# DEVELOPMENT AND EVALUATION OF RADIOIMMUNOLOGICAL METHODS FOR THE DETERMINATION OF OESTROGENS IN THE PERIPHERAL VENOUS PLASMA OF HUMAN SUBJECTS

by

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# ABSTRACT

The biochemistry of the oestrogens and their hormonal effects are outlined. The low levels of oestrogens in the peripheral venous plasma of men and non-pregnant women have necessitated the development of extremely sensitive assay techniques for their determination and advances in this field are reviewed. The principles of radioimmunoassay are outlined and the practical aspects discussed.

A hapten radioimmunoassay has been developed for the separate determination of oestrone and oestradiol-17β in plasma (method i) and this was subsequently modified to provide a more rapid assay for plasma 'oestrogen' (method ii) or oestradiol-17β (method iii). The conditions selected resulted from the investigation of several assay parameters: antiserum dilution, buffer composition and pH, incubation conditions, conditions for the separation of antibody-bound and free oestrogen and the effect of solvent residues.

The methods described have been evaluated in terms of the theoretical and practical assessment of errors and assay sensitivity and specificity.

These methods have been applied to the study of oestrogens in peripheral venous plasma from healthy individuals

and normal ranges established. The variation of oestrogen levels throughout the menstrual cycle and 24hr periods have been studied and compared with the findings of other workers. Limited application of these methods has been made to the determination of plasma oestrogen levels in dynamic studies and pathological conditions. The relative merits of the three methods and their clinical application are discussed.

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# CHAPTER I

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# OESTROGEN BIOCHEMISTRY

## A. INTRODUCTION

Endocrinology is a relatively young branch of the biological sciences whose emergence has come about through advances in the field of medicine, and whose growth has depended largely on recent advances in the fields of biochemistry and technology. A large number of hormones have now been isolated and characterized, many have been prepared synthetically and artificial substances have been found whose actions are similar to those of the native hormone.

There are many points of view with regard to the scope of endocrinology. Some regard it as that aspect of biology concerned with the chemical integration of the individual, while others prefer to retain the classical definition of Bayliss and Starling (1902) which limits endocrinology to the study of the ductless glands and their role in the physiology of the body.

The word endocrine is of Greek origin and means to 'secrete within'. It is difficult to formulate a precise definition for an endocrine gland since most cells possess some secretory capacity and contribute to the internal

environment of the organism. However, an endocrine system is recognized, consisting of a diverse group of tissues with the common property of producing chemical stimuli to other tissues. The glands involved can act independently of the nervous system but can also be influenced by nervous or psychological stimuli, both in the direction of increasing as well as decreasing or completely inhibiting their activity.

With regard to embryonic origin, the endocrine glands differentiate from all the germ layers. Those derived from the mesoderm (adrenal cortex, gonads) produce steroid hormones; those developing from the ectoderm or endoderm secrete hormones that are either modified amino acids, peptides or proteins.

Professor Starling originally used the term hormone (from the Greek word hormaein meaning to set in motion or spur on) to describe secretin and gastrin. The term was subsequently applied to the secretions of the anatomically recognized endocrine system: the pituitary, parathyroids, thyroid, pancreatic islet cells, the hormone-producing part of the gastrointestinal tract, adrenal cortices, gonads and the placenta. The components of the renin-angiotensin system and several new peptide hormones such as calcitonin and placental lactogen have since been added to the classical group of hormones.

The actions as well as the origins of blood-borne stimuli should be considered when deciding whether or not such stimuli should be classed as hormones. Some hormones, such as angiotensin II are formed from precursors in the circulation; others are formed by conversion from precursors in tissues, such as testosterone* in the female and dihydro-testosterone in many androgen-responsive cells.

Whatever their origin, hormones do not themselves take part in energy-producing processes. They are generally conveyed by the circulation and must be transmitted through intercellular tissue fluids in order to reach their target organs. They regulate processes such as growth, metabolic rate, blood chemistry and reproduction, adjustments that require duration rather than speed.

## B. ISOLATION AND IDENTIFICATION

### (i) Early History

Aristotle had taught that the female contribution to the embyro was the menstrual blood, the function of the ovaries remaining obscure. By comparative studies of the ovaries of different vertebrates Regnerus de Graaf (1672) recognized the true function of the mammalian ovary, although he incorrectly identified as eggs the follicles which today bear his name. Subsequently, in 1827, the Russian anatomist

* see Appendix II

von Baer saw the human ovum which, because of its large size, borders on naked eye visibility and hence could be detected with the primitive microscopes of that era.

The first ovariectomies in women were performed by Hauston, MacDonell and Battey in the 18th and 19th centuries. These operations were performed to remove ovarian tumours, without the realization that the ovary exerts an endocrine function.

In 1896 Knauer showed that ovarian transplants could restore the extinguished sexual cycle of spayed animals and concluded that apart from the production of ova, the female gonads exert an important function in regulating sexual behaviour. Subsequently, similar effects were observed when aqueous ovarian extracts were administered to spayed animals, and in 1912, Fellner reported that placental extracts also had this effect on ovariectomized rabbits, probably because the placenta contains ovarian hormone. Thus, it came to be realized that oestrus, the recurrent, restricted period of sexual receptivity in female mammals, is governed by an ovarian hormone which was referred to as an oestrogen (oestrus-producing compound).

A vast amount of work was undertaken to isolate this compound but progress was hampered by the lack of a convenient bioassay. Prior to 1923 most investigators used the uterus

of immature or spayed animals as an indicator of oestrogenic activity and so had to kill the animals for each test. It was of great importance therefore, when, based on the previous observation (Stockard and Papanicolaou, 1917) of regular cyclic variations in the vaginal epithelium of rodents, Allen and Doisy (1923) described their well-known test for oestrogenic substances.

Within a short time Doisy et al (1924) not only demonstrated oestrogenic activity in the fluid of Graafian follicles but were able to prepare highly active concentrates of it.

(ii) Unconjugated Oestrogens

In 1927, Aschheim and Zondek reported that the urine from many pregnant animals contained two hormones, one of which was similar to the oestrus-producing agent in the fluid of the Graafian follicle, whereas the other caused marked growth of ovarian follicles. The latter is now known to be chorionic gonadotropin (of placental origin). In 1929, Doisy et al and Butenandt independently succeeded in crystallizing the oestrogenic substance, which came to be known as oestrone, from human pregnancy urine. Thus, the first steroid hormone to be isolated was an oestrogen. Oestrone was subsequently isolated from human male urine (Dingemanse et al, 1938), beef

adrenal glands (Beall, 1939) and the bile of pregnant cows (Pearlman et al, 1947).

The next oestrogen to be isolated from human pregnancy urine was oestriol (Marrian, 1930; Doisy et al, 1930; Doisy and Thayer, 1931). Oestriol has also been isolated from human placental tissue (Huffman et al, 1940b).

Oestradiol was originally isolated by MacCorquodale et al, (1935) from the follicular fluid of sows ovaries. It was subsequently isolated from other sources including human pregnancy urine (Huffman et al, 1940a),human placental tissue (Huffman et al, 1940b), horse testes (Beall, 1940) and stallion urine (Levine, 1945). Initially this compound was designated α-oestradiol, but in 1950 the present rules of nomenclature were recommended and the name was changed to oestradiol-17β.

For many years these were thought to be the only oestrogens occuring in the human, although the mono and didehydro derivatives of oestrone, equilin, hippulin and equilenin had been found in animals (Girard et al, 1932a, 1932b). Thus, oestrone, oestradiol-17β and oestriol gained the title 'classic oestrogens'.

The isolation of these oestrogens involved great technical difficulties and no additional oestrogens were isolated from human sources between 1935 and 1955. However,

the development of chromatographic techniques made work with smaller amounts of steroid possible and several new oestrogens were found in subsequent years. Some of the oestrogens found in human pregnancy urine are indicated in Table 1.

Oestrone and oestriol were detected in the urine of non-pregnant women by Engel et al (1952) using the technique of countercurrent distribution, while specificity studies on a number of chemical methods developed for the estimation of urinary oestrone, oestradiol-17β and oestriol revealed that the classic oestrogens are present not only in the urine of fertile women but also in the urine of men and postmenopausal women. Other oestrogens which have been detected in non-pregnancy urine are 16-oxo-oestrone (Serchi, 1953), 16-epi-oestriol (Watson and Marrian, 1956) and 16α-hydroxy-oestrone (Loraine, 1958).

In 1958, Diczfalusy and Magnusson isolated and identified oestriol in cord blood and Oertel et al (1959) succeeded in isolating and identifying oestrone, oestradiol-17β and oestriol in human pregnancy plasma. Using pooled pregnancy plasma, Adlercreutz and Luukkainen (1968a) conclusively identified by combined gas-liquid chromatography and mass spectrometry oestrone, 2-methoxyoestrone and oestradiol-17β and tentatively identified 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16-epi-oestriol. The first reported attempt to measure free

| OESTROGEN | REFERENCE |
|---|---|
| 2-Hydroxyoestrone | Notchev and Stimmel, 1962. |
| 2-Methoxyoestrone | Loke and Marrian, 1958. |
| 6α-Hydroxyoestrone | Knuppen et al, 1966a. |
| 6β-Hydroxyoestrone | Breuer, 1964. |
| 11β-Hydroxyoestrone | Breuer, 1964. |
| 15α-Hydroxyoestrone | Knuppen et al, 1965a. |
| 15β-Hydroxyoestrone | Knuppen et al, 1966b. |
| 16α-Hydroxyoestrone | Marrian et al, 1957. |
| 16β-Hydroxyoestrone | Layne and Marrian, 1958. |
| 18-Hydroxyoestrone | Loke et al, 1959. |
| 2-Methoxyoestradiol-17β | Frandsen, 1959. |
| 6α-Hydroxyoestradiol-17β | Breuer, 1964. |
| 11-Dehydro-oestradiol-17α | Adlercreutz and Luukkainen, 1968a. |
| 15α-Hydroxyoestradiol-17β | Lisboa et al, 1967. |
| 15β-Hydroxyoestradiol-17β | Knuppen et al, 1966b. |
| 16-Oxo-oestradiol-17β | Layne and Marrian, 1958. |
| 17α-Oestradiol | Schott and Katzman, 1964. |
| 2-Methoxyoestriol | Breuer, 1964. |
| 15α-Hydroxyoestriol | Zucconi et al, 1967. |
| 16-Epi-oestriol | Marrian and Bauld, 1955. |
| 16,17-Epi-oestriol | Breuer and Pangels, 1959. |
| 17-Epi-oestriol | Breuer, 1960. |
| Oestratetraenol | Thysen et al, 1968. |

Table 1. Some oestrogens detected in, or isolated from, human pregnancy urine.

circulating oestrogens in normal as well as pregnant females was by Svendsen (1960) using a double isotope method. Oestradiol-17β was determined in plasma from men, fertile women and postmenopausal women by Korenman et al (1969) while Wu et al (1971) measured oestrone, oestradiol-17β and oestriol in plasma from pregnant and non-pregnant women and from men.

(iii) Conjugated Oestrogens

It has been known for many years that oestrogens are excreted in the urine in a conjugated form. As early as 1936, Cohen and Marrian isolated a conjugate of oestriol from pregnancy urine which was later shown to be an oestriol monoglucuronoside (Grant and Marrian, 1950). Carpenter and Kellie (1960, 1962) showed that the oestriol monoglucuronoside fraction of human pregnancy urine contains two isomers, oestriol-16α-glucuronoside and oestriol-17β-glucuronoside. Felger and Katzman (1961) suggested that pregnancy urine also contains a diglucuronoside. Another type of oestriol glucuronoside which was thought to be oestriol-3-glucuronoside was detected in pregnancy urine by Beling (1962). This compound was subsequently isolated from human pregnancy urine by Ladany (1968).

Other types of conjugated oestriol have been found in pregnancy urine, for example, Straw et al (1955) obtained evidence for the presence of a double conjugate of oestriol which contained both glucuronic and sulphuric acids, and Adlercreutz and Beling (1962) isolated a conjugate of oestriol which proved to be identical with oestriol-3-sulphate.

The work of Cohen and Bates (1949) and Oneson and Cohen (1952) showed that, quantitatively, the most important conjugate of oestrone in pregnancy urine is the glucuronoside. Both oestrone-3-sulphate (McKenna et al, 1961) and oestrone-3-glucuronoside (Diczfalusy, 1962) have been isolated from and identified in human pregnancy urine, and the presence of 17β-oestradiol-3-sulphate was also indicated (McKenna et al, 1961).

Rakoff et al (1943) demonstrated that 23-50% of the oestrogens in human pregnancy plasma are conjugated, as judged by bioassay. Using reverse isotope dilution and countercurrent distribution, Purdy et al (1961) characterized oestrone sulphate and concluded it to be an important circulating oestrogen in the human. Adlercreutz and Luukkainen, (1968a), using pooled pregnancy plasma, identified conjugated oestriol, oestrone, 2-methoxyoestrone, oestradiol-17β, 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 15α-hydroxy-oestrone. Conjugated 16β-hydroxyoestrone and 16-epi-oestriol

were tentatively identified.

(iv) Binding to Plasma Proteins

Not long after the isolation of the first oestrogenic hormone, Brunelli (1934) reported that a significant part of the oestrogenic activity in the blood of animals is associated with the globulin fraction of plasma. In 1943, Rakoff et al published results indicating that both free and conjugated oestrogens are bound to plasma proteins in pregnant women. Subsequent work showed that oestrogens are mainly associated with serum albumin (Bischoff et al, 1954) and with plasma protein fractions containing globulins (Sandberg et al, 1957) when fractionation is carried out according to the method of Cohn et al (1946).

Albumin is the main protein constituent of blood and has a tremendous capacity for binding a great number of substances. However, its binding affinity for oestrogens and other steroids is comparatively low and the complex dissociates very easily. Although conjugates of oestrone and oestradiol-17β are extremely water-soluble it has been shown that they too are bound to serum proteins and have a greater affinity for albumin than the free steroids (Sandberg et al, 1957). Sulphates, being dissociated as strong acids, might interact with proteins by electrostatic forces in addition to the forces

involved in the binding of the free steroids. The situation differs for glucuronosides since the glucuronoside moiety is only weakly acidic and has little effect on binding to albumin (Giorgi and Crosignani, 1969).

As far back as 1934, Brunelli had suggested that there was in plasma a globulin fraction with a high affinity for oestrogens. This hypothesis was confirmed by Rosenbaum et al (1966) and the protein has been purified and its physiochemical properties studied (Tavernetti et al, 1967; Barlow et al, 1969; Heyns et al, 1969; Rosner et al, 1969). A comparison of this protein's properties with those of a testosterone binding protein isolated by Mercier et al (1965) suggested that these binding proteins were one and the same. For this reason several authors prefer to call it a sex hormone binding globulin.

It was thought that the binding of oestrogens and other steroid hormones to plasma proteins constituted a means of transporting these steroids (Brunelli, 1934). This idea waned in popularity and it was suggested that the protein-steroid complex acts as a reservoir of steroid, a protector from catabolism and as a buffer against inundation of the extravascular spaces by the steroid hormone (Westphal, 1971a). Recently, there has been renewed interest in a possible transport function of steroid-binding plasma proteins in

connection with the entrance of the hormone into specific target cells and permeation of the nuclear membrane. Another possible function of the steroid-protein complex is the regulation of hormonal function by changes in concentration of the binding proteins, especially those with high binding affinity and low capacity, since binding to protein suppresses the biological activity of steroid hormones (Westphal, 1970).

(v) Association with Erythrocytes

Circulating oestrogens in blood are found not only in association with plasma proteins but also with erythrocytes. Albrieux (1941a, 1941b), investigating the distribution of blood oestrogens between cells and serum, reported a 2:1 distribution in non-pregnancy blood and a 1:1 distribution in pregnancy blood. Subsequently, Rakoff et al (1943) confirmed the pregnancy blood findings of Albrieux, but Roy and Brown (1960) and Maner et al (1963) reported that none of the classic oestrogens were associated with the erythrocytes of untreated pregnant subjects in contrast to males and non-pregnant females when given radioactive oestrogens intravenously.

In 1951 Bischoff et al showed that oestrone was 'activated' following incubation with mammalian erythrocytes and this 'activation' was found to be due to the transformation

of oestrone to oestradiol-17β (Gray and Bischoff, 1955). This observation suggested a penetration of oestrone into the erythrocyte and prompted study of the concentration of oestrogens in erythrocytes (Wall and Migeon, 1959; Migeon et al, 1959). Continuing their studies, Bischoff and Bryson (1960) investigated the problem of whether oestrogens actually penetrate the membrane of the red cells or are transported by it. They showed that the enzyme oestradiol-17β dehydrogenase is associated with the haemoglobin and that penetration must occur to account for the distribution in the intact cell.

## C. CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

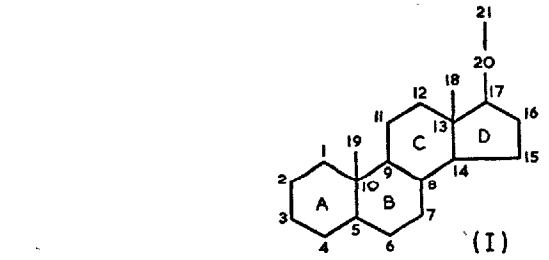
The naturally occuring oestrogens are steroid hormones responsible with other hormones for the development and maintenance of the female sexual organs and secondary sexual characteristics and also for the maintenance of the menstrual cycle and pregnancy.

The steroid hormones, derived from six isopentenyl pyrophosphate units, are based on the perhydrocyclopentane-phenanthrene nucleus which consists of a fully hydrogenated phenanthrene (rings A, B and C) to which is fused a 5-carbon cyclopentane ring (ring D). The constituents of the nucleus and those on the commonly occuring side chain are numbered

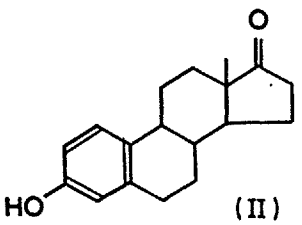
as indicated in Fig. 1.

The predominant natural oestrogens of man are oestrone, oestradiol-17β and oestriol, whose structures are shown in Fig. 1. Several other oestrogens representing oestrogen metabolites have been isolated in significant amounts from normal urine. Relevant information regarding the structure of the oestrogens was acquired rapidly in the period 1930-1934, the ultimate proof coming in 1948 with the total synthesis of oestrone by Anner and Miescher. They derive from the parent $C_{18}$ hydrocarbon, oestrane, which consists of the perhydrocyclopentanephenanthrene molecule with an angular methyl group at C-13; are characterized by the aromatic nature of ring A; the presence of an oxygen function at C-17 and a phenolic hydroxyl group at C-3. In contrast to many other steroid hormones there is no angular methyl group at C-10.

Ring A, being aromatic is planar, ring B having one double bond assumes a half-chair conformation and ring C exists in the chair form. Evidence from X-ray crystallography and optical rotation dispersion spectra (Fishman and Djerassi, 1956) suggests that ring D is a strained half-chair with C-15 close to the line projected through C-8 and C-10, and C-17 close to the line parallel to this through C-12 and C-16. A photograph of a Dreiding stereomodel of oestradiol-17β is shown in Fig. 2.




(I)

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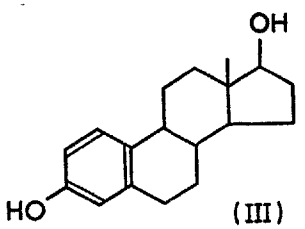
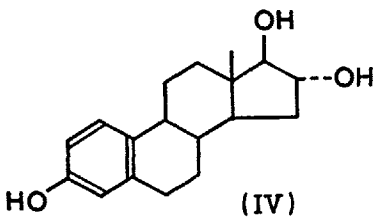



(III)




(IV)

Fig. 1. Structural formulae:-
I pregnane; II oestrone;
III oestradiol-17β;
IV oestriol.

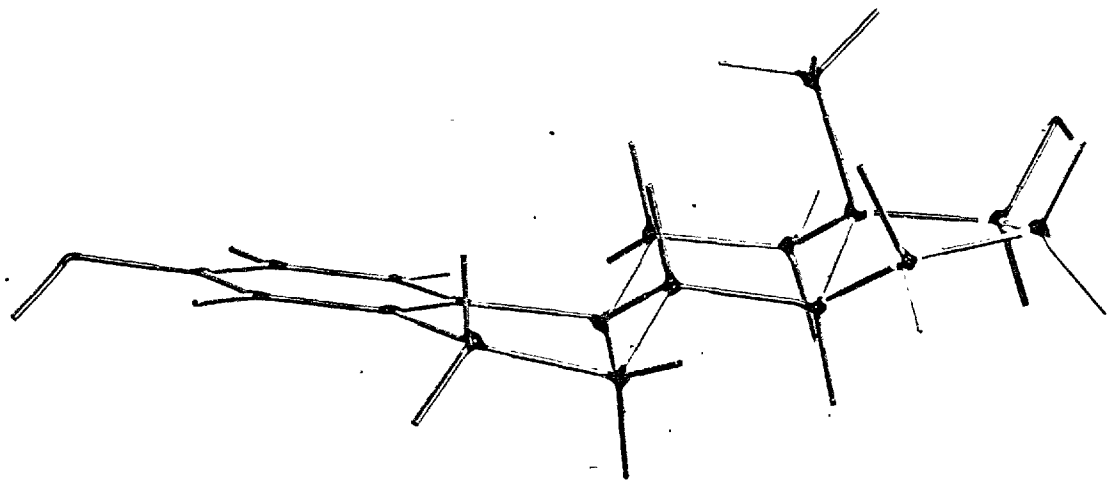
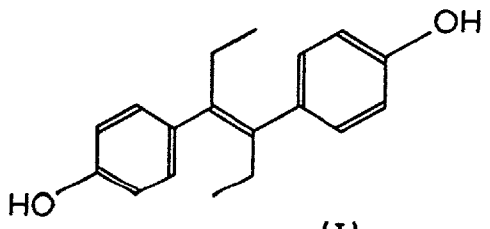


Fig. 2. Dreiding stereomodel of oestradiol-17β.

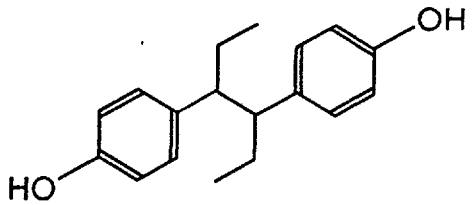
Stereoisomerism can occur in oestrogens as in other steroid hormones owing to groups attached to the ring carbon atoms, since these may be orientated below or above the plane of the ring (α and β respectively). The methyl group at C-13 has arbitrarily been given the β configuration and is the point of reference.

Early analytical work led to the conclusion that the phenanthrene ring is not essential for biological activity. This, together with the observation that oestrogens stimulate specific processes led Dodds and his colleagues to study carcinogen derivatives as potential artificial oestrogens. In 1938 Dodds et al synthesized diethylstilboestrol and other related compounds soon followed, notably hexoestrol and dienoestrol. The structural formulae of these compounds are shown in Fig. 3. They possess the biological properties of the natural oestrogens, are cheap to produce, active orally and have been widely used for the treatment of menstrual disorders, menopausal symptoms and carcinoma of the prostate gland.

The structural analogy of these compounds to the natural oestrogens is apparent from the formulae but, although molecular shape would seem to be a significant factor in relation to oestrogenic activity, it is not the only factor. A number of compounds having the same capacity to assume an orientation




(I)

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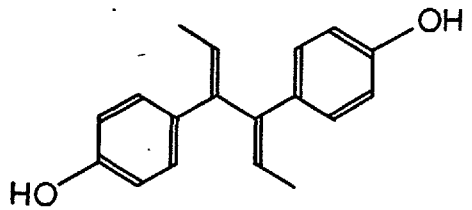


(III)

Fig. 3. Structural formulae:-
I diethylstilboestrol;
II hexoestrol;
III dienoestrol.

similar to the natural oestrogens lack biological activity. Schueler (1946) has suggested that oestrogenic activity may result if two hydrogen bonding groups are so situated in a molecule that they are 8.55 Å apart as is the case for oestradiol-17β.

The relationship between structure and biological function is further complicated by the variety of effects mediated by oestrogens, for they not only act directly, or in cooperation with other hormones to produce numerous effects on specific target organs but influence the chemistry of the body as a whole (Pincus, 1955; Boscott, 1962).

A general effect of the oestrogens is their promotion of tissue growth which is most pronounced in the accessory sex tissues but occurs in other tissues as well. A major role of the oestrogens is to control the growth and function of the uterus. Other growth effects are on the ovaries, cervix, Fallopian tubes, vagina, external genitalia and breasts.

The most widely known effect of oestrogens is their role in the control of the menstrual cycle, a series of interactions between hypothalamic, pituitary and ovarian factors giving rise to the characteristic sequence of follicular development, ovulation, corpus luteum formation and menstruation or pregnancy. Brown et al (1958) studied

the urinary excretion of gonadotropins, pregnanediol and oestrogen metabolites. They observed that oestrogen production rises during the follicular phase to reach a peak at mid-cycle, falls to lower levels, rises again during the luteal phase and returns to low levels during menstruation. The introduction of more sensitive assay techniques has enabled plasma oestrogen levels to be studied throughout the menstrual cycle. The pattern observed is essentially the same as that found in urine with the mid-cycle oestrogen peak occuring immediately prior to or coinciding with the LH peak (Mikhail et al, 1970). With the onset of menstruation the corpus luteum regresses and ceases to inhibit the pituitary gland which secretes FSH in increasing amounts. This stimulates follicular growth and increasing quantities of oestrogen are released from the growing follicles. As the oestrogen level rises, the endometrium, which at the end of menstrual disintegration is thin and poorly vascularized, thickens and the glandular and vascular patterns are restored. It has been suggested (Goebelsmann et al, 1969) that secretion of oestradiol-17β by the maturing follicles brings about the mid-cycle surge of both LH and FSH which is associated with ovulation itself.

The timing of the entry of a zygote into the uterus is very important if implantation is to occur. The movement of ova from follicle to site of implantation is influenced

considerably by oestrogens in a number of species (Bennett, 1969) and it may be that the post-ovulatory fall in oestrogen in man is associated with this. Another factor essential for implantation is the differentiation of the endometrium under the influence of luteal levels of oestrogen and progestogen into a tissue suitable for implantation.

At the biochemical level, pretreatment of an animal with oestradiol-17β causes the stimulation of various synthetic pathways in the isolated uterus; these include phospholipid, RNA and protein synthesis (Mueller, 1957; Mueller et al, 1958; Gorski et al, 1965). Mueller (1955) found that, in an 'in vitro' system, addition of 2-hydroxy- or 4-hydroxy-oestradiol-17β stimulates the incorporation of formate into protein in the rat uterus whereas oestrone, oestradiol-17β and oestriol do not.

Oestrogens appear to have effects on other hormones. There is evidence that administration of oestrogen causes an increased secretion of thyrotropin and a decrease in the rate at which thyroid hormone is used, associated with an increase in protein-bound iodine in plasma (Engbring and Engstrom, 1959). The binding of $^{14}$C-cortisol to transcortin in plasma is also increased by oestrogen administration (Sandberg and Slaunwhite, 1959; Mills et al, 1960).

Oestrogens are mildly anabolic and increase the retention

of nitrogen and sodium. Retention of the latter leads to a retention of water, particularly immediately prior to menstruation, which is believed to contribute to premenstrual tension. Other minerals such as calcium and phosphorus are also retained and this promotes the deposition of calcium in bone matrix and hastens the closure of the epiphyses (Budy, 1956). Oestrogens also have a re-epithelialization effect which is particularly marked in nasal and buccal mucosa and are mildly vasodilator, stimulating the peripheral circulation which can lead to hot flushes.

## D. SOURCE AND BIOSYNTHESIS

Oestrogens are secreted by the ovary, testis, placenta and the adrenal cortex. Under normal physiological conditions the ovary is the main site of oestrogen production in the human female. Oestrone and oestradiol-17β are constantly found in human ovaries and in most cases the concentration of oestradiol-17β exceeds that of oestrone (Short and London, 1961; Smith and Ryan, 1962). Oestriol has not been detected in every case. Oestrogens are found in follicular fluid as well as cyclic and pregnancy corpora lutea.

The presence of oestrogens in the ovary does not necessarily mean that they are secreted by the gland itself,

but demonstration of a higher oestrogen level in ovarian venous blood than in peripheral venous blood is a more positive indication. Varangot and Cedard (1959) constantly found oestrone and oestradiol-17β but seldom oestriol in the ovarian venous blood of women with non-endocrine gynaecological disorders, and the concentration of oestradiol -17β was 10 times greater than that found in peripheral plasma. The available experimental data points to oestradiol-17β as the major oestrogenic product secreted by human ovaries and oestriol as a peripheral metabolite.

The placenta plays a major role in the elaboration of the large amounts of oestrogen produced during pregnancy. In fact, towards the end of pregnancy its contribution is 100 - 1000 times that of the ovary. Oestrone, oestradiol-17β, oestriol and 16-epi-oestriol have been isolated from human placental tissue and various workers have shown that oestriol is quantitatively the most important (Mitchell and Davies, 1954; Diczfalusy and Lindkvist, 1956).

Indirect evidence for the production of oestrogens or oestrogen precursors by the human adrenal gland comes from the detection of small amounts of oestrone, oestradiol-17β and oestriol in the urine of castrated women (Bulbrook and Greenwood, 1957; Brown et al, 1959) and the observation that both male and female patients with certain types of adrenal

tumour can excrete abnormally large quantities of oestrogens (Simpson and Joll, 1938; Dohan et al, 1953). In 1969 Baird et al provided more direct evidence by studying the levels of oestrone and oestradiol-17β in peripheral and adrenal venous plasma.

Oestradiol-17β has been detected in human testicular tissue (Goldberg and Studer, 1937; Goldzieher and Roberts, 1952) and it has been shown that pooled human semen contains oestrone, oestradiol-17β and oestriol (Diczfalusy, 1954). Incubation studies with normal and pathological testicular tissue indicates that such tissue is capable of elaborating oestradiol-17β but the actual site is not known for certain.

When considering the sources of oestrogens one must not overlook the possibility of their arising by peripheral conversion of precursors in the blood. The conversion of testosterone to oestrogens does not appear to be entirely dependent upon the presence of endocrine organs since, when testosterone propionate was administered to ovariectomized, adrenalectomized women, oestrone and oestradiol-17β were found in their urine (West et al, 1956). The work of Grodin et al (1973) on the source of oestrogen production in postmenopausal women suggests that the principle oestrogen formed in these women is oestrone and that it is derived by aromatization of plasma androstenedione.

Historically, study of the biosynthesis of oestrogens begins with observations such as those of Nathanson and Towne (1939) and Dorfman and Hamilton (1939) which indicated the possible conversion of androgens, administered 'in vivo' to humans, to oestrogens. When labelled steroids became available it was shown that $^{14}$C-labelled testosterone is converted to $^{14}$C-labelled oestrone or oestradiol-17β (Heard et al, 1955; Baggett et al, 1956; Wotiz et al, 1956). These experiments helped tremendously in the understanding of oestrogen biosynthesis.

'In vitro' studies show that oestrogens may arise from $^{14}$C-labelled acetate in the ovaries of many species including man (Wotiz et al, 1955; Sweat et al, 1960; Ryan and Smith, 1961a) and from $^{14}$C-labelled cholesterol (Ryan and Smith, 1961c). Bloch, in 1945, showed that cholesterol can give rise to pregnanediol in the urine, which suggested that progesterone might be an intermediate between cholesterol and oestrogens. The conversion of progesterone to oestrogens was demonstrated 'in vivo' in a human subject by Davis and Plotz (1958) and 'in vitro' by Ryan and Smith (1961b). In this reaction 17-hydroxyprogesterone and androstenedione, both of which have been isolated from ovaries (Zander, 1958; Short, 1960) are intermediate products (Ryan and Smith, 1961d). Thus, evidence was obtained for a pathway from

cholesterol to oestrone and oestradiol-17β with progesterone, 17-hydroxyprogesterone and androstenedione as intermediates.

Three other compounds were also isolated in the experiments of Ryan and Smith (1961d): pregnenolone, 17-hydroxypregnenolone and dehydroepiandrosterone (DHA), giving support for the proposal of Neher and Wettstein (1960) that another pathway for the biosynthesis of oestrogens may exist including cholesterol, pregnenolone, 17-hydroxy-pregnenolone, DHA and androstenedione. Further support for this pathway comes from the observation of Axelrod and Goldzieher (1962) that 4-$^{14}$C-pregnenolone can act as a precursor of oestrone and oestradiol-17β in the human ovary.

Androstenedione and testosterone can also act as precursors of oestrogens in the presence of placental tissue (Longchampt et al, 1960; Ryan, 1959), ovaries Smith and Ryan, 1961; Baggett et al, 1956), corpora lutea (Wiest and Zander, 1961; Huang and Pearlman, 1963), and testes and adrenal cortex (Baggett et al, 1959). Meyer (1955) suggested that 19-hydroxyandrostenedione is an intermediate metabolite between androstenedione and oestrone. This view was supported by the work of Longchampt et al (1960) who demonstrated the conversion of 4-$^{14}$C-androstenedione to 19-hydroxyandrostenedione. The compound 19-oxo-androstenedione is aromatized to an even greater extent than 19-hydroxy-

androstenedione (Hayano et al, 1960; Morato et al, 1961), which suggests that both compounds may be intermediates in oestrogen biosynthesis. Current views on the biosynthesis of oestrogens in men and non-pregnant women are summarized in Fig. 4, the major pathway being that involving the 19-oxygenated derivatives of androstenedione.

There are two ways in which the angular methyl group at C-10 could be split off in the process of aromatization: (1) by removal of formaldehyde from the 19-oxo compound followed by spontaneous aromatization of ring A, and (2) by further oxidation of the 19-oxo compound to a 19-carboxy compound with subsequent loss of carbon dioxide; the 19-nor steroid thus formed could aromatize after introduction of another double bond in addition to that between C-4 and C-5. Support for the former hypothesis comes from the work of Breuer and Grill (1961) who, after incubation of either testosterone, androstenedione or 19-hydroxyandrostenedione with human placental microsomes, found formaldehyde and the corresponding oestrogen.

The dehydrogenation step at carbon atoms C-1 and C-2 in the process of aromatization has been studied in two laboratories with differing results. Axelrod and Goldzieher (1962) studied the conversion of androstenedione to oestrone by incubating with human and baboon ovaries and concluded that

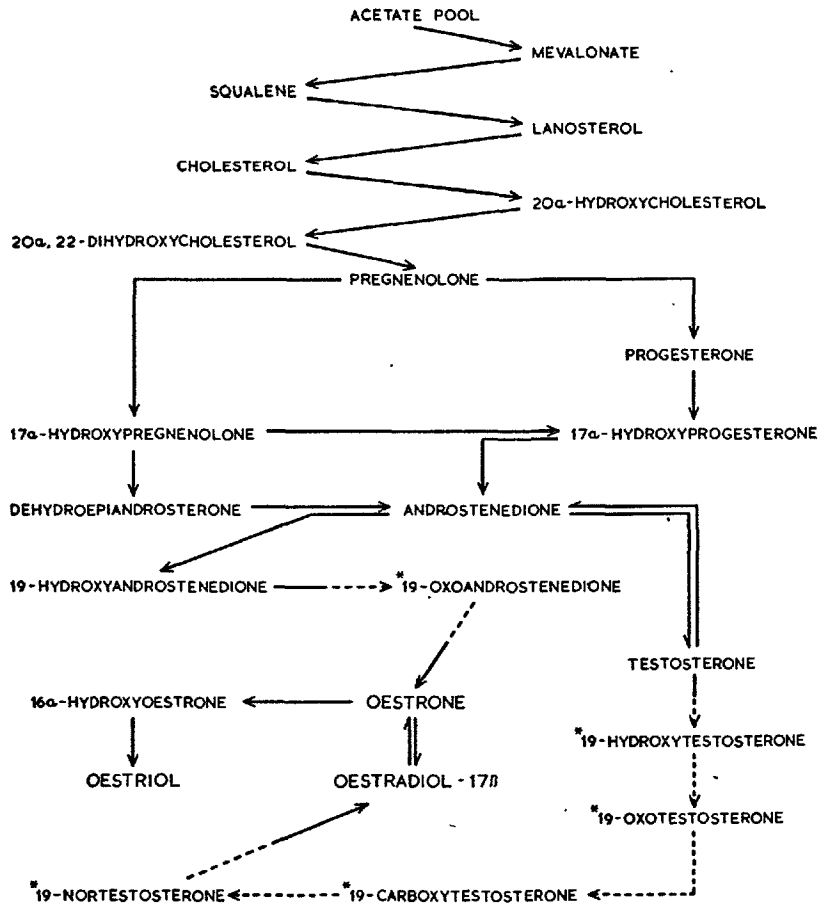


Fig. 4. Oestrogen biosynthesis in men and non-pregnant women.

* Indicates postulated intermediates.

the 1α hydrogen is eliminated together with the loss of carbon atom C-19. However, Morato et al (1962) studying the same reaction with a human placental preparation, concluded that it is the 1β hydrogen which is eliminated. The work of Brodie et al (1962) also indicates a preferential elimination of the 1β hydrogen during aromatization.

Substituted neutral steroids can also be aromatized. Thus, Ryan (1959) found oestriol after incubation of androstenetriol with the 10,000 g supernatant of human placenta and showed that 16α-hydroxytestosterone is an intermediate. Corresponding phenolic compounds can also be derived from 6α- and 6β-hydroxyandrostenedione which suggests that some of the hydroxylated phenolic steroids found in urine may originate not as metabolites of oestrone and oestradiol-17β but from the corresponding neutral steroids by aromatization.

## E. METABOLISM

As early as 1929, Fee et al perfused a heart-lung-kidney preparation with 'oestrin' and concluded that 'oestrin' is destroyed in the body. Zondek (1934) found that mammalian liver destroys oestrone, and since a boiled liver brei was inactive, deduced that oestrone had been destroyed enzymatically. These and similar experiments confirmed the idea that

oestrogens are inactivated by enzyme systems in animal tissues but identification of the products of inactivation had to await the availability of radioactively labelled oestrogens and advances in methodology.

A number of investigations have demonstrated the importance of the liver in oestrogen metabolism, and most of the metabolic changes affecting the oestrogen molecule in the human have been shown to occur in the liver. These changes include hydroxylation, oxidation, reduction and methylation, and possibly, epimerization, epoxidation (Breuer and Knuppen, 1961) and the formation of p-quinols (Hecker, 1958). Conjugation with glucuronic and/or sulphuric acid and the binding of oestrogens to protein also seem to be of physiological importance in the metabolism of oestrogens. Some probable pathways of oestrogen metabolism are indicated in Fig. 5. The concept of unique steroid metabolites cannot be applied to the oestrogens because of the complex interrelationships that exist among the members of this group.

The best known and most studied reaction in oestrogen metabolism is the interconversion of oestrone and oestradiol-17β. This reaction, together with the conversion of both these compounds to oestriol, was first demonstrated by Pincus and Pearlman in 1942 and confirmed by many workers in subsequent years. Owing to methodological inadequacies,

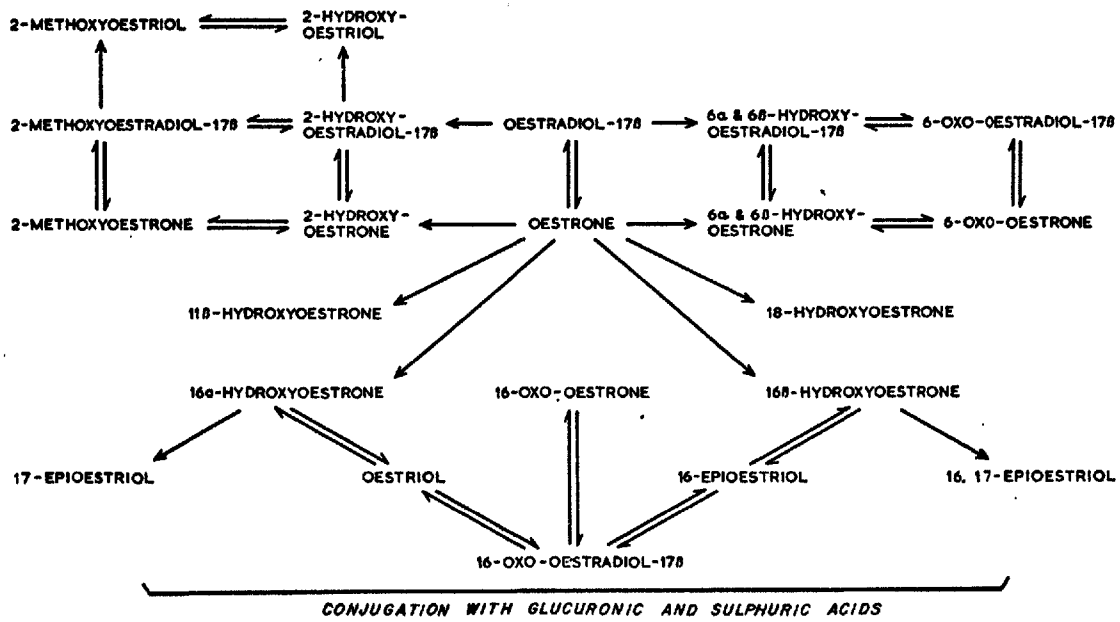


Fig. 5. Some probable pathways of oestrogen metabolism.

no reliable information on the quantitative aspects of these reactions became available until 1955, when Beer and Gallagher (1955a, 1955b) reported on the fate of different doses of isotopically labelled oestrone and oestradiol-17β administered to human subjects.

A more detailed study of the metabolism of the classical oestrogens was carried out by Brown (1957). He observed that the urinary levels of oestrone and oestradiol-17β took 2-4 days to return to control levels compared with 4-9 days for oestriol when oestrone or oestradiol-17β were injected intramuscularly. This would suggest that a considerable time is required for the conversion of oestrone and oestradiol-17β to oestriol, and because of this excretion of oestriol lags behind that of the other two. However, in 1964, Adlercreutz and Schauman suggested that the enterohepatic circulation of oestrogens, and especially of oestriol, is the main cause for the delayed excretion of oestriol following administration of oestradiol-17β.

The work of Brown (1957) also suggested that the conversion of oestradiol-17β to oestrone is faster than the reverse reaction which was confirmed by Fishman et al (1960a). Fishman and his colleagues made another important contribution to the field of oestrogen metabolism when they injected oestradiol-17β, labelled with tritium in the α-orientation

of C-17, into two women (Fishman et al, 1961). Most of the tritium appeared promptly in the body water, and the oestriol isolated from the urine of both patients contained only a small amount of radioactivity, thus indicating that oestrone is the real precursor of oestriol.

The state of ovarian function appears to have little influence on the way in which the three classical oestrogens are metabolised (Brown, 1957; Morse et al, 1963) but during pregnancy the situation seems more complicated. Davis et al (1963) were led to conclude that, between the 12th and 15th weeks of pregnancy, adaptive changes take place in the metabolism of oestrogens by maternal tissues which result in enhanced conversion of oestradiol-17β to oestriol. Wilson et al (1964) showed that, as in the case of the non-pregnant individual (Brown, 1957), oestriol is not metabolised to a significant extend during pregnancy.

Since the discovery that 2-hydroxyoestrone (Fishman et al, 1960b) and 2-methoxyoestrone (Kraychy and Gallagher, 1957) are metabolites of oestradiol-17β in man, the function and metabolism of the 2-substituted oestrogens has been investigated in many laboratories (Axelrod et al, 1961; Breuer et al, 1961a; Fishman, 1963). Kraychy and Gallagher (1957) suggested that methoxylation takes place in two separate stages, namely oxidation and subsequent methylation. Fishman et al (1960a) have shown that 2-hydroxylation does

occur in human tissues while enzymatic methylation of 2-hydroxyoestradiol-17β has been demonstrated by Breuer and his colleagues (Breuer et al, 1961a). Enzymatic demethylation of 2-methoxyoestrogens by rat liver was shown to be possible but only to a small extent (Mittermayer and Breuer, 1962).

In 1957, Mueller and Rumney showed that when 16-$^{14}$C-oestradiol-17β was incubated under aerobic conditions with mouse liver microsomes and reduced nicotinamide adenine dinucleotide phosphate a number of radioactive products more polar than oestradiol-17β were formed. One of the products proved to be identical with 6-oxo-oestradiol-17β, whilst the remaining phenolic substances were identified as 6'β'-hydroxyoestrone and 6'β'-hydroxyoestradiol-17β. The same metabolites were detected by Breuer et al (1959a) after the incubation of oestrone or oestradiol-17β with rat liver slices, but it was not until reference samples of known configuration became available that the experiment could be repeated when 6α- as well as 6β-hydroxyoestradiol-17β were identified as metabolites of oestradiol-17β (Breuer et al, 1961b). Incubation of 6-oxo-oestradiol-17β with rat liver slices also yields 6α- and 6β-hydroxyoestradiol-17β. Breuer et al (1960a) found that 6-hydroxyoestriol was formed when oestriol was incubated with rat liver but were unable to confirm its configuration.

Prior to 1960, no 6-oxo- or 6-hydroxy- phenolic steroids had been shown with certainty to occur in human urine. In order to find out whether human liver is capable of hydroxylating oestrogens at C-6, Breuer et al (1960b, 1960c) incubated human foetal liver slices with oestradiol-17β. Small amounts of 6-hydroxyoestradiol-17β were detected among the metabolites but the configuration of the 6-hydroxy group was not determined. However, experiments with 6-oxo-oestradiol-17β have shown that, like rat liver, human liver contains a 6α- and a 6β-hydroxysteroid dehydrogenase (Breuer et al,1961b).

Little information is available on the metabolism of the 7-, 11-, 15- and 18-substituted oestrogens. It is probable that 7α-hydroxylated phenolic steroids are present in human urine since 7α-hydroxy-DHA, which is a normal steroid metabolite in man (Starka et al, 1962), can be aromatized to 7α-hydroxyoestrone by human placental tissue (Cedard et al, 1964). Further experiments have shown that placental tissue contains a 7α-hydroxysteroid dehydrogenase capable of oxidizing 7α-hydroxyoestrone to 7-oxo-oestrone, in addition to a 7β-hydroxysteroid dehydrogenase (Cedard et al, 1964).

In 1962, C-11 substituted oestrogens had not yet been isolated from human urine but 11β-hydroxylation of neutral steroids had been shown to occur in the adrenal cortex (Hechter et al, 1951). This prompted Knuppen and Breuer

(1962a) to incubate oestrone with an ox adrenal homogenate. They isolated a metabolite of similar polarity to 16α-hydroxyoestrone which was subsequently identified as 11β-hydroxyoestrone. In preliminary experiments the metabolism of this oestrogen was investigated using rat liver slices (Breuer, 1962). It was found that 11β-hydroxyoestrone and 11β-hydroxyoestradiol-17β are readily inter-convertible and that both steroids can be oxidized to the corresponding 11-oxo-compounds. Since 11β-hydroxyoestrone has now been isolated from human urine (Breuer, 1964) it seems probably that oestrone and oestradiol-17β are metabolized to the corresponding 11-substituted compounds in man as well as in the rat.

Another of the newer oestrogen metabolites to be discovered is 15α-hydroxyoestrone which was first isolated after the incubation of oestrone with ox adrenal tissue (Knuppen and Breuer, 1964). Evidence suggesting that 15α-hydroxylation of phenolic steroids also occurs in human tissue has been obtained. 'In vitro' studies with human liver indicate the presence of both 15α- and 15β-hydroxysteroid dehydrogenases in this tissue (Knuppen and Breuer, 1966), in which case one would expect to find 15-hydroxy- and 15-oxo-oestrogens in human urine. In fact, 15α-hydroxyoestrone (Knuppen et al, 1965a), 15β-hydroxy-

oestrone, 15β-hydroxyoestradiol-17β (Knuppen et al, 1966b) and 15α-hydroxyoestradiol-17β (Lisboa et al, 1967), have now been isolated from human pregnancy urine. In 1967, Zucconi et al isolated 15α-hydroxyoestriol, a foetal metabolite, from pregnancy urine and from the urine of newborn infants.

In the course of the isolation of new Kober chromogens from pregnancy urine, Marrian and his colleagues obtained a phenolic steroid (KC-6A) which on treatment with sodium hydroxide yielded formaldehyde and 18-noroestrone (Loke et al, 1958). It was concluded that the phenolic steroid KC-6A was 18-hydroxyoestrone (Loke et al, 1958, 1959). Since it was known that the 18-hydroxylated $C_{21}$ steroids were of adrenal origin, Loke et al (1957) suggested that 18-hydroxyoestrone may be formed in the adrenal gland by 18-hydroxylation of oestrone. Accordingly, oestrone was incubated with ox adrenal homogenates (Loke et al, 1957) and small yields of a Kober chromogen resembling 18-hydroxy-oestrone in its chromatographic behaviour were obtained. Knuppen et al (1965b) demonstrated that human adrenal tissue can also hydroxylate oestrogens at C-18.

Key compounds in the metabolism of the 16-substituted oestrogens are 16-oxo-oestrone and 16-oxo-oestradiol-17β. The former compound can arise by partial oxidation of 16-oxo-oestradiol-17β (Lucis and Hobkirk, 1963) and, on incubation

with human liver slices, yields 16α-hydroxyoestrone, 16β-hydroxyoestrone, 16-oxo-oestradiol-17β, oestriol, 16-epi-oestriol, 17-epi-oestriol and 16,17-epi-oestriol (Breuer et al, 1959b). Partial oxidation of oestriol yields 16-oxo-oestradiol-17β (Levitz et al, 1958) which can itself be reduced to oestriol and 16-epi-oestriol (Levitz et al, 1960). The hydroxylation of oestrone to 16α-hydroxyoestrone, and the subsequent reduction of this compound to oestriol (Brown and Marrian, 1957; Nocke et al, 1961) seem to be quantitatively the most important reactions in the metabolism of the 16-substituted phenolic steroids. Other significant reactions are the oxidation of oestriol to 16-oxo-oestradiol-17β (Levitz et al, 1958) and the formation of 16β-hydroxy-oestrone with subsequent reduction to 16-epi-oestriol (Nocke et al, 1961). The formation of 17-epi-oestriol and 16,17-epi-oestriol (Nocke et al, 1961) ie not quantitatively important.

In 1963, Knuppen and Breuer reported the 'in vitro' aromatization of androstadienone to oestratetraenol which, as had already been shown can be converted to 16,17-epi-oestriol (Stimmel and Notchev, 1961; Knuppen and Breuer, 1962b). On chemical grounds it was suggested that this conversion occurs via the 16α, 17α-epoxide, a proposal supported by the work of Knuppen and Breuer (1962b). It

was not until 1968, however, that oestratetraenol was identified in human pregnancy urine (Thysen et al, 1968).

Since the first studies on oestrogen metabolism, it has been repeatedly suggested that the phenolic steroids are converted to non-steroidal compounds. This suggestion is based partly on the low recovery of phenolic steroids from the urine after administration of oestrone and oestradiol-17β (Beer and Gallagher, 1955a, 1955b) and partly on the rapid disappearance of phenolic steroids after incubation with animal tissues. The work of Knuppen et al (1961) indicates that liver preparations can convert oestradiol-17β and 2-methoxyoestradiol-17β to non-phenolic compounds.

In an effort to obtain more information about the nature of the degradation products of oestrogens, Jellink (1959) incubated human and rat liver slices with 16-$^{14}$C-oestrone. The results show that liver has a marked ability for converting oestrone into water-soluble, ether-insoluble metabolites. The fact that detectable radioactivity was absent from the $CO_2$ collected during the incubation indicates that ring D is not ruptured. It is possible that these water-soluble oestrogen degradation products do have an intact structure which, by extensive conjugation or hydroxylation of an unknown type, is extremely soluble in water.

Conjugation is an important process in the metabolism

of oestrogens. In organs where oestrogens are elaborated they occur predominantly in the free form, whereas in other parts of the organism they seem to be present in conjugated forms, as oestrogen glucuronosides or sulphates. Formation of glucuronosides occurs primarily in the liver in a reaction mediated by uridine diphosphate glucuronic acid (Isselbacher, 1956). The formation of glucuronosides is an efficient and irreversible process which is probably a means of inactivation and excretion of the steroid. In contrast, the formation of sulphates is relatively inefficient, the renal clearance is low and there is probably a reasonable amount of hydrolysis back to the free steroid. There is fairly good evidence that other conjugates such as phosphates occur in urine and blood but their biological significance is not clear.

## F. MECHANISM OF ACTION

The first step in the action of a hormone is its specific interaction with a responsive cell. Experiments with radioactively labelled hormones indicated that particular hormones are selectively concentrated by specific target cells and tissue, for example, oestrogens by the uterus (Jensen and Jacobson, 1962). In general, the tissues that respond most show the highest uptake of the hormone and retain it longest.

Much work has been carried out in order to elucidate the mechanism of action of hormones and three general mechanisms have been proposed. (1) Hormones may alter the permeability of the cell membrane or the membranes of intracellular organelles thus influencing the movement of materials and so conditioning the rate of some biochemical sequence. The membrane permeability hypothesis was first used with reference to the action of insulin (Levine and Goldstein, 1955). (2) Hormones exert a direct effect upon intracellular enzyme systems. There is evidence to support the hypothesis that many peptide and protein hormones bring about intracellular effects through the mediation of a second messenger, 3', 5'-cyclic adenosine monophosphate (Sutherland et al, 1968). It is thought that adenyl cyclase is somehow related to the hormone receptor at the cell surface. (3) Hormones may produce their effects by activating or suppressing certain genes. This hypothesis derives from studies on insect development, particularly the effect of ecdysone upon the salivary gland giant chromosome (Karlson, 1965). Such studies led to the idea that steroid hormones as a group may function as gene regulators.

With regard to the mechanism of action of oestrogens, the hypothesis receiving greatest support is that they act to regulate the expression of particular genes within target

cells (Talwar, 1969). Biochemical studies on the uterus and other oestrogen responsive tissues indicate that oestrogen stimulates the synthesis of messenger ribonucleic acid and protein (Gorski et al, 1965). Much data has been derived from experiments in which the actions of actinomycin D (which blocks transcription) and puromycin (which blocks translation) have been used. Such studies indicate that the gene locus is at least one of the points of action of the hormone.

An effect at the level of the gene is also indicated by studies of the distribution of radioactive oestrogen in target and non-target tissues. Tritiated oestradiol-17β binds strongly within the nuclei of the uterus, vagina, oviduct and mammary gland of the rat, within the nuclei of tissues associated with feedback control of sexual function and within the nuclei of the granulosa cells of the ovary and interstitial cells of the testis. No appreciable radioactivity is found in non-target tissues such as liver and diaphragm (Stumpf, 1969).

Jensen (1965) reported that centrifugal fractionation of uterine homogenates indicates that there are two intracellular sites for the 'in vivo' binding of oestradiol-17β. Nuclear-bound oestradiol-17β predominates but smaller amounts of the hormones are associated with a soluble macromolecule

in the cytosol. A similar distribution pattern is found when uteri are incubated with oestradiol-17β at 37°C (Jensen et al, 1967) but, when the incubation is carried out at 2°C, oestradiol-17β is found predominantly in the cytosol, moving to the nucleus as the temperature is raised (Jensen et al, 1968).

Toft and Gorski (1965, 1966) found that the oestradiol-17β receptor of the cytosol could be characterized as a 9.5S protein (now considered to be about 8S) by sucrose gradient ultracentrifugation. The complex extracted from the nucleus was shown to be different, as judged by its sedimentation behaviour, and was identified as a 5S protein (Jensen et al, 1972). Furthermore, it has been demonstrated that the 8S cytosol complex is reversibly de-aggregated to a smaller unit in the presence of 0.3M KCl and that this subunit of the cytosol complex can be differentiated from the nuclear complex by sucrose gradient ultracentrifugation in the presence of NaCl, when it sediments at about 4S (Erdos, 1968).

It is thought that, on entering the target cell, oestradiol-17β binds spontaneously with the 8S receptor protein forming a complex which subsequently de-aggregates to a 4S complex. The latter undergoes a temperature-dependent conversion to the 5S form but whether this

conversion occurs in the cytoplasm, at the nuclear membrane or within the nucleus remains to be elucidated, as does the sequence of events occuring between the arrival of oestradiol-17β in the nucleus and the triggering of protein synthesis.

In an investigation of the specificity of target tissue receptors, Jensen et al (1966) compared the behaviour of a variety of oestrogenic steroids with that of oestradiol-17β. It was found that 17α-methyloestradiol and 17α-ethynyloestradiol (as well as hexoestrol) resemble oestradiol -17β whereas oestriol shows a more transient binding. Alberga et al (1970) used chase experiments to test the ability of various compounds to displace $^{3}$H-oestradiol-17β bound in the uterus. Testosterone and progesterone had no effect, oestradiol-17α was much less active than oestradiol-17β, oestrone was less active than oestradiol-17α and diethylstilboestrol had an affinity similar to that of oestradiol-17β. Such experiments indicate the importance of the 17β-hydroxyl group and a phenolic group. As one would expect, it was found that binding affinity parallels biological activity.

# CHAPTER 2

# Chapter 2

# REVIEW OF METHODOLOGY

## A. INTRODUCTION

Progress in steroid biochemistry depends upon the development of techniques which enable the investigator to know what steriod he is dealing with and how much of it is present in the material being considered. Until the steroid becomes available in pure form, its assay must depend upon some relatively unique biological alteration which it produces, for example, cornification of the vaginal epithelium of rodents by oestrogens. Once the structure of the steroid is known, physical and chemical procedures can be developed for its detection and quantitative determination. Apart from the testing of new products in the pharmaceutical industry, bioassays have now been almost completely replaced by chemical and isotopic techniques for hormone assay. Many of the techniques which have been applied to steroid analysis are indicated in Fig. 6.

The formation of chemical derivatives with chromogenic or fluorescent properties can be used for the determination of groups of steroids of sufficient concentration in body fluids, for example, the Porter-Silber reaction for

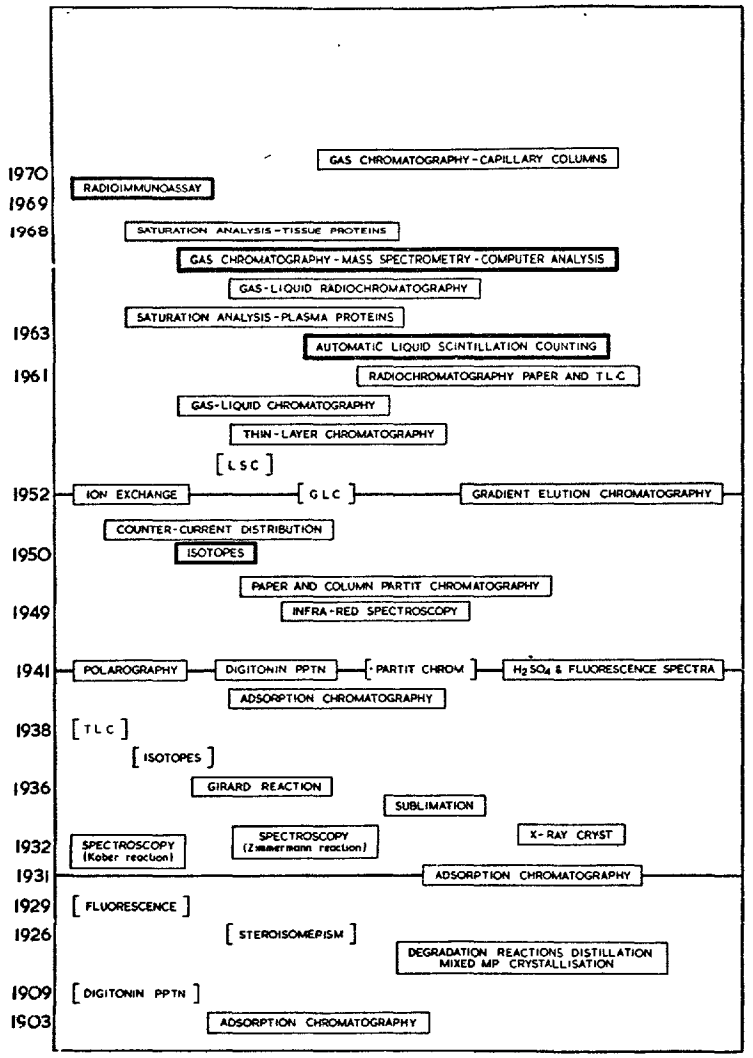


Fig. 6. Techniques used in steroid analysis.

17-hydroxycorticosteroids (Porter and Silber, 1950), the Zimmerman reaction for 17-ketosteroids (Zimmerman, 1936) and the Kober reaction for oestrogens (Kober, 1931).

Gas liquid chromatography (GLC), the principle of which is illustrated in Fig. 7, is an important technique in steroid biochemistry involving both separation and quantitation. It depends upon the partition of volatile compounds between a carrier gas and a liquid stationary phase. Steroids of different molecular weight or stereo-isomers can be separated fairly easily and derivatives, such as acetates and trimethyl silyl ethers, are often formed to accentuate differences. A detector of high sensitivity is required for most steroid analyses, the hydrogen flame ionization detector and electron capture detector being those most commonly used. Examples of the application of GLC to steroid determinations are the measurement of oestrogens in pregnancy urine (Wotiz and Martin, 1962) and in the plasma of non-pregnant women (Wotiz et al, 1967), plasma progesterone (Wyman and Sommerville, 1968), urinary testosterone (Vestergaard et al, 1966) and plasma testosterone (Collins et al, 1968). More recently, GLC with high resolution glass capillary columns has been developed for the simultaneous determination of a number of neutral steroids in urine (Ros and Sommerville, 1971).

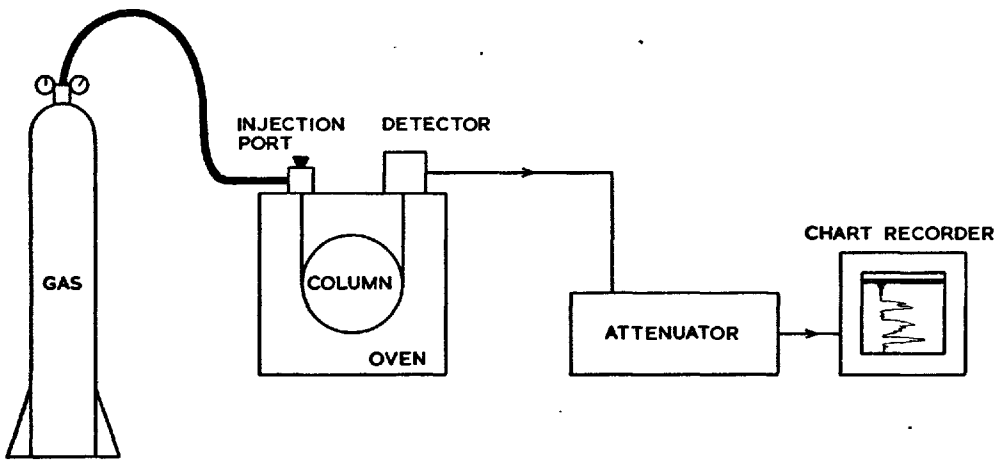


Fig. 7. Basic GLC system.

Another technique which has been applied to the measurement of steroids in blood and plasma is double isotope derivative formation. This technique involves addition of a radioactively labelled internal standard to the biological sample, and formation of various derivatives using reagents labelled with different radioisotopes and of known specific activity. Reagents which have been used include $^{3}$H-acetic anhydride, $^{35}$S-thiosemicarbazide, $^{35}$S-p-toluene sulphonic acid and $^{35}$S-p-iodobenzene sulphonyl chloride. The derivative formed is purified, after addition of carrier derivative, by multiple chromatographic steps and finally counted for both isotopes. From the counts obtained one can calculate back to find the amount of the compound that was present in the original sample. This technique has been applied to the determination of a number of steroids including aldosterone (Brodie et al, 1967) and oestrone and oestradiol-17β (Baird, 1968), which occur in very low concentrations in human peripheral blood.

A major advance in steroid methodology has been the introduction of saturation analysis techniques which depend upon the ability of certain binding proteins to combine specifically with a smaller molecule, the ligand. Examples of such binding pairs are antigen-antibody reactions, the binding of thyroxine, cortisol and gonadal hormones to

specific plasma globulins and the binding of steroid and peptide hormones to their cellular receptor sites. Some properties of proteins used for the assay of steroids are listed in Table 2.

Using a radioactively labelled ligand an assay system can be set up in which the combination of binding protein and labelled ligand can be inhibited by the introduction of very small quantities of unlabelled ligand. The resulting fall in protein-bound radioactivity can be used to establish a standard curve for the estimation of unknown amounts of the ligand over the picogram to nanogram range of concentration. The assay is called competitive protein binding when the binding reagent is a receptor protein or plasma globulin and radioimmunoassay when it is an antibody. Examples of the application of competitive protein binding are the assay of plasma aldosterone (Coghlan and Scoggins, 1967), cortisol and corticosterone in plasma (Murphy et al, 1963), oestrone and oestradiol-17β in blood (Corker and Exley, 1970), plasma progesterone (Riondel et al, 1965) and testosterone in male plasma (Horton et al, 1967).

A number of radioimmunoassay procedures have been described for the determination of steroids in peripheral venous blood or plasma. Examples are the radioimmunoassay of plasma oestrone and oestradiol-17β (Mikkail et al, 1970;

| PROPERTY | PLASMA PROTEIN | TISSUE PROTEIN | ANTIBODIES |
|---|---|---|---|
| ASSOCIATION CONSTANT | $10^7$-$10^{10}$ l/mole | high | very high |
| SPECIFICITY | | | |
| a) degree | good | better | best |
| b) relation to biological activity | high | very high | unknown |
| c) variability/stability | stable within species | stable within species | variable |
| STORAGE | very stable | unstable | very stable |
| TITRE | low | low | very high |
| PREPARATION | dilution (minutes) | cell fractionation (hours) | immunization (months) |

Table 2. Some characteristics of proteins used for the determination of steroids.

Emment et al, 1972), plasma oestriol (Tulchinsky and Abraham, 1971), plasma progesterone (Youssefnejadian et al, 1972a), plasma 17-hydroxyprogesterone (Youssefnejadian et al, 1972b) and testosterone in plasma (Furuyama et al, 1970; Collins et al, 1972).

## B. ESTIMATION OF OESTROGENS IN BLOOD AND PLASMA

Most methods for estimating oestrogens prior to 1955 involved bioassay techniques. A considerable amount of useful clinical information was accumulated on oestrogen excretion in health and disease but the results obtained were often of dubious quantitative significance. Specific determination of the individual oestrogens was not possible and had to await the introduction of various purification procedures, notably partition chromatography. Since 1955, reliable chemical methods, especially the Brown method(Brown, 1955), have been available for the assay of urinary oestrogens.

As the level of oestrogens in circulating blood represents the equilibrium between biosynthesis on the one hand and metabolism and excretion on the other, the determination of oestrogens in peripheral venous blood might be expected to provide different information on oestrogen physiology which complements that derived from urinary analysis. However, until recently, studies have been hampered by the lack of convenient procedures for the

assay of circulating oestrogens.

Intensive work on the Kober reaction led to the development of a colorimetric method sufficiently sensitive for the determination of the classical oestrogens in the blood of pregnant women (Roy and Brown, 1960). This technique was subsequently modified by Roy (1962) to include the fluorescence reaction of Ittrich (1960) resulting in a tenfold increase in sensitivity. Nachtigall et al, (1966) developed a rapid method for the estimation of plasma oestriol in the second half of pregnancy, quantitation being by fluorimetry. Other fluorimetric methods are those of Veldhuis (1953), Preedy and Aitkin (1957), Ichii et al (1963), Schrepfer and Nicholas (1965) and Goebelsmann and Gorwill (1970). Apart from the methods of Veldhuis (1953) and Ichii et al (1963) these methods involve hydrolysis; they all require large volumes of blood or plasma and operate at or below the lower limit of sensitivity for many samples of blood from non-pregnant women or men.

The development of GLC and ionization detection for the separation and measurement of small quantities of steroids presented a new approach to the determination of oestrogens in biological fluids. In 1964, Adlercreutz described a GLC method for the determination of conjugated oestrogens

in pregnancy blood. In 1965, Touchstone and Marawec reported a method for the measurement of free and conjugated oestrogens during pregnancy and, in 1970, Fischer-Rasmussen described a method for the measurement of free plus conjugated oestriol in pregnancy plasma.

The development of the electron capture detector by Lovelock (1961) led to an increase in sensitivity of GLC methods and, using heptafluorobutyrate derivatives and electron capture detection, Wotiz et al (1967) determined oestrone, oestradiol-17β and oestriol in hydrolysed plasma from non-pregnant subjects. Other methods involving electron capture detection have been described for the estimation of plasma oestrone (Attal et al, 1967) and plasma oestradiol-17β (Eik-Nes et al, 1965). The determination of oestrogens in blood by GLC has been reviewed by Adlercreutz and Luukkainen (1968b).

In 1960, Svendsen published a method for the determination of unconjugated oestrone and oestradiol-17β in plasma, based on the principle of double isotope derivative dilution. Using this method, the plasma levels of unconjugated oestrone and oestradiol-17β have been studied throughout pregnancy (Svendsen and Sørensen, 1964a) and the menstrual cycle (Svendsen and Sørensen, 1964b). Baird and Guevara (1969) have estimated unconjugated oestrone and

oestradiol-17β in peripheral plasma from non-pregnant females throughout the menstrual cycle, from castrated and post-menopausal women and from men, using the double isotope derivative dilution method of Baird (1968), which is more sensitive than that of Svendsen (1960).

A study of the role of oestrogens in the regulation of the menstrual cycle requires daily or even more frequent determinations of the concentration of oestrone and oestradiol-17β in blood. The above mentioned methods require large volumes of blood or plasma which precludes their use in studies where frequent samples from the same subject are required. However, the recently introduced saturation analysis techniques are very sensitive requiring only small volumes of plasma and hence overcome this problem.

The observation that many hormones are bound to specific globulins in blood and specific proteins in their target tissues before expression of biological activity led to the development of competitive protein binding techniques for the assay of many steroid hormones. Shutt (1969) reported a method for the estimation of plasma oestradiol-17β throughout the menstrual cycle using as the binding protein a soluble macromolecular protein from uteri of ovariectomized ewes. Pregnant rabbit uterine cytosol has been used for the study of oestradiol-17β levels in plasma

during the menstrual cycle (Corker and Exley, 1970), and by Korenman et al (1969) in men, postmenopausal women and normal women throughout the menstrual cycle. This approach has also been applied to the estimation of plasma oestrone in the normal menstrual cycle, in postmenopausal women and in men (Tulchinsky and Korenman, 1970).

The uterine binding proteins are temperature sensitive and relatively unstable. A more stable and readily prepared binding protein is provided by the oestradiol binding β-globulin of human plasma (Rosner et al, 1969), although it is less specific, reacting with several 17-hydroxysteroids (Heyns et al, 1969). Murphy ($1968_a$) observed that oestradiol-17β binding activity increases markedly during the third trimester of pregnancy and suggested the use of pregnancy plasma for the competitive binding assay of this steroid. Mayes and Nugent (1970) used this binding protein in a competitive binding system for the assay of oestradiol-17β in normal women throughout the menstrual cycle and in men. A similar assay was devised by Dufau et al (1970).

Antibodies, which belong to the γ-globulin class of proteins, constitute another group of binding proteins. In 1959, Lieberman et al reported that steroid-protein conjugates may be used as haptens for the production of

antibodies, yet it was 10 years before methods based upon the principle of radioimmunoassay were applied to the quantitative determination of steroid hormones in biological samples.

In 1969, Abraham reported the first radioimmunoassay method for the estimation of plasma 'oestrogen', in which polystyrene tubes were coated with antiserum. After equilibration with standards or unknowns, the free steroid was separated from that bound to antibody by pouring out the incubation mixture. Subsequently, Thorneycroft et al, (1970a) included a thin layer chromatographic step to enable the separate determination of oestrone and oestradiol-17β. Mikhail et al (1970) isolated oestrone and oestradiol-17β from plasma extracts by means of column chromatography on Sephadex LH 20. The fractions were incubated with polymerized antibody mixed with $^{3}$H-oestrone or $^{3}$H-oestradiol-17β, and the protein-bound and free components separated by centrifugation. Hotchkiss et al (1971) used thin layer chromatography to isolate oestradiol-17β, a liquid phase incubation system and dextran-coated charcoal for the separation of antibody-bound and free steroid. Microcolumns of Sephadex LH 20 were used for the isolation of oestrone, oestradiol-17β and oestriol from plasma extracts and dextran-coated charcoal was used for the separation of bound

and free components by Wu et al (1971). These methods all employ antisera raised against oestradiol-17β-hemisuccinate-bovine serum albumin. Oestriol has been determined in pregnant subjects by radioimmunoassay (Tulchinsky and Abraham, 1971), the antiserum used being one raised against oestriol-3,16α,17β-trisuccinate-human serum albumin. Microcolumns of celite were used to separate oestriol from oestrone and oestradiol-17β and again dextran-coated charcoal was used for the separation of the bound and free fractions. Recently more specific antisera have been raised using oestrogens conjugated to protein through C-6 in the B ring (Exley et al, 1971; Lindner et al, 1972; Kuss and Goebel, 1972; Jeffcoate and Searle, 1972).

## C. PRINCIPLES OF RADIOIMMUNOASSAY

While studying the metabolism of $^{131}$I-insulin in diabetes (Berson et al, 1956) it was observed that nearly all insulin-treated diabetics had insulin-binding antibodies. In this work, Berson and his colleagues showed that addition of increasing amounts of insulin to a fixed dilution of antiserum caused a gradual decrease in the fraction bound to Y-globulin, although the absolute amount had increased, and that $^{131}$I-insulin bound to Y-globulin could be displaced by addition of non-radioactive insulin to the system. The

recognition that the binding of labelled insulin is a quantitative function of the amount of insulin present when the antibody concentration remains constant, and that labelled insulin can be displaced by unlabelled insulin, formed the basis for the radioimmunoassay of insulin. Antisera to insulin were raised in guinea pigs and, in 1959, Yalow and Berson published the first results on human plasma insulin determined by the principle of radioimmunoassay. The next application of this technique was by Ungar et al (1959) for glucagon and during the 1960's it was applied to the assay of most peptide hormones..

Steroid hormones are not of themselves immunogenic. However, Landsteiner (1945) showed that certain small molecules, when covalently linked to proteins, become immunogenic. Thus the idea developed that, if a steroid were covalently coupled to a protein, an artifical immunogen would be produced capable of giving rise to antibodies with specificity not only for the protein carrier but also for the haptenic steroid. This has proved to be the case (Westphal, 1971b)and reference has already been made to a number of methods based upon the principle of radioimmunoassay for the determination of steroids in human peripheral venous blood.

The principle of radioimmunoassay is shown in Fig. 8. An unlabelled (non-radioactive) antigen in an unknown sample

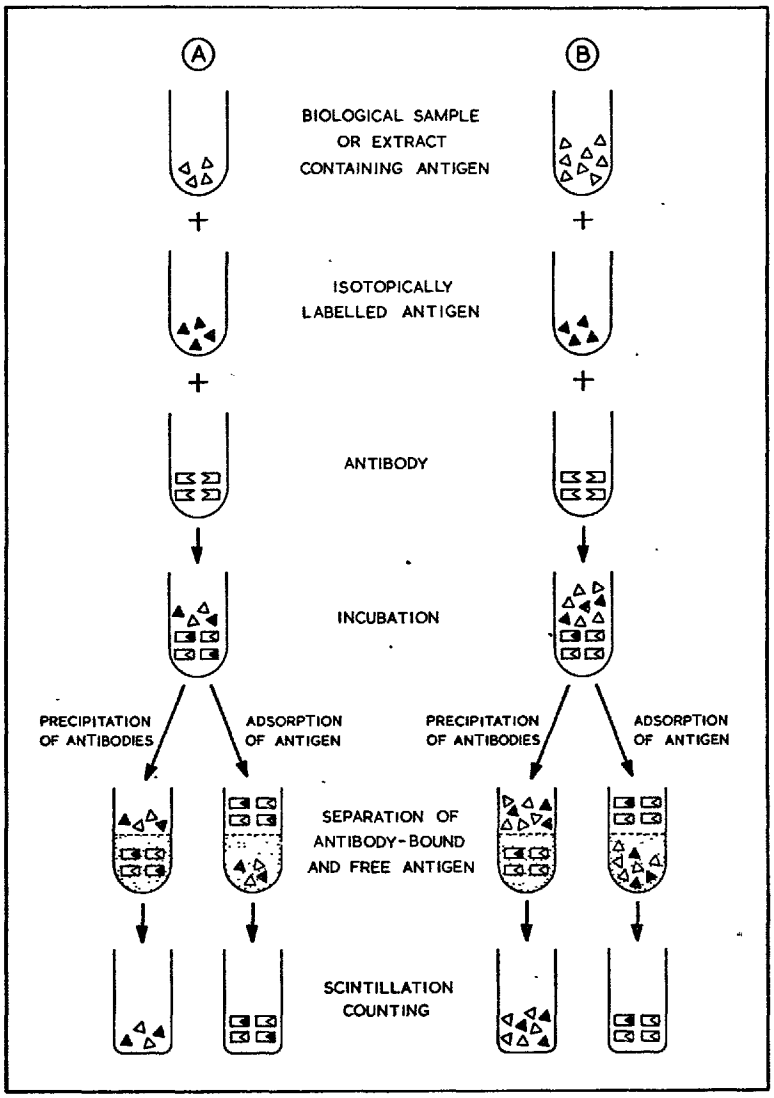


Fig. 8. The principle of radioimmunoassay. Scheme B involves twice as much unlabelled antigen as scheme A.

competes with labelled (radioactive) antigen for binding to antibody and so inhibits the binding of labelled antigen. The degree of inhibition observed with unknown samples is compared with that obtained with standard amounts of antigen. Although a number of mathematical models assume identical behaviour for labelled and unlabelled antigen, in practice the validity of the assay is dependent upon the identical behaviour of standard and unknown. The essential requirements for a radioimmunoassay include suitable reactants, namely labelled antigen and specific antiserum, and some technique for separating the antibody-bound and free antigen.

The binding of antigen to antibody appears to obey the law of mass action and, as antibodies belonging to the IgG class are considered to have two binding sites, it is necessary to assume that these are identical and function independently of one another. Considering a single type of antibody binding site and an antigen with a single reactive site, the equilibrium reaction can be written as follows:-

$$Ab + Ag \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AbAg \quad \ldots\ldots\ldots\ldots\ldots\ldots (i)$$

where, $k_{+1}$ = rate constant for forward reaction

$k_{-1}$ = rate constant for reverse reaction

Ab = unoccupied antibody binding sites

Ag = unbound antigen

and AbAg = antibody-bound antigen.

The association constant ($K_a$) is expressed by the following equation:-

$$K_a = \frac{k_{+1}}{k_{-1}} = \frac{[AbAg]}{[Ab][Ag]} \quad \ldots\ldots\ldots \quad (ii)$$

where, $[AbAg]$ = molar concentration of antibody-bound antigen

$[Ab]$ = molar concentration of free antibody binding sites

and, $[Ag]$ = molar concentration of free antigen.

If we let $Ab^o$ be the total molar concentration of antibody binding sites in the system, then:-

$$[Ab] = [Ab^o] - [AbAg]$$

Substituting for $[Ab]$ in equation (ii) above, we have:-

$$K_a = \frac{[AbAg]}{([Ab^o] - [AbAg])[Ag]}$$

and, $$\frac{[AbAg]}{[Ag]} = K_a ([Ab^o] - [AbAg]) \quad \ldots\ldots\ldots \quad (iii)$$

From equation (iii) we can see that the ratio of bound to free antigen is a linear function of the concentration of bound antigen. In reality, however, all antisera are heterogenous containing many different orders of antibody binding sites and no simple linear relationship can be

derived theoretically for such systems.

If the fraction of antigen bound is denoted by b so that $[AbAg] = b\,[Ag^o]$ where $[Ag^o] = [Ag] + [AbAg]$, and we substitute for $[AbAg]$ and $[Ag]$ in equation (iii) above, then:-

$$\frac{[AbAg]}{[Ag]} = \frac{b}{1-b}$$

$$= K_a\,([Ab^o] - b\,[Ag^o]) \quad \ldots\ldots \text{(iv)}$$

From equation (iv) it can be seen that $[AbAg] / [Ag]$ decreases as $b\,[Ag^o]$ increases, but, since b cannot exceed 1, the reduction in $[AbAg] / [Ag]$ may be hardly detectable if $[Ag^o]$ remains much less than $[Ab^o]$. Therefore, if we wish to determine an antigen concentration $[Ag^*]$, $[Ab^o]$ must not be much greater than $[Ag^*]$.

Rearranging equation (ii) we have:-

$$\frac{[AbAg]}{[Ag]} = K_a\,[Ab] \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(v)}$$

When setting up an assay, it is convenient to use an antiserum dilution which yields a bound to free ratio of approximately 1.0 when labelled antigen alone is present. In this case, we can see from equation (v) that the molar concentration of free antibody binding sites required is the reciprocal of the association constant. If the association constant of the antibody is less than $1 \times 10^6$ litres per mole, the amount of antibody required to obtain substantial binding

will be large, and if competition between labelled and unlabelled antigen is being measured, the presence of large amounts of free antibody will greatly reduce the effect of unlabelled hormone in the system. Similarly, if the labelled antigen is of low specific activity, high concentrations will be required to obtain readily measurable amounts of radioactivity; greater amounts of antibody will be required to maintain binding of labelled antigen at the desired level, and the response of the system to unlabelled antigen will again be small. Thus antibody of adequate binding affinity and labelled antigen of high specific activity are important if a good response to added antigen is to be achieved.

The association constant of an antibody is usually obtained from a Scatchard plot (Scatchard, 1949) which can be derived from equation (ii) as follows:-

Let, $n$ = antibody valency

$c$ = $[Ag]$

$[Ab']$ = total molar concentration of antibody molecules

$= ( [Ab] + [AbAg] )/n$

and, $r = [AbAg] / [Ab']$.

Then, $[AbAg] = r\,[Ab']$

and, $[Ab] = n\,[Ab'] - r\,[Ab']$.

Substituting in equation (ii) above, we have:-

$$K_a = \frac{r\,[Ab']}{c/n([Ab'] - r\,[Ab'])}$$

$$\frac{r}{c} = nK_a - rK_a \qquad \text{.....(vi)}$$

The Scatchard plot consists of a plot of r/c against r or [AbAg] / [Ab'][Ag] against [AbAg] / [Ab'] . In theory, a straight line graph is obtained with a slope equal to the association constant and intercept on the abscissa equal to the antibody valency. Since [Ab'] is constant in any one study a plot of [AbAg] / [Ag] against [AbAg] will yield the same information. A straight line is only obtained if there is a single species of antibody binding sites binding one antigen or antigenic determinant. In practice, it is extremely rare to find a population of antibodies which all possess exactly the same $K_a$ value. This means that the Scatchard plot will not be linear, which implies that at any given antigen concentration the radioimmunoassay system functions as one characterized by a single equilibrium constant, the value of which, given by the tangent to the curve at that antigen concentration, changes as the antigen concentration changes.

As has been mentioned already, the mathematical deri-

vations above assume that the antiserum used for an assay consists of a single species of antibody binding sites. In practice, such an antiserum has not yet been produced and so purification procedures have had to be introduced in a number of assays. Ekins et al (1969) and Rodbard (1970) have studied assay specificity and have shown that the relative potency of two cross-competing antigens in an immunoassay system is not constant but is a function of the bound to free ratio at which the relative potency estimate is made. This implies that assay specificity can be significantly increased or decreased by appropriate selection of assay reagents.

The various approaches to plotting radioimmunoassay response curves have not in general produced straight lines and so the variance in the estimate of the response at any one point is not constant. A number of people have attempted to overcome this difficulty, notably Rodbard et al (1968), who have applied weighted regression curve fitting techniques to appropriate transformations of their data. Rodbard claims that, with appropriate corrections, the logit of the response (Y) plotted against the log of the concentration of antigen is a straight line. Logit Y is $\log_B {}^{Y}/1-Y$, where $Y = {}^{B}/B^{O}$ and B and $B^{O}$ are the fractions of labelled antigen bound in the presence and absence of unlabelled antigen

respectively.

The theory underlying radioimmunoassay and other radioligand methods has been extensively developed by Berson and Yalow (1959, 1964), Ekins (1969), Borth (1970), Ekins and Newman (1970), Rodbard and Lewald (1970), Yalow and Berson (1970), Rodbard (1971) and Rodbard et al (1971). Rodbard and his colleagues have developed models for the prediction of the variance of the response around the dose-response curve (Rodbard, 1971), computer techniques to describe the equilibrium behaviour of complex, heterogeneous, cross-reactive antibodies involving any number (n) of antigens and any number (m) of antibodies (Feldman and Rodbard, 1971), and have described and made a computer simulation of the kinetic behaviour of radioimmunoassay systems prior to the attainment of equilibrium under a variety of conditions (Rodbard et al, 1971). Rodbard and Catt (1972) have also expanded the theory of radioimmunoassay to include the effect of the separation of bound and free fractions on the nature of the dose-response curve.

Mathematical models like those mentioned above have not been extensively applied outside the laboratories of the respective authors, perhaps because of their complexity. Chard (1971) has described a simple model which gives qualitative predictions which closely relate to the results

obtained in the setting up of a radioimmunoassay using a practical approach. This model is also based on the mass action equation (equation ii) and assumes that the reaction has reached equilibrium and that there is no significant difference between labelled and unlabelled antigen. If we let $[AbAg] = B$ and $[Ag^o]$ and $[Ab^o]$ are the total concentrations of antigen and antibody binding sites in the system, then from equation (ii):-

$$K_a = \frac{B}{([Ab^o] - B)([Ag^o] - B)}$$

$$= \frac{B}{[Ab^o][Ag^o] - B[Ag^o] - B[Ab^o] + B^2}$$

$$K_a([Ab^o][Ag^o] - B[Ag^o] - B[Ab^o] + B^2) - B = 0$$

$$[Ab^o][Ag^o] - B([Ag^o] + [Ab^o] + {}^1/K_a) + B^2 = 0 \quad ....(vii)$$

Equation (vii) can be solved for B by the general solution of a quadratic equation:-

$$\% \text{ bound} = (a - \sqrt{a^2 - b})c$$

where $a = [Ag^o] + [Ab^o] + {}^1/K_a$

$b = 4[Ab^o][Ag^o]$

and $c = {}^{50}/[Ag^o]$

Since the model is qualitative in essence, simple values can

be used for the different variables. This model has been used to study antibody dilution curves and standard curves which have been compared with those actually obtained in a method for oxytocin (Chard et al, 1970a, 1970b).

Ekins (1971) has deplored the way radioimmunoassays are set up in most laboratories: "I think the day of crude rule of thumb methods is passing, that somewhat more sophisticated methods for the choice of assay reagents, for the processing of assay data and for the routine validation of laboratory results are about to become the rule rather than the exceptional perogative of a few mathematically erudite laboratories". This is a very commendable attitude, but, for the majority of laboratories who do not have the resources, a simple model like that of Chard (1971) or a trial and error approach are the only possibilities.

## D. PRACTICAL ASPECTS OF RADIOIMMUNOASSAY

Protein hormones such as insulin and glucagon have circulating levels in the nanogram range and can be assayed in unextracted plasma or serum. In contrast, small peptide hormones such as oxytocin have basal circulating levels in the low picogram or even femtogram range. This means that the levels of the latter are often at or below the limit of detection of conventional radioimmunoassays. There are two

approaches to this problem: a) improve the sensitivity of the assay or b) extract and concentrate the substance to be assayed. There is a limit to the sensitivity of any radioimmunoassay which is determined by the affinity constant of the antiserum used, whereas an extraction and concentration procedure has no theoretical limits. Few if any antisera have been described yet which are capable of quantitating a single steroid in serum or plasma with complete reliability. At present, therefore, all radioimmunoassay systems for the measurement of steroids in blood require extraction of the compound prior to assay.

The principal steps of a steroid radioimmunoassay are:-

i) Extraction

ii) Separation or purification

iii) Incubation of extracts and standards with radioactively labelled (usually tritiated) steroid and antiserum

iv) Separation of antibody-bound and free steroid

v) Liquid scintillation counting of either antibody-bound or free steroid fraction, depending on the separation method

vi) Construction of a standard curve and calculation of results

Most workers add a known small amount of tritiated steroid (internal standard) prior to extraction so that losses can

be estimated. Some omit the separation and purification step.

i) Extraction

Ether is the solvent of choice for extraction in most steroid radioimmunoassays although benzene has been used for the extraction of oestrogens (Robertson et al, 1972). The levels of ether-extractable plasma steroids in peripheral venous plasma from men and women are indicated in Figs. 9 and 10 respectively. Most solvents, regardless of grade, give rise to a positive blank value which is not necessarily subtractable since substances in plasma extracts may even lower the blank. In an effort to reduce the blank value in oestrogen radioimmunoassays, the oestrogens have been extracted with fresh ether (Mikhail et al, 1970; Wu et al, 1971), fresh anaesthetic grade ether (Hotchkiss et al, 1971), cold ether (Moore and Axelrod, 1972), redistilled ether (Wright et al, 1973b) and ether redistilled after treatment with ferrous sulphate (Doerr, 1973). Apart from Hotchkiss et al (1971), who estimated procedural losses from the recovery of known amounts of oestradiol-17β or $^{14}C$-oestradiol-17β from plasma, all the above mentioned authors added tritiated internal standard(s) prior to extraction.

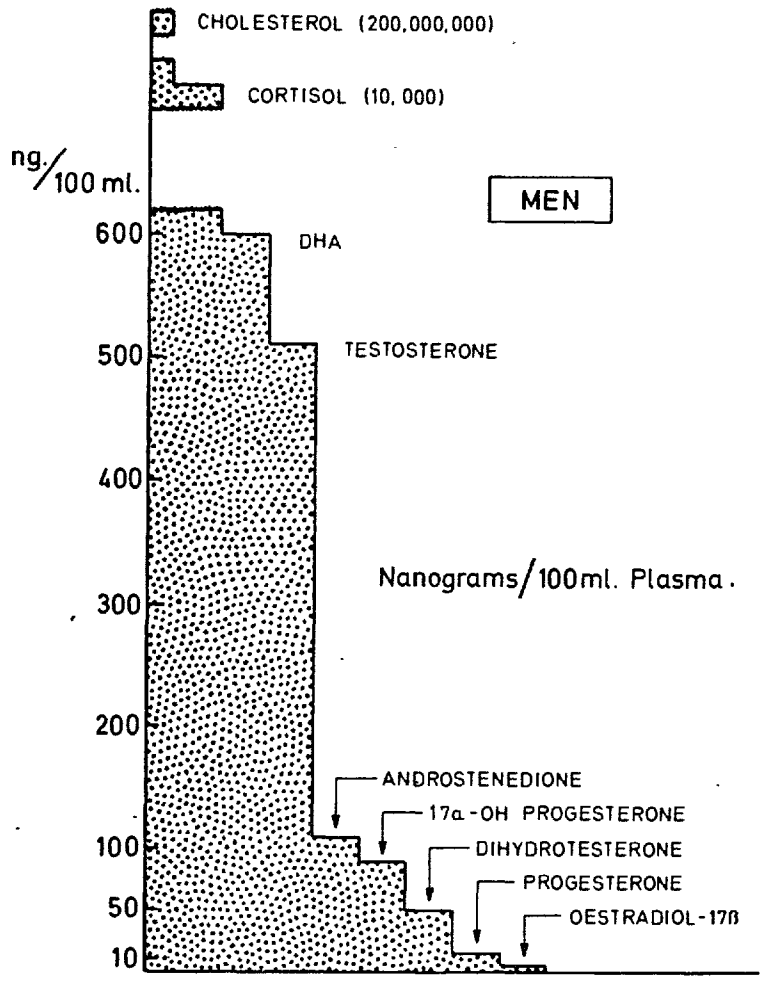


Fig. 9. Ether extractable plasma steroids in men.

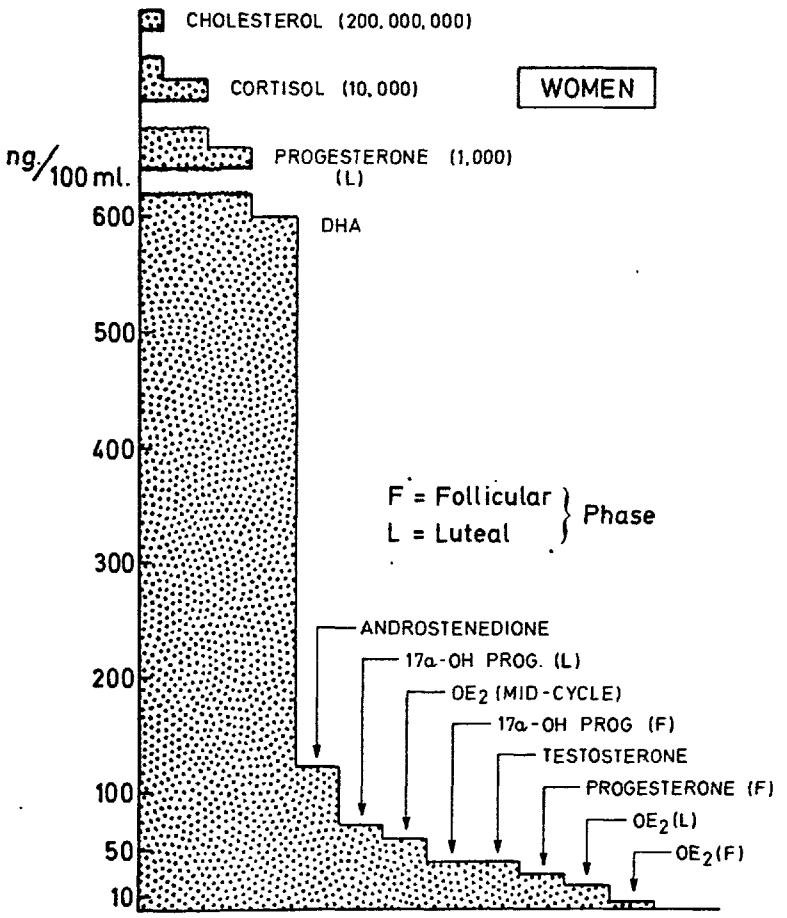


Fig. 10. Ether extractable plasma steroids in women.

ii) Separation or purification

By itself, the solvent blank has not been too great a problem. A much more serious problem is the blank value arising after separation by thin-layer chromatography (TLC). This is unfortunate since many satisfactory steroid separations have been evolved employing TLC on silica gel or other supports. Hotchkiss et al (1971) reported satisfactory blanks for their oestradiol-17β radioimmunoassay where oestradiol-17β is separated from cross-reacting components in the ether extract by TLC on Eastman Kodak plates but this does not appear to be a constant finding. On the other hand, Merck F 254 silica gel pre-coated plates do seem to give consistently low blanks. Doerr (1973) isolated oestradiol-17β from an ether extract by TLC on Merck F 254 silica gel plates, preventing oxidation of the oestradiol-17β with mercaptoethanol. Separation by column chromatography has proved to be of great value in steroid radioimmunoassays as good separations and low blank values are usually obtained. Column chromatography on Sephadex LH 20 is the most popular separation technique in the radioimmunoassay of oestrogens (Mikhail et al, 1970; Wu et al, 1971; Cameron and Jones, 1972; Moore and Axelrod, 1972; de Hertogh, 1973; Wright et al, 1973b). Chromatography on columns of celite has also been used (Robertson et al, 1972).

iii) Incubation of standards and unknowns with antiserum and labelled steroid

After extraction and purification, the assay proper can be performed. Standards and aliquots of the extract, in duplicate or triplicate, are incubated with antiserum and radioactively labelled hormone (tracer) and then the antibody-bound and free hormone fractions are separated. Peptide hormone radioimmunoassays employ γ-emitting $^{125}I$ or $^{131}I$ labelled tracer whereas steroid radioimmunoassays generally involve a β-emitting tritiated tracer. With regard to liquid phase radioimmunoassays for oestrogens, tracer can be added prior to the addition of antiserum (Cameron and Jones, 1972; Robertson et al, 1972; de Hertogh, 1973; Doerr, 1973) or subsequently (Hotchkiss et al, 1971; Wu et al, 1971; Wright et al, 1973b). The incubation is carried out at 4°C overnight by all the authors mentioned above except Wu et al (1971), who incubate for 2 hr only. In the case of the solid phase, coated-tube method as originally described by Abraham (1969) the extract, dissolved in buffer, is added to antibody-coated tubes followed by tracer and the incubation is carried out at room temperature for 2 hr. Both Mikhail et al (1970) and Moore and Axelrod (1972) add the appropriate tritiated oestrogen to a solution of polymerized antibodies, so that

a single addition of reagent is required, and incubate at room temperature for 2 hr. A slightly different approach has been reported by Nars and Hunter (1973) and Jeffcoate et al (1973), who propose the use of a radio-iodinated, rather than tritiated, oestrogen tracer with its accompanying advantages of increased sensitivity, shorter counting time and cheaper counting equipment.

iv) Separation of antibody-bound and free steroid

A means of separating antibody-bound and free antigen is a fundamental requirement of any radioimmunoassay. At the time of separation, the radioimmunoassay incubate is in a state of dynamic equilibrium. Separation systems which act rapidly to remove either the free antigen or antibody-bound antigen are preferable because they leave little time for readjustment of this equilibrium. An ideal method gives a clean separation, is reproducible and technically simple. A number of methods have been used and these fall into the following categories:-

a) electrophoretic and chromatoelectrophoretic,

b) gel filtration,

c) precipitation of antigen-antibody complex,

d) precipitation of free antigen, and

e) solid phase adsorption of antibody.

The choice is usually a matter of convenience and personal preference.

Electrophoretic and chromatoelectrophoretic methods have been important in radioimmunoassay since the pioneering report of Yalow and Berson (1961) on insulin. These techniques have limited use as they are only able to handle 25-200 µl of incubate and are unsuitable for steroid radioimmunoassays.

The difference in size between the antigen-antibody complex and the free hormonal antigen means that gel filtration on cross-linked dextrans, such as Sephadex, can be used to effect a separation. This technique has been used extensively in research on steroid hormone binding (Westphal, 1971c) but has seldom been applied to routine assays because of the time and attention required. However, Jiang and Ryan (1969) did use gel filtration in preliminary work on a radioimmunoassay for oestrogens.

Fractional precipitation using neutral salts, such as ammonium sulphate or sodium chloride, or organic solvents, such as alcohol or dioxane, provides a simple method for the separation of antibody-bound and free antigen. This method depends upon the fact that, at a critical concentration of the precipitant used, γ-globulins are insoluble while the free antigen remains in solution. Under these

conditions, separation of the bound and free fractions can be achieved by centrifugation or filtration. Jiang and Ryan (1969) found that antibody-bound and free tritiated oestradiol-17β could be efficiently separated by precipitation of the bound fraction by 50% saturation with ammonium sulphate.

Specific precipitation of the antigen-antibody complex is effected by the double antibody method which was first used in the radioimmunoassay of insulin by Morgan and Lazarow (1963). An antiserum prepared against the γ-globulin of the species used to raise the anti-hormone serum is used to precipitate the bound component. This system relies upon the knowledge that the antigenic sites of the first antibody are distinct from those involved in the antibody activity of the molecule. The main advantage of this method is that it gives a clean separation of bound from free hormone and is readily applicable to many different radioimmunoassays. Midgley and Niswender (1970) have applied this separation technique to radio-immunoassays for testosterone, progesterone, oestrone and oestradiol-17β.

The most common method used for the separation of antibody-bound and free steroid hormones is solid phase adsorption of the free steroid. Adsorbents which have

been used include charcoal, talc, micro-granules of silica, Florisil and certain resins. This method has the advantages of simplicity, economy and speed. However, all these adsorbents have some affinity for the antigen-antibody complex which must be decreased by prior treatment of the adsorbent or by addition of protein to the system. In the case of charcoal, Herbert et al (1965) have recommended pretreatment with dextrans of molecular weight from 10,000 to 250,000, depending on the size of the hormone to be assayed. The efficiency of a dextran-coated charcoal separation may vary according to the antiserum used. Abnormally low binding is often encountered when dextran-coated charcoal is used to examine an antiserum from an early bleed. This may be because the charcoal has a similar affinity for the free hormone as the antiserum itself so that the two compete for the hormone. With antisera obtained from later bleeds and having a greater affinity for the hormone, charcoal will be unable to compete significantly with the antiserum and separation will be complete. Adsorption of the free hormone with dextran-coated charcoal is the most popular separation technique in the field of oestrogen radioimmunoassay (Hotchkiss et al, 1971; Wu et al, 1971; Robertson, 1972; Doerr, 1973; Wright et al, 1973b).

Another development in separation techniques is the fixation of antibodies to an insoluble material. With this method, unbound antigen is easily separated from that bound to antibody by decanting or by centrifugation. Insoluble antibodies have been produced in a number of ways. One of the simplest methods is the conversion of soluble antibody to insoluble antibody aggregates with ethyl chloroformate or glutaraldehyde. Other approaches include coupling the antibodies to insoluble synthetic polymers such as Enzacryl AA or Protapol DJ/1 discs and adsorption to the walls of polypropylene or polystyrene tubes. Radioimmunoassays for plasma oestrogens have been described using antibodies polymerized with ethyl chloroformate (Mikhail et al, 1970), covalently bound to Enzacryl AA (Moore and Axelrod, 1972) and adsorbed to the walls of polystyrene tubes (Abraham, 1969).

In the case of assays designed for use with unextracted plasma or serum, the effect on the separation system of variations in total protein concentration in the incubate is important. Adsorption systems are particularly sensitive to total protein concentration as is precipitation of the bound components by salts. At present, this is not a problem with steroid radioimmunoassays, as the steroids are extracted prior to assay, but this point should be borne in mind if the setting up of a direct assay

on serum or plasma is undertaken.

v) Radioactivity measurement

Once the antibody-bound and free fractions have been separated, the relevant fraction (depending on the separation technique) is subjected to scintillation spectrometry - solid scintillation spectrometry for γ-emitting tracers and liquid scintillation spectrometry for β-emitting tracers.

vi) Construction of the standard curve and calculation of results

This is the final step of the assay procedure. A number of different plots have been used by workers in the oestrogen field. Examples of non-linear plots are: percentage of radioactivity in the free fraction versus log dose (Abraham, 1969); radioactivity bound versus dose (Hotchkiss et al, 1971); percentage of radioactivity bound versus dose (Mikhail et al, 1970) or log dose (Wu et al, 1971; Moore and Axelrod, 1972); relative binding ($B/B^o$, where $B^o$ = radioactivity bound in the absence of unlabelled hormone and B = radioactivity bound when known amounts of unlabelled hormone are added to the system) versus dose (Wright et al, 1973b); relative binding as a percentage versus log dose (de Hertogh, 1973). Doerr (1973) has applied a logit transformation to his response variable

$(^{B} - {}^{N}/_{B^{o}} - N$ where N = non-specifically bound radioactivity) and plots this against log dose thereby achieving a linear standard curve.

The response of the 'unknown' sample is expressed in the same form as the standards and its value is determined from the standard curve. Corrections are made for the proportion of the extract assayed, procedural losses and the volume of plasma or serum used. The final result is expressed in suitable units, for example, plasma LH concentration is expressed as ng of a specified LH reference standard per ml of plasma, plasma testosterone as ng/100 ml and non-pregnancy plasma oestrogens as pg/ml.

## E. ANTIGENS, IMMUNOGENS AND ANTISERA

The sensitivity and specificity of a radioimmunoassay depend largely upon the affinity and specificity of the antiserum used. Thus the production or acquisition of a suitable antiserum is of prime importance when setting up a radioimmunoassay. Although the production of a 'good' antiserum is often thought to be an art rather than a science, certain steps can be taken to improve the probability of success.

Antibodies are Y-globulins belonging to one of a number of classes of which IgG, IgM and IgA are the more

notable. In most mammalian species, the IgG group is qualitatively and quantitatively the most important. Every IgG molecule has two combining sites each consisting of the N-terminal sequence of one 'light' and one 'heavy' peptide chain and it is this group of antibodies that is usually involved in the radioimmunoassay reaction.

A substance which can combine with a specific antibody molecule is called an antigen, while one that can provoke an immune response is called an immunogen. The immunogenicity of a substance depends on both its size and its chemical composition. Although most large polypeptides are quite powerful immunogens, other compounds such as steroids, glycosides and some small peptides are non-immunogenic in their native state and must be conjugated to a larger molecule before they will stimulate an immune response.

Immunogenicity also depends upon the responsiveness of the animal, this being genetically determined for each immunogen. The choice of species for immunization is usually restricted to rabbits and guinea pigs. Rabbits are more hardy and easier to handle and bleed, whereas guinea pigs are less hardy and blood must be obtained by cardiac puncture. Guinea pigs are, however, cheaper to buy and maintain. Sheep have been used in a few instances and the potent antisera used by Abraham (1969)

and Thorneycroft et al (1970a) for the determination of plasma oestrogens were raised in this animal.

Although few critical studies have been made of the effect of the route of administration on the immune response, the evidence available suggests that the sites may be ranked in decreasing order of effectiveness as follows: into the lymph nodes, intra-articular, intradermal, intramuscular, intraperitoneal, subcutaneous and intravenous. However, it is not yet known whether the immunization procedure affects the production of antibodies of high avidity as distinct from antibodies in general.

The response to injected immunogen can be improved by the inclusion of adjuvants such as aluminium hydroxide or a mineral oil. The most widely used adjuvant is that introduced by Freund (1951). This consists of a light paraffin oil containing Arlacel A (mannide mono-oleate), which acts as an emulsifier, and mycobacteria. One of the ways in which adjuvant is believed to work is by releasing the immunogen slowly over the course of weeks, thereby preventing its rapid uptake into the circulation. It also facilitates phagocytosis of the immunogen by macrophage, causes the formation of local granulomatous lesions which may act as foci of antibody formation and causes both local and general stimulation of the reticuloendothelial system.

Antibody response seems to be virtually independent of immunogen dose over a wide range once a certain minimum quantity is exceeded. However, timing of injections is important in order to obtain the maximum response with the minimum of effort. Since one of the properties of an oily emulsion is to allow a sustained slow release of immunogen over a period of several weeks, it is pointless to inject at weekly intervals when Freund's adjuvant is used. Antibody levels rise relatively slowly after a primary injection, reaching a peak some 6 weeks later, as compared to approximately 10 days after each booster injection. A decision as to whether to bleed or to extend the immunization period rests upon the examination of trial bleedings of individual animals.

The presence of antibodies in immunized animals can be demonstrated by antibody titration studies. Constant amounts of labelled antigen are incubated with doubling dilutions of each antiserum. The distribution of radioactivity between antibody-bound and free fractions is ascertained and the percentage of radioactivity bound to antibody is plotted as a function of antibody dilution. The titration curve will indicate the working dilution at which the antiserum should be used in further tests for specificity and sensitivity. Usually the dilution chosen is such that 40-60% of the labelled antigen is bound in

the absence of unlabelled antigen.

The avidity of an antiserum can be assessed from a series of titration curves using different amounts of labelled antigen. Thus, an antiserum giving widely spaced curves when incubated with different amounts of antigen, for example 100, 50 and 25 pg, has a higher avidity than one that is unable to distinguish between these amounts. A point which should be noted, however, is that antiserum titration curves assess avidity for labelled antigen.

An essential step in determining the suitability of an antiserum for an assay is the construction of standard curves. This also allows for the estimation of the overall affinity constant of the antiserum when the data is expressed in the form of a Scatchard plot (see Chapter 2, Section C). When a large number of antisera are to be screened, it is impracticable to set up full standard curves. A simple preliminary procedure is to set up duplicate titration curves with and without the addition of a small constant amount of unlabelled antigen. Antisera with high sensitivity are then identified by a wide separation between the two curves.

Radioimmunoassay is based on competition between the antigenic determinants of the labelled and unlabelled

antigen for a limited number of antibody binding sites. Anything that compete for these sites, or in any other way affects the binding of antigen, will influence the results obtained unless the system is so arranged that standards and 'unknowns' are affected to the same degree.

Non-immunological factors that may affect the assay are acid or alkaline pH, high osmolality, high concentrations of plasma or other proteins or peptides, contamination of the incubate with materials such as charcoal or Fuller's earth, which adsorb antigen and, in some assays, the absence of certain cations such as calcium.

It should be noted that the antigenic determinant of a peptide hormone may not correspond to the amino acid sequence responsible for the biological effects of the peptide, and so immunological competition in the radio-immunoassay systen can result from the presence in the sample of biologically inactive fragments of the antigen, or partially denatured antigen containing the intact antigenic determinant, or fragments containing part of the determinant. Competition can also arise from other compounds containing structural groups which are identical with, or closely related to the antigenic determinant. In the case of steroids, the molecule is of such a size that the whole molecule probably acts as the determinant

but immunological competition can result from molecules of similar structure.

Organic molecules, which are non-immunogenic of themselves but which produce an immune response when they are attached to protein, are termed haptens. Activating agents are necessary in order to conjugate many molecules of biological importance to protein. Those most frequently used fall into two major classes: a) agents which activate carboxyl groups to form a peptide bond, for example, carbodiimides, alkyl chloroformates and isoxolium salts, and b) agents which form a bridge between amino groups, such as diisocyanates, diimidoesters and dihalonitrobenzenes. Sometimes conjugation cannot be accomplished directly, either because the hapten does not contain functional groups or because these groups must be left unsubstituted to improve the chances of obtaining a specific antiserum. In either case, it is necessary to synthesize or obtain an appropriate derivative of the hapten. The method of conjugation chosen should not distort the structure of the hapten since the antiserum produced will be required to react with the native molecule. The carrier may be a homologous protein or synthetic straight chain polymer, neither of which are themselves immunogenic, or a non-homologous and therefore immunogenic protein.

Steroids can be rendered immunogenic by coupling to

protein, but strong covalent bonding is necessary to prevent 'in vivo' lysis during the immunization procedure. Peptide bonds are biologically stable and can be formed between free carboxyl groups attached to the steroid molecule, using hydroxyl or ketone groups as anchoring points, and the ε-amino group of the lysine residues of albumin. Various approaches have been described for the formation of such bonds, for example, Lieberman et al (1959); Thorneycroft et al (1970b) and Goodfriend and Sehon (1970).

Most of the original anti-steroid antisera were produced following conjugation involving existing functional groups on the steroid. Those with a functional hydroxyl group were hemisuccinated or chlorocarbonated and then joined to serum albumin while, for conjugation through a ketone group, the oxime was formed first. Midgley and Niswender (1970) have obtained antisera to steroid derivatives conjugated at various positions to bovine serum albumin (BSA). These conjugates were prepared essentially as described by Erlanger et al (1967). The specificity of the antisera seemed to vary according to the position of conjugation.

As with other steroids, the first methods published for the radioimmunoassay of oestrogens employed antisera raised against oestrogen covalently linked via one or more functional groups to BSA. Antisera raised against oestradiol-17β-hemisuccinate-BSA have been used for the

radioimmunoassay of oestradiol-17β (Abraham, 1969; Hotchkiss et al, 1971), oestrone and oestradiol-17β (Mikhail et al, 1970; Cameron and Jones, 1972; Moore and Axelrod, 1972; de Hertogh, 1973; Wright et al, 1973b), oestrone, oestradiol -17β and oestriol (Wu et al, 1971) and oestrone, oestradiol -17α and oestradiol-17β Robertson et al, 1972). Doerr (1973) formed a hemisuccinate at the C-3 position, linked this to keyhole limpet haemocyanin and used this conjugate to raise an antiserum to oestradiol-17β.

A number of authors have expressed the view that the coupling of steroids to peptide carrier via one of the existing functional groups results in the masking or modification of this group which might mean a loss of important antigenic information and therefore the production of antisera of poor specificity (Lindner et al, 1972; Jeffcoate and Searle, 1972; Wright et al, 1973a). These workers have coupled 17β-oestradiol-6-(O-carboxymethyl)oxime to BSA and shown that the antisera resulting from immunization with this conjugate are more specific than those obtained with oestradiol-17β-hemisuccinate-BSA. Figs. 11 and 12 show diagrammatically the coupling of oestradiol-17β to BSA via a 17β-hemisuccinate and a 6-(O-carboxymethyl)-oxime linkage respectively. This aspect of steroid radioimmunoassay is the subject of intensive research at the

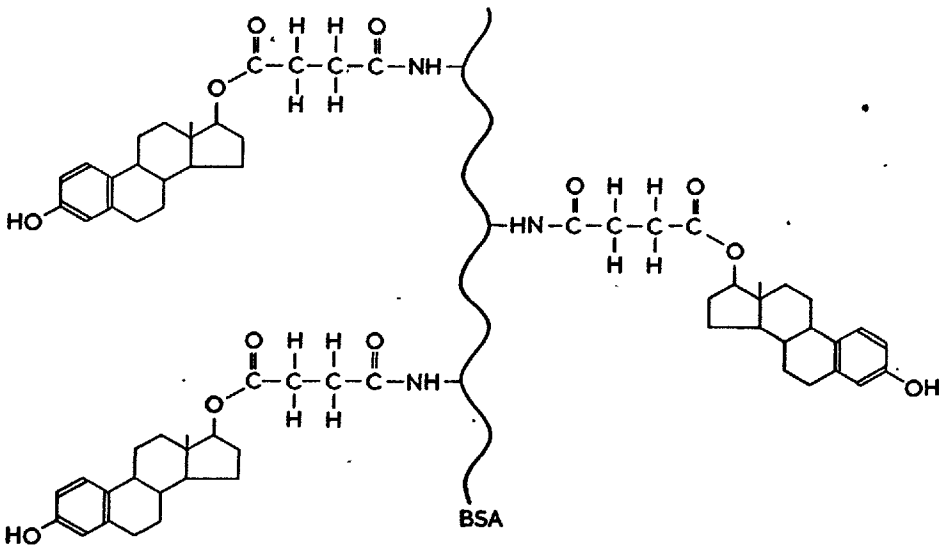


Fig. 11. Antigen: oestradiol-17β-hemisuccinate-BSA.

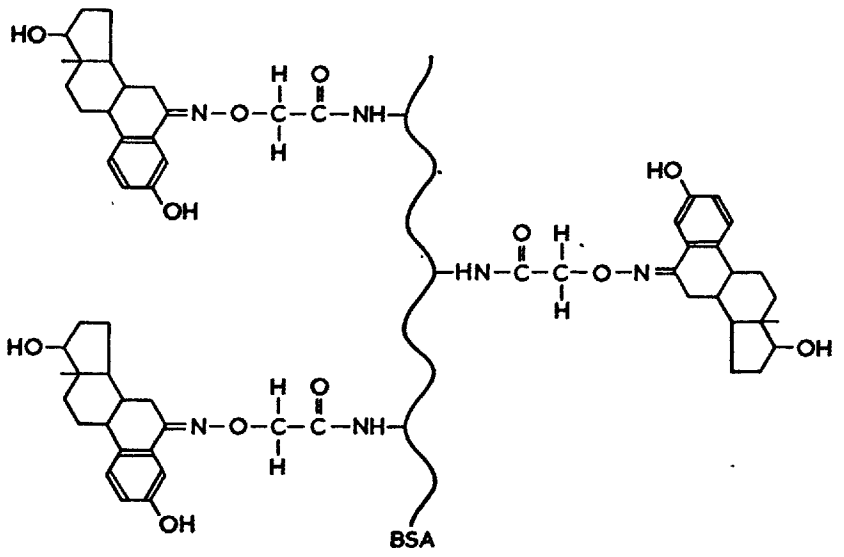


Fig. 12. Antigen: 17β-oestradiol-6-(O-carboxy-methyl) oxime-BSA.

present time and should lead to the preparation of new antisera with increased avidity and specificity.

# CHAPTER 3

# Chapter 3

# METHODS DEVELOPED FOR THE RADIOIMMUNOASSAY OF PLASMA OESTROGENS

## A. INTRODUCTION

The low levels of oestrogenic hormones in plasma from men and non-pregnant women have necessitated the search for an assay technique of greater sensitivity than those available up until the late 1960's. Thus, it came about that the radioimmunoassay technique, which had established itself as the method of choice for the assay of peptide hormones, was applied to the measurement of oestrogens in the non-pregnant female subject and in men.

During the present work, three methods have evolved: (1) a method for the separate determination of oestrone and oestradiol-17β in plasma, (2) a rapid assay for plasma 'oestrogen' and (3) a rapid method for the determination of oestradiol-17β in plasma. A number of assay parameters have been investigated.

## B. MATERIALS

### i) Solvents and chemical reagents

Acetone, benzene, diethyl ether (peroxide free), ethanol, methanol and toluene were all analar grade and,

apart from the diethyl ether and toluene, were redistilled prior to use. Water was glass distilled and deionized before use. Subsequently, analar water (BDH Chemicals Ltd., Poole, Dorset, England) was used. Isatin (2,3-indolinedione) and 1,4-diaminoanthroquinone were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. Sephadex LH 20 was obtained from Pharmacia, Uppsala, Sweden.

ii) Buffers

Phosphate buffered saline (PBS) was prepared by dissolving 1 g of $NaN_3$ and 9 g of NaCl in 305 ml of 0.2M $Na_2HPO_4 2H_2O$ and 195 ml of 0.2M $NaH_2PO_4 2H_2O$. The solution was made up to 1 l by addition of deionized distilled water or analar water and the pH adjusted to 7.0 if necessary. PBS solutions of pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 were prepared by adjusting the proportions of the phosphate solutions.

Tris-HCl and tricine-HCl buffered saline solutions were prepared by dissolving 1 g of $NaN_3$ and 9 g of NaCl in 1 l of a 0.1M solution of tris (tris(hydroxymethyl)-methylamine) or tricine (N-tris(hydroxymethyl)methyl-glycine) as appropriate and titrating to the desired pH with concentrated HCl.

Solutions containing 0.1% and 0.5% gelatine in PBS, tris-HCl and tricine-HCl buffered saline solutions were prepared by dissolving Knox Unflavoured Gelatine (Knox Gelatine Inc., Johnstown, N.Y. 12095, U.S.A.) in the buffer at 50°C and then cooling the solution. Subsequently, gelatine powder from BDH Chemicals Ltd. was used. All buffer solutions were stored at 4°C. PBS pH 7.0 was used for the assay methods described below.

iii) Standards

2,4,6,7-$^{3}$H-Oestrone (S.A. 100 Ci/mmole) and 2;4,6,7-$^{3}$H-oestradiol-17β (S.A. 100 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks, England. Solutions were prepared containing 10 mCi/ml in benzene:ethanol (95:5 v/v). Prior to each assay, 100 µl of the tritiated oestradiol-17β solution was removed, evaporated to dryness and redissolved in 10 ml of PBS containing 0.1% gelatine (PBS-0.1% gel). Solutions of tritiated oestradiol-17β in tris-HCl and tricine-HCl buffered saline containing 0.1% gelatine were prepared in the same manner. One hundred µl of this solution, containing approximately 20,000 dpm (27 pg) was used in the assay system. Internal standards to correct for procedural losses were prepared by evaporating 10 µl of the 10 mCi/ml standard to dryness and

redissolving in 10 ml of deionized distilled or analar water, so that 100 µl contained approximately 2,000 dpm.

Androstenedione, cortisol, cholesterol, dehydroepiandrosterone, oestradiol-17β, oestriol, oestrone, pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, testosterone and 5α-dihydrotestosterone were obtained from the Sigma London Chemical Company Ltd., Kingston-upon-Thames, Surrey, England. The following steroids were donated from the Medical Research Council Steroid Reference Collection, Westfield College, London, England: 2-methoxyoestrone, oestrone methyl ether, 16α-hydroxyoestrone, 2-methoxyoestradiol-17β, oestradiol-17β methyl ether, 16-oxo-oestradiol-17β, 17α-ethynyloestradiol, oestradiol-17α, 16-epi-oestriol, 17-epi-oestriol, 17α-ethynyltestosterone, 19-nor-testosterone, 17α-ethynyl-19-nor-testosterone and 19-nor-5α-dihydrotestosterone. Samples of 6-oxo-oestradiol-17β and 6-oxo-oestriol were obtained from Mr. M.W. Johnson, Department of Biochemistry, University of Liverpool, Liverpool, England. Oestrone and oestradiol-17β were serially diluted in ethanol or acetone to a concentration of 1 pg/ml, while the other steroids were diluted in acetone to convenient concentrations.

iv) Antisera

Dr. B.V. Caldwell, Worcester Foundation for Experimental

Biology, Shrewsbury, Mass., U.S.A. donated a sample of antiserum to oestradiol-17β-hemisuccinate-BSA. This antiserum had been partially purified by precipitation of antibodies to BSA and subsequent removal of all proteins, other than Y-globulins by treatment with Rivanol (6,9-diamino-2-ethoxyacridine lactate). A sample of antiserum to oestradiol-17β coupled to lysine residues of BSA via a 6-(O-carboxymethyl)oxime was kindly supplied by Dr. H.R. Lindner, Department of Biodynamics, the Weizmann Institute of Science, Rehovot, Israel. This antiserum was used without treatment. Both antisera were diluted 1:4 with PBS-0.1% gel and stored in 0.5 ml aliquots at -15°C. Prior to use, one aliquot of the required antiserum was further diluted 1:99 with PBS-0.1% gel (giving a 1 in 500 dilution of the original serum) and stored in 2.5 ml aliquots, one at 4°C and the remainder at -15°C. Final dilutions in the appropriate buffer containing 0.1% gelatine were made from the unfrozen sample of antiserum at the commencement of each assay.

v) Dextran-coated Charcoal

Norit A charcoal, obtained from the Sigma London Chemical Company Ltd., was washed with deionized distilled or analar water until all fine particles were removed and

thoroughly dried at 120°C. Dextran T 70, from Pharmacia, was dissolved in deionized distilled or analar water to a concentration of 100 mg/ml. To 500 ml of PBS, PBS-0.1% gel or tris or tricine buffered saline containing 0.1% gelatine, 1.25 g of charcoal and 1.25 ml of dextran T 70 solution were added and mixed thoroughly. The solutions were stored at 4°C. During use the charcoal was maintained in suspension by a magnetic stirrer (ChemLab Instruments Ltd., London, England).

vi) Radioactivity Measurement

Scintillation liquid was prepared by adding 500 ml of Triton X-100 (Koch-Light Laboratories Ltd.) to 1 l of toluene containing 3.0 g of PPO (2,5-diphenyloxazole from Koch-Light Laboratories Ltd.). All samples were added to disposable glass vials (Johnsen and Jorgensen (Trident) Ltd., London, England) containing 12 ml of scintillant. Each sample was stabilized for a minimum of 90 min at 2°C (see Fig. 13) and then counted for 4,000 counts or 4 min in an automatic liquid scintillation counting system - Nuclear Chicago Model 6860 (Mark I). The counting efficiencies were determined for each sample from calibration curves for an external standard channels ratio method using a $^{133}Ba$ source.

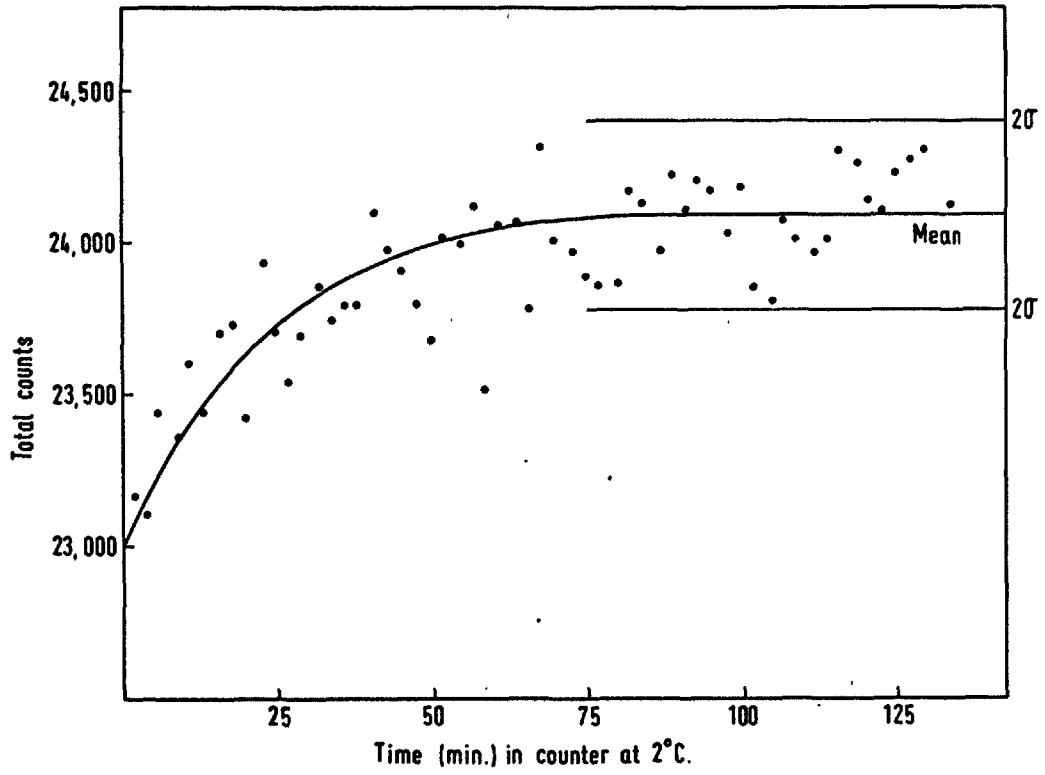


Fig. 13. Time course for the stabilization of tritium counts in toluene:Triton scintillant.

C. METHODOLOGY

i) The separate determination of plasma oestrone and oestradiol-17β

A flow diagram of the method is shown in Fig. 14.

a) Extraction

A disposable syringe was used to withdraw approximately 15 ml of peripheral venous blood which was transferred to a lithium heparin tube and centrifuged. The plasma was removed and processed immediately or stored at -15°C.

Internal standards - 2,000 dpm of tritiated oestrone and 2,000 dpm of tritiated oestradiol-17β in deionized distilled water - were added to 5 ml of plasma or 5 ml of deionized distilled water (method blank) and equal aliquots taken for liquid scintillation counting. The plasma was extracted with 2 x 15 ml and 1 x 10 ml of diethyl ether using a Rotamixer DeLuxe (Hooke and Tucker Ltd., New Addington, Croydon, England). The ether layers were transferred to a test tube by means of a Pasteur pipette and the pooled extracts dried at 40°C under nitrogen.

b) Column chromatography

The dried extract was dissolved in a few drops of benzene:methanol (85:15 v/v) containing a pink dye purified from 1,4-diaminoanthroquinone which is isopolar with

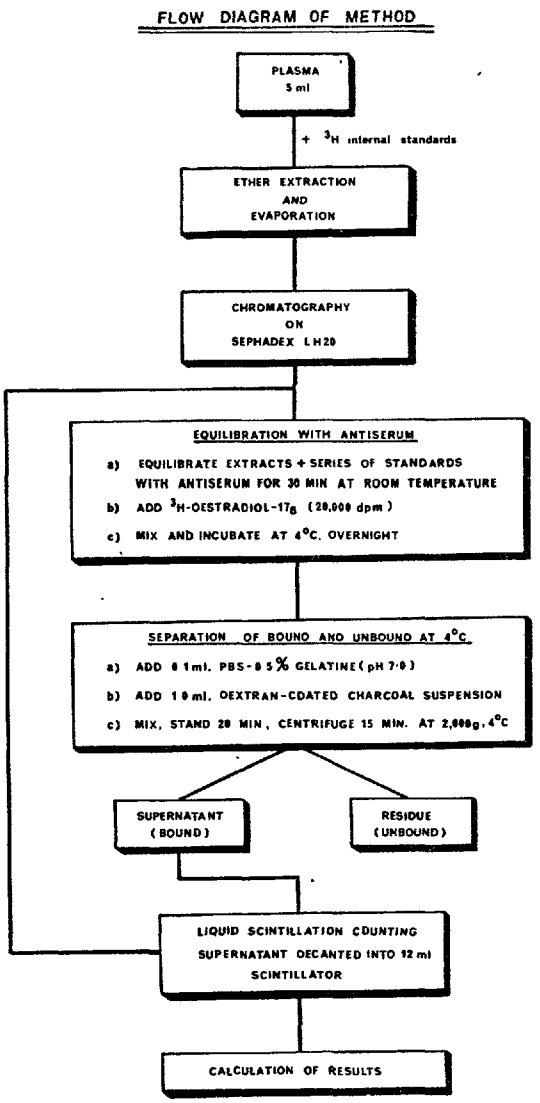


Fig. 14. Flow diagram of method i.

oestrone and isatin which runs with oestradiol-17β. The extract was transferred to a column of Sephadex LH 20 prepared in benzene:methanol (85:15 v/v) and occupying 80% of a disposable Pasteur pipette (Harshaw Chemicals Ltd., P.O. Box 4, Daventry, Northants., England). The same solvent mixture was used to elute the pink fraction containing oestrone and the yellow fraction containing oestradiol-17β. The separation achieved by this technique is shown in Fig. 15. These fractions were collected in separate counting vials, dried under nitrogen at 40°C and redissolved in 1 ml of ethanol. Ten per cent of the extract from female plasma and 20% of that from male plasma was removed in duplicate for assay. The remainder was evaporated to dryness and subjected to liquid scintillation counting in order to determine the experimental losses. The recoveries for the three methods are recorded in Table 3.

c) Assay procedure

The assay was performed in disposable glass tubes (75 x 10 mm). Two series of ethanolic standards - 10, 20, 50, 100 and 150 pg of oestrone and of oestradiol-17β prepared in triplicate - and aliquots of the plasma and water extracts prepared in duplicate were dried under nitrogen in a dry block heater (Tecam Dri-Block DB-3,

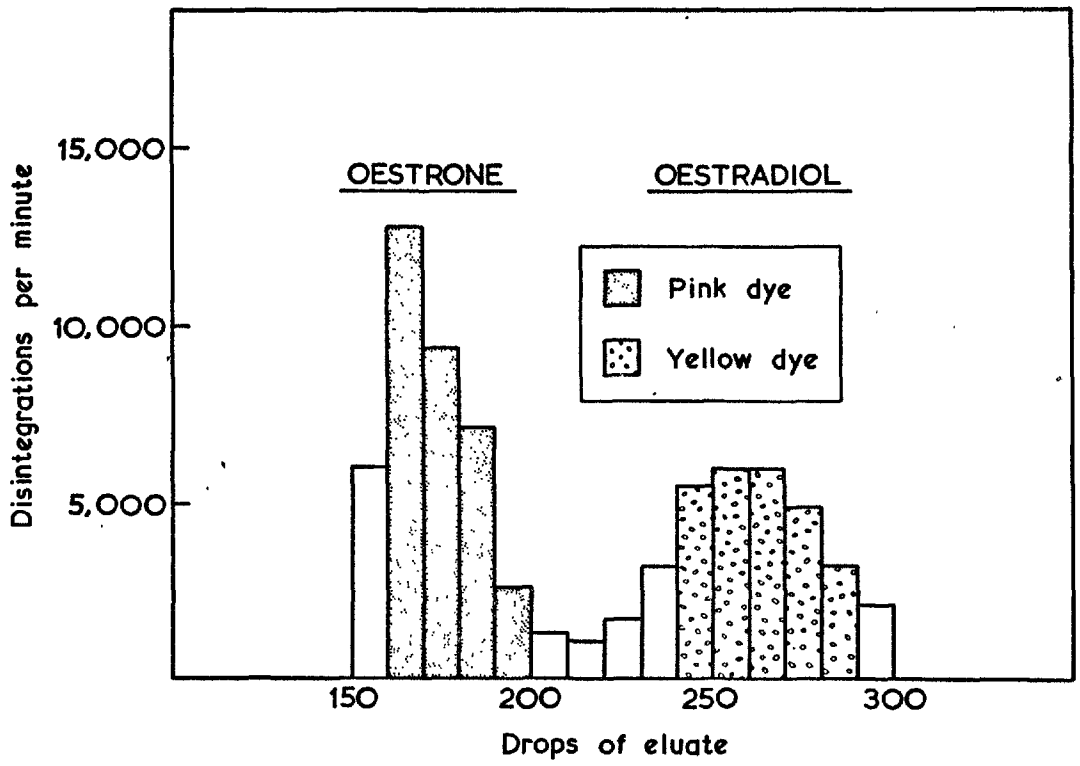


Fig. 15. Separation of tritiated oestrone and oestradiol-17β on Sephadex LH 20 using benzene:methanol (85:15 v/v).

| | NUMBER OF DETERMINATIONS | % RECOVERY | |
|---|---|---|---|
| | | MEAN | S.D. |
| Method i | | | |
| Oestrone | 100 | 54.7 | 10.4 |
| Oestradiol-17β | 100 | 52.5 | 9.4 |
| Method ii | | | |
| Oestradiol-17β | 100 | 82.7 | 9.6 |
| Method iii | | | |
| Oestradiol-17β | 100 | 87.3 | 14.6 |

Table 3. The recovery of tritiated oestrogen added to plasma.

Techne Cambridge Ltd., Cambridge, England) at 40°C. In addition, two tubes designated 00 and 0, which enable the percentage of labelled steroid bound to antibody and its subsequent displacement by non-labelled steroid to be determined, were prepared in triplicate with the standard curves. One hundred µl of antiserum to oestradiol-17β-hemisuccinate-BSA (17-HS antiserum) diluted with PBS-0.1% gel so that, in the absence of unlabelled steroid, 50-60% of the tritiated oestradiol-17β is bound (approximately a 1 in 25,000 dilution of the neat serum) was added to each tube, except the 00 tubes to which 100 µl of PBS-0.1% gel was added. The contents of each tube were mixed on a rotamixer and left to equilibrate for 30 min at room temperature. Then, 100 µl of tritiated oestradiol-17β in PBS-0.1% gel (approximately 20,000 dpm) was added. The contents of each tube were again mixed on a rotamixer and then incubated at 4°C overnight. The antiserum and tritiated oestradiol-17β were added with a 0.1/0.2 ml Biopette (Schwarz Bio Research, Orangeburg, New York, 10962, U.S.A.) which has a disposable nylon tip.

d) Separation of antibody-bound and free steroid

One hundred µl of PBS containing 0.5% gelatine (PBS-0.5% gel) was added to each tube with the biopette.

Next, 1 ml of dextran-coated charcoal in PBS (maintained in suspension by a magnetic stirrer) was added with an automatic pipetting syringe (F. Froud & Sons, 126 Dalston Lane, London, E.8.) to all except the 00 tubes which received 1 ml of PBS. The contents of each tube were mixed on a rotamixer, incubated for 20 min at 4°C and then centrifuged at 2,000 rpm (approximately 2,000 g) and 4°C for 15 min. The dextran-coated charcoal adheres to the bottom of the tube and the supernatant containg the bound fraction was decanted into a counting vial containing scintillation liquid and subjected to liquid scintillation counting.

e) Calculation of results

Standard curves for oestrone and oestradiol-17β were plotted in the form of disintegrations per minute (dpm) or percentage of the tritiated oestradiol-17β bound to antiserum versus dose of oestrogen and the results calculated according to the following equation:-

$$S = \left[ \frac{T_x}{\alpha} \cdot \frac{C_s}{C_x} \cdot \frac{\beta E_x}{E_s} - M \right] \frac{1}{V}$$

where S = result in pg/ml

$\alpha$ = aliquot taken for radioimmunoassay

$T_x$ = reading from standard curve (pg)

$C_s$ = cpm in recovery standard

$C_x$ = cpm in sample recovery

$\beta$ = aliquot taken for recovery determination

$E_s$ = counting efficiency of recovery standard

$E_x$ = counting efficiency of sample recovery

M = mass of internal standard

V = volume of plasma in ml.

Typical standard curves for oestrone and oestradiol-17β are shown in Fig. 16.

ii) The determination of plasma 'oestrogen'

This method measures oestradiol-17β plus oestrone. However, because of the lack of specificity of the antiserum, other circulating oestrogens will also contribute to the result. The method is essentially as method i above, but with the chromatographic step omitted. Five ml of plasma, obtained as above, or 5 ml of deionized distilled water were extracted with 1 x 15 ml and 1 x 10 ml of diethyl ether after addition of approximately 2,000 dpm of tritiated oestradiol-17β as internal standard. The pooled ether extract was evaporated to dryness at 40°C under nitrogen and then dissolved in 1 ml of ethanol. Duplicate aliquots (1/10 for female and 1/5 for male plasmas) were transferred to assay tubes and dried down together with a series of ethanolic oestradiol-17β standards (10, 20, 50, 100 and

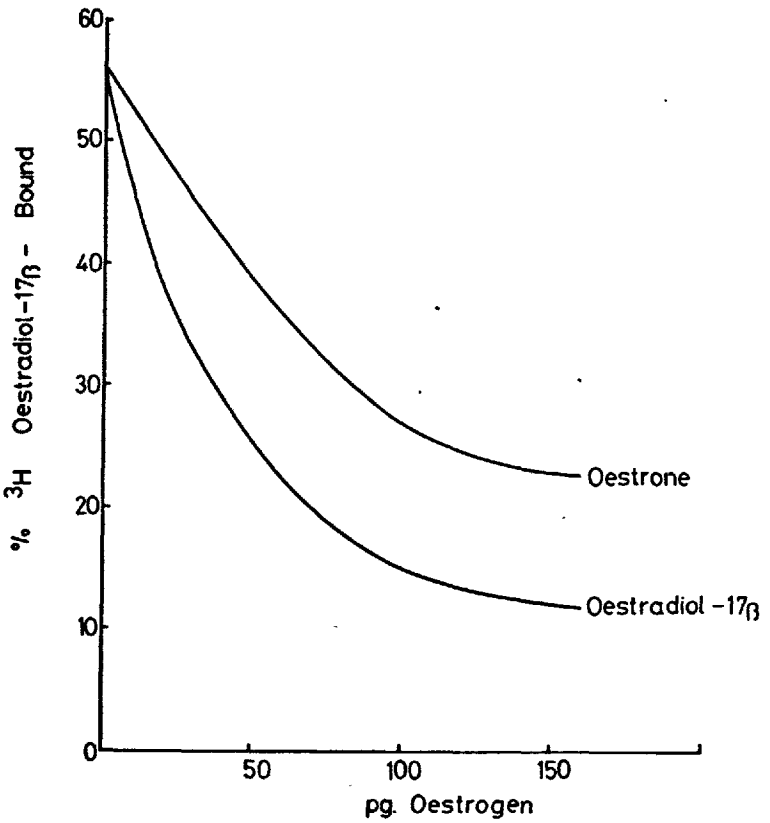


Fig. 16. Standard curves for oestrone and oestradiol-17β obtained with a 17-HS antiserum diluted 1:25,000.

150 pg) in triplicate. The remainder of the extract was evaporated to dryness and subjected to liquid scintillation counting so that procedural losses could be taken into account when the results were calculated. Tubes designated 00 and 0 were set up with the standard curve and the assay procedure, separation of antibody-bound and free oestrogen and calculation of results was carried out as in method i above.

iii) The rapid estimation of plasma oestradiol-17β

This method involves the use of an antiserum with a high degree of specificity towards oestradiol-17β. Tritiated oestradiol-17β (approximately 2,000 dpm, corresponding to 2.7 pg) was added to 5 ml of plasma or analar water which was then extracted with diethyl ether as described in method ii above. The pooled ether extract was evaporated to dryness at 40°C under nitrogen and the residue dissolved in 1 ml of acetone. Duplicate aliquots were removed for assay and the remainder taken to dryness before being subjected to liquid scintillation counting in order to determine the recovery.

Oestradiol-17β standards (10, 20, 50, 100, 150 and 200 pg), plasma extracts and method blanks were prepared

as in method ii above, but in acetone. Tubes designated OO and O were also set up in triplicate with the standard curve. One hundred µl of tritiated oestradiol-17β in PBS-0.1% gel (approximately 20,000 dpm) was added to each tube which was then rotamixed to ensure thorough mixing of the contents. Next, 100 µl of antiserum to 17β-oestradiol-6-(O-carboxymethyl)oxime-BSA (6-CMO antiserum) at the appropriate dilution (1:10,000 - 1:15,000) in PBS-0.1% gel was added to each tube except the OO ones which received 100 µl of PBS-0.1% gel. The contents of the tubes were rotamixed and then incubated overnight at 4°C. The incubation time can be shortened to 1-2 hr for clinical purposes.

For the separation of antibody-bound and free oestradiol-17β, 1 ml of dextran-coated charcoal in PBS-0.1% gel was added to each tube except the OO's which received 1 ml of PBS-0.1% gel. The contents were mixed thoroughly, incubated for 30 min at 4°C and then centrifuged for 15 min at 4°C and 2,000 rpm. The supernatant was decanted into counting vials containing 12 ml of scintillation fluid and subjected to liquid scintillation spectrometry. The results were calculated as in method i above. A standard curve for oestradiol-17β

with the 6-CMO antiserum is shown in Fig. 17.

## D. INVESTIGATION OF ASSAY CONDITIONS

The selection of the various assay conditions in the procedures described above was largely based upon a study of the following factors which are discussed below:-

i) Antiserum dilution

ii) Buffer composition and pH

iii) Incubation conditions

iv) Conditions for the separation of antibody-bound and free oestradiol-17β

v) Effect of solvent residues.

For the sake of brevity, the amount of tritiated oestradiol-17β bound to antibody will be referred to as the percentage or dpm bound.

### i) Antiserum dilution

The optimal working dilution for each batch of antiserum was investigated prior to use. The percentage bound by an antiserum at various dilutions was determined in the absence of unlabelled oestradiol-17β and in the presence of 200 pg of this oestrogen. The information was presented in one of two ways:-

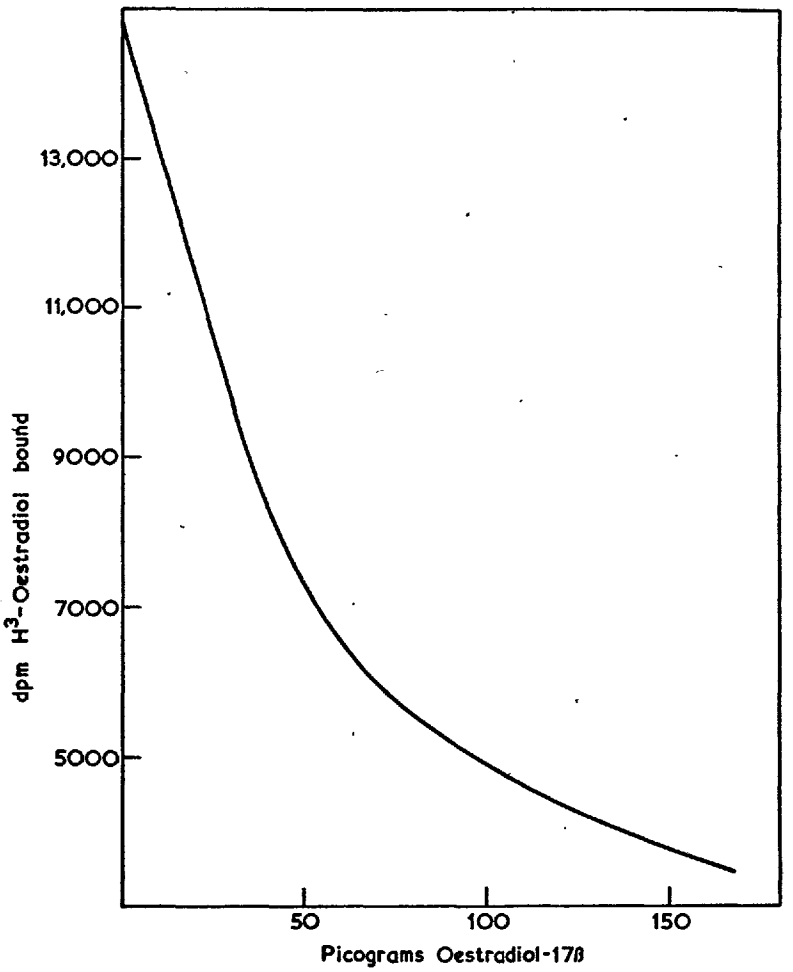


Fig. 17. Standard curve for oestradiol-17β obtained with a 6-CMO antiserum diluted 1:10,000.

a) The percentage bound at the 0 and 200 pg levels of unlabelled oestradiol-17β were plotted for each antiserum dilution so that straight lines with slopes proportional to the displacement resulted. That dilution corresponding to the line of maximum slope, that is maximum displacement, was then taken as the optimal working dilution. Fig. 18 shows the application of this approach for antisera to 17β-oestradiol-6-(O-carboxymethyl)-oxime-BSA and oestradiol-17β-hemisuccinate-BSA.

b) The percentage bound was plotted as a function of antiserum dilution for both the 0 and 200 pg levels, resulting in two curves. A third curve was derived by plotting the difference between these two curves (that is the displacement) and that dilution corresponding to the maximum of this third curve was taken as the optimum working dilution. The application of this approach is illustrated in Fig. 19.

It can be seen that the batch of antiserum to oestradiol-17β-hemisuccinate-BSA tested should be used at a dilution of 1:20,000 to 1:25,000 and that to 17β-oestradiol-6-(O-carboxymethyl)oxime-BSA at 1:10,000 to 1:15,000.

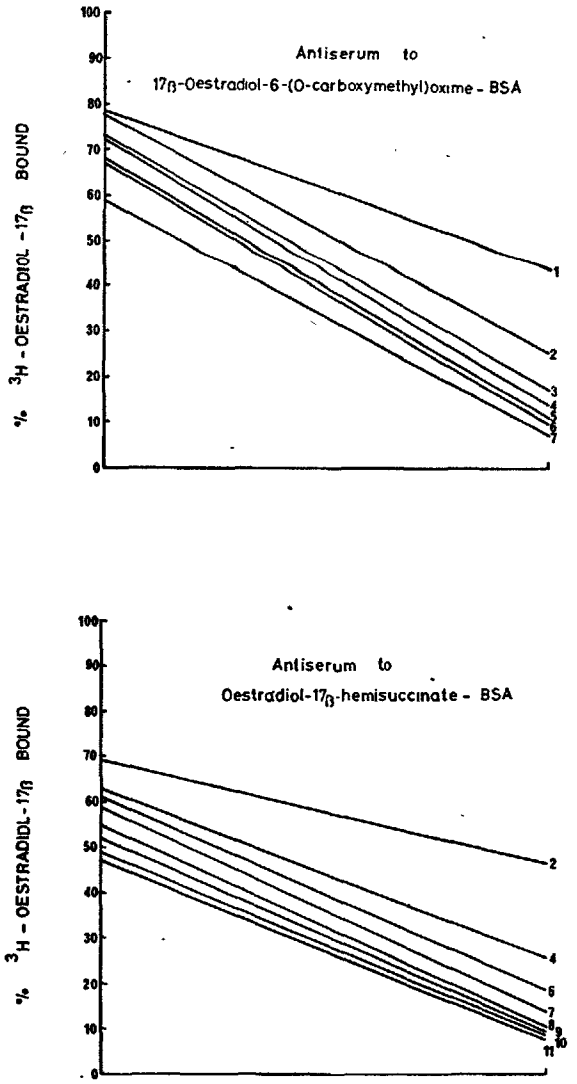


Fig. 18. Antisera dilution: effect on initial binding and displacement by 200 pg of oestradiol-17β.
Antisera dilutions:-
1 1:2,500
2 1:5,000

Fig. 18. Legend cont'd.

| | |
|---|---|
| 3 | 1: 7,500 |
| 4 | 1:10,000 |
| 5 | 1:12,500 |
| 6 | 1:15,000 |
| 7 | 1:20,000 |
| 8 | 1:25,000 |
| 9 | 1:30,000 |
| 10 | 1:40,000 |
| 11 | 1:50,000. |

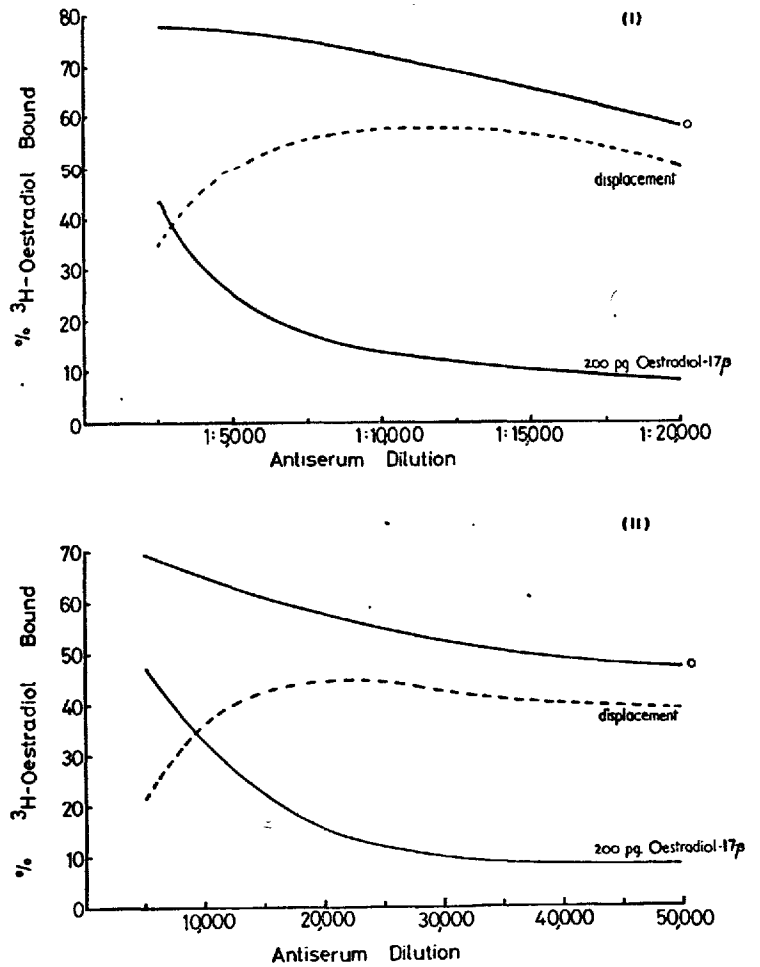


Fig. 19. Antisera dilution curves:-
I 6-CMO antiserum
II 17-HS antiserum.

ii) Buffer composition and pH

Both Hotchkiss et al (1971) and Abraham (1969) used 0.1M PBS pH 7.0, with and without gelatine respectively, as the diluent/solvent in their respective systems and most of the methods published subsequently followed this example. It was decided to examine the change induced in the initial binding of tritiated oestradiol-17β to the 17-HS antiserum and its displacement by 150 pg of oestrone or oestradiol-17β when the buffer and/or pH was varied. Solutions of 0.1M phosphate, tris-HCl and tricine-HCl buffers containing 0.9% NaCl and 0.1% $NaN_3$, covering a pH range of 6.0 to 9.0 in increments of 0.5 units were investigated. The results are illustrated in Fig. 20. That pH at which maximum separation between the zero and oestradiol-17β curves was observed is regarded as the optimum working pH. It can be seen that a change in pH of the PBS has very little effect on initial binding or displacement over the range 6.5 to 8.5 whereas tris-HCl buffered saline has an optimum at pH 8.5 and tricine-HCl buffered saline at 7.5. For the sake of interest, curves for oestrone are included. It is apparent that changing buffer and/or pH has little effect on the specificity of this antiserum with regard to oestrone. It was decided that little or no advantage

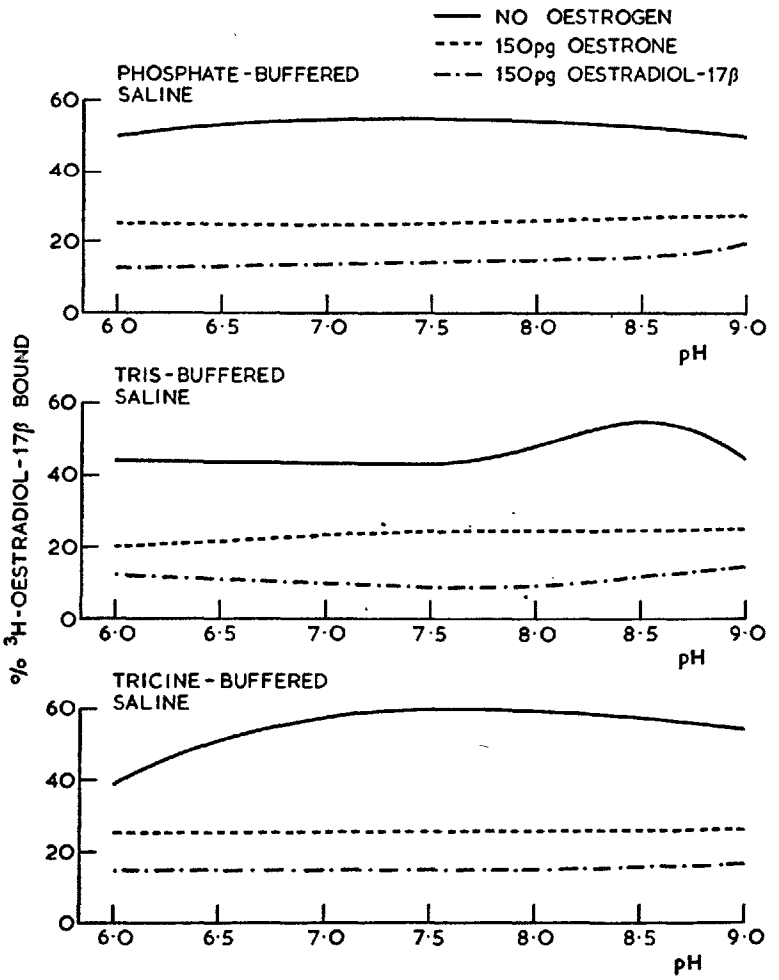


Fig. 20. Effect of buffer composition and/or pH on binding and displacement.

was to be gained by changing from PBS to another buffer system. Indeed, there might be some advantage in using a buffer the pH of which, when varied between 6.5 and 8.5, does not affect the assay system. Consequently, 0.1M PBS pH 7.0 was used subsequently.

iii) Incubation conditions

The classic conditions of Hotchkiss et al (1971) for the liquid phase radioimmunoassay of oestrogens involve evaporation to dryness of ethanolic standards and unknowns, incubation for 30 min with antiserum diluted with PBS-0.1% gel and an overnight incubation at 4°C with tritiated oestradiol-17β, also in PBS-0.1% gel. These conditions have been investigated for the 17-HS antiserum according to the scheme set out in Table 4. All final incubations were carried out at 4°C overnight. The results are recorded in Fig. 21.

It is obvious that low initial binding will preclude a large displacement of tritiated oestradiol-17β from the antiserum and it can be seen from Fig. 21 (E, F, G and I) that the absence of gelatine, which is thought to prevent non-specific binding to the walls of the assay tube, from the buffer markedly lowered the initial percentage binding,

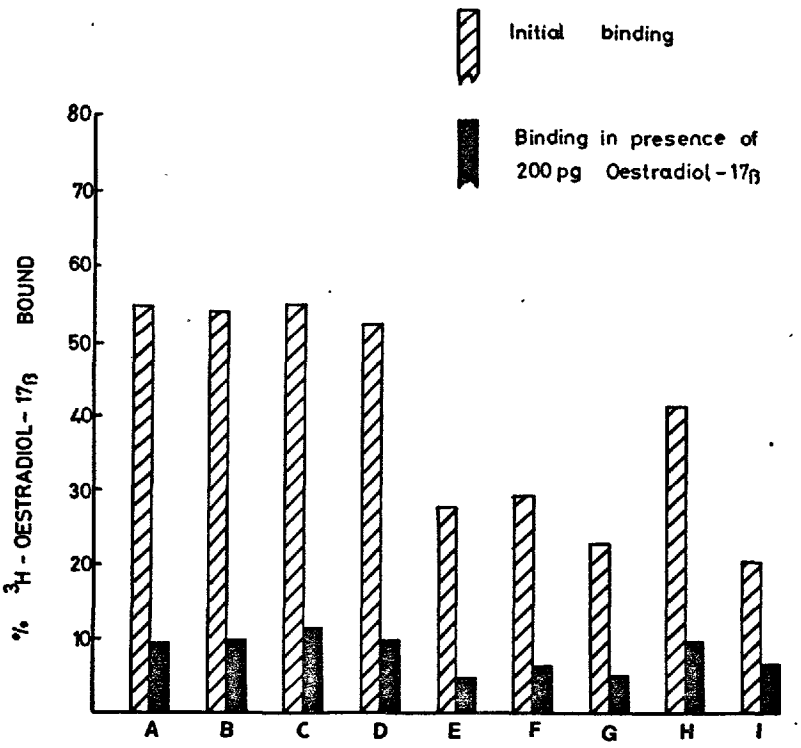


Fig. 21. Effect of varying solvent/diluent and conditions for setting up standard curves.
Key: see Table 4.

| CODE | SOLVENT/DILUENT | | | CONDITIONS |
|---|---|---|---|---|
| | Unlabelled oestradiol -17β | Tritiated oestradiol -17β | Antiserum | |
| A | Ethanol | PBS-0.1% gel | PBS-0.1% gel | Add AS, equilibrate ½ hr, add $*E_2$ |
| B | Ethanol | PBS-0.1% gel | PBS-0.1% gel | Add $*E_2$, equilibrate ½ hr, add AS |
| C | PBS-0.1% gel | PBS-0.1% gel | PBS-0.1% gel | Add AS, equilibrate ½ hr, add $*E_2$ |
| D | PBS | PBS-0.1% gel | PBS-0.1% gel | Add AS, equilibrate ½ hr, add $*E_2$ |
| E | Ethanol | PBS | PBS | Add AS, equilibrate ½ hr, add $*E_2$ |
| F | Ethanol | PBS | PBS | Add $*E_2$, equilibrate ½ hr, add AS |
| G | PBS | PBS | PBS | Add AS, equilibrate ½ hr, add $*E_2$ |
| H | Ethanol | Ethanol | PBS-0.1% gel | Dry down $*E_2$ with standard, redissolve in PBS-0.1% gel, add AS |
| I | Ethanol | Ethanol | PBS | Dry down $*E_2$ with standards, redissolve in PBS, add AS |

Table 4. Key to Fig. 21 showing solvent/diluent and conditions used.

$*E_2$ = tritiated oestradiol-17β

AS = 17-HS antiserum

a poor displacement resulting. The initial binding was also reduced when tritiated oestradiol-17β in ethanol was dried down with the standards and then taken up in PBS-0.1% gel or PBS (H and I respectively). A further disadvantage of this approach is the longer time required for drying down.

The order of adding antiserum and tritiated oestradiol-17β seems to be immaterial (A and B) although it is claimed (Rodbard et al, 1971) that preincubation of the antiserum with unlabelled oestradiol-17β leads to a steeper dose-response curve. In fairness, it should be pointed out that Rodbard and his colleagues preincubated for 8 hr as compared with 30 min in this case, but this is considered impracticable for routine assay purposes. Also, it was frequently observed that better standard curves resulted when the tracer and unlabelled standard were mixed prior to addition of antiserum.

The standards may be dissolved in ethanol, PBS-0.1% gel or PBS alone (A, B, C and D) without significant effect and the concentration of gelatine can be reduced to 0.05% (D). The advantage of dissolving the standards and unknowns in buffer is that the assay can proceed directly without the evaporation step. The disadvantages

are the lower solubility of steroids (88%) in PBS or PBS-0.1% gel as compared to ethanol and the less economic use of antiserum. With regard to the solubility aspect, there is the problem of incomplete solubilization of the unknown which one must assume is compensated for by the incomplete solubilization of the standard or vice versa. However, this is probably not a valid assumption especially where vastly different masses are involved. Also, small volumes of aqueous solvents containing gelatine tend to be more difficult to handle than organic solvents. In later work, acetone was used in place of ethanol so that the evaporation step might proceed more rapidly.

The temperature and duration of the incubation step have also been investigated for the 17-HS antiserum. Standard curves for oestrone and oestradiol-17β were set up and incubated at 4°C, room temperature and 37°C for 0.5, 1.0, 2.0 and 24.0 hr. The results are summarized in Fig. 22. It would seem that temperature has little effect on maximum binding and displacement only on the rate of attainment of this maximum. At 4°C there was a small increase in initial binding paralleled by an increase in displacement with 200 pg of oestrone or oestradiol-17β as the incubation time was prolonged, the largest increment occuring between 0.5 and 1.0 hr. Thus, with this batch of antiserum and

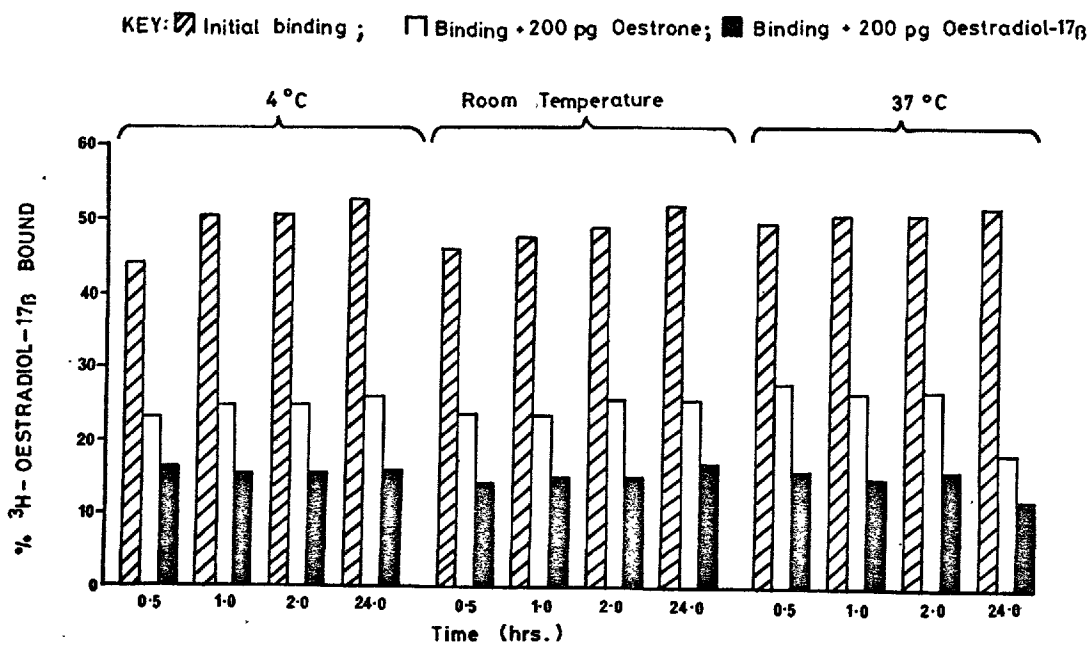


Fig. 22. Effect of incubation time and temperature on initial binding and displacement.

these conditions, incubation for 1.0 hr was found to be quite adequate although an overnight incubation was frequently more convenient. Again, at room temperature, there was a gradual increase in both binding and displacement with increased incubation time, while the initial binding was virtually at its maximum after 0.5 hr at 37°C, there being very little improvement in displacement as the incubation time was prolonged.

The effect of incubation time on initial binding at 4°C was investigated further for both the 17-HS and 6-CMO antisera. The results are recorded in Table 5. Binding increased rapidly in the first 5 min, then more slowly up until 2.0 hr after which very little increase in binding was observed.

iv) Conditions for the separation of antibody-bound and free oestrogen

Adsorption of free (unbound) oestrogen with dextran-coated charcoal was chosen as the separation technique because of its simplicity, universality and speed. Three aspects of this step were investigated: a) the concentration of gelatine, b) the efficiency of separation and c) the time between addition of dextran-coated charcoal

| TIME (hr) | INITIAL PERCENTAGE BINDING | |
|---|---|---|
| | Anti oestradiol-17β-hemisuccinate-BSA | Anti 17β-oestradiol-6-(O-carboxymethyl) oxime-BSA |
| 0 | 20.1 | 23.2 |
| 0.08 | 41.6 | 50.0 |
| 0.17 | 43.4 | 52.6 |
| 0.25 | 46.6 | 55.6 |
| 0.33 | 47.3 | 55.8 |
| 0.50 | 49.4 | 56.5 |
| 0.75 | 49.8 | 60.7 |
| 1.00 | 52.1 | 61.0 |
| 1.25 | 52.8 | 61.3 |
| 1.50 | 54.1 | 61.6 |
| 2.00 | 55.0 | 64.4 |
| 2.50 | 55.6 | 65.1 |
| 3.00 | 55.7 | 66.1 |
| 4.00 | 55.7 | 66.1 |
| 5.00 | 55.7 | 67.2 |

Table 5. Effect of incubation time on initial binding of tritiated oestradiol-17β to two antisera.

and centrifuging and between centrifuging and decanting.

a) Gelatine concentration

At the end of the incubation period, Hotchkiss et al (1971) added 100 µl of PBS-0.5% gel followed by 1 ml of dextran-coated charcoal suspended in PBS. How necessary is the addition of 0.5% gelatine in PBS ? Can this two step procedure be replaced by a single step where the dextran-coated charcoal is suspended in PBS-0.1% gel? These questions were investigated using standard curves for oestradiol-17β with the 17-HS antiserum and the results are presented in Fig. 23. It can be seen that the two step method of Hotchkiss et al (1971) offers little advantage over a one step process where dextran-coated charcoal is suspended in PBS alone (curves b and a respectively) apart from a small increase in the slope of the dose response curve over the 0-50 pg range. When the dextran-coated charcoal was suspended in PBS-0.1% gel, the standard curve (curve c) was displaced upward which means that shorter counting times can be used. Also, there was a further small increase in the slope of the initial part of the curve.

b) Efficiency of separation

The efficiency of adsorption of oestradiol-17β by

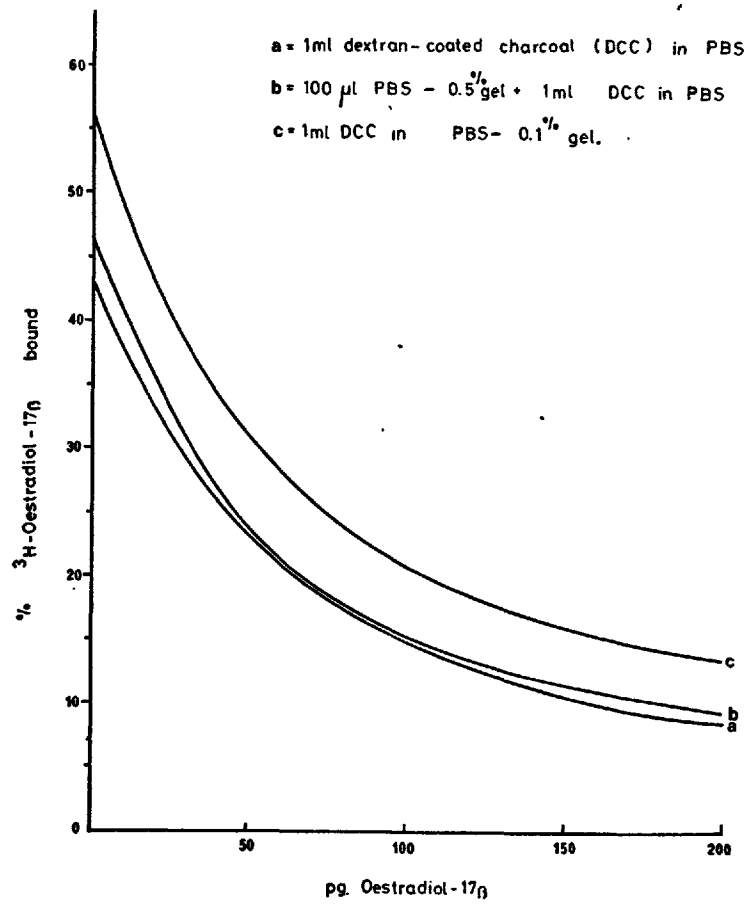


Fig. 23. Effect of gelatine concentration on dextran-coated charcoal separation technique.

dextran-coated charcoal in PBS-0.1% gel was assessed by determining the percentage of known amounts of tritiated oestradiol-17β adsorbed when incubated with dextran-coated charcoal for 20 min at 4°C. The results are presented in Table 6. Adsorption was efficient (98%) and independent of level over the range 8 - 500 pg.

c) Time lags

When an assay involves a large number of tubes, there can be a significant delay between addition of dextran-coated charcoal to the first and last tubes and also between decanting the first and last tubes. The effect of these time lags on binding and displacement with the 17-HS antiserum was examined and the results are illustrated in Figs. 24 and 25.

The effect of incubating with dextran-coated charcoal for varying lengths of time prior to centrifuging is shown in Fig. 24. The initial binding decreased rapidly as the incubation time was increased from 0 - 15 min, more slowly from 15 -30 min and very little as incubation was prolonged further. Binding in the presence of 150 pg of oestradiol-17β showed a similar, though less dramatic pattern. The overall effect was a decrease in displacement as incubation progressed, being rapid in the first 15 min, less rapid in

| Pg Tritiated oestradiol-17β added | % Tritiated oestradiol-17β adsorbed |
|---|---|
| 8.2 | 97.5 |
| 15.6 | 97.8 |
| 29.8 | 97.8 |
| 45.2 | 97.6 |
| 61.8 | 97.8 |
| 70.4 | 98.8 |
| 134.6 | 98.7 |
| 276.3 | 98.9 |
| 406.6 | 98.8 |
| 518.2 | 98.8 |

Table 6. Efficiency of dextran-coated charcoal in adsorbing tritiated oestradiol-17β.

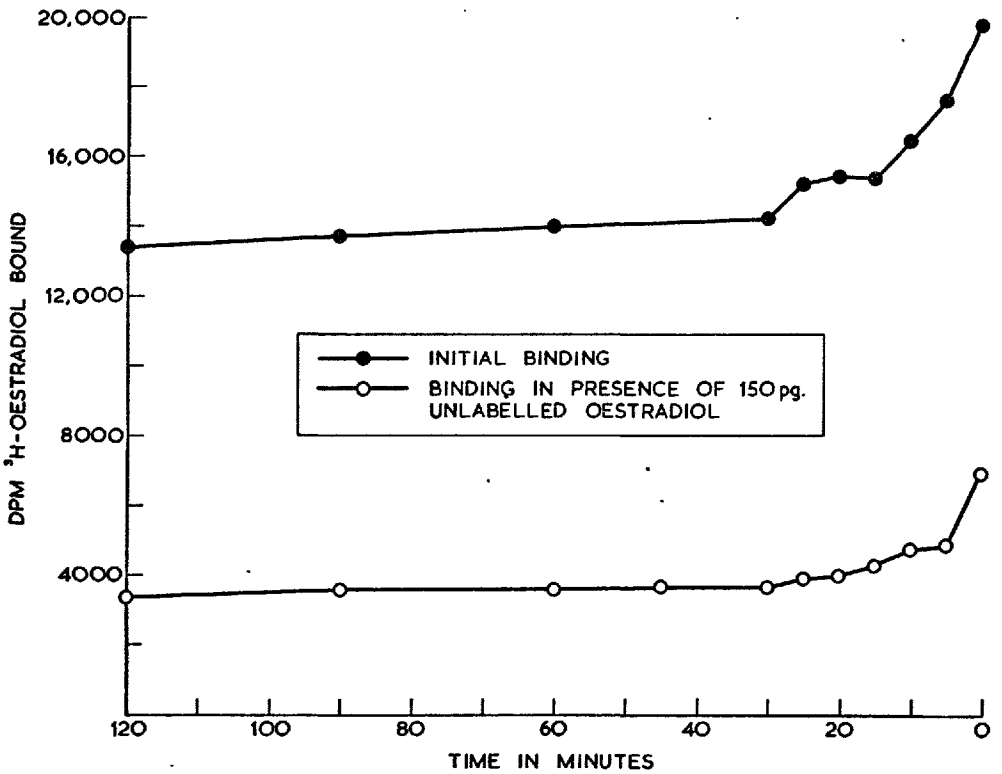


Fig. 24. Effect of varying time between adding dextran-coated charcoal and centrifuging.

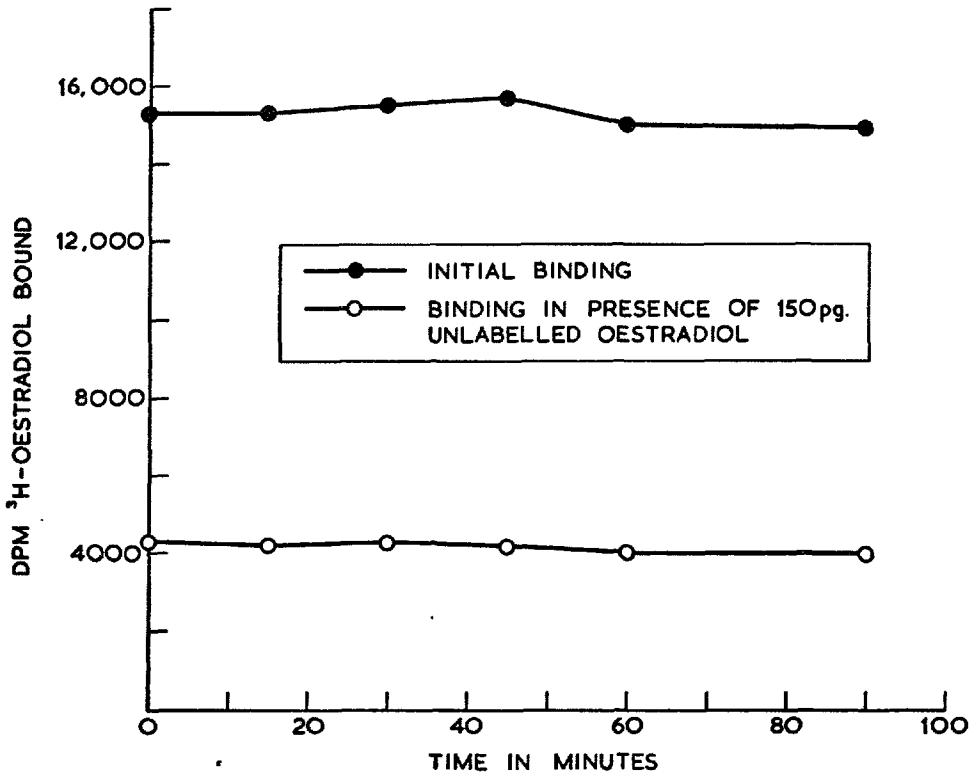


Fig. 25. Effect of varying time between centrifuging and decanting.

the next 15 min and very little thereafter. It therefore seemed advisable to allow incubation with dextran-coated charcoal to proceed for 20-30 min before centrifuging when large numbers of tubes were involved in an assay. Fig.25 shows the effect of leaving tubes after centrifuging for varying lengths of time at room temperature before decanting the supernatants. Both initial binding and displacement remained virtually unchanged when tubes were left for up to 1.5 hr. Thus, a considerable time may elapse between centrifuging and decanting without affecting the assay.

v) The effect of solvent residues

Solvents which may affect the assay are ether, ethanol or acetone and the column eluate (benzene:methanol 85:15 v/v). Standard curves were set up with the 17-HS antiserum for oestradiol-17β, with the following solvents added and evaporated to dryness: 1) nil, 2) 2 ml of ether, 3) 100 µl of ethanol, 4) 200 µl of ethanol, 5) 100 µl of column eluate, and 6) 200 µl of column eluate. The results are presented in Fig. 26. It can be seen that solvents can have a significant effect on the shape of the standard curve. However, these effects vary widely from batch to batch of solvent, some exerting a drastic effect on the standard curve and others hardly any.

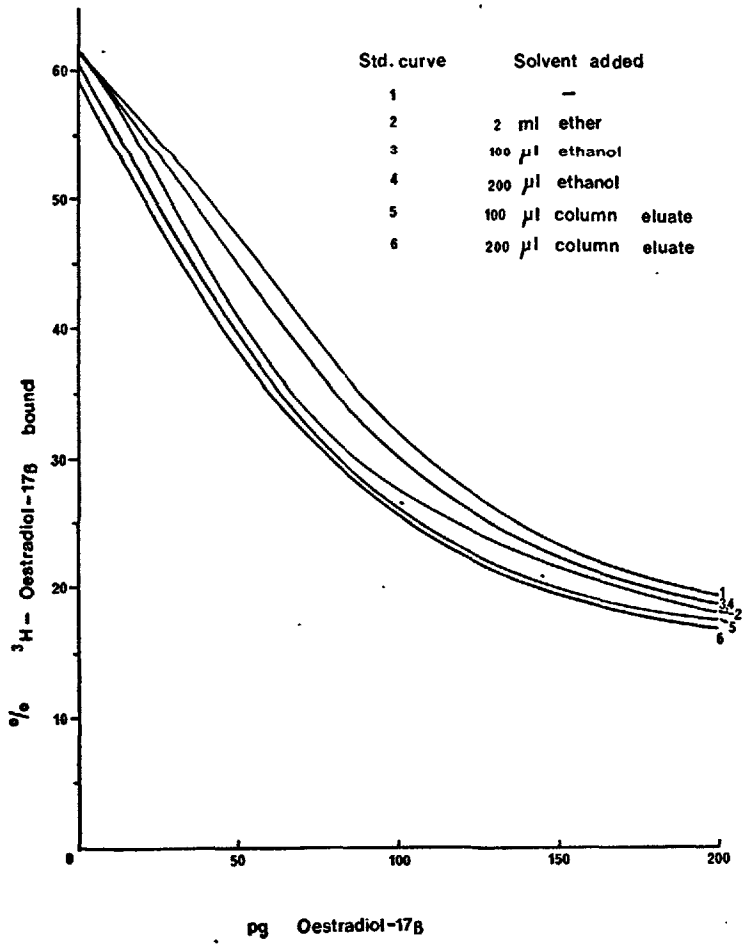


Fig. 26. Effect of solvent residues on standard curves prepared with a 17-HS antiserum.

Rather than adding solvent residues to standard curves, deionized distilled or analar water was extracted and taken through the method in the same manner as a plasma extract. The mean value of this method blank acted as a check on the solvents used, values greater than 10 pg/ml being considered indicative of poor solvent quality. In such a case, each solvent was examined individually, 100 ul of acetone or ethanol, benzene and methanol and 2 ml of ether being evaporated to dryness and assayed for oestradiol-17β equivalence. Thus, the offending solvent was identified and replaced.

The method blanks for assays spread over several months are recorded in Table 7. Apart from the method i oestrone blank, these values were considered acceptable. In retrospect, it was observed that the value of the oestrone blank dropped abruptly after August 1971 from a mean ± S.D. of 33 pg/ml ± 18 (n = 32) to 5 ± 7 (n = 16). A similar, though less dramatic change, was observed with the oestradiol-17β blank of method i, the corresponding values being 8 pg/ml ± 6 (n = 33) and 1 pg/ml ± 2 (n = 16). As the blank values were similar for methods ii and iii, which derive from assays before and after August 1971 respectively, it was thought that the quality

| | METHOD i | | METHOD ii | METHOD iii |
|---|---|---|---|---|
| | Oestrone | Oestradiol-17β | | |
| Pre August 1971 | | | | |
| Number | 32 | 33 | 94 | |
| Mean (pg/ml) | 33 | 8 | 7 | |
| S.D. | 18 | 6 | 7 | |
| Post August 1971 | | | | |
| Number | 20 | 20 | | 38 |
| Mean (pg/ml) | 5 | 1 | | 6 |
| S.D. | 5 | 2 | | 6 |

Table 7. Method blanks as calculated for 5 ml of deionized distilled or analar water taken through the procedure.

of either the benzene, methanol or possibly the Sephadex LH 20 as supplied was improved at this time.

The method blanks obtained after August 1971 compare favourably with those quoted in the literature. However, a direct comparison is not always possible because of the variety of ways in which results are expressed: pg (Mikhail et al, 1970; Doerr, 1973; Wright et al, 1973b), pg/sample (Wu et al, 1971) and pg/ml (Abraham, 1969; Hotchkiss et al, 1971; Kushinsky & Anderson, 1974) and the lack of information as to how these values were derived. In their publication, Kushinsky and Anderson (1974) discuss the problem of method blanks and advocate an accurate description of this assay parameter so that readers can compare this aspect of different methods more easily than is currently possible.

# CHAPTER 4

# Chapter 4

# E V A L U A T I O N

## A. INTRODUCTION

When determining oestrogen levels in plasma, it must be appreciated that a certain degree of inaccuracy or an error is involved. Each step of the method which involves sampling or measurement, for example, pipetting and determining radioactivity, and inherent properties of the method itself, such as specificity, contribute to this error. In addition, the size of the overall error has a considerable effect on the sensitivity of the method.

Errors can be examined in two ways:-

i) theoretically,

ii) practically.

The usual way of expressing errors theoretically is in terms of the standard deviation (S.D.) or coefficient of variation (C.V.) of the results obtained at individual steps. If n observations are made of a quantity x then:-

$$\text{S.D.} = \sqrt{\frac{1}{n-1}\sum(x - \bar{x})^2} \quad \ldots\ldots\ldots\ldots \quad \text{(i)}$$

$$\text{where } \bar{x} = \frac{1}{n}\sum x_n \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad \text{(ii)}$$

$$\text{and C.V.} = \frac{\text{S.D.}}{\bar{x}} \cdot 100\ \% \quad \ldots\ldots\ldots\ldots\ldots \quad \text{(iii)}$$

The more involved the method the larger in general the overall theoretical error will be. The error of a method is assessed practically from studies of accuracy and precision.

The methods described in Chapter 3 have been evaluated in terms of theoretical and practical errors, specificity and sensitivity.

## B. THEORETICAL ASSESSMENT OF ERRORS

In an attempt to determine the random error on individual samples, the random error was assessed for each term in the equation used for the calculation of results:-

$$S = \left[ \frac{T_x}{\alpha} \cdot \frac{C_s}{C_x} \cdot \frac{\beta E_x}{E_s} - M \right] \frac{1}{V} \quad \text{.....(iv)}$$

where $\alpha$ is the aliquot taken for radioimmunoassay and $T_x$ the reading from the standard curve; $C_s$ is the counts per minute (cpm) in the recovery standard and $C_x$ the cpm in an aliquot $\beta$ of the unknown; $E_x$ and $E_s$ are the counting efficiencies of the unknown and standard and M the mass of internal standard. V is the volume in ml of plasma (or water) extracted. S is the result in pg/ml.

An errors equation was derived for equation (iv) from the usual laws for the combination of additive and multiplicative independent normal errors:-

a) If

$$A = B + C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (v)$$

and B and C have errors of $\pm$ b and c respectively where b and c are the S.D. of B and C and a is the S.D. of A

then

$$a^2 = b^2 + c^2$$

Let the total random theoretical error (C.V.) on A be eA, then

$$eA = a/A$$

and

$$a^2 = e^2A.A^2$$

Substituting for a, b and c

$$e^2A = \frac{e^2B.B^2 + e^2C.C^2}{A^2}$$

and substituting for A

$$e^2A = \frac{e^2B.B^2 + e^2C.C^2}{(B + C)^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (vi)$$

b) If

$$A = B \,.\, C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (vii)$$

then

$$(a/A)^2 = (b/B)^2 + (c/C)^2$$

and

$$e^2A = e^2B + e^2C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(viii)}$$

c) If

$$A = B/C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(ix)}$$

then

$$(a/A)^2 = (b/B)^2 + (c/C)^2$$

and

$$e^2A = e^2B + e^2C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(x)}$$

d) If

$$A = (B-C)/D \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(xi)}$$

then

$$(a/A)^2 = \frac{b^2 + c^2}{(B-C)^2} + (d/D)^2$$

and

$$e^2A = \frac{e^2B.B^2 + e^2C.C^2}{(B-C)^2} + e^2D \quad \ldots\ldots\ldots\ldots \text{(xii)}$$

Referring to eq. (iv):-

let

$$\frac{T_x}{\alpha} \cdot \frac{C_s}{C_x} \cdot \frac{\beta E_x}{E_s} = \Delta \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(xiii)}$$

and

$$\frac{T_x}{\alpha} \cdot \frac{C_s}{C_x} \cdot \frac{\beta E_x}{E_s} - M = \mu \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(xiv)}$$

so that

$$\mu = \Delta - M \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(xv)}$$

Then

$$S = \frac{\mu}{V} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(xvi)}$$

and from eq. (x)

$$e^2S = e^2\mu + e^2V \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(xvii)}$$

Applying eq. (vi) to eq. (xv)

$$e^2\mu = \frac{e^2\Delta.\Delta^2 + e^2M.M^2}{\mu^2} \quad \ldots\ldots\ldots\ldots\ldots \text{(xviii)}$$

From eq. (xiii)

$$e^2\Delta = e^2T_x + e^2C_s + e^2E_x + e^2C_x + e^2E_s$$

Substituting for $e^2\Delta$ in eq. (xviii), we have

$$e^2\mu = \frac{[(e^2T_x + e^2C_s + e^2E_x + e^2C_x + e^2E_s)\ \Delta^2] + e^2M.M^2}{\mu^2} \quad \ldots\ldots \text{(xix)}$$

and substituting for $e^2\mu$ in eq. (xvii)

$$e^2S = \frac{[(e^2T_x + e^2C_s + e^2E_x + e^2C_x + e^2E_s)\Delta^2] + e^2M.M^2}{\mu^2} + e^2V \quad \ldots\ldots \text{(xx)}$$

As $\alpha$ and $\beta$ are constants, they do not have any error, but contribute to the overall error on S because of the form

of eq. (xx). The maximum error (C.V.%) on the terms in eq. (iv) were evaluated as follows:-

$C_s$, $C_x$, $E_s$ and $E_x$

There are two percentage errors in the term $C_s$, one from pipetting the standard into a counting vial and the other from liquid scintillation counting. The pipetting error using a Biopette was found to be 2.0 % and, as 4,000 counts were recorded, the counting error was about 1.6 % assuming Poisson statistics (the variance equals the mean in this case). The two errors were then combined as follows:-

$$e = (e_1^2 + e_2^2 \ldots\ldots e_n^2)^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(xxi)}$$

$$= (2.0^2 + 1.6^2)^{\frac{1}{2}}$$

$$= 2.56\ \%$$

There is no pipetting error for $C_x$ as either the eluate from the chromatography column is collected into a counting vial (method i) or the ether extract is transferred to a counting vial (methods ii and iii) and that remaining after aliquots have been taken for assay is counted. However, there is an error involved in the addition of ethanol or acetone (1.0 %) and in the removal

of aliquots for assay (2.0 %). These errors combine with a counting error of approximately 1.6 % to give an overall error on $C_x$ of 2.75 %.

The error in the counting efficiencies $E_s$ and $E_x$ is dependent upon the method used for evaluating the degree of quenching in a sample. This error was estimated to be 3.0 % using an external standard channels ratio method.

M and V

The amount of internal standard M added to the samples is determined by liquid scintillation counting of an aliquot of known specific activity. As the error in calculating dpm is 3.4 % and that on the specific activity 1.0 % these, combined with a 2.0 % pipetting error give an overall error of 4.1 %. The error on V was taken to be 5.0 % at the maximum.

$T_x$

The overall error on $T_x$ contains the errors involved in calculating dpm in the aliquots removed for assay and in the preparation of and reading from the standard curve. As each sample is counted for 4,000 counts and corrected for efficiency, the error in calculating dpm in each aliquot is 3.4 %. Combining this with a 2.0 % pipetting error gives

an overall error of 3.9 %. The error in reading from standard curves depends upon both the accuracy of preparing the curves and the position from which the reading is taken.

The reproducibility of standard curves is illustrated in Figs. 27, 28 and 29 for oestrone, oestradiol-17β with the 17-HS antiserum and oestradiol-17β with the 6-CMO antiserum respectively. The C.V. for a standard curve based on the mean of triplicate determinations ranges from 4.0 - 5.5 %. When eight standard curves are prepared in triplicate on the same day the range is 7.0 - 13.0 % for oestrone, 4.5 - 12.0 % for oestradiol-17β with the 17-HS antiserum and 5.0 - 8.0 % for oestradiol-17β with the 6-CMO antiserum. A larger increase in C.V. was observed when ten standard curves prepared in triplicate over a four month period were examined, the ranges being 11.0 - 29.0 % for oestrone and 16.0 - 28.0 % and 11.5 - 25.5 % for oestradiol-17β with the 17-HS antiserum and 6-CMO antiserum respectively.

S

The total random theoretical percentage error (eS), corresponding to six values of $T_x$ for the three methods, is presented in Table 8. When a standard curve based on the mean of triplicate determinations is used for the calculation of $e^2T_x$, the overall error (eS) on the

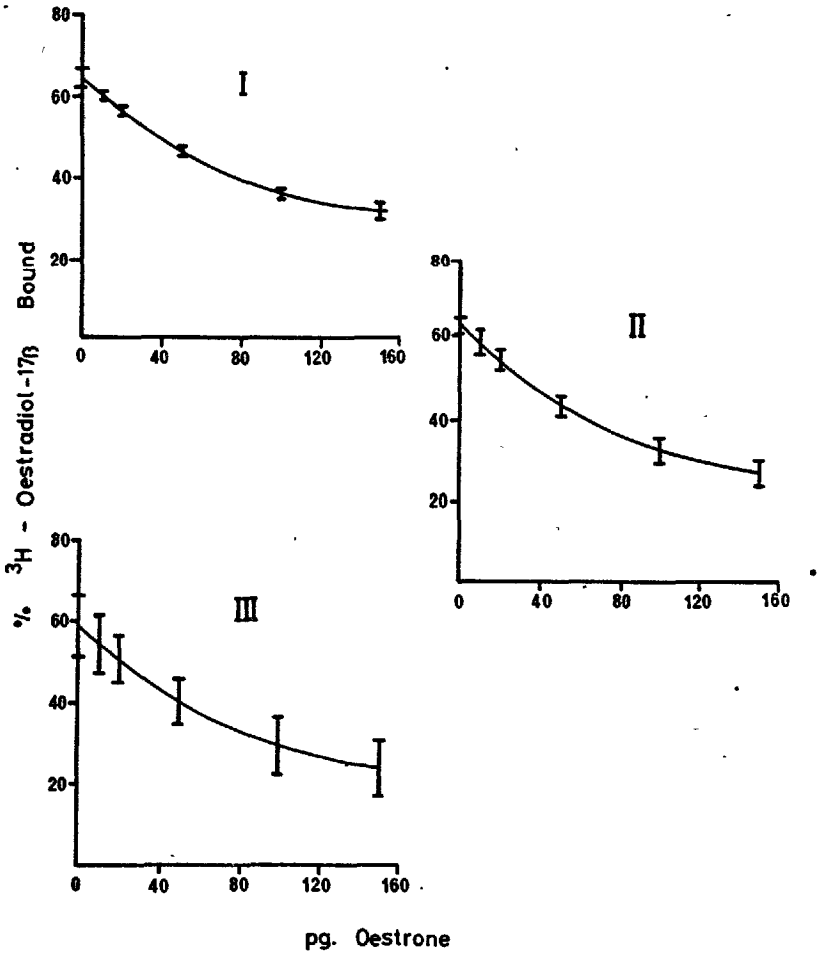


Fig. 27. Reproducibility of standard curves: oestrone with a 17-HS antiserum diluted 1:25,000.

I. Mean ± S.D. of single curve in triplicate.

II. Mean ± S.D. of eight curves in triplicate prepared on the same day.

III. Mean ± S.D. of ten curves in triplicate prepared over a 4-month period.

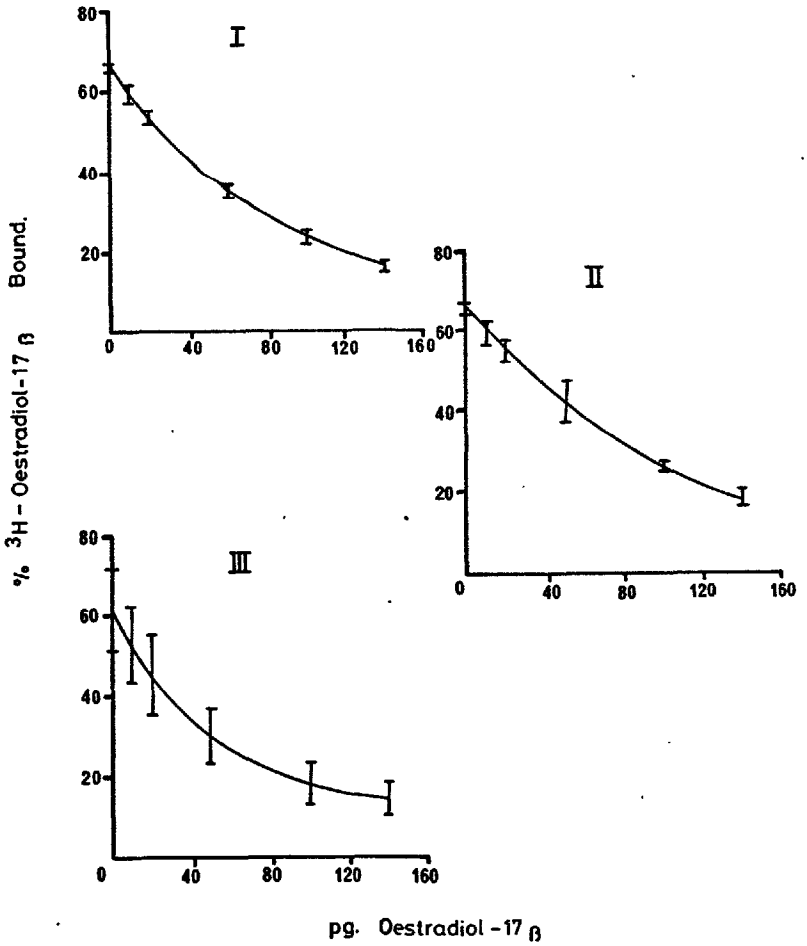


Fig. 28. Reproducibility of standard curves: oestradiol-17β with a 17-HS antiserum diluted 1:25,000.
Key as Fig. 27.

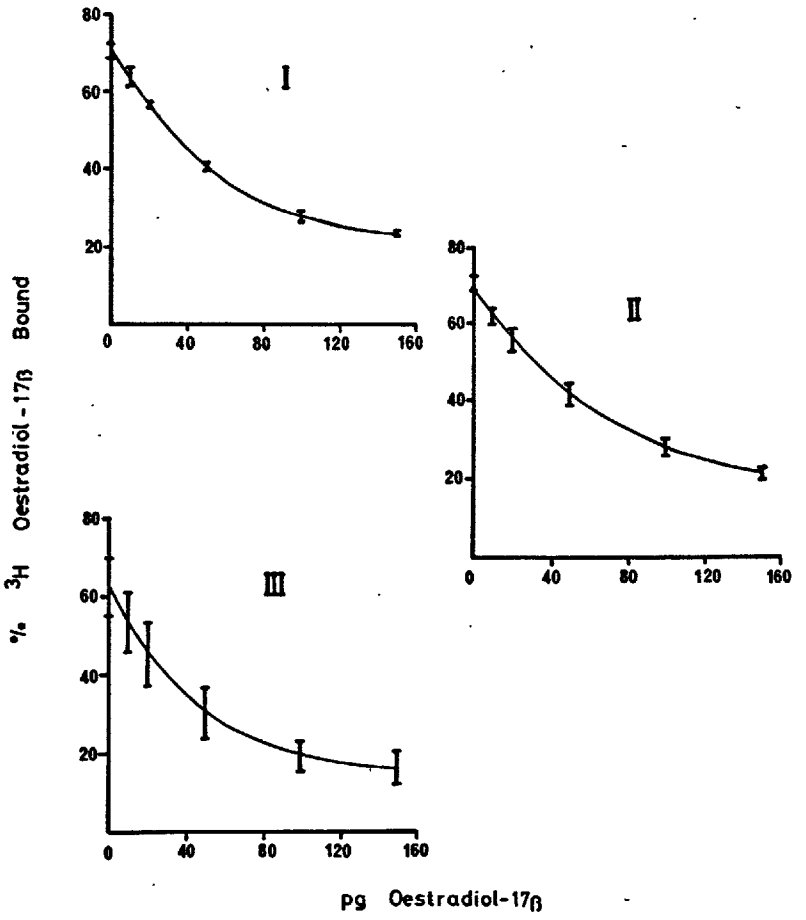


Fig. 29. Reproducibility of standard curves: oestradiol-17β with a 6-CMO antiserum diluted 1:12,500.
Key as Fig. 27.

| Reading on Standard Curve (pg) | TOTAL RANDOM THEORETICAL PERCENTAGE ERROR | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MALE PLASMA ($\alpha$ = 0.2) | | | | | | FEMALE PLASMA ($\alpha$ = 0.1) | | | | | |
| | 1 | 10 | 20 | 50 | 100 | 150 | 1 | 10 | 20 | 50 | 100 | 150 |
| Single Standard Curve | | | | | | | | | | | | |
| Method i | | | | | | | | | | | | |
| Oestrone | 11.9 | 9.3 | 8.7 | 8.7 | 8.6 | 8.9 | 10.2 | 9.2 | 8.7 | 8.6 | 8.6 | 8.8 |
| Oestradiol-17β | 11.9 | 8.7 | 8.8 | 9.4 | 9.1 | 8.8 | 10.2 | 8.6 | 8.8 | 9.4 | 9.1 | 8.8 |
| Method ii | 14.7 | 9.4 | 8.8 | 8.7 | 8.6 | 8.6 | 10.8 | 9.2 | 8.7 | 8.6 | 8.6 | 8.9 |
| Method iii | 16.0 | 8.9 | 8.8 | 8.9 | 9.0 | 8.7 | 11.2 | 8.7 | 8.7 | 8.8 | 9.0 | 8.7 |
| Eight Standard Curves prepared in Triplicate on same day | | | | | | | | | | | | |
| Method i | | | | | | | | | | | | |
| Oestrone | 13.5 | 10.7 | 11.8 | 11.5 | 14.4 | 14.0 | 11.5 | 11.6 | 11.7 | 11.5 | 14.3 | 14.1 |
| Oestradiol-17β | 11.6 | 9.4 | 10.3 | 14.0 | 13.0 | 13.7 | 9.9 | 9.3 | 10.2 | 14.0 | 11.4 | 13.7 |
| Method ii | 14.2 | 9.5 | 10.3 | 14.0 | 11.4 | 13.7 | 10.5 | 9.3 | 10.2 | 14.0 | 11.4 | 13.7 |
| Method iii | 16.0 | 9.2 | 10.6 | 10.9 | 10.9 | 10.7 | 11.2 | 9.1 | 10.5 | 10.9 | 10.8 | 10.7 |
| Ten Standard Curves prepared in Triplicate over a four month period | | | | | | | | | | | | |
| Method i | | | | | | | | | | | | |
| Oestrone | 20.7 | 15.7 | 14.3 | 16.0 | 25.4 | 30.2 | 17.4 | 15.5 | 14.2 | 15.9 | 25.4 | 30.1 |
| Oestradiol-17β | 25.5 | 19.8 | 23.4 | 25.2 | 29.2 | 26.6 | 21.2 | 19.5 | 23.2 | 25.2 | 29.1 | 26.6 |
| Method ii | 31.9 | 20.1 | 23.6 | 25.3 | 29.2 | 26.6 | 22.6 | 19.5 | 23.3 | 25.2 | 29.1 | 26.6 |
| Method iii | 27.0 | 16.5 | 20.2 | 21.4 | 23.6 | 26.8 | 18.4 | 16.1 | 20.0 | 21.3 | 23.5 | 26.8 |

Table 8. Total random theoretical percentage error of three methods for the radioimmunoassay of plasma oestrogens.

determination of oestrone or oestradiol-17β in male or female plasma is approximately 9.0 % for values of $T_x$ greater than 10 pg. The value of eS increases for values of $T_x$ less than 10 pg especially in the case of male plasmas. When $e^2T_x$ is calculated on the basis of eight standard curves prepared in triplicate on the same day, eS is increased to a maximum of 16.0 % and can exceed 30.0 % if standard curves prepared over several months are used as the basis for this calculation. The implication is that, in order to obtain the smallest error when determining oestrogens in plasma, a standard curve must be set up with each assay.

C. PRACTICAL ASSESSMENT OF ERRORS

The errors associated with the methods described were assessed from studies of the recovery of known amounts of oestrogen from analar water (accuracy) and of intra and inter assay variability (precision).

i) Accuracy

This term may be defined as the nearness with which a given analytical result approaches the true result. It involves the concepts of both precision and specificity since in order for a measurement to be accurate it must be both reproducible and unaffected by extraneous factors.

The accuracy of the three methods was assessed from replicate recoveries of known amounts of oestradiol-17β (methods ii and iii) or oestrone plus oestradiol-17β (method i) added to 5 ml of analar water. The results are recorded in Table 9. It can be seen that at levels of 50 pg and 100 pg oestrogen/ml water the C.V. was consistently less than 15 %. The values observed are similar to those quoted by other authors including Mikhail et al (1970), Wu et al (1971) and Moore and Axelrod (1972). At levels of 10 pg and 20 pg oestrogen/ml water the C.V. was considerably higher although the mean recovery (100 to 111.5 %) was better than expected considering that these levels are at or below the theoretical detection limit of the assay (see below).

ii) Precision

This reflects the reproducibility of a measurement and is often represented by the S.D. (or some multiple of the S.D.) of replicate estimations assuming that they are normally distributed about the mean. It should be noted that a precise result need not necessarily be an accurate one.

The intra assay precision for the determination of both oestrone and oestradiol-17β (method i) was assessed

| | Amount Added (pg/ml) | Mean Recovery (pg/ml) | S.D. | C.V.(%) |
|---|---|---|---|---|
| Method i | | | | |
| Oestrone | 10 | 10.6 | 4.4 | 41.5 |
| | 20 | 22.3 | 7.1 | 31.8 |
| | 50 | 51.2 | 5.2 | 10.2 |
| | 100 | 100.2 | 10.8 | 10.8 |
| Oestradiol -17β | 10 | 10.0 | 3.5 | 35.0 |
| | 20 | 21.0 | 6.2 | 29.5 |
| | 50 | 49.9 | 5.0 | 10.0 |
| | 100 | 99.2 | 13.5 | 13.6 |
| Method ii | | | | |
| Oestradiol -17β | 50 | 58.5 | 2.9 | 5.0 |
| | 100 | 111.7 | 4.9 | 4.4 |
| Method iii | | | | |
| Oestradiol -17β | 50 | 50.1 | 6.7 | 13.4 |
| | 100 | 98.7 | 12.2 | 12.4 |

Table 9. Accuracy of the methods as derived from replicate analyses (n = 12) of known amounts of oestrogen added to 5 ml of analar water.

from replicate determinations performed on plasma from male and female blood donors and the results are reported in Table 10. Using this approach, the C.V. varied from 13 to 27 % for oestrone and from 15 to 17 % for oestradiol-17β.

Another approach to assay precision is that proposed by Snedecor (1952) where values for the C.V. are derived from the following equation:-

$$C.V. = \left[\frac{\sum d^2}{2n}\right]^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(xxii)}$$

where

$$d = \frac{\text{highest - lowest value of duplicate}}{\text{lowest value of duplicate}} \times 100$$

and

$n$ = the number of duplicate determinations.

Both intra and inter assay precision were estimated in this way. Twenty plasma samples (range: 15 - 160 pg oestrone or oestradiol-17β/ml plasma) were determined in duplicate in one assay and then singularly in a second assay. Values for both intra and inter assay C.V.'s for the three methods are recorded in Table 11 and range from 8 to 9 % and 15 to 17 % respectively.

Direct comparison of the values for assay precision as found in this work with those of other workers is difficult owing to the different methods of assessment used.

| | OESTRONE | | OESTRADIOL-17β | |
|---|---|---|---|---|
| | Pg/ml Plasma Mean ± S.D. | C.V. (%) | Pg/ml Plasma Mean ± S.D. | C.V. (%) |
| Male Plasma | 73 ± 20 | 27.4 | 18 ± 3 | 16.7 |
| Female Plasma (day 8) | 87 ± 16 | 18.4 | 78 ± 13 | 16.7 |
| Female Plasma (day 23) | 166 ± 21 | 12.7 | 150 ± 22 | 14.7 |

Table 10. Replicate analyses (n = 12) on pools of male and female plasma.

| | INTRA ASSAY C.V. % | INTER ASSAY C.V. % |
|---|---|---|
| Method i | | |
| Oestrone | 9.3 | 15.9 |
| Oestradiol-17β | 8.2 | 14.1 |
| Method ii | | |
| 'Oestrogen' | 7.9 | 13.2 |
| Method iii | | |
| Oestradiol-17β | 8.8 | 13.8 |

Table 11. Intra and inter assay precision as determined by application of a procedure proposed by Snedecor (1952).

However, the numerical values reported in Tables 9 and 10 are in good agreement with those quoted by a number of authors including Abraham (1969), Mikhail et al (1970), Doerr (1973) and Kushinsky and Anderson (1974).

## D. SENSITIVITY

When discussing the concept of sensitivity, it is necessary to define one's terms as sensitivity has been used in connection with the slope of the dose-response curve (Yalow and Berson, 1970), the detection limit of the standard curve (Ekins and Newman, 1970) and the detection limit of the assay (Moore and Axelrod, 1972; Doerr, 1973; and Kushinsky and Anderson, 1974). As applied to an assay technique the term sensitivity commonly refers to the ability of a system to measure small amounts with acceptable precision. Thus, a technique is regarded as more sensitive if it enables smaller amounts to be measured with the same precision or the same small amount with greater precision.

Feldman and Rodbard (1971) defined sensitivity as the lowest concentration of unlabelled antigen which, when added to a solution containing no antigen, results in a significant change in the response variable. To be significant, this change must be equal to the S.D. of the response variable at

the point where the unlabelled antigen is zero, multiplied by a 'significant' value of Student's t (approximately 2). Another definition has been offered by Borth (1970), who describes the detection limit as the smallest concentration which is greater than, and statistically different from, the apparent concentration found in blanks. Assuming that the blank and sample variances are the same and using a one-sided t test at p = 0.05, the detection limit for single samples is approximately equal to the apparent blank concentration + 2.5 S.D.

The sensitivity of the three assay methods and their standard curves are reported in Table 12. It is evident that the sensitivity of the methods depends to a large extent upon the method of assessment. The values quoted in Table 12 (5 - 25 pg/ml) are in good agreement with those reported by Abraham (1969) and Kushinsky and Anderson (1974). However, sensitivities ranging from 1.8 pg/ml (Doerr, 1973) to 75 - 100 pg/ml (Moore and Axelrod, 1972) have appeared in the literature. Improved sensitivity would result if either the detection limit of the standard curve (which is dependent upon the precision of preparing standard curves) or the value for the method blank could be decreased.

| | DETECTION LIMIT OF STANDARD CURVE (pg) | DETECTION LIMIT OF ASSAY (pg/ml) | | |
|---|---|---|---|---|
| | | With reference to standard curve | | With reference to blank |
| | | $\alpha$ = 0.2 | $\alpha$ = 0.1 | |
| Method i | | | | |
| Oestrone | 4.0 | 7.4 | 14.8 | 17.5 |
| Oestradiol-17β | 4.0 | 7.6 | 15.2 | 6.0 |
| Method ii | | | | |
| Oestradiol-17β | 4.0 | 4.8 | 9.6 | 24.5 |
| Method iii | | | | |
| Oestradiol-17β | 12.0 | 13.7 | 27.4 | 21.0 |

Table 12. Assay sensitivity.

With reference to standard curve: sensitivity calculated on the basis of the detection limit of the respective standard curve and the mean recovery ($\alpha$ = aliquot of extract taken for assay).

With reference to blank: mean blank (pg/ml) + 2.5 S.D.

## E. SPECIFICITY

The specificity of an assay system refers to its ability to respond only to the hormone which the assay is intended to quantify. The specificity of a hapten radioimmunoassay depends upon three factors:-

i) the specificity of the antiserum,

ii) the specificity of the extraction/purification technique, and

iii) the concentration of cross-reacting compounds in the final extract.

If an antiserum has a high degree of specificity, the specificity of the extraction/purification technique and concentration of cross-reacting compounds in the final extract are of little consequence but, if the antiserum is not specific, these factors are of major importance.

During the initial development of radioimmunoassay systems for the determination of oestrogens in plasma, the only antisera available were those raised against antigens involving conjugation of oestrogen to protein via an existing functional group. It was soon realized, however, that the position of conjugation to the steroid nucleus could have a profound effect upon the specificity of the resultant antiserum (Midgley and Niswender, 1970) and the suggestion was made that conjugation of steroid to protein

through a position distal to structurally unique regions, for example C-6 or C-7, might result in significantly greater antiserum specificity. This supposition was subsequently shown to be correct (Exley et al, 1971; Jeffcoate and Searle, 1972; Kuss et al, 1973; Lindner et al, 1972; Wright et al, 1973a).

The specificities of the two antisera used in the work described were investigated in two ways. First, standard curves were set up for oestradiol-17β, 15 phenolic steroids and 14 neutral steroids. Care was taken to ensure that the volume of acetone used remained constant (100 µl) throughout this experiment. Standard curves were plotted and the percentage cross-reaction (CR %) calculated according to the method of Abraham (1969):-

Let

x = mass of unlabelled oestradiol-17β required to displace 50 % of the tritiated oestradiol-17β bound to antibody, and

y = mass of steroid required to displace 50 % of the tritiated oestradiol-17β bound to antibody.

Then

$$CR\ \% = \frac{x}{y} \cdot 100$$

Standard curves for the oestrogens (range 0-2 ng) and neutral steroids (range 0-10 µg) are presented in Figs. 30

and 31 for the 17-HS antiserum and Figs. 32 and 33 for the 6-CMO antiserum. The CR % as derived for each steroid tested is recorded in Table 13.

This method of examining antiserum specificity, which has been discussed in detail by de Lauzon et al (1973), although widely used, does not indicate what effect a steroid has on the estimation of oestradiol-17β when they are both present in a sample or extract. In an attempt to shed some light on this problem, mixtures of oestradiol-17β and neutral or phenolic steroids were assayed for oestradiol -17β equivalence. The results are recorded in Tables 14, 15, 16, 17, 18 and 19. Apart from cholesterol, the naturally occurring neutral steroids were tested at a level approximately equivalent to the maximum amount expected in 10 ml of non-pregnancy human peripheral venous plasma.

Both methods show that, as expected, the antiserum raised against oestradiol-17β coupled via the 6 position is considerably more specific than that coupled via the functional hydroxyl group at the 17 position. In general, those oestrogens with the larger CR %'s caused a greater over-estimate when oestradiol-17β was assayed in a mixture. Oestrone, 16α-hydroxyoestrone, 6-oxo-oestradiol-17β, 16-oxo-oestradiol-17β, oestradiol-17α, 17α-ethynyl-oestradiol, oestriol, 16-epi-oestriol and 17-epi-oestriol

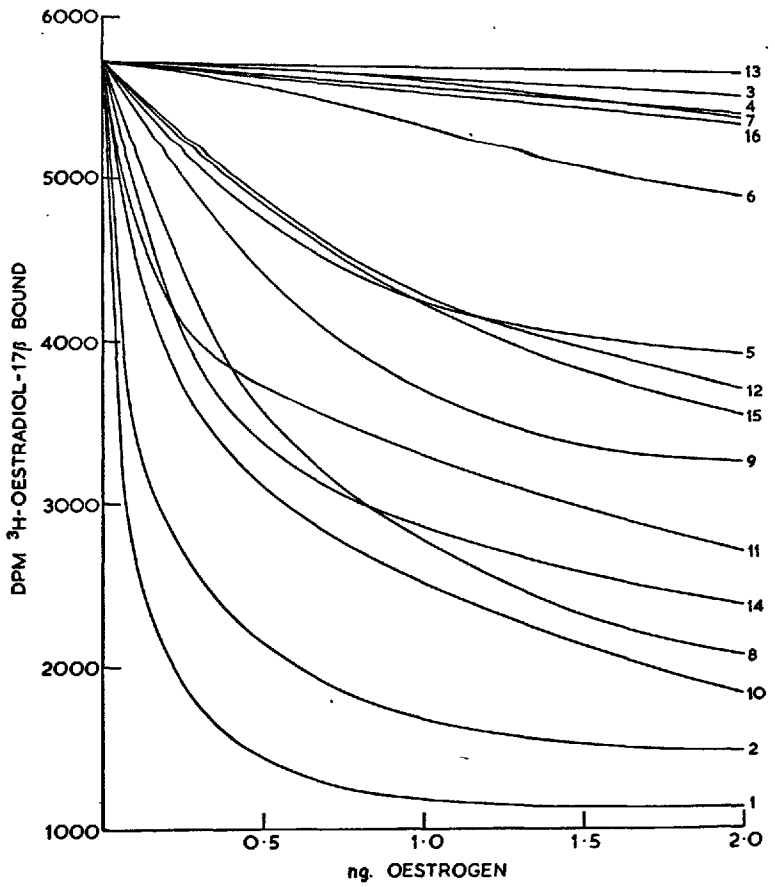


Fig. 30. Specificity of a 17-HS antiserum: oestrogen standard curves.
Key:-
1 Oestradiol-17β
2 Oestrone
3 2-Methoxyoestrone

Fig. 30. Legend cont'd.

4 Oestrone methyl ether
5 16α-Hydroxyoestrone
6 2-Methoxyoestradiol-17β
7 Oestradiol-17β methyl ether
8 6-Oxo-oestradiol-17β
9 Oestradiol-17α
10 17α-Ethynyloestradiol
11 Oestriol
12 6-Oxo-oestriol
13 16-Epi-oestriol
14 16,17-Epi-oestriol
15 17-Epi-oestriol
16 Oestetrol.

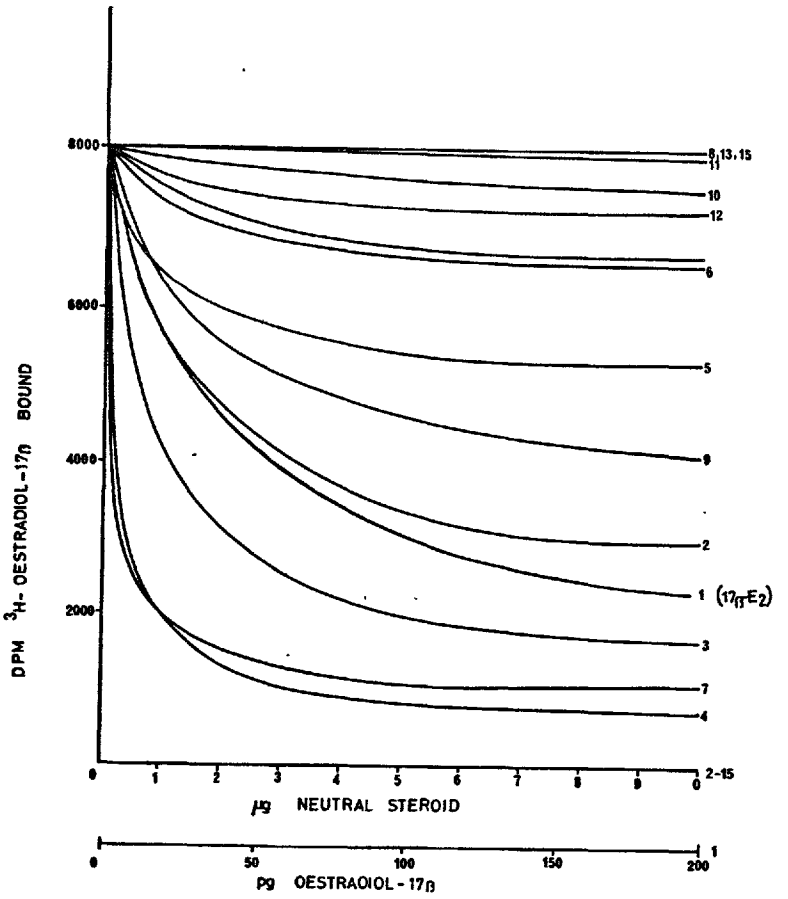


Fig. 31. Specificity of a 17-HS antiserum: standard curves for neutral steroids.
Key:-
1 Oestradiol-17β
2 Androstenedione
3 19-Nor-testosterone

Fig. 31. Legend cont'd.

4 19-Nor-5α-dihydrotestosterone
5 Dehydroepiandrosterone
6 Testosterone
7 5α-Dihydrotestosterone
8 Ethisterone
9 19-Norethisterone
10 Progesterone
11 17-Hydroxyprogesterone
12 Pregnenolone
13 17-Hydroxypregnenolone
14 Cortisol
15 Cholesterol.

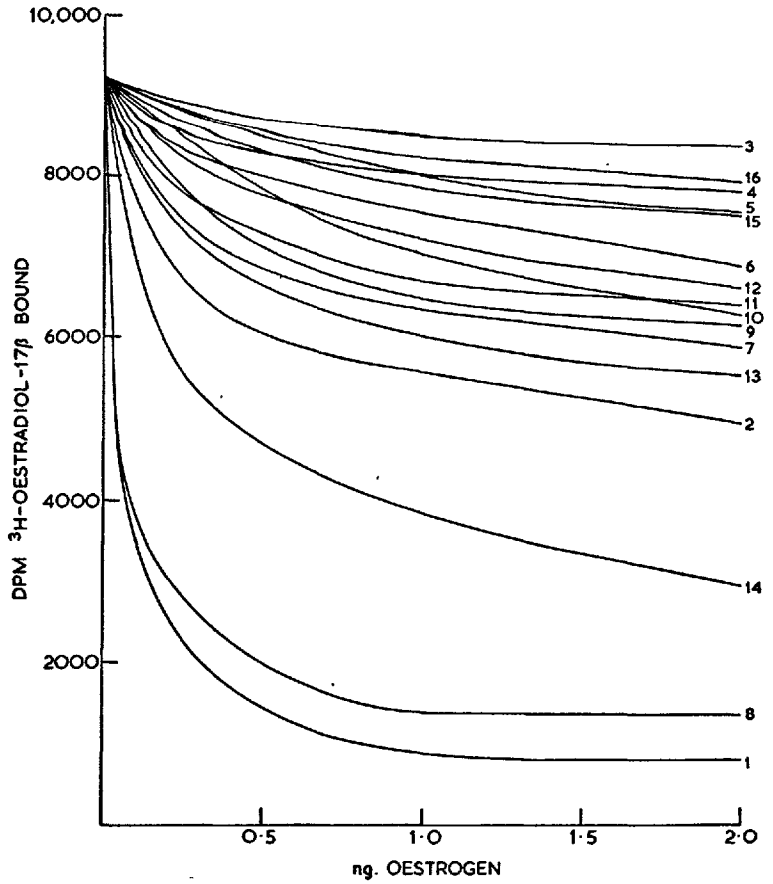


Fig. 32. Specificity of a 6-CMO antiserum: oestrogen standard curves. Key: as Fig. 30.

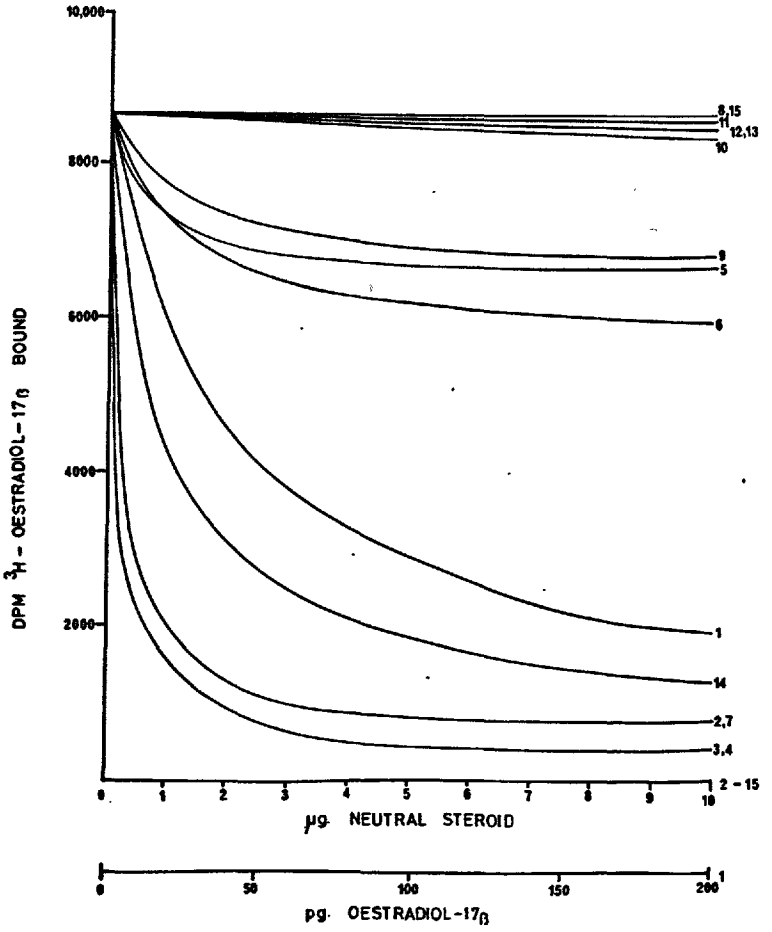


Fig. 33. Specificity of a 6-CMO antiserum: standard curves for neutral steroids. Key: as Fig. 31.

| STEROID | CR % 17-HS ANTISERUM | CR % 6-CMO ANTISERUM |
|---|---|---|
| PHENOLIC STEROIDS | | |
| Oestradiol-17β | 100.00 | 100.00 |
| Oestrone | 38.10 | 1.40 |
| 2-Methoxyoestrone | < 0.10 | < 0.05 |
| Oestrone methyl ether | < 0.10 | < 0.05 |
| 16α-Hydroxyoestrone | 1.30 | 0.14 |
| 2-Methoxyoestradiol-17β | < 0.10 | 0.80 |
| Oestradiol-17β methyl ether | < 0.10 | 0.10 |
| 6-Oxo-oestradiol-17β | 8.60 | 86.20 |
| 16-Oxo-oestradiol-17β | 2.40 | 0.80 |
| Oestradiol-17α | 11.40 | 0.90 |
| 17α-Ethynyloestradiol | 4.60 | 0.30 |
| Oestriol | 2.20 | 0.40 |
| 6-Oxo-oestriol | < 0.10 | 0.80 |
| 15α-Hydroxyoestriol | 0.10 | < 0.05 |
| 16-Epi-oestriol | 8.00 | 9.10 |
| 17-Epi-oestriol | 2.20 | 0.20 |
| NEUTRAL STEROIDS | | |
| Androstenedione | 0.0020 | 0.0300 |
| Cholesterol | < 0.0005 | < 0.0005 |
| Cortisol | < 0.0005 | 0.0050 |
| Dehydroepiandrosterone | < 0.0005 | < 0.0005 |
| 5α-Dihydrotestosterone | 0.0300 | 0.0400 |
| 19-Nor-5α-dihydrotestosterone | 0.0700 | 0.1000 |
| Ethisterone | < 0.0005 | < 0.0005 |
| 19-Norethisterone | < 0.0005 | < 0.0005 |
| Progesterone | < 0.0005 | < 0.0005 |
| 17-Hydroxyprogesterone | < 0.0005 | < 0.0005 |
| Pregnenolone | < 0.0005 | < 0.0005 |
| 17-Hydroxypregnenolone | < 0.0005 | < 0.0005 |
| Testosterone | < 0.0005 | < 0.0005 |
| 19-Nor-testosterone | 0.0050 | 0.0800 |

Table 13. The percentage cross-reaction (CR %) of a number of phenolic and neutral steroids with antisera raised against oestradiol-17β coupled to BSA via the 17 and 6 positions.

| 50 pg OESTRADIOL-17β + ADDED OESTROGENS | OESTRADIOL-17β EQUIVALENCE | | |
|---|---|---|---|
| | 20 pg | 50 pg | 100 pg |
| Oestrone | 65.5 | 90.5 | 140.0 |
| 2-Methoxyoestrone | 42.0 | 42.0 | 55.0 |
| Oestrone methyl ether | 50.0 | 56.5 | 58.0 |
| 16α-Hydroxyoestrone | 55.0 | 57.5 | 61.0 |
| 2-Methoxyoestradiol-17β | 55.5 | 57.5 | 59.0 |
| Oestradiol-17β methyl ether | 53.0 | 55.0 | 56.0 |
| 6-Oxo-oestradiol-17β | 46.5 | 59.5 | 76.0 |
| 16-Oxo-oestradiol-17β | 50.0 | 60.0 | 72.5 |
| Oestradiol-17α | 63.5 | 85.0 | 110.0 |
| 17α-Ethynyloestradiol | 57.0 | 65.0 | 91.5 |
| Oestriol | 55.0 | 65.0 | 75.5 |
| 6-Oxo-oestriol | 50.0 | 57.0 | 69.9 |
| 16-Epi-oestriol | 56.5 | 80.5 | 88.0 |
| 17-Epi-oestriol | 54.5 | 61.5 | 72.5 |
| Oestetrol | 53.5 | 59.0 | 59.5 |

Table 14. The effect of varying amounts of added oestrogen (20, 50 and 100 pg) on the determination of oestradiol-17β, with a 17-HS antiserum, in mixtures containing 50 pg of oestradiol-17β.

| 100 pg OESTRADIOL-17β + ADDED OESTROGENS | OESTRADIOL-17β EQUIVALENCE | | |
|---|---|---|---|
| | 50 pg | 100 pg | 200 pg |
| Oestrone | 140.0 | 192.0 | 239.0 |
| 2-Methoxyoestrone | 106.5 | 111.5 | 115.5 |
| Oestrone methyl ether | 111.0 | 115.0 | 116.0 |
| 16α-Hydroxyoestrone | 115.0 | 120.0 | 131.0 |
| 2-Methoxyoestradiol-17β | 110.0 | 124.0 | 126.0 |
| Oestradiol-17β methyl ether | 110.0 | 119.5 | 121.0 |
| 6-Oxo-oestradiol-17β | 124.0 | 130.0 | 149.5 |
| 16-Oxo-oestradiol-17β | 115.5 | 125.0 | 136.5 |
| Oestradiol-17α | 130.5 | 150.5 | 190.0 |
| 17α-Ethynyloestradiol | 120.5 | 136.0 | 159.0 |
| Oestriol | 125.5 | 126.0 | 140.0 |
| 6-Oxo-oestriol | 110.0 | 110.5 | 121.0 |
| 16-Epi-oestriol | 116.0 | 135.0 | 140.0 |
| 17-Epi-oestriol | 110.0 | 120.0 | 129.5 |
| Oestetrol | 115.0 | 121.0 | 124.5 |

Table 15. The effect of varying amounts of added oestrogen (50, 100 and 200 pg) on the determination of oestradiol-17β, with a 17-HS antiserum, in mixtures containing 100 pg of oestradiol-17β.

| 50 pg OESTRADIOL-17β + ADDED OESTROGENS | OESTRADIOL-17β EQUIVALENCE | | |
|---|---|---|---|
| | 20 pg | 50 pg | 100 pg |
| Oestrone | 51.0 | 59.5 | 65.0 |
| 2-Methoxyoestrone | 52.2 | 52.0 | 57.0 |
| Oestrone methyl ether | 52.5 | 56.0 | 66.0 |
| 16α-Hydroxyoestrone | 55.5 | 55.0 | 55.0 |
| 2-Methoxyoestradiol-17β | 54.5 | 57.0 | 60.5 |
| Oestradiol-17β methyl ether | 53.0 | 55.5 | 67.5 |
| 6-Oxo-oestradiol-17β | 73.5 | 92.0 | 132.5 |
| 16-Oxo-oestradiol-17β | 53.5 | 57.0 | 64.5 |
| Oestradiol-17α | 54.4 | 60.0 | 65.5 |
| 17α-Ethynyloestradiol | 56.0 | 57.5 | 63.0 |
| Oestriol | 57.5 | 58.0 | 69.0 |
| 6-Oxo-oestriol | 55.5 | 57.0 | 65.0 |
| 16-Epi-oestriol | 61.0 | 62.5 | 76.5 |
| 17-Epi-oestriol | 54.5 | 56.0 | 62.5 |
| Oestetrol | 52.5 | 54.4 | 55.0 |

Table 16. The effect of varying amounts of added oestrogen (20, 50 and 100 pg) on the determination of oestradiol-17β, with a 6-CMO antiserum, in mixtures containing 50 pg of oestradiol-17β.

| 100 pg OESTRADIOL-17β + ADDED OESTROGENS | OESTRADIOL-17β EQUIVALENCE | | |
|---|---|---|---|
| | 50 pg | 100 pg | 200 pg |
| Oestrone | 105.5 | 115.5 | 135.0 |
| 2-Methoxyoestrone | 100.0 | 103.5 | 110.5 |
| Oestrone methyl ether | 104.5 | 109.5 | 111.0 |
| 16α-Hydroxyoestrone | 100.5 | 101.0 | 107.5 |
| 2-Methoxyoestradiol-17β | 100.0 | 101.0 | 128.0 |
| Oestradiol-17β methyl ether | 107.0 | 108.0 | 116.0 |
| 6-Oxo-oestradiol-17β | 141.5 | 181.5 | 285.0 |
| 16-Oxo-oestradiol-17β | 105.5 | 105.5 | 119.5 |
| Oestradiol-17α | 109.5 | 114.5 | 125.5 |
| 17α-Ethynyloestradiol | 104.0 | 109.5 | 116.0 |
| Oestriol | 110.5 | 114.5 | 125.0 |
| 6-Oxo-oestriol | 102.0 | 106.0 | 125.0 |
| 16-Epi-oestriol | 113.0 | 121.5 | 130.0 |
| 17-Epi-oestriol | 106.0 | 104.0 | 111.5 |
| Oestetrol | 103.0 | 106.5 | 121.0 |

Table 17. The effect of varying amounts of added oestrogen (50, 100 and 200 pg) on the determination of oestradiol-17β, with a 6-CMO antiserum, in mixtures containing 100 pg of oestradiol-17β.

| STEROID | MASS ADDED | OESTRADIOL-17β EQUIVALENCE | |
|---|---|---|---|
| | | 50pg Oestradiol-17β | 100pg Oestradiol-17β |
| Androstenedione | 10 ng | 55.0 | 106.0 |
| Cholesterol | 1 µg | 56.0 | 109.0 |
| Cortisol | 1 µg | 70.0 | 119.0 |
| Dehydroepiandrosterone | 50 ng | 57.0 | 110.0 |
| 5α-Dihydrotestosterone | 10 ng | 47.0 | 104.5 |
| 19-Nor-5α-dihydrotesto-sterone | 10 ng | 58.0 | 107.5 |
| Ethisterone | 10 ng | 51.5 | 101.0 |
| 19-Norethisterone | 10 ng | 46.5 | 109.0 |
| Progesterone | 50 ng | 52.0 | 109.0 |
| 17-Hydroxyprogesterone | 10 ng | 45.0 | 101.0 |
| Pregnenolone | 10 ng | 47.0 | 107.0 |
| 17-Hydroxypregnenolone | 10 ng | 55.5 | 99.0 |
| Testosterone | 50 ng | 55.0 | 110.0 |
| 19-Nor-testosterone | 10 ng | 59.5 | 105.0 |

Table 18. The effect of various neutral steroids on the determination, with a 17-HS antiserum, of oestradiol-17β in mixtures containing 50 or 100 pg of oestradiol-17β.

| STEROID | MASS ADDED | OESTRADIOL-17β EQUIVALENCE | |
|---|---|---|---|
| | | 50pg Oestradiol-17β | 100pg Oestradiol-17β |
| Androstenedione | 10 ng | 52.0 | 97.0 |
| Cholesterol | 1 µg | 53.0 | 103.0 |
| Cortisol | 1 µg | 105.0 | 172.0 |
| Dehydroepiandrosterone | 50 ng | 55.0 | 98.0 |
| 5α-Dihydrotestosterone | 10 ng | 57.0 | 101.0 |
| 19-Nor-5α-dihydrotesto-sterone | 10 ng | 60.0 | 103.0 |
| Ethisterone | 10 ng | 47.0 | 91.0 |
| 19-Norethisterone | 10 ng | 55.0 | 90.0 |
| Progesterone | 50 ng | 55.0 | 112.5 |
| 17-Hydroxyprogesterone | 10 ng | 50.5 | 91.0 |
| Pregnenolone | 10 ng | 52.0 | 96.0 |
| 17-Hydroxypregnenolone | 10 ng | 53.0 | 102.0 |
| Testosterone | 50 ng | 62.0 | 107.0 |
| 19-Nor-testosterone | 10 ng | 70.0 | 110.0 |

Table 19. The effect of various neutral steroids on the determination, with a 6-CMO antiserum, of oestradiol-17β in mixtures containing 50 or 100 pg of oestradiol-17β.

all had cross reactions greater than 1.0 % with the 17-HS antiserum whereas, with the 6-CMO antiserum only three of the oestrogens tested, oestrone, 6-oxo-oestradiol-17β and 16-epi-oestriol had a CR % > 1.0. A CR % of 38.1 for oestrone with the 17-HS antiserum means that oestradiol-17β must be isolated by column chromatography prior to assay with this antiserum. Use was made of this lack of specificity, the antiserum being used to set up standard curves for oestrone as well as oestradiol-17β so that both oestrogens, after isolation by column chromatography on Sephadex LH 20, could be assayed in the same plasma sample (method i).

Wright et al (1973a) have examined the behaviour of a number of oestrogens on Sephadex LH 20. They observed that the elution curves with benzene:methanol (9:1 v/v) for 2-methoxyoestrone and 2-methoxyoestradiol-17β overlap that for oestrone and, similarly, those for 2-hydroxyoestrone, 16-oxo-oestrone, 16α- and 16β-hydroxyoestrone and 16-oxo-oestradiol-17β overlap that for oestradiol-17β. Unfortunately, not all of these oestrogens were available for cross-reaction studies but, from those that were, it was concluded that there was very little, if any, over-estimation of either oestrone or oestradiol-17β due to contamination with other oestrogens after they had been isolated on Sephadex LH 20.

The oestrogen showing the largest degree of cross reaction with the 6-CMO antiserum (86.2 %) was 6-oxo-oestradiol-17β as expected. A CR % of 9.1 with 16-epi-oestriol was more difficult to understand, however, Kuss et al (1973) also observed a measurable CR % (3.6) with this oestrogen when examining an antiserum raised against the same antigen. A CR % of only 1.4 for oestrone indicates that this antiserum is of sufficient specificity to allow oestradiol-17β to be assayed in an ether extract of non-pregnancy plasma without purification. However, if either antiserum is used for the estimation of oestradiol-17β in pregnancy plasma a separation step must be included, as is borne out by preliminary studies which are recorded in Table 20.

It can be seen from Table 13 that neither antisera cross-reacts to a significant extent with the neutral steroids. However, although the CR % of cortisol is $<0.0005$ and 0.005 with the 17-HS and 6-CMO antisera respectively, at a level of 1 µg it results in a significant over-estimate of oestradiol-17β, especially with the 6-CMO antiserum. Thus, although cortisol has virtually no detectable cross-reaction with these two antisera, its presence in the final extract could cause a serious over-estimate of oestradiol-17β

| SAMPLE | Ng OESTRADIOL-17β/ml PLASMA | | |
|---|---|---|---|
| | METHOD i | METHOD ii | METHOD iii |
| 1 | 7.74 | 17.15 | 12.52 |
| 2 | 12.79 | 33.27 | 27.63 |
| 3 | 15.64 | 31.93 | 28.76 |
| 4 | 16.21 | 27.33 | 25.92 |
| 5 | 22.82 | 40.57 | 32.24 |
| 6 | 17.38 | 32.15 | 25.55 |
| 7 | 18.75 | 35.76 | 30.21 |
| 8 | 26.00 | 29.33 | 27.35 |
| 9 | 23.51 | 44.54 | 39.67 |
| 10 | 7.61 | 34.07 | 15.04 |
| 11 | 1.18 | 7.61 | 5.54 |
| 12 | 0.51 | 5.73 | 5.23 |
| 13 | 1.98 | 28.24 | 12.25 |
| 14 | 12.04 | 50.71 | 26.26 |
| 15 | 1.92 | 13.81 | 7.63 |
| 16 | 17.30 | - | 34.36 |
| 17 | 3.41 | 18.66 | 9.92 |
| 18 | 3.30 | 19.14 | 10.68 |

Table 20. The levels of 'oestradiol-17β' in pregnancy plasma as determined by three methods.

especially in cases of increased cortisol production such as pregnancy (Martin and Martin, 1968), Cushing's syndrome (Murphy, 1968b) and advanced breast cancer (Jensen et al, 1968). Another source of interference in assays involving the 6-CMO antiserum could be 19-nor-testosterone if it were present in human plasma and in sufficient concentration.

In conclusion, it should be noted that because a steroid has a low CR % with a particular antiserum, it does not necessarily follow that this steroid will not interfere in an assay. Relative plasma concentrations in normal and pathological conditions together with the possible effects of pharmacological preparations must be taken into consideration when setting up an assay system.

# CHAPTER .5

# Chapter 5

# APPLICATION AND DISCUSSION

## A. INTRODUCTION

Normal ranges have been established for each of the three methods described and evaluated and compared with those of other authors. Studies have been made of the variations of oestradiol-17β, oestrone and 'oestrogen' throughout the normal menstrual cycle and during a 24 hr period. Limited application has been made to dynamic tests and pathological conditions. The relative merits of the three assays and their clinical application are discussed.

## B. NORMAL RANGES

A number of methods have been published for the determination of unconjugated oestrone and oestradiol-17β in human peripheral venous plasma or serum, several of which are reported in Table 21. Most of these methods have been applied to small and differing sample groups making comparisons difficult.

Methods i, ii and iii have been applied to the determination of oestrogens in the peripheral venous plasma of groups of healthy men and women and the results are

Table 21

| AUTHOR | METHOD | PLASMA VOLUME (ml) | SAMPLE | NUMBER | Pg/ml PLASMA (MEAN ± S.D. or RANGE) OESTRONE | OESTRADIOL-17β |
|---|---|---|---|---|---|---|
| Ichii et al (1963) | Fl | - | f | 9 | - | 320 |
| | | - | f:MC | 3 | - | 260 |
| | | - | m | 5 | - | 30 |
| Svendsen & Sørensen (1964) | DID | 10.0 | f:D1-10 | 18 | 193 ± 223 | 254 ± 166 |
| | | | 11-18 | 16 | 361 ± 264 | 260 ± 211 |
| | | | 19-26 | 2 | 225 ± 162 | 87 ± 59 |
| Abraham (1969) | RIA | 1.0 | f:F | 12 | - | 96 ± 42 |
| | | | L | 16 | - | 224 ± 29 |
| | | | m:P | 12 | - | 48 ± 14 |
| Baird & Guevara (1969) | DID | 20.0 | f:F | 9 | 60 ± 18 | 55 ± 21 |
| | | | MC | 13 | 170 ± 47 | 318 ± 98 |
| | | | L | 6 | 121 ± 27 | 191 ± 49 |
| | | | C | 6 | 116 ± 110 | 36 ± 32 |
| | | | PM | 6 | 71 ± 66 | 13 ± 2 |
| | | | m | 7 | 86 ± 93 | 26 ± 16 |
| Korenman et al (1969) | CPB | 3-5 | f:D1-10 | 15 | - | 66 ± 14 |
| | | | 11-20 | 12 | - | 124 ± 50 |
| | | | 21-30 | 6 | - | 137 ± 36 |
| | | | PM | 7 | - | 15 ± 8 |
| | | | m | 6 | - | 24 ± 7 |

Table 21 (cont'd)

| | | | | | | |
|---|---|---|---|---|---|---|
| Shutt (1969) | CPB | 4.0 | f:D2-10 | 5 | - | 180 |
| | | | 14-26 | 4 | - | 470 |
| Corker & Exley (1970) | CPB | 1.0 | f:F | 56 | - | 63 (26-93) |
| | | | MC | 6 | - | 332 (166-432) |
| | | | L | 59 | - | 143 (0-390) |
| Dufau et al (1970) | CPB | 4 or 8 | f:F | 12 | - | 10 - 100 |
| | | | MC | 12 | - | 350 ± 145 |
| | | | L | 12 | - | 190 ± 340 |
| | | | PM | 7 | - | 25 ± 11 |
| | | | m | 8 | - | 24 ± 20 |
| Mayes & Nugent (1970) | CPB | Estimated to contain 0.5-1.5ng | f:F | 10 | - | 62 ± 30 |
| | | | L | 10 | - | 220 ± 100 |
| | | | m | 15 | - | 30 ± 9 |
| Mikhail et al (1970) | RIA | 1-5 | f:MC | 8 | - | 338 ± 36 |
| | | | P | 7 | - | 100 ± 15 |
| | | | m | 6 | - | 74 ± 6 |
| Wu et al (1971) | RIA | 2.0 | f:D3 | 4 | 53 ± 4 | 21 ± 3 |
| | | | 27 | 4 | 79 ± 4 | 68 ± 5 |
| | | | H | 4 | 22 ± 3 | 5 ± 4 |
| | | | m | 4 | 41 ± 2 | 18 ± 2 |
| de Hertogh (1973) | RIA | 1.0 | one menstrual cycle | | 20 - 250 | 30 - 350 |
| Doerr (1973) | RIA | 5.0 | m | 51 | - | 18 ± 5 |

Table 21 (cont'd)

| | | | | | | |
|---|---|---|---|---|---|---|
| Dupon et al (1973) | RIA | 4.0 | f:F | - | - | 37 ± 10 |
| | | | MCP | 5 | 92 ± 30 | 249 ± 141 |
| | | | m:P | 8 | 27 ± 1 | 26 ± 2 |
| Korenman & Sherman (1973) | CPB | 3-5 | f:MCP | 13 | - | 380 |
| | | | L | - | - | 130 |
| Shaaban & Klopper (1973) | RIA | 0.5 | f:d-7 | 8 | - | 60 ± 51 |
| | | | 0 | 8 | - | 215 ± 67 |
| | | | +7 | 8 | - | 113 ± 72 |
| Shutt & Cox (1973) | CPB | 2.5-5.0 | f:F | 6 | - | 58 ± 16 |
| | | | L | 6 | 102 ± 57 | 128 ± 60 |
| Wright et al (1973b) | RIA | 1-3 | f:F | 11 | 102 ± 27 | 70 ± 41 |
| | | | L | 8 | 302 ± 67 | 167 ± 46 |
| | | | m | 7 | 51 ± 18 | 44 ± 30 |
| Korenman et al (1974) | RIA | 1-3 (serum) | f:PM | 12 | - | 11 (4-22) |
| | | | m | 10 | - | 21 (10-33) |
| Kushinsky & Anderson (1974) | RIA | 1.0 (serum) | f | 6 | 109 ± 61 | 126 ± 63 |
| | | | m | 6 | 32 ± 9 | 24 ± 11 |

Table 21. The levels of unconjugated oestrone and oestradiol-17β in normal men and non-pregnant women as determined by several authors.

Fl= fluorescence; DID= double isotope dilution; CPB= competitive protein binding; RIA= radioimmunoassay

Table 21. (legend cont'd)

f= female; m= male
F= follicular phase; MC= midcycle; MCP= midcycle peak;
L= luteal phase; PM= postmenopausal; C= castrate;
H= hypophysectomized; P= pool
D= day of cycle with reference to menses (D1= first day of menstrual bleeding)
d= day of cycle with reference to LH peak (d0= day of LH peak; - indicates before and + after LH peak).

presented in Tables 22, 23, 24 and 25. Again the sample groups are small and as the only reference point available for the female subjects was the first day of menses, the samples have been grouped with reference to this. The outcome of this is that the characteristic midcycle and luteal peak values of oestradiol-17β are masked by the remainder of their particular group (D11-18 and D19-30 respectively). It is also probable that few midcycle oestrogen peaks are included in the group D11-18 as random samples were used. Serial determinations are reported in Section C below which includes two complete menstrual cycles where, in addition to oestradiol-17β, LH, progesterone, 17-hydroxyprogesterone and 20α-dihydroprogesterone were determined.

The results obtained by methods i, ii and iii have been compared and, applying Student's t test, statistically significant differences were found for male plasma assayed by methods ii and iii ($p < 0.001$) and for female plasma D19-30 assayed by methods i and ii and i and iii ($p < 0.001$ and 0.05-0.02 respectively).

Comparison of the results obtained using method i with those reported in Table 21 is complicated in the case of female plasma samples by the small size and variety of

| | NUMBER OF SAMPLES | Pg/ml PLASMA | |
|---|---|---|---|
| | | MEAN ± S.D. | RANGE |
| Healthy men | 11 | 48 ± 15 | 29 - 75 |
| Healthy women | | | |
| days 1-10 | 18 | 117 ± 50 | 45 - 210 |
| days 11-18 | 14 | 162 ± 46 | 75 - 240 |
| days 19-30 | 10 | 156 ± 35 | 96 - 209 |

Table 22. The mean and range of values for oestrone (method i) in groups of healthy men and women.
(Day 1 = first day of menses)

| | NUMBER OF SAMPLES | Pg/ml PLASMA | |
|---|---|---|---|
| | | MEAN ± S.D. | RANGE |
| Healthy men | 12 | 28 ± 9 | 17 - 41 |
| Healthy women | | | |
| days 1-10 | 17 | 64 ± 52 | 18 - 214 |
| days 11-18 | 12 | 151 ± 76 | 53 - 264 |
| days 19-30 | 10 | 106 ± 51 | 26 - 180 |

Table 23. The mean and range of values for oestradiol-17β (method i) in groups of healthy men and women.
(Day 1 = first day of menses)

| | NUMBER OF SAMPLES | Pg/ml PLASMA | |
|---|---|---|---|
| | | MEAN ± S.D. | RANGE |
| Healthy men | 13 | 26 ± 12 | 15 - 50 |
| Healthy women | | | |
| days 1-10 | 14 | 82 ± 36 | 23 - 154 |
| days 11-18 | 13 | 197 ± 84 | 68 - 420 |
| days 19-30 | 8 | 216 ± 66 | 131 - 332 |
| Postmenopausal | 8 | 32 ± 20 | 9 - 66 |

Table 24. The mean and range of values for 'oestrogen' (method ii) in groups of healthy men and women.
(Day 1 = first day of menses)

| | NUMBER OF SAMPLES | Pg/ml PLASMA | |
|---|---|---|---|
| | | MEAN ± S.D. | RANGE |
| Healthy men | 39 | 33 ± 8 | 13 - 46 |
| Healthy women | | | |
| days 1-10 | 28 | 75 ± 36 | 20 - 172 |
| days 11-18 | 11 | 172 ± 77 | 76 - 340 |
| days 19-30 | 16 | 158 ± 65 | 41 - 242 |

Table 25. The mean and range of values for oestradiol-17β (method iii) in groups of healthy men and women.
(Day 1 = first day of menses)

sample populations which have been used and the lack of adequate information for the application of statistical analysis. In general, D1-10 has been compared with follicular phase plasmas, D11-18 with midcycle plasmas and D19-30 with luteal phase plasmas. Obviously this is not the best approach as borderline cases will tend to be omitted from studies of follicular, midcycle and luteal phase plasmas, while they will be included in the somewhat arbitrary grouping of samples with respect to the first day of menses.

Significant differences can be shown between the levels of oestrone found in female plasma (Table 22) on D1-10 with that found by Svendsen and Sørensen (1964) and on D19-30 with that found by Baird and Guevara (1969), Shutt and Cox (1973) and Wright et al (1973b), the respective p values being $< 0.01$, $0.05$, $< 0.05$ and $\leq 0.001$. With respect to oestradiol-17β significant differences can be shown between the results recorded in Table 23 and those reported by Svendsen and Sørensen (1964) for D1-10 ($p < 0.001$), Baird and Guevara (1969), Mikhail et al (1970) and Dufau et al (1973) for D11-18 ($p < 0.001$) and Abraham (1969), Baird and Guevara (1969), Mayes and Nugent (1970) and Wright et al (1973b) for D19-30 (p values: $< 0.001$, $< 0.01$, $< 0.01$ and $< 0.05$ respectively).

The level of oestrone as determined in male plasma (Table 22) was found to be significantly different from that reported in 1974 by Kushinsky and Anderson ($p < 0.05$) while, of the eight methods compared for difference with respect to oestradiol-17β levels in men, only one proved significant (Mikhail et al, 1970; $p < 0.001$).

## C. THE MENSTRUAL CYCLE IN HEALTHY INDIVIDUALS

The pattern of urinary oestrogen excretion in normally menstruating women had been well established by the end of the 1960's (Brown et al, 1958; Nocke and Breuer, 1963; Longhino et al, 1968). During the first 7 to 10 days of a normal 28-day cycle (day one being defined as the first day of menstrual bleeding) oestrone, oestradiol-17β and oestriol are excreted in small amounts. Values start to rise from about the 7th day and reach a well defined maximum at or near midcycle. This maximum is thought to bear some relationship to the time at which rupture of the follicle occurs. The midcycle peak is followed by a rapid decrease in oestrogen excretion followed by a second rise which takes place in the luteal phase at or about the 21st day of the cycle and is continued until shortly before the onset of menses when a rapid decrease in oestrogen excretion

occurs. The excretion of oestrogens in the urine would be expected to reflect the pattern of oestrogen levels in plasma.

Variations in plasma oestradiol-17β levels during the menstrual cycles of healthy women with regular cycles are shown in Figs. 34 to 38. The levels of oestrone and oestradiol-17β were determined by method i in cycles from two individuals and the findings are recorded in Figs. 34 and 35. Cycle I (Fig. 34) exhibits the expected pattern for oestradiol-17β with two characteristic peaks, one of 240 pg/ml at midcycle and a second of 210 pg/ml during the luteal phase. The variation in plasma oestrone was similar. A completely different pattern for the two oestrogens was observed in cycle II (Fig. 35) where a single oestradiol-17β peak of 240 pg/ml occurred on day 18 (with reference to first day of menses). Unlike cycle I, the ratio of oestradiol-17β to oestrone was consistently less than 1.0 except on day 18. This cycle appears to be anovulatory although absence of an LH surge and a plasma progesterone level of less than 5 ng/ml in the second half of the cycle would be a better index (Abraham et al, 1972). It is possible that many healthy women have an occasional anovulatory cycle.
Fig. 36 depicts changes in the plasma concentration of 'oestrogen' (cycle III) and oestradiol-17β (cycle IV) during

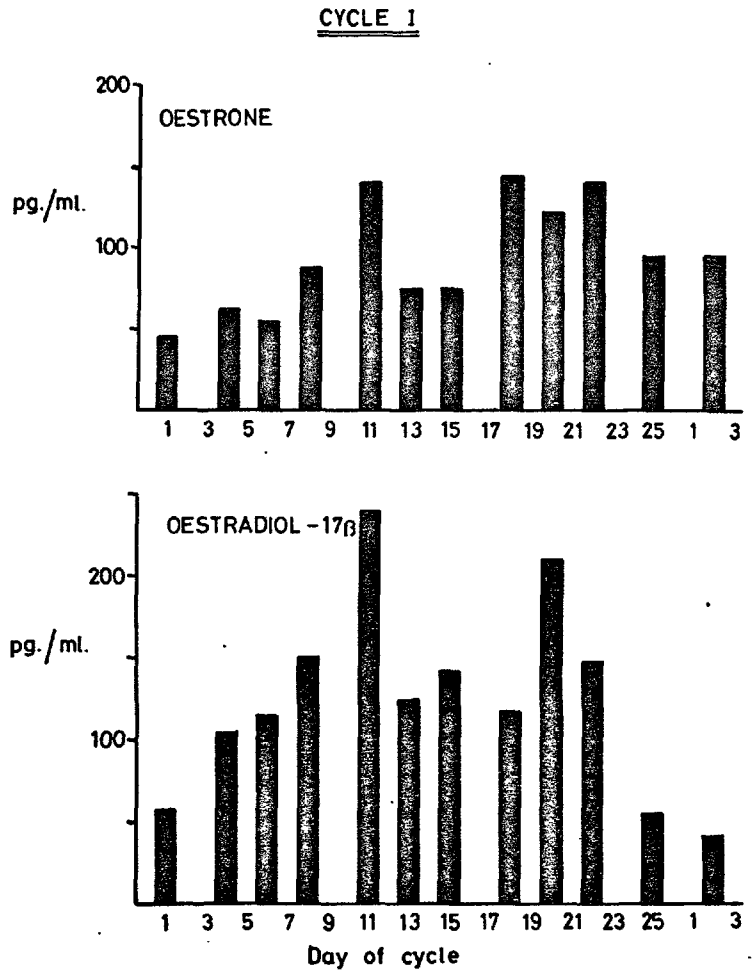


Fig. 34. Serial determinations of plasma oestrone and oestradiol-17β (method i) throughout the menstrual cycle: cycle I.

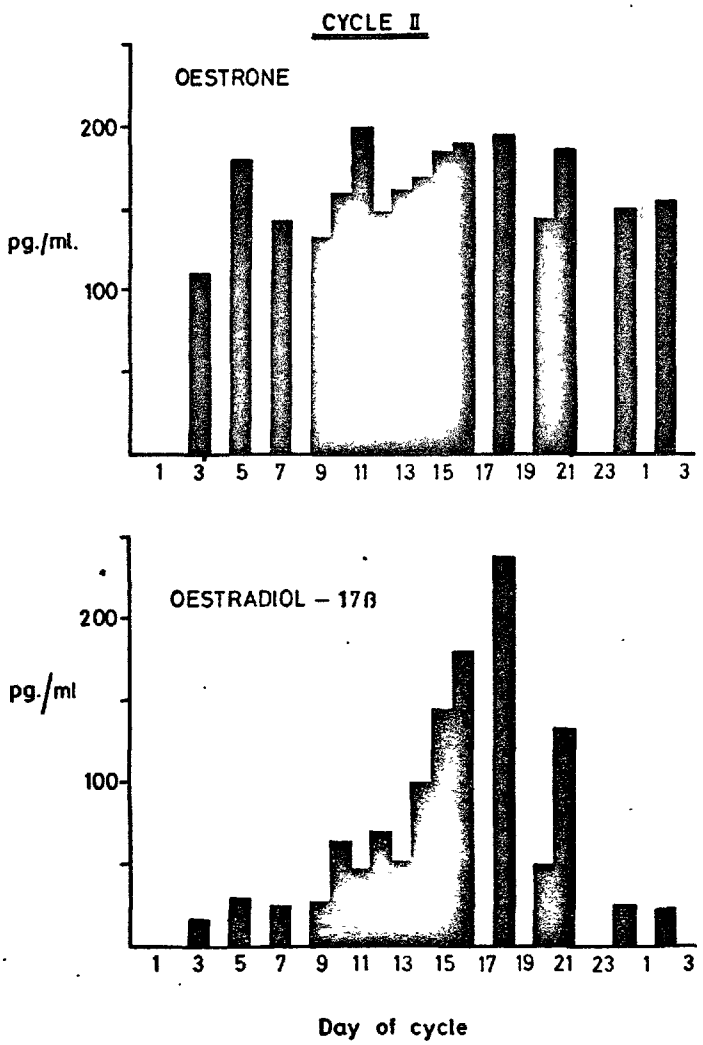


Fig. 35. Serial determinations of plasma oestrone and oestradiol-17β (method i) throughout the menstrual cycle: cycle II.

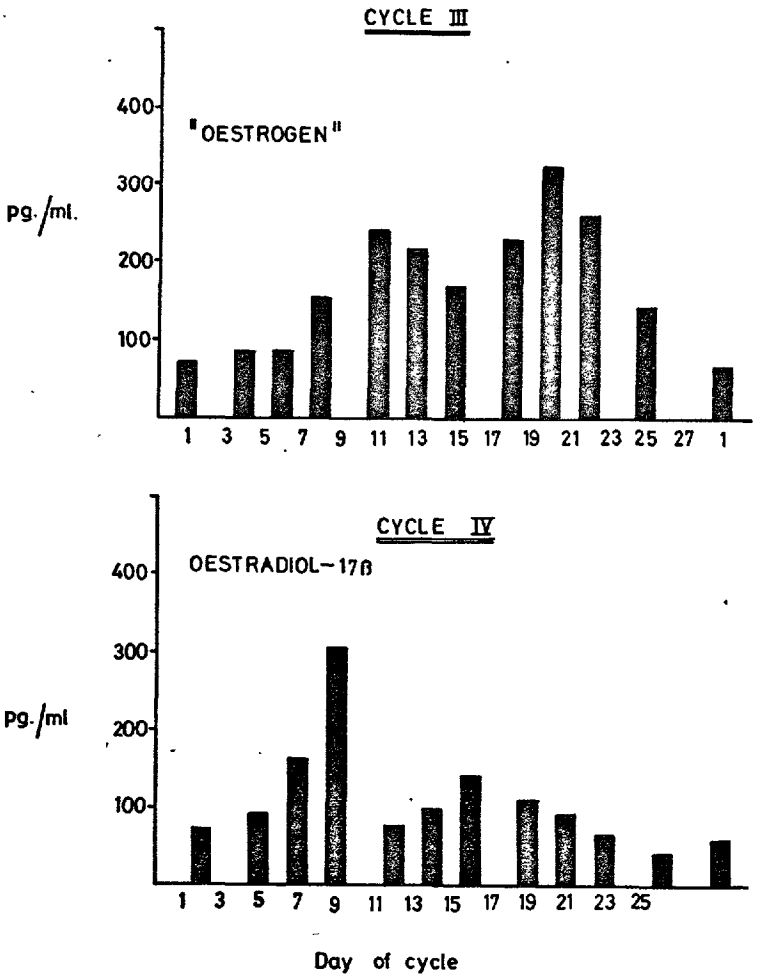


Fig. 36. Serial determinations of plasma 'oestrogen' (method ii) and oestradiol-17β (method iii) throughout the menstrual cycle: cycles III and IV respectively.

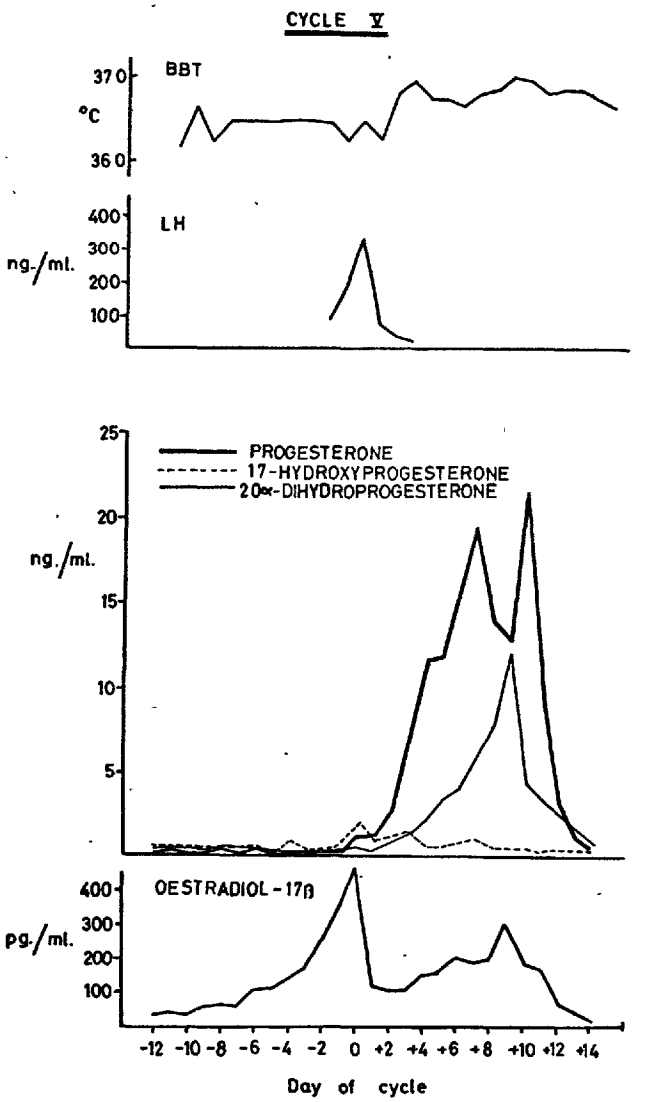


Fig. 37. Daily determinations of oestradiol-17β (method iii), progesterone, 17-hydroxy-progesterone, 20α-dihydroprogesterone and BBT throughout the menstrual cycle: cycle V.

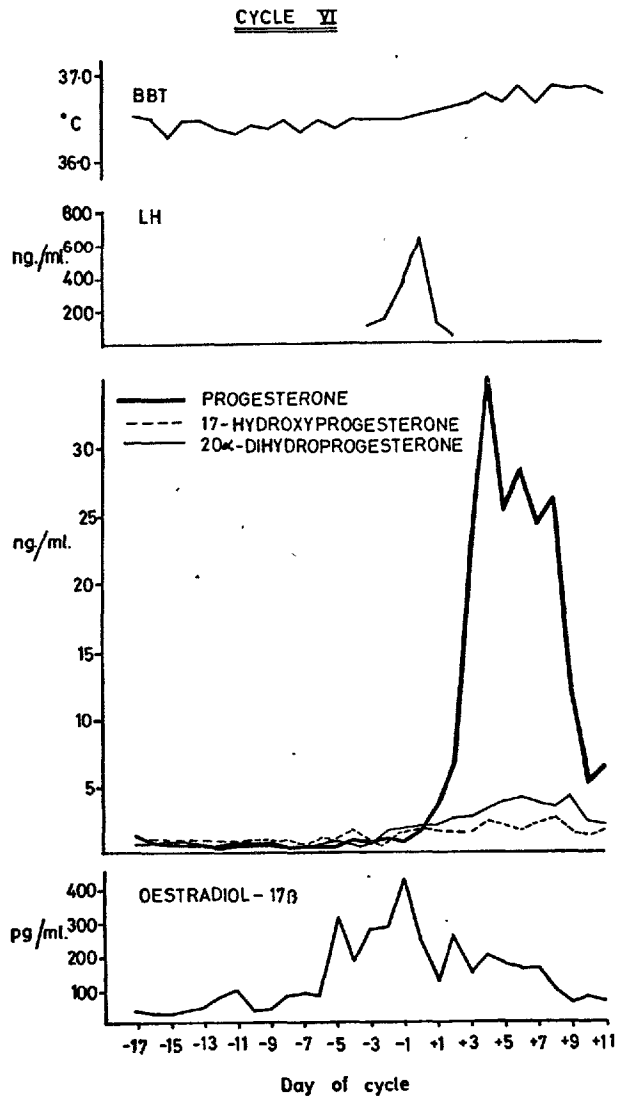


Fig. 38. Daily determinations of oestradiol-17β (method iii), progesterone, 17-hydroxy-progesterone, 20α-dihydroprogesterone and BBT throughout the menstrual cycle: cycle VI.

two further menstrual cycles. Once again biphasic patterns were observed. As daily sampling was not performed in any of these four cycles it is possible that the actual peak levels of oestrogen have been missed.

Daily determination not only of oestradiol-17β (method iii) but also of progesterone*, 17-hydroxyprogesterone and 20α-dihydroprogesterone were performed on cycles V and VI. In addition, basal body temperatures (BBT) and LH determinations around the time of ovulation were available. In cycle V plasma oestradiol-17β exhibited a biphasic pattern with a peak of 464 pg/ml on the same day as the LH peak (day 0). It fell rapidly and was elevated again during the luteal phase of the cycle with a peak of 300 pg/ml 9 days subsequent to the LH peak. The level fell rapidly from day +12 and menstruation occurred 3 days later. The pattern of oestradiol-17β in cycle VI is more complicated. There appear to be two peaks during the follicular phase, on days -5 and -1 with respect to the LH peak. It is probable that the latter is the true midcycle oestradiol-17β peak as this is considered to occur within 48 hr, and more commonly within 24 hr, of the LH peak (Korenman and Sherman, 1973). The level of oestradiol-17β was falling rapidly as the LH surge reached its peak and proceeded to rise again from day +1. By day +9

*I am grateful to Dr. E. Florensa for access, prior to publication, to data concerning the progestogens, plasma LH and BBT in these two cycles.

it had returned to follicular phase levels and menses occurred 3 days later. The plasma progesterone in both of these cycles was well in excess of the 5 ng/ml level 5-8 days after the LH peak considered indicative of an ovulatory cycle by Abraham et al (1972).

As only two cycles were studied in depth (cycles V and VI) caution must be exercised in any deductions made. However, it does seem probable that the progesterone level has begun to rise by the time that LH reaches its peak concentration as observed both by Abraham et al (1972) and Leyendecker et al (1972). It was observed in both cycles that the level of 17-hydroxyprogesterone is only slightly raised during the luteal phase of the cycle whereas that of 20α-dihydroprogesterone is raised to a greater extent. This would be expected on the grounds that 17-hydroxy-progesterone has a greater metabolic potential than 20α-dihydroprogesterone. In cycle V a small peak of 17-hydroxy-progesterone was noted on the day of the LH peak. Leyendecker et al (1972) also observed this in one of the cycles that they were studying. Abraham et al (1972) again observed a small peak of 17-hydroxyprogesterone on day 0 when they plotted the mean of nine menstrual cycles although this relationship did not hold for the individual cycles.

The variation of oestradiol-17β throughout the menstrual cycle has been the subject of or included in a number of research projects, for example, Baird and Guevara, 1969; Dufau et al, 1970; Dupon et al, 1973; Korenman et al, 1974; Shaaban and Klopper, 1973. Abraham et al (1972) and Leyendecker et al (1972) have studied the variation in plasma levels of progesterone, 17-hydroxyprogesterone, LH and FSH as well as oestradiol-17β and discussed the interrelationship of these steroids around the time of ovulation. The results presented are in agreement with the findings of the above mentioned authors.

## D. NYCTEROHEMERAL VARIATION

During recent years, much interest has been focussed on biological variation and rhythms, although the terminology has generally been confused. A circadian rhythm refers to the rhythmic repetition of certain phenomena at about the same time each day while the terms diurnal and nycterohemeral refer to occurrences during the day and during the night and day respectively.

When studying time-related changes in the levels of steroids, it must be realized that the frequency of sampling can have a profound effect upon the information derived.

The most common time interval used is 3-4 hr (Tulchinsky and Korenman, 1970; Korenman and Sherman, 1973; Mansfield et al, in press), although Leymarie et al (1974) sampled every 30 min and West et al (1973) every 20 min. It is also important to establish if the observed variation is indeed significant or could be accounted for by the inaccuracy of the method.

Having established that a time related variation does occur, the next step is to determine whether the variation is rhythmic or not. Most authors have investigated a variable number of subjects for a single 24 hr period. If the overall pattern is similar for each subject, one can infer that a rhythm may occur although, more often, the results are reported in terms of a variation (Tulchinsky and Korenman, 1970; Mansfield et al, in press). More direct evidence would be provided by sampling for more than one 24 hr period which may or may not be consecutive. Leymarie et al (1974) observed an identical variation in plasma testosterone when this steroid was investigated in one individual on two separate occasions, three months apart. Plasma progesterone, oestradiol-17β and LH have been investigated in samples taken every 3-4 hr for 4 days in the rhesus monkey (Spies et al, 1974). The results obtained were suggestive of diurnal rhythms in progesterone and

oestradiol-17β secretion during the luteal, but not the follicular phase of the menstrual cycle.

With regard to oestrogens in non-pregnant women during reproductive life, the problem of investigating nycterohemeral or diurnal variations is rather complicated, as there is considerable variation in the plasma levels of oestrone and oestradiol-17β throughout the menstrual cycle. Thus it is possible that any nycterohemeral variation would be masked by the day to day variation. Hence, any investigation of nycterohemeral or diurnal variation is best performed during the early follicular or mid-luteal phases.

Variation of the levels of plasma oestrone and oestradiol-17β, as reflected in 4-hourly samples taken for 24 hr, was investigated in two individuals and the results are recorded in Fig. 39. Inconsistent variations were observed and there was no obvious relationship between the two oestrogens. The fluctuations observed in plasma oestrone in subject I were considerably greater than oestradiol-17β in the same individual and both oestrogens in subject II. Fig. 40 shows the results of similar investigations with respect to oestradiol-17β in two more volunteers. Again no consistent pattern emerged although the levels varied considerably. The pattern observed was independent of the method used (subject III) giving support

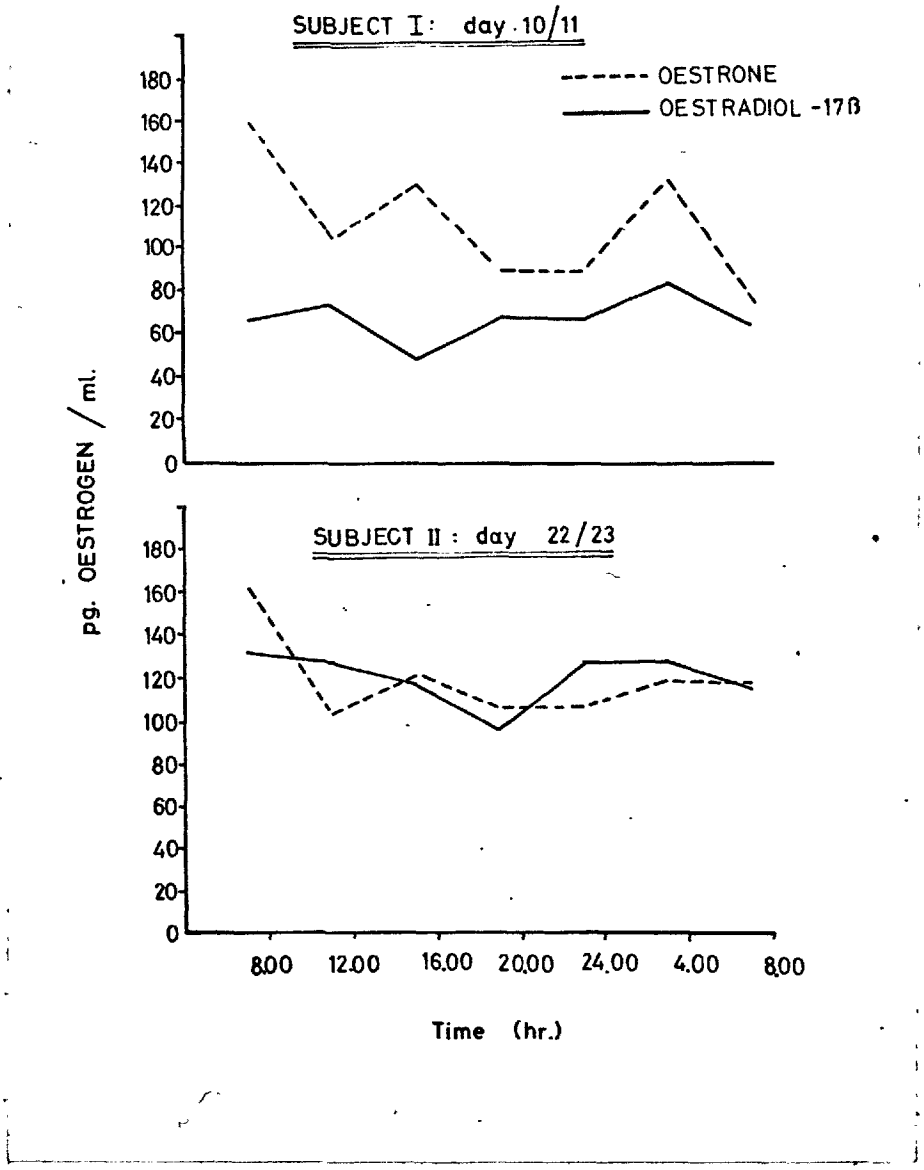


Fig. 39. Nycterohemeral variation. Plasma levels of oestrone and oestradiol-17β as determined in 4-hourly samples: subjects I and II.

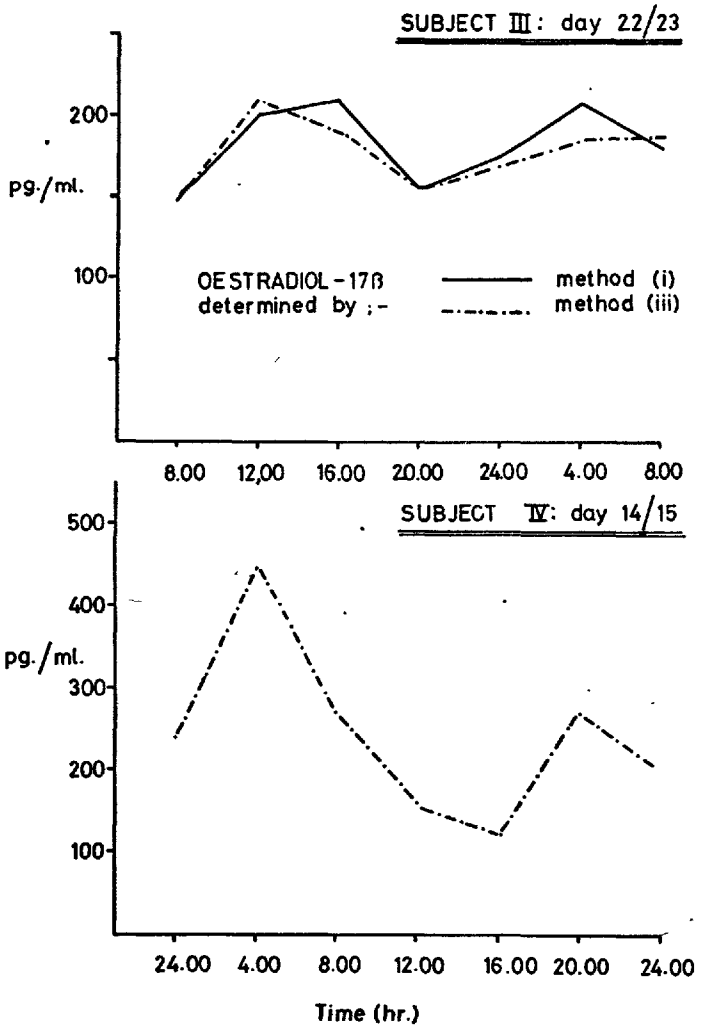


Fig. 40. Nycterohemeral variation. Plasma levels of oestradiol-17β as determined in 4-hourly samples: subjects III and IV.

for the specificity of the 6-CMO antiserum and validity of method iii. Subject IV is interesting in that it is probable that we are observing the changes in oestradiol-17β concentration around the time of its midcycle peak and this would completely mask any nycterohemeral variation or rhythm.

A lack of conformity among volunteers does not preclude there being a rhythm specific to the individual. To investigate this possibility two volunteers were venipunctured at 4-hourly intervals for periods of 48 and 60 hr during the course of a menstrual cycle study (see Section C above, subjects V and VI). Progesterone, 17-hydroxyprogesterone and 20α-dihydroprogesterone were measured in addition to oestradiol-17β. In subject V (Fig. 41), there is a tendency for oestradiol-17β to be high at 8 am and low during the evening. Any pattern for the progestogens is masked by the day to day changes associated with this part of the menstrual cycle (midcycle). In subject VI (Fig. 42), the oestradiol-17β variation is masked by the transitory rise on day -5 before the true midcycle peak on day -1. The variation in plasma progestogens is very interesting in this subject, 17-hydroxyprogesterone showing the largest variation with maximal levels at 8 am and minimal levels around midnight. Progesterone levels had a peak approximately 4 hr later

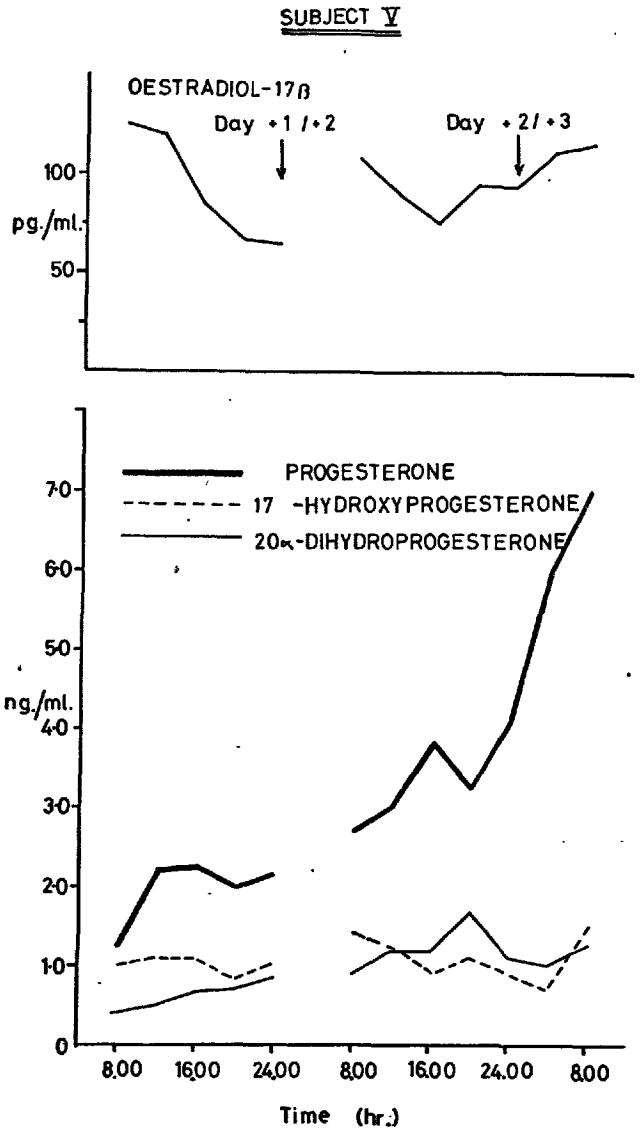


Fig. 41. Nycterohemeral variation. Plasma levels of oestradiol-17β (method iii), progesterone, 17-hydroxyprogesterone and 20α-dihydroprogesterone as determined in 4-hourly samples: subject V.

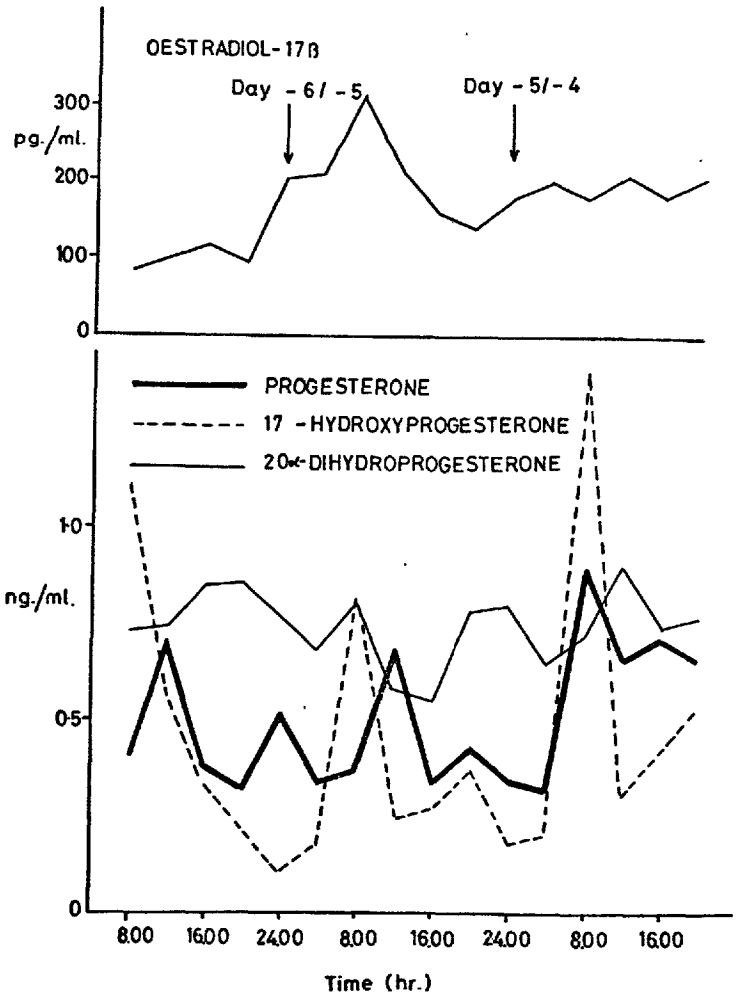


Fig. 42. Nycterohemeral variation. Plasma levels of oestradiol-17β (method iii), progesterone, 17-hydroxyprogesterone and 20α-dihydroprogesterone as determined in 4-hourly samples: subject VI.

while 20α-dihydroprogesterone had a tendency to mimic progesterone but being out of phase by approximately 8 hr. This was the only case where any type of rhythmic variation could be inferred.

Very little in the way of conclusion, apart from the observation that plasma oestrone and oestradiol-17β do vary throughout a 24 hr period and that the latter has a tendency to be high between 4 and 8 am, can be drawn from the above investigations which are too few for statistical analysis. What does emerge, however, is that the day to day changes in the levels of ovarian hormones during the menstrual cycle make any investigation of nycterohemeral variation extremely difficult. This may account for the lack of published data.

Baird and Guevara (1969) did attempt to investigate the diurnal variation of plasma oestrone and oestradiol-17β in five women and six men. Blood was drawn in the morning between 8 and 9 am and again in the evening between 4.30 and 5.30 pm. There were no significant differences in the five women. Five of the six men had lower levels of oestrone in the evening than the morning, and again five of the six men had no significant change in oestradiol-17β. The variation in plasma oestrone levels were investigated in four women and one man by Tulchinsky and Korenman (1970). They observed

that the lowest levels occurred between midnight and 4 am, there being a sharp rise to peak levels at 8-9 am. Korenman and Sherman (1973) investigated the variation in plasma oestrone and oestradiol-17β around the time of ovulation. They found large fluctuations in both oestrogens during the preovulatory phase but this degree of variation did not characterize the period following the LH peak.

## E. APPLICATION TO CLINICAL CONDITIONS AND DYNAMIC STUDIES

The levels of oestrone and oestradiol-17β have been determined in peripheral venous plasma from patients with a variety of clinical conditions. The findings are recorded in Table 26. A number of the patients studied were children and normal ranges for this population have not been established for the methods described. However, on the basis of the study by Angsusingha et al (1974) levels significantly greater than 10 pg/ml would not be expected in prepubertal males or females. What is obvious is that whenever feminization occurs in a male, or precocious puberty or premature thelarche in a female, the oestrogen levels are raised to adult female levels and in the case of patient 19 even to pregnancy levels.

Six patients with gynaecomastia were investigated and their oestradiol-17β levels were either at the high end of the

| PATIENT | | | DIAGNOSIS | Pg OESTROGEN/ml PLASMA | |
|---|---|---|---|---|---|
| Code No | Sex | Age (yr) | | Oestrone | Oestradiol -17β |
| 1 | M | 8 | Adrenal Tumour:?recurrence | <10 | 28 |
| | | | | <10 | <10 |
| | | | | <10 | 13 |
| 2 | M | 7 | Adrenal Tumour: feminizing | 201 | 303 |
| 3 | F | - | Adrenogenital syndrome | 287 | 287 |
| 4 | F | - | Breast cancer | 37 | 138 |
| 5 | F | - | Endocarcinoma of the cervix | - | 210 |
| 6 | M | - | Gynaecomastia | - | 51 |
| 7 | M | - | " | - | 54 |
| 8 | M | - | " | - | 39 |
| 9 | M | - | " | - | 44 |
| 10 | M | - | " | - | 37 |
| 11 | M | - | " | 166 | 89 |
| 12 | M | - | " | 68 | 117 |
| 13 | - | 12 | Hermaphroditism | - | 22 |
| 14 | - | 0.67 | Intersex | 25 | 146 |
| 15 | - | 13 | Intersex: mosaic | <10 | <10 |
| 16 | M | | Klinefelter syndrome and gynaecomastia | 131 | 101 |
| 17 | F | 1.5 | Precocious puberty | 71 | 408 |
| 18 | F | - | " " | 49 | 73 |
| 19 | F | 8 | Precocious puberty and mongolism | 281 | 1765 |
| | | 9 | | 1244 | 4122 |
| 20 | F | 2 | Premature thelarche | 25 | 184 |
| 21 | M | 9 | Sertoli cell tumour | <10 | 28 |
| 22 | F | - | Subfertility: $1^{o}$amenorrhoea | - | 103 |
| 23 | F | 29 | $2^{o}$amenorrhoea | <10 | <10 |
| 24 | F | 26 | " " | - | <10 |
| 25 | F | 31 | " " | <10 | 11 |
| 26 | F | - | " " | 84 | 129 |

Table 26. The levels of oestrone and oestradiol-17β as determined in a number of clinical conditions.

normal range (patients 6, 7, 8 and 9) or grossly elevated (patients 11 and 12). The first group tie in with the four patients investigated by Shuster and Brown (1962) where the urinary oestrogens were only slightly above the normal range. In a single case of Klinefelter's syndrome with gynaecomastia both oestrone and oestradiol-17β were grossly raised.

Only one of the four patients investigated for secondary amenorrhoea had plasma levels of oestrone or oestradiol-17β in the normal range while the one case of primary amenorrhoea had a normal level of oestradiol-17β and therefore, presumably, normally functioning ovaries.

The plasma levels of oestrone and oestradiol-17β have been determined in two subjects suffering from dysfunctional uterine bleeding on several days throughout a menstrual cycle. The results are recorded in Figs. 43 and 44. Both subjects had a normal biphasic oestradiol-17β pattern but the oestrone level was higher than oestradiol-17β in most of the samples examined. Baird (1969) observed significant changes in the oestrone:oestradiol-17β ratio throughout the normal menstrual cycle and it may be that dysfunctional uterine bleeding is characterized by an alteration of this ratio.

The radioimmunoassay of plasma oestrogens can be used not only as a diagnostic tool but also as a monitor of

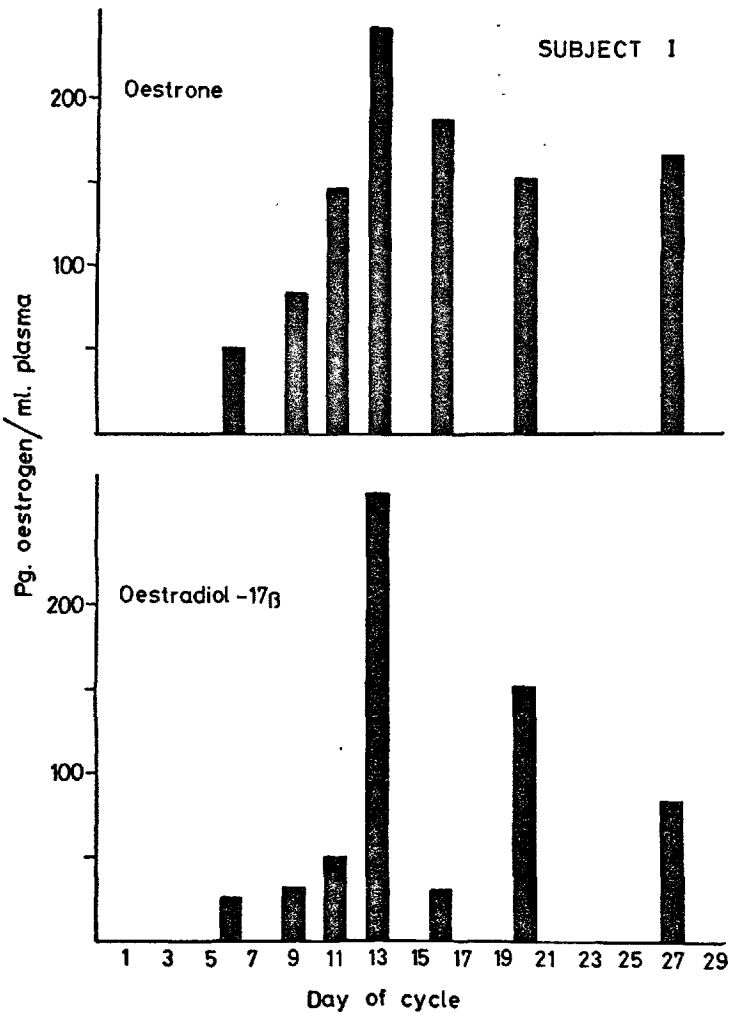


Fig. 43. Serial determinations of plasma oestrone and oestradiol-17β throughout the menstrual cycle of a patient with dysfunctional uterine bleeding: subject I.

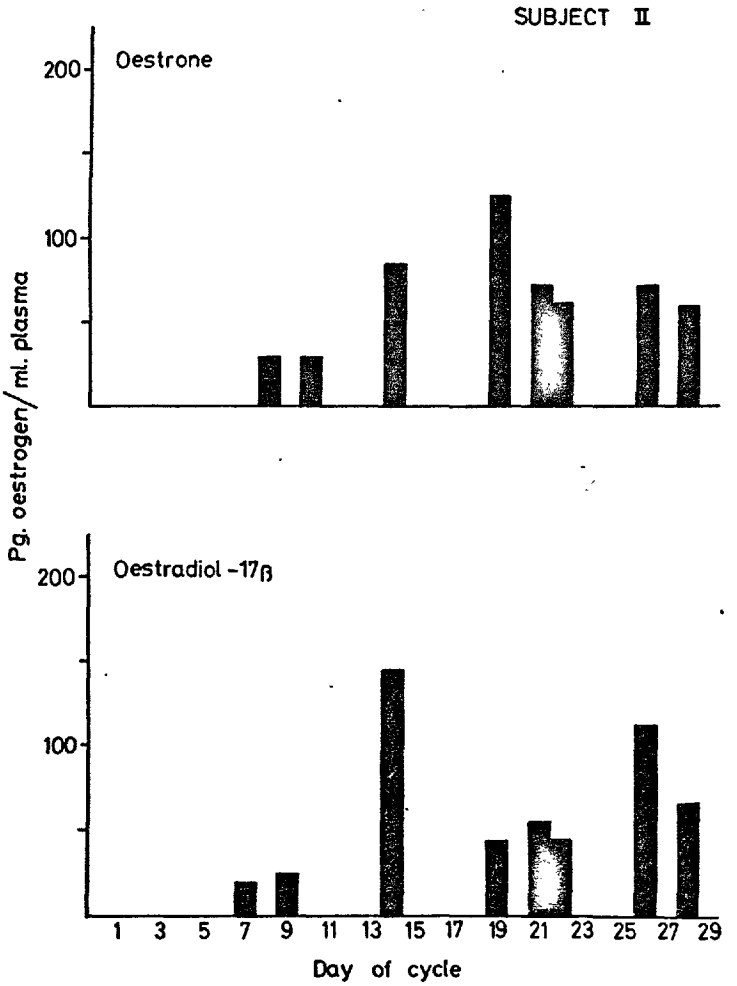


Fig. 44. Serial determinations of plasma oestrone and oestradiol-17β throughout the menstrual cycle of a patient with dysfunctional uterine bleeding: subject II.

treatment and it is probable that this is where it will prove most useful.

One patient suffering from gynaecomastia was treated with human chorionic gonadotropin (HCG) for 3 days followed by fluoxymesterone for a further 3 days. His oestradiol-17β level rose from a basal level of 54 pg/ml to 165 and 105 pg/ml on days 1 and 3 post HCG respectively, but had fallen to 32 pg/ml by the third day of fluoxymesterone treatment.

An extremely important application of the assay of plasma oestrogens is the monitoring of subfertile patients receiving gonadotropic preparations such as Pergonal*. A regime has been established whereby the effect on plasma oestradiol-17β of 5 vials of Pergonal (75 units each of LH and FSH per vial) is monitored. If no effect is observed at this level, 10 vials are tried. Once a good response to Pergonal is achieved treatment can begin. The patient is given 5 or 10 vials of Pergonal on alternate days until normal midcycle levels of oestradiol-17β are achieved (200 - 400 pg/ml). Subsequent to this HCG is given and the plasma levels of oestradiol-17β and progesterone are monitored. If pregnancy ensues both these steroid levels

* G.D. Searle & Co. Ltd., High Wycombe, Bucks., England.

continue to rise and menses does not occur. Fig. 45 shows the application of this regime to a patient who did become pregnant.

The radioimmunoassay of plasma oestradiol-17β has also been applied to some preliminary work on the physiological interrelationship between luteinizing hormone releasing factor (LHRF) and oestradiol-17β. Baseline levels of oestradiol-17β and the level at various times after the administration of 25, 50 or 100 μg of LHRF were determined in male and female volunteers. The results are recorded in Tables 27 and 28. There was little effect in the male volunteers except subjects D.C.A. and A.Mc. in whom a small rise in oestradiol-17β occurred approximately 8 hr after administration of 100 μg of LHRF (Table 27). In the case of the female volunteers an effect was more evident. Both subjects had a definite rise in plasma oestradiol-17β which began 3-4 hr after administration of 50 or 100 μg of LHRF and this rise was maintained for at least 4 hr (Table 28).

Newton and Collins (1972) administered synthetic LHRF to six women with oligomenorrhoea and four with secondary amenorrhoea. They found a 2- to 3-fold rise in oestradiol-17β occuring 3-8 hr after the rise in gonadotropins in seven

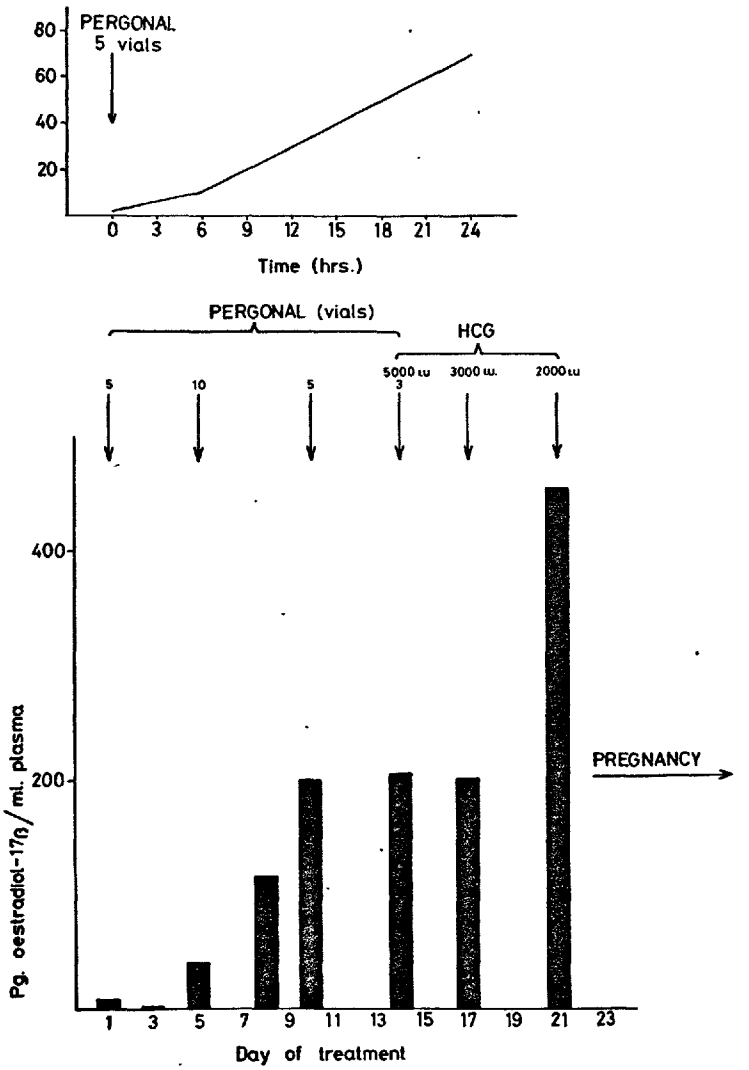


Fig. 45. Application of a regime for the treatment of subfertility with Pergonal.

Table 27.

| SUBJECT | LHRF DOSE | TIME (hr) | OESTRADIOL-17β (pg/ml) | SUBJECT | LHRF DOSE | TIME (hr) | OESTRADIOL-17β (pg/ml) |
|---|---|---|---|---|---|---|---|
| D.C.A. | 25μg | -0.25 | 33 | A.Mc. | 25μg | -0.25 | 31 |
| | | 0 | 42 | | | 0 | 13 |
| | | +2 | 39 | | | +2 | 31 |
| | | +3 | 36 | | | +3 | 34 |
| | | +4 | 35 | | | +4 | 32 |
| | | +8 | 34 | | | +8 | 42 |
| | | +24 | 21 | | | +24 | - |
| | | +32 | 22 | | | +32 | 22 |
| | | +48 | 30 | | | +48 | 16 |
| D.C.A. | 100μg | -48 | 34 | A.Mc. | 100μg | -48 | 37 |
| | | -24 | 36 | | | -24 | 45 |
| | | -0.25 | 46 | | | -0.25 | 56 |
| | | 0 | 44 | | | 0 | 41 |
| | | +1 | 39 | | | +1 | 40 |

Table 27 (cont'd)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | +2 | 43 | | | +2 | 45 |
| | | +3 | 34 | | | +3 | 40 |
| | | +4 | 42 | | | +4 | 37 |
| | | +8 | 63 | | | +8 | 46 |
| | | +24 | 45 | | | +24 | 36 |
| | | +32 | 46 | | | +32 | 43 |
| | | +48 | 42 | | | +48 | 38 |
| L.A. | 25µg | -48 | 21 | J.M. | 50µg | -0.25 | 18 |
| | | -24 | 24 | | | 0 | - |
| | | -0.25 | 31 | | | +2 | 19 |
| | | 0 | 27 | | | +3 | 17 |
| | | +2 | 33 | | | +4 | 10 |
| | | +3 | 28 | | | +8 | 18 |
| | | +4 | 38 | | | +24 | 22 |
| | | +8 | 26 | | | +32 | 20 |

Table 27 (cont'd)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | +24 | 21 | | | +48 | 12 |
| | | +32 | 16 | | | | |
| | | +48 | 10 | | | | |
| R.H. | 25µg | -48 | 28 | J.M. | 100µg | -24 | 17 |
| | | -24 | 16 | | | -0.25 | 26 |
| | | -0.25 | 20 | | | 0 | 33 |
| | | 0 | 26 | | | +1 | 30 |
| | | +2 | 27 | | | +2 | 21 |
| | | +3 | 13 | | | +3 | 30 |
| | | +4 | 14 | | | +4 | 32 |
| | | +8 | 17 | | | +5 | 34 |
| | | +24 | 14 | | | +6 | 36 |
| | | +32 | 27 | | | +7 | 34 |
| | | +48 | 13 | | | +8 | 33 |

Table 27. LHRF study: Male volunteers.

| SUBJECT | LHRF DOSE | TIME (hr) | OESTRADIOL-17β (pg/ml) | SUBJECT | LHRF DOSE | TIME (hr) | OESTRADIOL-17β (pg/ml) |
|---|---|---|---|---|---|---|---|
| MH | 50μg | -48 | 20 | MS | 50μg | -48 | 64 |
| | | -24 | 34 | | | -24 | 87 |
| | | -0.25 | 40 | | | -0.25 | 93 |
| Day 3 | | 0 | 37 | Day 10 | | 0 | 85 |
| | | +2 | 48 | | | +2 | 86 |
| | | +3 | 35 | | | +3 | 108 |
| | | +4 | 62 | | | +4 | 109 |
| | | +8 | 67 | | | +8 | 131 |
| | | +24 | 34 | | | +24 | 105 |
| MH | 100μg | -48 | 55 | MS | 100μg | -48 | 60 |
| | | -24 | 50 | | | -24 | 81 |
| | | -0.25 | 68 | | | -0.25 | 94 |
| Day 3 | | 0 | 69 | Day 10 | | 0 | 88 |
| | | +2 | 86 | | | +2 | 85 |
| | | +3 | 78 | | | +3 | 83 |
| | | +4 | 91 | | | +4 | 105 |
| | | +8 | 115 | | | +8 | 69 |
| | | +24 | 44 | | | +24 | 65 |

Table 28. LHRF study: Female volunteers.
(Day: with reference to menses)

of the patients. Since maximum serum levels of LH and FSH are considered to occur 20 - 30 min and 45 - 60 min after LHRF administration respectively (Nillius and Wide, 1972; Rebar et al, 1973; Hashimoto et al, 1973), the results on the female volunteers quoted above are in good agreement with this work. The variance of the response in serum gonadotropins to LHRF has been examined with respect to individuals (Hashimoto et al, 1973), phase of the menstrual cycle (Nillius and Wide, 1972) and dose (Rebar et al, 1973). One would expect this variation to be reflected in the response of oestradiol-17β but insufficient data is presented to support this contention.

## F. DISCUSSION

Specificity, accuracy and other criteria for the three methods described in this thesis have been discussed in the appropriate sections. As with other indices of hormone production there is no single method which can be recommended for all purposes; the choice of method depends upon the type of information sought and the urgency with which the result is required.

Method i may be the procedure of choice for most research projects in that it affords a reasonably specific and

sensitive method for the separate determination of oestrone and oestradiol-17β. However, depending upon access to the liquid scintillation counter, the minimum time for completion of this assay is 2.5 days, whereas it may be important in clinical practice to obtain the result as quickly as possible for day-to-day monitoring. This has already been illustrated in Section E of this chapter with regard both to dynamic tests and the management of patients in whom ovulation is to be induced by the administration of gonadotropins. A knowledge of the plasma concentration of oestrogen immediately before ovulation is of great value in selecting the time of administration of HCG and in selecting the dosage. If the HCG is administered prematurely the developing follicle may be luteinized and ovulation may not occur; if the dose is too high there is an increased risk of hyperstimulation of the ovary and of multiple pregnancy. Method ii (for the determination of plasma 'oestrogen') was developed for this type of application and when more specific antisera became available (Chapter 2, Section E) it was possible to proceed to method iii which provides a rapid determination of plasma oestradiol-17β.

A major problem in the application of methods for the determination of plasma steroids in general is the possible

existence of nycterohemeral rhythms or random variations. This problem has been studied and discussed (Chapter 5, Section D) and it would appear that the variations which do occur are less marked than in the case of certain other steroids such as plasma cortisol and testosterone. Furthermore, the success with which method iii has been applied to the management of patients indicates that plasma oestradiol-17β levels constitute a valuable index irrespective of the time of sampling. However, in view of the limited information on nycterohemeral variations it might be advisable to take the blood samples at approximately the same time each day if this is practicable. It is very unlikely that the result of a random blood sample can be of value either in the diagnosis of rare endocrine disorders or for the purposes outlined above and strong cases can be made for serial determinations. Both with regard to the choice of method and the timing of venipuncture, there must be close cooperation between the clinician and the biochemist and this applies equally to routine clinical application and the design of any research project.

As in the case of other steroids, there is considerable discussion about the relative merits of plasma and urinary

assays. With regard to the oestrogens, it should be emphasized that secreted oestradiol-17β is the precursor of a large number of oestrogens of which approximately 30 are present in the urine of the non-pregnant woman. On the other hand, most current methods for urinary oestrogen assay measure the three classical oestrogens and it is probable that the specific determination of oestradiol-17β in plasma may give different information from that obtained by urinary assay. Thus, in certain conditions - for example, Kleinfelter's syndrome, where urinary oestrogens are in the normal range, it is possible that future application of a plasma oestrogen method may indicate abnormal production of oestradiol-17β or of other oestrogens. Although both plasma and urinary assays can be criticized as being less direct indices of oestrogen production than the calculation of oestrogen secretion rates this latter approach is too time-consuming for clinical application. In special projects where secretion rates are to be calculated, method i could be used to advantage.

It is certain that application of these methods must be carried out on a much larger scale both with regard to reproductive physiology and pathology before their value can be assessed with confidence. At this stage in our

knowledge, it would appear that serial analyses are of value in dynamic tests, whereas, plasma oestrogen assays have not yet been applied on a systematic scale in an attempt to obtain a biochemical classification of menstrual disorders.

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# APPENDICES

# Appendix I

## ABBREVIATIONS

| | |
|---|---|
| BBT | Basal body temperature |
| BSA | Bovine serum albumin |
| cpm | Counts per minute |
| CR % | Percentage cross-reaction |
| C.V. | Coefficient of variation |
| DHA | Dehydroepiandrosterone |
| dpm | Disintegrations per minute |
| GLC | Gas-liquid chromatography |
| HCG | Human chorionic gonadotropin |
| LHRF | Luteinizing hormone releasing factor |
| PBS | Phosphate buffered saline |
| PBS-0.1% gel | Phosphate buffered saline containing 0.1% gelatine |
| PBS-0.5% gel | Phosphate buffered saline containing 0.5% gelatine |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| S.D. | Standard deviation |
| TLC | Thin-layer chromatography |
| 6-CMO antiserum | Antiserum raised against 17β-oestradiol -6-(O-carboxymethyl)oxime-BSA |
| 17-HS antiserum | Antiserum raised against oestradiol-17β-hemisuccinate-BSA |

# Appendix II

## NOMENCLATURE

| Trivial Name | Systematic Name |
|---|---|
| Androstadienone | Androsta-4,16-dien-3-one |
| Androstenedione | Androst-4-ene-3,17-dione |
| 6α-Hydroxyandrostenedione | 6α-Hydroxyandrost-4-ene-3,17-dione |
| 6β-Hydroxyandrostenedione | 6β-Hydroxyandrost-4-ene-3,17-dione |
| 19-Hydroxyandrostenedione | 19-Hydroxyandrost-4-ene-3,17-dione |
| 19-Oxo-androstenedione | Androst-4-ene-3,17,19-trione |
| Androstenetriol | Androst-5-ene-3β,16α,17β-triol |
| Cholesterol | Cholest-5-en-3β-ol |
| 20α-Hydroxycholesterol | Cholest-5-ene-3β,20α-diol |
| 20α,22-Dihydroxycholesterol | Cholest-5-ene-3β,20α,22-triol |
| Cortisol | 11β,17α,21-Trihydroxypregn-4-ene-3,20-dione |
| Dienoestrol | 3,4-Bis(4-hydroxyphenyl)-2,4-hexadiene |
| Diethylstilboestrol | 3,4-Bis(4-hydroxyphenyl-3-hexane) |
| Dehydroepiandrosterone | 3β-Hydroxyandrost-5-en-17-one |
| 7α-Hydroxydehydroepi-androsterone | 3β,7α-Dihydroxyandrost-5-en-17-one |

| | |
|---|---|
| 5α-Dihydrotestosterone | 17β-Hydroxy-5α-androstan-3-one |
| 19-Nor-5α-dihydrotestosterone | 17β-Hydroxy-5α-oestran-3-one |
| Ecdysone | 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one |
| Equilenin | 3-Hydroxyoestra-1,3,5(10),6,8-pentaen-17-one |
| Equilin | 3-Hydroxyoestra-1,3,5(10),7-tetraen-17-one |
| Ethisterone | 17α-Ethynyl-17β-hydroxyandrost-4-en-3-one |
| 19-Norethisterone | 17α-Ethynyl-17β-hydroxyoestra-4-en-3-one |
| Hexoestrol | 3,4-Bis(4-hydroxyphenyl)-hexane |
| Hippulin | 3-Hydroxyoestra-1,3,5(10),8-tetraen-17-one |
| Lanosterol | Lanosta-8,24-dien-3β-ol |
| Oestradiol-17α | Oestra-1,3,5(10)-triene-3,17α-diol |
| Oestradiol-17β | Oestra-1,3,5(10)-triene-3,17β-diol |
| Oestradiol-17β methyl ether | Oestra-1,3,5(10)-triene-3,17β-diol 3-methyl ether |
| 2-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-2,3,17β-triol |
| 2-Methoxyoestradiol-17β | Oestra-1,3,5(10)-triene-2,3,17β-triol 2-methyl ether |
| 4-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-3,4,17β-triol |

| | |
|---|---|
| 6α-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-3,6α, 17β-triol |
| 6β-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-3,6β,17β-triol |
| 6-Oxo-oestradiol-17β | 3,17β-Dihydroxyoestra-1,3,5(10)-trien- 6- one |
| 11-Dehydro-oestradiol-17α | Oestra-1,3,5(10),11-tetraene-3, 17α-diol |
| 11β-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-3,11β, 17β-triol |
| 11-Oxo-oestradiol-17β | 3,17β-Dihydroxyoestra-1,3,5(10)-trien-11-one |
| 15α-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-3,15α, 17β-triol |
| 15β-Hydroxyoestradiol-17β | Oestra-1,3,5(10)-triene-3,15β, 17β-triol |
| 16-Oxo-oestradiol-17β | 3,17β-Dihydroxyoestra-1,3,5(10)-trien-16-one |
| 17α-Methyloestradiol | 17α-Methyloestra-1,3,5(10)-triene-3,17β-diol |
| 17α-Ethynyloestradiol | 17α-Ethynyloestra-1,3,5(10)-triene-3,17β-diol |
| Oestratetraenol | Oestra-1,3,5(10),16-tetraen-3-ol |
| Oestriol | Oestra-1,3,5(10)-triene-3,16α, 17β-triol |
| 2-Hydroxyoestriol | Oestra-1,3,5(10)-triene-2,3,16α, 17β-tetrol |
| 2-Methoxyoestriol | Oestra-1,3,5(10)-triene-2,3,16α, 17β-tetrol 3-methyl ether |
| 6α-Hydroxyoestriol | Oestra-1,3,5(10)-triene-3,6α,16α, 17β-tetrol |
| 6β-Hydroxyoestriol | Oestra-1,3,5(10)-triene-3,6β,16α, 17β-tetrol |

| | |
|---|---|
| 6-Oxo-oestriol | 3,16α,17β-Trihydroxyoestra-1,3,5(10)-trien-6-one |
| 15α-Hydroxyoestriol (Oestetrol) | Oestra-1,3,5(10)-triene-3,15α,16α,17β-tetrol |
| 16-Epi-oestriol | Oestra-1,3,5(10)-triene-3,16β,17β-triol |
| 16,17-Epi-oestriol | Oestra-1,3,5(10)-triene-3,16β,17α-triol |
| 17-Epi-oestriol | Oestra-1,3,5(10)-triene-3,16α,17α-triol |
| Oestrone | 3-Hydroxyoestra-1,3,5(10)-trien-17-one |
| Oestrone methyl ether | 3-Hydroxyoestra-1,3,5(10)-trien-17-one 3-methyl ether |
| 2-Hydroxyoestrone | 2,3-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 2-Methoxyoestrone | 2,3-Dihydroxyoestra-1,3,5(10)-trien-17-one 2-methyl ether |
| 6a-Hydroxyoestrone | 3,6α-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 6β-Hydroxyoestrone | 3,6β-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 6-Oxo-oestrone | 3-Hydroxyoestra-1,3,5(10)-triene-6,17-dione |
| 7α-Hydroxyoestrone | 3,7α-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 7-Oxo-oestrone | 3-Hydroxyoestra-1,3,5(10)-triene-7,17-dione |
| 11β-Hydroxyoestrone | 3,11β-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 11-Oxo-oestrone | 3-Hydroxyoestra-1,3,5(10)-triene-11,17-dione |
| 15α-Hydroxyoestrone | 3,15α-Dihydroxyoestra-1,3,5(10)-trien-17-one |

| | |
|---|---|
| 15β-Hydroxyoestrone | 3,15β-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 16α-Hydroxyoestrone | 3,16α-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 16β-Hydroxyoestrone | 3,16β-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 16-Oxo-oestrone | 3-Hydroxyoestra-1,3,5(10)-triene-16,17-dione |
| 18-Hydroxyoestrone | 3,18-Dihydroxyoestra-1,3,5(10)-trien-17-one |
| 18-Noroestrone | 3-Hydroxygona-1,3,5(10)-trien-17-one |
| Pregnanediol | 5β-Pregnane-3α,20α-diol |
| Pregnenolone | 3β-Hydroxypregn-5-en-20-one |
| 17-Hydroxypregnenolone | 3β,17α-Dihydroxypregn-5-en-20-one |
| Progesterone | Pregn-4-ene-3,20-dione |
| 17-Hydroxyprogesterone | 17-Hydroxypregn-4-ene-3,20-dione |
| 20α-Dihydroprogesterone | 20α-Hydroxypregn-4-en-3-one |
| Testosterone | 17β-Hydroxyandrost-4-en-3-one |
| 16α-Hydroxytestosterone | 16α,17β-Dihydroxyandrost-4-en-3-one |
| 19-Carboxytestosterone | 17β-Hydroxyoestra-4-en-3-one-10-carboxylic acid |
| 19-Hydroxytestosterone | 17β,19-Dihydroxyandrost-4-en-3-one |
| 19-Oxo-testosterone | 17β-Hydroxyandrost-4-ene-3,19-dione |
| 19-Nor-testosterone | 17β-Hydroxyoestra-4-en-3-one |

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