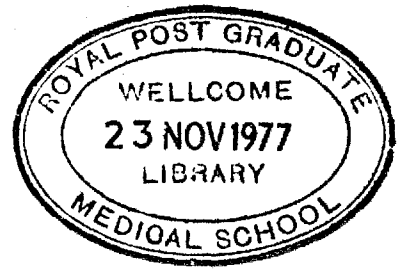
MEASUREMENTS OF UNCONJUGATED HORMONAL STEROIDS IN BIOLOGICAL FLUIDS

A THESIS



Submitted for the Degree of

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JOHANN MATTHIAS KJELD
Cand. med. et chir.

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ABSTRACT OF THESIS

Radioimmunoassay methods have been developed for the study of the levels of unconjugated testosterone (T), 5α-dihydrotestosterone (DHT), oestradiol (E_2) and progesterone (P) in human body fluids. Special methods were devised for the study of T and DHT conjugates in the same media.

Various factors related to specimen handling were investigated and several relevant facts discovered. A sharp rise of unconjugated T and DHT levels in stored urine samples was investigated further and evidence was found for the existence of a hitherto unknown T and DHT conjugates in urine.

In healthy individuals serum and urine specimens were obtained during the same time period. Unconjugated T, DHT and E_2 levels were measured in the samples and their relationship studied.

In a group of women on oral contraceptives significant elevations of unconjugated DHT levels were found both in serum and urine. Serum E_2 levels in this group were found to be similar to those of postmenopausal women.

No T glucuronide was found in two human amniotic fluid specimens of the 16th and 18th week of gestation.

Serum T levels in males were studied by frequent sampling and both diurnal and episodic fluctuations observed. The effect on serum T levels in males by infused human pituitary luteinizing hormone and follicle stimulating hormone were studied to establish their role in the control of serum T levels.

Finally, serum unconjugated E_2 and P were studied in a group of women with amenorrhoea before and during a first course of treatment with clomiphene citrate. Serum luteinizing hormone and prolactin were also measured. Serum E_2 levels were found to be the most sensitive index of responsiveness to the drug.

The thesis presents several new aspects of sex steroids in body fluids which are of immediate practical and theoretical importance. These, together with evolved methodology, should create a fertile soil for further work.

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ABBREVIATIONS USED IN THESIS

BSA	=	bovine serum albumin
CPM	=	counts per minute
CV	=	coefficient of variation
DHT	=	5α-dihydrotestosterone
DHT*	=	radiolabelled 5α-dihydrotestosterone - also 3H-DHT
DHTG	=	5α-dihydrotestosterone glucuronide
DHTS	=	5α-dihydrotestosterone sulphate
E_1	=	oestrone
E_2	=	oestradiol
E_2*	=	radiolabelled oestradiol - also 3H-E_2
E_3	=	oestriol
EE_2	=	ethynyl oestradiol
FSH	=	follicle stimulating hormone
h	=	hour
HCG	=	human chorionic gonadotrophin
i.m.	=	intra-muscular
i.v.	=	intravenous
LH	=	luteinizing hormone
min	=	minute
OC	=	oral contraceptive
P	=	progesterone
P*	=	radiolabelled progesterone
RIA	=	radioimmunoassay
S.A.	=	specific activity
SD	=	standard deviation
T	=	testosterone
T*	=	radiolabelled testosterone - also 3H-T
TG	=	testosterone glucuronide
TLC	=	thin layer chromatography
TS	=	testosterone sulphate
U.V.	=	ultraviolet
$\bar{x}$	=	mean value

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S E C T I O N 1

introductory

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CHAPTER 1

GENERAL INTRODUCTION

The work in this thesis involves the use of radioimmunoassays to study several aspects of sex steroids as reflected in human body fluids.

The introduction of labelled steroid compounds, first with carbon-14 and later with tritium of very high specific activity (see Jensen and Jacobsen, 1962) has changed the whole field of steroid research. The detection of beta radiation by liquid-scintillation counting discovered in 1950 (Reynolds et al., 1950; Kallmann and Furst, 1950) is similarly a cornerstone in modern steroid research. These techniques have eventually led to the evolution of enormously sensitive quantitative assays for steroidal compounds. The detection limits of some radioimmunoassays reach 1 picogram (10^{-12}gm).

In the following, radioimmunoassays are used to study areas of steroid biochemistry left by earlier pioneers equipped with less sensitive and far more cumbersome techniques. Recent progress in fields related to the steroids, such as in polypeptide hormones (gonadotrophins) and pharmacology (contraceptives, anti-hormones) have also created new problems and some of them are tackled in this work.

Secretion of sex hormones is chiefly controlled by the pituitary gonadotrophins which are under control of the hypothalamic releasing factors. This constitutes a feed back mechanism, the so-called hypothalamo-pituitary-gonadal axis. This system, however, does not account for many commonly known phenomena, for instance, the

episodic fluctuations of serum testosterone or the high oestradiol levels in the late follicular phase of the menstrual cycle, when both serum LH and FSH are very low. This may of course mean that serum levels of unconjugated sex hormones only partially reflect gonadal function.

While the clinical effects of many pharmacological agents are fairly well documented, information on their biochemical effects is scarce and therefore risk factors involved in their long term use may still exist. This is particularly true for the contraceptives, where, in view of the daily use by millions, the problem calls for urgent attention.

The structure of the steroid nucleus has been known for over 30 years and one or another steroid compound is probably found in every living cell. Yet very little is known about the biological role of these various compounds, and the mechanism of action of steroid hormones at the molecular level is still an enigma. Metabolic pathways of steroid hormones in the animal body are gradually emerging but relatively little is known about various steroid esters or conjugates which, if only for their quantity in biological fluids, are thought to play a significant role in steroid hormone metabolism and, eventually, their mechanism of action (Lebeau and Baulieu, 1973; Gottfried, 1972).

Steroids occur in two main forms in biological fluids, that is, conjugated (chiefly as glucuronides and sulphates) and unconjugated. In serum the unconjugated fraction exists as two sub-fractions, the protein-bound and the non-protein-bound. A confusion prevails in the clinical use of the words "free" and "total" in regard to steroid

levels in serum and urine. The word "free" has been used to designate the unconjugated fraction in urine or the unconjugated non-protein-bound fraction in serum. Similarly the word "total" has been used to designate the total steroids in urine after hydrolysis of the conjugates or the unconjugated fraction in serum including both the protein- and non-protein-bound fractions, but excluding the conjugated fraction.

There are two main steroid hormone synthesizing tissues in the body, the adrenal cortex and the gonads. In addition, in pregnant women the placenta is an energetic sex hormone producer from steroid precursors. The sex hormones and their metabolites are mainly excreted in the urine, 80% of the radioactivity of intravenously injected isotopically labelled oestradiol and testosterone being recovered in the urine within 2 days (Sandberg and Slaunwhite, 1956; Van Kammen et al., 1975). The earliest functional assessment of the male gonads and adrenal cortex by chemical methods were the urinary 17 oxosteroids (ketosteroids) and 17 oxogenic (ketogenic) steroids respectively. The ovarian function has been assessed by the total urinary oestrogen content, namely conjugated and non-conjugated C-18 (A ring aromatic) compounds. These methods used colorimetric detection of the respective compounds in urine after hydrolysis by various methods. Although outdated now because of lack of specificity these clinically well established methods are still in general use.

The relative value of blood and urinary measurements is a long-standing source of controversy (Marrian, 1955). It is, perhaps, sensible to adopt the attitude of Chattoraj and Wotiz, (1975)

suggesting that a complete and true picture of the secretory activity of an endocrine gland is likely to be obtained by investigating both blood and urine. Further, to arrive at a proper diagnosis, such observations may need to be supported by determining blood and urine levels, or both, before and after an endocrine organ has been stimulated by a trophic hormone or suppressed by suitable agents.

For the gonads the picture is complicated by peripheral conversion of sex hormone precursors, such as dehydroepiandrosterone and androstenedione secreted by the adrenals as well as the gonads. The adrenals also secrete small amounts of androgens and oestrogens directly into the circulation. While the rate of conversion of the precursors is different in the sexes, little is known about the factors that govern it.

In clinical medicine the functional status of the adrenal cortex has in recent years been effectively assessed by measurements of cortisol levels in plasma and urine. The unconjugated ("free") fraction in urine has been found an even more sensitive index of hypersecretion. Evidence also suggests that urinary unconjugated cortisol reflects the non-protein bound plasma levels, which are purported to be the effective fraction at the cellular level (Burke, 1971). In other words, the kidney may be regarded as an ultrafilter and integrator producing an excellent index of the effective levels of the unconjugated non-protein bound cortisol fraction in plasma. Analogous measurements of unconjugated sex hormone levels have not been possible until recently because of the very small quantities encountered (ng/ml).

In the work presented here unconjugated sex steroids have been measured during the same time period in serum and urine in order to establish a possible correlation.

The sex steroids studied were testosterone (T), 5α-dihydrotestosterone (DHT) and oestradiol (E_2). Progesterone (P) has also been measured in some studies.

DHT measurements were included as recent studies indicate that DHT may be the main androgen effector at the subcellular level. This was originally suggested by Wilson, (1972), and Liao et al., (1972), based on the findings in many androgen-responsive tissues of intranuclear retention of DHT even when T was added to the medium. According to the hypothesis, T, which is converted peripherally by the enzyme 5α-reductase into DHT, serves together with androstenedione, as a precursor or prehormone to DHT and possibly other 5α-reduced metabolites. The circulating levels of DHT (which has about twice the androgenic effect of T in some bioassays) may be seen to exert hormonal effects in organs poor in 5α-reductase activity. Further evidence for this mechanism of androgen action has been accumulating and is excellently reviewed by Williams-Ashman, (1975), and Wilson, (1975).

When establishing the assays some work had to be devoted to studies on sample collection and sample storage. Furthermore when trying to measure the non protein bound serum T fraction by dialysis, the results of preliminary studies compelled us to abandon that technique. However, the important findings in this abortive attempt led to some special studies on T conjugates in serum and urine. These have enabled us to present evidence for a new T conjugate in urine which has not been described previously.

CHAPTER 2

A SHORT HISTORICAL REVIEW

2.1 Concepts

2.1.1. Endocrine secretion

The knowledge about the role of the testis in the reproductive process goes back to antiquity. Aristotle used gonadal extirpation in the study of the physiology of reproduction as early as 300 B.C. (Steinberger and Steinberger, 1975). Castration has been practiced for centuries both in domestic animals and humans. Aside from sterility, characteristic effects sought were submissiveness and changes in body composition, and, in humans, the high pitched voice of the eunuchs served well in religious choirs.

In 1792 John Hunter published his observations on the effects of castration on the vesiculae seminales. His work is of importance as the first scientific approach in what was later to become known as endocrinology.

Anatomy was the main medical science in the middle ages and that might have been the reason for the long held belief that regulation of male characteristics was mediated by nerves extending between the testis and the male reproductive organs. However, in 1849 an experimental demonstration of an internal secretion by the testis was given by Berthold in his classical experiment in which castration effects in the fowl were prevented by transplantation of the testes to an ectopic site.

Berthold's experiment was in fact the first experimental demonstration of the existence of endocrine secretion. The conceptual importance of his work was, however, only slowly realized. For a

time endocrinology was of somewhat doubtful repute owing to the idea of rejuvenation or revirilization by testicular hormones. The use of testicular extracts and testis transplants in men in the late 19th and early 20th centuries is the source of folklore and jokes still alive with the present generation.

Modern endocrinology was finally born and baptized in the momentous work of Bayliss and Starling in 1902, on the control of pancreatic secretion by the intestine.

The oestrogenic endocrine activity of the ovary was established just before 1900 and further studies went along the now established lines. Extirpation of the ovaries prevented oestrus in animals but if simultaneous transplantation was performed oestrus took place as before.

2.1.2. Isolation of hormones and structure

As with concepts so it took a long time for progress in the material world. In 1935, or 86 years after Berthold's experiment, David, Dingemanse, Freud and Laqueur isolated testosterone from bull testicles and thereby brought forth by chemical means an argument for the existence of the type of substance Berthold's experiment had indicated.

The same year, 1935, MacCorquodale, Thayer and Doisy isolated 17B-oestradiol from porcine ovaries, implying the ovary as a specific internal secretory gland.

During a period of less than 10 years tremendous progress was made in the field of hormonal steroids. Butenandt in Germany and Doisy, Veler and Thayer in the U.S.A. isolated oestrone from human pregnancy urine in 1929. Marrian in London isolated oestriol from

the same source in 1930. In 1931 Butenaudt had isolated androsterone from human male urine. By 1938 all the most important adrenal and gonadal steroids known to-day had been isolated.

The structure of the steroid nucleus was elusive to the chemists of those times, but by a concerted effort from several laboratories the cyclopentano-perhydro-phenanthrene structure had been established by 1935. These discoveries and the steroid chemistry involved are entertainingly described by Fieser and Fieser, (1959).

After the isolation and elucidation of the structure of the steroids the way was paved for further research. However, it was not until in the early nineteen fifties that a rapid progress set in again. This was due primarily to the introduction of radioactive isotopes for labelling steroid compounds. The use of carbon-14 and later tritium labelled compounds has led to the isolation and characterization of new sex steroids, their metabolites and conjugates, the calculation of production and secretion rates, the establishment of plasma and tissue binding, and the localization and fate in tissues. Some of these are the subject of the following sections.

2.1.3. Biosynthesis and metabolism

Sex steroids are synthesized in the gonads and adrenals from two carbon (acetyl radicals) precursors (Srere et al., 1950; Brady, 1951).

The general chain of synthesis is as follows (Fig. 2.1.):

acetate → mevalonic acid → squalene → cholesterol → pregnenolone

→ 17α-hydroxypregnenolone → dehydroepiandrosterone →

androstenedione ⇌ testosterone

↓ ↓

19-hydroxyandrostenedione → 19-hydroxytestosterone

↓ ↓

oestrone ⇌ oestradiol

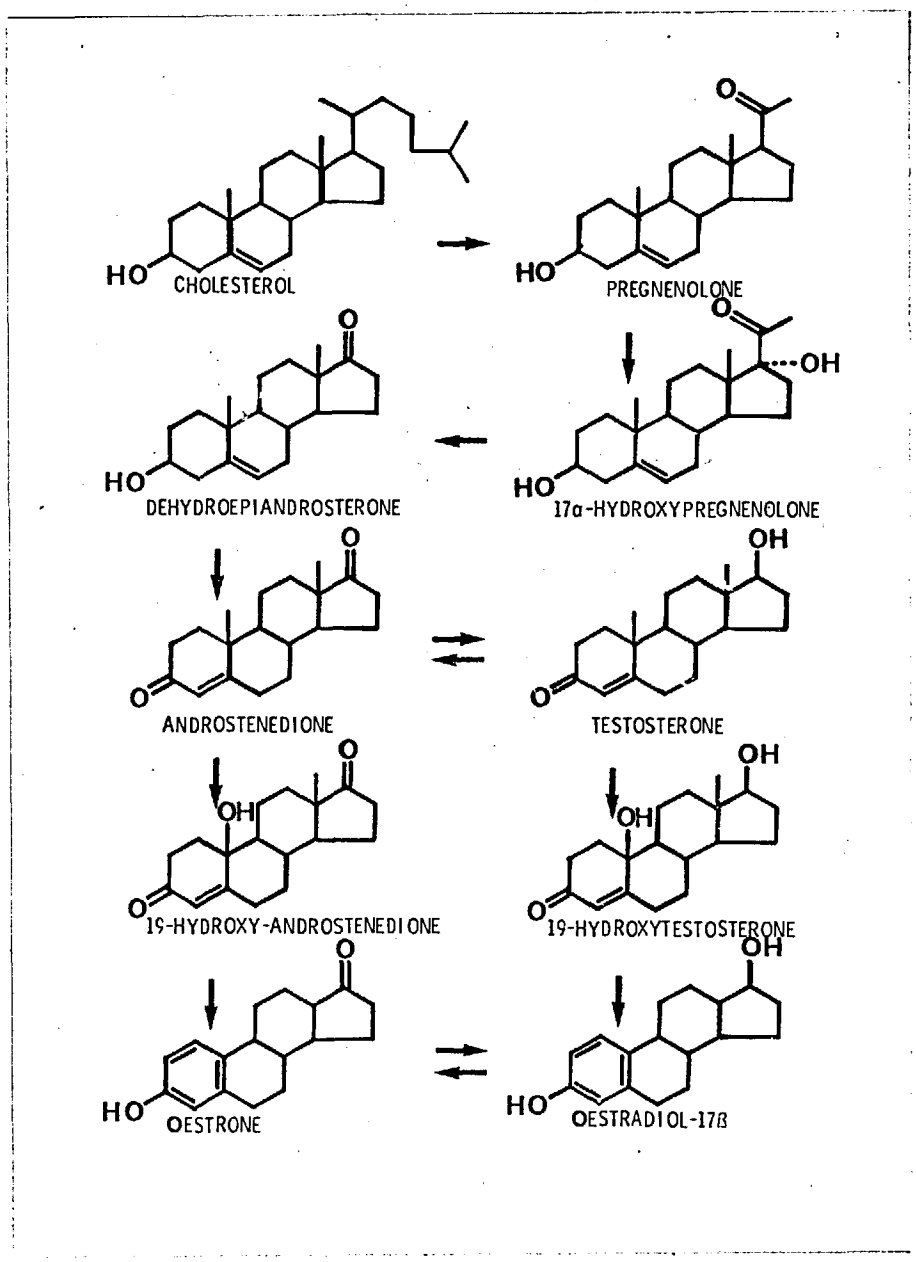
The ovaries are the main source of plasma oestrone (E_1) and oestradiol (E_2) in the female, more than 95% of E_2 stemming from that source (Baird and Fraser, 1974) and the testicles the main source of plasma testosterone in males, (over 95%) (Baird et al., 1969). Dehydroepiandrosterone in plasma derives mainly from the adrenals in both sexes while up to 50% of androstenedione, which is almost totally of adrenal origin in males, may derive from the ovaries (Bardin et al., 1969).

The earliest evidence for the formation of oestrogens from androgens in humans was reported by Steinach et al. in 1936. They found increased urinary oestrogen excretion after testosterone administration to normal men. However, it was not until isotopically labelled compounds became available that the real pathway shown above could be worked out (Meyer, 1955; Smith and Ryan, 1961; Slaunwhite and Samuels, 1956).

The urinary metabolites of the sex hormones have been studied by injecting radiolabelled tracers of the free hormone into the blood stream. The main metabolites for testosterone are 5β- or 5α-androstane compounds (etiocholanolone, androsterone etc.) and oestriol for the oestrogens (Robel et al., 1966; Beer and Gallagher, 1955).

It has been known for a long time that the sex steroids and their urinary metabolites are mainly excreted in the form of conjugates (Funk et al., 1929). Conjugates have also been isolated from human blood. The sex steroids are conjugated mainly to glucuronides and

Fig. 2.1.



Structural formulas of the steroid compounds involved in the later stages of sex hormone synthesis. The arrows indicate the sequence of transformation.

sulphates but other conjugates have been described. In urine the glucuronide fraction is preponderant. This is possibly related to differences in renal clearance, as glucuronides are cleared about 20 - 40 times faster than sulphates into the urine (Bongiovanni and Eberlein, 1957; Kellie and Smith, 1957).

Because of lack of sensitive methods little is known about serum levels of individual conjugates of sex steroids in normal man or woman. Urinary levels have mostly been measured by non-specific methods (Dicsfalusy and Levitz, 1970; Solomon and Bhavani, 1970).

Conjugates of sex steroids are found in bile and an entero-hepatic circulation does exist (Sandberg & Slaunwhite, 1957 and 1965). Conjugation takes place in the gut, the liver, kidneys and other organs, but, at least sulphate conjugates, are also secreted by the gonads and adrenals (Saez et al., 1967). Except for purposes of urinary excretion, the role of the conjugates still remains obscure. However, steroid sulphates and glucuronides can be metabolized directly i.e. the steroid moiety metabolized without breaking the conjugating bond, the conjugates themselves thus being enzymic substrates (Baulieu et al., 1963; Knapstein et al., 1967).

2.1.4. Control mechanisms

The experiments of Smith, 1926 and Zondek and Ascheim, 1927 showed that the gonads were under some control from the pituitary gland. Based on morphological studies two main trophic factors were identified, follicle stimulating hormone (FSH) and the interstitial cell stimulating hormone (ISCH), which was later shown to be identical with the luteinizing hormone (LH). A third hormone principle to be isolated from the pituitary is prolactin (lactogenic

hormone, mammotrophic hormone) later to be shown to be identical to a hormone called luteotrophic hormone. Human pituitary FSH and LH have been highly purified and shown to be glycoproteins of approximately 32,000 and 28,000 molecular weight, respectively. They are composed of two chemically distinct subunits, referred to as alpha and beta subunits, joined by non-covalent bonds (Saxena and Rathnam, 1971; Rathnam and Saxena, 1971; Sairam et al., 1972). In humans prolactin is structurally similar to growth hormone, and was difficult to separate from that hormone, but has been definitely identified as a separate hormone. The full structure of prolactin from sheep pituitary glands has been established (Li et al., 1970). It is a single chain polypeptide composed of 198 amino acids with molecular weight of 23,000.

Early measurements of gonadotrophin levels in blood and urine from castrates showed high levels and treatment with sex hormones brought these levels down. A simple inhibitory feedback mechanism was thus established between the pituitary and the gonads. However in recent years it has become increasingly clear that this mechanism is only a part of a very complex control system involving the hypothalamus.

The periodic oestrus in animals or menstrual cyclicity in the female primate has been a challenging enigma for the endocrinologist.

Pfeiffer in 1936 showed that ovarian transplants in spayed female rats had cyclic activity but not in castrated male recipients. However, if the male rats were castrated at birth and then received ovarian grafts cyclicity was observed. Pfeiffer therefore thought that the pituitary was conditioned by some testicular factor in the neonatal period.

Harris and Jacobsohn, in 1952, grafted male rat pituitaries into hypophysectomised female rats and were able to show that the pituitary was not permanently masculinized as the rats resumed oestrus cycles. This experiment revealed two important facts. Firstly, the hypothalamus was the organ that had been conditioned or imprinted, secondly, the pituitary functioned properly only in close connection with the hypothalamus, thus corroborating earlier findings of a portal blood flow from the hypothalamus to the pituitary (Green and Harris, 1947, 1949). Barraclough later showed that the central nervous system could be masculinized by a single injection of testosterone into neonatal rats, (Barraclough, 1966).

Recently it has been shown that there is a qualitative feed back of gonadal steroids on gonadotrophin release as well as a quantitative one. Thus, androgen treatment of castrated rats (males or females) leads to a change in the FSH molecule, which becomes bigger and has a longer half-life in the circulation (Peckham et al., 1973; Bogdanove et al., 1975).

One of the latest theories is based on studies of sex-linked liver enzymes in rats subjected to various treatments. A pituitary feminizing factor (FF) is postulated and neonatal testicular androgens programme the hypothalamus to secrete a feminizing factor inhibitory factor (FFIF) in males during adult life (Gustafsson et al., 1975).

The hypothalamus is now known to secrete the so called hypophysiotrophic hormones (releasing factors) which are transported via the hypothalamic-hypophysial portal system to the anterior

pituitary. Two of these factors (neurohormones) have been isolated from animal hypothalami, the thyrotrophin-releasing factor (TRF) in 1969 and the luteinizing hormone-releasing factor (LRF) in 1971.

LRF, which releases both LH and FSH, was isolated from porcine (Schally et al., 1971) and ovine (Burgus et al., 1972) hypothalami. Its structure is identical in these two species (Matsuo et al., 1971; Burgus et al., 1972) being the decapeptide-amide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. This compound which has been variously named, luteinising hormone releasing factor (LRF), luteinising hormone releasing hormone (LHRH), and gonadotrophin-releasing hormone (GnRH), has been obtained by total synthesis and is presently undergoing clinical trials. A great number of synthetic analogues of LRF have been synthesized and are being tested for biological activity. The aims are twofold: a) to obtain a compound with stronger and longer lasting effects than LRF for therapeutic purposes, b) to obtain a compound which antagonizes the effects of LRF, thus developing a drug which might be useful in fertility control.

Releasing factors have not yet been isolated in pure form from human sources. As TRF and LRF are each identical in two unrelated animal species (pig and sheep), and these are active in man as well as common laboratory animals, it is likely that human hypothalamic releasing factors will turn out to be very similar to those isolated from animals.

The discovery that highly purified preparations of LRF, and later the synthetic compound itself, release both LH and FSH has cast doubts on the existence of a distinct hypothalamic hormone for FSH control (Schally et al., 1971 and 1976). McCann (1974), however, points out that large increases in plasma LH can be produced by stimulating the

preoptic region without any effect on FSH. Also, in male castrates treated with testosterone, LH levels are brought down dramatically, while FSH levels decrease only slightly. This would seem to indicate a separate hypothalamic FSH factor or that the pituitary responds with variable sensitivity and in a variable way to the same hypothalamic factor. At the time of writing the existence of an FRF, distinct from LRF remains to be proved or disproved.

Prolactin, unlike FSH and LH, is mainly under an inhibitory control by the hypothalamus which secretes the prolactin inhibitory factor (PIF). The existence of PIF was first clearly demonstrated by Everett in 1954, who found that pseudopregnancy in rats occurred after transplantation of the pituitary gland under the renal capsule. The existence of PIF has since been confirmed by a number of investigators. Kamberi et al., (1971) cannulated the pituitary stalk vessels in donor rats and infused the portal blood into the portal vessels of recipient rats. When donor rats were given dopamine via the third ventricle a marked decrease in prolactin levels was observed in the recipient rats. No change in prolactin levels occurred in the recipient rats when dopamine was infused directly into their pituitary portal vessels. The decrease in prolactin levels in the recipient rats when infused with portal blood from dopamin treated donors was therefore due to a prolactin-inhibiting factor. Recently dopamine has been shown to act directly on the hypophysis, blocking prolactin release (MacLeod and Lemeyer, 1974; Takahara et al., 1974).

A prolactin releasing factor (PRF) has been postulated and some investigators have described it in hypothalamic tissue (Valverde et al., 1972). Administration of the thyrotrophin releasing hormone

(TRF), a synthetic tripeptide, increases prolactin concentrations in peripheral blood.

The secretion of the hypothalamic releasing factors is under neural control probably mediated through adrenergic transmitters. Ovulation can be caused by electrical stimulation of the hypothalamus, but not by stimulating the pituitary (Harris 1948). More recently Fink and Jamieson,(1976) have measured increased LRF in the pituitary stalk blood of rats during the application of an electrical stimulus to the medial preoptic area. Evidence from both in vitro and in vivo studies implicates dopamine in the control of hypothalamic factors (Kamberi et al.,1970a, 1970b). Prolactin levels are sensitive to drugs affecting catecholamine metabolism. Thus administration of L-dopa (the precursor of dopamine)or of iproniazid (a monoamine oxidase inhibitor) reduce serum prolactin levels, whereas drugs, such as chlorpromazine and reserpine, which inhibit hypothalamic catecholamine activity,increase serum prolactin levels (see review by Meites and Clemens, 1972). Several ergot derivatives reduce serum prolactin levels markedly and have found therapeutic use.

Thus the neurohormones are under the control of neural and metabolic stimuli. This has been called the "open loop" control. Their secretion is also influenced by the sex steroid levels in blood (long loop),or their corresponding pituitary hormones (short loop). Elevation of circulating LH and FSH levels in menopausal women or in castrated animals does not support a primary role for the short feedback mechanism. Lastly the neurohormone secretion may be inhibited by their own concentration in the hypothalamus (ultra short loop) (Davidson 1969; Motta et al., 1969; Schally et al., 1972).

The complexity of this control system is further increased as oestradiol and progesterone can have both stimulating and inhibiting effects on gonadotrophin secretion. Oestrogen treatment, for instance, enhances the pituitary response to LRF (Arimura and Schally, 1971; Jaffe and Keye Jr., 1975). In ovariectomized animals a single injection of oestrogen produces a fall in plasma LH levels. A second injection of either progesterone or oestrogen given on the second or third day after the priming dose produces an altogether different response with a marked rise of LH reaching a peak value 5 - 6 hours after the injection. (Caligaris et al., 1968, 1971).

Although not studied to any extent, some synergism with regard to sex hormone production has been shown to exist between the gonadotrophins (Kjeld et al., 1976, and ref. therein, also Chapter 7).

The association of steroids with plasma proteins had been known for a long time. In 1956 Daughaday found that over 95% of unconjugated 17-hydroxycorticosteroids were bound to plasma proteins and about 65% of their glucuronides. Further studies showed a high capacity low specificity albumin binding and high specificity low capacity globulin binding. A similar phenomenon has been found to exist for the sex steroids (Pearlman and Crepy, 1956; Marcier et al., 1966). A beta-globulin, called sex hormone binding globulin (SHBG) or testosterone-oestradiol binding globulin (TeBG), with a relative specificity for 17B-hydroxylated C_{18} and C_{19} stercids has been identified. The concentration of SHBG in serum is increased by oestrogens and thyroid hormones but decreased by androgens. Increased levels are found in pregnancy, during contraceptive treatment with ethynylestradiol, and in hyperthyroid states.

Decreased levels are found in many cases of hirsutism and in hypothyroidism (Anderson, 1974). The role of SHBG remains highly speculative.

2.1.5. Effects and mode of action of sex steroids

The primary function of the sex steroids is related to reproduction. They exert a trophic effect on the respective genital tracts and play a fundamental role in gametogenesis. This latter role of the sex steroids is, so far, less studied and little understood.

The influence of sex hormones is not solely confined to the sex organs. After sex steroids became available as pure compounds numerous studies were conducted into their general (non-sex organ) effects. Only a few well established ones will be listed here.

The androgens have a generalized anabolic effect, causing increased protein formation, particularly of muscle and bone. Administration of androgens results in the retention of nitrogen, phosphorus and potassium, together with a lowering of the respiratory quotient, indicating increased utilization of dietary or body fat for protein synthesis. Psychological effects are mainly related to aggression contrary to the submissiveness of the eunuch. The anabolic-androgenic activity ratio varies for different androgens. This was noted as early as 1935 (Kochakian, 1946), and several steroids with high anabolic ratio have been synthesized and found various clinical uses.

Oestrogens affect body weight and composition, lipid, carbohydrate, protein and mineral metabolism. They affect the cardio-

vascular and reticuloendothelial systems, influence capillary fragility, and also other endocrine systems such as the thyroid and adrenals.

No coherent or unifying explanation has, as yet, been offered for all these diverse effects of the sex hormones. However, it may be that their effects are exerted to a different degree at a very basic level in the metabolism of every cell. This has been shown by studies which indicate that sex steroids cause both hypertrophy and hyperplasia in accessory sex glands (Mueller, 1971; Williams-Ashman and Reddi, 1971).

By refined techniques with radioactive precursors it has been shown that labelled RNA and protein in the uterus and prostate were formed in a matter of minutes after sex hormone injection into castrated rats. DNA content also increased more slowly indicating nuclear replication. DNA and RNA polymerases have both been shown to rise, but in otherwise healthy tissue the effects pertaining to hyperplasia wear off under continued hormone treatment thus indicating an internal control within the cells regarding responsiveness to sex steroids (Williams-Ashman, 1975; Mueller, 1971).

Studies on the mode of action of sex steroids in target organs at the cellular and subcellular level have recently entered into a new era of intense activity. The birth of this era dates back to the pioneering studies of Jensen, 1958; Glascock and Hoekshra, 1959 and Jensen and Jacobsen, 1962. These workers labelled oestrogenic hormones with carrier free tritium to a very high specific activity and were able to study, for the first time, the disposition in animals of physiological doses of the hormones. Using oestradiol-^{3}H, Jensen and Jacobsen demonstrated that uterus, vagina and anterior pituitary

possessed a unique affinity for oestradiol (E_2) which was incorporated into these tissues without a chemical transformation. It was soon recognized that these tissues contain characteristic E_2 binding components or receptors, later to be identified as macromolecular protein.

This work set off a wave of studies on the tissue uptake of E_2 and, in turn, similar studies were begun with other steroid hormones.

For the androgens a specific uptake has been established by the accessary sexual glands, such as the prostate, seminal vesicles and epididymis. It was found that the nuclei of these organs selectively retained 5α-dihydrotestosterone (DHT) rather than T (Bruchousky and Wilson, 1968; Anderson and Liao, 1968). As this was observed after the injection of tritiated T, it seems that, in these organs, T is reduced to its 5α-metabolite, DHT, before it exerts its action in the nuclei. The enzyme 5α-reductase is found in the microsomal fraction (endoplasmic reticulum) of cells and is firmly bound to the subcellular structure. Thus DHT and E_2 seem to be the two sex hormonal effectors, respectively, at the nuclear level in the accessory sex organs.

The uptake of the sex steroids by the nuclei involves at least two separable steps, a binding to a cytoplasmic protein receptor, and then a transport to and subsequent uptake by the nuclei. Purified nuclear preparations in vitro do not take up the steroids unless a cytoplasmic preparation is added.

The anti-sex-hormones cyproterone acetate and clomiphene seem to exert their effects at this receptor level, as they compete with the respective sex hormones for the binding sites on the cytoplasmic receptors, both in vivo and in vitro (Liao and Fang, 1969; King and

Mainwaring, 1974). The anti-sex hormone agents do, however, also compete for receptors in the hypothalamus and the pituitary, thus affecting the feedback control mechanism. These agents may, furthermore, compete for enzymes in sex steroid synthesis or degradation.

The testicular feminization syndrome in humans and its animal counterparts, the Tfm mouse and the Stanley-Grumbeck rat, now seems to be a disease of androgen insensitivity. The disorder is thought to be a congenital abnormality or absence of cytoplasmic androgen receptors. This has been incisively studied in the kidney of pseudo-hermaphrodite variants of the mouse and rat (Bardin et al., 1973).

In conclusion it can be said that most of the effects of sex steroids can be accounted for by their influence on DNA replication and transcription in the nuclei. This is further supported by the abolition of the sex hormone response by actinomycin-D, an inhibitor of RNA synthesis. That the mechanism of action may, however, be even more complex is suggested by the inhibitory effects of puromycin and cycloheximide on the RNA formation induced by sex hormones. These agents are known inhibitors of protein synthesis and it has therefore been postulated that an initial synthesis of a protein is critical (Jensen et al., 1974).

Nothing is known about how the sex hormones eventually get out of the nucleus and, in turn, the cell. In the intensive studies outlined above on sex hormone target organs, investigators have paid little attention to sex steroid conjugates. Neither their formation nor their possible action at the subcellular level has been studied adequately. In ordinary bioassay systems (see later) conjugates are

not active per se, but it has been proposed that they can have a more subtle action in modifying the availability of hormonal steroids (Baulieu et al., 1968).

Finally attention is directed to the inherent difference in the tissue response to sex steroids at different ontological stages. The sensitivity of the hypothalamus to androgen stimuli in the neonatal period has already been mentioned, but similar studies on foetuses at early stages show this characteristic more clearly. Thus castration of male foetuses or their treatment with the antiandrogenic agent, cyproterone acetate, has indicated that androgens elaborated by the foetal testis are crucial stimuli for the Wolffian duct, the urogenital sinus, and the genital tubercle, to differentiate initially into structures characteristic of the normal male genital tract. This important subject is reviewed by Jost, 1970.

2.2 Methods of assay

2.2.1 Androgens

Bioassays. The earliest methods for assessing the androgen content of a sample were related to their only known property, that is their androgenic effects. These biological methods served both as qualitative and quantitative assays. Although indispensable, bioassays have inherent problems related to the use of animals. Such factors as age, weight, breed, care, etc. have to be standardized to get comparable results. The growth of the capon's comb has been the most commonly used index for androgenicity (Dorfman, 1962).

The capons are either injected intramuscularly or the test material is prepared for direct inunction into the comb. Various bioassays on mammals, chiefly rodents, have also been used. Most of them involve measuring weight increments of the seminal vesicles or the prostate, or both, after injection of the test material into castrated animals. Testosterone and 5α -dihydrotestosterone have the strongest androgenic activity in these systems, the latter about two times stronger than the former. Detection limits are in the range of 10 - 20ug.

In 1935 the League of Nations Committee adopted 0.1mg of androsterone as equivalent to one international unit of androgen activity.

Colorimetric assays. In 1935 Zimmermann demonstrated that pure ketonic steroids such as androsterone, testosterone and oestrone etc. could be quantitatively determined by reacting with m-dinitrobenzene in alkaline solution. Maximum absorption of the pigment is at about 520nm when the carbon group involves carbon 17. Although applied to measure 17-ketosteroids (17-oxosteroids; 17KS) in plasma this method has mainly been applied for urinary measurements. The principal urinary 17-KS are androsterone, etiocholanolone, dehydroepiandrosterone, 11-keto(or oxo) androsterone, 11-ketoetiocholanolone and 11B-hydroxy-androsterone, which comprise about 90% of the 17-KS. These substances originate from various tissue precursors and, even if measured individually after chromatographic separation, they do not represent a single precursor. As about half to two thirds of the 17-KS in urine are of adrenal origin they reflect testicular function poorly (Fraser et al., 1941; Mason and Engström, 1950).

The above mentioned compounds exist almost totally as conjugates of glucuronides and sulphates, which have to be hydrolysed before their final measurement. This has been accomplished with various preparations of the enzymes B-glucuronidase and sulphatases or by acid hydrolysis. This step is liable to several errors and the commonly used differential hydrolysis (glucuronides versus sulphates) might not be valid (Bradlow, 1970).

Methods based on the Zimmermann reaction, measuring total 17-KS in plasma or urine, were used for over 20 years as the sole means of chemically assessing testicular function, and, although outdated, these methods are still in use in clinical chemistry.

Fluorimetry. Androgens have not been measured by fluorimetry to the same extent as oestrogens and corticosteroids. However, the first method capable of measuring unconjugated testosterone (T) in plasma made use of fluorimetry for final detection (Finkelstein, Forchielli and Dorfman, 1961). This method required 50 ml of plasma, exployed 72 hour paper chromatography followed by enzymic conversion of T to oestradiol, which was measured fluorimetrically after a second chromatography step. Average normal T values for males by this method were about 5ng/ml. Normal female values were over-estimated as the detection limit of the assay was about 1ng/ml.

Double isotope derivative techniques. These methods made use of radiolabelled (^{3}H or ^{14}C) steroids of high specific activity and purity. A radiolabelled portion of the steroid to be measured is added to the sample which is to be analysed. After extraction and extensive purification of the sample a suitable reagent labelled with a second

isotope is brought in. This reagent must form a stable derivative of the compound which is being analysed. Further purification follows until a stable ratio between the two isotopes is reached. From the specific activity of the second compound, the stochiometry of the reaction between the two compounds and the radioactive recovery of the first compound added the quantity in the original sample is calculated. Acetic anhydride (^{3}H or ^{14}C) or thiosemicarbazide (^{35}S) have been used to form the derivatives (Hudson et al., 1963; Riondel et al., 1963).

With these methods, using about 10ml of plasma, about 500pg/ml could be detected with reasonable precision. The methods are however, lengthy, complex, tedious and costly, all of which has curtailed their general use.

Gas Chromatography. This powerful technique was introduced by James and Martin in 1952. The technique involves an inert gas being used as the mobile phase in a chromatography system. Volatile compounds are thus relatively easily measured by this technique, and methods for making volatile derivatives of less volatile compounds have been devised. Detection of the separated compounds is accomplished by non-specific devices, either the flame-ionization detector or the more selective and sensitive electron-capture detector. Examples of testosterone assays using these techniques are those of Guerra-Garcia et al., 1963, and Brownie et al., 1964.

Gas chromatography is less cumbersome than the double isotope derivative methods. However, for steroid measurements samples need to be considerably processed before final detection, which itself is a relatively slow process. The method is therefore not suitable for any large number of measurements.

Competitive protein binding (CPB). This type of method belongs to a methodological group which has been variably named as saturation analysis, displacement assays or radioligand assays (Ekins and Newman, 1970; Murphy, 1970). The radioimmunoassay (RIA) which is the subject of the next section, belongs to this group. The principle of these methods is that varying amounts of the measured compound (ligand) competes with a constant amount of a radiolabelled portion of that same substance (radioligand) for binding sites on a third substance, a binding protein. Since this binding protein is also added in constant amounts in the assay, the degree of displacement, or, conversely, the binding of the radioligand, becomes a measure of the quantity of the unlabelled substance in the sample.

In recent years the sex hormone binding globulin (SHBG) has been used by a number of investigators as the binding protein in T and DHT assays (Mayes and Nugent, 1968; Kato and Horton, 1968; Ito and Horton, 1970; Tremblay et al., 1970). These assays are sensitive enough to measure picogram levels in small samples. Specificity, however, is only obtained by at least one or two chromatography steps. Another reported problem is the non-specific and capricious blank experienced by many investigators.

Radioimmunoassay (RIA)

General discussion. This assay method uses antibodies as the binding protein and was originally used to measure polypeptide hormones by Berson and Yalow in 1959. Steroids are not antigenic, however, when a steroid is coupled as a hapten to a macromolecular antigen, the

antibodies induced against that antigen will specifically bind the steroid. This hapten principle has been known since the work of Landsteiner and associates (Landsteiner and Scheer, 1936), but was successfully exploited for steroids by Erlanger et al. in 1957 (and 1959) in attempts to produce steroid antibodies for their anti-hormonal effects. The methods of these and other workers were later to be widely used to produce antisera for use in steroid RIAs.

The specificity of the antibodies is dependent on the position of conjugation of the steroid molecule to the macromolecule, the part of the molecule furthest from the bond playing the greatest part in the specificity (Midgley and Niswender, 1970). Labelling the steroid with a gamma-emitting isotope, such as ^{125}Iodine, instead of the commonly used beta-emitter, tritium, reduces reagent cost and might increase sensitivity because of increased counting efficiency. The relative merits of this approach are discussed by Hunter et al., 1975. Coupling the antibodies to a solid phase or support (sephadex, plastic tubes etc.) has been used, and also might allow the repeated use of the same antibodies.

RIA of steroids has already revolutionized steroid research and, from what has just been said, it can be seen that the use of the principles involved can be improved. It can be safely predicted that RIA will be the method of choice for measuring steroids for at least the next decade, both in research and in clinical practice. This is due to its specificity, sensitivity, speed and relative procedural simplicity. RIAs immediate acceptance is best displayed by the prolific literature on the subject and also the intensive commercial activity surrounding it during the last 2 or 3 years.

At the time of writing RIAs for testosterone (T) have been published by many workers experimenting with various procedural possibilities. Being of higher specificity, antisera raised against T conjugated to a protein through the 3-keto group (T-3-oxime-protein) have generally been used. Two of the first methods to be described were those of Niswender and Midgley, 1970, and Furuyama et al., 1970. 5α-dihydrotestosterone (DHT) cross-reacts 40-70% (against T) with the T-3-oxime-protein antisera and these have therefore been used for DHT assays following chromatography (Coyotupa et al., 1972; Barberia and Thorneycroft, 1974). An assay for DHT using antiserum against DHT linked to bovine serum albumin through position 1 of the steroid, has been described (Bauminger et al., 1974). However, preliminary chromatography was needed as the antiserum cross-reacted 10% with T.

2.2.2. Oestrogens

As brief description of analytical principles involved in individual types of methods has already been given in the previous section, this section will be correspondingly shorter.

Bioassays. Bioassays of oestrogens are mainly based on the changes taking place in the cell structure of the lining of the vagina in animals during oestrus. These cells become cornified and easily distinguished from those of the resting period, or of the permanent condition of spayed animals. Spayed mice or rats have mostly been used in one or another variation of the Allen-Doisy test (Allen and Doisy, 1923). Increase in uterine weight of spayed rodents has also been used (for review see Emmens, 1962).

Colorimetry. The earliest chemical method for oestrogens was a colorimetric one described by Kober in 1931. This method involved heating with concentrated sulphuric acid, water dilution, and the use of phenol to form a red colour. The method was subject to instability and non-specific interference. A number of methods has been published based on the same principle. A well known one is that of Brown (Brown, 1955).

Ittrich, in 1958, improved the technique by extracting the red colour with chloroform containing 2% p-nitrophenol. This technique avoided a lot of the non-specific chromogens and also made fluorescence measurements of the chromogen feasible.

Fluorimetry. When Marrian in 1930 isolated oestriol from human pregnancy urine he observed a green fluorescence when the crystalline substance was heated with concentrated sulphuric acid. This property of oestrogens has been put into use for their quantitation by several workers (see review by O'Donnel and Preedy, 1967). Fluorimetry is more sensitive than colorimetry and down to 5ng can be estimated. However, because of the minute amounts of unconjugated oestrogens in body fluids, hydrolysis of oestrogen conjugates is a necessary step in both these methods, which thus measure the "total" amount of either all the oestrogens (E_1 E_2 etc.) or the individual ones following separation after hydrolysis.

Double isotope derivative techniques. The first method of this kind for oestrogens was developed by Svendsen in 1960. This method made use of pipsyl chloride - ^{35}S for derivative formation and oestradiol ^{131}I-pipsylate as internal standard. Several later methods

used carbon-14 or tritium labelling for internal standard, and acetic anhydride for derivative formation. These methods can detect quantities in the picogram range, but are complex, cumbersome and very time-consuming.

Gas chromatography. As for androgens, methods using this technique were quickly developed (Vanden-Heuvel, 1960; Wotiz and Martin, 1961). However, as for the isotope methods just mentioned, these methods are too time-consuming and cumbersome for general use, and, although still in use, will soon become obsolete except as reference methods.

Competitive protein binding (CPB). The earliest attempts to measure 17β-oestradiol (E_2) by CPB was that reported by Murphy (1968) using SHBG. However, as this protein is non-specific and binds to several androgens much more strongly than to E_2, this method, even after extensive chromatography, gave erratic results and was not used much. Another approach using specific oestrogen-binding proteins from uteri was more successful (Korenman et al., 1969). This method was capable of measuring unconjugated oestradiol and oestrone using 2 - 3ml of plasma. However, difficulties were experienced. The cytosol preparation of the binding proteins was unstable, and many investigators had great problems with non-specific blanks.

Radioimmunoassays (RIAs). The first RIAs of oestrogens were those described for E_2 by Abraham, (1969) and Midgley et al. (1969). These methods used antisera raised against E_2-17-hemisuccinate-BSA (bovine serum albumin). As these antisera were not specific for the individual oestrogens (the A ring governing the specificity, see above

under T) a chromatography step to separate the oestrogens had to be included. This could be used to advantage for the simultaneous measurements of two or more of the oestrogens (Mikhail et al., 1970).

Antisera raised against oestrogens coupled to bovine serum albumin through the 6 positions have been used by several workers, and are relatively specific for the individual oestrogens (Exley et al., 1971). This type of E_2-antiserum has recently been used in an E_2-RIA without prior extraction or chromatography (Jurjens et al., 1975).

2.2.3. Levels in plasma and urine.

In this section two tables are presented, Table 2, 1 and Table 2, 2 for quantitative orientation. The values given in the tables are selected from recent literature. For reasons of space the tables are not comprehensive, and where several values have been reported, a value amongst those most closely agreeing was selected for presentation.

2.3. Dynamic aspects

2.3.1. Production and clearance.

The various steroid hormones of animal origin have relatively short half-lives in the body, that is, half of an administered dose is eliminated from the body in a matter of hours (Sandberg and Slaunwhite, 1957, 1958; Slaunwhite and Sandberg, 1958). These and other studies also showed that intravenously injected radiolabelled hormones disappear rapidly from the blood stream. After an initial

TABLE 2.1.

Concentrations in plasma (or serum; ng/ml) and urinary excretion (ng/24h) of estrogens in normal humans

	Reproductive females				Pregnancy, last 5 weeks		Postmenopausal		Males	
	PLASMA		URINE							
	Foll.	Luteal	Foll.	Luteal	Plasma	Urine	Plasma	Urine	Plasma	Urine
Unconj. E_1	(b) 7.9×10^{-2}	(b) 1.1×10^{-1}	(d) 4.3×10	(d) 2.9×10^{2}	(1) 1.2×10	(d) 2.7×10^{4}	(h) 3×10^{-2}		(b) 6.2×10^{-2}	(a) 1.5×10^{2}
Total E_1			(g) 5×10^{3}	(g) 1.4×10^{4}	(f) 6×10^{4}	(g) 1.2×10^{6}		(g) 2.5×10^{3}		(a) 10^{4}
Uncong.E_2	(b) 6.6×10^{-2}	(b) 1.4×10^{-2}	(c) 3.2×10	(c) 4.6×10	(1) 2.6×10	(d) 6.7×10^{3}	(h) 1.5×10^{-2}		(a) 2.3×10^{-2}	(a) 6×10
Total E_2			(g) 2×10^{3}	(g) 7×10^{4}	(f) 2×10^{4}	(g) 4.2×10^{2}		(g) 6×10^{2}		(a) 2.7×10^{3}
Unconj.E_3		(k) 1.2×10^{-1}	(d) 6.5×10	(d) 2.8×10^{2}	(1) 6	(d) 10^{5}	(k) 7×10^{-3}		(a) 6.5×10^{-3}	(a) 1.3×10^{2}
Total E_3			(g) 6×10^{3}	(g) 2.2×10^{4}	(f) $1.1 \times 10^{-}$	(g) 2.2×10^{7}	(g) 3×10^{3}	(g) 3.3×10^{3}		(a) 8×10^{3}

Levels in children under 10 years of age are: E_2 2×10^{-2}(c)

E_1 = estrone; E_2 = estradiol; E_3= estriol.

(a) Pentikainen et al.,1975; (b) Korenman et al.,1969; (c) Faiman and Winter, 1974; (d) Desfosses et al., 1973; (f) Preedy and Aitken, 1957; (g) Loraine and Bell, 1966; (h) Judd et al., 1974; (k) Powell and Stevens, 1973; (1) Camacho and Migeon, 1963.

TABLE 2.2.

Concentrations in plasma (or serum; ng/ml) and urinary excretion (ng/24h) of androgens in normal humans

	Reproductive females				Pregnancy, last 5 weeks		Postmenopausal		Males	
	PLASMA		URINE							
	Foll.	Luteal	Foll.	Luteal	Plasma	Urine	Plasma	Urine	Plasma	Urine
Unconj.T	(j) 3.1×10^{-1}	(j) 2.9×10^{-1}			(n) 1.0		(h) 2×10^{-1}		(j) 5.1	
T-sulf.	(m) 1.8×10^{-1}								(m) 1.1	(m) 10^{4}
T-Gluc.			(l) 8×10^{3}							(l) 10^{5}
Unconj.DHT	(j) 1.6×10^{-1}						(j) 1.7×10^{-1}		(j) 4.7×10^{-1}	
Androst-enedione	(m) 1.7						(h) 7.5×10^{-1}		(m) 1.3	
DHEA	(m) 5.0								(m) 5.0	

Levels in children under 10 years of age are: T 1.4×10^{-1}(i) and DHT 5×10^{-2}(i)

T = testosterone; DHT = 5 -dihydrotestosterone; DHEA = dehydroepiandrosterone

(h) Judd et al, 1974; (i) Coyotupa et al, 1972; (j) Barberia and Thorneycroft, 1974; (l) Camacho and Migeon, 1963; (m) Bardin and Santen, 1975; (n) Vermeulen et al, 1971.

rapid fall in concentration a constant exponential disappearance rate was observed with half-lives of about 60-90 minutes (Peterson, 1959). Unconjugated steroid hormones in plasma are bound to various proteins, and testosterone (T), for instance, is known to be bound to a different extent and with different affinity to at least three plasma proteins, namely, sex hormone binding globulin, corticosteroid binding globulin and albumin. Plasma levels of these proteins can influence the plasma concentration of T.

Thus the value of a single plasma determination of a steroid hormone represents a momentary balance between several contributing factors which are likely to be of a very complex nature. Here we will be concerned with two of them, the entrance of the hormone into the circulation (plasma pool), and its removal from it.

First, the plasma concentration of a hormone depends partly on its rate of secretion into the circulation by the tissue(s) producing it. Sex steroids, as mentioned earlier, are not solely produced by the gonads; a small fraction of them is produced by the adrenals, and by peripheral conversion of precursors such as androstenedione and dehydroepiandrosterone. This extragonadal fraction is not of significance for plasma T levels in males, or oestradiol (E_2) in females. However, it becomes very significant for T levels in females and E_2 levels in the male where over 50% comes from sources other than the gonads (Baird et al., 1969; Kelch et al., 1972).

This diverse source of origin has led to an approach involving the concept, plasma production rate (PR), which is the net sum of all secretion of the hormone into the blood stream (see later).

Secondly, the plasma concentration depends partly on the rate at which the hormone is eliminated (cleared) from the circulation. Again several mechanisms are involved being mainly metabolism (conjugation) in tissues, and to a smaller extent an excretion by the kidney and the gut, etc. The plasma metabolic clearance rate (MCR) includes all the various secretory and metabolic elimination processes in the overall net clearance of the plasma pool. As for clearance concepts in general the MCR has the dimensions of a volume per unit time, or, in other words, the dimensions of flow (Riggs, 1970).

The relationship between the production rate (PR) the plasma concentration (C) and the metabolic clearance rate (MCR) is expressed mathematically by the equation:

$$PR \equiv C \times MCR$$

If a substance was secreted exclusively into the blood stream by one organ, and eliminated (cleared) exclusively by another excreting organ, then PR of that substance would equal the secretion rate and MCR would equal the clearance of the excretory organ. Thus the secretion rate and the excretion rate of the respective organs would be identical. This probably never strictly applies in practice. Nevertheless the earliest attempts to assess the plasma production rates of sex hormones used radiolabelled tracers and urinary measurements. These methods are called isotopic dilution methods. The principle was originally suggested by Pearlman et al., (1954).

As the sex hormones are eliminated from the circulation as various different metabolites through a number of routes, no single excretory metabolite can represent the plasma production rate. However, this difficulty can be overcome if a radiolabelled dose of the hormone under investigation is injected into the circulation and its recovery assessed in an excreted measureable compound derived uniquely from the plasma pool of that hormone. These isotope dilution techniques have been widely used to measure production rates of oestrogens (Fishman et al., 1962; Gurpide et al., 1962; Vande Wiele et al., 1968) and testosterone (Korenman et al., 1963; Hudson et al., 1966). The urinary metabolites used were the glucuronides. The first indication that this technique might not be valid for androgens was provided by Korenman and Lipsett, (1964), who demonstrated that testosterone was synthesized in a tissue compartment from plasma dehydroepiandrosterone and androstenedione, and was conjugated before re-entering the plasma. This finding thus indicated that testosterone glucuronide was not uniquely derived from plasma testosterone, and that the urinary isotope dilution technique overestimated the testosterone blood production rate in humans, especially in the female where over 50% of the plasma testosterone is derived from precursors.

The validity of the estimation of blood production rates involving urinary metabolites is dependent upon various assumptions concerning methodology, pathways, and sites of metabolism. Consequently, the physiological significance of the values obtained by this method suffers from uncertainty. A second approach to the determination of production rates was the steady state infusion technique

introduced into the steroid field by Tait and his co-workers (Tait and Burstein, 1964; Baird et al.,1969). In this procedure an infusion of the isotopically labelled hormone is carried on until a steady state is reached. At steady state the equation, presented above, applies and from the infusion rate and the radioactive concentration of the infused hormone the MCR is calculated. When the MCR is known the endogenous blood production rate is calculated from the endogenous concentration in blood (or plasma) and the MCR.

The production rates obtained by this technique agree reasonably well with the results of the isotope dilution method in many cases, but, as mentioned earlier, PR of testosterone in women was overestimated by the latter method and is about 3 - 4 times lower with the infusion technique. So it would seem that the infusion technique would be more reliable in cases where a plasma hormone is to a large extent produced by peripheral precursors.

The sources of individual hormones are best studied by arteriovenous concentration differences across organs. Such studies have provided useful information about the contribution of individual glands to the plasma pool. Thus adrenal vein concentrations of testosterone and oestradiol in normal men and women are only slightly above peripheral concentrations, suggesting that little of these steroids arose from the adrenal gland (Baird et al.1969). Plasma testosterone levels fall markedly in men following castration (Gandy and Peterson,1968),and oestradiol levels are low in castrated and postmenopausal women suggesting that the respective gonads are the major source of these hormones in man. More direct evidence

for testicular contribution to androgen and oestrogen levels in blood was obtained by Kelch et al., (1972) and Pazzagli et al., (1974). who studied hormone levels in the spermatic vein versus peripheral plasma. The contribution of the ovaries to plasma androgens and oestrogens in humans was similarly studied by Lloyd et al., 1971.

5α -dihydrotestosterone (DHT) in males stems mainly from peripheral conversions of testosterone (60%) and androstenedione (15%). DHT in females derives almost totally from peripheral conversion - 88% from androstenedione and 7% from testosterone (Bardin and Santen, 1975).

Table 2, 3 shows some blood production rates in men and women. The oestradiol values are from Baird et al., 1969 and the androgen values from Bardin and Santen, 1975. Dehydroepiandrosterone production rates are taken from Kono, 1966.

2.3.2. Function tests.

The so-called suppression and stimulation tests have been in common clinical use for adrenal diseases for the last decade. With the recent advances in sex hormone assays such functional tests are becoming possible in gonadal disorders. The tests used so far have mainly involved stimulation tests with trophic hormones, such as human chorionic gonadotrophin (HCG) and human menopausal gonadotrophin (HMG). These agents have been used to treat infertility in women and a preliminary test involving administration of these agents and subsequent assessment of the urinary oestrogen response has been used to select patients for the treatment (Gemzell and Johansson, 1971).

TABLE 2.3.

Blood production rates of some sex steroids (ug/day)

Steroid	men	women	
Oestradiol	40	F	81
		L	270
Oestrone	150	F	110
		L	243
Testosterone	7000		250
Dihydrotestosterone	343		41
Androstenedione	2250		2600
Dehydroepiandrosterone	7000		7000

F = follicular phase;
L = luteal phase.

In males HCG has been used to assess Leydig cell function by measuring urinary or plasma T levels after its intramuscular injection and more recently after its intravenous administration. (Oertel and Eik-Nes, 1959; Lipsett et al., 1966; Maurer et al., 1973).

The drug clomiphene citrate (1(p-(β-di-ethylaminoethoxy) phenyl) - 1, 2-diphenyl-2-chloroethylene citrate), originally identified as an anti-oestrogen, has been used successfully to stimulate ovarian function. It was introduced into clinical use by Greenblatt et al., (1961) for the treatment of anovulatory disorders. Oral administration results in increased plasma E_2 levels in normal women (Robertson et al., 1971), and increased plasma T levels in normal men (Bardin et al., 1967; Marshall et al., 1972), but not in hypopituitary patients. Blood levels of LH and FSH are increased in both sexes during clomiphene administration, but prolactin levels are probably unaffected (Kjeld et al., 1975). While clomiphene could thus have its primary effect at the hypothalamic level, its exact mode of action remains obscure.

The recently available luteinizing hormone-releasing factor (LHRH) may be of some help in elucidating disorders of the hypothalamic-pituitary-gonadal axis. Increased plasma T levels in normal men have been reported after intravenous or intramuscular administration of LHRH (Kley et al., 1974; Judd et al., 1974). The use of LHRH administration as a diagnostic tool in various clinical disorders is presently under intensive investigation.

S E C T I O N 2

methodology

CHAPTER 3

SUMMARY

In this section the backbone of the methodology of the thesis is presented. The radioimmunoassays (RIAs) evolved have been specially designed so as to reduce sample size, labour and consumption of materials without sacrificing sensitivity or precision. Individual methods are described first and the RIA of testosterone is described in detail as the prototype of the assay procedures. Descriptions of other assays are shorter with emphasis on particulars relating to the respective assays.

Normal ranges obtained by the methods are given following their description, and, for testosterone, a group of hirsute women are included.

Special methods, involving, for instance, a particular chromatography procedure or synthesis of compounds, will be dealt with in the appropriate chapter.

Materials, unless specially described elsewhere, are listed in the second part of this chapter together with their sources.

3.1. METHODS

3.1.1. Testosterone in serum ("direct assay"; without chromatography)

Reagents

Solvents

All solvents were Analar grade and used without further purification.

Thin layer chromatography (TLC)

TLC was done on aluminium sheets (20 x 20cm) precoated with Silica gel containing fluorescent indicator (Merck, art. no. 5554). Unless otherwise stated the solvent system used for testosterone (T) and 5α dihydrotestosterone (DHT) was cyclohexane: ethyl acetate (3:2, V/V).

Buffers

Two buffer solutions were used differing in their bovine serum albumin (BSA) content:

PO_4-buffer, 0.1M, pH 7.5		and	Working buffer (0.2% BSA)	
KH_2PO_4	4.5g		BSA	0.1g
K_2HPO_4	11.6g		PO_4-buffer	100ml
Merthiolate	0.1g			
BSA	1.0g			
NaCl	9.0g			
Dist. H_2O	1000ml			

Dextran-Charcoal solution

This reagent was made of the following constituents:

Norit A, activated charcoal	250mg
Dextran T-70	25mg
PO_4-buffer	100ml

Constituents were added to a stoppered glass bottle which was shaken until the dextran was dissolved and then cooled to $4^{o}C$.

Steroids

T was obtained from Sigma and DHT from Steraloids. Both steroids were subjected to rigorous purity tests by replicate RIA before and after TLC. 5α-androstane-3β, 17β diol, 5α-androstane-3α, 17β-diol, androstenedione, 17α-testosterone (epitestosterone) and 5β-androstane-3α, 17β-diol were each assayed for T and DHT, and found to contain less than 0.5% of either.

T standards

20-30mg of T were weighed in glass vials and dissolved in methanol to give a concentration of 2.5mg/ml. This solution is diluted with methanol to a concentration of 25ng/ml.

Of this solution 0.4, 0.3, 0.2 and 0.2ml are pipetted into four glass vials and dried under gentle air stream. The residues of the first three vials are dissolved in 5ml of working buffer, but the fourth one in 10ml. This constitutes four standard solutions of 200, 150, 100 and 50pg/0.1ml. Two further standards, 25 and

10pg/0.1ml are made by diluting the 50pg/0.1ml standard two and five times, respectively.

These standard solutions are stored at 4°C when not in use, and will then keep for at least six months.

Radioligand (labelled T; T*)

(1, 2, 6, 7(n) - 3H) testosterone (T*) from the Radiochemical Centre, specific activity (SA) approximately 100Ci/mmol, was diluted in ethanol to a radioactive concentration of 50uCi/ml. This preparation is kept at 4°C as a stock solution. When tested on TLC this T* has been found to be of satisfactory purity (≃ 98%) on purchase and was 95% pure after 6 months' storage as above. The stock solution is diluted 5 times, and from this latter solution the working solution was made by 100-fold dilution in working buffer to give approximately 7,000 counts per min (CPM)/0.1ml. This last solution is referred to as the T* solution.

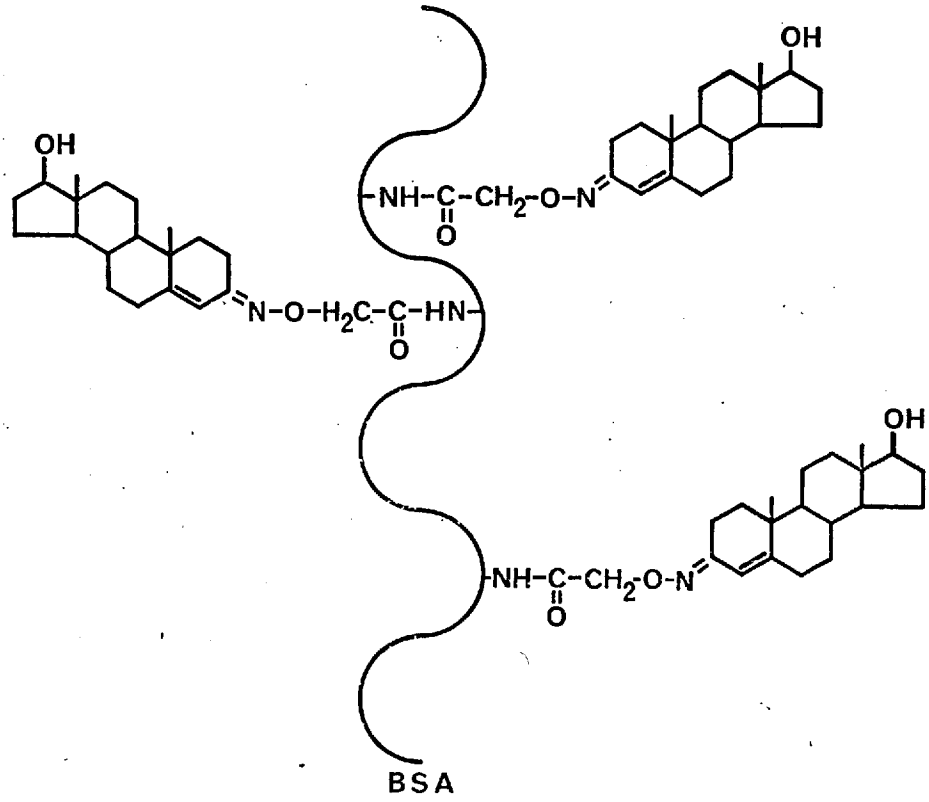
Internal standard solution

This was prepared by a ten-fold dilution of the T* solution and was added to samples to correct for procedural losses of T.

Antiserum

The testosterone (T) antigen, T-3-carboximethyl oxime bovine serum albumin (T-3-CMO-BSA; see Fig. 3.1.) was prepared by the method of Erlanger et al. (1957). Using U.V. spectra and respective optical densities (Erlanger et al., 1957) it was estimated that about 34 molecules of T were coupled to each BSA molecule.

Fig. 3.1.



A schematic presentation of the antigen, testosterone-3-carboxymethyl-oxime-bovine serum albumin (T-3-CMO-BSA), prepared to raise testosterone antisera.

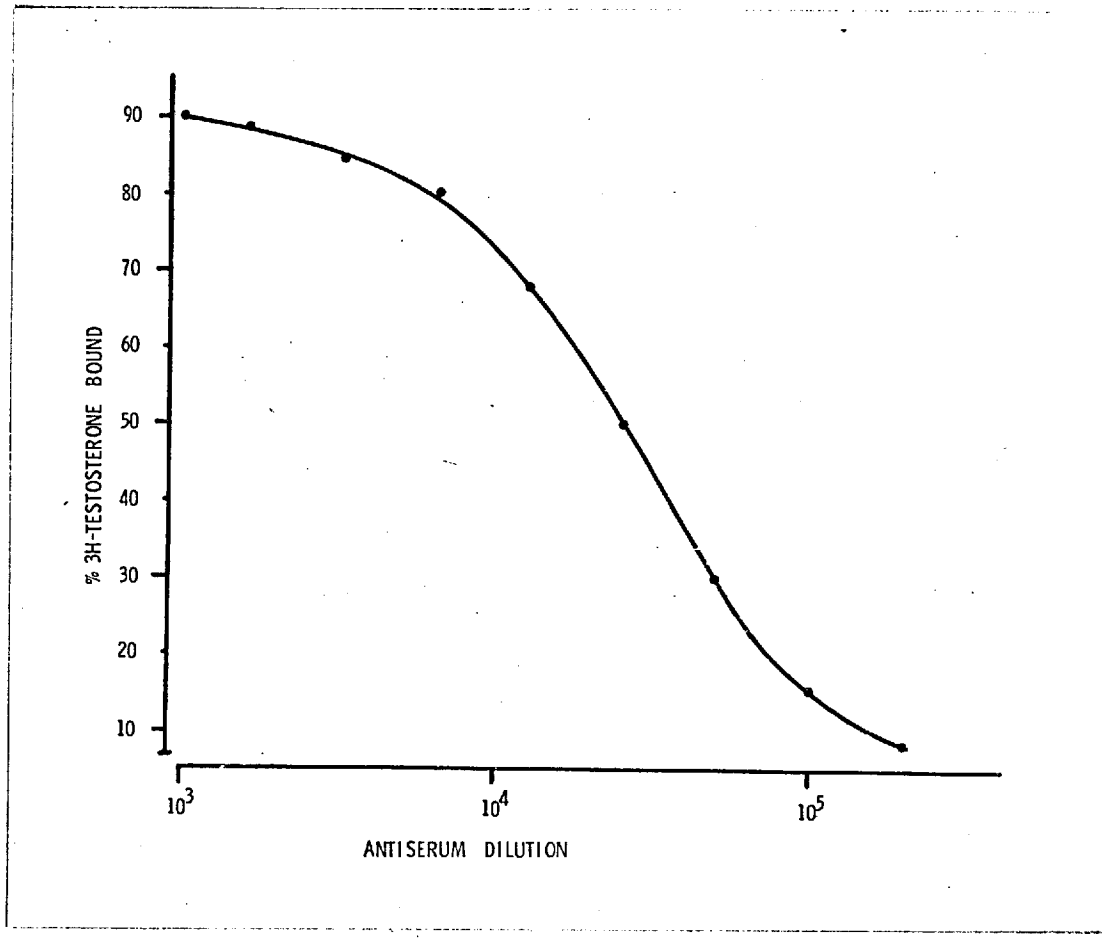
Antisera were raised in male New Zealand rabbits by i.m. injection. The antigen was dissolved in isotonic sodium chloride solution and an equal amount of complete Freund's adjuvant added, and made to a fine emulsion by mixing. 2mg of antigen were injected at multiple sites each time, first at weekly intervals for 4 weeks, and then once a month. Four months after the first injection two out of three rabbits so treated had developed antisera that could be used for assay, a dilution of about 1:20,000 giving roughly 50% binding of T* (see Fig. 3.2.). Antisera were diluted 1:5 in working buffer and stored at -20°C. In this stock solution, thus stored, the antiserum has been found stable (no loss in T* binding affinity), for at least two years. The stock solution was further diluted 1:20 and aliquotted in anacups, which were kept frozen until used.

The specificity of the antiserum was investigated by measuring the displacement of T* caused by increasing masses of various steroid compounds more or less structurally related to T. The results are shown in Fig. 3.3. Table 3.1. shows the calculated cross-reaction of these steroids and of T-sulphate and T-glucuronide, which showed negligible displacement of T* when 0.1 - 10ug were added. 5α -dihydrotestosterone (DHT) cross-reacts 66%, and this affinity of the antibody for DHT was exploited in the DHT assay described below.

Radioactivity measurement

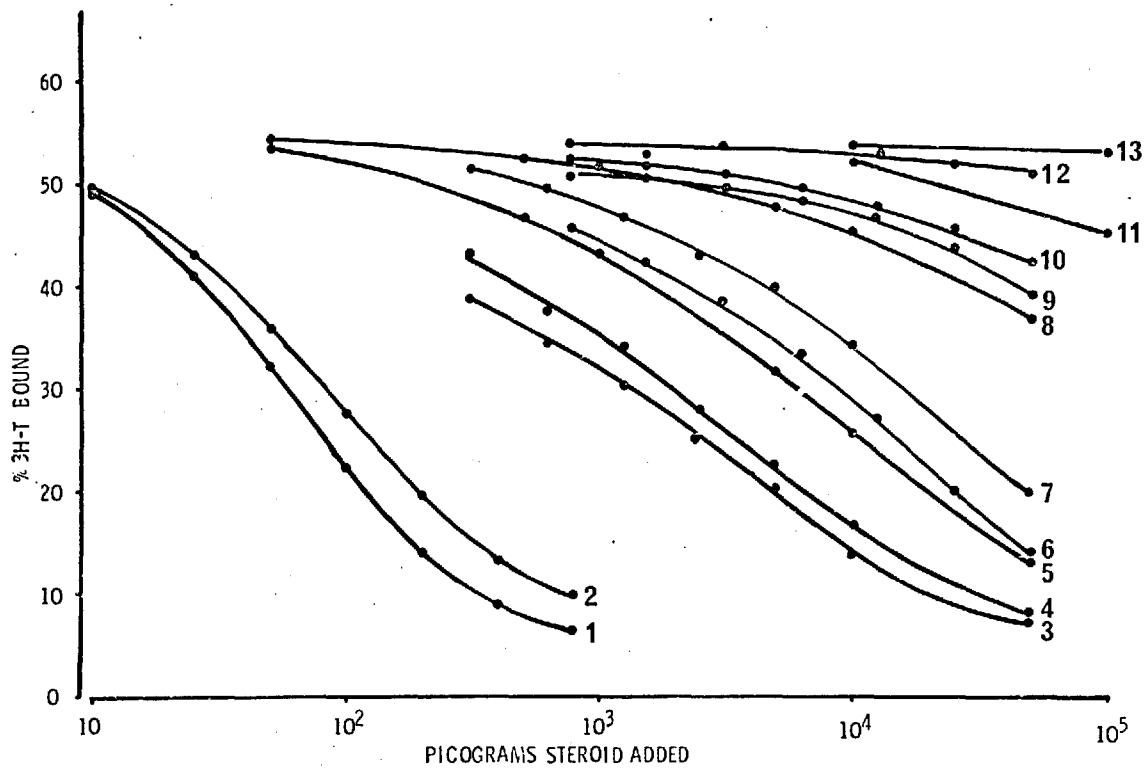
Aliquots to be counted were put into disposable plastic vials (counting vials) and 10ml of scintillator fluid added. The scintillator fluid was prepared by dissolving 9.6g of

Fig. 3.2.



A dilution curve for the testosterone (T) antiserum showing the per cent binding of tritiated testosterone (3H-Testosterone, T*) (ordinate) with increasing dilution of the antiserum (abscissa). The amount of labelled T was 6100 counts per min (CPM) or about 27 pg. The antiserum dilution (abscissa) is the dilution before the addition of the antiserum to the assay tubes. The final dilution is three times greater.

Fig. 3.3.



The comparative ability of increasing amounts of various steroids (abscissa) to reduce the per cent binding of tritiated testosterone, 3H-T (ordinate), to testosterone antibodies raised against T-3-CMO-BSA.

1	testosterone (T)	8	androsterone
2	5α dihydrotestosterone	9	testosterone acetate
3	5α-androstane-3β,17β-diol	10	dehydroepiandrosterone
4	5α-androstane-3α,17β-diol	11	estradiol
5	androstenedione	12	cyproterone acetate
6	17α-testosterone (epi-T)	13	cholesterol
7	5β-androstane-3α,17β-diol		

TABLE 3.1.

Cross-reaction of various steroids with anti-testosterone-3-CMO-BSA antiserum. Where applicable the cross-reaction has been calculated according to the method of Abraham et al.,(1970) where the amounts of T and other steroid (S) causing 50% displacement are used for calculation; thus $\frac{T}{S} \times 100 = \%$ cross-reaction.

Name of steroid	% cross-reaction
1. Testosterone	100
2. 5α -dihydrotestosterone	66
3. 5α -androstane-3β , 17β diol	3
4. 5α -androstane-3α , 17β diol	2
5. Androstenedione	0.7
6. 17α -testosterone (epitestosterone)	0.5
7. 5β -androstane-3α ,17β -diol	0.3
8. Androsterone	< 0.1
9. Testosterone acetate	< 0.1
10. Dehydroepiandrosterone	< 0.1
11. Estradiol	< 0.1
12. Cyproterone acetate	< 0.01
13. Cholesterol	< 0.01
14. Testosterone sulphate	< 0.01
15. Testosterone glucuronide	< 0.01

2,5-diphenyloxazole (PPO) and 0.12g of 2-(1-naphtyl)-5-phenyloxazole (α-NPO) in 1600ml of toluene and adding 800ml of Triton X-100. An automatic liquid scintillation spectrometer, Intertechnique SL36, was used for counting. This instrument had automatic background subtraction and a thermostatic cooling system (4°C) for the sample cabinet and photomultipliers. Samples were cooled at 4°C in the dark for a minimum of 60 min and then counted for a total of 10000 counts. The counting efficiency in this system was approximately 35%.

Glassware

The centrifuge tubes used for ether extraction and other non-disposable glassware were soaked overnight in Divo-Lab No.1 detergent, washed in warm water and left overnight in dilute hydrochloric acid (0.01M). They were then washed twice in warm tap water and finally two times in distilled water. Bacteriological tubes were used without washing and disposed of after use.

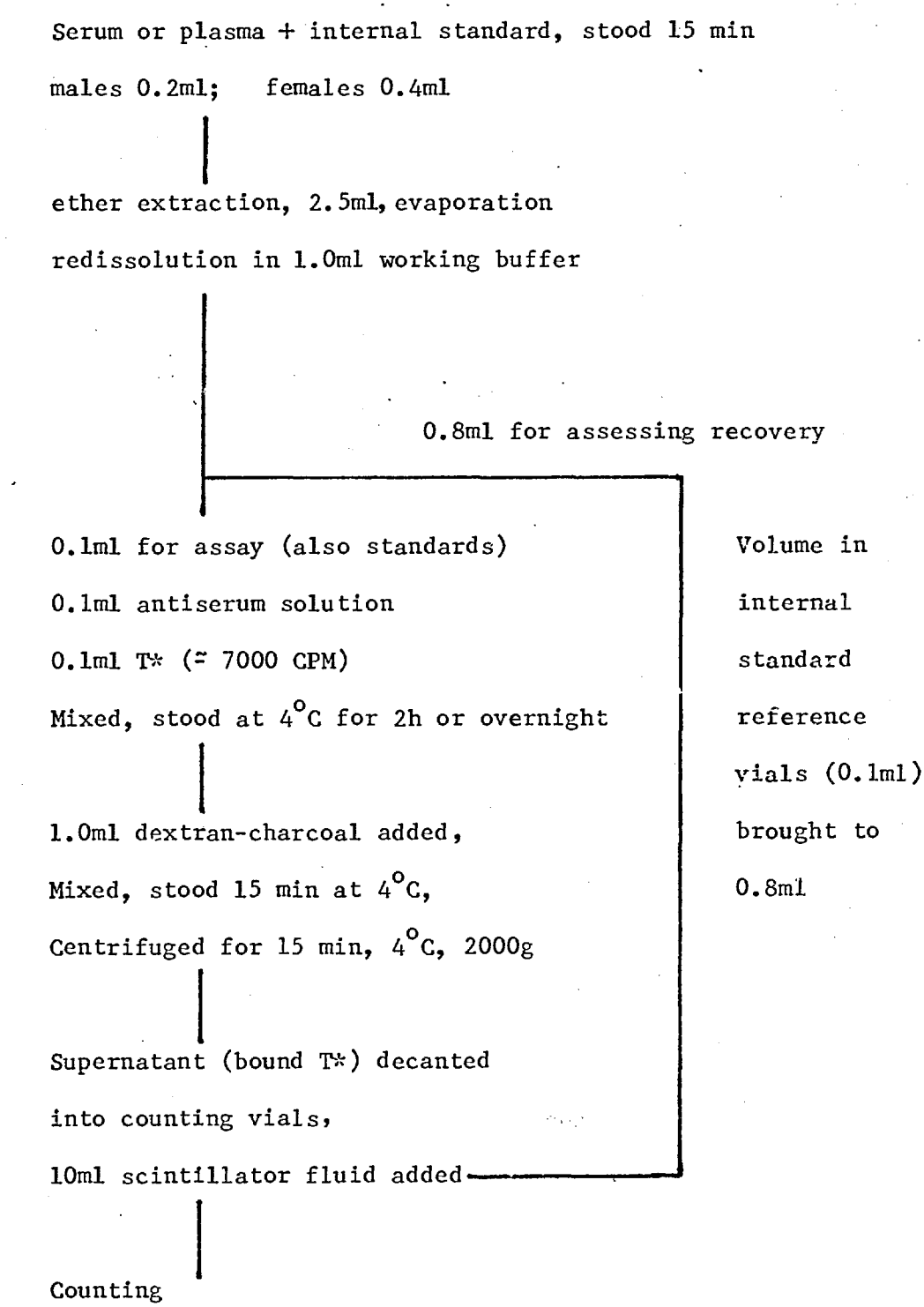
Method for serum or plasma

For a quick appraisal of volumes and steps involved in the method a flow chart is given on the next page.

Assay procedure

Serum or plasma samples, 0.2ml for males and 0.4ml for females, were put into conical rimless centrifuge tubes (12ml capacity). 0.1ml of internal standard (I.S.) solution was added to each tube and to three counting vials which served for reference when assessing

A flow diagram for serum T determination by radioimmunoassay



recovery. The centrifuge tubes were shaken and left at room temperatue for 20 min. Two water blanks, and high and low value serum pools, each in duplicate, were included in every batch of the assay. Diethyl ether, 2.5ml, was now added by an automatic dispenser to the tubes, which were then shaken on a vortex mixer for 20 seconds. The ether was transferred to rimless bacteriological tubes (12.5 x 50mm) which were placed in a thermostatic block at 40 - 45°C.

The dried extract was redissolved in 1.0ml of working buffer by vortex shaking for 15 seconds. Of this solution 0.1ml was transferred to disposable plastic tubes (Luckham LP3) and 0.8ml to counting vials, to which 10ml scintillator fluid was added. The scintillator fluid was also added to the I.S. reference vials after their content (0.1ml) had been brought to 0.8ml by working buffer. These vials were shaken to mix the content and counted to assess recovery of extraction.

The standard solutions, 10-200pg/0.1ml, were now added in duplicate to LP3 plastic tubes. Working buffer, 0.1ml, was added to a further four tubes, two for zero binding (no unlabelled T) and two for assessing the total radioactivity added to each tube (see below). To the LP3 plastic tubes, standards and unknowns (see above), 0.1ml of an appropriate dilution of the antiserum was then added followed by 0.1ml of the T* solution. The tubes were shaken briefly on a rotamixer and left at 4°C for at least 2 hours or, generally, overnight.

The next step was the separation of bound and unbound T* (and T). The dextran-charcoal solution was kept in homogeneous suspension on a magnetic stirrer and 1.0ml of it added to each tube except the "total radioactivity" tubes, to which 1.0ml working buffer was added instead. The tubes were left for 15 min at 4°C, and then centrifuged,

at 4°C, at approximately 2,000g for 10 min.

After centrifugation the supernatant was decanted into counting vials to which 10ml of scintillator fluid was added. The vials were shaken briefly and counted.

Standard curve and calculation of results.

A typical standard curve is shown in Fig. 3.4. The standard curves were generally plotted as CPM. A standard of 400pg can be included but sensitivity will diminish for higher readings of the curve.

After obtaining readings from the standard curve and the recovery fraction, calculated from the internal standard reference vials, the final results were expressed as ng/ml and calculated thus:

$$T, ng/ml = \frac{\text{reading of unknown} - H_2O \text{ reading}}{\text{Recovery fraction}} \times 1/20; \ (\times 1/40, \text{ females})$$

Characteristics of the method

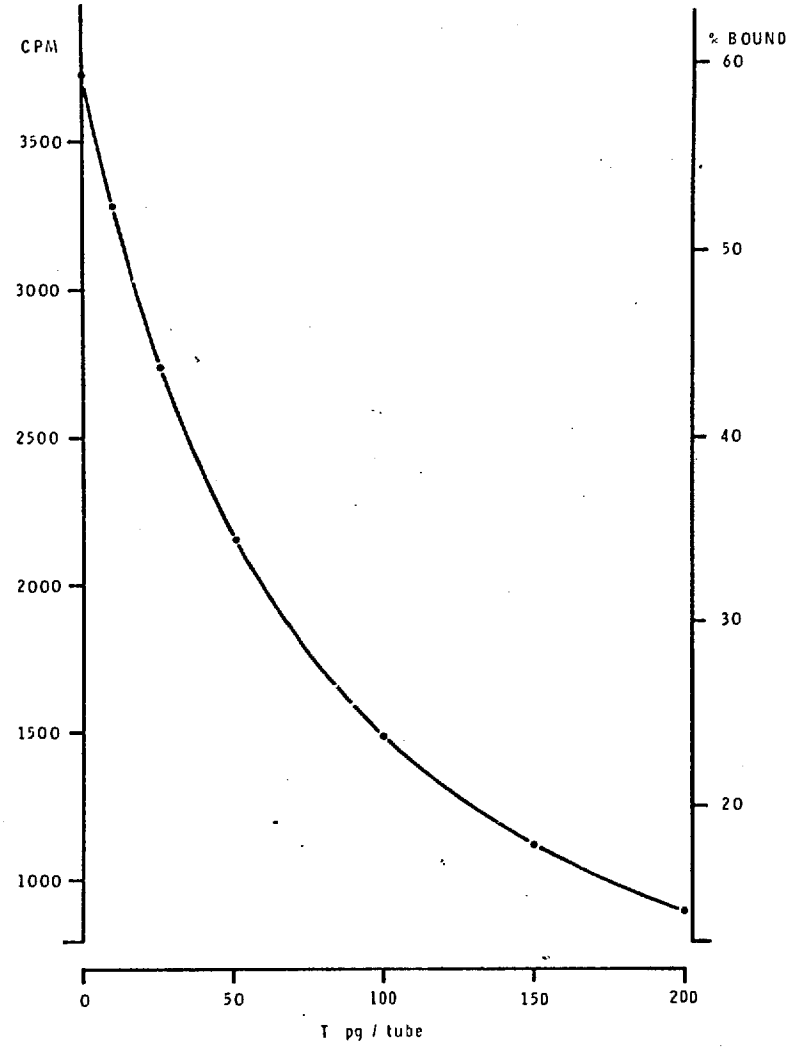
Serum versus plasma

Serum and plasma (heparinized) samples were obtained from one bleeding from 3 males and 3 females. The samples were measured and plasma and serum from each individual gave identical results.

Water blanks and detection limits

In 25 assays the reading of the water blank (reagent blank) was 0.83 ± 0.93 ($\bar{x} \pm$ SD) with a range of 0 - 3.0pg. In 18 of these assays it was <1.0. As the within assay variation of blank values was always smaller, 2 - 3pg should be detected with 95% confidence using the volumes described.

Fig. 3.4.



A standard curve in the testosterone (T) assay. Curves were generally plotted as counts per min (CPM) on the ordinate but the corresponding per cent binding is shown on the right hand side.

Precision

The reproducibility of the standard curves was assessed by comparing values for duplicates of standards in 10 assays. The lowest standard, 10pg, had the highest coefficient of variation (CV) of 3.39%.

Twelve replicate assays of a male sample in one assay batch showed 5.53 $\pm$ 0.28ng/ml or a CV of 5.2%. The between assay variations was estimated from two serum pools run through every assay (n = 25). The values obtained were 6.69 $\pm$ 0.60 and 2.15 $\pm$ 0.32 with CV of 11 and 14%, respectively.

Recovery and accuracy

The recovery of the internal standard was 88.9 $\pm$ 3.25%. Unlabelled T added to both serum and water in amounts up to 8ng was recovered 100% after internal standard correction had been applied. The accuracy signifies the real quantity of the compound measured (T) in the medium, and is thus related to the specificity of the method. As no specific methods are available to remove testosterone from serum so that blank values could be assessed, the unspecific blank of serum measurements is better judged by values from individuals likely to be low in serum T, such as postmenopausal women, castrated individuals, and children (see below).

Specificity

The relative affinity of the T-antiserum for various steroids has already been shown (Fig. 3.3., Table 3.1.). DHT is the only related steroid to cross-react more than 3%. However, high

concentrations of steroids which crossreact only slightly could interfere significantly in the assay. A TLC step was therefore included in the assay after the ether extraction. The method is the same as described below in the combined T and DHT assay, and separates DHT and, less completely, the androstanediols from testosterone. When samples from nine healthy males were measured, with and without chromatography, values of 5.53 ± 1.27 and 6.32 ± 1.31ng/ml were obtained respectively. Thus, as expected, the method is not specific for testosterone. The average difference of 0.79 is, however, largely accounted for by DHT which in the same samples had an average value of 0.67ng/ml, and would be expected to account for 0.4 - 0.5ng/ml in the assay without chromatography.

For female samples the chromatography step causes a greater reduction in values. When chromatography was included in the measurement of samples from 11 normal females an average value of 0.208ng/ml was obtained as compared to 0.55ng/ml for 20 normal females without chromatography (Table 3.2.). Although DHT in females is about 0.1ng/ml and so could account for a substantial part of the discrepancy, there seems to be relatively more of substances capable of interfering in the assay in female serum than in the male one.

Application and Comments

The method described is similar to that described by Collins et al. (1972), with several modifications. Volumes of ether for extraction are smaller and quickly evaporated. Keeping the standards in a buffer solution for ready use greatly speeds up the assay and should increase inter-assay precision. The recoveries of the internal standard were highly reproducible and can therefore be left out, except in

TABLE 3.2.

Serum T_k (ng/mℓ) values obtained by the radioimmunoassay method for T with and without chromatography

Blood was withdrawn 8 - 11 a.m.

	Females, healthy reproductive age irrespective of phase of cycle	Females, healthy postmenopausal	Females, hirsute 15 - 42y	Males, healthy 25 - 46y	Children, boys with asthma, 8 - 11y
	W I T H O U T		C H R O M A T O G R A P H Y		
$\bar{x} \pm$ SD	0.55 ± 0.17		1.0 ± 0.38	6.35 ± 1.33	0.19 ± 0.08
range	0.2 - 0.84		0.45 - 1.87	3.5 - 9.3	0.1 - 0.3
n	20		29	20	11
	W I T H		C H R O M A T O G R A P H Y		
	(foll.phase)				
$\bar{x} \pm$ SD	0.21 ± 0.05	0.2 ± 0.08	0.85 ± 0.48	5.29 ± 1.32	
range	0.13 - 0.30	0.10 - 0.33	0.25 - 1.64	4.0 - 7.7	
n	11	7	7	14	

the reference pools, when the assay is done for routine clinical purposes. With these modifications up to 60 samples can be processed by one technician in a single day.

The normal values, given in Table 3.2., are similar to theirs without chromatography and, for males, about 20% higher than the values reported by them and others using chromatography prior to RIA (Coyotuba et al. 1972; Barberia and Thorneycroft, 1974).

The assay has been in clinical use for over 2 years and proved of help in gonadal and adrenal disorders. In two out of three female cases with adrenal carcinoma and elevated plasma cortisol, serum T levels were above 4.0ng/ml. In primary or secondary male hypogonadism values have invariably been below the normal range. Two castrated males, untreated, with carcinoma of the prostate had values of 0.15 and 0.22ng/ml.

The circadian and episodic fluctuation of serum T levels in males by this method will be the subject of a special study in Chapter 7. Chapter 6 discusses serum T levels in females as small circadian and episodic fluctuations in females have been described (Rosenfield and Helke, 1974). Reports on variations of plasma or serum T levels during the menstrual cycle have been conflicting, finding no rise (Dupon et al. 1973; Kato and Horton, 1968) or small but significant rise during the midcycle (Goebelsmann et al., 1974; Epstein et al., 1975).

3.1.2. Simultaneous assay of unconjugated T and DHT in serum or urine

General

This assay is similar to the one for T above except for the inclusion of the thin layer chromatography (TLC) step and larger volumes of samples needed. Same reagents were used and, additionally,

tritiated DHT (DHT* or 3H-DHT; SA 47 Ci/mmol) which, prepared as T* above, was used as a radioligand in the DHT assay and as internal standard to assess recovery of DHT. DHT standard solutions were prepared as T standards above. Judged by cross-reaction studies the T-antiserum had a relative affinity of 66% for DHT (Fig. 3.3.). Approximately 50% binding of DHT* was obtained at antiserum dilutions of 1:5,000. A standard curve from DHT standards was plotted in an identical fashion as for T (Fig. 3.4.).

A flow diagram of the method is presented on the next page and the only part of the methodology that needs to be described in this chapter is the TLC step.

Thin layer chromatography (TLC)

Silica gel precoated on aluminium foils (20 x 20cm) was used (Merck, art. no. 5444). The ether extract of the samples was redissolved in 100µl of acetone and applied to the plates followed by a further 100µl acetone wash of the bacteriological tubes in which the ether had been evaporated. T and DHT markers (5 - 10mg) were applied on the margin of each sheet, spaced 2.5cm from the samples. The TLC sheets were run in universal chromatank (Shandon, in cyclohexane : ethylacetate (3:2 V/V) until the solvent front reached the upper edge (100 - 120 min). After drying, the margin of the sheets, containing the markers, was cut off and T and DHT visualized by spraying with a 5% solution of molybdophosphoric acid in methanol. The Rf values, in this system, for T and DHT, were 0.24 and 0.4 respectively. The T and DHT zones were now cut out and then the individual samples. These were eluted from the plates with diethyl ether (3ml), which was transferred to bacteriological tubes, evaporated, and the residue redissolved in 1.0ml and 0.5ml of working buffer for T and DHT respectively. For T

A flow diagram for the method of simultaneous T and DHT determination in serum or urine

Serum; males 0.5ml, females 1.0ml
Urine; males 2.0ml, females 2.0ml
Internal standards (T* and DHT*) added
Stood for 30 min at room temperature

↓

Diethyl ether extraction, 5 volumes,
evaporation, redissolution in acetone,
0.1 ml x 2 applied on TLC plates

↓

After run, TLC plates cut, eluted with ether,
evaporated, redissolved in buffer, T 1.0ml, DHT 0.5ml

↓

aliquots for recovery estimation,
0.8ml (T) and 0.25ml DHT (volume
brought up to 1.20ml with buffer

↓

0.1ml (T), 0.2ml (DHT) for assay (also standards for T
and DHT assays; 0.1ml buffer added to DHT standard tubes)
0.1ml antiserum (dil. ~ 1:15,000 for T, 1:60,000 for DHT)
0.1ml T* and DHT* to respective set of tubes,
mixed, stood overnight at $4^{o}C$

↓

1.0ml dextran-charcoal added,
mixed, stood 15 min at $4^{o}C$
centrifuges for 15 min at $4^{o}C$, 2000g

↓

Supernatant (bound T* and DHT*) decanted
into counting vials, 10ml scintillant added

↓

Counted

Volumes of internal standard reference vials brought up to 1.2ml

and DHT 0.1 and 0.2ml were taken for the assays and 0.8 and 0.25ml for recovery determinations respectively.

Characteristics of the method

Water blanks and detection limits

In 12 assays the water blank readings were 2.9 $\pm$ 2.2 (0 - 8) and 5 $\pm$ 3.3 (0.2 - 11) pg for T and DHT respectively. Accordingly detection limits at the 95% confidence level are about 5 and 7pg for T and DHT respectively. Within assay blank values had a lower scatter. Calculated from blank duplicates in the assays above the mean difference and its S.D. were 2.2 $\pm$ 1.7 (T) and 2.63 $\pm$ 1.6 (DHT); that is, considerably smaller variances.

Precision

Reproducibility of the DHT standard curve was similar to the standard T curve described above. Precision for the urinary T and DHT measurements was assessed by running a male urine sample through 9 assays and by measuring it 10 times in the same assay batch. Results were respectively 0.296 $\pm$ 0.044 (CV = 14.8) and 0.290 $\pm$ 0.018 (CV = 6.2) ng/ml for T and 74.2 $\pm$ 14.3 (CV = 19.2) and 67.5 $\pm$ 6.2 (CV = 9.2) pg/ml for DHT. A urinary pool with high DHT concentration was prepared and run through 12 assays. Values obtained were 694 $\pm$ 72 (CV = 10.3) pg/ml.

A male serum sample was run through 8 consecutive assays and values for T and DHT were 2.56 $\pm$ 0.22 and 0.365 $\pm$ 0.041 ng/ml respectively.

Recovery

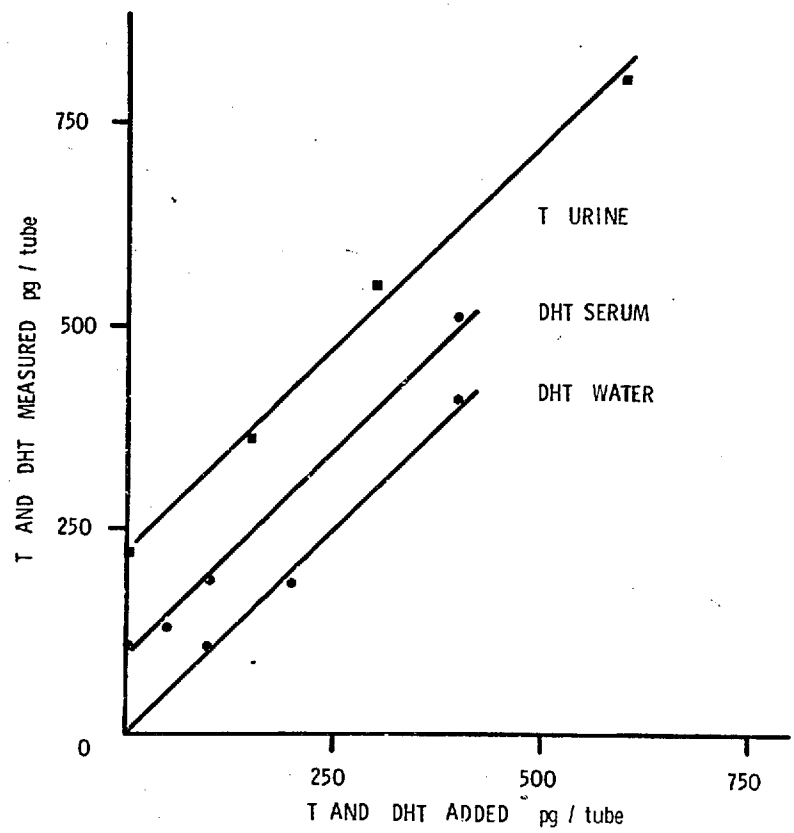
Recovery of the internal standards was 67.3 ± 5% and 61.5 ± 8% for T* and DHT* respectively. Unlabelled T and DHT were added to urine and serum as well as water and the recovery (corrected by internal standard) was approximately 100% in all cases. Fig. 3.5. shows the recovery of T from urine and DHT from serum and water.

Specificity

As described under the T method above the antiserum is highly specific for T and DHT. However, to distinguish between the two, a chromatography step is needed. The chromatography system used gave a good separation with Rf values of 0.24 and 0.4 for T and DHT respectively. The three 5-dihydro-diols, which were tested for cross-reaction with the antibody, had Rf values of 0.27, 0.25 and 0.14 for the 5α-3α, 5α-3β and 5β-3α configurations respectively. Thus the chromatography system separated from DHT all the steroid compounds known to cross-react more than 1% with T against the antiserum. The separation of the 5 diols from T was, however, less adequate, but of lesser significance, because of the relatively low affinity of these compounds for the antiserum, compared to T.

Urinary unconjugated T was measured in 10 samples with and without TLC. Values obtained without TLC were 30 - 50% higher than when TLC was included. However, this could be largely accounted for by urinary unconjugated DHT (see Chapter 6). When the TLC plates were run twice in the same solvent system, thus better separating compounds with similar Rf values (when $Rf < 0.5$), T and DHT values for both serum and urine were the same as after a single run.

Fig. 3.5.



Recovery of various amounts of testosterone (T) and 5α-dihydrotestosterone (DHT) when added to serum, urine or water.

A further test of specificity was done by running 8 urinary samples in two consecutive TLC systems, the system described above followed by chloroform: water (95:5) which effectively separated the androstanediols from T and DHT. Results obtained by this consecutive double run were about 4% lower on average but not significantly different from those obtained with the standard system described above.

Application and comments

Urinary measurements are dealt with in Chapter 6.

Serum T values obtained with the above method for normal men, women in the follicular phase of the menstrual cycle, postmenopausal and hirsute women are shown in Table 3.2. Values, except for DHT in females, agree reasonably well with those obtained by comparable methods. Table 3.3. shows normal values reported by 6 groups of workers including the present ones (morning values, see Chapter 6).

Table 3.3.

Normal values (ng/ml) in males and females (serum T and DHT) obtained by 6 groups of workers

n	Males T	Males DHT	n	Females T	Females DHT	Ref.
12	5.15 ± 1.72	0.50 ± 0.14	12	0.42 ± 0.1	0.22 ± 0.04	(a)
14	5.29 ± 1.32	0.57 ± 0.17	11	0.194 ± 0.05	0.09 ± 0.04	(b)
20	5.14 ± 1.2	0.47 ± 0.17	10	0.307 ± 0.1	0.16 ± 0.05	(c)
16	4.9 ± 1.6	1.3 ± 0.8	18	0.22 ± 0.07	0.34 ± 0.17	(d)
7		0.36 ± 0.08	5		0.19 ± 0.06	(e)
12	5.73 ± 1.9		10	0.53 ± 0.1		(f)

(a) Trembley et al., 1970; (b) Present work; (c) Barberia and Thorneycroft, 1974; (d) Coyotupa et al., 1972; (e) Bauminger et al., 1974; (f) Collins et al., 1972.

Serum DHT levels in 6 postmenopausal women were 118 $\pm$ 79.4 pg/ml. Although measured in few individuals the values obtained for post-menopausal women do not indicate a real difference from normal reproductive women. Coyotupa et al., (1972) reported postmenopausal levels slightly higher than those in females of the reproductive age, while Barberia and Thorneycroft (1974) reported slightly lower levels. Judd et al. (1974) reported a mean T value of 0.198 ng/ml in peripheral blood of postmenopausal women and a 15-fold higher one in the ovarian vein indicating a high production of T by the post-menopausal ovary.

The group of 29 hirsute females is a heterogeneous one consisting of both the polycystic ovaries syndrome and idiopathic hirsutism of different degrees of severity. In agreement with other workers there is a considerable overlap of serum T values of the hirsute group with those of normal women (Kirschner and Bardin, 1972). While the mean values of samples from normal females and the hirsute ones are significantly different ($p < 0.001$), both with and without chromatography, there seems to be little to choose between the two methods as regards discerning between the two groups of normal and hirsute females. The 7 hirsute females whose T values were measured by chromatography had DHT values of 0.13 $\pm$ 0.05 ng/ml indicating that serum DHT values are similarly an imperfect parameter to differentiate between the two groups.

In the above series the definition of hirsutism has been excessive facial and body hair, where the patient had facial shaving at least once a month. As there is of course no sharp clinical demarcation line between normal and slighly hirsute females a perfect chemical parameter differentiating between the two groups is probably not to be found. It might be more pertinent to correlate the serum androgen levels with the severity of the hirsutism using, for instance,

the clinical index proposed by Ferriman and Gallway (1961). Although such detailed correlation was not undertaken in the present series of hirsutism, such correlation was nevertheless strongly indicated by all the high serum T values (1.3ng/ml or higher) coming from severely hirsute patients.

Because of higher blank values when the TLC step is included in the assay, precision and sensitivity at lower ranges is less than in the "direct" T assay (without TLC). This has been compensated for by increasing the sample volumes. The TLC step is time consuming and, when included, the time taken to finish an assay is almost double that taken to finish the direct T assay. As it has not shown any particular advantages in clinical use over the direct serum T assay, the "TLC assay", in spite of its increased specificity, is not recommended for routine clinical use. The blank values are even more troublesome in the DHT assay, and in order to get a reasonable precision one has to secure 30pg or more in the immunoassay incubation tube. Recently, the DHT assay has been modified using 3H-T(T*) instead of 3H-DHT (DHT*) as the radioligand. This has brought the blank values down to those for the T assay (with TLC), and consequently increased the sensitivity and precision at low DHT levels.

The two related compounds, 5α-androstane-3β, 17β-diol and 5α-androstane 3α, 17B-diol, already shown to cross-react 3 and 2% respectively with T, had Rf values close to that of T in the TLC system. These compounds could therefore interfere somewhat in the T assay. Serum levels of 5α-androstane 3α, 17B-diol have been measured and found to be about 0.2 and 0.6ng/ml in females and males respectively (Strickland et al., 1973). If the other compound is

assumed to have levels of similar order of magnitude, these compounds cannot cause significant increases in the serum T measurements. The levels of the unconjugated forms of these compounds in urine are, however, unknown, and some minor elevation in urinary T levels could have been caused by them.

In order to find if the difference between urinary T values obtained with and without TLC was caused by some specific compound in urine, a 4ml urine sample was extracted and run in the standard TLC system and the sample strip then cut into 14 pieces, which were eluted and measured for T by the standard RIA. A distinct T peak was found and a smaller one corresponding to DHT but no others. It thus appears that the non-specific fraction in the urinary measurements by the direct T assay, is caused mainly by DHT.

3.1.3. Oestradiol in serum ("direct assay", without chromatography)

General

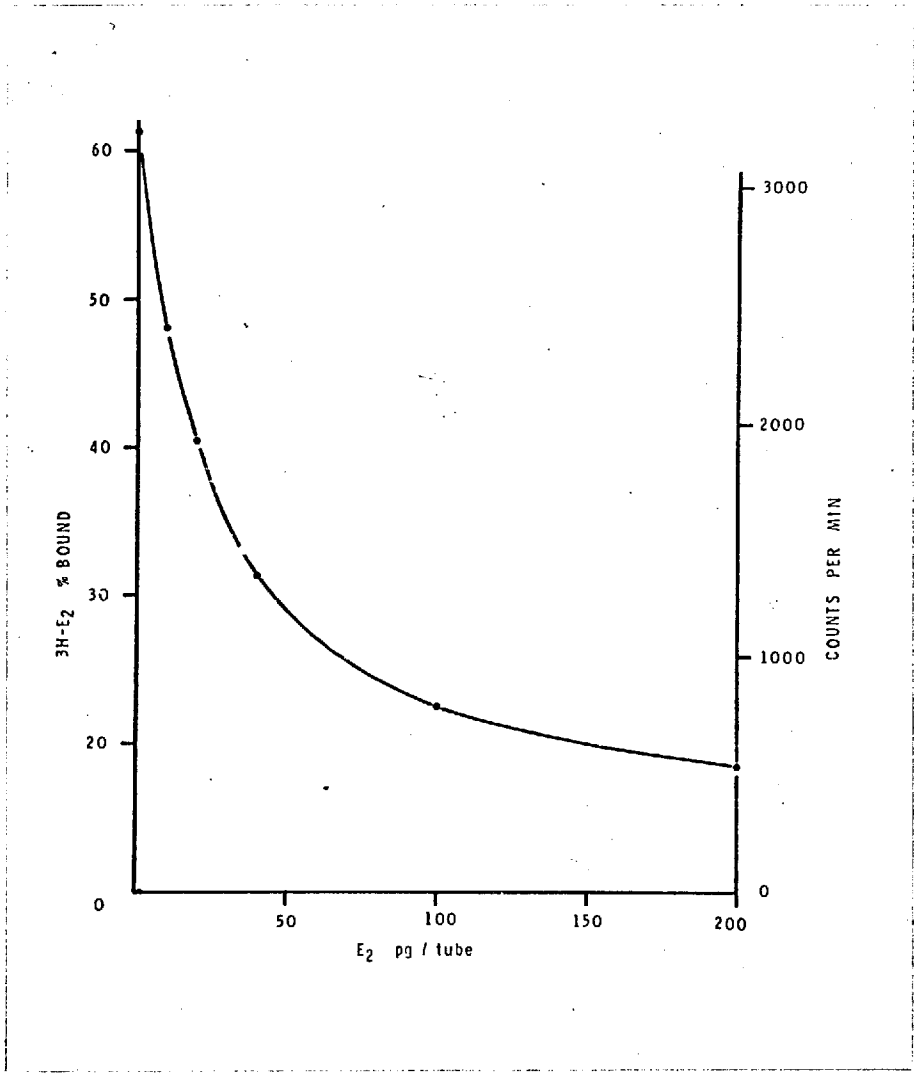
The assay for unconjugated serum oestradiol (E_2) was similar to the T assay in general and the same general reagents were used. Consequently only two specific reagents, the radioligand and the anti-serum will be described briefly.

A flow diagram of the method is presented on the next page and Fig. 3.6. shows a typical standard curve.

Radioligand and standards

The (2, 4, 6, 7(n) - 3H) oestradiol was purchased from the Radiochemical Centre, Amersham, (SA = 90 curies/mmol). The labelled

Fig. 3.6.



A standard curve for oestradiol showing per cent binding of tritiated oestradiol, 3H-E_2 (ordinate) versus increasing amounts of unlabelled E_2. Counts per min (CPM) which are generally used for plotting are shown on the right hand side.

A flow diagram of the RIA method for serum E_2

serum or plasma + internal standard, stood 15 min, 0.5ml

males and females

↓

ether extraction, 2.5ml x 2, evaporation, redissolution

in 0.5ml working buffer

↓

0.25ml for assessing recovery

0.2ml for assay (also standards, 5 - 150pg)

0.1ml antiserum solution, 1/30,000 dil.

0.1ml E_2*, ~7,000 CPM

Mixed, stood at 4°C for 2h or overnight

↓

1.0ml dextran-charcoal added

Mixed, stood 15 min at 4°C

Centrifuged for 15 min at 4°C, 2,000g

↓

Supernatant (bound E_2*) decanted

into counting vials

10ml scintillator fluid added

↓

Counting

Volumes (also in internal standard reference vials) brought to 1.2ml

oestradiol (E_2*) in the assay was prepared for use in the E_2 assay as was T* above in the T assay, similarly preparing an internal standard for recovery assessment by 10-fold dilution of the E_2* solution (the E_2* solution corresponds to the T* solution in 3.1.1.) Unlabelled E_2 was tested for purity, as was T above, and standards made from 5 - 200pg/0.2ml.

Antiserum

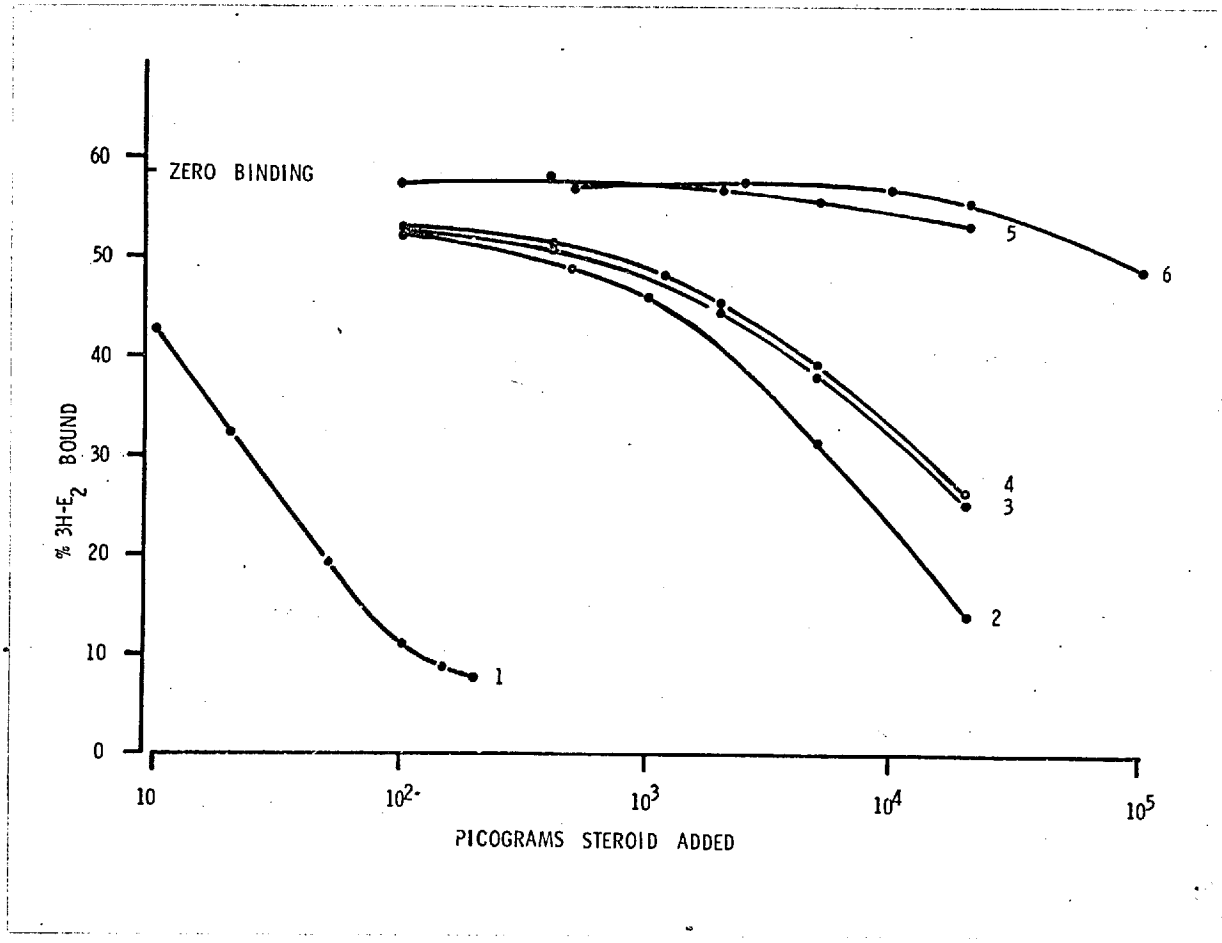
The antiserum was a gift from Dr. E. Youssefnejadian, Institute of Obstetrics and Gynaecology, Chelsea Hospital for Women, Dovehouse Street, London, S.W.3. This antiserum was raised in rabbits against oestradiol-6-carboxymethyloxime-bovine-serum albumin (E_2-6-CMO-BSA). Dilution curves showed 50% binding of E_2* (~7,000 CPM) at a dilution of 1:30,000. Cross-reaction of 5 steroids was tested and results are shown in Fig. 3.7. Oestriol (E_3) cross-reacts 0.41., oestrone (E_1) and ethynyl-oestradiol (EE_2) 0.19 and 0.16% respectively. It is a point of interest that the phenolic A ring seems to play an important role in determining the affinity of the antiserum towards the steroid compounds. The three compounds mentioned above (Fig. 3.5; no. 2.3 and 4) all have the phenolic structure of E_2 and show a very slight cross-reaction, whereas DHT (6) and mestranol (5), which has the 3-hydroxyl group etherified by a methyl group (but otherwise the same structure as EE_2), show much less cross-reaction.

Characteristics of the method

Water blanks

In 13 consecutive assays the water blank readings were 0.58 ± 0.37pg ($\bar{x} \pm$ SD) with a range of 0 - 1.25.

Fig. 3.7.



The comparative ability of increasing amounts of a few steroids (abscissa) to reduce the per cent binding of tritiated oestradiol, 3H-E_2 (ordinate), to oestradiol antibodies raised against E_2-6-CMO-BSA.

Precision

Reproducibility of standard curves was assessed by duplicates from 10 consecutive assays. The 5pg standard had the highest coefficient of variation of 5.4%.

Nine replicate assays of a serum pool in the same assay batch showed 51.3 $\pm$ 4.85 (CV = 9.4). Two serum pools run through 13 consecutive assays provided interassay precision at two levels of 7.52 $\pm$ 2.39 (CV = 32%) and 30.3 $\pm$ 33.8 (CV = 11.2%) pg/ml.

Recovery

The recovery of the internal standard in 20 assays was 84.7 $\pm$ 5.0%. Unlabelled E_2 added to various sera and to water in amounts up to 400pg/ml gave 100% recovery. To obtain 100% recovery of unlabelled E_2 from water the albumin concentration of the working buffer was critical, the recovery dropping to 70 - 80% when a buffer of 0.1% BSA was used. This phenomenon did not apply to serum where 100% recovery was obtained at 0.1. BSA concentration.

Specificity and accuracy

The relative affinity of the E_2-antiserum for a few more or less related steroids is shown in Fig. 3.7. E_3 and E_1 both cross-react less than 1% compared with E_2. To test the accuracy and the specificity of serum measurements 10 serum samples from various individuals of both sexes and serum pools were measured by including a TLC step in the method. This TLC system, described later in this section under urinary measurements, effectively separates E_3 and E_1 from E_2. When results from measurements with and without chromatography were compared no difference was detected.

Application and comments

Values obtained from a normal population are presented in Table 3.4. and are similar to those reported by other workers using techniques such as the double isotope derivative method (Baird, 1968), competitive protein binding (Korenman et al., 1969) and radioimmunoassays (Mikhail et al., 1970; Emment et al., 1972; Abraham et al., 1972). The serum levels of unconjugated E_2 in pregnancy agree closely with those reported by Loriaux et al., (1972) and Dawood and Ratnam, (1974). As demonstrated in Fig. 3.6. the antiserum used in this assay was highly specific for E_2. Judged by the chromatography studies no purification beyond the ether extraction was necessary for serum assays. Finding no significant difference whether or not a chromatographic step is included in the assay is in agreement with Doerr et al., (1973) and Bolton and Rutherford, (1976). The latter workers, however, found evidence for low levels of 6-Keto-oestradiol-17β in pregnancy plasma, a compound that might cross-react 150 - 500% with E_2 in assays using antisera raised against E_2-6-CMO-BSA. In the present series two pregnancy sera were measured with and without chromatography. No significant difference in results by the two methods was found. England et al., (1974), using an antiserum raised against a BSA conjugate of an 11β-hemisuccinate derivative of E_2, found that chromatography was not needed for serum E_2 measurements.

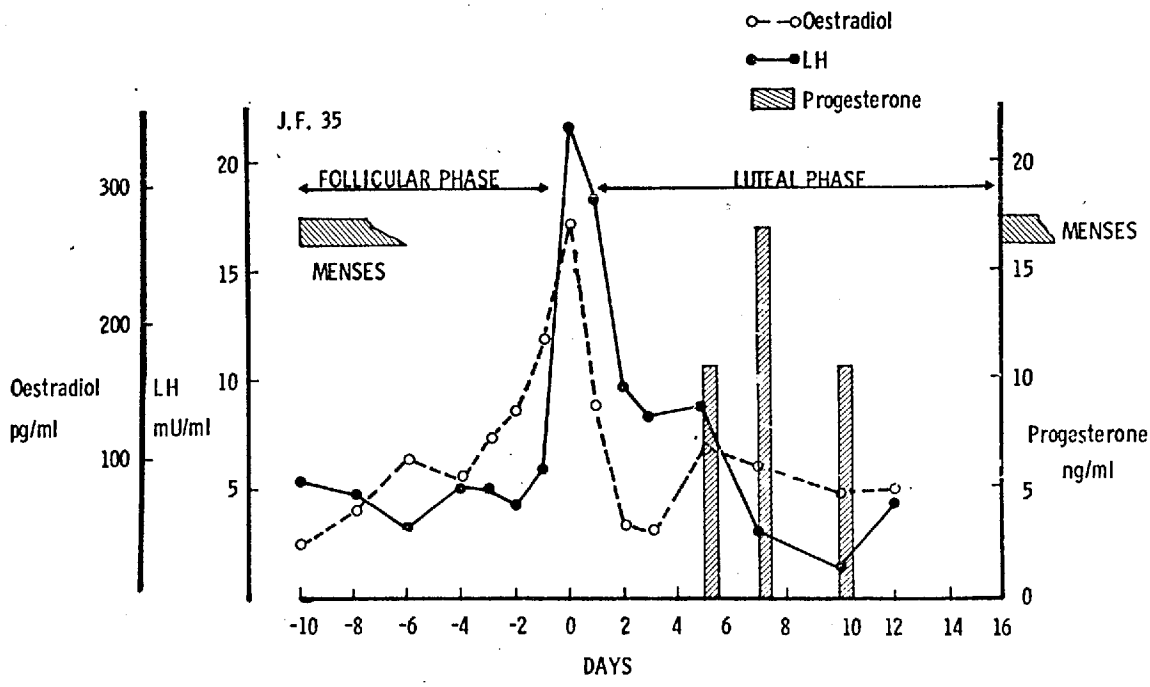
Levels were studied with frequent sampling during 5 normal menstrual cycles. Fig. 3.8. shows serum E_2 levels during a normal cycle and its relation to serum LH and progesterone levels. In one woman, E_2 levels were measured on every 3rd day during 3 consecutive cycles. A similar pattern was found in all 3 cycles.

TABLE 3.4

Normal values of unconjugated serum E_2 (pg/ml) obtained by the RIA method described

	F E M A L E S					M A L E S
	Reproductive age			Postmenopausal	Late pregnancy	24 - 37 years
	Days of cycle				ng/ml	
	1 - 10	11 - 20	21 - 30	-	-	-
$\bar{x} \pm$ SD	61 ± 30.6	129 ± 64	94 ± 33	11.3 ± 3.4	17.5 ± 4.8	22 ± 9.2
range	14 - 138	51 - 276	54 - 160	6 - 17	11.2 - 27.5	11 - 42
n	20	20	17	7	10	10

Fig. 3.8.



Serum levels of oestradiol (E_2), luteinizing hormone (LH) and progesterone (P) during a normal menstrual cycle.

Progesterone levels during the follicular phase averaged 0.32ng/ml.

3.1.4. Unconjugated Oestradiol in Urine

The procedure used for assaying free E_2 in urine is the same as for serum except for the inclusion of a TLC step and larger sample volumes for extraction of 3.0ml for either sex.

Thin layer chromatography (TLC) for separation of oestrogens

The same silica gel plates were used as for the T and DHT assays described before. The solvent system used was benzene : diethyl ether (50:50). This system effectively separates the classical oestrogens, E_1, E_2 and E_3, which had Rf values of 0.04, 0.52 and 0.8 respectively. The marker (10-20mg E_2) was visualized either by an ultraviolet light or spraying with 5% molybdophosphoric acid in methanol.

Characteristics of the urinary E_2 method

Water blanks

In five assays the overall mean and S.D. of duplicate water blanks readings was 1.76 ± 1.72pg.

Precision

Seven replicate determinations of a serum pool in one assay produced 341 ± 21.5pg/ml (CV = 6.3%). Two pools run in duplicate through 5 assays produced 332 ± 36.7pg/ml (CV = 11%) and 10.8 ± 1.6pg/ml (CV = 14.7%).

Recovery

The recovery of the internal standard was $60.4 \pm 9.4\%$ ($\bar{x} \pm$ SD). To a male urine 100, 200 and 400pg were added and recoveries were 81, 98 and 105% respectively.

Specificity and accuracy

Ten urinary samples from normal males and females and pregnant women were measured with and without TLC. Measurements without TLC were 1.4 (pregnancy urine) to 2.8 times higher than when TLC was included. Hence there are in urine, unlike in serum, compound(s) capable of interfering or cross-reacting with the binding of E_2* to the antiserum. A second chromatographic step, cyclohexane : ethyl acetate (3:2), was added for the E_2 determination of 5 urinary samples. Results were not different from those obtained with the standard procedure.

Application and comments

The results of measurements of unconjugated E_2 in urine will be a subject of Chapter 6 in the experimental section.

The very different results obtained with and without TLC in urine as opposed to serum are interesting and point to compounds in urine cross-reacting with E_2* for the antiserum. Compounds such as C-6 oxygenated derivatives of oestrogens would be expected to cross-react in the direct assay. Such compounds have been found in human pregnancy urine (Knuppen et al., 1966). These compounds are, however, generally much more water soluble than the parent compound (Marrian and Sneddon, 1960) and would perhaps be extracted more poorly from the urine.

3.1.5. Progesterone in serum

The assay for serum progesterone is in principle similar to the T assay described in the first section of this chapter. There are however modifications which will be outlined below.

Antiserum

The antiserum was a gift from Dr. Youssefnejadian, Chelsea Hospital for Women, London, S.W.3. It was raised in rabbits against progesterone-11α-hemiglutaric acid-BSA. Dilution curves showed 50% binding of tritiated progesterone (P*), approximately 7000 CPM, at a dilution of 1:5000. The cross-reaction of a few steroids with progesterone was tested. 11α-hydroxy-progesterone cross-reacted 100%, 17α-hydroxy-progesterone 11% and cortisol 0.01%.

Radioligand and Standards

(1, 2, 7, 7 - $3H_4$) progesterone (S.A. = 110Ci/mmol) was purchased from the Radiochemical Centre, Amersham, and prepared for use as described for T*. Progesterone standards were prepared in solutions as for T, ranging from 10 - 400pg/0.1ml.

Assay procedure

The assay flow chart is the same as the one given for serum T except that a petroleum ether with a boiling range 40 - 45°C is used for extraction of the sera and 0.2ml and 0.4ml are generally extracted for female and male sera respectively.

Characteristics of the method

Water blanks

In 10 consecutive assays water blank readings were 1.88 ± 2.33 with a range from 0 to 6pg.

Precision

Two pools were measured in 9 consecutive assays showing values of ($\bar{x} \pm$ SD) 7.65 ± 0.95pg/ml (CV = 12%) and 0.75 ± 0.14pg/ml (CV = 19).

The intra-assay coefficient of variation (CV) was estimated from the average CV of triplicate determinations of these pools in five assays and were 7 and 12.7 respectively for the high and low pool.

Recovery

The recovery of P* in the internal standard averaged 68.6% with a standard deviation of 8.5. Adequate recovery (≃ 70%) of the internal standard in the petroleum ether extract needed at least 20 seconds shaking on the vortex mixer, but reached a maximum of only 80% after 45 seconds shaking. Diethyl ether extraction was more efficient, averaging about 88%, but, for reasons to be described shortly, could not be used. When unlabelled progesterone was added to sera and water, 100% recovery was invariably obtained.

Specificity and accuracy

As the antiserum is not specific for progesterone, cross-reaction by other steroids was expected in the direct assay, without chromatography prior to the RIA itself. When diethyl ether was used for extracting the samples and the extract then assayed directly, an average value of 1.2ng/ml was obtained for normal males, and females in the follicular phase of the menstrual cycle. A TLC step was therefore included using precoated silica gel plates (Merck, 5554) and cyclohexane : ethyl acetate (3:2). Using this procedure values were 3 times lower for the above mentioned samples. However, for samples above 8ng/ml (women in the luteal phase), no significant difference in results was observed. In an attempt to avoid the TLC step the less polar solvent, petroleum ether, was tried, and values were then not significantly different from those obtained after the

inclusion of the TLC step above. Thus the finally chosen method seems to be an accurate one, that is measuring the real amount of progesterone in serum.

Application and comments

Values obtained from normal males and females are presented in Table 3.5. The values are in reasonable agreement with those reported by other investigators using RIA (Youssefnejadian et al., 1972; Abraham et al., 1971), and competitive protein binding (Johansson, 1969). Four normal menstrual cycles were followed by taking blood samples every 2nd or 3rd day and measuring E_2, P and LH. Serum P levels had invariable risen on the first day after the LH peak, and rose to reach peak levels on the 7th or 8th day after the LH peak. Fig. 3.8. shows the relation of the serum P levels to those of LH and E_2. The use of petroleum ether to extract serum samples has been in common use in competitive protein binding assays for serum P. These assays make use of the affinity for P of the serum cortisol binding globulin (CBG), and, petroleum ether extracts 80 - 90% P from serum, whereas cortisol is extracted 0.25% (Johansson, 1969). This author also reported that about 14% of 17α -hydroxy-progesterone was extracted from serum by petroleum ether. Thus, in the present assay petroleum ether has minimized the cross-reaction of more polar compounds such as 17α -hydroxyprogesterone (serum levels 0.5 - 2.0ng/ml) and cortisol, which, because of its high serum concentration ($\simeq$ 100ng/ml), could otherwise cross-react in the assay. The selection of the boiling range 40 - 45 is of practical advantage as petroleum ether of that boiling range is quickly evaporated at temperatures which are non-deleterious to P.

TABLE 3.5

Normal levels of serum progesterone obtained by the present RIA method (ng/ml)

	F E M A L E S		M A L E S
	Days of menstrual cycle		
	1 - 13	14 - end of cycle	24 - 36 years
$\bar{x} \pm$ SD	0.39 ± 0.15	8.05 ± 5.54	0.34 ± 0.23
range	0.13 - 0.59	1.6 - 18.6	0.1 - 0.8
n	27	32	7

3.2. MATERIALS

3.2.1. Equipment

Liquid-scintillation spectrometer, Intertechnique, Type SL 36; Intertechnique Ltd., Unit 5, Victoria Road, Portslade, Sussex BN4 1XQ, U.K.

Fraction collector, LKB Ultrorac (TM) 7000; LKB-Producter AB, Stockholm-Bromma 1, Sweden.

Heating blocks, Type BT2; Grant Instruments Ltd., Barrington, Cambridge CB2 5QZ, U.K.

Universal U.V. lamp (254 and 360 nanometers); CAMAG, Muttenz, Switzerland.

Shandon Universal TLC chromatank.

3.2.2. Reagents

Radiolabelled compounds

(1, 2, 6, 7(n)-3H) Testosterone, Code TRK.402; 5α -Dihydro (1 , 2 (n)-3H) Testosterone, Code TRK. 395; (2, 4, 6, 7(n)- 3H) Oestradiol, Code TRQ. 553 and Progesterone-1, 2, 6, 7-H_3(n), Code TRK 413 were all obtained from The Radiochemical Centre, Amersham, Buckinghamshire HP7 9LL, U.K.

Testosterone β-D-glucuronide, (Testosterone-1, 2-3H(n)), 40-60 Ci/mmole, Cat. no. NET 291 was purchased from New England Nuclear (NEN) Chemicals GmbH, D 6072 Dreieichenhain, Siemenstrasse 1, West Germany.

Non-labelled steroid compounds

Testosterone; 5α-androstane-3β, 17β-diol; 5α androstane-3α, 17β-diol; 5β-androstane-3α, 17β-diol; androstenedione; androsterone; dehydroepiandrosterone; testosterone glucuronide; testosterone acetate; oestradiol; oestrone; oestriol; ethynyl-oestradiol; mestranol; progesterone; 11α-hydroxy-progesterone; 17α hydroxyprogesterone; were all purchased from Sigma (London) Chemical Co., Kingston, Surrey, KT2 7BH, U.K.

5α-dihydrotestosterone; 17α-testosterone (epitestosterone) and cholesterol were obtained from Steraloids Ltd., 31, Radcliffe Road, Croydon, U.K.

Cyproterone acetate was a pharmaceutical preparation (Androcur) from Schering Chemicals Ltd., Burgess Hill, Sussex, U.K.

Various Chemicals

All general reagents such as salts, acids etc. were obtained from British Drug Houses (BDH), Poole, Forset, U.K. and, unless stated otherwise, were analar grade.

Thiomersal (Merthiolate), (BDH)
Ethylenediaminotetracetic acid (EDTA), (BDH)
p-chloromercuribenzoic acid, (Sigma)
D-Saccharic acid, monopotassium salt, (Sigma).

Triton X-100, Cat. no. T-6878, (Sigma).
PPO(2, 5-diphenyloxazole); Koch-Light Laboratories Ltd.,
Colnbrook SL3 OBZ, Buckinghamshire, England.

α-NPO (2-α(1-naphtyl)-5-phenyloxazole)-5-phenyloxazole), scintillation grade; Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, Scotland.

Bovine serum albumin (BSA), crystallized; Armour Pharmaceutical Company Ltd., Eastbourne, U.K.

Sulphur trioxide pyridine complex, technical, cat. no. 5755-6 (for synthesis of testosterone sulphate); Aldrich Chemical Company Inc., 264, Water Road, Wembley, Middlesex, U.K.

Carboxymethoxylamine hemihydrochloride, Cat. no. Cl. 340-8 (for synthesis of testosterone-3-carboxymethyl-oxime), (Aldrich Chem. Co.).

For coupling of T-3-CMO to BSA:
Tri-n-butylamine; Kodak, Kirkby, Liverpool L33 7UF, U.K.
Isobutyl chlorocarbonate (isobutyl chloroformate) (Sigma).

Solvents

All solvents were obtained from BDH, Hopkins and Williams, Chadwell Heath, Essex, U.K. or James Burrough Ltd., 60, Montford Place, London S.E.11, and, unless stated otherwise, were of analar grade.

Toluene (for scintallation counting), sulphur free, Product No. 30454 (BDH).

Miscellaneous

TLC aluminium sheets, Silica gel F254, pre-coated 20cm x 20cm, layer thickness 0.25mm, cat. no. 5554/0025; E. Merck, D 61 Darmstadt, Germany, supplied through BDH.

Sephadex A25 (DEAE); Pharmacia (Great Britain) Ltd., Paramount House, 75 Uxbridge Road, London W5 5SS.

Sephadex CM25 (Pharmacia)

Dextran T-70 (Pharmacia)

Norit A activated charcoal (Sigma)

Plastic tubes, disposable, Code no. LP3; Luckham Ltd., Labro work, Victoria Gardens, Burgess Hill, Sussex, U.K.

Counting vials, plastic, disposable, (Intertechnique).

Freund's complete adjuvant; Difco Laboratories, West Molesey, Surrey KT8 OSE, U.K.

S E C T I O N 3

experimental

SCOPE

This section consists of six chapters dealing with various aspects of sex hormones in human body fluids. Here a short description is given of the aims and themes of each chapter. Results will be summed up briefly and discussed further in the last chapter (Chapter 9).

The first chapter of this section, Chapter 4, deals with some problems in relation to the measurements used in later chapters. It was necessary to study the possible effect of various common anticoagulants on the radioimmunoassays used, since information on this was not found in the literature where plasma has mainly been used for measurements of steroid levels in peripheral blood. There is also a paucity of data on the concentration of unconjugated steroids within the red blood cell and it was therefore uncertain whether haemolysis affected serum testosterone measurements. Finally, the effect of storage of samples on sex hormone measurements had to be studied.

An observation described in this chapter, namely a sharp rise of unconjugated testosterone (T) and 5α-dihydrotestosterone (DHT) in urinary samples on standing at 18^{o} or 37^{o}C led to the subject of the ensuing chapter, which gives an account of studies towards the elucidation of this phenomenon. Some physico-chemical properties of T-sulphate and T-glucuronide are investigated and special methods devised to study T and DHT conjugates in urine and other biological

fluids. The results of these studies gave solid evidence for a new labile conjugate of T and DHT in urine which has not been described before.

Chapter 6 is a study of unconjugated sex hormone levels in serum and urine in a group of normal males and females and normal females on oral contraceptives. The aim was, firstly, to look for any correlation between the individual hormones, T, DHT and oestradiol (E_2), in either serum or urine, and, secondly, to look for a correlation between serum levels and urinary excretion of each individual hormone, that is, to see if urinary excretion might reflect unconjugated serum levels.

A further objective was to evaluate possible circadian variations in the hormonal levels. Two urine specimens were therefore collected from each individual, a day and an overnight specimen, and blood was withdrawn in the morning and in the afternoon.

Chapter 7 is a further study on the diurnal variations in serum T levels in males, suggested by the results of the previous chapter, together with a closer look at episodic fluctuations observed in preliminary studies. An experiment was undertaken in an attempt to establish the role of the pituitary gonadotrophins in the control of serum unconjugated T levels in males. Human pituitary FSH and LH were infused, either separately or combined, into seven normal males and the response of serum T levels studied.

Chapter 8 is a study of the levels of serum E_2 and progesterone(P) in amenorrhoea of various aetiology and the effect of treatment with

clomiphene citrate. The study involves an analysis of the basal serum levels of the steroid hormones, their response to the drug, and their relation to the simultaneously determined levels of the trophic hormones, LH and prolactin.

Chapter 9 constitutes a general summary of the results with a brief discussion of their significance and relevance to related research.

CHAPTER 4

Aspects of specimen handling: serum versus plasma; testosterone concentration in erythrocytes; effects of storage and attempts to curb them.

INTRODUCTION

It has been customary to use plasma for measurements of unconjugated steroids in peripheral blood, thus obtaining a quick separation from the corpuscular elements. This is followed by storage at -20°C until assayed. While this is probably a sound procedure, no investigation seems to have been done to assess the possible effects of the various anticoagulants on steroid levels in the samples. Similarly there are no reports on whether results differ between plasma and serum. Hence, while not known, it is possible that serum might be the superior choice.

Van der Molen and Groen (1968) found evidence for the presence of 17β-hydroxy steroid dehydrogenase activity in blood and erythrocytes capable of affecting the interconversion of androstenedione (A) and testosterone (T) within 3h at 37°C. About 8% conversion (A → T) was found for erythrocytes and 7% for whole blood. Higher values were found if glucose and other co-factors were added. Little if any conversion took place in plasma. Horton and Tait (1966) on the other hand, did not detect any appreciable conversion of tritiated androstenedione incubated in whole blood for 1h at room temperature. Migeon et al. 1962 presented evidence for an oestradiol

17β-hydroxysteroid dehydrogenase activity in human erythrocytes. These studies suggested that there might be differences between serum and plasma samples related to the quicker separation of the erythrocytes from plasma.

In some of the investigations presented in this thesis the use of serum samples was preferred. A pilot study was therefore done to find if such a difference existed and how great it was.

Another question, related to the one above, is the concentration of unconjugated steroids within the erythrocytes. Early studies with injection of large amounts of unlabelled T and U.V. measurements, indicated small amounts (4%) of steroid within red blood cells (West et al., 1951). Touchstone and Murawec (1965), using gas chromatography found no oestrogen in the erythrocytes, neither conjugated nor free. However, Sandberg and Slaunwhite (1957), and Migeon et al. (1959), injecting labelled oestrogens into women, found substantial amounts of injected radioactivity associated with the red cells. The latter authors showed that this activity was in a state other than as free steroids or their glucuronides. More recently Mulder et al. (1972) have measured the conversion rate of dehydroepiandrosterone (DHA) and its sulphate by intact and haemolyzed erythrocytes, and concluded that the erythrocyte membrane was more permeable to free steroids than their conjugates.

In order to assess the possible effects of haemolysis on serum samples and because of considerations relating to the radioimmunoassay itself, as the recovery of the internal standard from the red cell homolysate, a pilot study was done, measuring the T concentration in haemolysed red cells.

Finally, the effect of storage of serum and urinary samples on their unconjugated sex hormone levels was studied. Such studies have apparently not been reported before. It was originally intended to measure serum unbound T levels (non-protein bound T) by equilibrium dialysis, directly measuring the T levels by radioimmunoassay (RIA) in the dialysate. Using cuprophane membranes (Technicon), it was shown in preliminary studies that the incubation time could be reduced from 16h (Visking tubes) to about 5h (cuprophane). In earlier studies, using equilibrium dialysis and tritiated T for the estimation of serum unbound T (Vermeulen et al., 1971; Rivarola et al., 1968), control measurements of serum unconjugated ("total unconjugated") T levels in the samples, after the same incubation time (16h) at 37°C, were apparently not done.

When measurements of urinary unconjugated androgen hormones (T, DHT) were begun, no report had appeared on values or methods. Since then, one communication has appeared on urinary unconjugated T levels (Andino et al., 1976), but none on DHT. In that communication no mention was made of effects of storage of samples.

Urinary unconjugated E_2 has been measured by at least four groups of investigators (Desfosses et al., 1973; Edwards and Jeffcoate, 1974; Kono et al., 1974; Pentikäinen et al., 1975). Desfosses et al. studied the effect of storage at room temperature on E_2 levels in 3 female urine samples and found little change for up to 26h. Values reported by these groups, however, varied widely, especially in male urine where the highest values were about 20-fold the lowest ones.

In view of this relatively unexplored ground it was mandatory to investigate the effect of storage of urinary samples on their unconjugated (free) sex hormone content before embarking upon urine collections.

MATERIALS AND METHODS

Materials

Serum, red blood cells and urine were obtained from normal individuals of the hospital staff.

Serum was prepared from peripheral blood which was left at room temperature for 2 - 3h and then centrifuged at approx. 1500g for 10 min and the supernatant collected by pasteur pipette. Heparinized or EDTA treated blood was centrifuged (1500g) within an hour of collection and the plasma separated. Serum or plasma were then either frozen and kept at -20°C until used or assayed forthwith.

Urine samples were either freshly voided ones or refrigerated during collection and frozen as soon as possible on completing the collection. No preservatives were added at the collection stage. In all incubation studies, however, involving serum or urine, merthiolate was added to a concentration of 0.02 - 0.05%.

Red blood cells were prepared from heparinized blood. The plasma was separated and the cells were then washed once in 2 volumes of isotonic saline. The cells were then lysed by freezing and thawing 3 times.

The β-glucuronidase inhibitor, saccharo-1, 4-lactone was prepared by boiling a 5% solution of potassium saccharate (Sigma) for 30 min (Roy, 1970).

Methods

The determinations of individual hormones (T, DHT, E_2) were done by the methods described in Chapter 3. When results are described it is indicated whether the direct (without chromatography) assay is used or whether chromatography is included.

Urinary testosterone glucuronide (TG) concentration was assessed by the direct T assay after hydrolysis of TG by β-glucuronidase. The β-glucuronidase preparation was a gift from Miss F. Kahn, Department of Chemical Pathology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS. The enzyme was prepared from limpets (Patella Vulgata) according to Fotherby and Love, 1960.

The testosterone sulphate (TS) in urine was assessed by the direct T assay following solvolysis by the method of Burstein and Lieberman (1958).

RESULTS

1. Plasma versus serum

Unconjugated T measurements were done on serum and plasma prepared from the same blood specimen. Three individuals were bled for this purpose, two females and one male. Two plasma preparations were made from each bleeding, one by the addition of EDTA and one by heparin. Samples were measured in duplicates by the direct T assay. Results are shown in Table 4.1.

Table 4.1.

Comparison of T levels in serum and plasma prepared by addition of heparin or EDTA. Results (mean of duplicates) are in ng/ml.

Subject	Serum	Plasma (heparin)	Plasma EDTA
Female 1	0.74	0.70	0.76
Female 2	0.38	0.49	0.42
Male	8.4	8.4	8.7

Identical measurements were done for unconjugated E_2 and no difference in values observed.

2. Testosterone content of red blood cells (RBC)

RBC were obtained from two females and one male. After lysis of the cells the content was measured for T by the direct assay. Results are presented in Table 4.2.

Table 4.2.

T concentration in lysed red blood cells versus plasma. Values are in ng/ml

Subject	Plasma	Red blood cells
Female 1	0.4	0.00
Female 2	0.38	0.06
Male	5.73	0.52

The recovery of T* (internal standard) from the red cell haemolysate was not significantly different from that of plasma.

3. The effect of storage of serum samples on their unconjugated sex hormone levels.

Using the direct serum assays for serum T and E_2, preliminary studies showed that while serum E_2 levels did not rise when samples were incubated for 18h at 37°C, serum T levels showed inconsistent rises. Serum T and DHT were therefore studied further including the TLC step in the measurements (see Chapter 3). Results from such study in a group of male and female samples, 6 of each, are shown in Table 4.3 and Fig. 4.1. No rise in serum T levels was observed in females and while the average T levels in males were higher after the incubation, three samples showed no rise. DHT levels, on the other hand, rose in 5 out of 6 samples from both sexes and the average of the male samples after incubation was significantly ($p < 0.05$) higher than before the incubation.

4. Storage of urine samples.

1) Effects on unconjugated T and DHT levels.

Four male urinary samples were stored at room temperature (18°C) and aliquots taken for T and DHT determinations (chromatography) each day for four days. The same urines were incubated at 37°C for 18 and 40h and, similarly, aliquots taken for T measurements. Results are shown in Fig. 4.2. where the per cent rise in levels is plotted against time. The rise is more marked for DHT than T and temperature dependent as T levels rose 3 - 4 times faster at 37°C that at 18°C.

Female urine samples, when tested at 37°C for 18h, showed a rise for both T and DHT which was similar to that in the male samples (Fig. 4.3.).

TABLE 4.3.

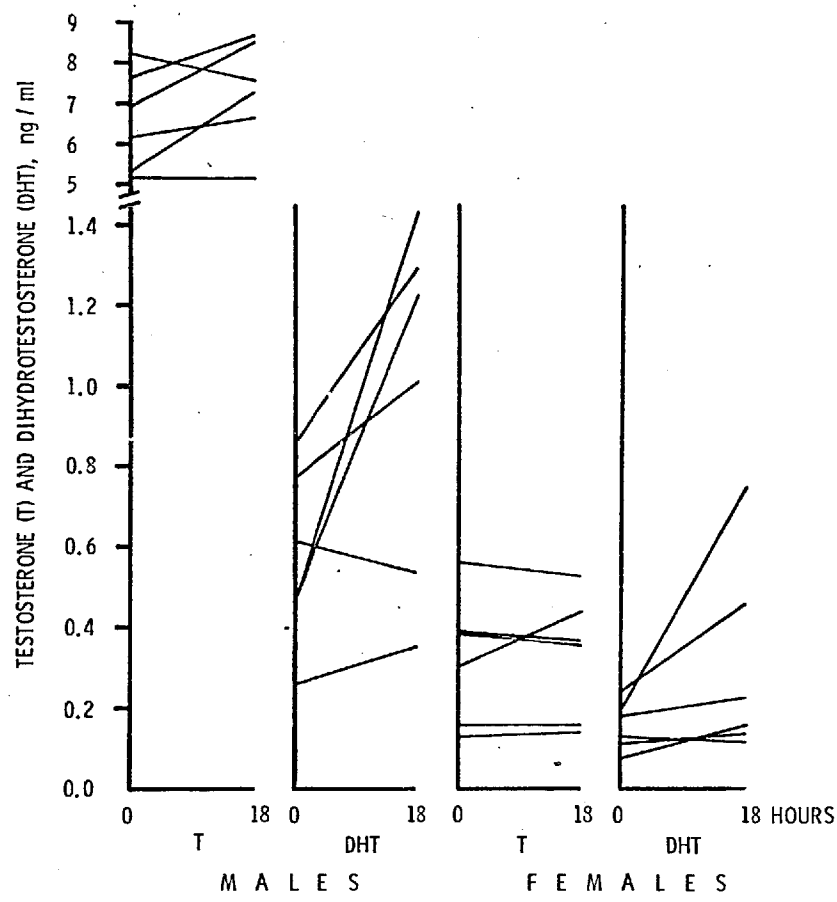
The effect of incubating serum samples for 18h at 37°C on their T and DHT levels (mg/ml)

Samples from normal males and females

	MALES					FEMALES			
	T		DHT			T		DHT	
Sample No.	before	after 18h at 37°C	before	after 18h at 37°C	Sample No.	before	after 18h at 37°C	before	after 18h at 37°C
1	8.33	7.61	0.862	1.294	1	0.38	0.35	0.246	0.457
2	7.60	8.71	0.617	0.543	2	0.40	0.36	0.197	0.756
3	5.19	5.13	0.261	0.349	3	0.16	0.16	0.110	0.137
4	6.23	6.65	0.687	1.086	4	0.13	0.14	0.177	0.226
5	6.94	8.64	0.456	1.438	5	0.57	0.53	0.129	0.113
6	5.36	7.37	0.466	1.236	6	0.30	0.44	0.076	0.159
mean	6.61	7.35	0.558	0.991*		0.32	0.33	0.155	0.308
S.D.	1.25	1.34	0.210	0.441		0.16	0.15	0.062	0.253

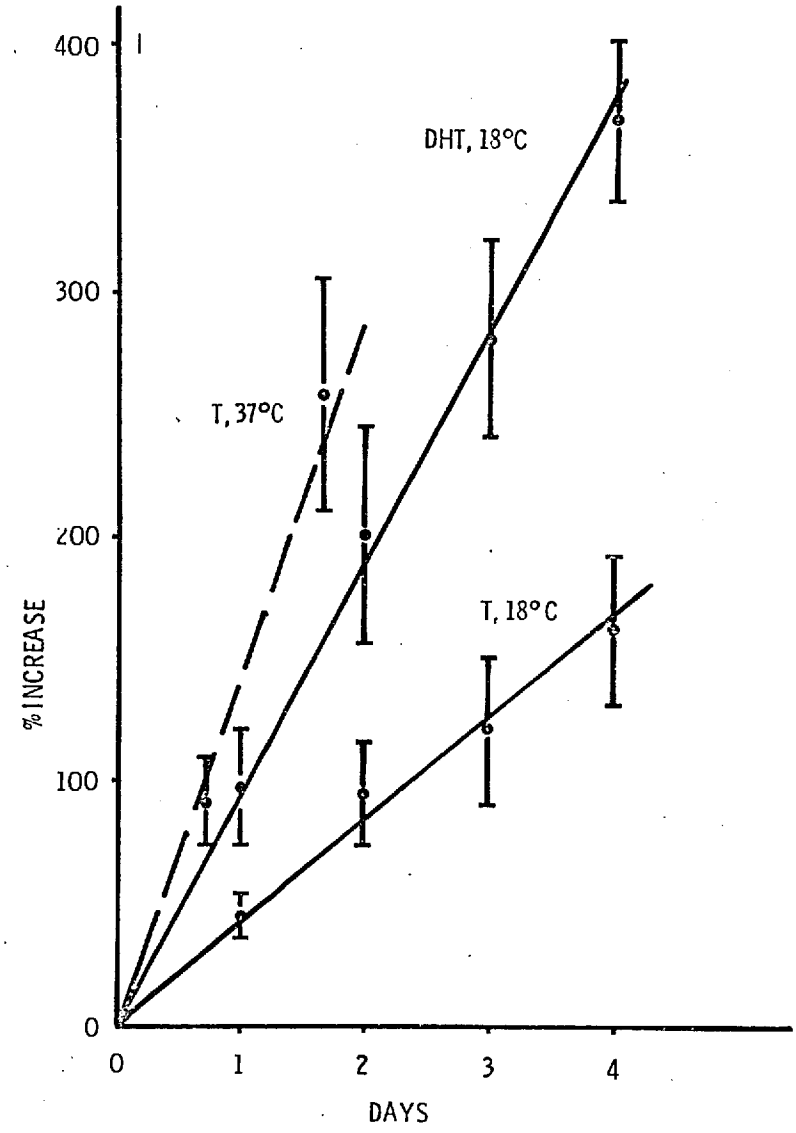
* Significantly different ($P < 0.05$) from the mean concentration before incubation.

Fig. 4.1.



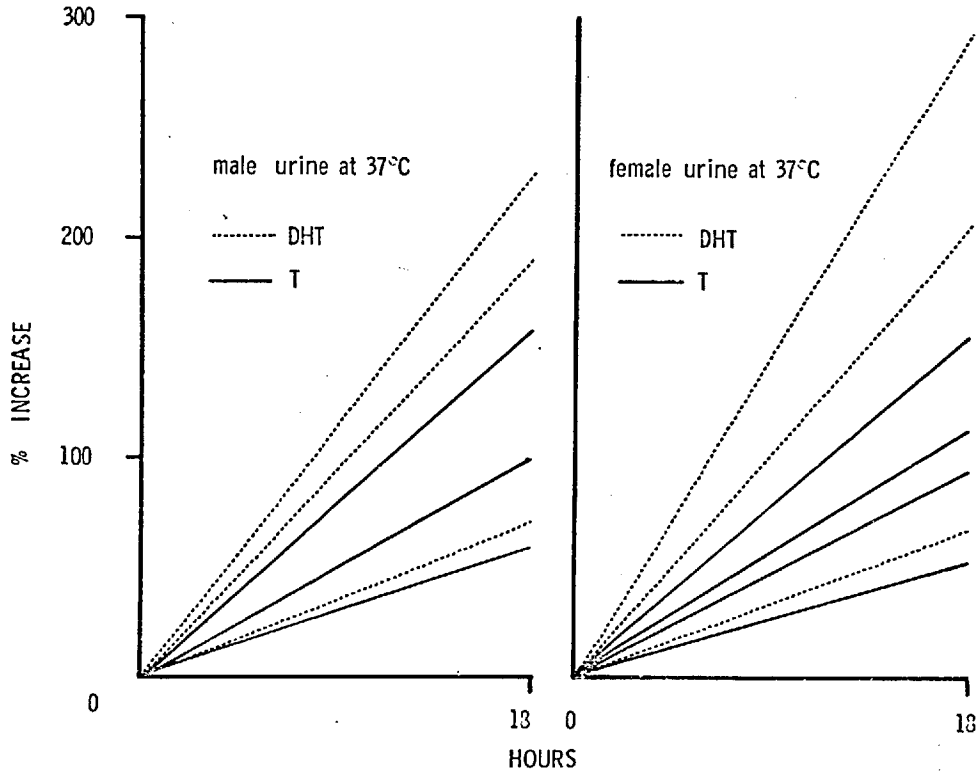
Changes in serum T and DHT levels (ordinate) after 18h incubation of the samples at 37°C. The data are the same as in Table 4.3.

Fig. 4.2.



Per cent increase of unconjugated T and DHT as ordinate with time as abscissa, in 4 male urine samples left at room temperature (18°C) or at 37°C. The vertical bars indicate the standard deviation.

Fig. 4. 3.



The per cent increase of T and DHT (ordinate) in male and female urine samples after incubation at 37°C for 18h (abscissa).

Urine samples stored at -20°C showed no rise in T and DHT after 6 months' storage. Samples stored at 4°C showed an average rise of 6% on the first day, but no rise after that for up to 10 days.

2) Effects on oncunjugated E_2 levels

Two ml aliquots of 8 urinary specimens, 4 female and 4 male, were incubated at 37°C for 18 and 40h and then measured for E_2 by RIA including the TLC step. Results are presented in Table 4.4 and Fig. 4.4. Except in 2 cases, a six-fold rise or more was seen after 18h incubation, and a further rise, although generally somewaht slower, after 40h incubation.

No rise of free E_2 was detected in frozen urine samples for up to six months. Refrigeration at 4°C similarly prevented E_2 rise for at least 5 days.

5. Urine samples: effects of high temperature; addition of chemicals; pH effects

Aliquots of two urine samples were put in test tubes which were then left in boiling water for 30 min. Unconjugated T levels rose 3-fold in the heated specimens and these showed minimal rise of T when stood at 37°C for 18h. However, levels of T-glucuronide and T sulphate showed about 30% decrease in the heated specimens, indicating considerable destruction of immunoassayable T in urine by the treatment.

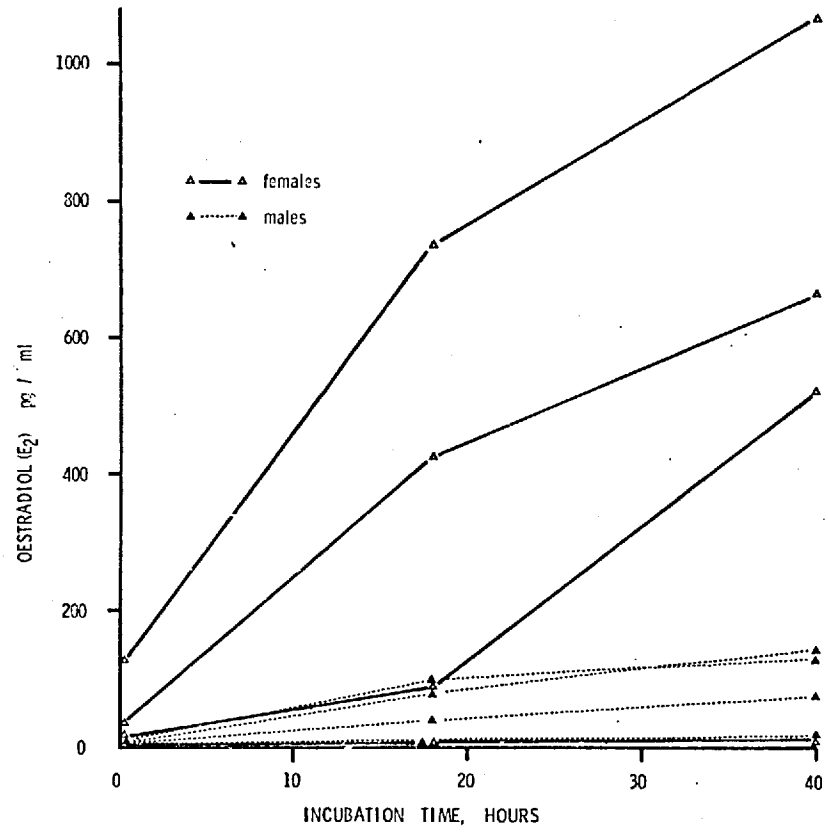
A few enzyme inhibitors and metal ions were added to urine samples as well as bacteriostatic agents in various amounts. These were EDTA, parachloro-mercurybenzoic acid, saccharo-1, 4-lactone, sodium sulphite, magnesium chloride, zinc chloride, streptomycin

Table 4.4.

The effect of incubating urinary samples at 37°C on their unconjugated E_2 levels (pg/ml). Values are the mean of duplicate measurements.

Sample	Basal	18h	(18h/basal)	40h	(40h/basal)
Female No. 1	11	86	(7.8)	420	(47)
" " 2	31	425	(13.7)	660	(21)
" " 3	117	738	(6.3)	1188	(10)
" " 4	5.4	5.9	(1.1)	13	(2.4)
Male " 1	6.8	43	(6.3)	76	(11)
" " 2	9.0	88	(9.8)	146	(16)
" " 3	6.3	9.9	(1.6)	8.6	(1.4)
" " 4	9.9	97	(9.8)	139	(14)

Fig. 4.4.



The effects of storage of urinary samples at 37°C for 18 and 40h (abscissa) on their unconjugated E_2 concentration (ordinate). Values are the same as in Table 4.4.

sulphate, sodium azide and merthiolate. None of these reagents had any effect on the T rise observed in urine when stored at 37°C.

The effect of pH was tested by dividing a urine specimen of a ph 5.9 into three aliquots, bringing the pH to 8.6 and 2.0 in two of them, the third serving as a control. These were stood at room temperature and aliquots taken daily for 4 days and frozen until assayed for T and DHT (TLC). Samples of pH 8.6 showed a slower initial rise for both T and DHT and there was no increase on the 4th day. Samples of pH 2.0 had all increased about 4 - 5-fold from the values of the control samples but showed no further increase with time (see Fig. 5.8 in Chapter 5).

DISCUSSION

The studies described in this chapter were done primarily in preparation for work to be reported in later chapters. They do, however, raise several interesting points, both theoretical and practical.

Although compared on only few samples, plasma and serum seem to be interchangeable with respect to T and E_2 measurements, that is, if separation from red cells takes place within 3h.

The results of the measurements of the red cell haemolysates do indicate that there is no unconjugated T within the cells. If washing for instance had been more thorough, the level in the male sample would probably have approached zero. A similar situation seems to exist with regard to oestrogens in red cells (Touchstone and Murawec, 1965). A direct measurement of the T content of red cells

does not seem to have been done before. The immediate practical consequences are that gross haemolysis of blood samples would dilute the serum and give lower T concentrations. How permeable to steroids the red cell membrane is and whether blood samples are likely to be exposed to factors which alter that permeability are questions which remain to be answered.

The theoretical aspects are even more interesting. Is it, for instance, an active mechanism, akin to the sodium pump, that keeps the steroids out of the cell or is it a lack of steroid receptors in the erythrocytes that causes the low intracellular concentration? Or, are steroids perhaps within the cells but in some unknown conjugated form? The studies of Sandberg and Slaunwhite (1967) and Migeon et al. (1959) injecting labelled oestrogens into women, showed substantial amounts of injected radioactivity associated with the red cells. However, using labelled glucocorticoids for administration, Kornel et al. (1970) were able to show that much of this radioactivity could be washed out, indicating loose adsorption to the cell membrane. As enzymic activity in red cells, relating to steroids, is not bound to the cell membrane (Migeon et al., 1962), it seems as if we are faced, in erythrocytes, with enzymes without the presence of the substrates.

The inconsistent rise of serum T and the significant rise of serum DHT on storage at 37°C meant that an intended dialysis technique for serum unbound T had to be abandoned. The observed rise challenges results obtained by such methods in the past, where samples were incubated at 37°C for 16h. The cause of the rise remains to be found. It is possible that the steroids are formed from precursors by enzymatic activity in serum. However, in the case of

DHT, the enzyme 5α-reductase is known to be largely an intracellular NADPH dependent enzyme, firmly bound to the microsomal fraction (Wilson and Gloyna, 1970). Also, investigations have so far shown that steroid related enzyme activity resides almost entirely in the erythrocytes (Van der Molen and Groen, 1968; Migeon et al., 1962). Hydrolysis of serum conjugates is therefore a more likely possibility.

The sharp rise of both unconjugated T and DHT in urine samples on storage has not been reported before. A relatively cell and protein free fluid like urine was not expected to have great enzymic activity, so the rise was somewhat unexpected. Studies with enzyme inhibitors pointed against enzymic activity as the cause of the rise. β-glucuronidase activity, for instance, was unlikely as it is effectively inhibited by saccharo 1, 4-lactone (Roy, 1970).

The pH studies suggested an acid labile conjugate and it was decided to look further into that possibility. Those investigations are described in the next chapter.

The even greater rise of unconjugated E_2 on storage at 37°C is similarly difficult to explain, but hydrolysis of an E_2-conjugate is perhaps most likely. Desfosses et al. (1973) found only minimal rises in E_2 after storage of 3 urine samples at room temperature for 26h. Their results do not have to be at variance with the present ones as two samples in the present study showed minimal rise. Unfortunately the effect of storage at room temperature was only studied by the direct RIA method for E_2 which showed only modest rise in two samples. Repeated studies at 37°C have, however, confirmed the unconjugated E_2 rise described above.

The two individuals whose urine samples showed no rise were a male taking ampicillin and a female on oral contraceptives. Ampicillin is known to lower urinary excretion of total oestriol in pregnant women (Willmann and Pulkinen, 1971) but there is no report on effects of oral contraceptives on conjugated or unconjugated endogenous E_2 in urine. Chapter 6 describes the effects of oral contraceptives on urinary unconjugated E_2 excretion, but the cause of the observed E_2-rise in urine on standing and the reason for the absence of such a rise in some cases remains to be investigated further.

For all the sex hormones, T, DHT and E_2, the question arises 'how much free hormone does the kidney excrete?' It seems to be a possibility that a considerable part of the unconjugated hormones is formed in the urinary tract after renal excretion.

As no way was found to curb the rise of the unconjugated hormones in urine on storage by preservatives, the only way left was to refrigerate and/or freeze the samples. During a 24h collection the samples should preferably be refrigerated immediately after voiding during the collection period and then frozen. Such collection method was adopted in all the studies on unconjugated sex steroids described in Chaper 6.

CHAPTER 5

Studies of conjugates: testosterone glucuronide and testosterone sulphate; conjugates in urine and serum; analytical techniques for the study of sex steroid conjugation; evidence for a new labile conjugate of testosterone and 5α-dihydrotestosterone in human urine.

INTRODUCTION

In the previous chapter a striking rise in unconjugated sex steroids was described in urine when it was left standing at room temperature, and a still greater one at 37°C.

Apart from its theoretical interest, the phenomenon bore directly on the intended measurements of the free hormones in urine, and it was therefore decided to study it further.

Preliminary investigations on T and DHT in Chapter 4 were against an enzymic process and rather suggested a labile conjugate to be the source of the released free hormones. Other possibilities existed. It was for instance possible that a non-covalent complex of the hormones with some urinary compound was gradually broken up, or that the hormones were formed from structurally related precursors, enzymatically or not.

As regards steroid conjugates in general in biological material, a wide variety has been described in the literature. A list of these with extensive references is presented in a review by Bernstein et al., 1970. These include sulphates, glucuronides, N-Acetylglucosaminides, acetates, sulphatides, phosphatides, nucleosides and

nucleotides, and conjugation with glutathione, glycine and taurine. However, the existence of many of these conjugates awaits confirmation and only the first three are well established.

For testosterone (T) two conjugated forms are well established in plasma and urine, namely T-Sulphate (TS) and T-glucuronide (TG), bearing an ester and glycosidic bond respectively (see reviews by Dessypris, 1975, and Solomon and Bhavani, 1970).

Results in Chapter 4 suggested that the release of unconjugated T and DHT in urine on storage might stem from an acid labile compound. In one of the first reports indicating androgen conjugates in urine, Funk et al. (1929) acidified the urine to pH 4 - 4.5 (Congo red) before continuous extraction with chloroform. These workers later found more activity in the extracts when urine was treated with strong HCl and heated, (Funk and Harrow, 1930). It was soon realized that heating (boiling) was disadvantageous, in that it caused artefacts and destroyed the steroids. Solvolysis procedures, where urine is acidified to pH 1 and continually extracted at room temperature with a solvent, have been in common use for steroid sulphates since the work of Liebermann and associates, who showed that by these mild methods sulphates were rapidly hydrolyzed, while glucuronides remained little affected (Lieberman and Dobriner, 1948). The method described for dehydroepiandrosterone sulphate (Burstein and Lieberman, 1958) has been widely adopted for other C-19 steroid sulphates. Although used for TS no characterization of the hydrolysis of that compound or TG seems to have been undertaken, neither using solvents nor using acid alone.

The literature contains two reports on unconjugated T in urine released by acidification to pH 1 without the use of solvents. Dulamanis et al. (1964) acidified the urine with HCl, incubated it

for 16h at 40°C and measured T by a double-isotope derivative technique. Van der Molen et al. (1966) treated the urine similarly, but do not state which acid was used for adjusting the pH. This latter group used gas liquid chromatography to measure T and reported about 10 times lower values than the former group for 24h urine collections from normal individuals. Neither group attempted identification of the compound.

The work described in this chapter was an attempt to answer some of the questions raised above. The first experiments involved studies on the sulphate and glucuronide conjugates of T which were initially the suspected cause of the unconjugated hormone release, and a method for their separation on TLC was developed. This was followed by various studies on urine including the effect of pH described in the previous chapter. This led eventually to the development of a separation technique on Sephadex ion exchange column using sodium chloride gradient elution.

MATERIAL AND METHODS: GENERAL CONSIDERATIONS

Urine and serum samples were from normal males and females used either immediately after collection or frozen until used. Two amniotic fluid specimens were supplied by Mr. H. Gordon, Consultant in Obstetrics and Gynaecology, Hammersmith Hospital, London W12 OHS.

Unless otherwise stated, chemicals and other materials used are as listed in Chapter 3.

1. Measurement of T and DHT

The T and DHT methods described in Chapter 3 were used as specified in each experiment. In studies involving acidification a small amount, 15 - 30mg, of sodium bicarbonate ($NaHCO_3$) was added to all tubes used to dry down the ether extract. The sodium bicarbonate did not affect the RIA when added in amounts up to 50mg. It, however, neutralized the acid which at high concentrations affected the assay by causing lower readings.

The sodium bicarbonate also prevented possible acid hydrolysis during the evaporation procedure as preliminary studies showed that substantial amounts (20 - 60%) of TG and TS were extracted by diethyl ether at high concentrations of acid.

The β-glucuronidase preparation was described in the previous chapter. About 400 phenolphthalein units (Fishman et al., 1948) were used for each tube.

Solvolysis was done with ethyl acetate as the solvent as described by Burstein and Lieberman (1958) with a constant extraction for 20h.

2. Radiolabelled conjugates

Synthesis of testosterone sulphate (TS) and tritiated testosterone sulphate (T*S).

TS was prepared by a modification of the method of Sobel and Spoerri (1941). To a small conical glass stoppered tube, containing 0.3ml pyridine, 5mg of pyridine sulphur trioxide and 2mg of T were added. The tube was left at room temperature for 4 days. The TS

was then separated from the unesterified steroid by TLC (precoated Silica gel plates, Merck, art. no. 5554) in a system of chloroform: methanol : water 80:20:1 as used by Klein and Girond, 1965. In this system T had an Rf value of 0.71 and the sulphate 0.16. When examined under U.V. light only two spots were seen. The TS spot was identified further by the methylene blue reaction (Crépy and Judas, 1960) and by its U.V. absorption spectrum after extraction into methanol.

T*S was made similarly. 100uCi of T* in ethanol was first dried down under a gentle air stream and pyridine (0.3ml), pyridine sulphur trioxide (5mg), and T (1mg) were then added.

Tritiated testosterone glucuronide (T*G) was purchased from New England Nuclear Co. (see Chapter 3) and purified on TLC (see below) before use.

3. Thin layer chromatography (TLC) of T (and DHT) conjugates

a) Extraction of conjugates

For extraction of conjugates the method originally described for human urine by Edwards et al. (1953) as modified by Kornel (1963) was used (method F). This involved adjusting the pH of urine to 1, saturation with $(NH_4)_2SO_4$ and extraction with an ether : methanol (3:1) mixture which was then evaporated at 40°C under gentle air stream. The more easily obtainable and cheaper analar grade methanol was used instead of ethanol. Sodium bicarbonate was added, as above, to the tubes in which the mixture of extraction solvents was evaporated.

b) TLC procedure

Precoated silica gel plates (Merck) were used throughout. The dried residue from the extraction was dissolved in methanol and applied (0.1ml x 2) on the plates. The plates were run in a solvent system of chloroform : methanol : water (70:30:2). In this system TG, TS, T and T-acetate had average Rf (standard deviation) values of 0.21 (0.07), 0.59 (0.09), 0.80 (0.02) and 0.84 (0.02) respectively. Adding various amounts of ammonium hydroxide did not alter Rf values appreciably.

Markers (5 - 10ug of the conjugates or free steroid) were applied on either margin, 2cm from the edge of the plate, spaced 2cm from the nearest sample. Markers were detected under ultra-violet light after each run. It was noted that the urinary conjugates sometimes had Rf values slightly different from those of the markers. This was ascribed to other compounds in the urinary extracts and was more marked for TG than for TS.

T*S and T*G were added to urine samples and their recovery from the TLC plates ranged from 64 - 78% being similar for both conjugates.

4. Column chromatography on DEAE Sephadex ion exchanger

The method used is a modification of that used by Hähnel (1965) for urinary oestrogens. Hähnel's method has been shown to separate authentic T and dehydroisoandrosterone conjugates (Hobkirk and Davidson, 1971).

DEAE Sephadex, A25, (Pharmacia) was left in distilled water overnight and columns (30 x 0.5cm) were then made. A sodium chloride gradient elution was used as follows: two reservoirs were used, one containing the NaCl Solution (Res. 1) and the other for the mixing of NaCl and water (Res. 2). The Res. 2 was placed on a magnetic

stirrer, 100ml of distilled water added and stirred continuously. It was then closed by an airtight lid perforated by two tubings, one for intake of the NaCl solution from Res. 1 and the other leading to a peristaltic pump, pumping the content of the mixing reservoir on to the column. A typical concentration gradient (sodium expressed in mM/l), using 0.5M NaCl in Res. 1, is shown in Fig. 5.9.

Elution patterns were dependent on the concentration of sodium chloride used in Res. 1 and on the width of the columns used. The internal diameter of the glass tubes used for making the columns varied slightly which was enough to show an effect.

1.0ml of urine was applied to the columns and eluted at a rate of 0.75ml/min. Fractions of 3ml were collected.

In an experiment involving Sephadex CM 25 the procedures were identical.

SPECIFIC EXPERIMENTS AND RESULTS

1. Effect of increasing concentration of H_2SO_4 on hydrolysis of authentic TS and TG.

TS and TG solutions were prepared in working buffer. Aliquots were distributed into centrifuge tubes and constant volume of H_2SO_4 solutions of increasing strength added. The tubes were then sealed and incubated at room temperature ($18^{\circ}C$) for 16h (duplicates) and at $40^{\circ}C$ for 48h. T in a solution of working buffer was added to 4 tubes (duplicates) and similarly subjected to increasing H_2SO_4 concentration at $40^{\circ}C$ for 48h. The T (DHT) content of all tubes was

then measured by the direct T assay not using the internal standard.

Results are presented in Fig. 5.1. TS is more easily hydrolyzed by the acid at both temperatures. TG does not hydrolyze at room temperature until the acid is about 1.0M, but TS starts to hydrolyze at about 0.4M. At the higher temperature and longer incubation both conjugates start hydrolyzing at lower molarity of the acid and the hydrolysis reaches completion at about 1.25M for TS and 2.25M for TG.

T is almost unaffected by the strong acid at 40°C.

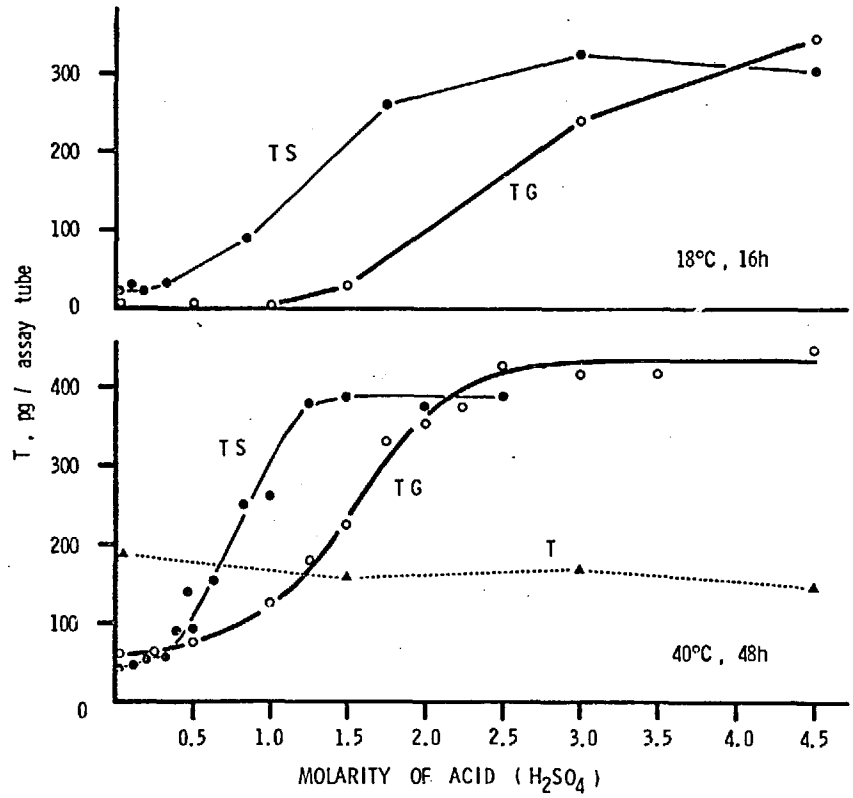
2. Effects of HCl on TS and TG

The effect of increasing concentration of HCl on TS and TG hydrolysis was tested as for H_2SO_4 above (1.) but only at 37°C for 18h. Results are shown in Fig. 5.2. It can be seen that HCl causes hydrolysis of both the conjugates below a molarity of 1.0. TS is more susceptible to the hydrolyzing effects of HCl and is almost completely hydrolyzed at 1.5M.

3. Comparison of the effects of HCl and H_2SO_4 on unconjugated T release by urine.

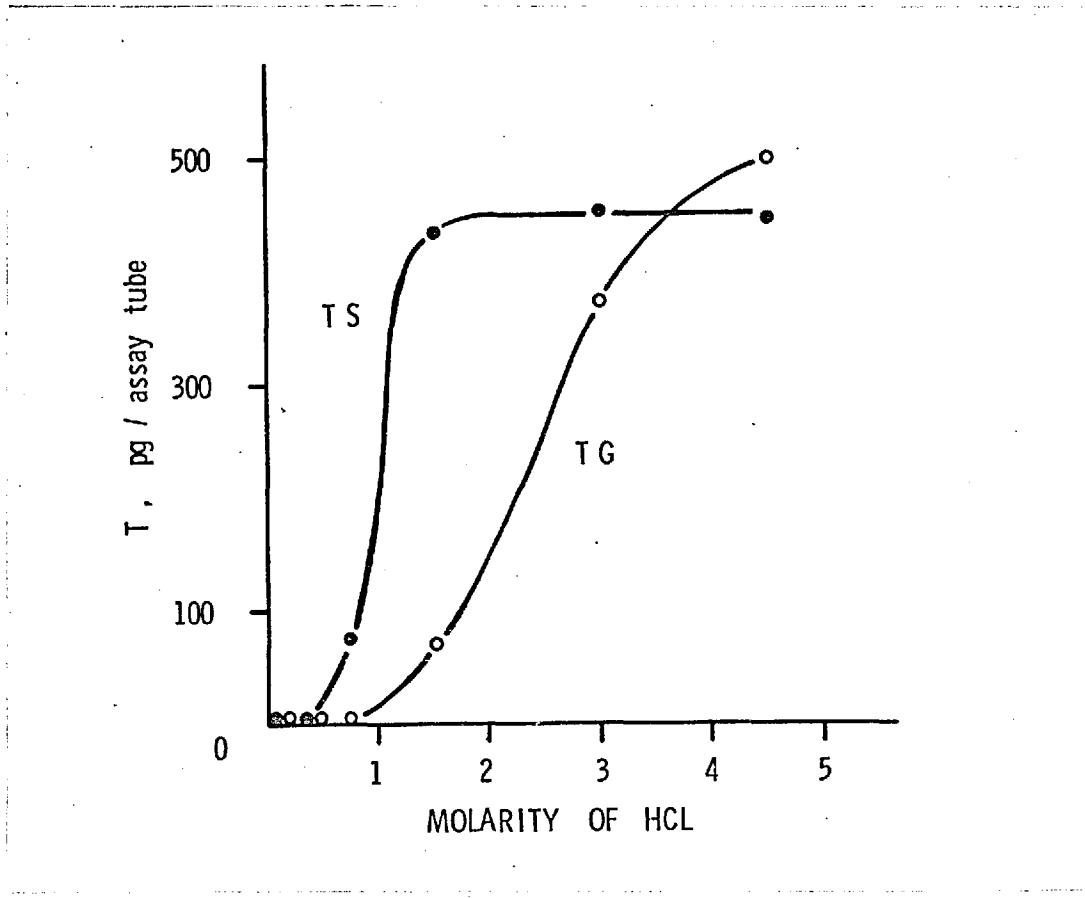
Urine samples were aliquotted in several centrifuge tubes which were adjusted to various pH's by adding either HCl or H_2SO_4. These were then left at 37°C for 16h and measured for T (DHT) by the direct T method. Results are shown in Fig. 5.3. HCl can be seen to have a greater T-releasing effect than H_2SO_4 at a comparable pH.

Fig. 5.1.



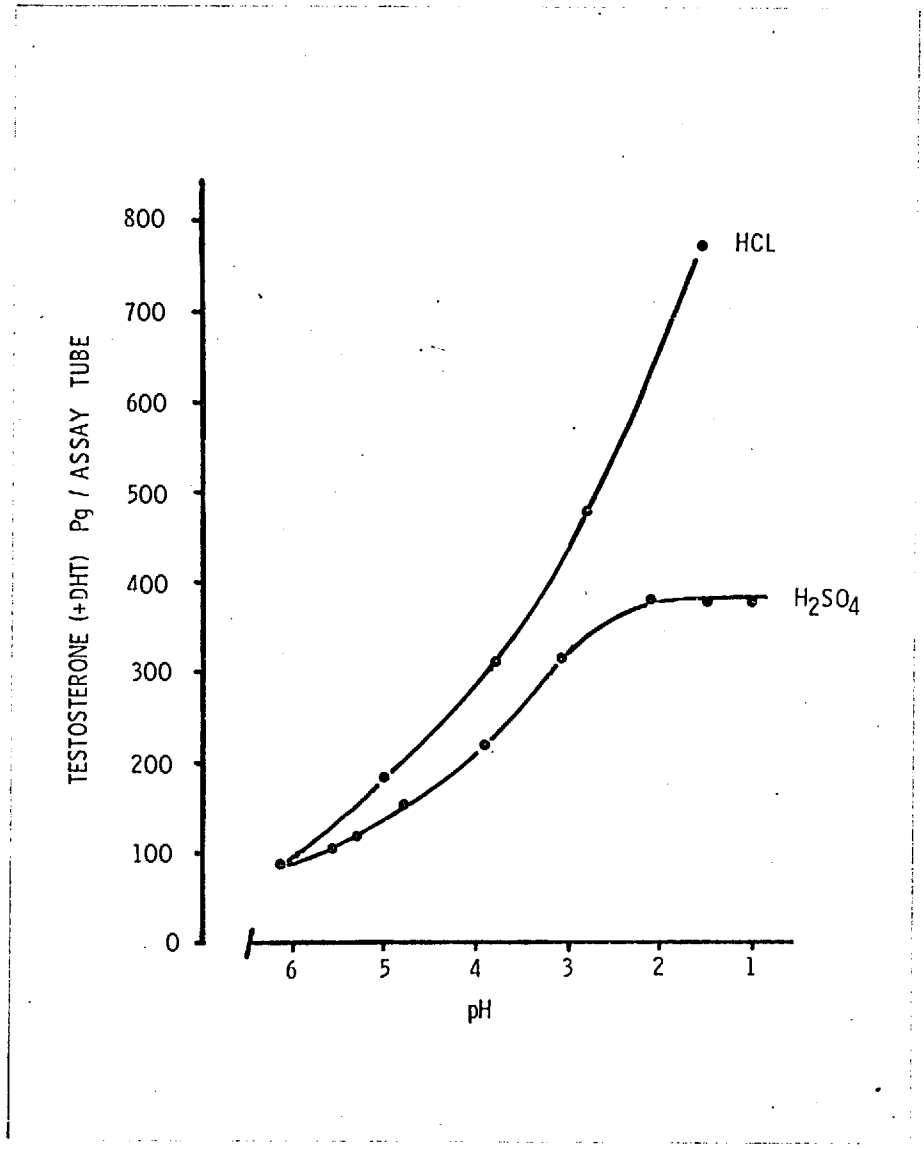
Effect of increasing concentration of sulphuric acid (abscissa) on the hydrolysis of testosterone sulphate (TS) and testosterone glucuronide (TG) measured as increase in unconjugated testosterone (T) in the assay tubes (ordinate). The upper graph shows changes at 18°C (room temperature) for 16h, the lower at 40°C for 48h. T has been added to four tubes in the latter experiment and results show no appreciable destruction.

Fig. 5.2.



Effect of increasing concentration of hydrochloric acid (abscissa) on the hydrolysis of TS and TG at 37°C for 18 hours as measured by unconjugated T release (ordinate).

Fig. 5.3.



Effects of decreasing pH (abscissa) on testosterone (T) release (ordinate) in urine caused by hydrochloric acid (HCl) or sulphuric acid (H_2SO_4).

4. TLC of urinary conjugates and their visualization by acid hydrolysis followed by RIA.

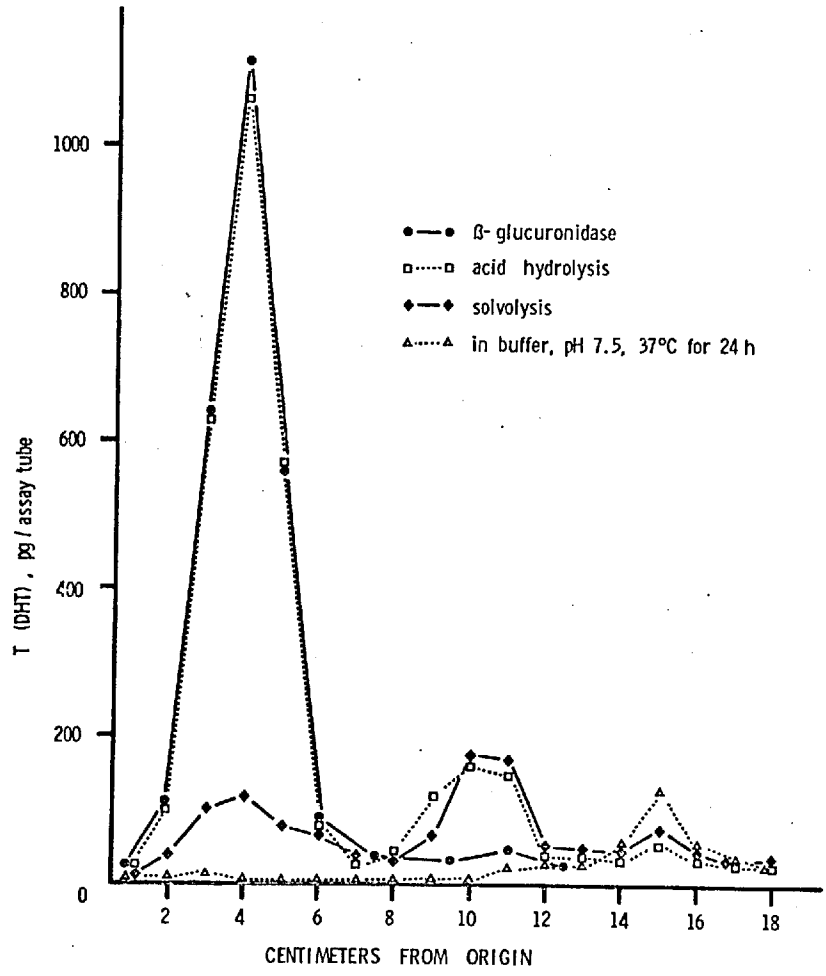
One ml of male urine was extracted for conjugates and run on TLC. The sample path was cut out and then cut into 18 one cm long pieces. Each piece was put into a centrifuge tube and eluted in methanol which was dried down under gentle air stream at 40°C. The residue was dissolved in 1.0ml of working buffer and 0.2ml aliquots from each tube put into 4 sets of 18 tubes. Each of these 18 tube sets were subjected to different treatment. β-glucuronidase in acetate buffer (pH 4.7) was added to the first set, the second set was made 3M H_2SO_4, the third subjected to solvolysis and the fourth had buffer added to make the volume equal to those of the other sets (0.4ml). All tubes were left at 37°C for 24h and then measured for T (DHT) by the direct T assay.

Results are presented in Fig. 5.4. The first peak, approximately 4 cm from the origin, is the TG, the second (~ 10.4cm) the TS and the third (15cm) the free T (DHT). The acid (3M H_2SO_4) and β-glucuronidase both hydrolyze TG (DHTG) but whereas the β-glucuronidase preparation has no effect on the TS (DHTS) it is hydrolyzed by the acid.

The solvolytic procedure hydrolyses TS and also a substantial part of the TG. The control tubes, containing buffer solution and having no specific treatment beyond the incubation at 37°C, showed only the peak corresponding to the free steroid(s).

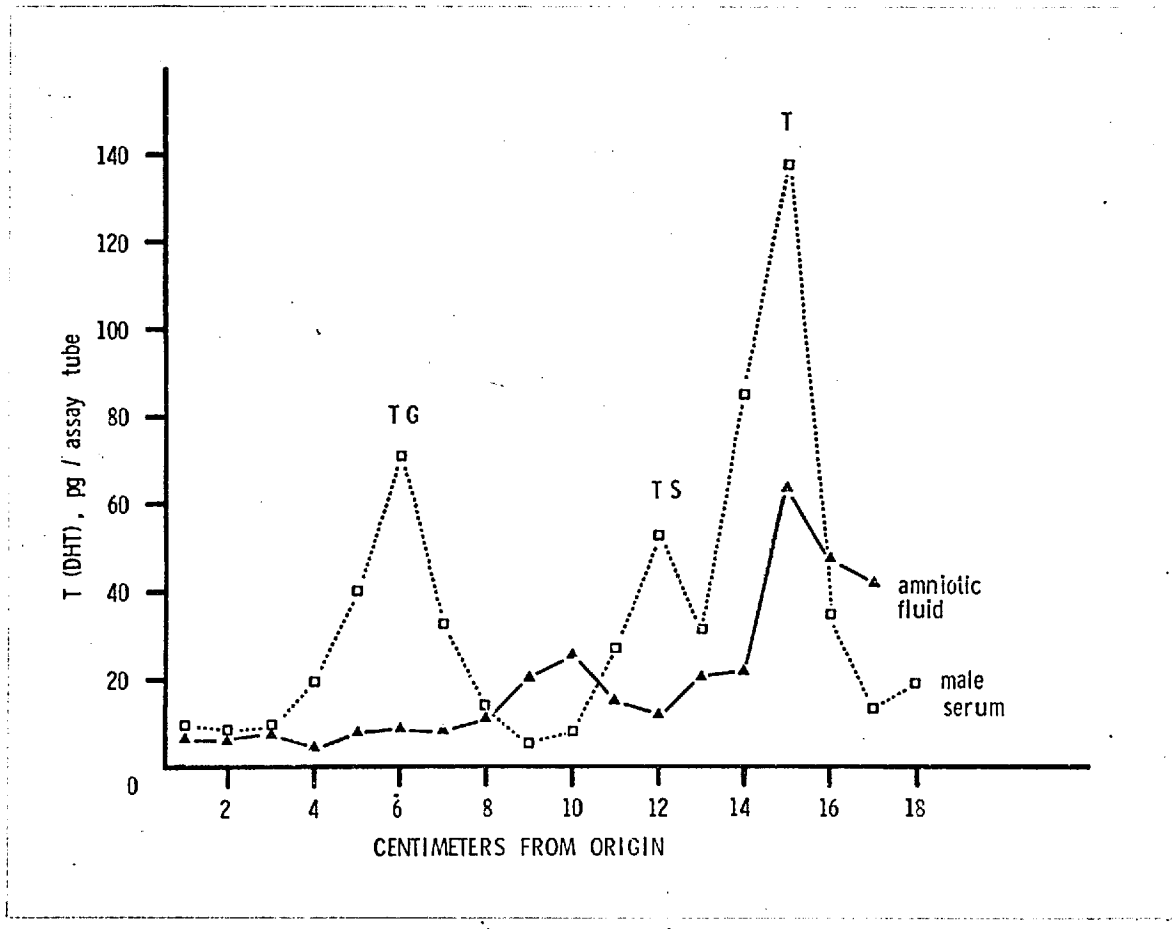
Examples of the use of this technique on serum and amniotic fluid are given in Fig. 5.5. Two amniotic fluid specimens from the 16th and 18th weeks of pregnancy were studied and neither showed any T (DHT) glucuronide peaks.

Fig. 5.4.



Thin layer chromatography (TLC) of T (DHT) conjugates extracted from male urine (see text). The TLC path has been cut into one cm long pieces (abscissa) separately eluted and measured for unconjugated T (DHT) (ordinate) after four different forms of treatment. The first and largest peak is testosterone (T) and dihydrotestosterone (DHT) glucuronide, the second T (DHT)-sulphate and the last and smallest one unconjugated T (DHT).

Fig. 5.5.



T and its conjugates in amniotic fluid (18 weeks gestation) and in male serum as studied by thin layer chromatography followed by acid hydrolysis. (See Fig. 5.4).

5. The stability of T*S and T*G in urine and in buffer; stability of TS and TG in buffer.

T*S and T*G were added to tubes containing urine specimens and working buffer. Tube contents were extracted by ether immediately after the addition, and after incubation at 37°C for 16h. Radioactivity in the ether extract before and after incubation showed no difference in neither the tubes containing buffer nor those containing urine.

Buffer solutions of TS and TG were incubated for 16g at 37°C and left at room temperature for 3 days. No rise in free T levels was detected by the direct T assay.

6. Heat (37°C) and acid lability of solvent extracts of urine.

Three fractions were made from two urine samples by extracting a) with 5 volumes of ether; b) with 5 volumes of ether and methanol (3 + 1 ; no acid added); c) urine left after these extractions. Solvent extracts were dried down and dissolved in working buffer. The three fractions were then each divided in 3 portions of which one was left at 37°C for 16h, one was made 0.1N with respect to H_2SO_4 and one frozen, serving as control. The fractions were then measured for T by the direct T assay. No rise of T occurred in fraction a), neither at 37°C nor after acidification. Fractions b) and c) both showed similar rise of T (DHT), about 4-fold after the acid treatment and about 2-fold after the incubation at 37°C.

7. Effects of increasing H_2SO_4 concentration at various temperatures on T (DHT) release by urine.

A urine sample was divided into 3 sets of aliquots (0.1 ml) to which H_2SO_4 solutions of increasing strength were added. The first set was incubated at room temperature for 16h, the second at 37°C for 16h and the third at 40°C for 48h. These were then measured with the direct assay for T (DHT). Results are shown in Fig. 5.6.

Being of special interest the first part of the abscissa (concentration of acid) is expanded. A pH scale is also given (not linear!) representing simultaneous pH measurements of the samples.

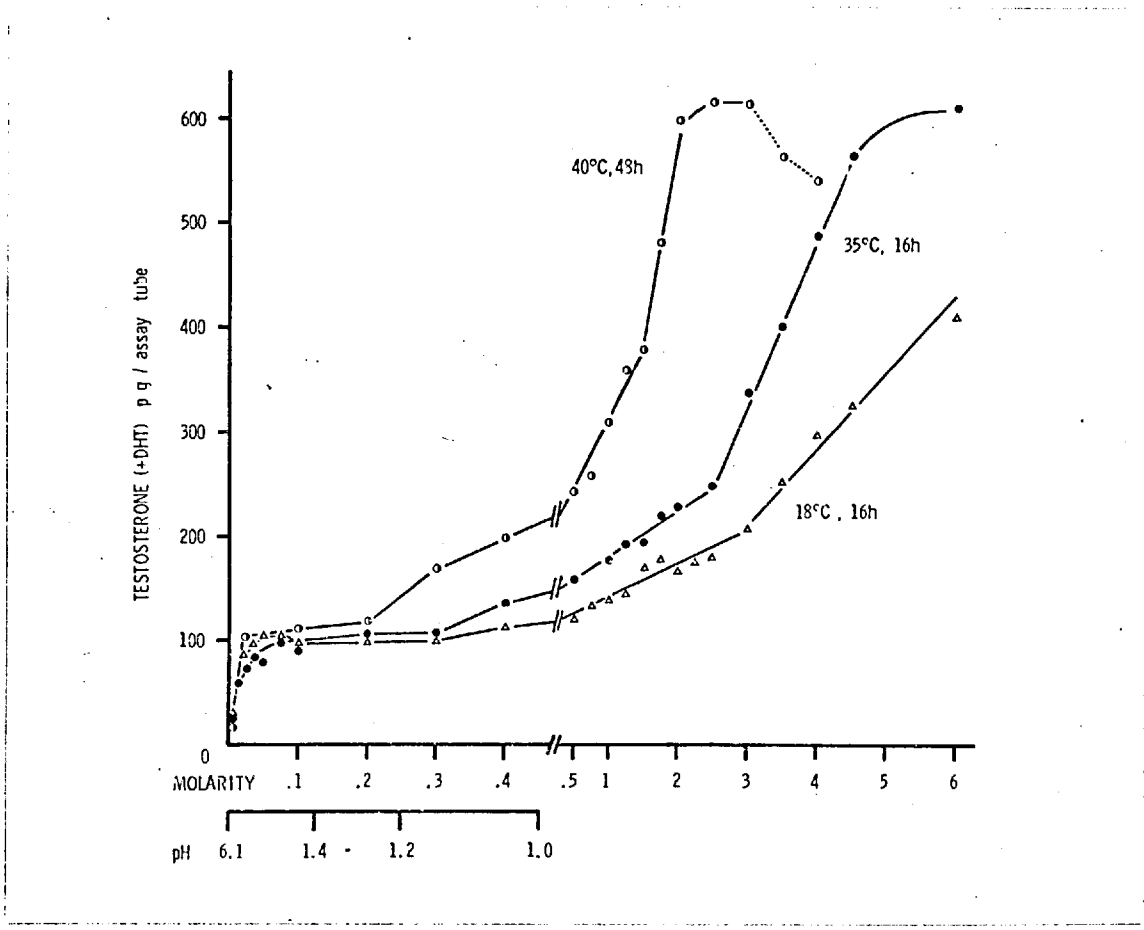
It can be seen that after an initial rapid rise of T a plateau is reached for up to about 0.4M acid in the samples incubated at room temperature. Although shortened it is still well visible for the samples incubated at 40°C.

Two linear rate increases are visible in all the incubation sets. In the 40°C incubation, the last two tubes with the highest acid concentration have slightly lower T content than the three previous ones.

8. Time course of hydrolysis of TS and TG and T release in urine in 3M H_2SO_4 at 37°C.

TS and TG were prepared in working buffer and aliquotted in centrifuge tubes. A urine pool was similarly aliquotted. Aliquots in duplicates were then made 3M with respect to H_2SO_4 at various time intervals and tubes sealed and icubated at 37°C. They were then measured by the direct T assay without internal standard. Results are shown in Fig. 5.7. It may be noted that the hydrolysis of TS is first to reach completion (6 hrs.) and that the hydrolysis pattern

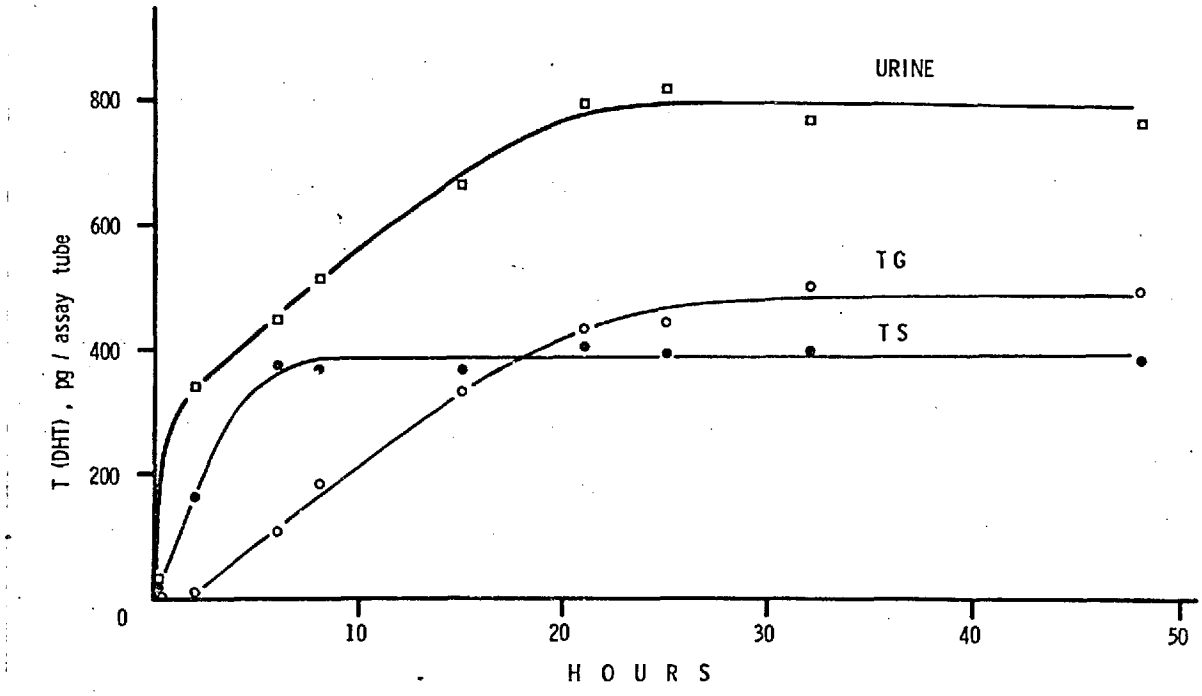
Fig. 5.6.



The effect of increasing concentration of sulphuric acid, H_2SO_4 (abscissa) at three different temperatures, for 16 or 48 hours on unconjugated T (DHT) release (ordinate) in human urine sample.

The first part of the abscissa is expanded to show effects at low molarities of the acid and a pH scale (not linear) with four readings is included.

Fig. 5.7.



T (+ DHT for urine) release (ordinate) by testosterone glucuronide (TG), testosterone sulphate (TS) and urine, subjected to 3M H_2SO_4 at 37°C for increasing length of time (abscissa).

of urine after the initial fast rise corresponds closely to that of TG reaching a completion at about 22 hrs.

9. The time course of T-release in urine made 0.05M H_2SO_4

A urine sample was divided into aliquots made 0.05M H_2SO_4 (pH approximately 1.8) and left at room temperature (18°C). Duplicate aliquots were extracted at 10 to 60 min after the acidification together with untreated controls and measured in the direct T assay. Results are shown in Fig. 5.8. Rise in unconjugated T (DHT) levels occurred within 10 min after the addition of the acid but no further rise with time was observed in the acidified aliquots. There was no significant rise in the untreated control aliquots.

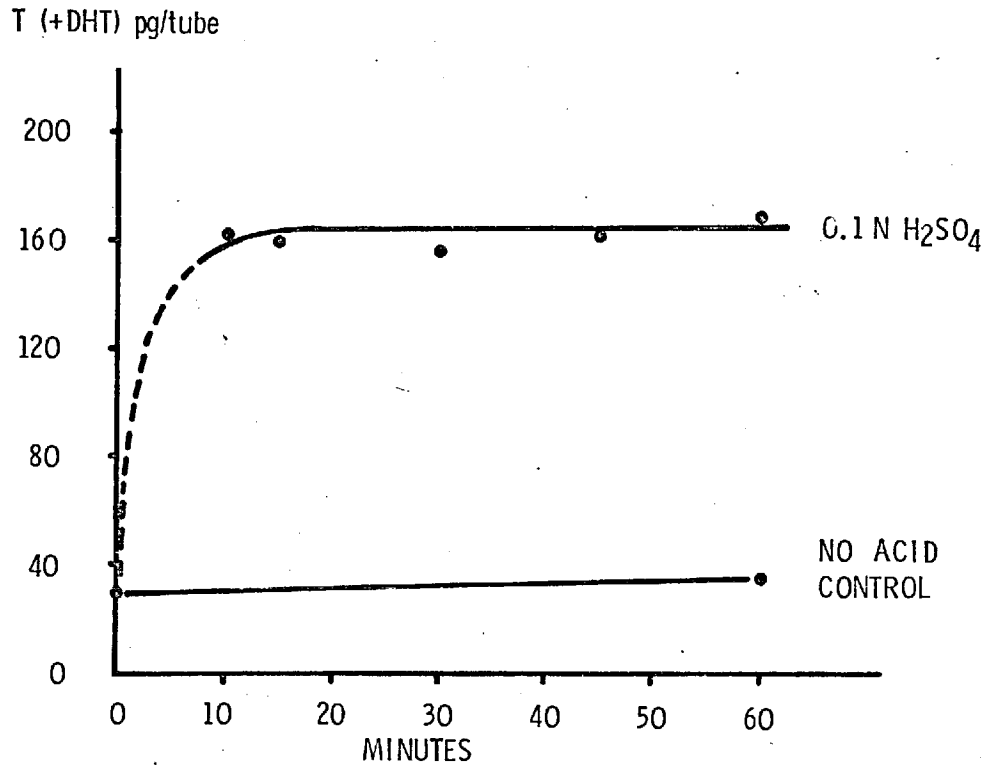
10. Separation of T*S and T*G on ion exchange column.

The tritiated conjugates were dissolved in buffer and applied directly together with T* on a Sephadex DEAE column. On a second column T*G and T* were applied in a urine sample. Radioactivity in the fractions (3ml) was counted and results are shown in Fig. 5.9. Unconjugated T is eluted first followed by TG and TS in that order. The results show that when added in urine, there tended to be an earlier elution of the conjugates from the column with no effect on the free steroid. Recovery of added radioactivity was over 90%.

11. Recovery of T* from urine studied by the ion exchange column.

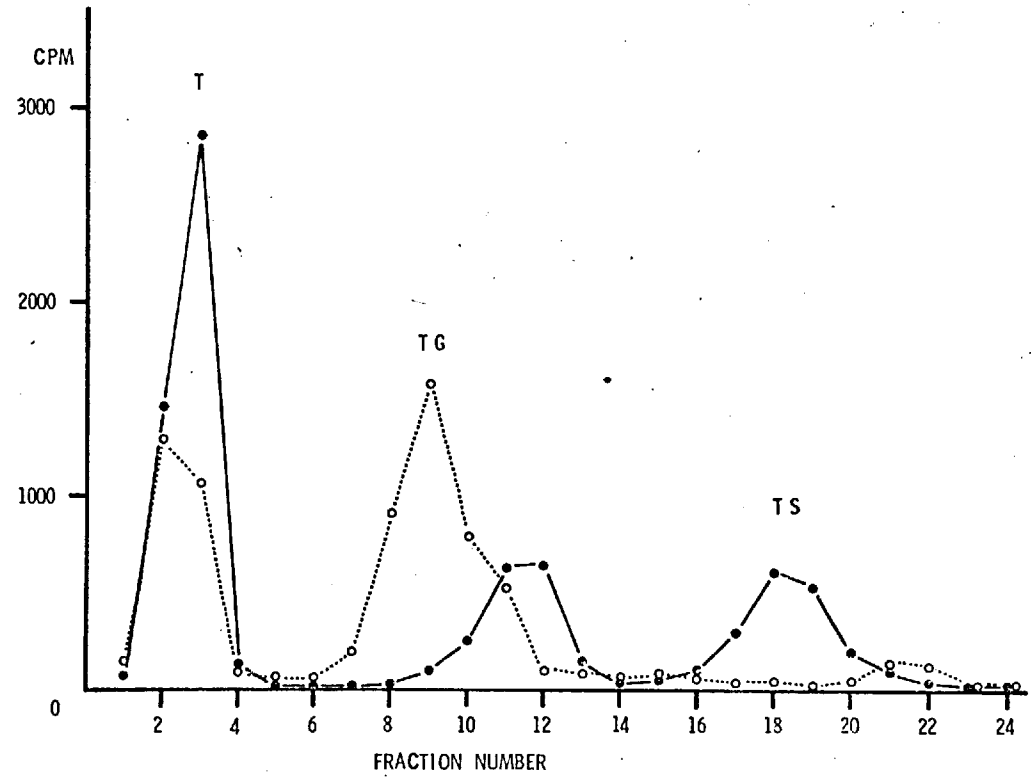
T* (25,000 CPM) was added to a urine sample which was incubated at 37°C for 6h and then applied on the Sephadex DEAE column. Radioactivity in the elution fractions showed only one peak corresponding to unconjugated T.

Fig. 5.8.



T (DHT) release (ordinate) by a urine sample made 0.1 N H_2SO_4 and stood at room temperature for increasing length of time (abscissa). Control samples, to which no acid was added are included.

Fig. 5.9.



Chromatography of tritiated testosterone (T), tritiated testosterone glucuronide (TG) and tritiated testosterone sulphate (TS) on DEAE Sephadex (A-25) column. Radioactivity in CPM (ordinate) is plotted against the number of the 3ml fractions collected (abscissa).

o —— o elution pattern after the application of the 3 compounds when dissolved in buffer.

0 ... 0 elution pattern for T and TG after their application when dissolved in urine.

12. Chromatography of urinary conjugates on ion exchange column.

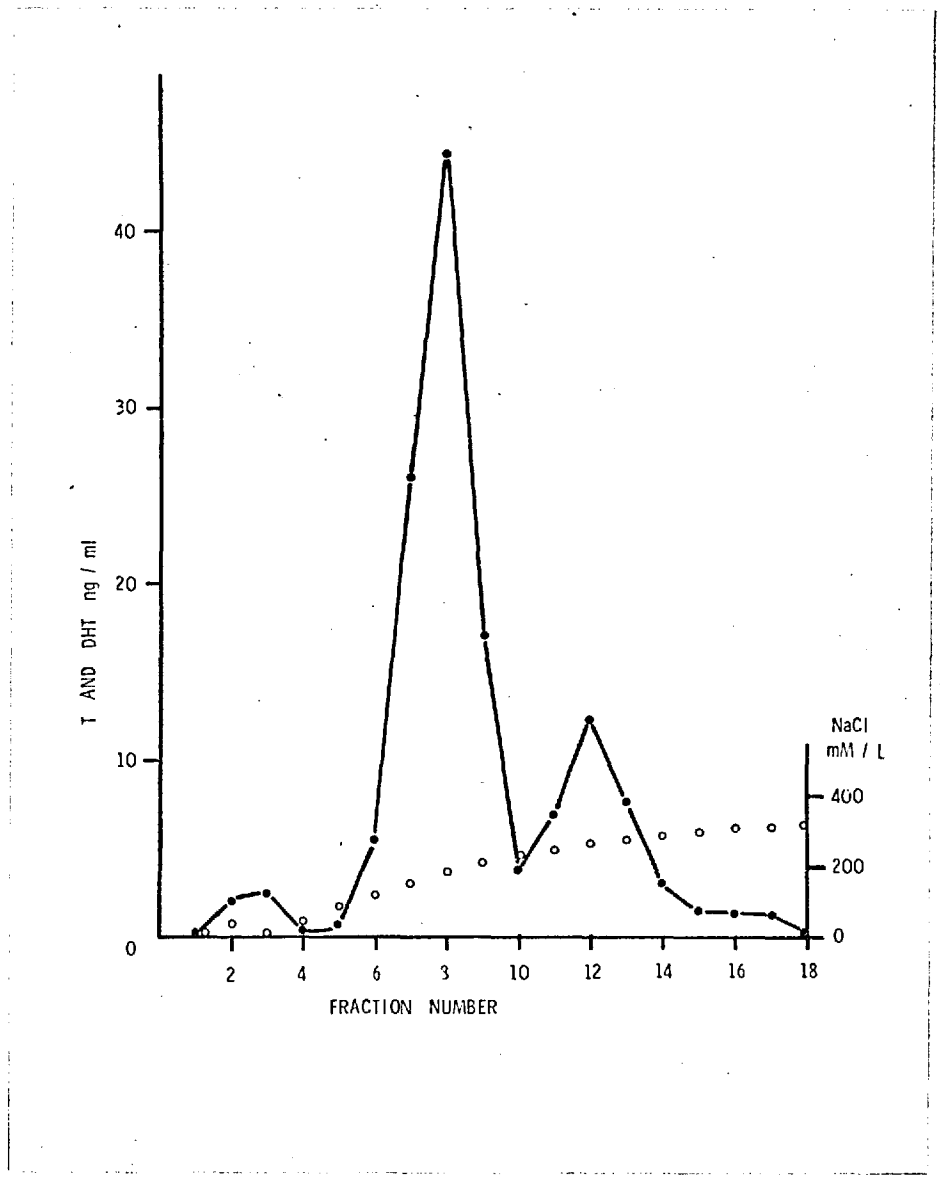
One ml of male urine was applied on the anion exchange column and eluted with a NaCl gradient (0.5mM/l in Res.1). Aliquots of elution fractions were made 3M H_2SO_4 and stood at 40°C for 20h and then measured with the direct T assay (without int. standard except in 3 tubes). Sodium concentration in each fraction was measured by flame photometry. Results are shown in Fig. 5.10; 30 fractions were measured, but as no appreciable T was measured in later fractions, only the first 18 are shown in the Figure. The T (DHT) concentration on the ordinate is given as ng/ml eluent based on an average recovery of 80%. The circles show the sodium concentration in mM/l (millimoles/litre).

Aliquots from the elution fractions of the column above were taken into two sets of 18 tubes, which were then subjected to two kinds of treatment. The first set was made 0.05M H_2SO_4 and stored at room temperature for 20 min followed by ether extraction. The other set was stored at 40°C for 20h before extraction. Both sets were measured by the direct T assay (including int. standards in 3 tubes in each set). Results are shown in Fig. 5.11. including the results from the previous experiment (Fig. 5.10.).

In order to contain all concentrations in the graph, and show clearly small but significant concentration changes, the ordinate has been given a logarithmic scale.

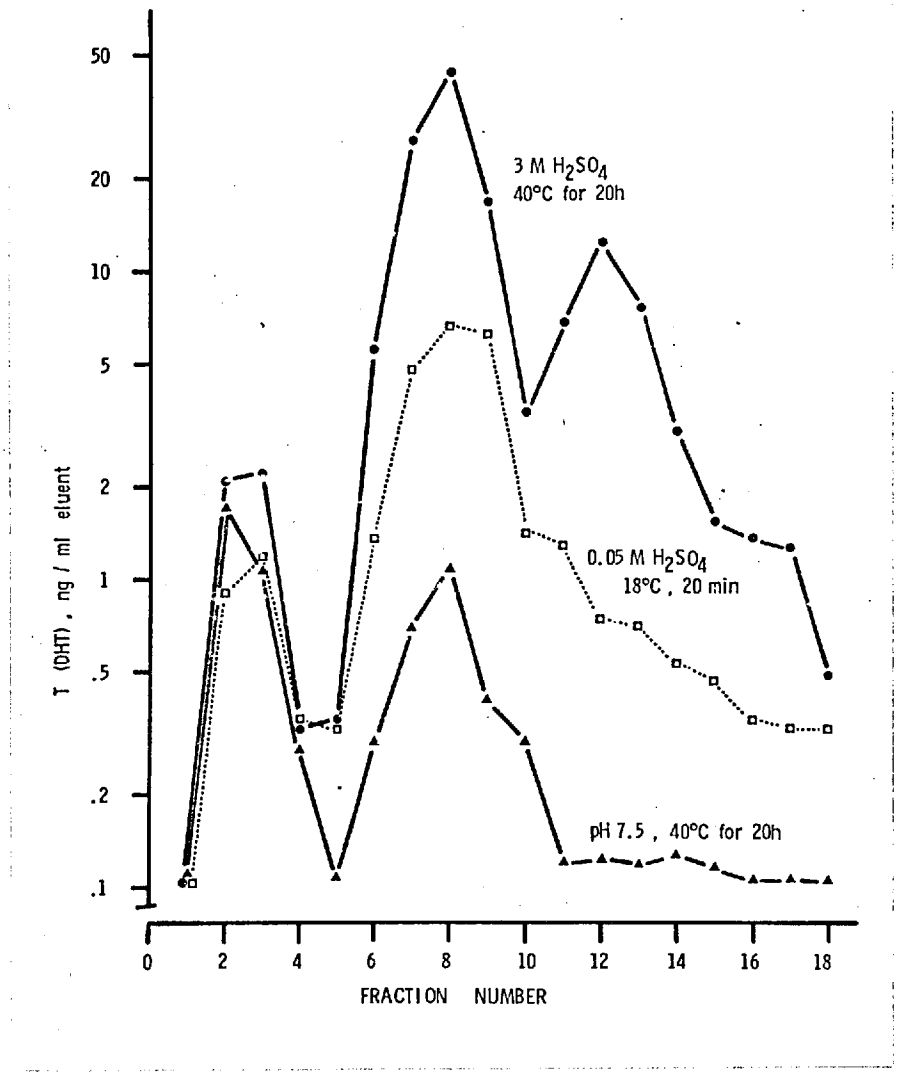
There is a clear rise, both after acidification and storage at 37°C, which in both cases peaks in the fractions which contain the TG peak. The acid treatment also caused a slight rise in the fractions containing the TS peak. This latter rise was noted in other experiments and was always small but variable.

Fig. 5.10.



Chromatography of a male urine sample on Sephadex (A-25) column. Aliquots of elution fractions (abscissa) have been subjected to acid hydrolysis and then measured for T + DHT, the concentrations of which in one ml of eluent are read on the ordinate. The small circles show the sodium concentration in mM/L, ordinate scale on the right. The peaks are as in Fig. 5.9 T, TG and TS from left to right.

Fig. 5.11.



Chromatography of a male urine sample on DEAE Sephadex (A-25) column. Same as Fig. 5.10 except that the y-axis has been given a logarithmic scale and aliquots of fractions have been subjected to two additional forms of treatment before assaying free T (DHT).

A urine sample was run on a cation exchanger column (Sephadex CM25) and fractions collected and analysed as above. One big peak was detected corresponding to the free steroid.

13. T and DHT contents in peaks.

Peaks from either TLC or column chromatography were studied by extracting aliquots from peak fractions after acid hydrolysis (3M H_2SO_4 at 40°C for 20h) and applying the extract to siliga gel plates run in the same system as used for the simultaneous T and DHT assay (see Chapter 3). When the paths in these runs were cut into 18 one cm pieces, eluted and measured by the direct T assay, only two peaks, corresponding to T and DHT, were found.

Quantitative measurements of T and DHT based on two male urine samples showed a DHT/T ratio of about 1/4 for the glucuronide peaks, 2/3 for the sulphate peaks and 1/6 for the rise caused by acidification to 0.05M H_2SO_4.

DISCUSSION

The studies reported in this chapter indicate the existance in human urine of a labile T and DHT conjugate hitherto unknown.

Summarizing the findings briefly it has been shown that

a) unconjugated T and DHT are released in intact urine at ambient temperatures and that this release is probably related to a very acid labile urinary fraction, releasing T and DHT at H_2SO_4 concentration of 0.05M (pH 1.8) within 10 min.

b) the T release is not caused by urinary TG or TS judged by hydrolysis characteristics of TG and TS both in urine and buffer solutions

c) the labile compound has polar solubility characteristics

d) a gentle chromatographic separation technique (neither acidification nor extraction) shows a distinct T (DHT) peak revealed by the use of the properties mentioned in (a) above

e) the labile compound forms a substantial part of the total urinary T and DHT excretion.

Perhaps the first indication of the existence of a very acid labile T and DHT compound in urine was provided by the early studies of Funk, Harrow and Lejwa (1929). These investigators adjusted the urine to a pH of 4.0 - 4.5 and extracted it continously with chloroform for 6 hours. The separated chloroform layer was then evaporated and the residue dissolved in olive oil, which was injected into castrated cocks to assess the effect of the male hormone (testiculin) on the comb size.

As to the nature of the compound, one can only speculate as yet. Methods need to be developed for better separation and eventual isolation of the compound(s). An obvious problem is the lability of the compound. In the first attempts to separate the compound on TLC, its outstanding acid lability was not realized and, because of its almost quantitative recovery the acid extraction procedure was adopted (Kornel, 1963). Further experiments with no pH adjustment prior to extraction followed by TLC or other separation technique seem justified. Some extension of the column technique, however, seems more attractive.

From compounds described in the literature only those synthesized by Wotiz et al. (1959) seem to fit the characteristics described. One of these, 3,5-androstadiene-3,17-dione, 3-enol-glucosiduronic acid (17-oxo-androsta-3, 5 dien-3-yl-β-D-glucopyranosiduronic acid) has been reported to be highly acid labile (Wakabayashi, 1961). Such 3-enol β-glucuronide has been suggested to exist for androstene-3, 17-dione, based on the isolation of the latter compound from a β-glucuronidase hydrolysate of urine from individuals injected with testosterone (Schubert, 1958). From our own preliminary studies on progesterone in urine, which as androstenedione, has no hydroxyl group for conjugation, it can be inferred that some form of conjugation exists for urinary progesterone. These studies showed a 2 - 3-fold rise in urinary free progesterone on incubation of urine at 37°C for 18 hrs.

In a detailed study of the direct metabolism of dehydroisoandrosterone glucuronide Knapstein et al. (1967) injected 3H-dehydroepiandrosterone - 14C - 3 - glucuronide ($^{3H}/14C = 2.02$) into a normal female and reported the isolation of eleven steroid glucuronides from the urine having the same $^{3H}/14C$ ratio as the precursors. Amongst these metabolites were androsterone and etiocholanolone glucuronide and the authors therefore concluded that the direct conversion of dehydroepiandrosterone-3-glucuronide to such metabolites necessitated the intermediacy of the 3,5-dienol-3-glucuronide. The labile compound described here might be this missing link.

In favour of that possibility is the similar elution pattern of the labile compound to that of TG from the sephadex ion exchange column combined with its lability (Wakabayashi, 1961).

If, however, the 3-enol glucuronide form does exist for T, the 17β-hydroxyl group offers another site for conjugation, thus forming a double conjugate, a conjugation form which does occur naturally among oestrogens. Such double T conjugate would presumably have a distinct chromatography peak, which has not been seen in the present studies.

Although at present unidentified, this labile compound seems to have hydrolysis characteristics distinct enough from TS and TG for making its quantitative determination in terms of T (or DHT) possible. Thus the difference of T concentration in urine before and after acidification (0.05M H_2SO_4) should equal its quantity. This has been done in urine samples from six normal individuals and indicates about 5-fold difference in excretion between the sexes (T + DHT, direct assay).

An interesting feature emerging from these studies is the difference in hydrolytic effect between H_2SO_4 and HCl. This difference might explain the discrepancy in the results obtained for the "pH 1 hydrolyzable" conjugate by Dulmanis et al. (1964) and van der Molen et al. (1966). The latter authors did not state which acid was used for the pH adjustment, but the present studies raise the possibility that H_2SO_4 has been used by them thus resulting in lower values than those reported by Dulmanis et al., who used HCl. The choice of a pH 1 is apparently an arbitrary one and the present results show that there is no pH value at which a selective and quantitative hydrolysis of only TS takes place when either HCl or H_2SO_4 are used.

The methodology devised makes a powerful tool for the analysis of steroid conjugates and invites various interesting studies. There should be no immediate problems in studying serum and other

biological fluids as well as tissue homogenates. The TLC method has already been used to study some tissue homogenates and an example of its application for amniotic fluid and serum is presented. As recoveries were not monitored in these studies, values shown in Fig. 5.5. represent only a rough relation of quantities. No T glucuronide (TG) was found in the two amniotic fluid specimens studied. This has not been described before in the literature. The absence of TG might be related to the age of the foetuses as both specimens were from the earlier half of pregnancy (16 and 18 weeks). It could, however, be a reflection of a characteristic foetal steroid metabolism, contrary to the situation in later life, when T glucuronide is found both in urine and blood. T sulphates have been found to be the main conjugated fraction in previable foetuses after perfusion with labelled T (Benagio et al., 1968). It is a possibility that TG is more efficiently cleared from the amniotic fluid than the unconjugated steroid or its other conjugates.

The presence in urine of conjugates, resistant to the combined hydrolytic effect of the concentrations of acid, incubation time and temperatures used, is unlikely. As shown in Fig. 5.6. and Fig. 5.7. there is no tendency for values to increase beyond a certain level reached, signifying the completion of the hydrolysis. In Fig. 5.6. unconjugated T (DHT) values, after 48h incubation at 40^{o}C, in the two tubes with the strongest acidity, have come down from a level reached by the previous ones. This probably indicates a destruction of the immunoassayable T (DHT). Such a destruction is perhaps more likely to occur in urine than in "purer" media, as it has never been detected in buffer solutions subjected to similar incubation conditions.

This is illustrated in the lower part of Fig. 5.1. where neither the T released from the conjugates nor the T added show any destruction after the same incubation conditions. It is therefore unlikely that any T-release versus T-destruction equilibrium has been at play.

CHAPTER 6

Unconjugated testosterone (T), 5α-dihydrotestosterone (DHT) and oestradiol (E_2) in simultaneously collected serum and urine samples; levels in normal reproductive women and men, women on oral contraceptives, post-menopausal and hirsute women; relationship between hormone levels.

INTRODUCTION

Unconjugated sex hormones exist in serum in two states, that is as non-protein bound (unbound) and bound to protein, mainly sex hormone binding globulin (SHBG; approximately 60% for T) and albumin (approximately 40% for T). Only a minor fraction of T and E_2 is in the free state or unbound (0.5 - 2.0%; Vermeulen et al., 1971; Fisher et al., 1974). The biological activity of sex hormones has been purported to be related to the size of this fraction. If this were so the size of this fraction might correlate better with clinical situations than do the total unconjugated levels (protein bound and non-protein bound).

Methods developed so far to assess this fraction are indirect and cumbersome, and it was thought worthwhile to look for another approach.

The use of urinary free (unconjugated) cortisol as an index of adrenal function was first suggested by Cope and Hurlock (1959) and has now been successfully used for several years in clinical practice

(Burke and Beardwell, 1973). This success might be due to the function of the kidney as an integrating ultra-filter, with the unconjugated urinary levels reflecting (and correlating with) the unbound levels in blood (Burke and Beardwell, 1973).

By analogy with the experience for cortisol, it seemed justified to attempt similar measurements for the sex steroids. Further, as serum T levels fluctuate considerably in men (see Chapter 7) during the day, a T measurement on a signle blood specimen might not be reliable in borderline cases and urinary excretion could be a better clinical index.

Measuring T and DHT simultaneously in both serum and urine was also of interest for other considerations. A specific retention of T and DHT by the mouse kidney has been shown (Bardin et al.,1973; Bullock and Bardin, 1975) and kidneys from all species contains appreciable amounts of 5α-reductase activity (King and Mainwaring, 1974b). T/DHT ratios in urine could therefore reflect 5α-reduction of T by the kidney when these were compared to those of the serum.

Despite the discouraging results described in Chapter 4 on the stability (or instability rather) of the unconjugated sex hormones in urine, it was decided to measure them in simultaneously collected urine and serum specimens in two groups of normal individuals, males and females, taking due care to refrigerate samples during collection and freeze them immediately thereafter until measured.

In order to gain some knowledge on the renal handling of the unconjugated hormones, urinary creatinine was measured and correlated with the hormone levels. Creatinine measurements also served as a check on the urine collections.

Unconjugated E_2 was measured in serum and urine to study its relation to T and DHT and to obtain reference measurements for another group included in the studies, namely, women on oral contraceptives (OC).

Sex hormone binding globulin (SHBG) is well documented to be raised in women on OC containing synthetic oestrogens (Van Kammen et al., 1975; Briggs, 1975). It has been postulated that changes in SHBG concentration in serum might influence the peripheral effects of sex hormones (Anderson, 1974). It was therefore of interest to study the serum levels versus urinary excretion of unconjugated hormones in a group of such women. Both total unconjugated and non-protein bound plasma levels of cortisol have been reported to be elevated in these women (Burke, 1970).

Briggs (1976) in an exhaustive review of the literature on the biochemical effects of OC cites reports on changes in plasma testosterone, but levels of DHT in women on OC have apparently not been reported. While serum T levels during OC ingestion have been found elevated (Briggs and Briggs, 1972; Easterling et al., 1974; Tremblay and Duke, 1974) reports on serum E_2 levels in women on OC have been conflicting. Some reports find sustained low levels of E_2 equivalent to follicular levels of a normal cycle (Mishell et al., 1972; Briggs and Briggs, 1972), other reports record still lower levels (Johansson et al., 1972; Johansson, 1975). Preliminary results obtained by the direct E_2 radioimmunoassay described in Chapter 3 showed a sharp reduction in serum unconjugated E_2 levels in women taking OC.

The mechanism of action of OC is still unknown, but has been thought to be either an inhibition of gonadotrophin release or a

direct effect on the ovary and the other female genital organs. While OC do lower serum and urinary gonadotrophin levels (Briggs et al., 1970; review), the reports cited above on sex steroids together with reports on elevated serum levels of other steroids such as aldosterone and cortisol (Beckerhoff et al., 1973; Burke, 1970; Layne et al., 1962) suggest that the OC have their primary effect on steroid metabolism. Such metabolic effect might in turn influence the pituitary release of gonadotrophins.

Serum values of T and DHT obtained in hirsute women and postmenopausal women have been presented in Chapter 3 under the appropriate methods. In this chapter small groups of each of these subjects were studied for urinary values of the 3 unconjugated sex hormones (T, DHT, E_2).

MATERIAL AND METHODS

Subjects participating in this study and making up the normal groups were healthy individuals from the hospital staff, including males aged 25 - 37 years, premenopausal women consisting of a group not taking oral contraceptives (OC) and a group on OC aged 18 - 34, and postmenopausal women aged 55 - 63. The premenopausal women not taking OC (normal group) were all within the first 10 days of the follicular phase. The women taking OC (contraceptive group) were at different dates from their last menstrual bleeding and had been taking contraceptives for at least two months. A group of hirsute women were outpatients of the hospital and on no medication at the time of urine collection.

The OC used were Gynovlar 21(TM), containing 50ug ethynyl oestradiol (EE_2) + 3mg norethisterone acetate (2 subjects); Eugynon 50(TM) containing 50ug EE_2 + 500ug dl-norgestrel (2 subjects) and, Ovran (TM) containing 50ug EE_2 + 250ug d-norgestrel (5 subjects).

Serum samples were collected between 09.00 and 11.00 and between 16.00 and 18.00 to give 6 hours between bleedings. Urine samples were collected from 20.00 to 08.00 during the previous night and from 08.00 to 20.00 during the day of blood collection. Participants had instructions to refrigerate urine samples after voiding and keep them refrigerated during the period of collection. Urine samples were then frozen immediately after reception and kept frozen (-20°C) until measured. Serum was separated from the corpuscular elements within three hours from bleeding and kept frozen at -20°C until measured.

Serum and urinary levels were measured by the methods described in Chapter 3 as indicated under Results.

Urinary creatinine measurements were based on the Jaffe reaction according to the method of Folin and Wu (1919) adapted for the Autoanalyzer (Technicon).

RESULTS

1. Serum levels

   (i) Serum levels of unconjugated T and DHT in normal males and females in the early follicular phase of the menstrual cycle.

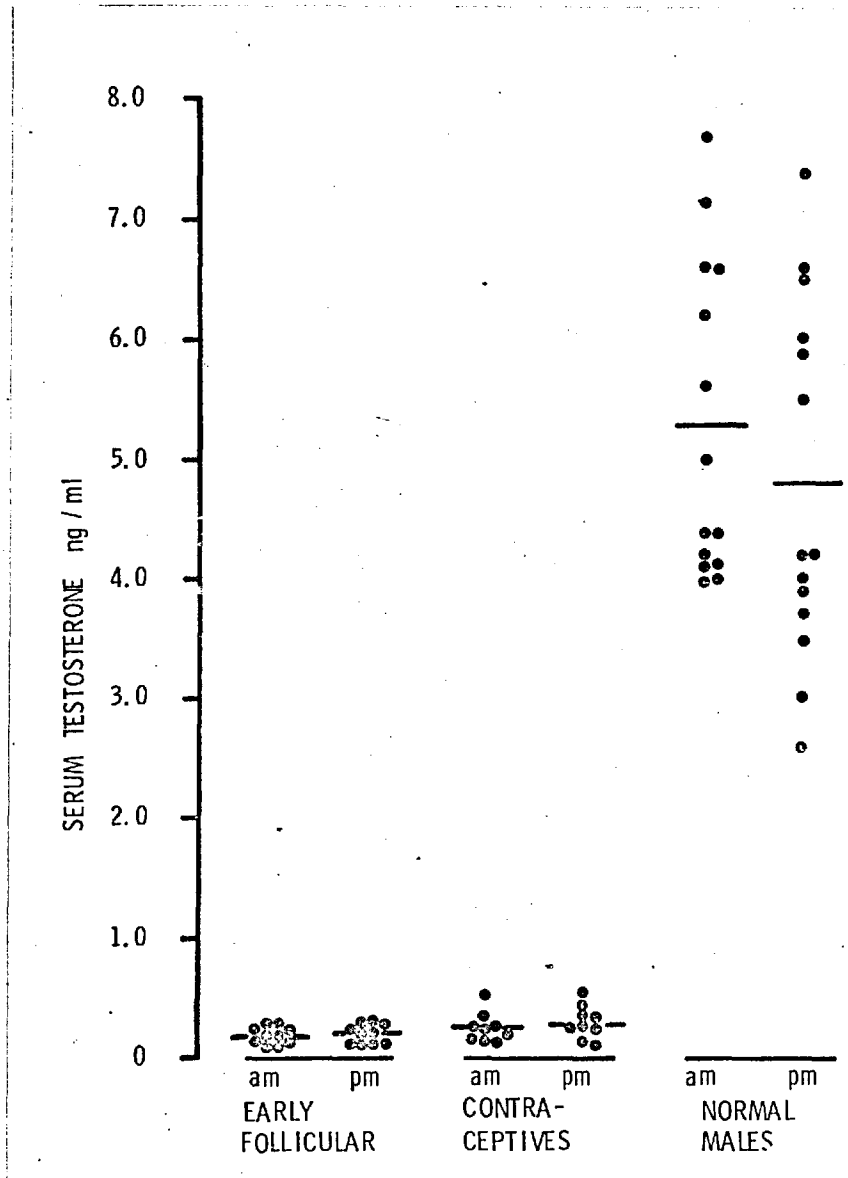
Serum levels were measured by the simultaneous T and DHT assay including TLC. Results are presented in Table 6.1., Fig. 6.1. and Fig. 6.2.

Table 6.1.

Serum levels of T (ng/ml) and DHT (pg/ml) in normal females (follicular phase) and males in the morning (09.00 - 11.00 am) and afternoon (16.00 - 18.00 pm)

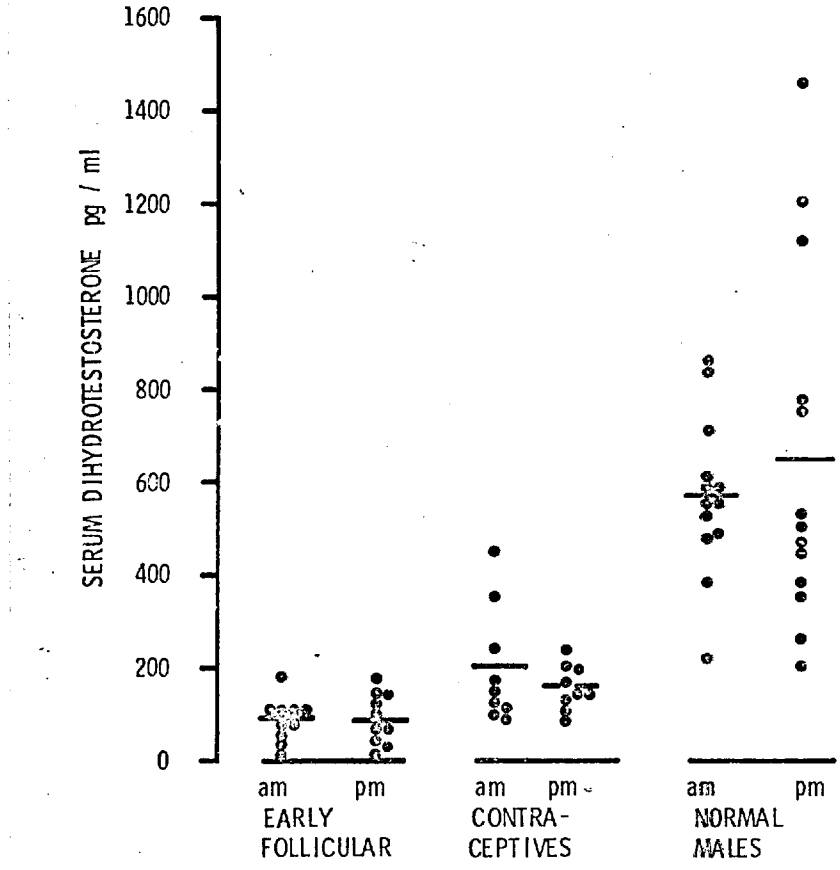
	F E M A L E S						M A L E S					
	T			DHT			T			DHT		
	am	pm	mean	am	pm	mean	am	pm	mean	am	pm	mean
mean	0.19	0.22	0.21	91	90	91	5.29	4.79	5.06	570	651	610
st.dev.	0.05	0.06	0.05	44	48	42	1.32	1.50	1.19	171	391	251
range	(0.08 to 0.29	0.14 to 0.34	0.13 to 0.30	11 to 180	6 to 177	24 to 179	4.0 to 7.7	2.6 to 7.4	3.3 to 7.2	221 to 860	202 to 1460	212 to 1033
(n)	-	(10)	-	-	(10)	-	-	(14)	-	-	(14)	-

Fig. 6.1.



Serum testosterone (T) in three groups of healthy individuals; normal women in the first 10 days of the menstrual cycle, women on oral contraceptives and normal males. The horizontal bars indicate mean values.

Fig. 6.2.



Serum levels of dihydrotestosterone (DHT) in three groups of healthy individuals. The groups are the same as in Fig. 6.1. The horizontal bars indicate mean values.

Although mean morning and afternoon values of unconjugated T and DHT in males showed slight differences, neither was significant at the 5% level. The T/DHT ratio was approximately 2.3 in females and 8.2 in males. Males had about 24 and 7 times higher levels of T and DHT respectively.

Correlation coefficients between hormone levels are presented in Table 6.2. Although a positive correlation was found between mean serum T and DHT levels in the 10 females ($r = 0.53$) and 13 males ($r = 0.50$) neither was significant at the 5% level when tested by the t test. In the males, but not in the females, a significant correlation was found in the overall values (a.m. and p.m.) and results are presented in Fig. 6.3. The regression line was calculated by the least squares method and had the equation $y = 2.19 x + 3.67$; the correlation coefficient (r) was 0.46; $p < 0.02$ ($n = 26$).

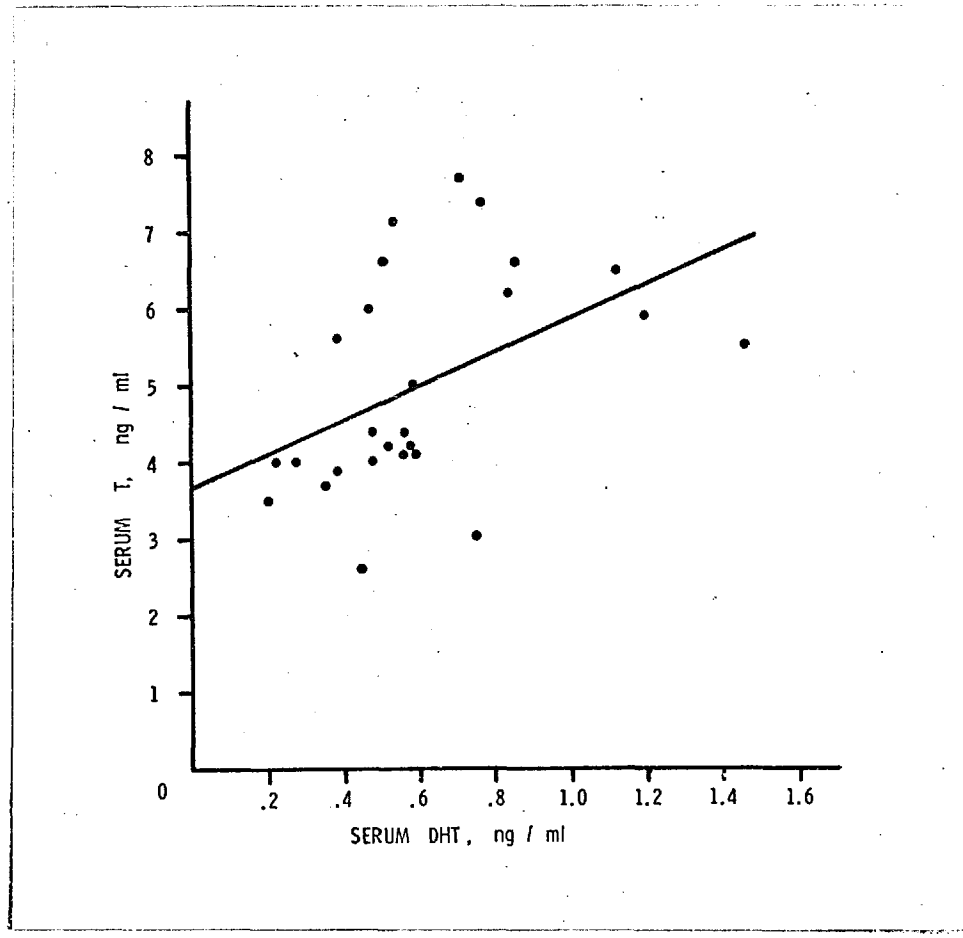
As both morning and afternoon values in 13 males are plotted in Fig. 6.3. it represents both inter and intra-individual variation of T/DHT ratios. To investigate whether there was a significantly greater variation between individuals than at different times in the same individual, both the means and differences between morning and afternoon T/DHT ratios in each individual were obtained (Table 6.3.). The variances of the means and the differences were then calculated and compared using the variance ratio test (two sided F test; Armitage, 1971). As shown in Table 6.3. the two variances were found to be significantly ($p < 0.025$) different with the lower variation for differences within the individuals. These results suggest the existence of an individual T/DHT constant which varies from one individual to another.

Table 6.2.

Linear correlation coefficient (r) between unconjugated hormone levels in three groups of individuals in both serum and urine. n = number of individuals. p is given where correlation was significant at the 5% level.

		Serum			Urine		
group	hormones	r	n		r	n	
normal men	T/DHT	0.50	13		0.90	11	$p < 0.001$
	E_2/DHT	0.60	13	$p < 0.05$	0.15	8	
	T/E_2	0.44	13		0.24	8	
normal women (early foll.)	T/DHT	0.53	10		0.80	10	$p < 0.01$
	E_2/DHT	0.74	10	$p < 0.02$	0.60	7	
	T/E_2	0.27	10		0.59	7	
women on oral contraceptives	T/DHT	0.23	9		0.90	9	$p < 0.001$
	E_2/DHT	0.21	9		0.25	9	
	T/E_2	0.09	9		0.11	9	

Fig. 6.3.



Relationship between serum T (ordinate) and DHT (abscissa) levels in 13 normal males. Levels from a morning and an afternoon specimen are plotted for each individual. The regression line (least squares) has the equation $y = 2.19x + 3.67$; $r = 0.46$; $n = 26$; $p < 0.02$

Table 6.3.

Serum T and DHT levels (ng/ml) in normal males in the morning and in the afternoon. T/DHT ratios have been calculated for each individual and the variances of their means and differences are compared by the variance ratio test (F)

Subject	morning			afternoon			mean of T/DHT (am + pm/2)	absolute difference
No.	T	DHT	T/DHT	T	DHT	T/DHT	ratios	of ratios
1	5.0	0.60	8.3	4.2	0.52	8.1	8.20	0.2
2	4.0	0.22	18.2	3.5	0.20	17.5	17.85	0.7
3	5.6	0.39	14.4	6.0	0.47	12.8	13.60	1.6
4	4.1	0.59	7.0	7.4	0.77	9.6	8.30	2.6
5	6.2	0.84	7.4	6.5	1.10	5.9	6.65	1.5
6	4.0	0.48	8.3	2.6	0.45	5.8	7.05	2.5
7	7.7	0.70	11.0	6.6	0.51	12.9	11.95	1.9
8	6.6	0.86	7.7	5.9	1.21	4.9	6.30	2.8
9	4.4	0.57	7.7	3.7	0.35	10.6	9.15	2.9
10	7.2	0.53	13.6	3.9	0.38	10.3	11.95	3.3
11	4.2	0.58	7.2	4.0	0.27	14.8	11.00	7.6
12	4.1	0.56	7.3	3.0	0.76	4.0	5.65	3.3
13	4.4	0.49	9.0	5.6	1.47	3.8	6.40	5.2
mean			9.78			9.31	9.54	2.78
st. dev.			3.51			4.36	3.57	1.97
variance			12.32			19.01	12.73	3.70

$F = 12.73/3.70 = 3.44;\quad p < 0.025$

(ii) Serum unconjugated oestradiol (E_2) levels in normal females and males.

The serum E_2 levels were measured in the same samples as in (i) above with the direct E_2 assay (without chromatography). Results are presented in Table 6.4. and Fig. 6.4. where the mean values of the am and pm specimens have been plotted.

There was no difference between morning and afternoon specimens, neither in females nor males. Mean female values were about 1.8 times higher than the male ones and the difference was significant at the 1% level.

A significant linear correlation ($p < 0.05$) was found between serum E_2 and DHT levels in both sexes (Table 6.2.). Fig. 6.5. shows this relationship in normal males and females. While a positive correlation was found between serum E_2 and T (see Table 6.2.) it was not significant in either sex when tested by the t test.

(iii) Serum T, DHT and E_2 levels in normal females on oral contraceptives.

Serum samples from nine normal females on oral contraceptives (contraceptive group) were measured for T, DHT and E_2. Samples were collected in the morning and evening as above. Values are presented in Table 6.5. and plotted together with values from other groups for comparison in Fig. 6.1. (T), Fig. 6.2. and Fig. 6.4.

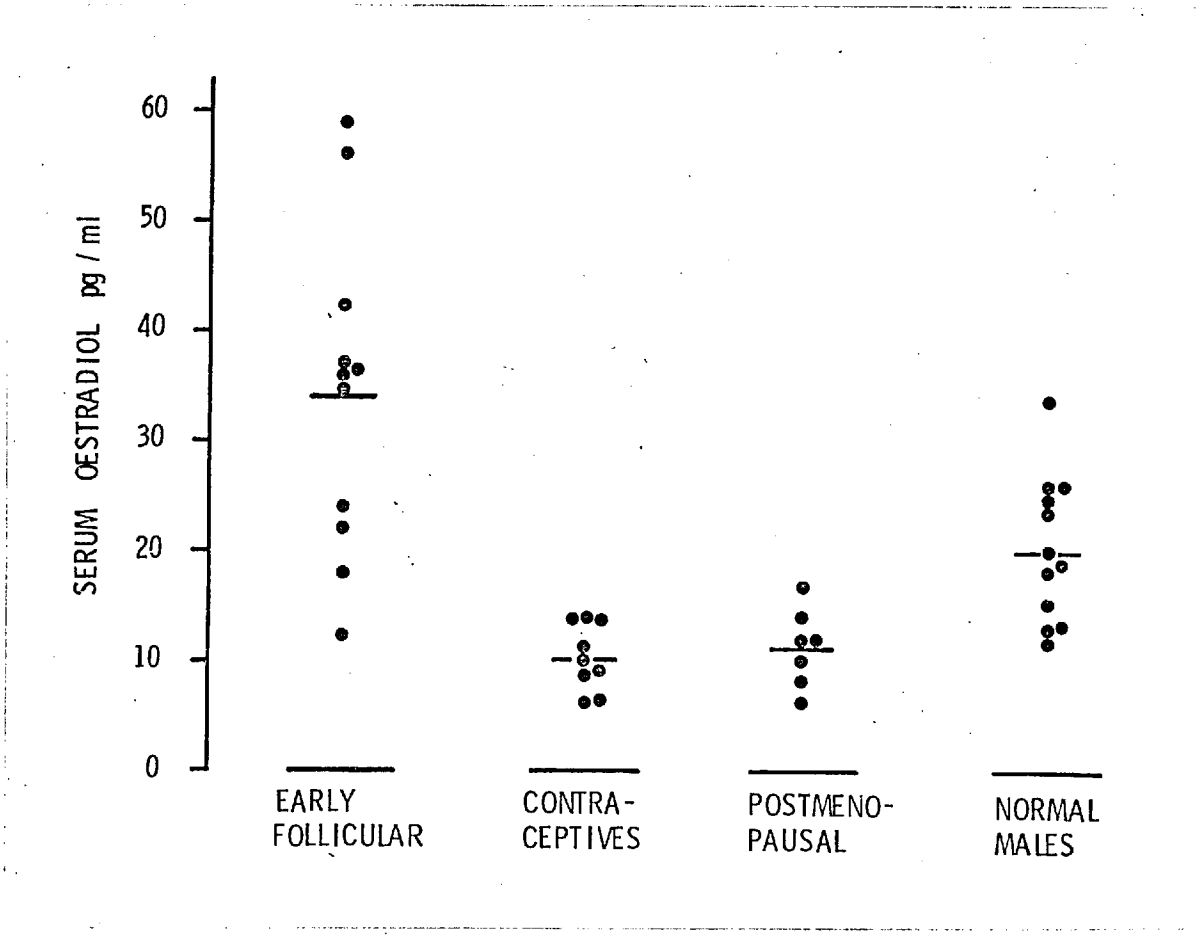
As shown in Table 6.5. the means of both serum T (Fig. 6.1.) and DHT (Fig. 6.2.) are higher in the contraceptive group than in the normal group by about 1.3 and 2.0 ($p < 0.01$) times respectively. The table also shows that mean serum E_2 levels are about 3 times lower ($p < 0.001$) in the females on contraceptives than in those not taking

Table 6.4.

Serum E_2 levels (pg/ml) in normal females (early follicular) and males

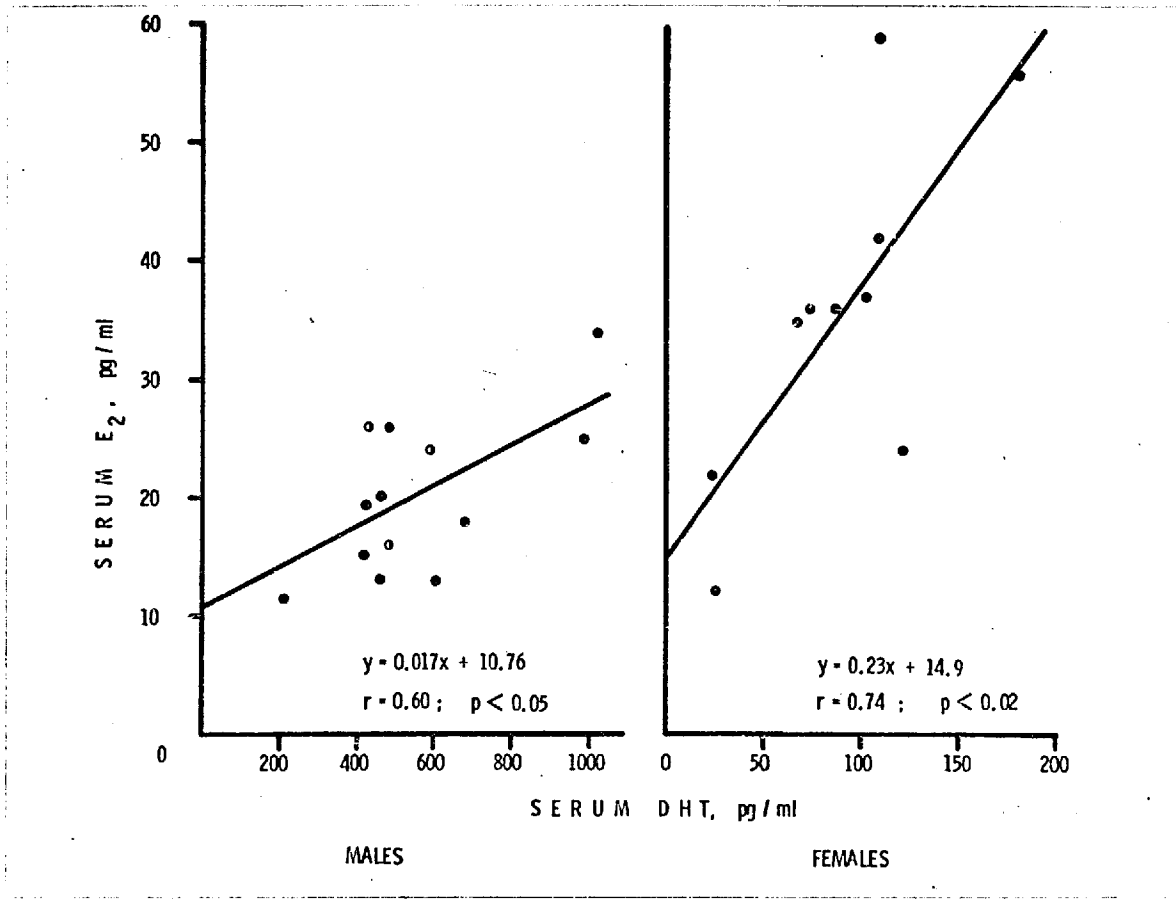
	F E M A L E S			M A L E S		
	am	pm	mean	am	pm	mean
mean	29	31	34	19.5	18.8	19.2
st. dev.	13	18	15	7	9.5	6.7
range	10 - 52	14 - 66	12 - 59	12 - 36	8 - 42	12 - 34
(n)	(7)	(7)	(11)	(11)	(11)	(11)

Fig. 6.4.



Serum oestradiol (E_2) levels in 4 groups of individuals. It may be noted that E_2 levels in women on oral contraceptives (OC) are in the range of postmenopausal women. The horizontal bars indicate mean values.

Fig. 6.5.



Relationship between serum E_2 (abscissa) and DHT (ordinate) in 13 normal males and 10 females in the first 10 days of the follicular phase of the menstrual cycle. Except for 3 half-filled points in the male series, serum levels are the mean of two values obtained from each individual, one in the morning and one in the afternoon, 6 hours apart. The 3 half-filled points in the male series were values from single blood specimens obtained in the morning.

Table 6.5.

Serum T (ng/ml), DHT and E_2 (pg/ml) levels in normal females on oral contraceptives

	T			DHT			E_2		
	am	pm	mean	am	pm	mean	am	pm	mean
mean	0.26	0.29	0.28[a] (0.21)*	204	161	181[b] (91)*	9.5	12	10.3[c] (34.0)*
st. dev.	0.13	0.14	0.11	133	44	71	5	3	3.2
range	0.12 - 0.52	0.90 - 0.55	0.11 - 0.46	100 - 454	95 - 238	108 - 325	4 - 16	9 - 15	6 - 14
(n)	-	(9)	-	-	(9)	-	(6)	(6)	(9)

*Corresponding mean values from the control group (early follicular; Table 6.1. and Table 6.4.)

a) No significant difference ($p > 0.05$) when compared to the control group.

b) A highly significant difference ($p < 0.01$) when compared to the control group.

c) A very highly significant difference ($p < 0.001$) when compared to the control group.

contraceptives. Fig. 6.4. shows that the serum E_2 levels of the contraceptive group are in the same range as those of postmenopausal women and lower than those of normal males. Correlation between serum E_2 and DHT was poor as were correlations between E_2 and T and between T and DHT. Table 6.2. shows the linear correlation coefficient (r) between hormonal levels in the contraceptive group and the two "normal" groups, males and females. It may be noted that all the serum r values are at least two times lower in the contraceptive group than in the other groups.

Results from the contraceptive group did not point to any difference between morning and afternoon levels for any of the hormones.

2. Urinary excretion.

(i) Urinary excretion of unconjugated T and DHT in normal males and females in the early follicular phase of the menstrual cycle.

Urine samples were measured by the simultaneous T and DHT assay (Chapter 3). Results are shown in Table 6.6. and plotted together with values from other groups of individuals in Fig. 6.6. and Fig. 6.7.

It can be seen that the 24h urinary excretion of T and DHT in males is about 4 and 3.5 times higher respectively than in females. Whereas night and day values are identical in males, those in females tend to be lower during the night.

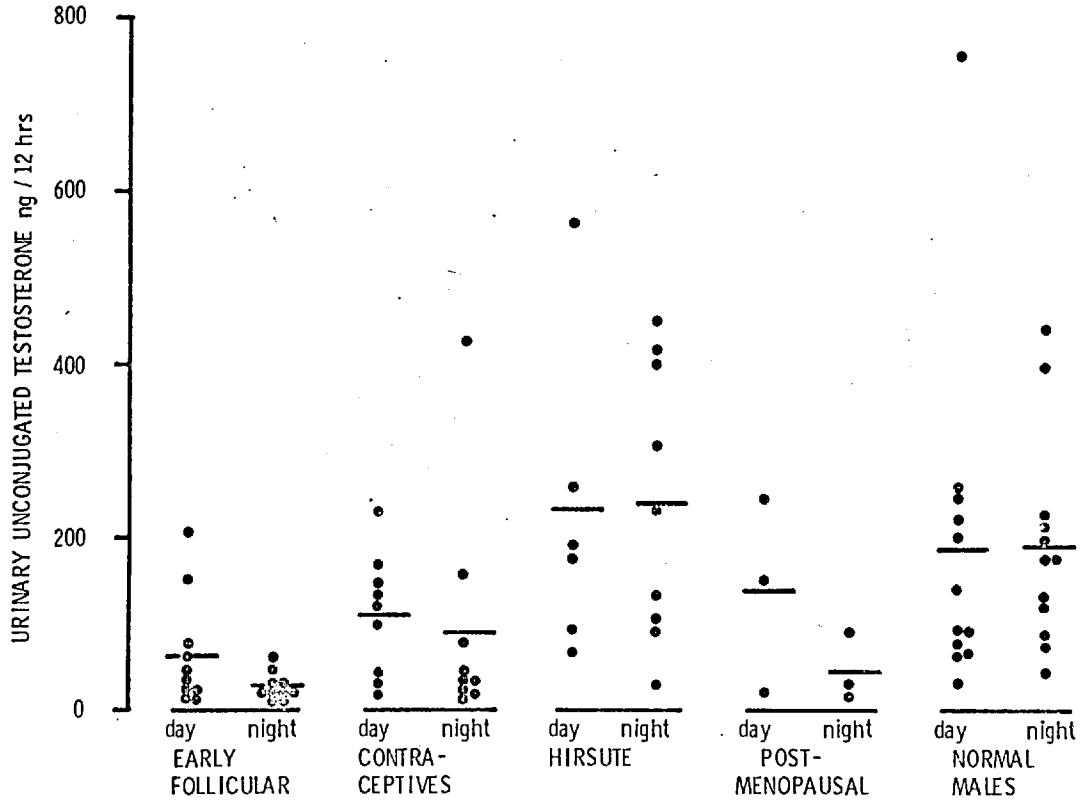
The relationship between the T and DHT excretion is shown in Fig. 6.8. and the r values for each group in Table 6.2. Values from females on oral contraceptives (see below) have been included in Fig. 6.8. A very highly significant overall linear correlation ($p < 0.001$) was found for unconjugated T and DHT excretion in the three groups.

Table 6.6.

Urinary excretion of unconjugated T and DHT (ng/12 or 24h) in normal females and males.

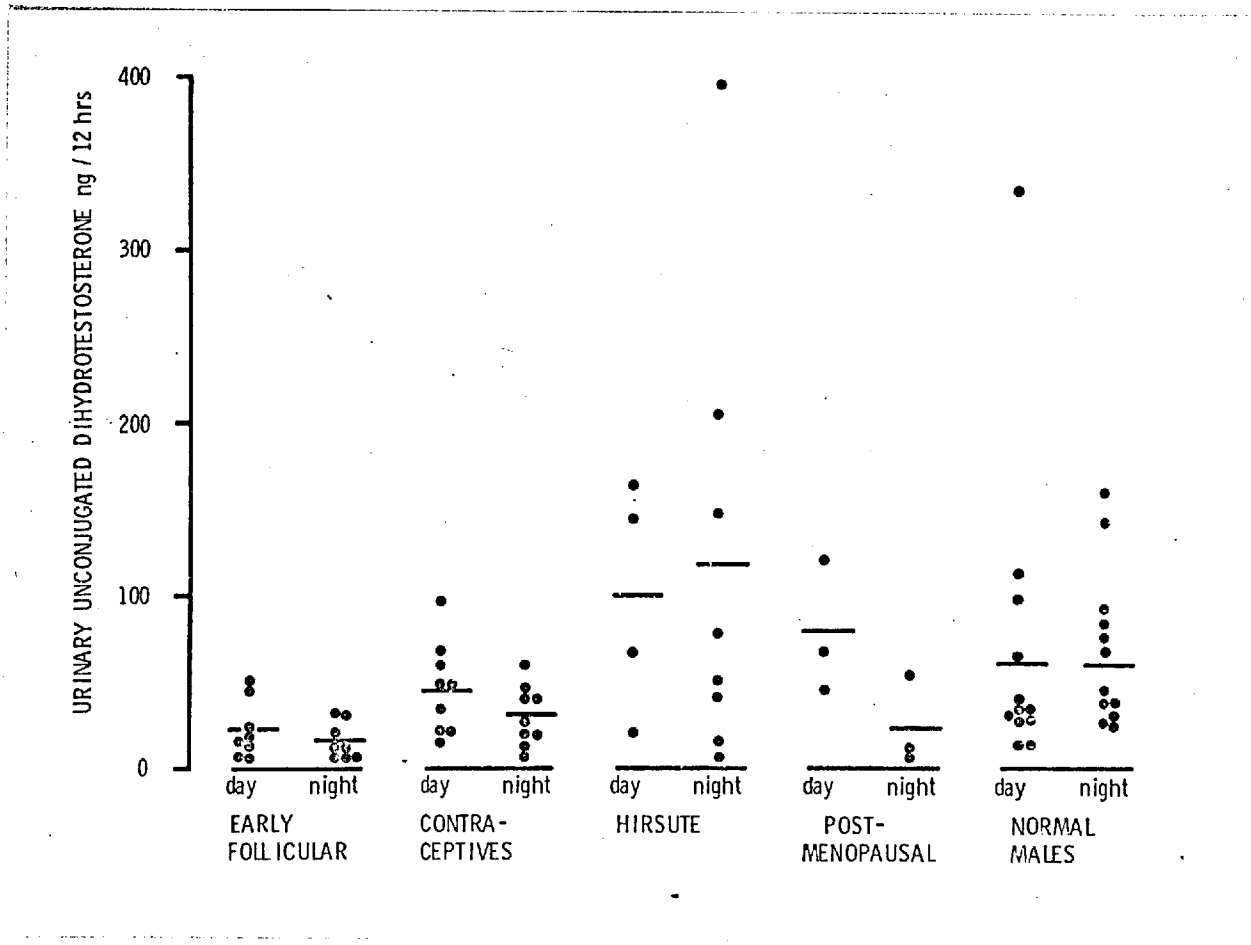
	F E M A L E S						M A L E S					
	T			DHT			T			DHT		
	day	night	total	day	night	total	day	night	total	day	night	total
mean	63.7	28.6	92.3	23	16.1	39	187	190	377	69	69	138
St.dev.	64.2	13.4	76.1	16.6	11.5	23.6	195	122	292	89	45	103
range	(13.0 to (202	13 to 58	33 to 260	5 to 50	5 to 33	10.6 to 82	30 to 756	46 to 442	76 to 1154	13 to 333	26 to 160	51 to 408
(n)	-	(10)	-	-	(8)	-	-	(12)	-	-	(12)	-

Fig. 6.6.



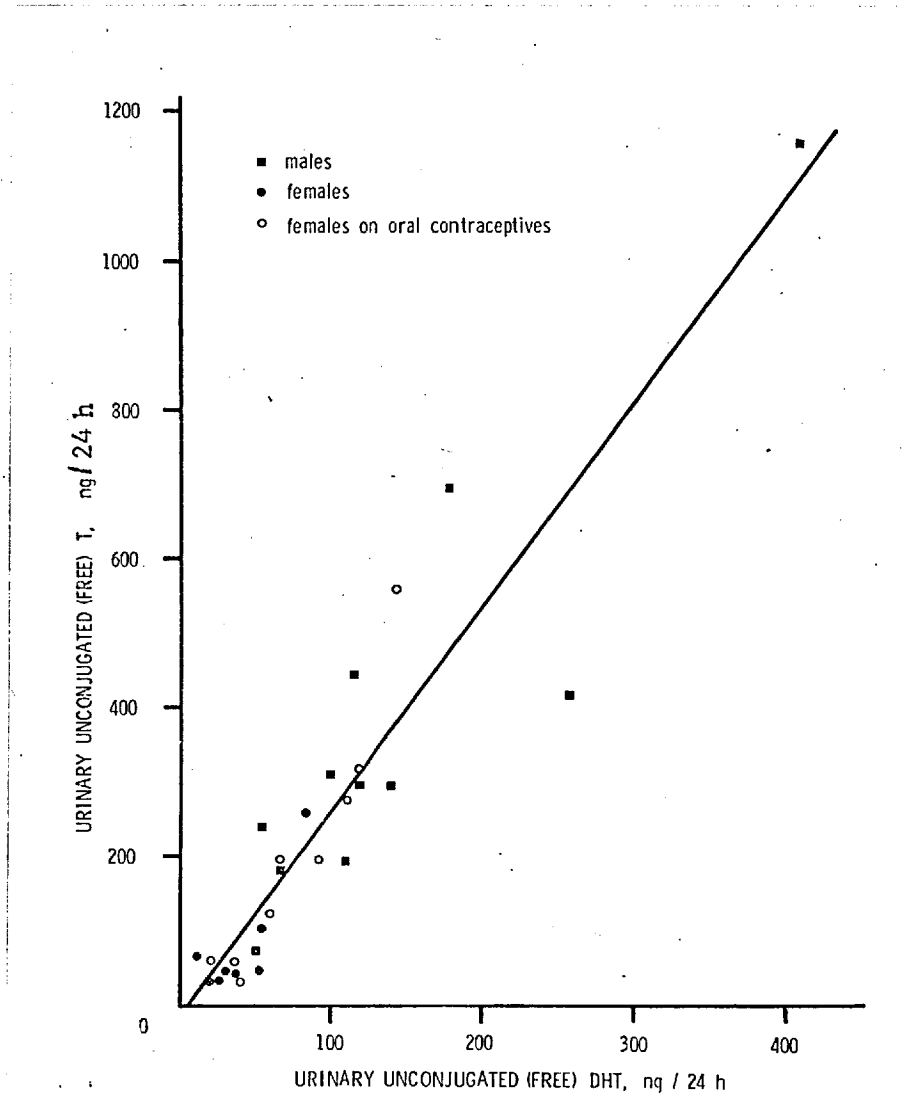
Urinary excretion of unconjugated testosterone (T) in five groups of individuals. The horizontal bars indicate mean values.

Fig. 6.7.



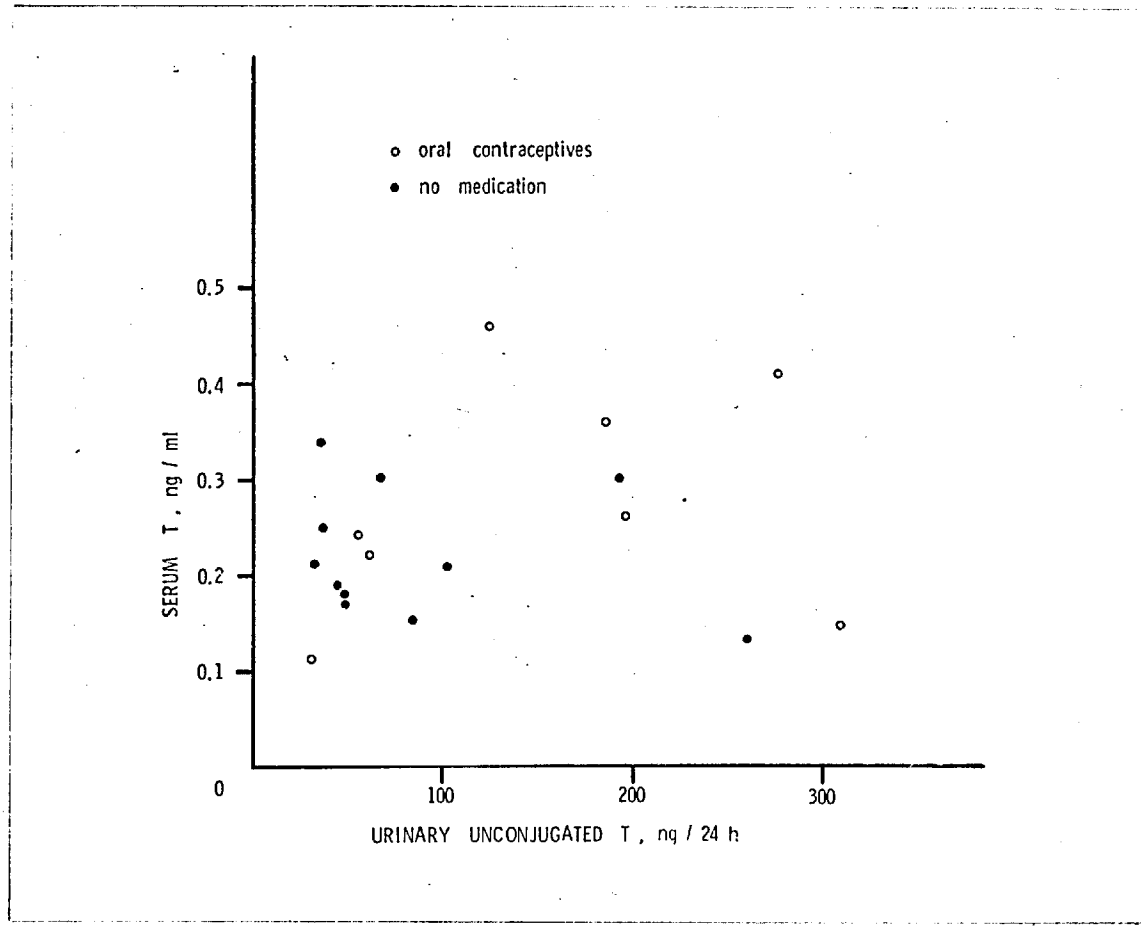
Urinary excretion of unconjugated dihydrotestosterone (DHT) in five groups of individuals. The horizontal bars indicate mean values.

Fig. 6.8.



Relationship between urinary excretion of unconjugated T (ordinate) and DHT (abscissa) in normal males and females and females on oral contraceptives. The regression line (least squares) has the equation $y = 2.75x - 13.06$; $r = 0.92$; $n = 28$; $p < 0.001$

Fig. 6.9.



Relationship between unconjugated serum T levels (ordinate) and unconjugated urinary T excretion (abscissa) in normal females and normal females on oral contraceptives. The linear correlation coefficient (r) for all values (oral contraceptives + no medication) was 0.23

Correlation between unconjugated urinary excretion and unconjugated serum levels was poor for either T or DHT in either sex. Fig. 6.9. shows the relationship between urinary unconjugated T excretion and unconjugated serum T levels in normal females and in females on oral contraceptives.

(ii) Urinary excretion of unconjugated E_2 in normal males and females (early follicular phase).

Urine samples were measured by the E_2 method described in Chapter 3 using TLC. Results are shown in Table 6.7.

The average 24 hours excretion of unconjugated E_2 is 2.7 times higher in females than in males. Females showed a wide variation in excretion values and the difference between the sexes was not significantly different at the 5% level. Night and day collections showed no consistent difference in values. In males levels of urinary unconjugated E_2 correlated poorly with the unconjugated T and DHT levels. This correlation was better in the female group (Table 6.2.). Unconjugated urinary E_2 also correlated poorly with serum levels of unconjugated E_2.

(iii) Urinary excretion of unconjugated T, DHT and E_2 in females on oral contraceptives.

Urine samples from these women were measured as for normals above. Results are presented in Table 6.8., Fig. 6.6. and Fig. 6.7.

The average 24h excretion of both T and DHT is about 2 times greater in these females than in the normal (control) group, not taking contraceptives. The difference was, however, significant ($p < 0.05$) only for DHT. Mean unconjugated E_2 excretion was 2.7

Table 6.7.

Urinary excretion of unconjugated E_2 (ng/12h or 24h) in normal males and females (early follicular)

	F E M A L E S			M A L E S		
	day	night	total	day	night	total
mean	13.5	10.5	24	3.4	5.5	8.9
St. dev.	19.5	9.9	25	1.2	4.9	5.0
range	2.2 - 62	3.4 - 30	5.7 - 75	2 - 5.2	0.8 - 16.4	3.2 - 19.3
(n)	-	(10)	-	-	(8)	-

Table 6.8.

Excretion of urinary unconjugated T, DHT and E_2 (ng/12 or 24h) in healthy females on oral contraceptives

	T			DHT			E_2		
	day	night	total	day	night	total	day	night	total
mean	109	91	200(92.3)**	46	30	76*(39)**	4.0	4.8	8.8(24)**
st. dev.	71	134	167	27	18	42	2.2	4.6	6.2
range	(15 - 321)	(16 - 428)	(31 - 561)	(15 - 97)	(5 - 59)	(20 - 142)	(2.2 - 8.2)	(15 - 11.9)	(3.8 - 20.1)
(n)	-	(9)	-	-	(9)	-	-	(6)	-

* Significantly ($p < 0.05$) higher than the corresponding value for the control group (see below)

** Corresponding mean values from Table 6.6. and Table 6.7. for normal females in the early follicular phase not ingesting contraceptives (control group).

times lower in this group than in the control group above, but the excretion in the control group fluctuated widely and the difference was not significant at the 5% level.

(iv) Urinary excretion of unconjugated T, DHT and E_2 in postmenopausal and hirsute women.

Excretion values of unconjugated T and DHT in postmenopausal women are shown in Fig. 6.6. and Fig. 6.7. and were not significantly different from those of the normal early follicular group. Day collections from these postmenopausal women showed higher T and DHT levels than the night collections.

Four postmenopausal women had average urinary unconjugated E_2 excretion of 12.4 ± 4.0 (S.D.) ng/24 hours.

Fig. 6.6. and Fig. 6.7. show levels of unconjugated T and DHT in several overnight and a few day collections of urine from hirsute women. All these women had serum T levels (direct assay) higher than 0.9ng/ml. It may be noted that there is a wide scatter of excretion values in these women with mean values for both T and DHT higher than those for male urines.

Five of these hirsute women had average unconjugated urinary E_2 excretion of 231 ± 257 (S.D.) ng/24 hours (range 24 - 575). Serum unconjugated E_2 in this group of five ranged from 25 to 71 pg/ml.

3. Urinary creatinine and its relation to the urinary unconjugated sex hormones.

Creatinine was measured in the day and night urine collections from 6 of the normal females, 6 of the normal females on oral contraceptives, and 6 of the group of normal males. The contraceptive

group had lower average ($\pm$ S.D.) creatinine excretion of 0.825 ($\pm$ 0.24)g/24h versus 0.99 ($\pm$ 0.20)g/24h in the normal group. The creatinine excretion in the males was 1.55 ($\pm$ 0.35)g/24h.

The relationship between the creatinine excretion and the excretion of unconjugated T, DHT and E_2 was studied. Whereas E_2 excretion in each group showed poor correlation with the creatinine, both T and DHT showed a significant correlation ($p < 0.02$) when all samples were pooled. The relationship between urinary unconjugated T and creatinine is shown in Fig. 6.10. Values of day and night collections from all three groups are included except one sample from a male in which duplicates of testosterone values were unacceptable.

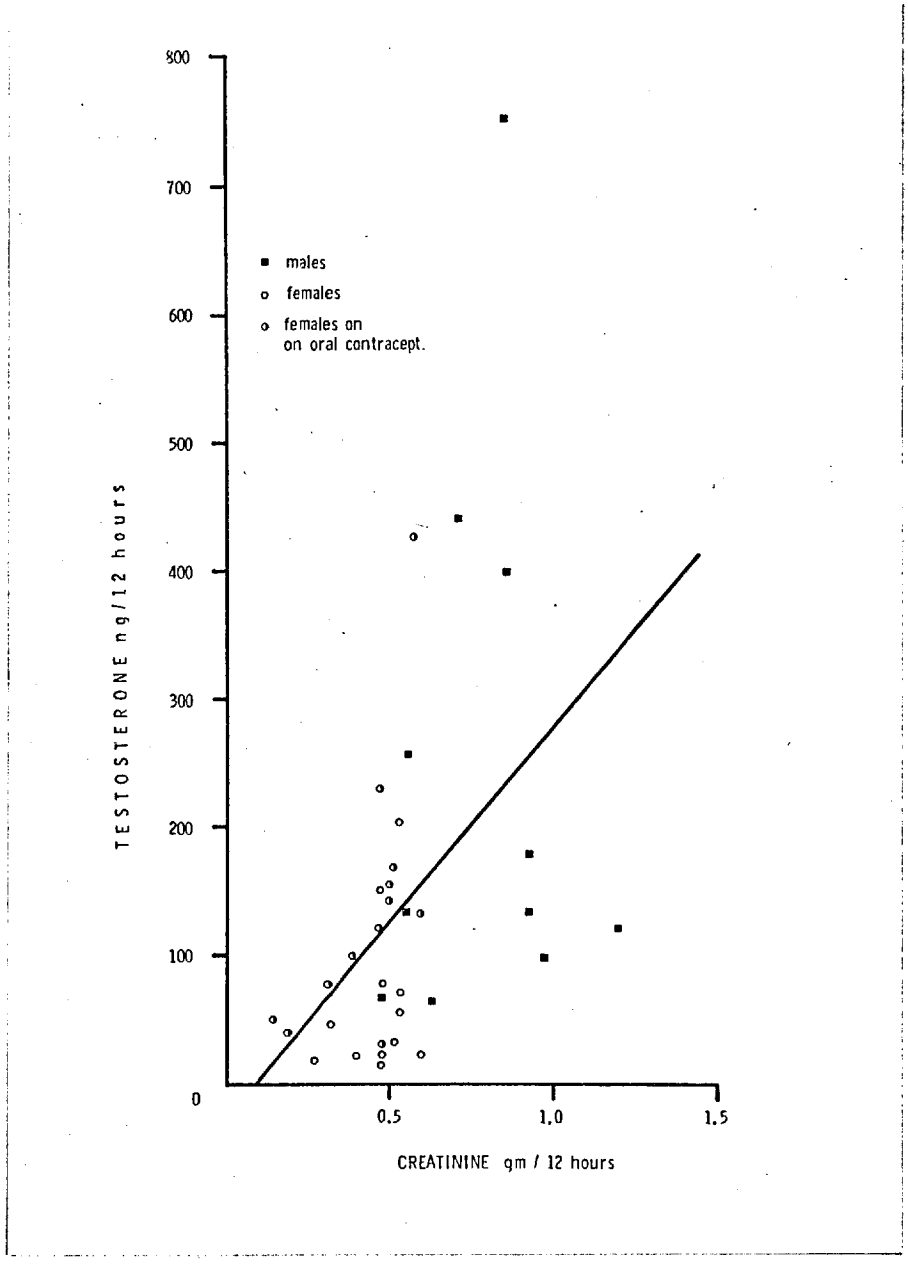
DISCUSSION

Serum levels

The serum levels of unconjugated T and DHT presented here agree well with those reported by others (see Table 3.3.) except for serum DHT in women (the "normal group") where the present values are lower. This discrepancy could be related to the fact that all the women in the normal group were in the early follicular phase of the menstrual cycle, as can be seen from the low serum E_2 values. This is of interest in connection with the significant correlation found between serum E_2 and DHT levels in both sexes.

The results of Coyotupa et al. (1972) indicated that serum DHT levels were low in the first 5 days of the menstrual cycle, then increasing, being higher during the luteal phase. Subsequent studies by others on serum DHT levels throughout the menstrual cycle, have not shown such changes (Guerrero et al., 1976; Abraham et al., 1974).

Fig. 6.10.



The relationship between urinary unconjugated (free) testosterone excretion (ordinate) and urinary creatinine excretion (abscissa) in normal males and females and females on oral contraceptives. The equation for the regression line (least squares) is $y = 295.6x - 20.3$; $r = 0.43$; $n = 35$; $p < 0.02$

It is unlikely that methodological factors are entirely responsible for the lower DHT levels in females, as the male values agree closely with others. It would appear therefore that further studies are warranted on DHT levels and their correlation with E_2 levels during the menstrual cycle.

Only serum T levels in males showed any appreciable difference between morning and afternoon specimens. As the time interval between the a.m. and p.m. blood collection was only 6 hours a further study with longer interval was carried out to evaluate this difference (see following chapter).

The much smaller T/DHT variation within male individuals than between them is in agreement with the findings of Pirke and Doerr (1975). These authors measured the serum T and DHT levels in one individual in samples taken every 15 - 30th minute for 24 hours and found a close parallelism between the two hormones. The data presented in Table 6.2 suggest that there is in males an individual T/DHT constant which varies from one individual to another. The significance of this constant needs further study and might be related to body build, prostate weight or other individual characteristics.

In women on oral contraceptives (OC) serum T and DHT levels were higher than in the normal group not taking OC, and the difference for serum DHT was highly significant. This elevation could possibly partly be accounted for by the elevated serum SHBG in women on OC. A similar OC regime as used by the contraceptive group in the present study (50ug ethynyloestradiol) has been found to raise the SHBG levels by about 100% (Briggs, 1975; van Kammen, 1975). It is difficult to explain, however, on this basis why DHT is elevated so much more that T even considering that DHT is bound more strongly to SHBG. The DHT results need confirmation but are

corroborated by the finding of elevated unconjugated DHT in urine and will be discussed a little further in connection with the urinary results (see below). Serum DHT levels in women on OC have apparently not been reported before.

The serum unconjugated E_2 levels were significantly ($p < 0.001$) lower in the contraceptive group than in the control group (early follicular) and similar to the levels in postmenopausal women. This is at variance with some other workers who have reported serum E_2 levels in women ingesting OC to be similar to those of the early follicular phase in normal cycles but without the midcycle and luteal peaks (Mishell et al., 1972 Briggs and Briggs, 1972; Longcope et al., 1974). Johansson et al. (1972) found lower serum E_2 levels in women during treatment with 5 different contraceptive preparations than in women in the follicular phase of a normal cycle. None of the treated groups in this series however, had levels as low as postmenopausal women simultaneously studied. The low serum E_2 levels found in women on OC in the present study as compared with the early follicular control group is probably related to the high specificity of the antiserum used in the present method (Chapter 3). All the above reports on serum E_2 used antisera raised against oestradiol coupled to bovine serum albumin (BSA) through the 17 position. These antisera are not specific for E_2 and the methods therefore needed a chromatographic separation step, which might not have separated cross-reacting compounds adequately from E_2. Thus in two of the reports neither Sephadex LH-20 nor celite columns separated ethynyloestradiol (EE_2) adequately from E_2. (Johansson et al., 1972; Longcope et al., 1974).

This reduction of E_2 levels if confirmed in larger groups of women on OC could have some bearing on the badly understood mechanism of action of oral contraceptives. Furthermore, it might become feasible to "titrate" more accurately the minimal effective dose of OC needed for contraception by using the reduction in serum E_2 and rise in DHT levels as an index.

Urinary levels

In Chapter 4 it was shown that both unconjugated T and DHT in urine rose about 2-fold or more when urine samples were left at 37°C for 18 hours. In view of these findings it could be speculated whether the kidney excretes any unconjugated T and DHT at all; that is, it is possible that the unconjugated levels have been released in the urinary tract from the labile compound described in Chapter 5. To study this possibility a 2ml urine specimen was obtained from the renal pelvis during a renal transplant operation and measured in duplicate for T (direct assay). A low but definite reading (15pg) was obtained on the standard curve. As the patient had been heavily hydrated for operational purposes the concentration in this case was meaningless and the positive reading merely pointed to the existence of unconjugated T (DHT) in urine from the renal pelvis. If measurements of free sex hormones in urine become a feasible procedure in clinical endocrinology a special study would have to be undertaken to assess the release of the unconjugated hormone during storage in the urinary tract.

If such a release of unconjugated hormones, measured in the present study, occurred to any appreciable extent, one might have expected a greater release during the night (longer stay in the bladder

at 37°C) and higher values in the overnight collections. This was not seen, but might of course have been offset by lower excretion values during the night.

A point of interest is the T/DHT ratio in urine as compared to that of serum. The average ratio in male urine was 2.7 versus 8.3 in serum. In the normal females these were 2.3 and 2.3 respectively. If one assumes glomerular filtration (see below) only of the unconjugated hormones one would, because of the higher affinity of SHBG for DHT, have expected an increase in T/DHT ratios in urine as compared to serum, and more so in women who have about 2 times higher SHBG levels than men (Anderson et al., 1972). This reasoning would therefore, in spite of the excellent correlation between unconjugated urinary T and DHT (Fig. 6.8.), suggest a different excretion mechanism for the two hormones. T might, for instance, be more readily reabsorbed in the tubules, or DHT secreted after 5α-reduction of T. This, however, remains highly speculative at the present stage of knowledge of the renal handling of unconjugated steroids.

No significant differences were found in unconjugated T, DHT or E_2 levels between night and day collections. In view of known circadian fluctuations for serum T in males, with higher levels occurring during the late night and early morning, such differences could have been expected. It is however possible that the collection periods were not ideally timed to "catch" such fluctuations. The results do suggest that day and night collections are about equivalent with respect to measurements of urinary unconjugated T, DHT and E_2.

The author is aware of only one report on urinary unconjugated T levels in humans comparable to the present studies. Andino et al.,

(1976) measured urinary free (unconjugated) T and androstenedione excretion in 9 normal males and 22 normal females. Results for T were 250 (range 20 - 650) and 800 (200 - 2600) ng/24h for females and males respectively. These values are higher than the present ones of 92 (33 - 260) and 377 (76 - 1154) ng/24h for females and males respectively. No mention was made of how samples were handled.

The levels of urinary unconjugated E_2 in the normal groups agree well with those found by Desfosses et al. (1973) and Kono et al. (1974), but those of Jeffcoate and Edwards (1974) are much higher both for males (100ng/24h) and females (100ng/24h). The results of urinary unconjugated E_2 obtained by Kono et al. in 3 consecutive samples in one male (mean 4.8ng/24h) agree well with the present male values, but those by Pentikäinen et al. (64 ng/24h) are again higher. Only Desfosses et al. studied effects of storage. The discrepancy observed is probably caused by different handling of the urine samples (see Chapter 4).

Higher average urinary excretion of both unconjugated T and DHT was found in the women on OC than in the normal group, but only the DHT excretion was significantly different. These excretion values are in accordance with the higher serum values of the hormones in the OC group and the comparatively higher DHT than T levels.

These urinary findings suggest that the elevation of unconjugated serum T and DHT is not caused primarily be raised serum SHBG and thus increased serum binding capacity during OC ingestion, as more unconjugated T and DHT seems to be available for the kidney for excretion. Unless the OC have some special effect on the excretion of steroids by the kidney it seems likely that other organs are also

exposed to increased levels of circulating androgens, the effect of which, however, must be blocked by the contraceptives as virilizing symptoms do not prevail during OC use.*

EE_2 does not bind to SHBG as do the sex hormones (Raynaud et al., 1973) and any displacement of the measured hormones from SHBG by EE_2 is therefore unlikely. EE_2 does on the other hand bind to uterine cellular receptors (Raynaud et al., 1973) and it seems a possibility that the elevated androgen levels in urine of women ingesting OC might be caused by competitive inhibition of such cellular androgen binding sites in the kidney consequently losing more unconjugated steroids into the urine.

The above findings seem to be in agreement with those of Tremblay and Dube (1974) who found increased serum levels of non-TeBG (SHBG) bound testosterone in women on d-norgestrel-ethynyl-oestradiol when compared to a normal control group. These authors found lower values than normal for this non-TeBG bound fraction in women taking norethindrone-mestranol. It is therefore possible that the progestogens in the combined OC may have some effect on the amount of unconjugated T and DHT excreted by the kidneys. In the present small group of women no such effect was clearly seen, but this possibility needs a separate study.

No correlation was found between the excretion and serum levels of the unconjugated hormones within the relatively narrow ranges of each group studied. However, if the three groups, normal men and women, and women on OC, are taken together, thus spanning a much wider range, a rough correlation at least can be seen.

* cases of hirsutism have been reported

In the hirsute females the average urinary levels of T, DHT and E_2 were all greatly elevated. A diagnostic value of urinary measurements in these women would be debatable. They all had elevated serum T levels as an excellent index of their clinical status whereas the urinary values overlapped with those of the non hirsute females. While the urinary unconjugated E_2 levels were elevated, serum E_2 levels were in the normal range (21 - 71 pg/ml) suggesting some anomaly in the renal excretion of unconjugated E_2 in these women as compared to the other groups.

The significant correlation between creatinine and both unconjugated T and DHT in urine suggests excretion by glomerular filtration. Other factors as body build and weight may be responsible for the correlation and further study in this regard is necessary.

The results of the urinary determinations of unconjugated levels of T, DHT and E_2, here presented, do not suggest that they have much to offer for routine clinical use. Besides the instability of the urine specimens described in Chapter 4, values obtained in the different groups overlapped considerably. The thin layer chromatography (TLC) step used also makes the urinary assays unattractive. This time consuming step can probably be omitted for T and DHT as they are the only urinary steroids detected (Chapter 3) by the antiserum used, and their levels correlated closely (Fig. 6.8.). A simplified "direct" urinary "T plus DHT" assay might be preferable and could be useful in certain sex hormone related disorders still to be investigated in that regard.

CHAPTER 7

Serum testosterone levels in normal men: circadian and episodic fluctuations; response of serum testosterone to infused hLH and HFSH.

INTRODUCTION

Factors governing serum testosterone (T) levels or testosterone production by the testis are little known. Recent methodological progress (Christensen and Mason, 1965) has made it possible to study separately the effect of the trophic hormones in vitro on the two components of the testis, the interstitial tissue (Leydig cells) and the tubules (Sertoli cells and spermatogenic epithelial cells). Investigators using these techniques have confirmed the specific effect (cyclic AMP rise) of LH on the interstitial tissue and of FSH on the tubular tissue (van der Molen et al., 1973). While in vitro experiments have proved that the Leydig cells produce T, there is still controversy regarding T production by the tubules (Lacy, 1973). Hafiez et al. (1972) have shown that, in vitro, prolactin and LH have a synergistic action in stimulating testicular T production by the rat testis. Data are still lacking, however, on the synergistic action of the various trophic hormones on the separated testicular components with regard to T production.

In vivo experiments have been hampered by a lack of sufficiently pure pituitary gonadotrophin preparations. However, animal experiments have indicated that FSH on its own does not increase T production in the intact male rat (van der Molen et al., 1973) but does augment the response of the rabbit testis produced by LH (Johnson and Ewing, 1971).

Few data are available on the effect of pituitary gonadotrophins on serum T levels in man. It has nevertheless been long accepted from animal experiments that LH is the main trophic hormone promoting the production of T by the Leydig cells of the human testis. While good responses are seen in serum T levels after intramuscular and intravenous administration of human chorionic gonadotrophin (HCG) (Nieschlag et al., 1971; Anderson et al., 1972; Maurer et al., 1973; Vivanco et al., 1973), LH itself can only be inferred to have the same effect as it has not been rigorously proved to do so (Hall, 1970). Little is known about the effect of FSH on the serum T levels in man but it has been associated with circadian variations in serum T levels (Faiman and Winter, 1971).

A circadian rhythm in plasma T levels is now generally accepted (Faiman and Winter, 1971; Rose et al., 1962; Barberia et al., 1973). It has however been debated (Boon et al., 1972) and superimposed episodic fluctuation described (Schiavi et al., 1974).

The serum T measurements in males in Chapter 6 showed lower average value for the afternoon specimens and suggested a circadian variation. This was therefore studied further with a longer time interval between blood collections.

An earlier study in this laboratory on the 17β-hydroxyandrogen (Anderson, 1970) response to infused hLH in man (Marshall et al.,1973) showed that only one out of four male subjects gave a definite response. Using the same preparation of hLH this study was extended using an identical infusion procedure but using in addition a preparation of hFSH suitable for intravenous administration. In four of the infused subjects circadian and episodic changes were studied by frequent sampling throughout a separate control day.

MATERIALS AND METHODS

1. Subjects

Eighteen healthy men, aged 24 - 41, were each bled between 8 and 9 a.m. and 8 and 9 p.m. and the serum measured for T.

Seven healthy male subjects, members of the hospital staff, aged 26 to 37, volunteered for the infusion study. They were not fasting and remained recumbent during the infusions which all lasted for 4h. The FSH infusions were begun at 10.00 hours, the others between 12.00 and 14.00 hours. During the basal control period the subjects carried on with their normal activities. In subjects 1, 2 and 3, basal T values were obtained on two occasions 12h apart on the day previous to the hFSH infusion. Serum T levels were measured before, during and up to 60h after the hFSH infusion in these three subjects. In subjects 4 and 5, basal T values were obtained 13 times on a separate control day. These two subjects also had serial serum T level determinations during a day of a combined infusion of hFSH and hLH and a day of infusion of hLH alone. In subjects 6 and 7 basal T values were similarly obtained 8 times on a control day and serum T levels were measured serially before, during and until 16h after a combined hFSH and hLH infusion.

2. Infusion procedures

From one arm, using an indwelling catheter, serum samples were taken and on the other arm the infusion was administered into the anti-cubital vein through an indwelling needle.

(i) <u>FSH infusions</u> The hFSH preparation infused was the MRC's Human Pituitary FSH Clinical - 70/10, batch F_1. This stems from a "washed CM1" fraction prepared from human pituitary extracts by Dr. W.R. Butt (Butt et al., 1961). The biological activity using ovarian augmentation bioassay in mice (2nd IRP HMG as standard) was

113.6 IU/ampoule, each ampoule containing approximately 1mg of the pituitary fraction. When measured by our FSH-RIA referred against the MRC pituitary standard 69/104 (Bangham et al., 1973), each ampoule was found to contain 70 IU. A total of 4 ampoules was given in each infusion. The contents of the ampoules to be infused were first dissolved in 2.5ml isotonic saline (initial solution). Then 0.1ml of this initial solution was diluted to 2.0ml from which 0.5ml was set aside for hormone assay and the remainder given as a test dose. Ten minutes later 1.0ml of the initial solution was given as a loading dose and then the remainder made up to 50ml in isotonic saline and infused over 4h by a constant rate pump. Subject No.2 was an exception who had 25% of the total dose as a loading dose and the remainder similarly in a constant rate infusion.

(ii) LH infusions The hLH infused was the MRC's Human Pituitary LH Clinical-70/67, Batch L_1. This material is the "DEAE I" fraction prepared from human pituitary glands by Dr. A.S. Hartree (Hartree, 1966). The biological activity of this preparation estimated by increase in ventral prostate weight against the 2nd IRP HMG gave an approximate value of 600 IU of LH per ampoule, each ampoule containing 0.1mg of the pituitary fraction (DEAE I). Measured by our LH-RIA against the MRC pituitary standard, 60/104, each ampoule was found to contain 252 IU. Two ampoules were given in each infusion. Using similar procedure as described for the FSH infusion, 2% of the total dose were given as a test dose, 25% as an initial loading dose and the rest infused at a constant rate for 4h.

(iii) Combined infusions The same doses as above were given of each gonadotrophin and the same quantities mixed together in 50ml isotonic saline and infused over the 4h.

3. Assays

(i) Serum T was measured by the direct (without chromatography) radioimmunoassay (see Chapter 3).

(ii) Serum LH and FSH were measured by double antibody RIA (Marshall et al., 1972, 1973) results for both gonadotrophins being expressed as U/l compared with the MRC Pituitary Standards, 69/104 (Bangham et al., 1973). Normal ranges for adult men were 2.0 - 8.0 U/l and 1.6 - 8.6 U/l for LH and FSH respectively.

4. Analysis of results

In order to assess mathematically the effect of the gonadotrophins, serum T levels after the infusions have been compared with the diurnally corresponding basal levels, where these have been obtained. This has been done by comparing the mean T levels over 0 - 16h commencing with the end of the infusions. Because of episodic fluctuations and the inability to obtain samples at the same time of day the levels were derived by measuring the areas below the curves in mm^2 and dividing it by the time scale (mm) and the number of mm per concentration unit on the ordinate.

RESULTS

A. Circadian variation in serum T

Serum T levels were measured in the early morning (8 - 9 a.m.) and in the late evening (8 - 9 p.m.) in 18 normal men. The results of this study are shown in Table 7.1. Levels were significantly lower ($p < 0.05$) in the evening.

TABLE 7.1.

Serum testosterone (T) levels (ng/ml) in 18 normal men measured from 8 to 9 a.m. and from 8 to 9 p.m.

	a.m.	p.m.
mean	6.29	5.16
st.dev.	1.41	1.57
range	3.5 - 9.3	2.0 - 7.8

B. Infusion studies

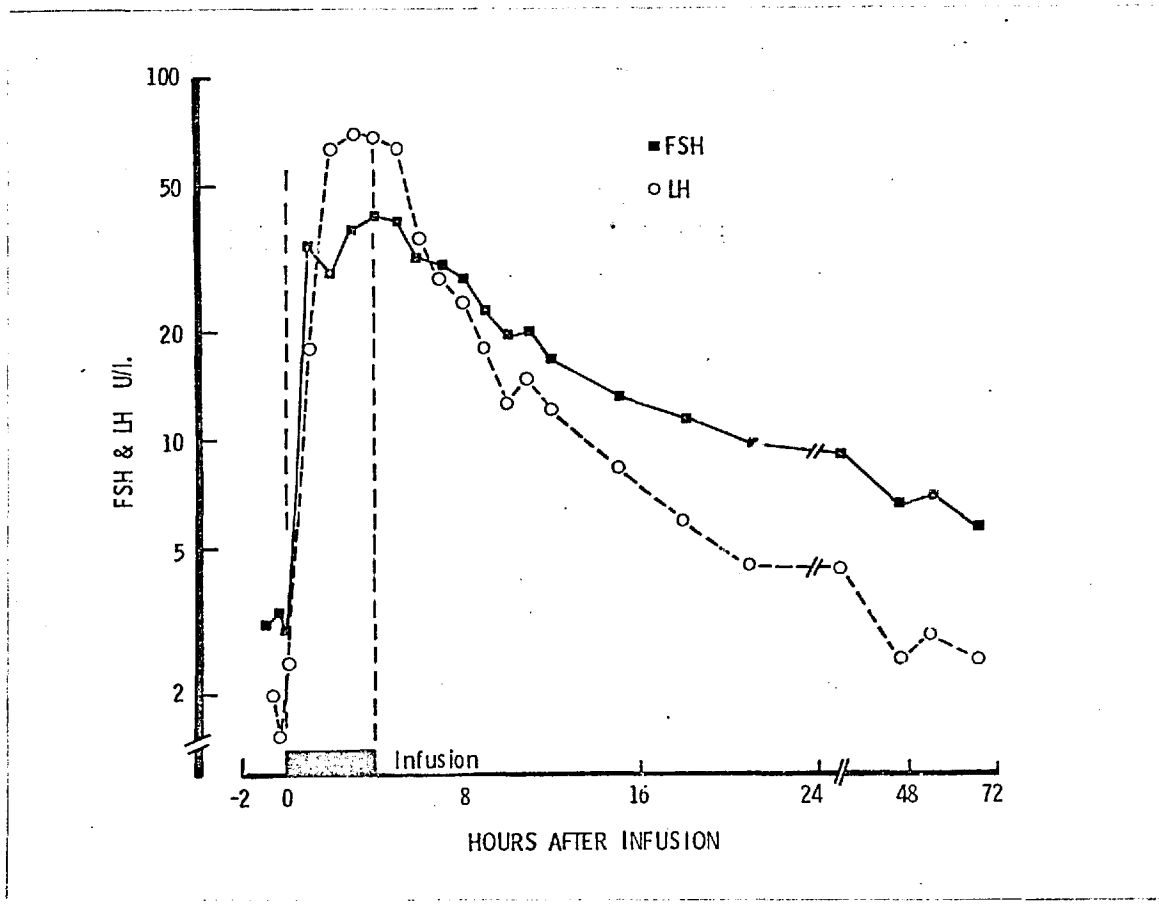
(1) Changes induced in serum levels of FSH and LH.

The basal gonadotrophin levels immediately before the infusions were 2.5 - 3.9 U/l for FSH and 1.5 - 3.0 U/l for LH. In all cases equivalently raised levels were attained during the infusions or a rise to more than 10 (for FSH) and 18 (for LH) times the basal level. Serum levels of LH rose by about 4 U/l during the FSH infusion but FSH did not show a significant rise during the LH infusion. Changes of gonadotrophin levels during and after the infusions are shown in Fig. 7.1. for subject 4. Similar changes were observed for all subjects.

(2) Serum T levels.

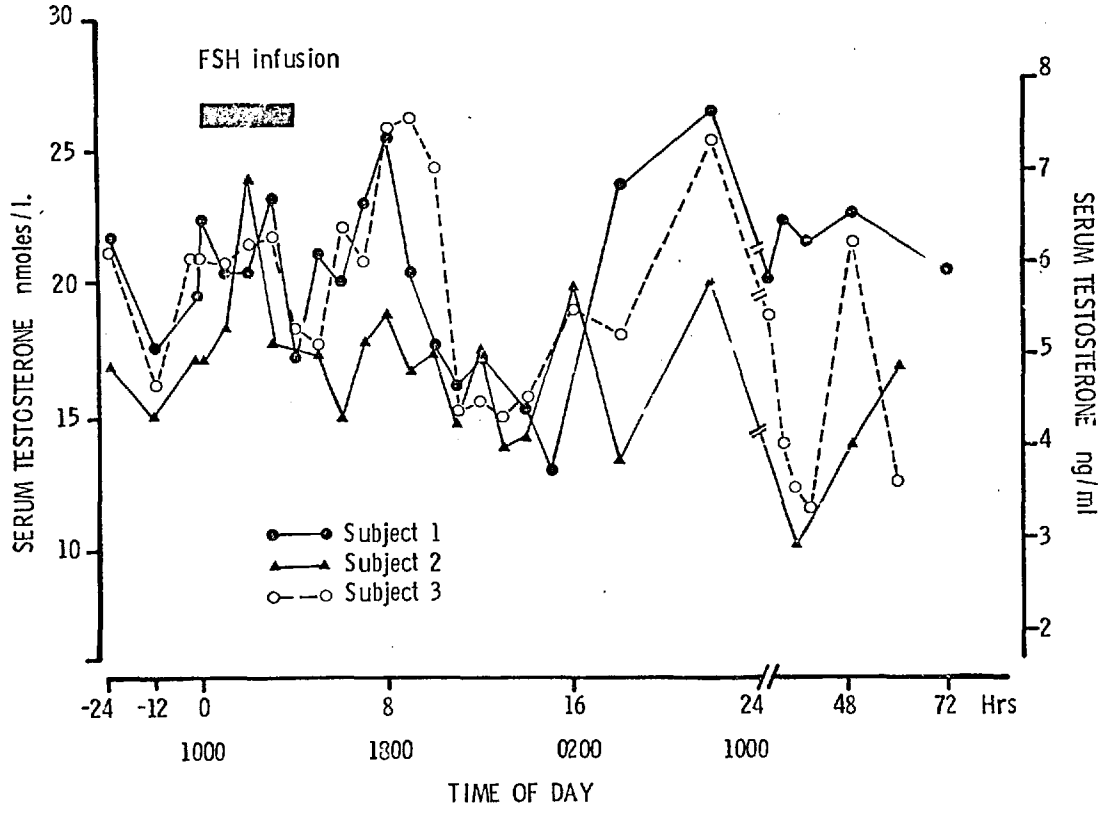
(a) The basal serum T values during the 24h control periods. Subjects 1 - 3 who had basal T levels measured only once in the morning and evening all showed lower values in the evening (Fig. 7.2.). Subjects 4 - 7 who had several measurements throughout the control period showed episodic fluctuations superimposed on a circadian rhythm

Fig. 7.1.



Serum levels of FSH and LH in subject 4 before, during and after a standard 4h combined infusion of human pituitary FSH and LH. Similar pattern of serum levels of FSH and/or LH was observed in all subjects, having either a single (LH or FSH) or combined (LH + FSH) infusions.

Fig. 7.2.



Serum T levels before, during and after intravenous infusion of hFSH into 3 normal male subjects. The first two values are from the previous day.

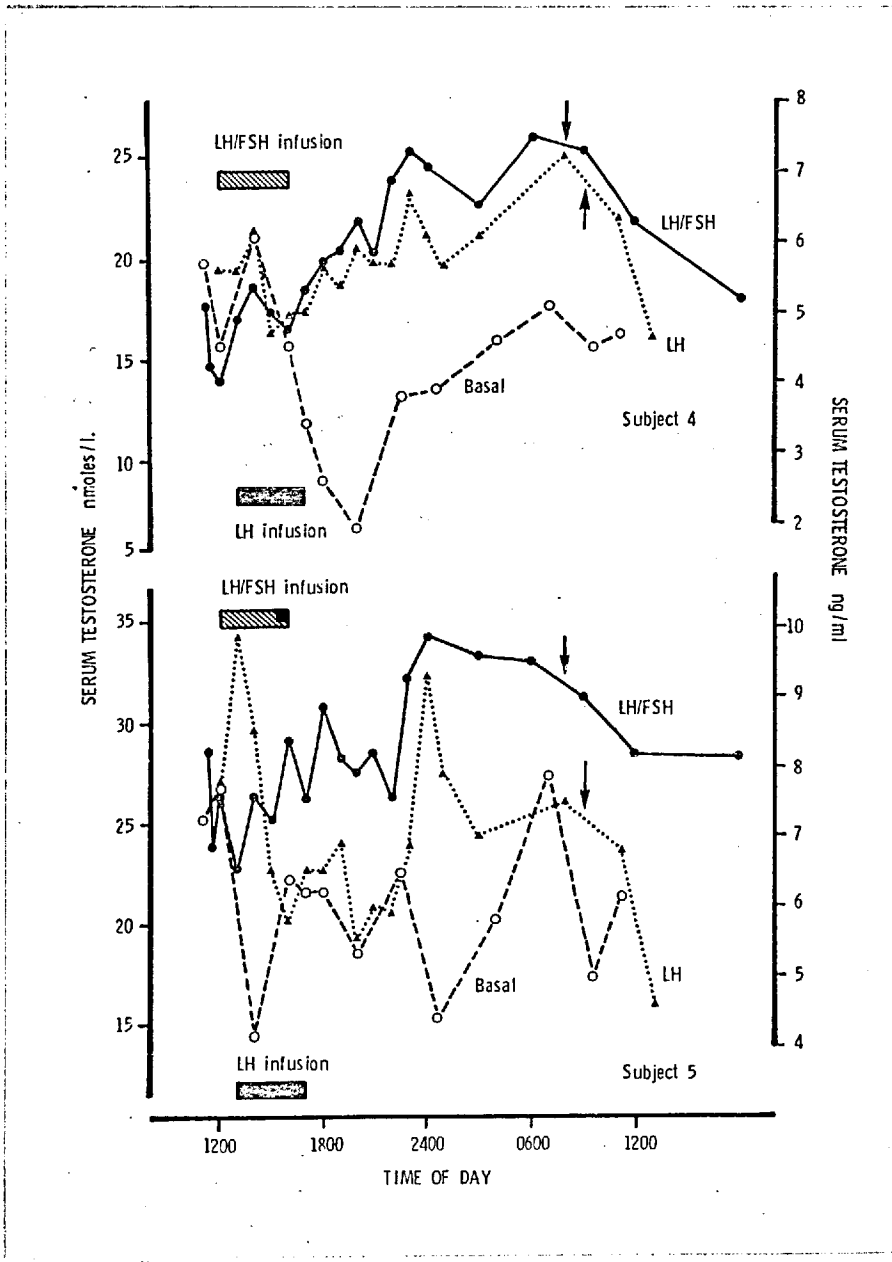
with high nocturnal and morning values Figs. 7.3 and 7.4. The greatest deviation from the mean value was 55% in subject 4 in whom the highest levels were approximately three times the lowest ones.

(b) The FSH infusion In subjects 1 - 3 no response in serum T levels was seen after the FSH infusion beyond the changes to be expected from episodic fluctuations, while the subjects continued to show the usual circadian rhythm (Fig. 7.2.).

(c) The LH infusion After the LH infusion the mean serum T rose by 55.5 and 16.9% over the basal day in subjects 4 and 5 respectively (Fig. 7.3., and Table 7.2.). This rise did not start until after the infusion, was gradual and abolished the circadian depression in the evening.

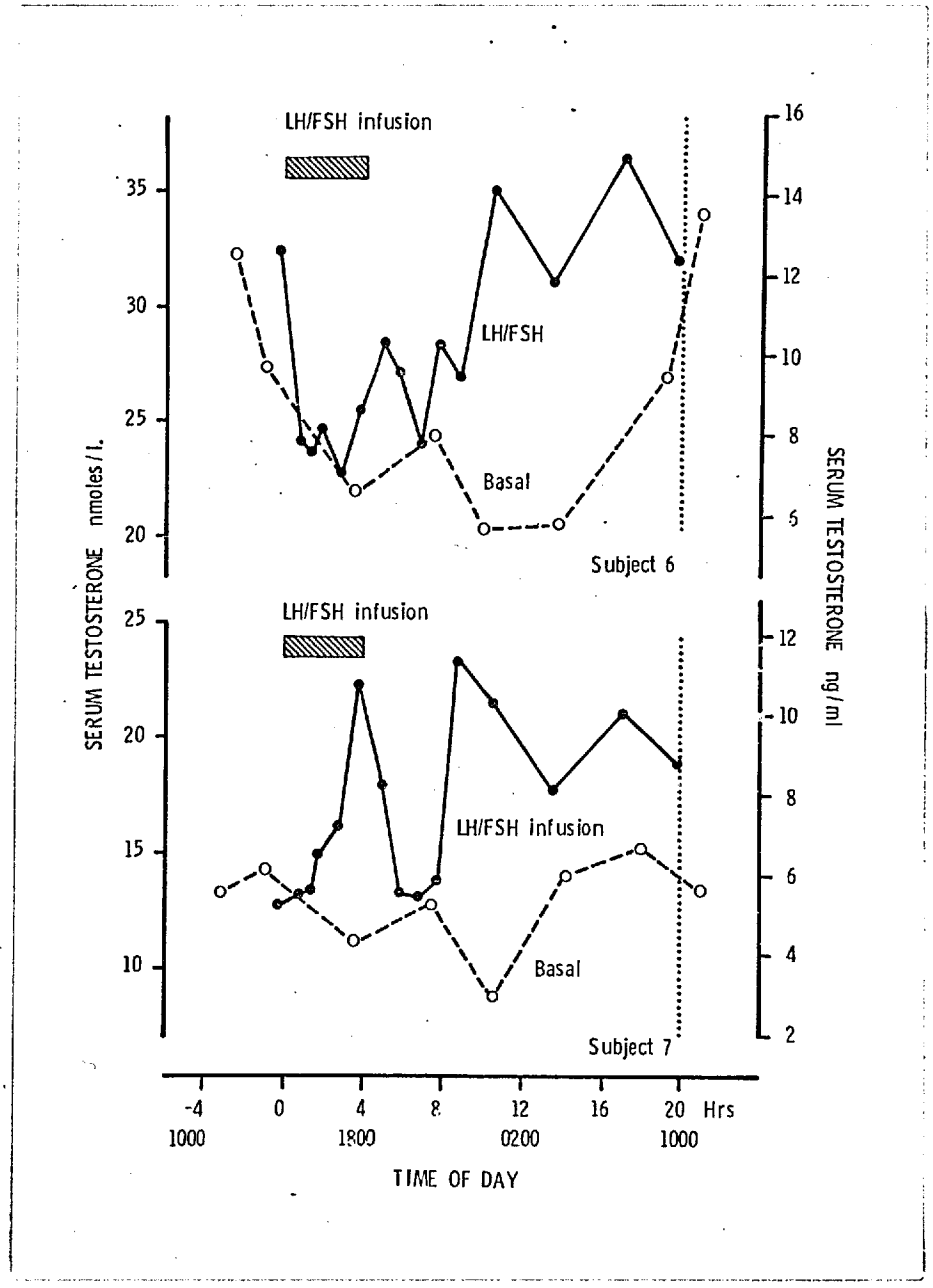
(d) The combined FSH/LH infusion In subjects 4 - 7 after the combined infusion, there was a significant response in serum T levels by all subjects (Figs. 7.3 and 7.4; Table 7.2.). The rise was sluggish, started only after the end of the infusion and reached a maximum after approximately 7h, then levelling off at a raised level, the whole response lasting for at least 16h and abolishing the circadian evening depression. In subject 4 the response looked similar to the response after LH alone but had the higher mean level of 6.39 or 68.3% rise above the basal day value (Table 2.). Subject 5 had a rise of 46.6% above the basal day values, a response significantly ($P < 0.001$) higher than after the infusion of LH alone. Subjects 6 and 7 had rises of 35.2% and 39.7% respectively above their basal day values.

Fig. 7.3.



Serum T levels in subjects 4 and 5 during a) a basal day, b) a hLH-infusion day and c) a combined hLH/hFSH-infusion day. The vertical arrows indicate 16h from the end of the infusion (see Table 7.2.).

Fig. 7.4.



Serum T levels in subjects 6 and 7 during a) a basal day and b) a combined hLH/hFSH-infusion day. The vertical dotted lines indicate 16h from the end of the infusions (see Table 7.2.).

Table 7.2.

Mean * serum testosterone (T) levels during 0 - 16h after 4-h infusions of hLH and hLH + HFSH compared with basal levels over the corresponding period of another day

(from data of Figs. 7.3 and 7.4)

Subject No.	Basal day T.ng/ml	LH infusion day		(LH + FSH) infusion day	
		T.ng/ml	%rise over basal levels	T.ng/ml	% rise over basal levels
4	3.80	5.91	55.5($P < 0.001$)	6.39	68.3($P < 0.001$)
5	5.94	6.94	16.9($P < 0.01$)	8.70	46.6($P < 0.001$)
6	6.56	-	-	8.86	35.2($P < 0.02$)
7	4.56	-	-	6.38	39.7($P < 0.001$)
Mean	5.22	6.42	36.2	7.58	47.45

* Mean values were derived by measuring the area under the serum T curves in mm^2 and then dividing by the appropriate time scale (mm) and the number of mm per concentration unit.

DISCUSSION

The measurem ents of the basal serum T levels showed a definite circadian rhythm with higher levels in the late morning and the lowest generally in the early evening at about 10 p.m. This is in agreement with the majority of reported studies (Anderson, 1970; Fairman and Winter, 1971; Rose et al., 1972; Barberia et al., 1973; Schiavi et al., 1974). The cause of this diurnal rhythm remains unknown as is the cause of the episodic fluctuations which were so marked in subjects 4 and 5. Similar fluctuations have been observed by other investigators (Evans and MacLean, 1971; Boon et al., 1972; Alford et al., 1973; Schiavi et al., 1974). Faiman and Winter (1971) showed that there is probably no adrenal involvement in this circadian rhythm, and while they found a correlation between serum FSH and plasma T changes, Alford et al. (1973) could not find any such relationship.

While FSH did not elevate T levels on its own, LH seems to be able to do so to a varying extent. The findings in the two LH infusions may be regarded as in reasonable agreement with earlier findings (Marshall et al. 1973) where only one response was found after 4 identical LH infusions. This, together with the the higher and consistent responses after the combined infusions, strongly suggests a synergism in action between the two gonadotrophins with regard to T production.

Because of the sluggish and prolonged response seen after these infusions the abrupt episodic fluctuations seen in normal subjects are unlikely to depend on fluctuations in gonadotrophin secretion. Further, levels such as those reached in the infusions have not been described in normal man although the gonadotrophin levels are known to

fluctuate episodically. One can not, however, rule out some ratio (FSH/LH) sensitive mechanism not reflected in the presently attained blood levels of LH and FSH.

Maurer et al. (1973), infusing HCG intravenously over 3h in men observed a serum T response after 30 min from the start of the infusion when using either 100 or 500 IU total dose. When they used only 50 IU an initial depression and no overall rise was observed. Kley et al. (1974) reported various responses in serum T levels after 8h intravenous infusion of luteinizing hormone releasing hormone (LH-RH) where plasma LH rose 3 to 4 times above basal values and FSH to a lesser extent.

Larger doses of gonadotrophins administered might have elicited greater and earlier responses. However, the gonadotrophin levels attained during the infusions are higher than those seen in normal males and still higher levels would be grossly unphysiological. In conclusion, the results presented point to a multifactorial control mechanism of serum T levels in man where FSH ~~plays a definite if permissive~~ may have a role. LH is probably the main factor but needs some co-factors for its effect to take place and, besides, has its T secretory effects modified by other hormones or agents still unknown.

CHAPTER 8

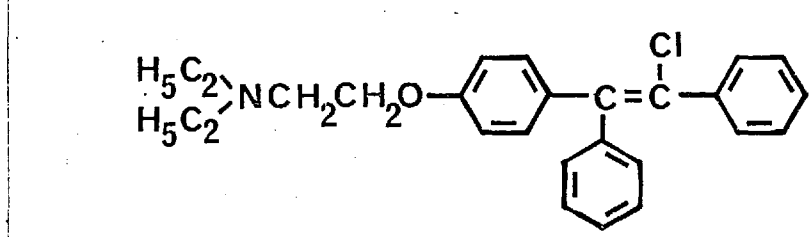
Amenorrhoea: serum levels of oestradiol, progesterone, luteinizing hormone and prolactin; four types of responses to clomiphene administration; serum oestradiol as an index of clomiphene responsiveness.

INTRODUCTION

Clomiphene is a non-steroid compound (for structural formula see Fig. 8.1.) and was originally studied because of its anti-oestrogenic effects in animals. It was introduced for the treatment of infertility in women in 1961 by Greenblatt et al. Whereas the response in amenorrhoeic women of serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and progesterone to clomiphene administration has been dealt with in several papers (Ross et al., 1970; Marshall and Fraser, 1971; Friedman, 1973; Radwanska and Swyer, 1974), the response of serum oestradiol (E_2) and its relation to the other responses is less well known. Further, although amenorrhoea associated with galactorrhoea has been studied extensively there is only scanty information on serum prolactin in ~~"functional"~~ other amenorrhoeas or on its response to clomiphene administration.

It was therefore decided to study the serum levels of LH, E_2, progesterone and prolactin in a first course of clomiphene in women with functional amenorrhoea. In an attempt to facilitate diagnosis at this early stage and as a guide to further clomiphene treatment the various responses have been classified.

Fig. 8.1.



CLOMIPHENE

The structural formula of clomiphene (1- p-(β-diethylaminoethoxy) phenyl -1,2-diphenyl-2-chlorethylene). The ethylene unit in the structure gives rise to two isomers, cis and trans, of which the former is more biologically active. The preparation commonly used, clomiphene citrate (Clomid), is a mixture of the two isomers.

PATIENTS AND METHODS

Patients

Twenty patients representing with apparently "functional" amenorrhoea of three months duration or more were chosen for study; patients with clear evidence of a pituitary tumour, other endocrine disorders, ovarian failure or organic gynaecological lesions were excluded.

Further studies of these patients have revealed the following: one patient proved, at laparoscopy, to have the polycystic ovary syndrome (Table 8.1); two had primary amenorrhoea, one due to isolated gonadotrophin deficiency associated with otherwise normal pituitary function, the other a hypothalamic syndrome with associated growth hormone deficiency and short stature; in the other patients, psychological precipitating factors were generally found (Table 8.1), but in five of them the amenorrhoea remained unexplained.

All patients were euthyroid by conventional clinical and biochemical criteria (serum protein bound iodine or thyroxin and/or serum thyroid stimulating hormone). Pelvic examination was normal in all patients except the one with polycystic ovaries.

Test procedures

Clomiphene citrate was given as a dose of 2 mg./kg./day (to the nearest 25 mg.) for seven days except for the patient with the polycystic ovary syndrome who had 50 mg. of clomiphene daily for five days. Blood was taken for two consecutive days when starting treatment and then daily on days 6 to 17, and always between 0800 and 1000 hours. None of the patients had side effects during treatment. In repeated courses, clomiphene was given in an identical way using only measurements

TABLE 8.1.

The aetiology of amenorrhoea in patients grouped according to their response to clomiphene

Responses to clomiphene	Diagnosis	No.
"Normal" (ovulation and menses)	Post pill amenorrhoea	3
	Anorexia nervosa	2
	Polycystic ovary syndrome	1
	Unknown	1
Partial (menses only, no ovulation)	Anorexia nervosa	2
	Unknown	3
Minimal (hormonal response but no ovulation or menses)	Anorexia nervosa	3
	Depressive episode	1
	Unknown	1
Nil	Isolated gonadotrophin deficiency*	1
	Hypogonadotrophic amenorrhoea +	1
	Hypothalamic amenorrhoea*	1

* Primary amenorrhoea.

+ Secondary amenorrhoea without evidence of pituitary tumour.

of basal body temperature or serum progesterone as indications of ovulation, and pregnancy tests when the menses were delayed.

Hormone measurements

The methods for measuring serum E_2 (direct assay) and progesterone are described in the methodological section, Chapter 3.

Serum LH was measured by a double antibody radioimmunoassay (Marshall et al., 1972). Using the MRC pituitary standard 69/104 the normal range for the follicular and luteal phases was 2 to 8 U./l. and for the mid-cycle peak 22 to 33 U./l.

Serum prolactin was measured by double antibody radioimmunoassay using antiserum raised against human prolactin. Human prolactin for labelling was supplied by the National Institutes of Health, Bethesda, Maryland (VLs No.1). The normal female range was 2 to 25ug/l. Intra-assay and inter-assay coefficients of variation were 14 per cent and 21 per cent respectively. Human prolactin 72/4/9, which was used as a standard, and the antiserum were kindly supplied by Professor H. Friesen.

RESULTS

Types of responses to clomiphene

After reviewing the hormonal and menstrual responses, seven have been set aside as normal responses since they were associated with both menstruation and ovulation, the latter being inferred when the serum progesterone rose to over 2.0 ng/ml on the first day after the LH peak or higher values on subsequent days (Table 8.2., Fig. 8.2.). As shown in Table 8.3. the other responses are then readily grouped into one of three types of subnormal responses, namely "partial" (menses without

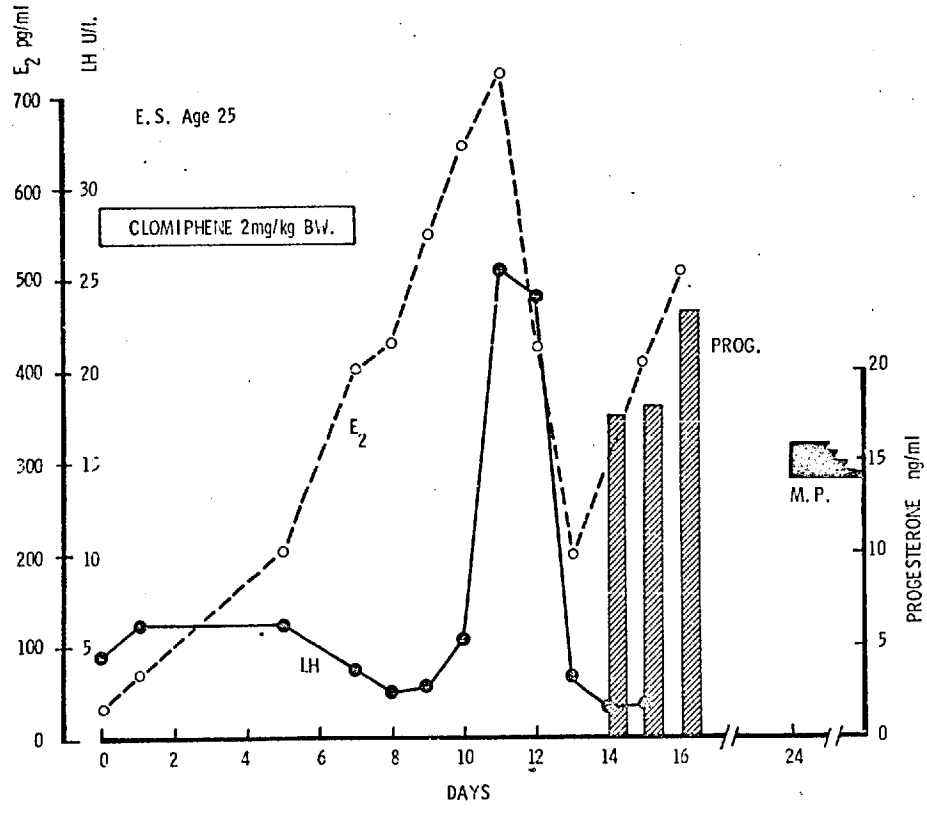
TABLE 8.2.

Data about patients who responded to clomiphene citrate by ovulation and menstruation

No.	Diagnosis	Age	Serum LH (U./l.)				Serum E_2 (pg/ml)		Serum progesterone		Day of onset of menses	Duration of luteal phase (days)
			Basal	Primary peak	Secondary peak	Day of secondary peak	Basal	Peak	Highest levels measured (ng/ml)	Days after LH peak		
1.	Post pill amenorrhoea	26	4.1	6.2	26	12	30	726	23.5(7.6)*	5	25	13
2.	Post pill amenorrhoea	25	3.8	15.5	55.0	14	27	1306	16 (4.3)	3	26	12
3.	Post pill amenorrhoea	23	3.0	-	12.5	11	25	1480	4.9(4.3)	4	20	9
4.	Anorexia nervosa	24	4.5	12.5	32	12	27	1045	8.0(4.3)	4	19	7
5.	Anorexia nervosa	18	3.3	7.6	16	16	20	3784	3.9(1.5)	1	20	4
6.	Polycystic ovary syndrome	25	3.4	7.2	6.0	12	6.2	676	10.8(4.3)	3	21	9
7.	Unknown	25	6.4	10.5	30	12	23	228	4.5(4.3)	2	23	11

* Mean normal plasma progesterone for the given day after the LH peak in spontaneous ovulatory cycles (Ross et al., 1970) are shown in parentheses.

Fig. 8.2.



Hormone responses seen in an amenorrhoic subject who had a "normal" response to a standard clomiphene test.

M.P. = menstrual period; PROG. = progesterone.

TABLE 8.3.

Data about response to a clomiphene citrate test in 20 patients with "functional" amenorrhoea.
The dose of clomiphene citrate used was 2mg/kg/day for 7 days in all but one patient.
The latter had polycystic ovaries and was only given 50mg of clomiphene citrate daily for 5 days.

Type of response to clomiphene	No.	Serum levels (median and range)								
		LH (U./litre)			Oestradiol (pg/ml)		Highest level of progesterone measured (ng/ml)	Basal prolactin level (ug/litre)	Days to secondary peak of LH	Duration of luteal phase (days)	Day of onset of menstruation
		Basal	Primary	Secondary	Basal	Peak					
Normal (ovulation and menses)	7	3.9 (3.0-6.4)	10.5 (6.2-15.5)	26 (6.0-55.0)	25 (6.2-30.0)	726 (228-3784)	8.0 (3.9-23.5)	7.2 (4.7-80)	12 (11-16)	9 (4-13)	21 (19-26)
Partial (menses only, no ovulation)	5	3.3 (0.55-0)	6.2 (5.0-8.4)	12 (5.7-14.0)	20 (11-80)	267 (139-435)	1.5 (0.9-2.0)	6.8 (5.8-22)	13 (10-15)	-	27 (19-47)
Minimal (hormonal response but no ovulation or menses)	5	1.5 (1.0-3.2)	6.2 (3.8-12.0)	-	26 (18-36)	375 (166-455)	1.2	5.4 (3.8-6.2)	-	-	-
Nil	3	1.0	1.0	-	6.2 (5.6-15.0)	-	0.6	3.4 (2-9.6)	-	-	-

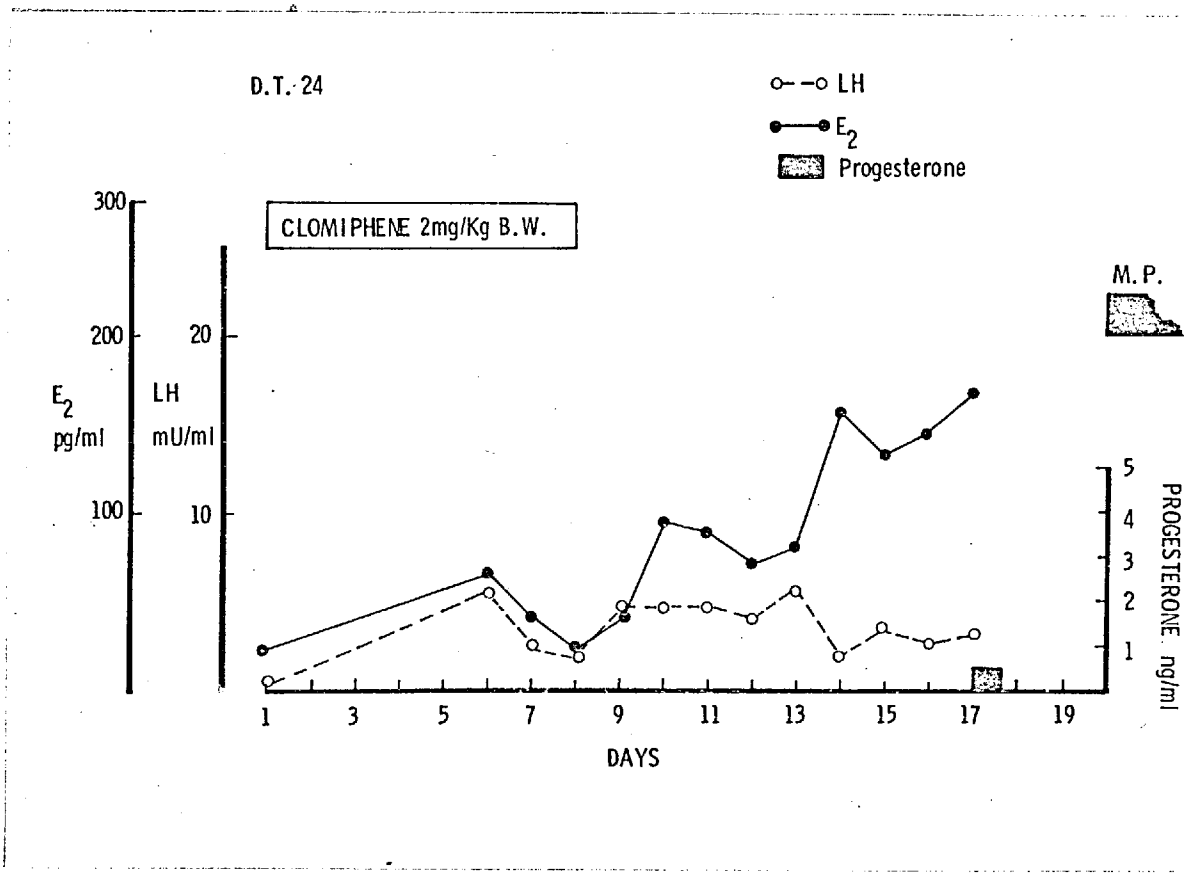
ovulation), "minimal" (some hormonal response but neither ovulation nor menses) and "nil" (no response at all).

The "normal" response. The hormonal responses of a representative patient are shown in Fig 8.2. while Table 8.2 presents for all seven such patients the main features of the "normal" responses to clomiphene. In this group menses always occurred by the 26th day after starting clomiphene. From Table 8.2. it may be noted that basal values and responses in this group tended to be higher than in the subnormal groups. E_2 and progesterone levels reached in the responses were, with one exception, higher than the corresponding ones in spontaneous cycles and they did not, with the same exception, overlap with the responses of the subnormal groups.

The "partial" response. Five patients menstruated after clomiphene administration but did not ovulate as judged by their serum progesterone level remaining 2ng/ml or less (Table 8.3.). All these patients had a second LH peak but as for other responses in this group they were generally smaller than those in the normal group (Fig. 8.3.). The menstrual response was irregular and tended to be delayed. All patients in this group later gave "ovulatory" responses in repeated clomiphene courses and pregnancies eventually occurred.

The "minimal" response. The five patients in this group had generally low basal serum LH levels and no secondary LH peak together with a negligible progesterone rise. However, they all responded with a considerable rise in serum E_2 although these elevations were either of short duration during the clomiphene administration or protracted. Four patients in this group are known to have had normal responses on further clomiphene administration.

Fig. 8.3.



Hormone responses seen in an amenorrhoic subject who had a "partial" response to a standard clomiphene test.

M.P. = menstrual period.

The "nil" response. Here basal serum levels of both LH and E_2 were subnormal and no responses to clomiphene were detected. Two of the three patients had primary amenorrhoea of pituitary-hypothalamic origin (Table 8.1.)

Serum prolactin levels

In the group with normal responses, four patients had initial prolactin levels within the normal range and three had had elevated levels (47 to 80ug/l.; Table 8.3). The levels were followed throughout the test in one with normal basal levels and two with high levels. While the higher levels fluctuated there was no trend of change during the test.

Among the patients who showed subnormal responses to clomiphene, none had initially high prolactin levels. In three of these, followed throughout the cycle, no changes were noted.

DISCUSSION

The ease of administration, relatively minor side effects and efficiency in restoring ovulation and menses often make clomiphene citrate the first treatment in "functional" amenorrhoea associated with infertility. Eighty per cent of such patients can be expected to ovulate and approximately 50 per cent become pregnant (Kase, 1973). The findings in our small group of patients seem to confirm these figures.

The hormonal responses to a first course of clomiphene can give clues to diagnosis. Measurements of total hormone excretion in urine, though often used in the past, may not satisfactorily reflect the effective levels of the hormones at the target cells. This applies

particularly to the sex steroids some of which are secreted into the blood stream both free and as conjugates which are then cleared at a different rate into the urine (Baulieu and Robel, 1970). We therefore measured the unconjugated E_2 in serum during and after standard courses of clomiphene.

Although the basal serum E_2 levels showed tenfold differences between comparable individuals serial serum E_2 measurements provided the most sensitive index of the response to clomiphene. Thus when the serum E_2 levels rose to values above 460pg/ml ovulation always followed. Further, the E_2 responses allowed us to define in the first clomiphene course a group of patients that neither ovulated nor menstruated ("minimal" response) but who were still able to respond normally as 80 per cent of them ovulated later after repeated courses of clomiphene. The finding of considerably higher peak levels of E_2 in our normal group than in spontaneous cycles is in agreement with the results obtained by Robertson et al. (1971).

The striking finding of four short luteal phases in seven of the normal response group also supports the claim that in many cases clomiphene needs to be supplemented by some luteotrophic agent or progesterone itself to improve prospects of pregnancy (Kistner, 1966; Rabau et al., 1968; Greenblatt et al., 1971; Keller, 1972; Insler et al., 1973; Jones, 1973). Repeated clomiphene-induced cycles have to be studied further, however, to establish the overall incidence of this phenomenon.

While the highest progesterone level measured in the groups of patients with subnormal responses was 2.0ng/ml, the lowest progesterone level measured in the normal response group during the luteal phase was

1.9ng/ml on the first day after the LH peak. The value of 2.0ng/ml on the first day, combined with later values if obtained, was therefore used as the dividing point between the normal and subnormal responses. The normal progesterone values for the luteal phase (see Chapter 3) have a similar range (approximately 1.0 to 15 ng/ml) to those given by Ross et al. (1970). As these authors studied a larger number of luteal phases their mean values have been given for reference in Table 8.2. and it will be seen that the present values for the corresponding day are always higher. This is in agreement with Robertson et al. (1971) who reported higher levels of serum progesterone after clomiphene administration than those seen in spontaneous cycles. Although this may be related to the clomiphene dose given, the present results would suggest that in the normal clomiphene response serum progesterone levels higher than those in spontaneous cycles must occur before ovulation can be inferred. This in turn might have some bearing on the incidence of luteal insufficiency or short luteal phase found in our study and that of Van Hall and Mastboom (1969).

Basal levels of the hormones measured gave little information about the potential response to clomiphene although no response is likely to follow very low levels of both serum LH and E_2. No correlation was found between duration of amenorrhoea and hormonal responses.

It is perhaps of interest that all three patients with elevated prolactin levels showed a normal response to clomiphene, and the drug did not appear to have any effect on prolactin levels.

CHAPTER 9

GENERAL SUMMARY AND CONCLUSIONS

In this final chapter results in the experimental (3rd) section will be summed up and their significance discussed a little further than already done in the individual chapters.

The methodological section needs little further discussion as radioimmunoassays of steroids are already a well-established procedure. The methods used in the thesis have been streamlined considerably and simplified, thus enabling large batches to be measured quickly and reproducibly.

Much of the work presented is new, that is, methods and measurements have not been reported before in the literature. The methods used in Chapter 5 to study the conjugation pattern of testosterone (T), for instance, have been specially elaborated for that purpose and represent a new approach which can probably be applied to other steroids as well. The measurement of unconjugated T in urine has been published only recently (Andino et al., 1976), but there are no reported measurements of unconjugated DHT in urine, nor of serum DHT in women on oral contraceptives (OC). Most of the preliminary work in Chapter 4 was necessary to obtain background information relevant to methodology.

Chapter 4 describes many results which almost all call for further investigation. Briefly, it was found that 1) red cells probably contain no unconjugated testosterone (T) or 5α-dihydrotestosterone (DHT), 2) levels of unconjugated serum T rose inconsistently in male sera but not at all in female sera, and DHT rose in

sera from both sexes on incubation of the samples at 37°C, 3) unconjugated T, DHT and oestradiol (E_2) levels all rose in urine samples stored at 37°C.

T content of red cells was only measured in three individuals, and needs further investigation. The immediate practical consequences are that gross haemolysis will dilute the unconjugated T concentration in plasma or serum. The easily accessible red cells have been largely neglected in the study of steroid cell interactions. The author is only aware of one group of workers that has done any amount of work recently on red cells and steroids. Mulder et al. (1973) showed that free steroids penetrated quickly into reconstituted resealed red cells, compared with the rather slow penetration of steroid sulphates. Apart from the mechanisms involved in keeping the steroids out of the cell it might be rewarding to study this characteristic in various endocrine disorders.

The inconsistent rise of T and the more pronounced one of DHT in serum samples, when incubated at 37°C, led to the abandonment of an intended dialysis technique to measure unbound T in serum. This observation raises doubts as to the accuracy of earlier studies on unbound T levels obtained by this technique. Although only T and DHT were studied, the results raise the question whether this might not occur for other steroids as well. Some of these, such as the androstanediols have, as has DHT, a high affinity for SHBG (Baulieu et al., 1970), and could displace T and generally create a different steroid environment at the end of the dialysis (16h) compared to that in the original sample.

The sharp rise of unconjugated sex steroids in urinary samples on storage posed a problem for their collection and handling. Only a minimal rise was found when stored at 4°C, and none at -20°C. Urinary samples were therefore refrigerated during collection and frozen immediately after reception.

Two out of eight urine samples stored at 37°C showed only minimal rises of unconjugated E_2. The samples belonged to a male who had been taking ampicillin, and a woman on oral contraceptives. The striking difference suggests that some quantitatively important change is caused by these agents in E_2 metabolism. It is known that ampicillin administration to pregnant women reduces total urinary oestrogen excretion (Adlercreutz et al., 1975), but no studies on urinary E_2 conjugates in women taking oral contraceptives have been reported. It is inferred that conjugates might be involved, in view of the findings for urinary T and DHT to be discussed below.

Chapter 5 describes the results of investigations into the causes of the T and DHT elevations in stored urine samples discussed above.

The study produced solid evidence for the existence in urine of a labile T and DHT-containing compound, probably a conjugate, but having physical and chemical characteristics different to T glucuronide or T sulphate.

The acid lability of this compound combined with the similarity of its elution to that of T glucuronide from the ion-exchange column used, makes it possible that the compound is the 3,5-dienol-3-glucuronide of T (and of DHT). The existence of such compounds was indicated by the studies of Knapstein et al., (1967) on the direct

metabolism of dehydroepiandrosterone-3-glucuronide (see discussion in Chapter 5; direct metabolism is a term originally coined by Dr. E.E. Baulieu (1963) meaning a metabolic change in the steroid moiety of a steroid conjugate without the bond of conjugation being broken).

Evidence for the natural existence of such compounds has not been available to date, but a few have been synthesized and a great acid-lability reported for one of them (Wotiz et al., 1959; Wakabayashi et al., 1961). After administration of adrenosterone (androst-4-ene 3,17-dione, 11β-ol) to rats, 11β-hydroxy-17oxo-androsta-3,5-dien-3-yl-B-D-glucopyranosiduronic acid was identified in their urine (Wotiz and Fishman, 1963).

The results in Chapter 5 suggest that the acid-lability of the compound may be used for its quantitative measurement in terms of T, and that column chromatography may serve as a tool to indicate its presence in tissues or fluids. As it is still a possibility that the T release observed is caused by more than one compound, each with similar characteristics in regard to acid lability and elution characteristics from the anion-exchange column, further separation and eventual isolation procedures need to be developed. This might be hampered by the outstanding lability of the compound(s) in urine, even at neutral pH. In any event its further characterization might yield valuable information on the intermediary metabolism of steroids, and its quantitative measurement might prove to be of some diagnostic value.

Similar compounds may exist for other steroids. Preliminary studies indicate that progesterone is released in urine on storage at 37°C in a similar fashion to T.

Studies on T release in urine by increasing concentrations of hydrochloric acid and sulphuric acid showed a much stronger effect of hydrochloric acid, which also hydrolyzed T glucuronide and T sulphate more efficiently than did sulphuric acid. This may explain discrepancies in the literature concerning "ph 1 hydrolyzable" urinary T conjugate (Van der Molen et al., 1966; Dulmanis et al., 1964).

Chapter 6 describes measurements of unconjugated T, DHT and E_2 in silultaneously obtained serum and urine specimens from three groups of individuals. These were normal males and females and normal females on oral contraceptives. Small groups of hirsute and postmenopausal women were also included in this study.

In the male group a significant overall correlation was found between serum T and DHT levels. The variation in T/DHT ratios was significantly greater between individuals than within individuals suggesting a characteristic T/DHT constant for each individual. The significance of such a constant needs further investigation.

A significant correlation was found between serum DHT and E_2 in both normal males and females but not in females on oral contraceptives. This is of interest as these two hormones are the main androgen and oestrogen effectors, respectively, at the subcellular level (Wilson, 1975). This will be discussed a little further below in relation to the hormonal levels found in the women on oral contraceptives.

Unconjugated T and DHT in urine showed a high degree of correlation. Levels of unconjugated urinary E_2 correlated poorly with either T or DHT. Urinary T and DHT levels were about four times higher in normal males than normal females in the early follicular phase of the menstrual cycle. A significant correlation was found between urinary creatinine and either T or DHT, but not with E_2.

Within each of the three main groups studied, levels in urine for each individual hormone correlated poorly with serum levels. However, higher serum levels in a group were accompanied by higher urinary levels. Urinary levels of unconjugated T, DHT and E_2 therefore seem to reflect only large variations in unconjugated serum levels.

In women ingesting oral contraceptives serum and urine T levels were higher than in the normal group but did not reach statistical significance whereas DHT levels did ($p < 0.01$) for serum, $p < 0.05$ for urine). Serum E_2 levels were significantly lower ($p < 0.001$) in the contraceptive group, and urine E_2 levels also lower but not significantly.

The serum E_2 levels in the women ingesting oral contraceptives were in the postmenopausal range. Such a reduction in the endogenous serum E_2 levels has not been reported before, a fact probably related to the specificity of the E_2 antiserum used in the present study. It is possible that the low endogenous E_2 levels produced by the contraceptives may be a part of the antiovulatory effect of the OC. The suppression of the E_2 levels would for instance abolish any positive E_2 feedback effect on the pituitary (Jaffe and Keye jr., 1975; Yen et al., 1975).

The higher urinary levels of T and DHT in the contraceptive group suggest that these women have elevated levels of these androgens in serum available for renal excretion and that these elevations are not a secondary effect caused by elevated SHBG in serum resulting in increased serum capacity only. Assuming a similar availability of the androgens to other tissues these women seem to be exposed to high androgen levels, the effects of which, in the absence of signs of virilism, must be blocked by the contraceptive drugs.

These results call for a new interpretation of the effects of oral contraceptives in the body. As in our small group ethynyl oestradiol (EE_2) was a common ingredient of the oral contraceptives and no obvious differences were related to the amount of the progestational agent in the contraceptive preparations, EE_2 is presumably the main cause of the differences observed between the normal and the contraceptive groups. It seems a possible explanation that EE_2 in normal women upsets a normal balance between androgens and oestrogens which the body tries to restore by increased androgen levels. Such mechanism might explain the adrenal hypertrophy caused by E_2 administration in rats (Afolabi et al., 1976), the adrenals being called upon to produce more androgens to balance the increased exogenous E_2 levels. Whether administered EE_2 and E_2 produce similar changes in serum levels of androgenic steroids needs further investigation.

It has been found that E_2 and DHT have a synergistic action in restoring normal sexual behaviour in castrated male rats (Baum and Vreeberg, 1973; Larsson et al., 1973) and a synergistic effect of E_2 and DHT on the growth of the magnum of the chick oviduct has been described (Palmiter and Haines, 1973). In man such a synergism combined with an inherent balance in the sex steroid environment may exist and the significant correlation of E_2 and DHT levels in both sexes found in the present study supports that assumption. The elevated levels of serum DHT in women on oral contraceptives could mean a disturbance of a physiological DHT/E_2 ratio.

The results presented in Chapter 6 on the measurements of unconjugated sex steroids in serum suggest that a closer study on the relationship between serum levels of individual sex steroids is

warranted. Such approach might yield valuable information on the mechanism of action of these hormones and eventually give some clues as to the molecular interactions involved.

In males the administration of EE_2 leads to decreased plasma concentration of T (Clark et al., 1973; Forchielli et al., 1965). This could mean a different response of the two sexes to the administration of EE_2. However, this could be dose related as Clark et al. (1973) used twice as high EE_2 doses (50ug b.i.d.) as those used in the present study and that of Tremblay et al. (1974); and Forchielli et al. (1965) used still higher doses of EE_2. It should be of interest to study serum DHT levels in males after EE_2 ingestion (for instance, during treatment of prostate cancer) to see if they rise as in females.

The antiandrogenic properties of EE_2 which are indicated by the present results are in accordance with the efficiency of this drug in combination with cyproterone acetate (an antiandrogen and progestagen) in the treatment of hirsutism (Zielske et al., 1971; Ismail et al., 1974; Hammerstein et al., 1975).

Chapter 7 gives an account of studies on serum T levels in males, their fluctuation during 24 hours and responses to infusions of human pituitary LH and FSH preparations. In agreement with most investigators a circadian rhythm was found with lower levels in the late evening and superimposed episodic fluctuations of considerable magnitude ($\pm$ 50% of the average).

The infusion of FSH alone did not elicit any significant rise in serum T, but two LH infusions did so to a varying extent. Combined infusions of both LH and FSH produced significant serum T rise (35 - 68%) in 4 subjects and greater rise in 2 of them than after LH

alone. The serum T responses were gradual, reaching a maximum 7 - 8h after the end of the infusion. It was concluded that the gonadotrophins could not be the cause of the sharp episodic fluctuations in serum T levels seen during a normal day. Serum T levels must, therefore, be governed by other unknown factors. The infusion studies also suggested a synergistic action between LH and FSH in elevating serum T levels. These findings are in agreement with those recently reported by Rowe et al. (1975) who showed that LH is likely to be the main factor controlling serum T levels in males, but other factors may obscure a direct relationship between endogenous serum levels of these two hormones.

Chapter 8, the final chapter, is a study of serum E_2 levels and progesterone in amenorrhoic women before and during a first course of clomiphene citrate. Serum LH and prolactin were measured simultaneously in the samples. The hormonal criteria of a "normal" or "ovulatory" response, and three grades of subnormal anovulatory responses, were defined in twenty patients after clomiphene. The hormonal responses were estimated by frequent serum measurements and by the menstrual response. Seven patients had a normal or ovulatory response with a late rise of serum progesterone and subsequent menses. Their serum luteinizing hormone (LH) and oestradiol (E_2) responses were greater than in spontaneous menstrual cycles. Five patients gave only partial responses, with menstruation and no prior rise of progesterone. Their serum LH and E_2 responses were generally lower than in the normal group but had the same pattern except for an absence of the luteal phase rise in E_2.

Five patients who did not menstruate showed smaller and more variable responses. The remaining three showed no hormonal responses. The second and third groups had ovulatory responses on subsequent clomiphene courses, whereas no improvement was shown by the last group.

The serum E_2 responses offered the most sensitive index of the responsiveness to clomiphene whereas basal values were of little value. Response values in the normal group suggested that higher progesterone levels need to be reached after clomiphene administration than in spontaneous cycles for ovulation to be inferred. The serum prolactin showed no consistent response during the clomiphene test.

As for the oral contraceptives, little is known about the mechanism of action of clomiphene. It may have a direct action on the gonads, or, a more generally held belief, act on the hypothalamus with subsequent release of FSH and LH which in turn stimulate the gonads. Both mechanisms could possibly be at work simultaneously.

In a recent paper by Miechi et al., (1975) clomiphene citrate was administered i.m. (5 and 10 mg) to normal males and a maximal serum testosterone response (2-fold rise) was seen within 60 min. FSH response was maximal at 60 min and LH at 30 min after the injection. In view of the results of Chapter 7 such an early rise of serum T could not be caused by the LH and FSH rises and would indicate a direct effect of clomiphene on the testis or, possibly, an increased sensitivity of the testis to the gonadotrophins. Such mechanism might explain the present finding that the E_2 levels were the most sensitive index for predicting the responsiveness to clomiphene treatment.

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