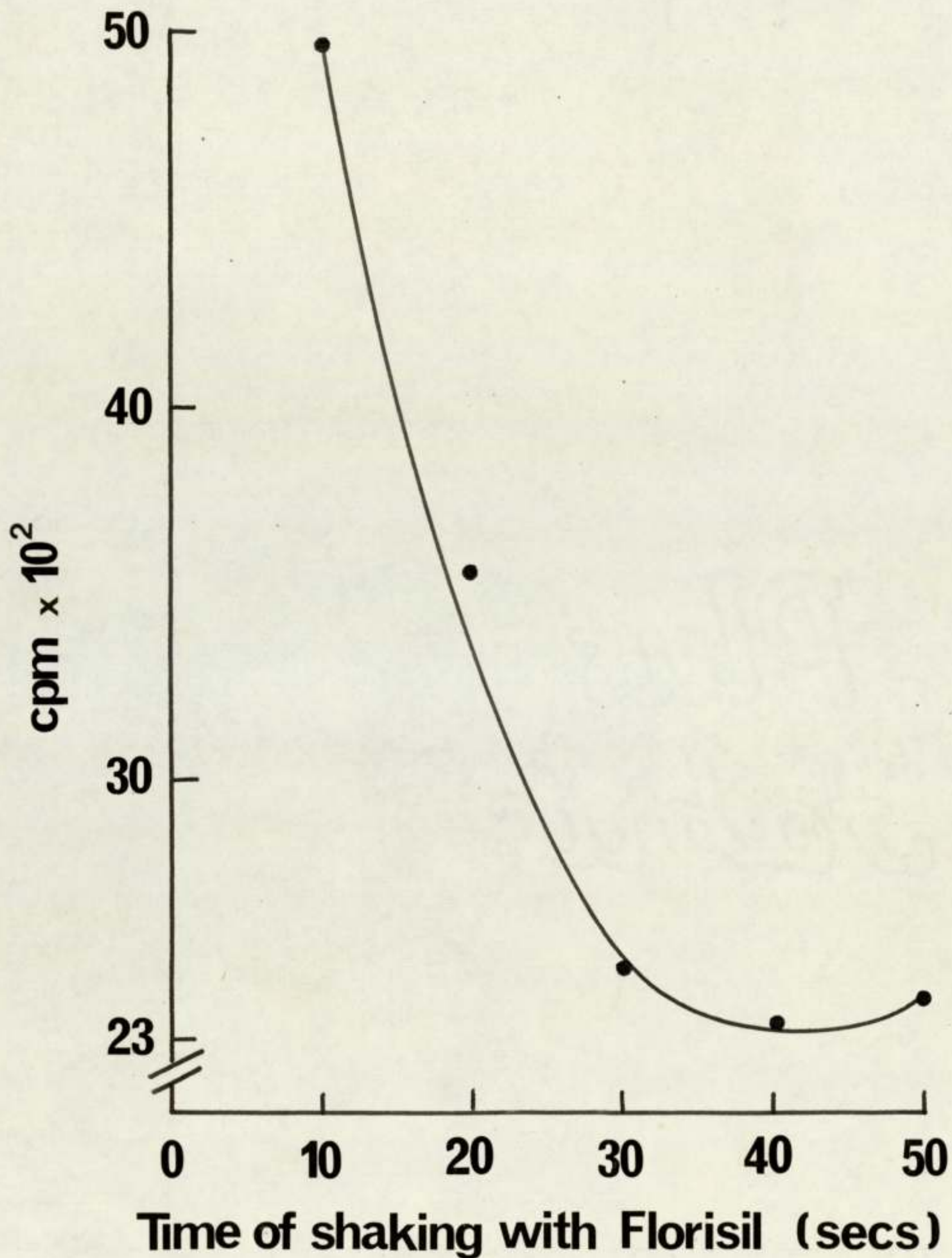


FIGURE 18.

Variation in counts in supernatant solutions containing 0.5% dog plasma, tritiated corticosterone and 1.25 ng progesterone, after shaking with Florisil for different times.

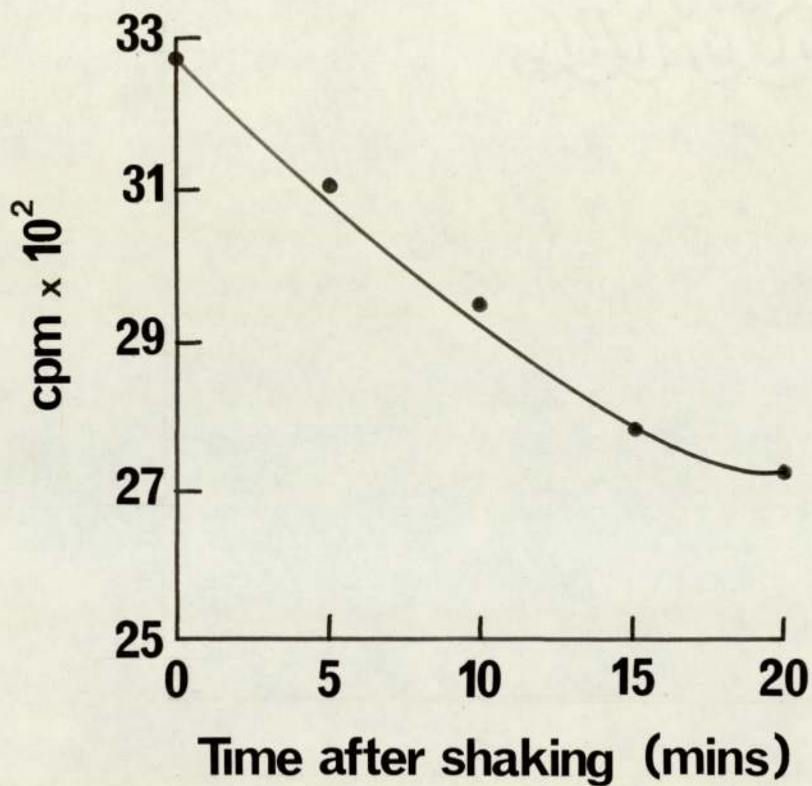


FIGURE 19.

Variation in count in supernatant solutions containing 0.5% dog plasma, tritiated corticosterone and 1.25 ng progesterone. Solutions were shaken with Florisil and then allowed to stand for the times shown.

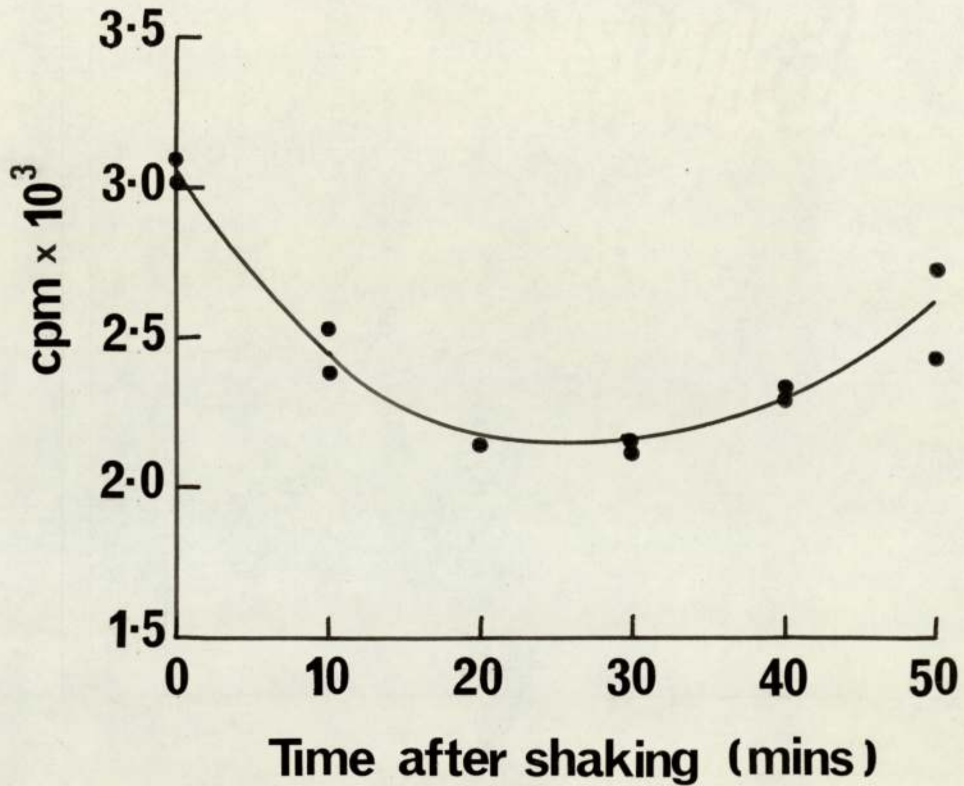


FIGURE 20.

Variation in count in supernatant solutions containing 0.5% dog plasma and tritiated corticosterone. Solutions were shaken with Florisil and then allowed to stand for the times shown.

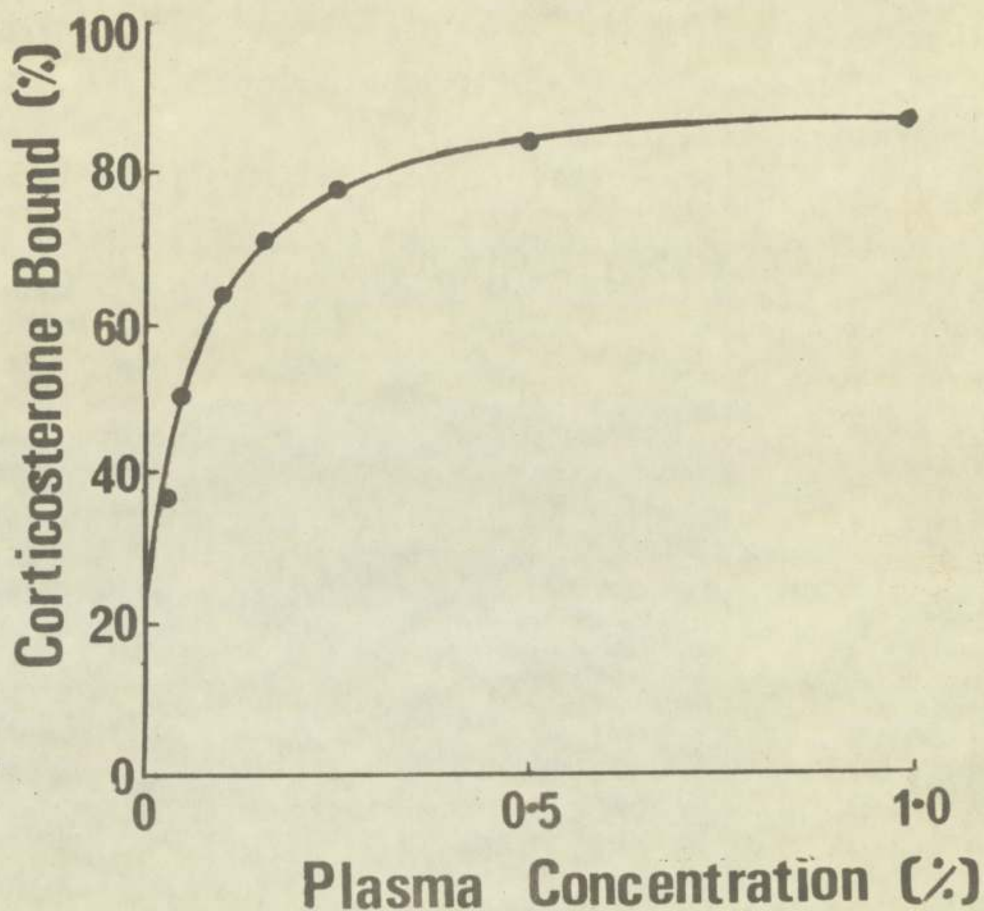


FIGURE 21 Percentage of tritiated corticosterone bound to different concentrations of plasma after shaking with Florisil. Plasma was from a woman treated with ethinyl oestradiol

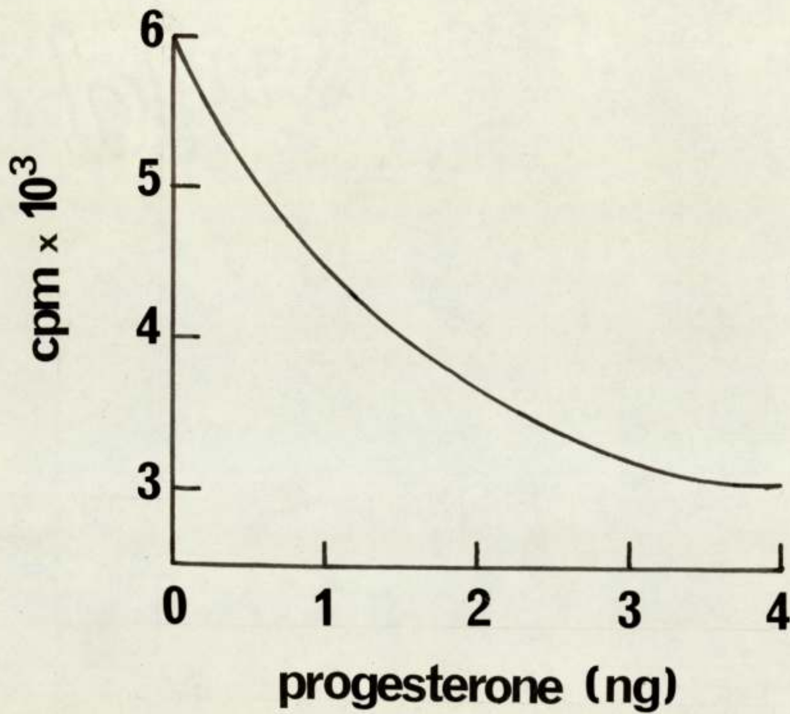


FIGURE 22.

Standard cpb curves for progesterone using tritiated corticosterone, Florisil and plasma (0.1%) from a woman treated with ethinyl oestradiol.

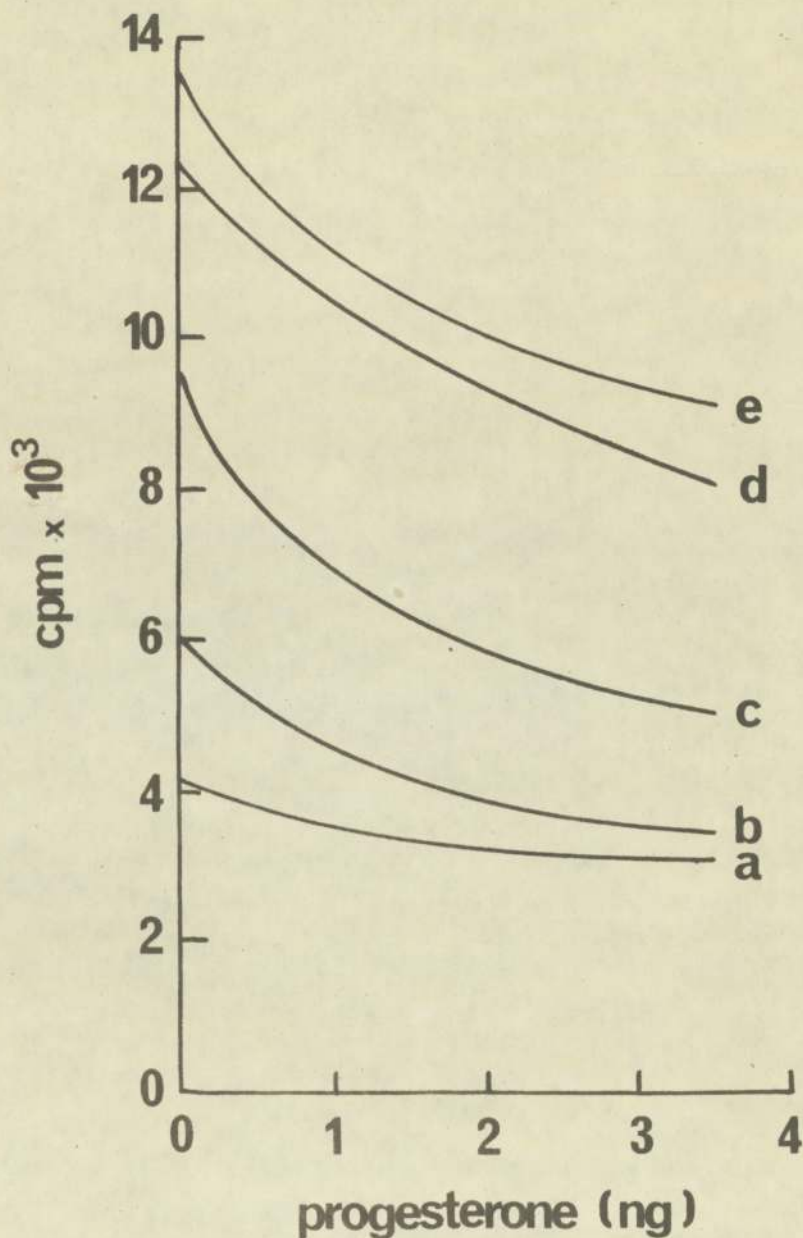


FIGURE 23

Standard cpb curves for progesterone using tritiated corticosterone, Florisil and different concentrations of dog plasma, (a) 0.25%, (b) 0.5%, (c) 1.0%, (d), 2.0%,(e) 2.5%

O.E.263 A

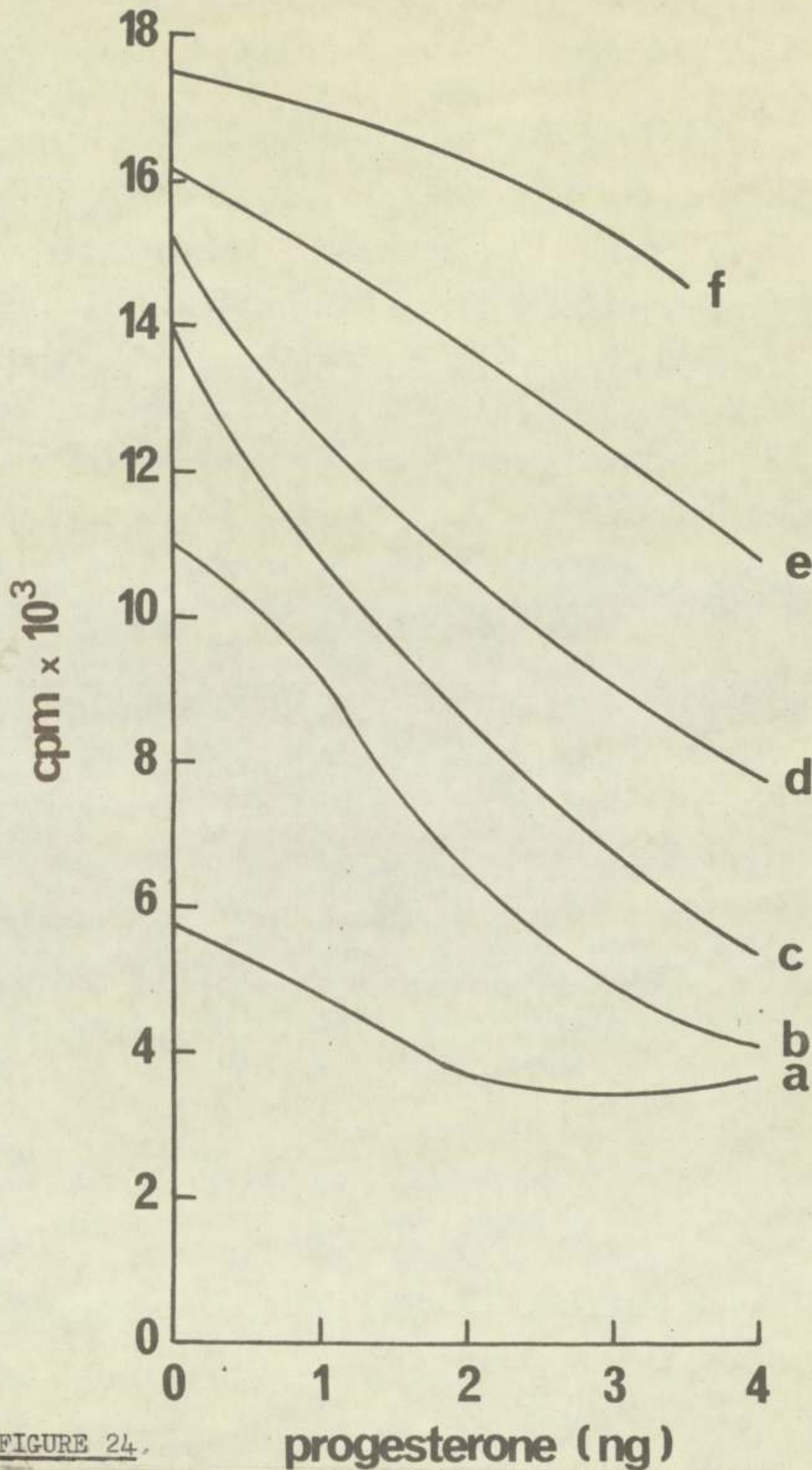


FIGURE 24.

Cpb curves for progesterone using tritiated corticosterone, Florisil and different concentrations of plasma from a woman (late pregnancy) (a) .0625%, (b) 0.125%, (c) 0.2%, (d) 0.25%, (e) 0.5, (f) 1.0%

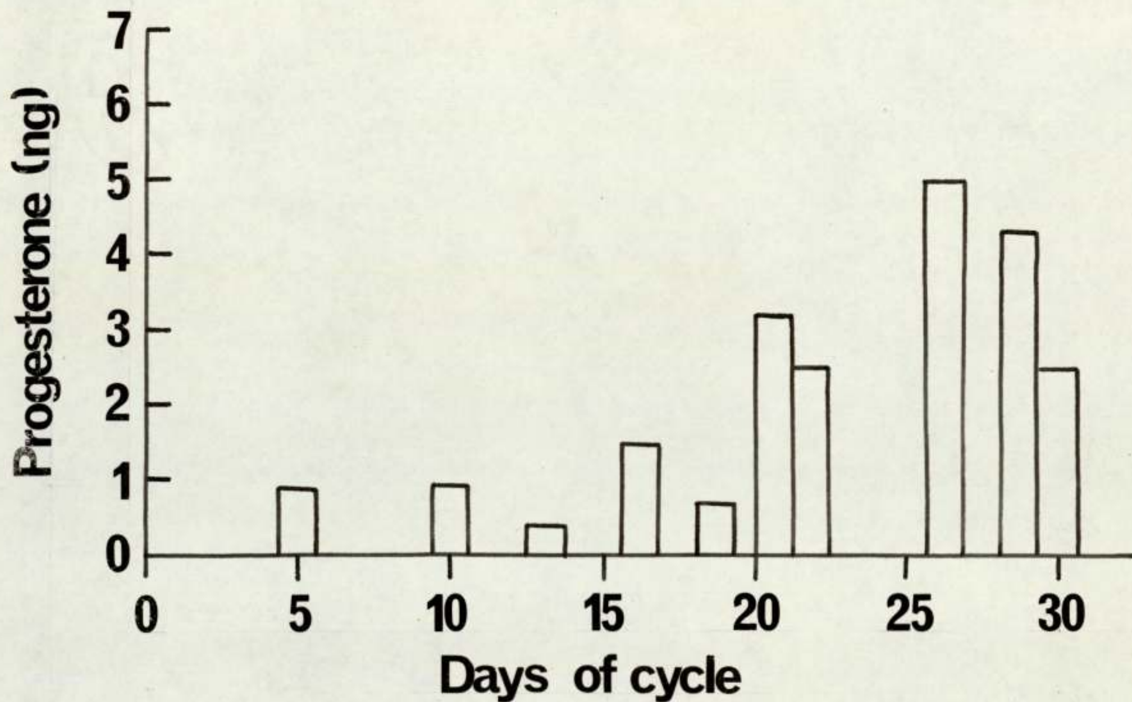


FIGURE 25.

Progesterone (ng/ml) in blood of a normal rhesus monkey during one cycle.

ABBREVIATIONS.

FSH	Follicle stimulating hormone
LH	Luteinising hormone
HCG	Human chorionic gonadotrophin
ACTH	Adrenocorticotrophic hormone
17α-Hydroxypregnanolone	3α, 17α-dihydroxy-5β-pregnane-20-one
Pregnanediol	5β-pregnane-3α, 20α-diol
Pregnenolone	3β-hydroxypregn-5-en-20-one
17α-Hydroxypregnenolone	3β, 17α-dihydroxypregn-5-en-20-one
Progesterone	pregn -4-ene-3, 20-dione
17α-Hydroxyprogesterone	17α-hydroxy-pregn-4-ene-3, 20 dione
Androstenedione	androst-4-ene-3, 17-dione
Androstenediol	androst-4-ene-3β, 17β-diol
Dehydroepiandrosterone (DHA)	3β-hydroxyandrost-5-en-17-one
Testosterone	17β-hydroxyandrost-4-en-3-one
Aetiocholanolone	3α-hydroxy-5β-androstan-17-one
Oestriol	oestra-1,3,5 (10)-triene-3, 16 , 17β-triol
Oestrone	3-hydroxyoestra-1,3,5 (10)-trien-17-one
Oestradiol-17β	oestra-1,3,5 (10)-triene-3,17β-diol

Cortisol	11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione
Corticosterone	11β, 21-dihydroxypregn-4-ene-3, 20-dione
Aldosterone	11β, 21-dihydroxy-18-al-pregn-4-ene-3, 20-dione
Pregnanetriol	5β-pregnane-3α, 17α, 20α-triol
Pregnenetriol	pregn-5-ene-3β, 17α, 20α-triol
Dexamethasone	9α-fluoro-16α-methylprednisolone
Prednisolone	11β,17α, 21-trihydroxypregna-1, 4-diene-3, 20-dione
PPO	2, 5-diphenyloxazole
Dimethyl POPOP	1, 4-bis-2 (4-methyl-5-phenyloxazoyl) benzene

Acknowledgments.

I wish to thank my supervisors, Professor A. J. Matty and Professor W. R. Butt, for their advice and encouragement during the preparation of this thesis.

I am grateful to Dr. A. C. Crooke for permission to carry out the work in his Department and for many helpful discussions.

Dr. C. S. Corker kindly made available to me his methods before publication, and supervised some of the work in his laboratory.

All the members of the Endocrine Dept., Birmingham & Midland Hospital for Women have been generous with their help and advice and it has been a pleasure to work with them.

REFERENCES

Allan, R. N. and Alexander, M. K. (1968): J. Clin. Path. 21, 691.

Abraham, G. and Odell, W. (1970) In: Peron F. and Caldwell B. V. Eds. Immunological Methods in Steroid Determination, Worcester Foundation Symposium. (In press)

Antoniades, H. N., Pennell, R. B., Slaunwhite, W. R. Jr. and Sandberg, A. A. (1957): J. Biol. Chem. 229, 1071.

Ausiello, D. A. and Sharp, G. W. G. (1968): Endocrinology 82, 1163.

Baird, D. T. (1968): J. Clin. Endocr. 28, 244.

Barakat, R. M. and Ekins, R. P. (1961): Lancet 2, 25.

Barrett, G. C. (1962): Nature, 194, 1171.

Beardwell, C. G., Burke, C. W. and Cope, C. L. (1968): J. Endocr. 42, 79.

Bischoff, F. and Stauffer, R. D. (1957): Am. J. Physiol. 191, 313.

Bowman, R. E. and de Luna, R. F. (1968): Anal. Biochem. 26, 465.

Brante, G. (1949): Nature, 163, 651.

Braunsberg, H. and James, V. H. T. (1961): J. Clin. Endocr. 21, 1146.

Bray, G. A. (1960): Anal. Biochem. 1, 279.

Brodie, A. H., Shimizu, N., Tait, S. A. S. and Tait, J. F. (1967): J. Clin. Endocr. 27, 997.

Brown, J. B. (1954): J. Obstet. Gynaec. Br. Commonw. 66, 795.

Brown, J. B. (1955): Biochem. J. 60, 185.

Brown, J. B., Evans, J. H., Adey, F. D., Taft, H. P. and Townsend, L. (1969): J. Obstet. Gynaec. Br. Commonw. 76, 289.

Brown, J. B., Macnaughton, C., Smith, M. A. and Smyth, B. (1968): J. Endocr. 40, 175.

Brown, J. B. and Matthew, G. D. (1962): Recent Prog. Horm. Res. 18, 337.

Brown, P.S. (1955): J.Endocr. 13, 159.

Brown, P.S. (1956): J.Endocr. 14, 129.

Brunelli, B. (1935): Arch. Intern. Pharmacodynamie, 49, 262.

Burger, H.C., Kent, J.R. and Kellie, A.E. (1964): J. Clin. Endocr. 24, 432.

Burke, C.W. (1969): Brit. Med. J. 2, 798.

Bush, I.E. (1952): Biochem. J. 50, 370.

Bush, I.E. (1957): In: G.E.Wolstenholme. Ed. Ciba Foundation Colloquia on Endocrinology, Blakiston, Philadelphia, Vol.XI,p.263.

Butt, W.R. (1967): In: The Chemistry of the Gonadotrophins, American Lecture Series, pub. C.C.Thomas, Springfield, Ill. p.96.

Butt, W.R., Crooke, A.C. and Cunningham, F.J. (1961): Biochem. J. 81, 596.

Butt, W.R., Crooke, A.C., Cunningham, F.J. and Palmer, R.F. (1963): J. Endocr. 26, 303.

Butt, W.R. and Kinnear, A.A. (1956): J. Endocr. 13, xxviii.

Butt, W.R., Morris, P., Morris, C.J.O.R. and Williams, D.C. (1951): Biochem, J. 49, 434.

Casey, J.H. and Kellie, A.E. (1964): J. Endocr. 29, iv.

Coghlan, J.P. and Scoggins, B.A. (1967): J. Clin. Endocr. 27, 1470.

Corker, C.S. and Exley, D. (1969): J. Endocr. 43, xxx.

Corker, C.S. and Exley, D. (1970): Steroids, 15, 469.

Corker, C.S. (1970): Personal communication.

Cox, R.K. (1969): J. Reprod. and Fert. 19, 386.

Crooke, A.C. (1970): Brit. Med. Bull. No. 1, 26, 17.

Crooke, A.C., Butt, W.R. and Bertrand, P.V. (1966):
Acta endocr. (Kbh) Supp. 111.

Crooke, A.C., Butt, W.R., Bertrand, P.V. and Morris, R. (1967a):
Proc. R. Soc. Med. 60, 656.

Crooke, A.C., Butt, W.R., Bertrand, P.V. and Morris, R. (1967b):
Lancet 2, 636.

Crooke, A.C., Butt, W.R., Bertrand, P.V. and Morris, R. (1968):
Lancet 2, 180.

Crooke, A.C., Bertrand, P.V., Butt, W.R. and Morris, R. (1970):
In: Irvine, W.J. ED. Reproductive Physiology, E. & S. Livingstone Ltd., Edinburgh, p. 47.

Crooke, A.C., Butt, W.R., Palmer, R., Morris, R., Edwards, R.L., Taylor, C.W. and Short, R.V. (1963): Brit. Med. J. 1, 1119.

Crooke, A.C., Butt, W.R., Palmer, R.F., Bertrand, P.V., Carrington, S.P., Edwards, R.L. and Anson, C.J. (1964):
J. Obstet. Gynaec. Br. Commonw. 71, 571.

Crooke, A.C., Butt, W.R., Palmer, R.F. and Morris, R. (1962):
Acta endocr. (Kbh) Suppl. 67, 132.

Crooke, A.C., Butt, W.R., Palmer, R.F., Morris, R., Edwards, R.L. and Anson, C.J. (1963): J. Obstet. Gynaec. Br. Commonw. 70, 604.

Cruickshank, J.M. (1970a): J. Obstet. Gynaec. Br. Commonw. 77, 640.

CRuickshank, J.M. (1970b): J. Obstet. Gynaec. Br. Commonw. 77, 644.

Cruickshank, J.M. and Alexander, M.K. (1970): Br. J. Haematology (In press).

Cruickshank, J.M., Morris, R., Butt, W.R. and Crooke, A.C. (1970):
J. Obstet. Gynaec. Br, Commonw. 77, 634.

Daughaday, W.H. (1958): J. clin. Invest. 37, 511.

Daughaday, W.H., Holloszy, J. and Kozak Mariz, I. (1961): J. Clin. Endocr. 21, 51.

De Moor, P. and Steeno, O. (1963): J. Endocr. 28, 59.

Diczfalusy, E. and Palmer, R.F. (1967): Europ. Rev. Endocrin. Suppl. 2, part 2, p. 337.

Diczfalusy, E. and Magnusson, A.M. (1958): Acta endocr. (Kbh), 28, 169.

Doe, R.P., Dickinson, P., Dinneman, H.H., and Seal, V.S. (1969): J. Clin. Endocr. 29, 1.

Dufau, M., Dulmanis A., Catt, K.J. and Hudson, B. (1970): J. Clin. Endocr. 30, 351.

Edelman, I.S. and Fimognari, G.M. (1968): Recent Prog. Hormone Res. 24, 1.

Ekins, R.P. (1960): Clin. Chim. Acta. 5, 453.

Everett, B.J. and Herbert, J. (1969): Nature 222, 1065.

Few, J.D. (1968): J. Endocr. 41, 213.

Fotherby, K. (1962): J. Endocr. 25, 19.

Frick, J. and Kincl, F.L. (1969): Steroids 13, 495.

Gemzell, C.A., Carlborg, L., Johansson, E.D.B. and Roos, P. (1970): J. Obstet. Gynaec. Br. Commonw. 77, 58.

Gemzell, C.A., Diczfalusy, E. and Tillinger, K.G. (1958): J. Clin. Endocr. 18, 1333.

Gemzell, C.A., Diczfalusy, E. and Tillinger, K.G. (1959): Acta Obstet. Gynaec. Scand. 38, 465.

Grad, B. and Khalid, R. (1968): J. Geront. 23, 522.

Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963): Biochem J. 89, 114.

Hagerman, D.D. and Williams, K.L. (1969): Am. J. Obstet. Gynec. 104, 114.

Hallberg, M.C., Zorn, E.M. and Wieland, R.G. (1968): Steroids 12, 241.

Hancock, K.W., Scott, J.S., Stitch, S.R., Level, M.J., Oakey, R.E. and Ellis, F.R. (1970): Lancet 2, 482.

Harris, W. (1961): In: Control of ovulation, Villes, C.A. Ed. pub. Pergamon Press, New York, p. 56.

Horton, R., Kato, T. and Sherins, R. (1967): Steroids 10, 245.

Hudson, Coghlan, J., Dulmanis, A., Wintour, M. and Ekkel, I. (1963): Aust. J. exp. Biol. med. Sci. 41, 235.

Ittrich, G. (1958): Hoppe-Seyl, Z. 312, 1.

James, V.H.T. and De Jong, M. (1961): J. Clin. Path. 14, 425.

James, V.H.T., Townsend, J. and Fraser, R. (1967): J. Endocr. 37, xxviii.

Jiang, N.S., Ryan, R.J., Albert, A. (1971): Unpublished data.

Johansson, E.D.R. (1969): Acta endocr. (Kbh) 61, 592.

Johansson, E.D.B. (1970): Karolinska Symposia, 2nd Symposium: Steroid assay by protein binding, Diczfalusy, E., Ed. p.188.

Johansson, E.D.B., Neill, J.D. and Knobil, E. (1968): Endocrinology, 82, 143.

Kato, T. and Horton, R. (1968): Steroids 12, 631.

Kinard, F.E. (1957): Rev. Sci. Instr. 28, 293.

Kliman, B. and Peterson, R.E. (1960): J. Biol. Chem. 235, 1639.

Klopper, A. (1957): J. Obstet. and Gynaec. Br. Commonw. 64, 504.

Klopper, A., Wilson, C., Cooke, I. (1969): J. Endocr. 43, 295.

Klopper, A,, Michie, E.A. and Brown, J.B. (1955): J. Endocr. 12, 209.

Korenman, S.G, (1968): J. Clin. Endocr. 28, 127.

Korenman, S.G. (1969): Rec. Progr. Horm. Res. 25, 602. (In discussion, Murphy, B.E.P.).

Korenman, S.G., Perrin, L.E. and McCallum, T.P. (1969): J. Clin. Endocr. 29, 879.

Korenman, S.G., Tulchinsky, D. and Eaton, L.W. Jr. (1970): Karolinska Symposia, 2nd Symposium: Steroid assay by protein binding, Diczfalusy, E., Ed. p. 291.

Lipede, A.B. (1970): Personal communication.

Loraine,J.A. (1958): In: The Clinical Application of Hormone Assay, pub. E.&.S. Livingstone Ltd., Edinburgh, p. 221.

Lunenfeld, B. (1963): J. int. Fed. Gynaec. Obstet. 1, 153.

Lunenfeld, B. and Rabau, E. (1967): In: Wood, C., Ed. Proc. V. Wld. Congr. Gynaec. Obstet. pub. Butterworths, Australia, p. 294.

Maeda, R., Okamoto, M., Wegienka, L.C. and Forsham, P.H. (1969): Steroids 13, 83.

Mahesh, V.B. and Greenblatt, R.B. (1961): Nature, 191, 888.

Martin, B.T., Cooke, B.A. and Black, W.P. (1970);
J. Endocr. 46, 369.

Mattingly, D. (1962): J. clin. Path. 15, 374.

Mayes,D., Furuyama, S., Kem, D.C. and Nugent, C.A. (1970):
J. Clin. Endocr. 30, 682.

Mayes, D. and Nugent, C.A. (1968): J. clin. Path. 15, 374.

Metcalfe, M.G. (1963): J. Endocr. 26, 415.

Morley, A.A. (1966): Lancet, 2, 1220.

Morris, R. (1959): Acta endocr. (Kbh) 32, 596.

Morris, R. and Leeson, S.A. (1969): J. med. Lab. Tech. 26, 243.

Moshang, T. Jr., Rudd, B.T., Eberlein, W.R. and Bongiovanni, A.M.
(1970): Steroids 15, 195.

Mozes, M., Bogokowsky, H., Antebi, E., Lunenfeld, B., Rabau, E.,
Serr, D.M., David, A. and Salomy, M. (1965): Lancet, 2, 1213.

Murphy, B.E.P. (1964): Nature, 201, 679.

Murphy, B.E.P. (1967): J. Clin. Endocr. 27, 973.

Murphy, B.E.P. (1968): J.clin. Endocr. 28, 343.

Murphy, B.E.P. (1969): In: Rec. Progr. Horm. Res. 25, 563.

Murphy, B.E.P., Engleberg, W. and Pattee, C.J. (1963):
J. Clin. Endocr. 23, 293.

Murphy, B.E.P. and Pattee, C.J. (1964): J. Clin. Endocr. 24, 919.

Nachtigall, L., Bassett, M. Slagle, S., Hogsander, U. and Levitz, M.
(1966): J. Clin. Endocr. 26, 941.

Neill, J.D., Johannson, E.D.B., Datta, J.K. and Knobil, E. (1967): J. Clin. Endocr. 27, 1167.

New, M.I., Gross, J.M. and Peterson, R.E. (1968): Acta endocr. 58, 77.

Nugent, C.A. and Mayes, D. (1966): J. Clin. Endocr. 26, 1116.

Oyakama, E.K. and Levedahl, B.H. (1958): Arch. biochem. biophys. 74, 17.

Palmer, R.F. (1964): J. Obstet. and Gynaec. Br. Common. 71, 744.

Parlow, A.F. (1961): In: Albert, A. Ed. Human Pituitary. Gonadotrophins, pub. C.C. Thomas, Springfield, Ill. p. 300.

Patterson, M.S. and Greene, R.C. (1965): Anal. chem. 37, 854.

Pearlman, W.H., Crepy, O. and Murphy, M. (1967): J.Clin. Endocr. 27, 1012.

Pepper, H. and Lindsay, S. (1960): Proc. Soc. Exp. Biol. and Med. 104,145.

Pickett, M.T. and Kellie, A.E. (1962): Acta endocr. (Kbh) 41, 129.

Pincus, G. (1965): Control of Fertility, Academic Press, New York. p.276.

Preedy, J.R.K. and Aitken, E.H. (1961): J. biol. Chem. 236, 1300.

Rakoff, A.E., Paschis,K.E. and Cantrow, A. (1943): Am. J. Obstet. Gynec. 46, 856.

Reeves, B.D., de Souza, M.L.A., Thompson, I.E. and Diczfalusy, E. (1970): Acta endocr. (Kbh) 63, 225.

Riondel, A., Tait, J.F.,Gut, M., Tait, S.A.S., Joachim, E. and Little, B. (1963): J. Clin. Endocr. 23, 620.

Riondel, A., Tait, J.F., Tait, S.A.S., Gut, M. and Little, B. (1965): J. Clin. Endocr. 25, 229.

Robinson, R.G. and Fanestil, D.D. (1970); Karolinska symposia: 2nd symposium: Steroid assay by protein binding, Diczfalusy, E., Ed. p. 275.

Rosenfield, R.L. (1969): Steroids 14,251.

Rosenfield, R.L., Eberlein, W.R. and Bongiovanni, A.M. (1969): J. Clin. Endocr. 29, 854.

Rudd, B.T., Rosenfield, R.L., Bongiovanni, A.M. and Eberlein, W.R. (1969); Steroids 13, 227.

Sandberg, A.A., Slaunwhite, W.R. Jr. and Antoniades, H.N. (1957): Recent Prog. Horm. Res. 13, 209.

Saroff, J., Keenan, R.E., Sandberg, A.A. and Slaunwhite, W.R. Jr. (1967): Steroids 10, 15.

Seal, U.S. and Doe, R.P. (1965): Steroids 5, 827.

Shutt, D.A. (1969): Steroids 13, 69.

Slaunwhite, W.R. Jr. and Sandberg, A.A. (1959): J. Clin. Invest. 38, 384.

Spencer-Peet, J., Daly, J.r. and Vivien Smith (1965): J. Endocr. 31, 235.

Steelman, S.L. and Pohley, F.M. (1953): Endocrinology 53, 604.

Steelman, S.L., Segaloff, A. and Anderson, R.N. (1959): Proc. Soc. Exp. Biol. Med. 101, 452.

Steelman, S.L., Segaloff, A. and Mayes, G.M. (1958): Archs. Biochem. Biophys. 78, 262.

Stenlake, J.B., Davidson, A.G., Williams, W.D. and Downie, W.W. (1970): J. Endocr. 46, 209.

Strott, C.A. and Lipsett, M.B. (1968): J. Clin. Endocr. 28, 1426.

Strott, C.A., West, C.D., Nakagawa, K., Kondo, T. and Tyler, F.H. (1969): J. Clin. Endocr. 29, 6.

Szego, C.M. and Roberts, S. (1946): Proc. Soc. Exp. Biol. Med. 61, 161.

Szego, C.M. and Roberts, S. (1947): Endocrinology 41, 322.

Szego, C.M. and Roberts, S. (1953): Recent Prog. Hormone Res. 8, 419.

Szego, C.M. and Roberts, S. (1956): J. Biol. Chem. 221, 619.

Turner, J.C. (1967): In: Sample preparation for liquid scintillation counting. pub. The Radio Chemical Centre, Amersham, p.9.

Van Baelen, H., Heyns, W. and De Moor, P. (1967): J. Clin. Endocr. 27, 1056.

Van der Molen, H.J.and Green, D. (1965): J Clin. Endocr. 25, 1625.

Westphal, U. (1955): Endocrinology 57, 456.

Westphal, U. (1957): Arch. Biochem. 66, 71.

Westphal, U. and Ashley, B.D. (1958): J. Biol. Chem. 233, 57.

Wotiz, H.H., Charransol, G. and Smith, I.M. (1967): Steroids 10, 127.

Yalow, R.S. and Bearson, S.A. (1960): J. Clin. Invest. 39, 1173.

Yoshimi, T. and Lipsett, M.b. (1968): Steroids 11, 527.