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BIOCHEMICAL STUDIES ON CONJUGATES
OF ANDROSTENEDIONE

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ABSTRACT

Biochemical Studies on Conjugates of Androstenedione

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The presence of androstenedione in blood and urine in a conjugated form has been demonstrated and methods for estimating these compounds have been devised. In urine, the mean excretion of total androstenedione (nmol/24h), both free and conjugated was 195 in 11 male subjects and 77 in 11 female subjects. Excretion values were determined in subjects with various endocrinopathies. For a group of postmenopausal subjects, a good correlation was found between the total urinary excretion of androstenedione and the mean plasma androstenedione concentration during the same period, indicating that the measurement of total urinary androstenedione is a useful reflection of plasma values and thus of the production of androstenedione.

The types of androstenedione conjugate in urine have been investigated by enzymic and acid hydrolysis. The greater proportion of androstenedione in urine is in a conjugated form. There is evidence for at least 2 conjugates, the 3-enol glucuronide and the 3-enol sulphate. Androstenedione 3-enol sulphate has been synthesised and physically characterised. Enzymic and acid hydrolysis of this conjugate has been studied.

The presence of androstenedione in a conjugated form in the peripheral circulation has been demonstrated. In 7 normal subjects values for conjugated androstenedione of 1.3-3.7 nmol/l (mean 2.3) were found, representing a mean of 45.6% of the concentration of unconjugated androstenedione in the same samples. The origin of this conjugated androstenedione has been investigated. From data on the concentration of the conjugate in sequential peripheral blood samples and in adrenal venous blood, it is concluded that the conjugate is a direct secretion product of the human adrenal gland.

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Abbreviations and trivial names

The following abbreviations have been used in this manuscript:

ACTH: adrenocorticotrophin.

Androstenedione: androst-4-ene-3,17-dione.

Androstenedione 3-enol glucuronide: 17-oxoandrost-3,
5-dien-3-yl- β -D-glucopyranosiduronate.

Androstenedione 3-enol sulphate: 3 β -sulphoxyandrost-3,
5-dien-17-one.

c.p.m.: counts per minute. The efficiency of the
scintillation counters used in this study was around 50%.

C.V.: coefficient of variation.

I.V.C.: inferior vena cava.

I.U.: international units.

R.D.: redistilled.

Units: S.I. units have been used throughout this work.

If values from other workers have been quoted in the text
and the original units were not S.I. units, these values
have been converted to S.I.

SECTION 1

1.1 Introduction

Androstenedione is an important adrenal and gonadal secretory product. The role which androstenedione plays in human steroid endocrinology is as a prehormone for testosterone and oestrone, particularly in the female, where it is a major source of these steroids. The androgenicity of a steroid hormone, with the structure of androstenedione was first recognised in 1935 (Butenandt and Kudzus, 1935; Ruzicka and Wettstein, 1935; Toschopp, 1935).

The secretion of androstenedione in the human, by the adrenal cortex, the ovaries and the testis is now well documented. Weiland, De Courcy, Levy, Zala and Hirschmann (1965) were the first to demonstrate direct secretion of androstenedione by the adrenal cortex. Secretion is episodic and responsive to the same stimulus as cortisol, ACTH, and exhibiting the same circadian rhythm (Tunbridge, Rippon and James, 1973).

The ovary, in premenopausal subjects, secretes androstenedione (Horton, Romanoff and Walker, 1966; Baird, 1971; Baird, 1976). The relative amounts of androstenedione secreted by the ovary and the adrenal cortex vary depending on the time of day and the stage of the menstrual cycle (Abraham, 1974). Different groups of workers have found various amounts of androstenedione in blood at different times of the cycle. For example, Vermeulen and Verdonck (1976a) found higher levels of androstenedione in the blood at the time of the LH surge, but other workers have not confirmed these findings (e.g. Guerrero, Aso, Brenner, Cekan, Landgren, Hayenfeldt and Diczfalusy, 1976). In most of these studies androstenedione has only been measured in one blood sample, on each day of the cycle, but because of the episodic secretion of androstenedione these results may not be very

meaningful. Judd and Yen (1973) found that the increase in values of plasma androstenedione at mid cycle varied from subject to subject. Evidence from short profiles indicated that androstenedione secretion by the ovary, as well as the adrenal cortex, is episodic, and that this episodic secretion is more marked in patients with hirsutism (James, Rippon and Jacobs, 1976).

In the postmenopausal woman, there are differing views as to whether androstenedione is secreted by the ovary and the adrenal cortex or only by the adrenal cortex. Vermeulen (1976a, b) and Judd, Judd, Lucas and Yen (1974) have found that in ovariectomized postmenopausal women, values for androstenedione in the peripheral plasma were lower than those obtained from normal postmenopausal women, indicating that the postmenopausal ovary is responsible for about 30% of the production of androstenedione. Hutton, Jacobs, James, Murray and Rippon (1977a, b) concluded though, from studies on normal postmenopausal women that there was no change in the hormone levels or patterns after ovariectomy, and that the major glandular source of steroids after the menopause was the adrenal cortex.

The secretion of androstenedione by the testis has been demonstrated by the measurement of androstenedione in spermatic venous blood (Fiorelli, Borrelli, Forti, Gonnelli, Pazzagli and Serio, 1976; Hammond, Ruukonen, Kontturi, Kosela and Vihko, 1977). Hammond et al. (1977) found values of androstenedione in spermatic blood 12 times those in peripheral venous blood. These samples were taken, though, whilst the subjects were under anaesthesia and this may have affected the values obtained, although the peripheral values were within the range reported for normal subjects. Fiorelli et al. (1976) found no significant correlation between levels of testosterone and androstenedione and dihydrotestosterone in either spermatic or peripheral

venous blood. Also no correlation was found between androstenedione and testosterone in the peripheral venous blood of men.

To elucidate the mechanism for the nocturnal rise in testosterone, Judd, Parker, Rakoff, Hopper and Yen (1973) studied 4 men on 3 nights, at weekly intervals, administering dexamethasone (2 mg) on the third night. These data suggested that androstenedione like testosterone, is secreted episodically in increasing amounts during the night, but that though the testosterone rise is as a result of increased testicular secretion, the increasing androstenedione secretion was of adrenal origin.

An important role of androstenedione is as a prehormone for other androgens and the oestrogens. In both men and women, androstenedione significantly contributes to the blood production rates of other steroids. About 60% of circulating testosterone in women is formed by the peripheral conversion of androstenedione (Horton and Tait, 1966), and in both men and women peripheral conversion of androstenedione to oestrogens occurs in tissues outside the endocrine glands, e.g. skin, liver, muscle and fat (Grondin, Siiteri and Macdonald, 1973; Edman and Macdonald, 1976; Longcope, Pratt, Schneider and Fineberg, 1978). This conversion of androstenedione to oestrone extraglandularly is the most important source of oestrogen in the postmenopausal female.

The studies for determining the production and secretion rates of androstenedione have used radiolabelled isotopes. These studies have their limitations, since to measure the production rate a good estimate of the plasma concentration is required, as the production rate is the product of the plasma concentration and metabolic clearance rate of the steroid. Hence, to accurately determine the production rate it is important to obtain a good estimate of the mean plasma level throughout the 24h period. This requires a large number of samples to

be taken because of the episodic secretion and the circadian variation of androstenedione. Blood production rates and metabolic clearance rates of androstenedione have been measured by several groups of workers. Horton and Tait (1966) found that the metabolic clearance of androstenedione was $2,330 \pm (107 \text{ S.E.})$ l/day indicating that considerable hepatic metabolism occurs. The blood production rate of androstenedione in the female was 3.4 mg/day and in the male 1.4 mg/day. From the conversion rates which they determined they concluded that in the female essentially no androstenedione arises from blood testosterone, but that 60% of the plasma testosterone resulted from the peripheral conversion of androstenedione present in plasma in the female. Rivarola, Sacz, Meyer, Jenkins and Migeon (1966) measured the metabolic clearance rates and blood production rates of androstenedione under basal conditions and ACTH and HCG stimulation. The blood production rate, under basal conditions, was 2.1 mg/day in male subjects and 2.7 and 3.6 mg/day in female subjects and these values were increased with ACTH stimulation. Like Horton and Tait (1966), they also found that in females 50% of the blood testosterone production rate was accounted for by transformation of blood androstenedione.

The estimation of androstenedione in plasma by radioimmunoassay and other methods has been reviewed by Friedrich, Jaeger-Whitegiver, Bieder, Keller and Schindler (1975), but since then other radioimmunoassay methods for estimating androstenedione have been reported. These have included alumina column chromatographic purification (James, Braunsberg, Rippon, Andino and Parker, 1977; Parker, Grover and Odell, 1978) and thin layer chromatographic purification (Vermulen and Verdonck, 1976b) of the steroid, prior to estimation. Other methods have been reported which have not included a chromatographic purification stage but some of these have not been validated. The method

used for the determination of androstenedione in plasma in this thesis is a non chromatographic assay which has been fully validated (Goodall, Rippon and James, 1979).

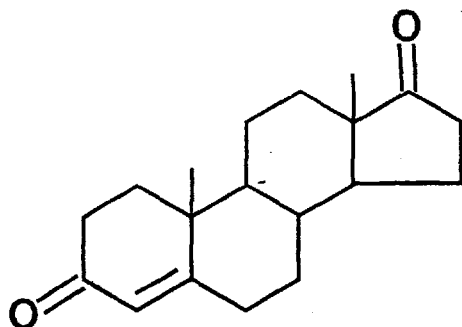
Plasma levels of androstenedione in males and females are of the same order (Friedrich et al., 1975), although values in men would appear to be slightly lower than the values obtained from female subjects. The time during the 24h period of plasma sampling, though, must be taken into account. Examples of mean values obtained are for male, 4.0 nmol/l and female 4.9 nmol/l (Thornycroft, Ribeiro and Stone, 1973), and male 3.1 nmol/l and female 4.1 nmol/l (Friedrich et al., 1975). In an extensive study of normal female subjects, a range of 0.9-7.0 nmol/l was found by James, Rippon and Jacobs (1976) who correlated the values with the age of the subjects.

In the study of normal and abnormal androgen secretion, it is important to be able to evaluate androstenedione secretion accurately. The circadian rhythm of androstenedione secretion and the ability of the adrenal cortex to secrete androstenedione after ACTH stimulation are well documented. The marked fluctuations in androstenedione in plasma which occurs during a 24h period have been shown using the technique of steroid profiling (James, Tunbridge, Wilson, Hutton, Jacobs, Goodall, Murray and Rippon, 1978; James, Tunbridge, Wilson, Hutton, Jacobs and Rippon, 1978). Therefore, the time during the 24h period, at which a blood sample is taken, must be considered before any interpretation can be made of the result. An accurate determination of the concentration of androstenedione in plasma is also required for the assessment of production and secretion rates of androstenedione, and this requires the taking of several blood samples, during a 24h period, which needs a large amount of patient co-operation and is expensive.

The possibility of using the measurement of androstenedione in urine has therefore been explored. At present no unique metabolite of androstenedione is known to exist and the initial aim of the work was to investigate the measurement of androstenedione and its conjugates in urine. The major metabolites of androstenedione, androsterone and aetiocholanolone, are not unique as they are also derived from other androgens, e.g. testosterone.

The initial approach to the problem (Andino, James, Parker and Rippon, 1976) was to investigate the amount of unconjugated androstenedione in human urine, as by analogy with urinary free cortisol this might be expected to reflect plasma levels. These workers concluded that the amount of unconjugated androstenedione that was present in urine does reflect the integrated plasma levels of androstenedione, and could prove to be a useful index of androstenedione production. There is in the literature, though, much evidence from other workers (e.g. Schubert and Frankenberg, 1964; Longhino, Tajic, Vedris, Jankovic and Drobnjak, 1968; Schollberg and Hubl, 1968) that androstenedione is present in urine in a conjugated form, probably as the 3-enol glucuronide and the 3-enol sulphate (Fig. 1.1). These conjugates of androstenedione are present in amounts in considerable excess of those present in the unconjugated form. Spontaneous hydrolysis of these conjugates can occur resulting in spuriously high values of androstenedione in the unconjugated fraction. This has been found for other steroids present in urine (Kjeld, Puah and Joplin, 1977). A method has therefore, been developed which measures the total amount of androstenedione, both conjugated and unconjugated, that is present in urine.

The chemical bonding in the conjugates of androstenedione is likely to be different to the usual type of linkage between steroids



androstenedione

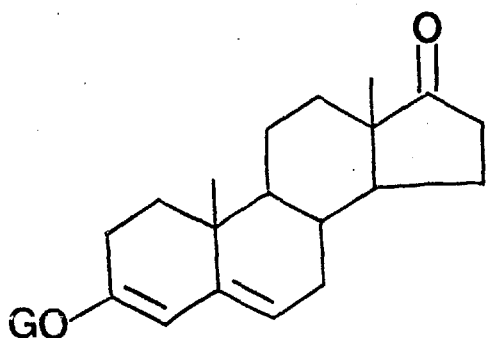
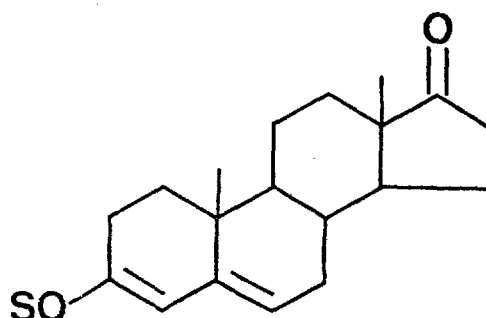
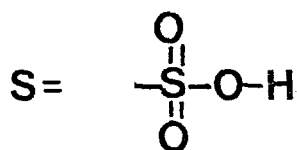
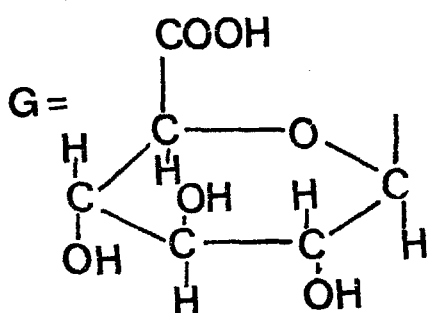
androstenedione
3-enol glucuronideandrostenedione
3-enol sulphate

Fig1.1 The structure of androstenedione and its conjugates

and the conjugating acid. The steroid is usually conjugated to sulphuric or glucuronic acid through an hydroxyl group already present in the molecule, e.g. dehydroepiandrosterone sulphate (Bernstein, Dusza and Joseph, 1970). As androstenedione contains no such grouping, enolization of a ketone group has to occur prior to conjugation. This is most likely to happen with the ketone to form a 3,5-diene structure with glucuronic or sulphuric acid linked through the oxygen at 3 position. Both the 3-enol glucuronide and the 3-enol sulphate have been synthesised. The 3-enol glucuronide of androstenedione has been prepared by two groups of workers (Wotiz, Smakula, Lichtin and Leftin, 1959; Matsui, Kawase and Okada, 1974) and the 3-enol sulphate by Oertel, Teiber and Rindt (1967). The synthetically prepared steroids have been well characterised using physical chemical methods, which show the presence of the enol bonding. More studies have been reported on the hydrolysis and stability of the glucuronide than the sulphate (Wotiz, 1962; Wakabayashi, Wotiz and Fishman, 1961).

Matsui et al. (1974) after extensive studies on the stability of androstenedione 3-enol glucuronide, commented that care must be taken when attempting to hydrolyse these conjugates, due to the possible formation of polar artifacts. As yet, the isolation of the conjugates of androstenedione from urine has not been reported and this may be due to these conjugates being unstable as a result of the enolic bonding of the steroid to the conjugating acid.

Oertel et al. (1967) prepared the 3-enol sulphate of androstenedione to study the effect of incubation of this conjugate with placental microsomes. Their findings suggested that the 3,5-dienol sulphate of androstenedione could be converted biosynthetically to oestrone sulphate, and that the biosynthesis of oestrogens may occur from dehydroepiandrosterone sulphate via androstenedione sulphate.

This concept of the metabolism of conjugates without the loss of the acid grouping conjugated to the steroid has been called direct metabolism and has been investigated for other steroids (Solomon and Bhavnani, 1970). Oertel and co-workers, after studies on the direct metabolism of dehydroepiandrosterone conjugates (Knapstein, Rindt, Wendleberger and Oertel, 1967 a; Knapstein, Rindt, Wendleberger and Oertel, 1967 b) suggested that the conversion of these conjugates to reduced metabolites required intermediates with a 3,5-dieneol structure.

Evidence for the direct metabolism of androstenedione enol glucuronide has been reported by Wotiz (1962), who tentatively concluded that direct metabolism could occur, after studying the products of the incubation of androstenedione enol glucuronide with rat liver mince. Wotiz and Fishman (1963) have also identified the presence of enol glucuronide conjugates of androst-4-ene-3,11,17-trione in rat urine. The evidence of these workers could indicate that there is a more important role for these conjugates in the body than just an excretory mechanism, and that biosynthetic pathways may be present for steroid conjugates as well as for the unconjugated steroids.

The only report of the presence of conjugated androstenedione in plasma, is that of Rhamy and Hadd (1973), who identified androstenedione enol glucuronide from the plasma of a woman with an interstitial cell ovarian tumour. A method has, therefore, been devised to demonstrate the existence of conjugated androstenedione in the plasma of normal subjects. This method has also been used to measure the amounts of conjugated androstenedione present in plasma from subjects undergoing adrenal suppression or stimulation, in an attempt to determine the source of this conjugated steroid in plasma.

The aim of the work in this thesis has been twofold. Firstly develop a reliable method for the measurement of the total amount of

androstenedione in urine, both unconjugated and conjugated. Using this method the excretion of androstenedione in normal subjects and subjects with pathological disorders has been determined and whether urinary androstenedione was a good index of androstenedione production. The second aim of the work, has been to explore the physiology of these conjugates and to determine their source and possible significance. This, initially, involved an investigation of the type and relative amount of the conjugates of androstenedione present in urine. To be able to assess the recovery of these conjugates of androstenedione from biological fluids during hydrolysis and to determine their stability under conditions of storage, radiolabelled conjugates are required. Attempts have, therefore, been made to synthesise radiolabelled androstenedione 3-enol sulphate. Preliminary experiments indicated that androstenedione was present in plasma, in a conjugated as well as an unconjugated form. A method has, therefore, been developed for the estimation of the amounts present in blood, and using this assay to estimate the levels of conjugated androstenedione in the peripheral and adrenal vein blood from normal subjects and subjects with pathological disorders, the source and possible role of this conjugated androstenedione in blood has been investigated.

1.2 Materials

The following materials are those which have been used throughout the work in this thesis, unless otherwise stated.

(i) Chemicals

All chemicals, except where stated, were supplied by Fisons Scientific Apparatus Ltd., Loughborough, England.

Androstenedione and other crystalline steroids were supplied by Steraloids Ltd., Croydon, England.

[1,2,6,7-³H] androstenedione (104Ci/mmol) and [7(n)-³H] andro-

stenedione (10 Ci/mmol), as well as other radiolabelled steroids were obtained from the Radiochemical Centre Ltd., Amersham, England. The purity of the radiolabelled steroids was checked at regular intervals (every 2 to 3 months) by paper chromatography (Bush A) and compared with unlabelled androstenedione. If the tritiated steroid was impure, i.e. contained approximately less than 95% androstenedione, about 1 month's supply of label was purified prior to use.

Human gamma globulin, HG11 and Sulphatase, aryl sulphatase from Helix Pomatia, containing β -glucuronidase, Type H1 were purchased from Sigma London Chemical Company Ltd., Poole, Dorset, England.

β -glucuronidase from Esch. Coli. was obtained from API Laboratory Products Ltd., Philpot House, Rayleigh, Essex, England.

Bovine Plasma Albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, England.

Amberlite XAD-2 resin was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, England.

Sephadex LH20 was obtained from Pharmacia Ltd., Prince Regent Road, Hounslow, Middlesex, England.

(ii) Reagents.

0.05M Borate buffer pH 8.0 was prepared from boric acid (6.2 g) made up to 21, with distilled water containing 2% methanol and the pH adjusted using aqueous sodium hydroxide.

Phosphate buffer pH 6.4 was prepared from di-sodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.37 g) and sodium di-hydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2.87 g) made up to 250 ml with distilled water.

0.2M acetate buffer pH 4.7 was prepared from sodium acetate ($\text{NaCO}_2\text{CH}_3 \cdot \text{H}_2\text{O}$, 4.01 g) and glacial acetic acid (1.17 ml) and made up to 250 ml with distilled water.

Concentrated carbonate solution was prepared from sodium hydroxide (anhydrous, 270 mg) and sodium bicarbonate (anhydrous, 720 mg) in distilled water (103.5 ml).

Scintillation solution was prepared by dissolving p-terphenyl (6 g) and di-methyl POPOP (0.08 g) in 21 of sulphur free toluene containing 2% methanol.

Methanol (S.L.R.) was redistilled prior to use.

Ethyl acetate (A.R) was redistilled immediately prior to use, if not a blank value was often detectable if redistilled water was put through the hydrolysis procedure.

The silica gel used for the columns, for the eluates from paper chromatography, was Merck silica gel No. 7734 mesh 70-325 which was purified before use by washing in 6M hydrochloric acid, until the washings were colourless, washing with distilled water to constant pH, washing with hot methanol twice and finally washing with freshly redistilled dichloromethane. The gel was dried in a vacuum oven at 40°.

(iii) Apparatus.

Extraction tubes were manufactured by Quickfit and Quartz Ltd., Stone, Staffordshire, Type MF 24/ O/4 with stoppers type SB10.

Glass tubes, 58 mm x 16 mm, with plastic closures, in which urine samples were hydrolysed, were purchased from Fisons Scientific Apparatus Ltd.

Glass assay tubes, 50 mm x 10 mm with plastic closures, controlled neck shell vial No.3/G/D/O8/O15/O2/A were supplied by F.B.G. Trident Ltd., Temple Cloud, Bristol, England. Assay tubes were rinsed with methanol prior to use.

Racks for holding assay tubes during the decanting stage are

Analmatic racks type A902/3 fitted with adaptors type A902/3A supplied by Denley, Daux Road, Billingshurst, Sussex, England.

Paper for chromatography was supplied by Whatman Lab Sales Ltd., Springfield Mill, Maidstone, Kent, England, and was washed prior to use by running in a chromatography tank with methanol for at least 24h.

Silica gel thin layer chromatography plates Merck (Art No. 5553) were supplied by B.D.H. Chemicals Ltd., Poole, Dorset, England.

Radiochromatographic imaging system (β -graph) and strip scanner were manufactured by Panax Equipment Ltd., Willow Lane, Mitcham, Surrey, England.

Constriction pipettes were manufactured by H.E. Pedersen, 7 Sommerstedgade, DK1718, Copenhagen V, Denmark, and supplied by F.C. Copely Ltd., Colwick Industrial Estate, Nottingham, England.

Compu-Pet dispenser, dual micro model was obtained from William R. Warner Ltd., Eastleigh, SO5 3ZQ, England.

Liquid scintillation counting vial inserts MKII were supplied by Hughes & Hughes Ltd., Elms Industrial Estate, Church Road, Harold Wood, Romford, RM3 OHR, England.

Liquid scintillation counters. Beckmann liquid scintillation system No. LS200B was used for counting tritium and carbon 14 and ICN tracer Lab coru/matic 200 was used for counting tritium.

The Hamilton digital diluter/dispenser was manufactured by Hamilton Bonaduz AG, CH-7402 Bonaduz, Switzerland.

1.3 Methods

(i) Paper chromatography.

Paper chromatographic systems have been used for the purification of extracts from plasma and urine after hydrolysis. The paper which has been used throughout the work in this thesis was Whatman No. 2

chromatography paper, which was cut into 1.5 cm strips. Residues of extracts, for paper chromatography, were redissolved in methanol: dichloromethane (1:1, 3 drops) and applied to the paper and run in the system for the required time, after equilibration for a minimum of 1.5h. The areas of activity on the strip were located using the β -graph scanner and the androstenedione eluted from this 3-4 cm area, by folding the strip into four longitudinally, placing it in a column which contained silica gel and washing through with methanol (6 ml). These columns which were 6 mm in diameter, contained 30 mm of silica gel and were washed between assays with 95% aqueous methanol (2x5 ml), hot methanol (approximately 55^o, 2x5 ml) and methanol (2x5 ml).

The following paper chromatographic systems have been used; Bush A (Neher, 1964), heptane:methanol(R.D.):water(R.D.), 5:4:1, Bush B3 (Neher, 1964) petroleum ether(A.R. 100-120):toluene(A.R.):methanol (R.D.):water(R.D.), 33:17:40:10, Bush B 23-9 (Mckenna and Rippon, 1964) di-iso propyl ether:t-butanol:hexane(R.D.):benzene:0.88 ammonia solution:water(R.D.), 250:160:37:62:50:450 and a modified Bush B23-9 system, di-iso propyl ether:t-butanol:hexane(R.D.):benzene:0.88 ammonia solution:water(R.D.), 250:250:37:62:50:450.

(ii) Radioimmunoassay of androstenedione in plasma and serum samples.

Androstenedione has been measured using a radioimmunoassay technique in samples of plasma, serum and urine. A simple non-chromatographic radioimmunoassay for plasma androstenedione, which has been validated by comparison with a method which used chromatography, has been used for the measurement of the plasma samples in this thesis (Goodall, Rippon and James, 1979). This assay involved the extraction of an aliquot of plasma with hexane:ether (4:1, 4 ml), evaporation

of an aliquot of this extract, incubation of the residue with the antibody in buffer solution and separation of the bound and the free fractions with a solution of polyethylene glycol in borate buffer. The antibody was obtained from Dr. W. Schopman and was raised in rabbits immunized with androstenedione 11- hemisuccinate conjugated to bovine serum albumin. The characteristics of the assay are given in Table 1.1.

(iii) Crossreactions of the antibody.

The crossreactions of the antibody with cortisol, androsterone and aetiocholanolone have been measured. The crossreaction with cortisol was assessed as cortisol and androstenedione are secreted by the adrenal gland in response to the same trophic stimulus, and hence it was important to establish that the pattern obtained during sequential sampling was as a result of androstenedione in the plasma and not cortisol. The crossreaction with cortisol as assessed by the method of Abrahams (1969) was 0.0018%. The crossreactions of androsterone and aetiocholanolone were assessed as these steroids are present, in a conjugated form, in greater amounts than androstenedione in urine and as complete separation is not obtained between androstenedione and androsterone in a Bush B3 system and between androstenedione and aetiocholanolone in a Bush A system. The crossreaction of the androstenedione antibody with androsterone was 0.34% and with aetiocholanolone was 0.14%.

(iv) Radioimmunoassay of extracts after paper chromatography.

Residues of plasma and urine samples have been assayed, after hydrolysis and paper and silica gel column chromatography by the following method:

³H Androstenedione, which had been added to the samples prior to hydrolysis so that correction can be made for methodological losses,

Table 1.1 Characteristics of the Radioimmunoassay
for plasma androstenedione

Within assay variation	C.V. of 8.8% (N=24)
Between assay variation	C.V. of 10.8% at 4.2 nmol/l (N=23)
	C.V. of 9.8% at 1.9 nmol/l (N=12)
Mean recovery of tritiated androstenedione from plasma	96.6% (C.V. of 4.1%. N=28)
Non specific blank	8.7 (range 6.4-12.5) as % of total counts added (N=15)
Value when distilled was put through assay procedure	3 pg (N=9)
Sensitivity	5 pg (N=10)

was contained in the column eluates. Hence, the amount of radio-labelled androstenedione contained in the aliquot of eluate to be assayed has to be altered so that the amount is the same as that added to the standard quantities of androstenedione. To estimate the methodological losses of androstenedione during the chromatographic stage, 1 ml of the column eluate was transferred to a plastic counting vial and the solvent evaporated, under a stream of nitrogen, at 40°. Scintillator (2 ml) was added to the vials, and the counts estimated in a liquid scintillation counter, together with 2 vials which contained aliquots of the same recovery standard as had been added to the plasma or urine at the beginning of the experiment.

Further aliquots of the column eluates, usually 2x0.5 ml, were pipetted into assay tubes. Standard quantities of androstenedione 10,20,40,80,160,320 pg in methanol (0.1 ml) were pipetted into assay tubes from individual working solutions. ^3H Androstenedione (5,000 c.p.m.) in methanol (0.1 ml) was added to the tubes containing standards and to 2 tubes for the estimation of non specific binding. Two further aliquots were placed in plastic counting vials and counted, at the same time, as the eluates from the columns. From the amount of radio-activity in the residue from the column eluates and the activity added to the standard quantities of androstenedione, it is possible to calculate the amount of ^3H androstenedione in a solution of methanol which has to be added to the aliquots of the eluates to be assayed, so that the tubes containing the samples contain the same amount of activity as is contained in the tubes with standard quantities of androstenedione. After the counts had been adjusted 10% ($\frac{V}{v}$) propylene glycol in methanol (0.1 ml) was added to all the tubes including the 2 tubes for the assessment of non specific binding. The contents of the tubes were evaporated, in a vacuum oven at 40°, until

only the propylene glycol residue remained.

The assay was then continued as for the assay of androstenedione in plasma samples (Appendix), except labelled androstenedione was not added in the buffer solution of antibody. The amount of androstenedione in the samples was then calculated, correcting for the methodological losses from the chromatography stage.

(v) Preparation of extracts of urine.

The method first used to prepare extracts of urine, containing both conjugated and unconjugated steroids, was that of Edwards, Kellie and Wade (1953), in which ammonium sulphate (half the volume of urine in weight) was dissolved in the aliquot of urine which was extracted with ether:ethanol (3:1, 3 times). This method proved satisfactory with small volumes of urine, e.g. 10 ml, but when larger volumes were used a residue of ammonium sulphate remained, after evaporation of the solvent and this interfered with the chromatography of the extract. This may have been because the extract was not completely dry when the residue was redissolved for chromatography.

A more recent method for the preparation of total extracts from urine is one using Amberlite XAD-2 resin columns (Bradlow, 1968). Small columns of Amberlite XAD-2 resin were prepared by slurring the resin in water. The resin was washed by a gentle upward flow of distilled water, sufficient to keep the resin suspended, for about 2h to remove any fine resin. For 50 ml of urine, columns (1 cm in diameter) were prepared with 5 g of resin and for the smaller quantities of urine micro columns (diameter 6 mm) containing 3 cm of resin were used. The urine to be extracted was allowed to percolate through the column slowly and the column was then washed with twice the volume of water as urine. The column was allowed to drain completely dry and an extract was collected by washing the column with 2.5 times the volume

of methanol as urine. The methanolic extract was evaporated under reduced pressure. The columns were washed with water and methanol and prior to being used again.

Recently, Bradlow (1977) has reported that some workers have been finding problems with the recovery of conjugates from the columns with more recently manufactured batches of Amberlite XAD-2 resin. This problem has not been encountered in this work, and when urines have been used which were from patients who had been infused with ^{14}C androstenedione and ^3H oestrone, it was found that in 3 experiments with different urines 84, 93 and 93% of the radioactivity was recovered.

SECTION 2

Conjugated androstenedione in urine

2.1 Introduction

The possible existence of androstenedione in urine was first suggested by Schubert (1958), who identified androstenedione, after hydrolysis of material isolated from the urine of individuals who had been injected with testosterone. Schubert postulated that the androstenedione was linked to the glucuronic acid through an enol linkage. Soon after this publication, Wotiz et al. (1959) prepared synthetically the 3-enol glucuronide of androstenedione and using this synthetic compound showed that it was hydrolysed rapidly at acidic pH and by incubation with β -glucuronidase (Wakabayashi et al., 1961). The properties of androstenedione 3-enol glucuronide have been studied in greater detail by Matsui et al. (1974) and using an adaption of the method of Wotiz et al. (1959) androstenedione 3-enol glucuronide and ^{14}C androstenedione 3-enol glucuronide were prepared and their stability studied under various conditions. Incubation of ^{14}C androstenedione 3-enol glucuronide in human urine at 37° resulted in only 49% hydrolysis and the authors concluded that when this compound was hydrolysed in urine either enzymatically or non-enzymatically, appreciable amounts of polar artefacts were formed. These workers found that androstenedione 3-enol glucuronide was solvolysed almost quantitatively to androstenedione with acidic ethyl acetate, with 87% of the radioactivity being recovered as androstenedione. On incubation of ^{14}C androstenedione 3-enol glucuronide with various preparations of β -glucuronidase, they found that only 64-71% of the conjugate was hydrolysed. The authors concluded that ^{14}C androstenedione 3-enol glucuronide could undergo non enzymic transformations which might be erroneously interpreted as

enzymic transformations and in order to establish the biochemistry of the 3-enol glucuronide, cautious investigations are required, with regard to its chemical stability. Stahl and Dörner (1972) when investigating the amounts of conjugated androstenedione in urine from pregnant and non pregnant women found that the amount of conjugated androstenedione cleaved, was dependent upon the source of the β -glucuronidase preparation used. They concluded that this was due to the different amounts of sulphatase present in the enzyme preparations, although this could be a reflection of the breakdown of the conjugate to polar artefacts, by the differing preparations.

In the investigation of the conjugates of androstenedione in urine and attempts to develop a method to measure these conjugates, mild conditions have been used and mixtures incubated for a minimum time, as it is likely that these problems could occur with the enol sulphate conjugate as well as with the 3-enol glucuronide conjugate. The only reference to enol sulphate conjugate of androstenedione is by Oertel et al. (1967) who have synthesised androstenedione enol sulphate, though the stability of this compound under different conditions was not assessed.

Various groups of workers have measured androstenedione in urine, usually by extracting the free, incubation with β -glucuronidase followed by hydrolysis, combining the extracts from the three stages and estimating the total amount of androstenedione. In the first reports of androstenedione in urine (Lieberman, Dobriner, Hill, Fieser and Rhoads, 1948; Millar, Rosenkrantz and Dorfman, 1953) the androstenedione was obtained from urine as a crystalline solid and physically characterised. Various physical chemical methods have been used for estimating the amount of androstenedione in urine, e.g. fluorimetry (Stahl and Dörner, 1971), ultra violet absorption (Schubert et al., 1964), gas chromato-

graphy (Dalzell and Elattar, 1974), competitive protein binding assay after reduction to testosterone (Waalder, Thorsen, Støa and Aarskog, 1974), and a colorimetric method using androstenedione 2, 4-dinitro-phenylhydrazine (Korneev, 1973).

Several groups of workers have established normal ranges for urinary androstenedione (Schubert et al., 1964; Tajic, 1965; Longhino et al., 1968; Schollberg et al., 1968; Stahl and Dörner, 1971). Stahl and Dörner (1971) measured values after only β -glucuronidase hydrolysis. Schollberg et al. (1968) had reported in the previous paper (Hubl and Schollberg, 1968) higher values for normal male and female subjects but they modified their method for the second paper.

Androstenedione has been determined in urine from subjects with various endocrine diseases. Tajic (1965) measured androstenedione in the urine of patients with hirsutism, Cushing's disease and Addison's disease, and Hubl, Siebert, Büchner and Hering (1971) and Demchenko and Serdechnaya (1977) measured androstenedione excretion in men suffering from hypogonadism. Androstenedione in urine has been measured by various groups of workers at differing times during the female menstrual cycle (Longhino et al., 1968; Schollberg, Seiler and Wagner, 1969; Longhino, Drobnjak, Krk and Vedris, 1972; Yanbelevich and Nikolaeva, 1977). Androstenedione excretion during pregnancy has been measured by Stahl and Dörner (1972), Dörner, Stahl, Götz, Rossner and Halle (1971) and Longhino and Cebahar (1976). Two groups of workers have measured androstenedione in the urine of children. Dalzell et al. (1974) measured the androstenedione only after β -glucuronidase hydrolysis but not acid solvolysis, by gas chromatography. Waaler et al. (1974) also measured urinary androstenedione in children after β -glucuronidase hydrolysis, and found a good correlation with bone age.

In these previous studies, no evidence has been given as to whether total urinary androstenedione excretion was a good indication of the circulating levels and the production of androstenedione. Attempts have therefore been made to develop a reliable method for the measurement of androstenedione in urine, measuring the amounts of androstenedione present after hydrolysis, by radioimmunoassay. Preliminary experiments were carried out by extracting urine aliquots with hexane:ether to remove any free androstenedione, incubating the urine with β -glucuronidase, further extraction with hexane:ether and hydrolysis according to the method of Burstein and Lieberman (1958). The results of these experiments indicated, as other workers had found, that the amount of androstenedione that was released by acid solvolysis was greater than that released by β -glucuronidase and that only a small portion of androstenedione present in urine is unconjugated.

To investigate the type of conjugates in more detail, the conditions of hydrolysis for enzymic and non enzymic cleavage have been optimised. The types of conjugate present have also been investigated by t.l.c. and Sephadex LH20 column chromatography.

A method has been developed for the measurement of the total amount of androstenedione in urine and this method used to estimate the levels in groups of normal and pathological subjects. For a group of post-menopausal subjects urinary androstenedione excretion has been correlated with plasma values of androstenedione.

2.2 Incubation of urine with β -glucuronidase (E. Coli)

The β -glucuronidase preparation used in these experiments was from E. Coli. The optimum conditions for the cleavage of conjugated androstenedione in urine have been determined in a series of experiments by estimating the amounts of androstenedione, liberated under varying

conditions, by radioimmunoassay after chromatography. This is not the best method for estimating these values as it would be more meaningful if the recovery of androstenedione 3-enol glucuronide had been assessed using radiolabelled conjugate but this was not available.

(i) Optimization of temperature and time

Aliquots of urine (0.5 ml) from the same urine pool were extracted with hexane:ether (4:1, 5 ml). Phosphate buffer (1 ml), ^3H androstenedione (15,000 c.p.m.) in methanol (0.1 ml) and β -glucuronidase (0.01 ml) were added and the mixtures incubated at the following temperatures: 37° , 50° and room temperature ($20.5\text{--}23.8^\circ$) for varying times between 0.5 and 24h. After incubation, the mixture was again extracted with hexane:ether (4:1, 5 ml). The extracts were purified by paper (Bush B3) chromatography and the amount of androstenedione estimated by radioimmunoassay.

These results (Fig. 2.1) indicate that the amount of androstenedione released from urine by β -glucuronidase does not decrease with prolonged incubation, although it is possible that androstenedione 3-enol glucuronide could break down to polar artefacts without androstenedione being formed as an intermediate. The optimum time for incubation of urine specimens with β -glucuronidase was 6h at 37° . The amount of androstenedione 3-enol glucuronide being converted to androstenedione at room temperature by β -glucuronidase, even at 24h, has not reached the amount released at 37° or 50° , although from the slope of the curve it would appear that these values have not yet reached a plateau.

(ii) Optimization of the amount of β -glucuronidase added

To aliquots of urine (0.5 ml), pre-extracted with hexane:ether (4:1, 5 ml) was added phosphate buffer (1 ml) and ^3H androstenedione (15,000 c.p.m.) in methanol (0.1 ml). Aliquots of β -glucuronidase were added (0.01, 0.02, 0.04 ml; 0.01 ml of β -glucuronidase is equivalent to 2 I.U. or 400 Fishman units). β -glucuronidase was not added to two aliquots. After mixing, the urine-buffer mixture was incubated at 37° for 6h. The mixture was again extracted with hexane:ether (4:1, 5 ml)

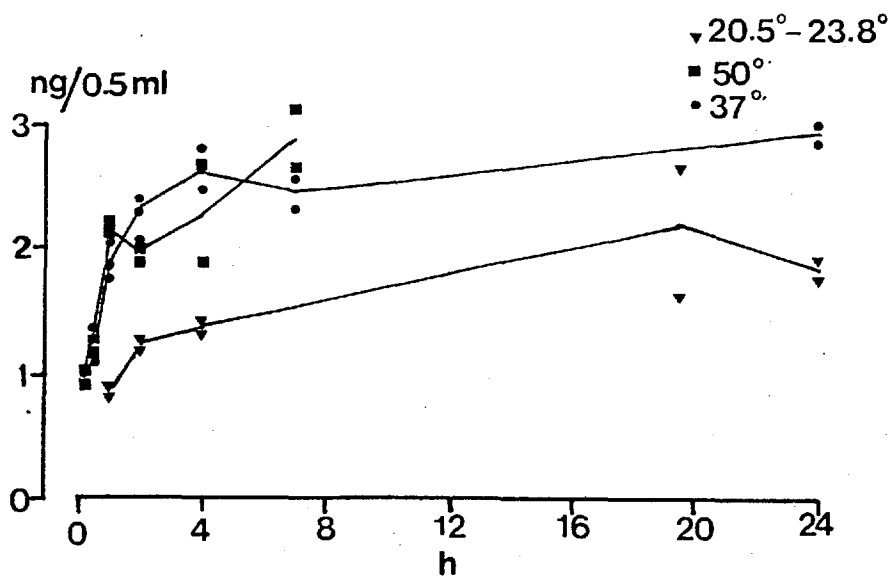


Fig.2.1 Incubation of urine at various temperatures with β -glucuronidase

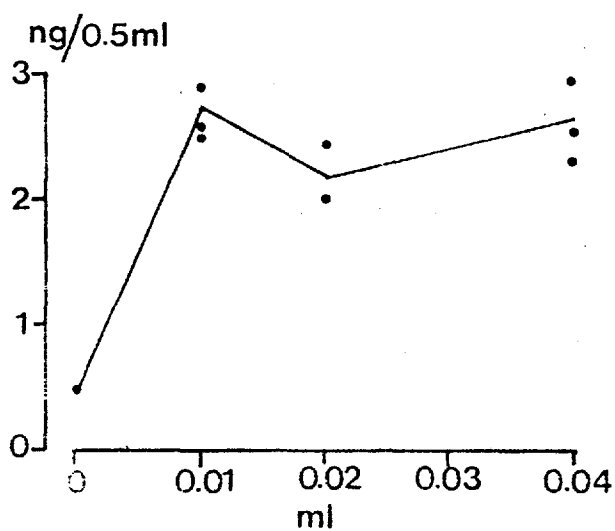


Fig.2.2 Incubation of urine with various amounts of β -glucuronidase

and the extracts assayed by radioimmunoassay, after Bush B3 paper chromatography.

The results shown in Fig. 2.2 indicate that 0.01 ml of β -glucuronidase was sufficient to release all the androstenedione conjugated to glucuronic acid present in the urine. Non enzymatic release of androstenedione also occurs under these conditions, 0.48 ng/0.5 ml for this urine sample compared to 2.7 ng/0.5 ml of urine when 0.01 ml of β -glucuronidase is added to the incubation mixture.

(iii) Effect of sodium sulphate on the hydrolysis of androstenedione conjugates

Furuya (1977) reported that increased amounts of 17-hydroxycorticosteroid glucuronides were hydrolysed when sodium sulphate was added to the medium for incubation with β -glucuronidase preparations from bovine liver. In order to determine whether the same effect occurred for the cleavage of conjugated androstenedione from urine with β -glucuronidase (*E. Coli*) a comparison has been made with aliquots of urine incubated with a buffer solution containing sodium sulphate (80 g/l) compared with a buffer solution that did not contain sodium sulphate. The amounts of androstenedione released from the urine samples to which sodium sulphate had been added were lower than the amounts released from the samples to which no sodium sulphate had been added. The inhibitory effect of adding sodium sulphate to the incubation medium, may be due to the different type of bonding in androstenedione 3-enol glucuronide, as it is a planar structure compared with the equatorial or axial bonding of glucuronic acid to other steroid molecules.

2.3 Incubation with sulphatase (Helix Pomatia)

In an attempt to investigate whether androstenedione was present in urine, in a form conjugated to sulphuric acid, aliquots of urine have been incubated with sulphatase (*Helix Pomatia*). This preparation also contains β -glucuronidase, hence the aliquots of urine were incubated with β -glucuronidase (*E. Coli*), prior to incubation with sulphatase.

Aliquots of urine (0.5 ml) to which ^3H androstenedione (30,000 c.p.m.) in methanol (0.1 ml) had been added were extracted with hexane: ether (4:1, 5 ml). Phosphate buffer (1 ml) and β -glucuronidase (0.01 ml) were added and the mixture was incubated at 37° for 6h. After extraction with hexane:ether (4:1, 5 ml), the pH of the solution was adjusted to 5, using M acetic acid. A further aliquot of tracer and sulphatase (18.6 I.U.) in acetate buffer (0.1 ml) were added and the mixture incubated at 37° for 15h. The mixture was again extracted with hexane:ether (4:1, 5 ml). The amount of androstenedione in each of the extracts was estimated by radioimmunoassay, after Bush B3 paper chromatography.

The results (Table 2.1) indicate that incubation with sulphatase causes release of androstenedione. The values obtained are lower, though, than those obtained when aliquots of the same samples were incubated with acid to hydrolyse the androstenedione conjugated to sulphuric acid. This could be for two reasons, either hydrolysis with sulphatase was causing more of the conjugated androstenedione to be converted to polar artefacts or as the conditions for the hydrolysis of androstenedione from urine had not been optimized using sulphatase, the maximum release under the conditions being used may not have been obtained. The experiment was repeated with a further stage added. After the urine-buffer mixture had been incubated with sulphatase a further aliquot of recovery tracer was added, the pH of the mixture was adjusted to 1, incubated at 37° for 15h and extracted with hexane: ether (4:1, 5 ml).

Table 2.1 Incubation of urine with β -glucuronidase and sulphatase after extraction of any unconjugated androstenedione. Androstenedione (nmol/24h) in each fraction.

Sample	Free	β -glucuronidase	Sulphatase
1	27.6	36.4	64.7
2	33.9	31.1	28.0

Comparison of total amount of androstenedione (nmol/24h) obtained by differential hydrolysis and acid hydrolysis.

Sample	Differential hydrolysis	Acid hydrolysis
1	129.4	222.4
2	93.0	153.8

The amounts of androstenedione (nmol/24h) obtained in each fraction were: unconjugated 7.0, β -glucuronidase 11.2, sulphatase 9.8, and acid hydrolysis 7.7. These results indicate that as further amounts of androstenedione are released by acid hydrolysis after incubation with sulphatase, the optimum conditions for hydrolysis with sulphatase have not yet been established, and that breakdown to polar artefacts was not occurring.

Sulphatase (*Helix Pomatia*) hydrolyses dehydroepiandrosterone and aetiocholanolone sulphates but not androsterone sulphate. It will therefore, hydrolyse sulphates of a $3\beta, \Delta^5$ and $3\beta, 5\alpha$ configuration but not sulphates with a $3\alpha, 5\alpha$ and $3\alpha, 5\beta$ configurations. Androstenedione enol sulphate has Δ^3, Δ^5 configuration and would be planar at the 3 position. Hence, it is a matter of speculation as to whether this is the best type of sulphatase to use (Bradlow, 1970; Roy, 1970), though oestrone sulphate has a planar structure and this is hydrolysed by this type of sulphatase.

2.4 Chromatography of urine extracts

To investigate further the types of conjugates of androstenedione that are present in urine, total extracts of urine have been prepared and chromatographed either by thin layer or Sephadex LH20 column chromatography systems to separate unconjugated steroids from those conjugated as mono or disulphate and mono glucuronide.

(i) Thin layer chromatography

Thin layer chromatography using aluminium or glass backed silica plates has been used to investigate the type of conjugates of androstenedione in urine samples from normal and postmenopausal subjects, and urine samples from postmenopausal subjects who had been infused with ^{14}C androstenedione and ^3H oestrone. The solvent system used for these experiments was dichloromethane:methanol:water (70:30:2).

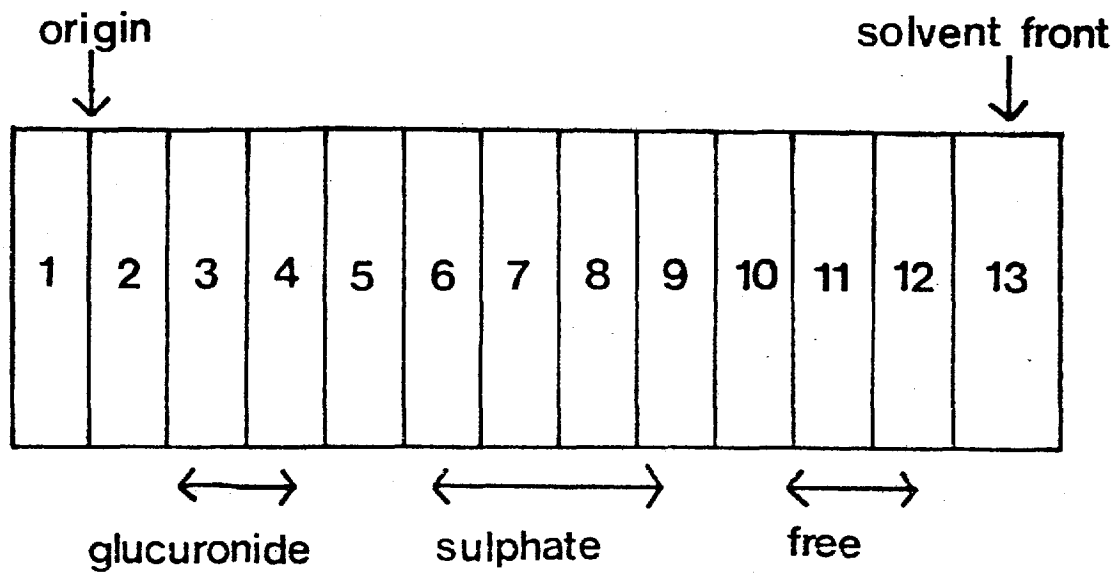


Fig 2.3 Division of t.l.c. plate, showing the separation of the conjugates

A similar solvent system was used by Puah, Kjeld and Joplin (1978) for the separation of conjugates of testosterone from both plasma and urine.

The complete urine extract was redissolved in dichloromethane: methanol (1:1), applied to a silica plate and chromatographed. The plate was divided into 13 areas as shown in Fig. 2.3. These areas were 1.5 cm wide, except for area 13 which was 2 cm wide. The unconjugated androstenedione, androstenedione enol sulphate and androstenedione 3-enol glucuronide ran in this system as indicated. The free and conjugated steroids were run as markers in separate lanes on the same plate, when an extract was chromatographed. For unconjugated androstenedione, both radio labelled and unlabelled steroids were used as markers. The androstenedione 3-enol glucuronide used as a marker was a generous gift from Dr. M. Matsui and the androstenedione enol sulphate was synthesised. The unlabelled steroid markers were detected using an ultra violet lamp.

The silica gel from each of the areas was scraped off the glass backed plates and when aluminium backed plates were used the individual areas of the plates were cut into small pieces. The steroids were extracted with methanol (3 x 1.5 ml), the combined extracts were evaporated under nitrogen and the residue redissolved in phosphate buffer (0.5 ml). The mixture hydrolysed in an ethyl acetate-saline mixture as for the total hydrolysis of urine aliquots but all the ethyl acetate layer was taken for radioimmunoassay. For the residues which contained only unlabelled androstenedione only one paper chromatographic purification stage (Bush B3) was carried out. When total extracts were made from urine samples from subjects who had been infused with ^{14}C androstenedione and ^3H oestrone, an aliquot of the purified residue was counted to determine the specific activity of the androstenedione in each area of the plate, and the extracted residue from each area of the plate was purified in both a Bush A and a Bush B3 paper chromatographic system, to ensure that complete separation from the other metabolites, e.g. androsterone and aetiocholanolone, was obtained prior to counting of the residue. Radioactive tracers of these steroids were chromatographed on separate strips in the same system, for both these paper chromatographic stages to show that a good separation was obtained. Of the 6 ml of extract eluted after chromato-

graphy, 4.5 ml was taken for counting after evaporation of the organic solvent in scintillation vials and 0.5 ml was diluted sufficiently so that the amount of androstenedione could be assessed by radioimmunoassay and the specific activity of the androstenedione in each area of the plate could be estimated.

To assess the stability of the conjugates of androstenedione, as these conjugates are very acid labile, a total urine extract was chromatographed on a thin layer silica plate. The plate was divided into areas and each area extracted with methanol (3x2 ml). Half of the extract from areas 3 and 4 were combined to give the glucuronide fractions and from areas 7, 8 and 9 to give the sulphate fraction. The combined glucuronide and sulphate fractions were evaporated to dryness under nitrogen and rechromatographed, the plates were divided into areas and extracted with methanol. The remaining half of the extracts from areas 3, 4, 7, 8 and 9, the extracts from the other areas of the first plate and the extracts from the two plates on which the sulphate and glucuronide fractions were re-run were evaporated under nitrogen, hydrolysed and the amount of androstenedione estimated by radioimmunoassay after Bush B3 paper chromatography. The amounts of androstenedione obtained in the areas, shown in Table 2.2, indicate that androstenedione conjugated to sulphuric acid is more stable than that conjugated to glucuronic acid. In the first chromatographic separation, the ^3H androstenedione tracer ran in areas 11 and 12, but on rechromatography of the extracts, the plate was run for slightly longer and the ^3H androstenedione tracer was in area 13. For the sulphate fraction the percentage decomposition was 4.1% and for the glucuronide 84%. These results indicate that the recovery of the steroid from the t.l.c. stage is good but this indicates that the decomposition of the androstenedione conjugated to glucuronic was probably occurring during the extraction of the glucuronide conjugate

Table 2.2 Amounts of androstenedione (ng) in areas of a thin layer plate from a total extract of urine. Part of the residues from the areas containing the glucuronide and sulphate conjugates were rechromatographed to assess the stability of these conjugates. (Amounts are corrected for equivalent amount of residue.)

Area of plate	Initial chromatography	Rechromatography	
		Areas 3,4.	Areas 7,8,9.
1	5.3	<0.3	<0.4
2	2.4	<0.4	<0.6
3	15.2	5.2	1.0
4	26.1	1.3	0.7
5	2.6	<0.3	<0.5
6	8.1	<0.3	<0.4
7	15.4	0.5	<0.3
8	42.9	0.6	4.2
9	17.1	<0.4	38.0
10	1.9	0.5	26.6
11	6.3	<0.3	1.0
12	1.5	<0.3	<0.4
13	2.4	17.5	3.0

androstenedione
ng/area

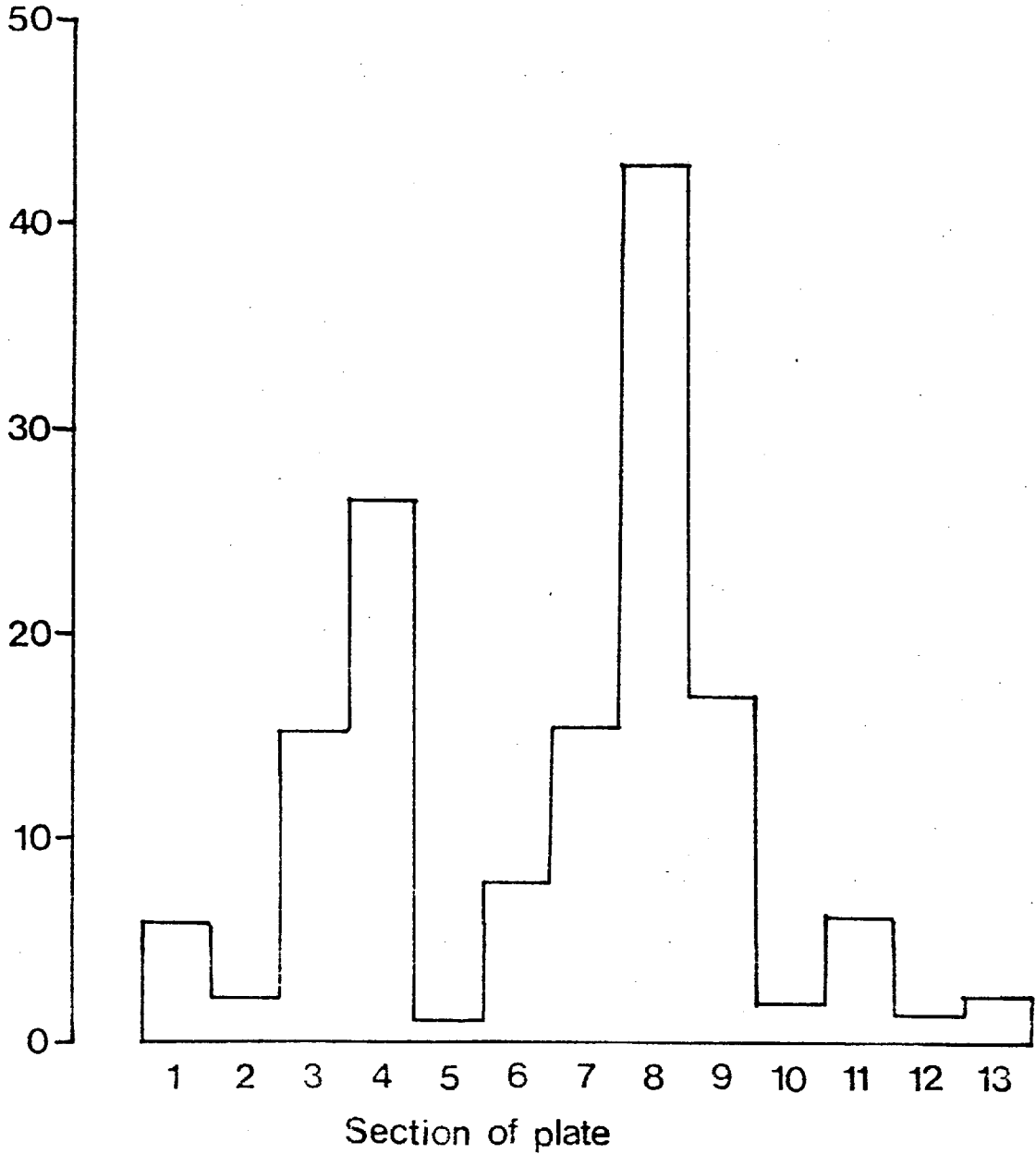


Fig2.4 T.l.c. of a urine extract from a normal subject

from the t.l.c. plate or during the evaporation of the solvent, prior to rechromatography. Some hydrolysis probably occurs on the plate because of the relatively high amount of unconjugated androstenedione present when this method is used compared to the amount that can be extracted from urine samples with hexane:ether. In an attempt to reduce this decomposition, the residue has been left as a dry residue on the plate or after evaporation of solvent for as short a time as possible.

The values for the amount of androstenedione present in the areas of the plate from a normal subject are shown in Fig. 2.4. This is typical of the pattern seen in normal subjects. The ratio of the amount of androstenedione in the unconjugated fraction compared to the amounts in the glucuronide and sulphate fractions, is lower than that seen in some other subjects. The higher values may be a reflection of the conditions of storage of the urine or the preparation of the extract. The relative amounts in the conjugated and unconjugated fractions corresponds well with the amounts of androstenedione that have been found by the extraction of urine aliquots with hexane:ether (4:1) to remove the free androstenedione, incubation with β -glucuronidase and acid hydrolysis. Both these methods indicate that the majority of androstenedione in urine is in a conjugated form. The amount conjugated to sulphuric acid being greater than that conjugated to glucuronic acid and only a small proportion of the androstenedione excreted in urine is in an unconjugated form.

An extract of urine from a postmenopausal subject has been chromatographed in the same way. This subject (aged 50) who had undergone an ovariectomy 4 months previously, was administered dexamethasone (1.5 mg a day) for a week prior to an infusion of unlabelled androstenedione (250 μ g/h for 6h). The urine sample that was chromatographed was from a urine collection made for 12h after the start of the

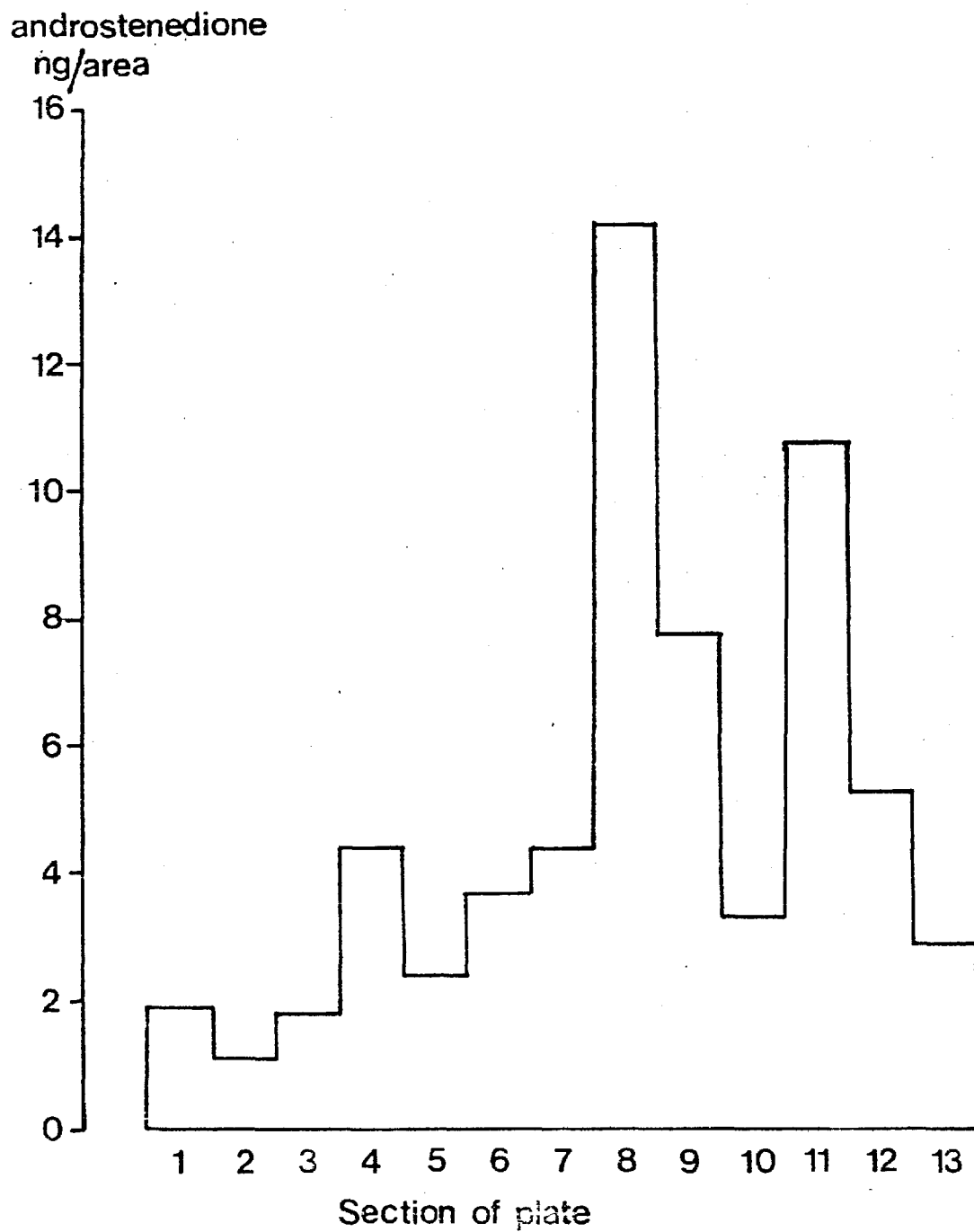


Fig2.5 T.l.c. of a urine extract from a subject infused with androstenedione

infusion and the results are shown in Fig. 2.5. The pattern is the same as that for normal subjects. For the 9h prior to the infusion and for the 24h from the start of the infusion, blood samples were taken at $\frac{1}{2}$ h intervals and the plasma androstenedione values determined. Before the infusion the levels were below the limit of detectability of the assay, during the infusion they rose to 6.2 nmol/l and after the infusion had ceased were again undetectable. These results indicate that the conjugates found in urine are a reflection of the androstenedione in the peripheral circulation.

It has been possible to analyse by thin layer chromatography urine extracts from patients who have been infused with ^{14}C androstenedione and ^3H oestrone as part of a study of the conversion of androstenedione to oestrone in women with endometrial cancer (Reed, Hutton, Baxendale, James, Jacobs and Fisher, 1979; Reed, Hutton, Beard, Jacobs and James, 1979). By analysing these urines it has been possible to determine the specific activities of the conjugates for the different conjugates. This has been carried out on urine samples from five patients, 1 from a normal postmenopausal subject, 2 from subjects with endometrial cancer and from 1 subject with a thecoma on samples obtained before and after operation. The pattern of the specific activities was similar in all 5 samples and is shown for 1 subject in Fig. 2.6. The androstenedione conjugated to sulphuric acid has a much higher specific activity than that conjugated to glucuronic acid or was present in urine in an unconjugated form.

(ii) Column chromatography

Another approach to the investigation of the conjugated androstenedione in urine has been by column chromatography. There are many reports in the literature on the fractionation of conjugates both in

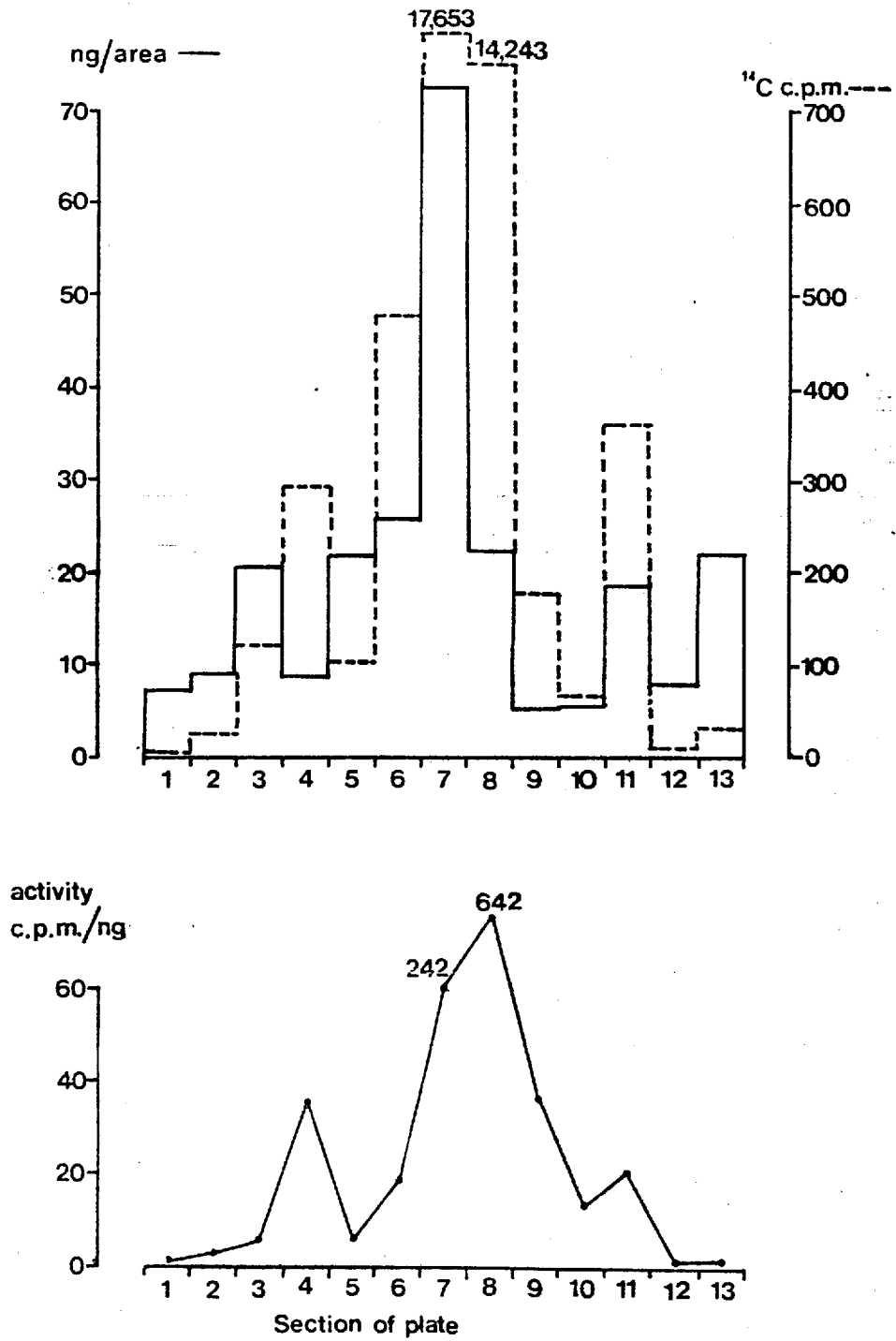


Fig 2.6T.l.c. of a urine extract from a subject who was infused with ^{14}C androstenedione

urine and plasma on columns of various packing materials and resins. Sjovall, Sjovall and Vihko (1968) reported the separation of mono sulphate fractions from the unconjugated and glucuronide fractions using column chromatography with Sephadex LH20 and chloroform:methanol (1:1) system containing 0.01 mol/l of sodium chloride. This method has been used to separate the different types of androstenedione conjugates in a urine extract from a postmenopausal lady (aged 50) with a well differentiated adenocarcinoma of the endometrium, who had been infused with 30 μCi of ^{14}C androstenedione and 10 μCi of ^3H oestrone.

The residue from the urine extract was redissolved in chloroform:methanol (1:1) containing 0.01 mol/l of sodium chloride and chromatographed on a Sephadex LH20 column prepared in the same solvent, collecting 2 ml fractions. 50 μl of each fraction was taken for counting in small glass tubes with scintillator (1.5 ml).

The amount of counts in these aliquots is shown in Fig. 2.7a. Sjovall et al. (1968) suggested that the conjugates were eluted from the column in the order free, glucuronide, sulphate and disulphate. This would indicate that the fractions containing unconjugated steroids are numbers 11-20, those containing glucuronides 21-29, and sulphates 33-41. Fractions 61-150 contained no significant counts above background and hence there was no evidence for the presence of disulphate conjugates of androstenedione. The fractions containing radioactivity were investigated further, as this radioactivity reflected the amounts of all the steroidal metabolites of the androstenedione and oestrone which were conjugated to either glucuronic or sulphuric acid.

Fractions 11-50 were evaporated to dryness and redissolved in methanol (2 ml). 100 μl of each of these fractions was hydrolysed at pH 1 for 16h at 37° after being added to phosphate buffer (0.5 ml) and 30% saline solution (1 ml) in equilibrium with ethyl acetate (1.5 ml). 0.5 ml of this ethyl acetate extract was added to methanol (3.5 ml) and 2 x 0.5 ml of this methanol extract was taken for radioimmunoassay by the method used for the measurement of androstenedione in plasma.

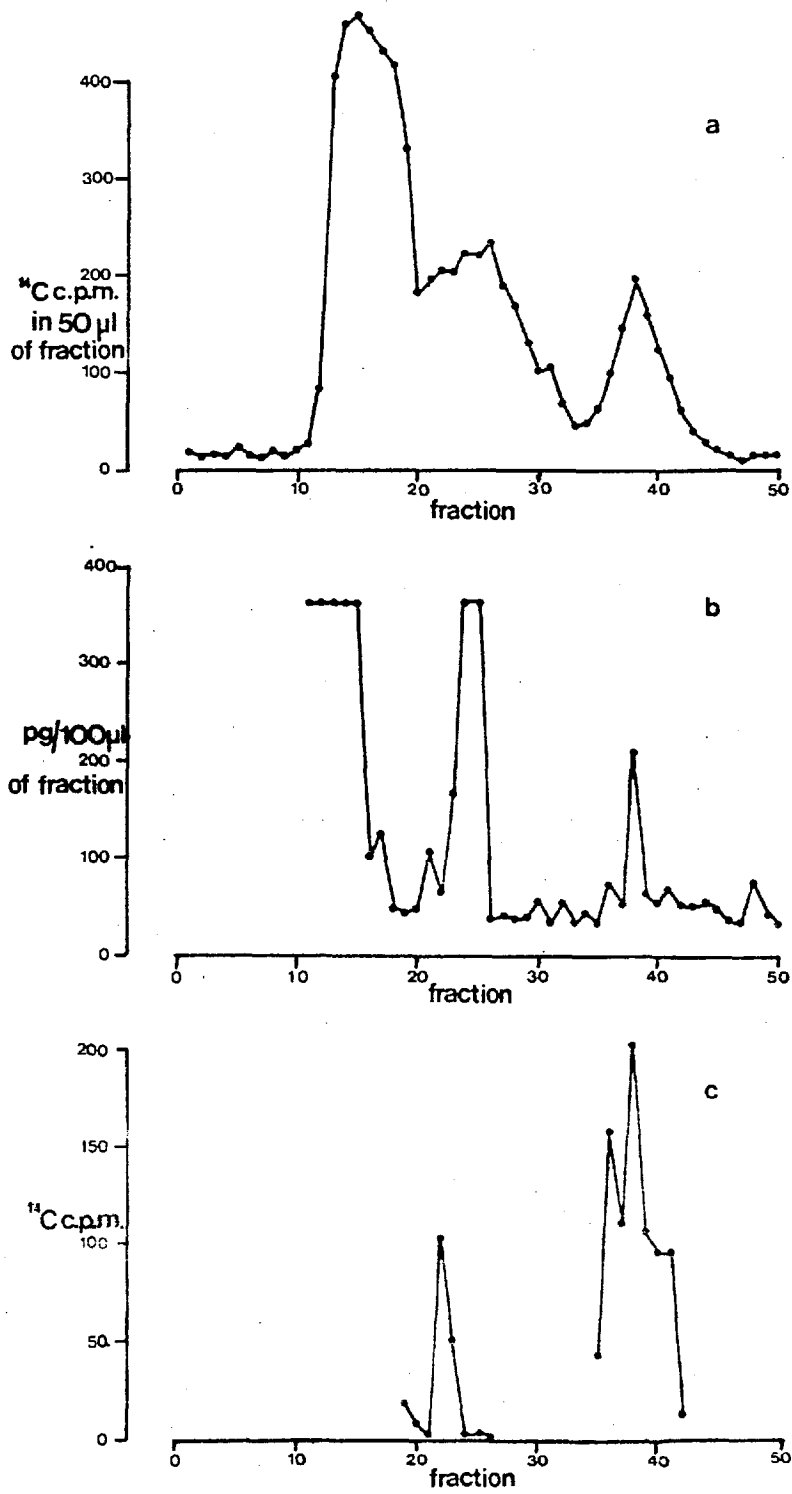


Fig2.7 Column chromatography of urine extract

The amounts of androstenedione in 100 μ l each of these fractions is shown in Fig. 2.7b. In fractions 11-16 and 24 and 25, there was probably an artefact present which interfered with the radioimmunoassay and gave spuriously high values.

To investigate further the pattern of conjugates for fractions 19-26 and 35-42, the remaining aliquot of these fractions from the column was hydrolysed and purified by chromatography in Bush A and Bush B3 paper systems, 4.5 ml of the elute from the paper was taken for counting. The results are shown in Fig. 2.7c. The counts in each fraction were very low, the maximum for the glucuronide being 17.3 c.p.m. above background and that for the sulphate fraction being 50.2, before correction for recovery. Although these results cannot be regarded as being accurate, they indicate that conjugated androstenedione in urine is derived from androstenedione which is in the peripheral circulation. These results also indicate, as did the results of t.l.c. of urine extracts, that there are two types of conjugates of androstenedione present in urine, and that a greater proportion of the conjugated androstenedione in urine is linked to sulphuric acid than glucuronic acid.

2.5 Stability of the conjugates

The stability of androstenedione conjugates under various conditions of storage has been assessed, by measuring the amount of free androstenedione that was extractable into hexane:ether (4:1). Andino et al. (1976) measured the amount of unconjugated androstenedione in urine, in an attempt to assess whether this was a good reflection of plasma androstenedione values. The problem with measuring unconjugated androstenedione in urine, is that decomposition of the conjugated androstenedione present could occur during the time of the collection or storage of the urine sample. Kjeld et al. (1977) measured levels of

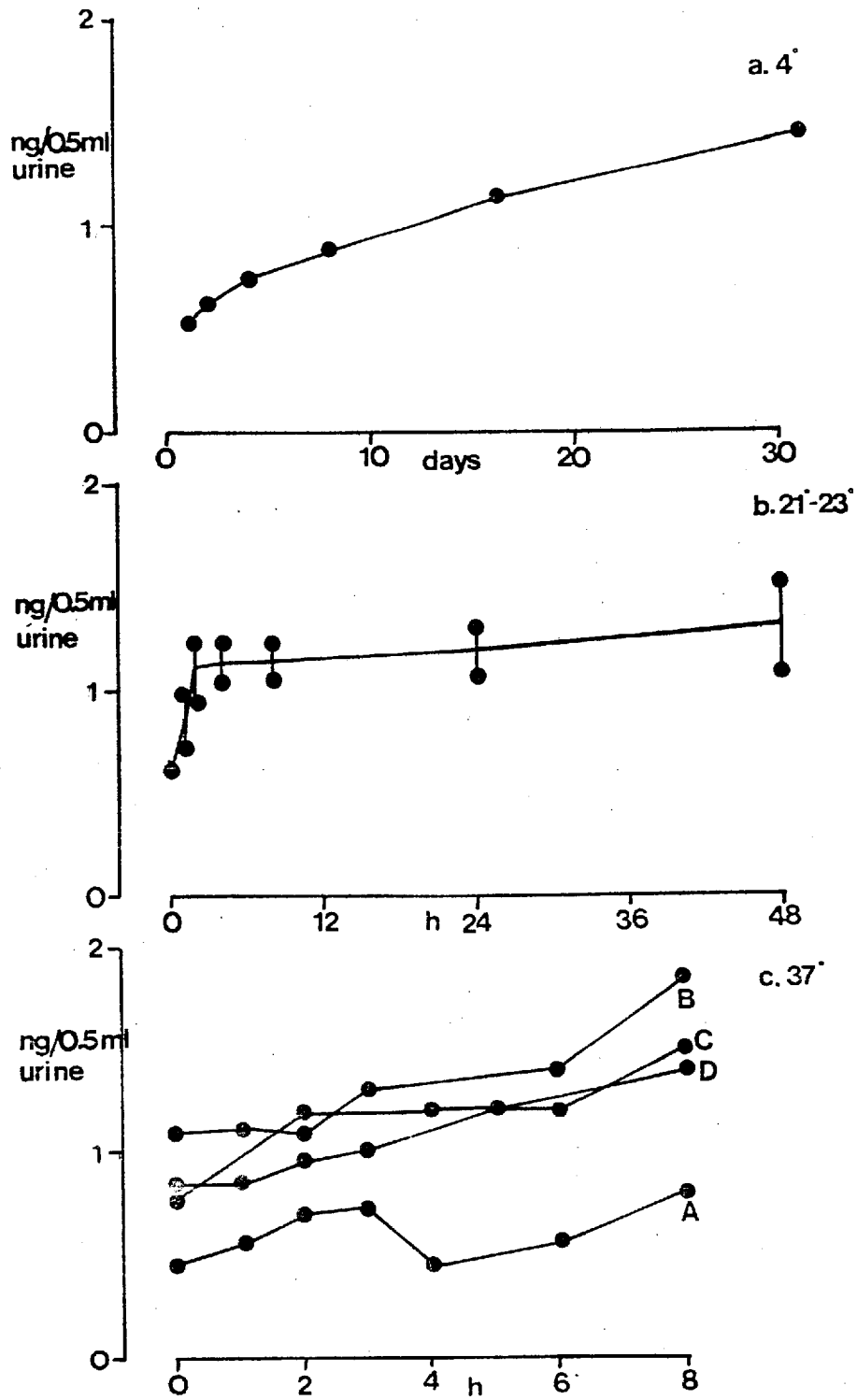


Fig2.8 Effect of conditions of storage

unconjugated testosterone, 5 α -dihydrotestosterone, oestradiol and progesterone in human urine before and after storage at various temperatures and found that at -20°C and 4°C for up to 5 days no change occurred in the levels, but for testosterone there was a 100% increase in 2-3 days at 18°C and 5 α -dihydrotestosterone around 200% increase. Unconjugated androstenedione has been assessed in urine pools stored at 4°C, room temperature and 37°C.

(i) Storage at 4°C

Aliquots of a urine pool (0.5 ml) were kept at 4°C for 1-31 days. ³H androstenedione tracer (30,000 c.p.m.) in methanol (0.1 ml) was added and allowed to equilibrate with the urine for at least $\frac{1}{2}$ h before extraction of the unconjugated androstenedione with hexane:ether (4:1, 5 ml). The amount of androstenedione in the extract was estimated by radioimmunoassay after paper (Bush B3) chromatography.

These results are shown in Fig. 2.8a. The amount of unconjugated androstenedione in the urine increased by 260% from day 1 to day 31.

(ii) Storage at room temperature (21-22°C)

Urine aliquots (0.5 ml) into which ³H androstenedione tracer (30,000 c.p.m.) in methanol (0.1 ml) had been added were left at room temperature for 1-48h. The unconjugated androstenedione was extracted with hexane:ether (4:1, 4 ml, 3 ml) and the androstenedione in the extract estimated, after paper (Bush B3) chromatography by radioimmunoassay.

Fig. 2.8b shows the values obtained, which indicate that the unconjugated androstenedione in the urine increased by 110% from 0-48h.

(iii) Incubation at 37°C

Due to the lability of the urinary conjugates of androstenedione, it could be supposed that the unconjugated androstenedione present in a urine specimen is a result of the breakdown of conjugates, rather than excretion of unconjugated androstenedione. Decomposition of conjugated androstenedione could also occur in the bladder, and this could make the

interpretation of the amount of unconjugated androstenedione in a urine specimen less meaningful as it would be necessary to determine how long a particular specimen had been in the bladder. In an attempt to determine whether decomposition of androstenedione conjugates does occur, under simulated bladder conditions, urine specimens were collected from four normal volunteers.

Aliquots of these urines (2 x 0.5 ml), after ^3H androstenedione (30,000 c.p.m.) in methanol (0.1 ml) had been added, were incubated at 37° for 0-8h. The mixture was extracted with hexane:ether (4:1, 5 ml) and the amount of androstenedione estimated in the extract by radio-immunoassay after paper (Bush B3) chromatography.

The results are shown in Fig. 2.8c. Increases of 74, 66, 96 and 69% of the original value were found at 8h, indicating that there is an increase in value for urinary unconjugated androstenedione under conditions which are similar to those in the bladder. A considerable part of the unconjugated androstenedione in a urine specimen could, therefore, be due to the hydrolysis of conjugated androstenedione. The results of these experiments indicate that if an estimation of unconjugated androstenedione in a urine sample is to be interpreted objectively, care must be taken to see that the storage and collection of the urine specimens is carefully controlled otherwise spuriously high values may result.

2.6 Development of a method for estimating the total amount of androstenedione

As the measurement of unconjugated androstenedione could lead to spuriously high results, a more reliable estimation of the amount of androstenedione in urine is required. As androstenedione is conjugated to the glucuronic and sulphuric acid by an enol linkage, these conjugates would be hydrolysed by acid and the use of β -glucuronidase is not necessary (Matsui et al., 1974). The method which has been developed was an adaptation of the method for hydrolysis of steroid sulphates

(Burystein and Lieberman, 1958), and involves incubating the urine at 37° overnight in a buffer-saline mixture at pH 1 in equilibrium with ethyl acetate. The method has been used to estimate urinary androstenedione excretion in subjects with various endocrinopathies.

(i) Hydrolysis of conjugates at varying pH

Aliquots of urine (1 ml) were extracted with hexane:ether (4:1, 5 ml), to remove the unconjugated androstenedione. Phosphate buffer (1 ml) and ^3H androstenedione (15,000 c.p.m.) in methanol (0.1 ml) were added to the urine and the pH of aliquots (in duplicate) was adjusted to values between 6.4 and 1.0 using 0.1 M and M hydrochloric acid. After incubating at 37° for 20.5h, the mixtures were extracted with hexane:ether (4:1, 5 ml) and the amount of androstenedione estimated in the extracts by radioimmunoassay after paper (Bush B3) chromatography.

As indicated by the results in Fig. 2.9, there is an increasing cleavage of the conjugated androstenedione to give androstenedione with increasing acidity. This is expected because of the type of conjugation between androstenedione and glucuronic or sulphuric acid.

(ii) Optimization of time of incubation for hydrolysis

These experiments were carried out to determine the minimum time required for incubation at 37° , to achieve maximum cleavage of the conjugated androstenedione. These experiments would also indicate whether any decrease in yield occurred with increasing time due to the formation of polar artefacts.

(a) At pH 1 and extraction with hexane:ether

To aliquots of a urine (0.1 ml) ^3H androstenedione tracer (50,000 c.p.m.) in methanol (0.1 ml) and phosphate buffer (0.4 ml) were added. The pH of the mixture was adjusted by adding M sulphuric acid (3 drops) and the mixture incubated at 37° for 0-24h. Concentrated carbonate solution (1 ml) was added to neutralize the mixture and the androstenedione extracted with hexane:ether (4:1, 5 ml). The urine-buffer mixture was frozen in a dry ice-ethanol bath and the hexane:ether layer decanted.

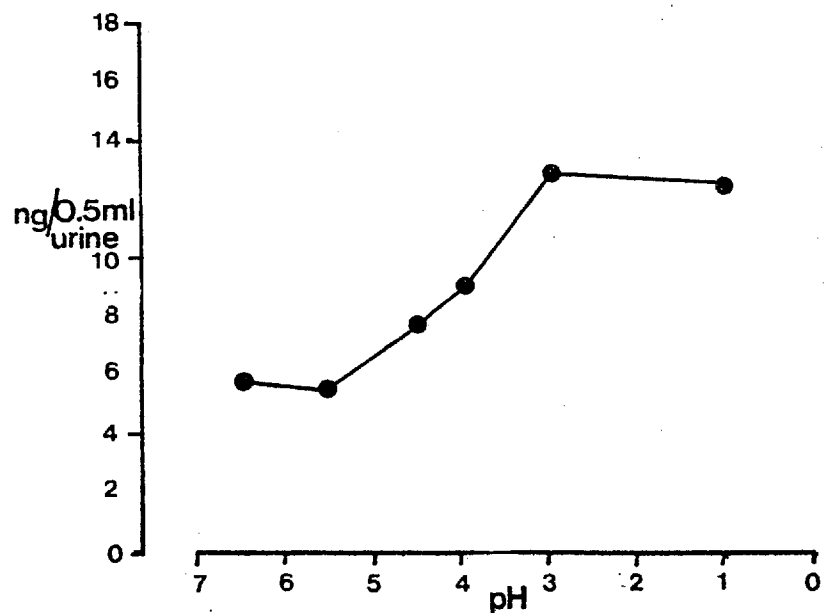


Fig2.9 Hydrolysis at varying pH

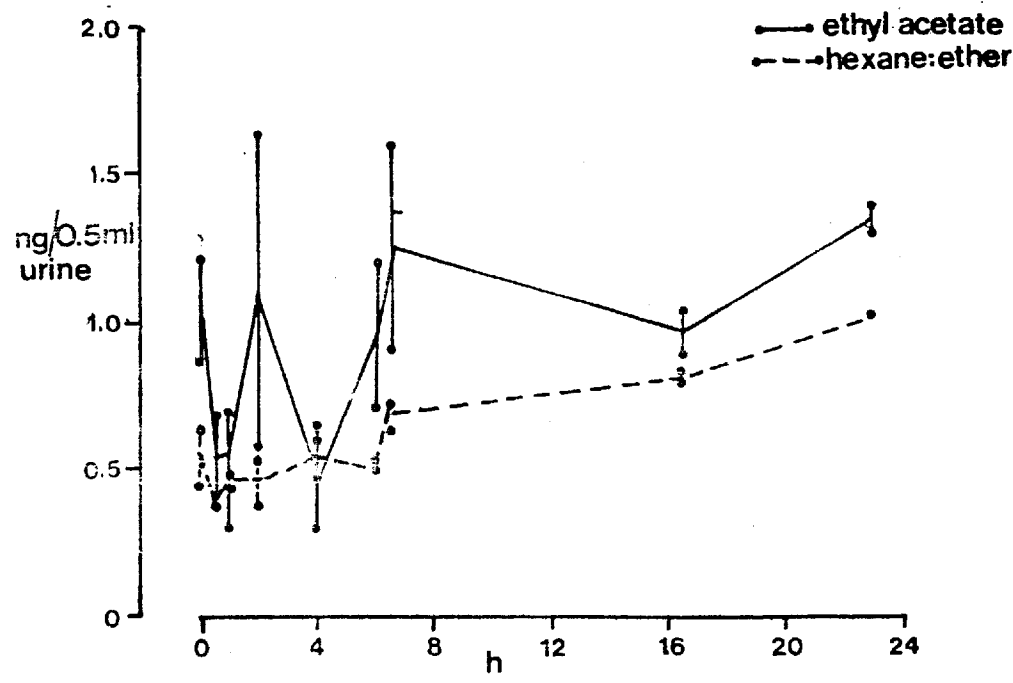


Fig 2.10 Incubation of urine with acid for various times

(b) At pH 1 in 20% saline solution and ethyl acetate

Aliquots of urine were hydrolysed in 20% saline solution in equilibrium with ethyl acetate, using the method given below, except after the required incubation time, the extracts were neutralized with concentrated carbonate solution (1 ml). The extracts from both experiments were purified by Bush B3 paper chromatography and assayed.

The results are shown in Fig. 2.10 and indicate that no breakdown of androstenedione to polar artefacts with increasing time at 37° occurred, although this does not eliminate the possibility that polar artefacts are being formed without androstenedione as an intermediate. The duplicates obtained for the hydrolysis of the conjugates by the method using ethyl acetate are not very good. This may be because, although concentrated carbonate solution was added some of the mixtures may still have been acidic and as the ethyl acetate layer retains a large amount of water and hydrolysis could still have occurred after the incubation time. The other indication that this may have been the problem is that the duplicates for the aliquots which were incubated for 16.5 and 24h are better than those for the other times. These extracts (16.5 and 24h) were evaporated and chromatographed immediately after the hydrolysis had finished and were not left in contact with the acidic phase. The duplicates obtained for the aliquots of urine hydrolysed at pH 1 and extracted with hexane:ether are better than those hydrolysed with ethyl acetate and maybe because hexane:ether is not such a polar mixture and does not retain as much of the acidic phase. The results from this experiment indicate that the optimum time for hydrolysis is around 16h in saline-ethyl acetate and up to 24h in pH 1 urine-buffer mixture.

(iii) Method

This method has been used for the assessment of the total amount of androstenedione, both conjugated and unconjugated, in urine.

An aliquot of a urine collection, normally a 24h collection (0.1 ml or 0.5 ml) was made up to 0.5 ml with phosphate buffer. ^3H androstenedione (50,000 c.p.m.) in methanol (0.1 ml) were added and the pH of the solution was adjusted to 1 using M sulphuric acid. 30% ($^w/v$) aqueous sodium chloride (1 ml) and ethyl acetate (1.5 ml) were added and the mixture shaken for 10 minutes. After incubating, for 16h at 37° , the mixture was shaken for a further 10 minutes and the ethyl acetate layer (0.5 or 1.5 ml) separated and evaporated to dryness at 40° , under a stream of nitrogen. The residue was purified by paper (Bush B3) chromatography and the amount of androstenedione determined by radioimmunoassay.

(iv) Validation of this assay

All samples were assayed in duplicate and the intra-assay variation was assessed from 32 different urine specimens, for which the C.V. was 10.3%. The inter-assay variation was assessed from urine pools which were stored deep frozen and the amount of androstenedione assessed in successive assays. The C.V. for pool 1 was 15.0% ($N=18$) at 77.6 nmol/24h and for pool 2 was 14.0% ($N=12$) at 59.7 nmol/24h. These pools have also been used to assess the stability of urine specimens which have been stored deep frozen at -20° . The results obtained from these pools for periods of up to 6 months are shown in Fig. 2.11 and indicate that there is no significant variation over this period in the total amount of androstenedione present in the urine.

Water was routinely assayed with each batch of samples and the value was normally below the lowest standard. If the ethyl acetate was not freshly redistilled, this could cause an increase in the blank value to a detectable amount; when this occurred the samples in the batch were reassayed.

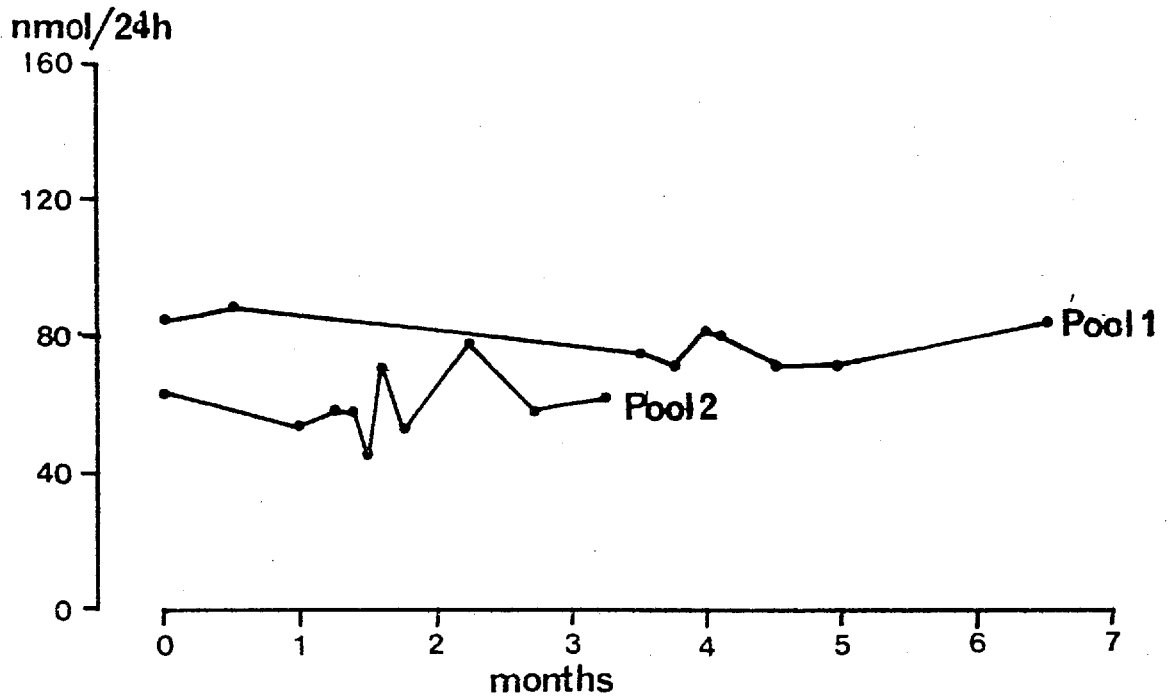


Fig2.11 Variation of total amount of androstenedione
in urine with storage at -20°

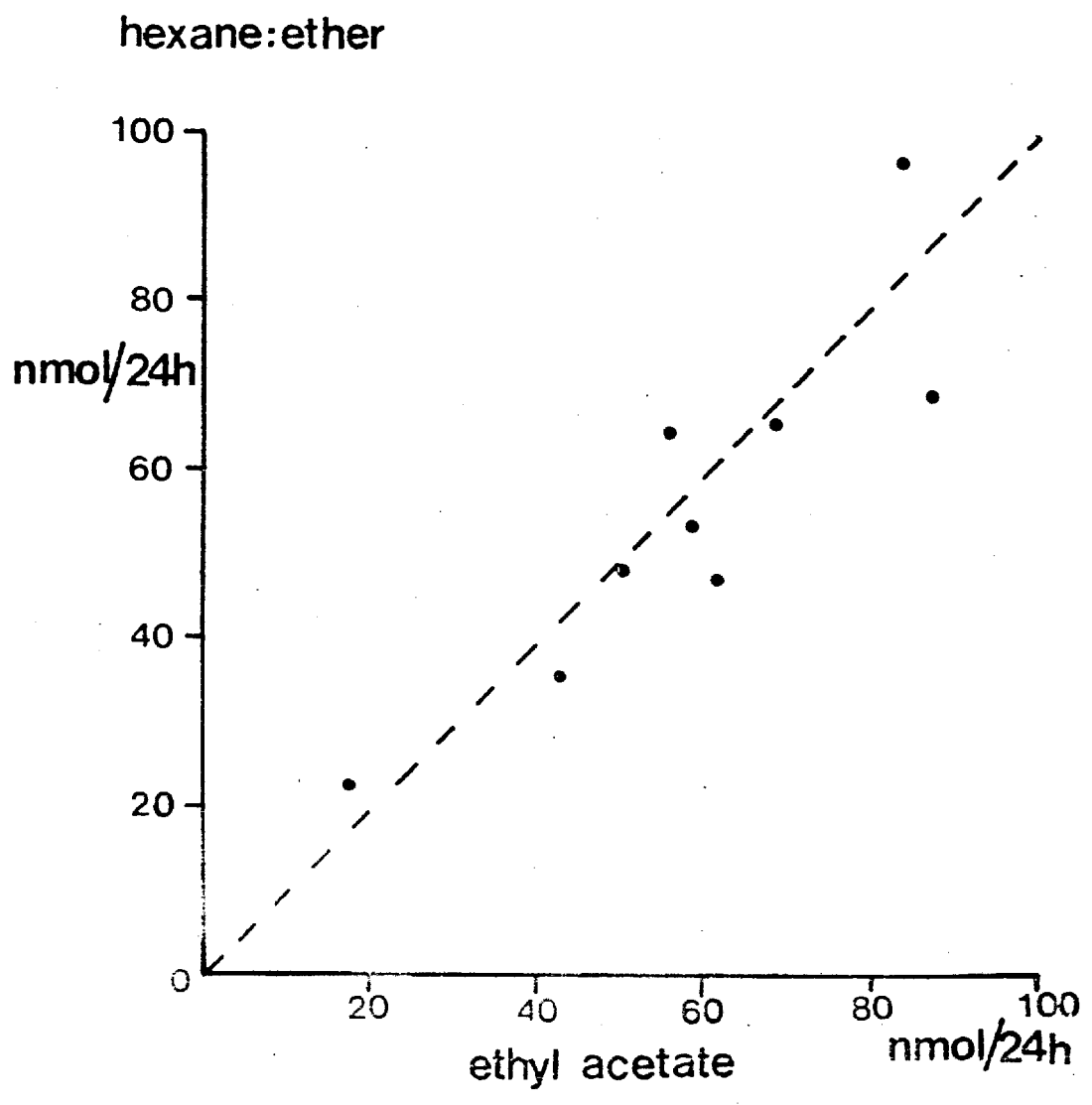


Fig2.12 Comparison of hydrolysis methods

(v) Comparison of different methods

Attempts have been made to simplify the assay. The method by which the conjugates were hydrolysed in the presence of ethyl acetate and saline solution has been compared to one in which the pH of the diluted urine was adjusted, as this process would be simpler to use than the method using ethyl acetate. Aliquots of urine were assayed in duplicate by the method using ethyl acetate-saline as outlined previously and also in duplicate using the following procedure:

To urine (0.1 ml) was added phosphate buffer (0.4 ml) and ^3H androstenedione tracer (50,000 c.p.m.) in methanol (0.1 ml). The pH of the mixture was adjusted to 1 using M sulphuric acid (3 drops). The urine-buffer mixture was incubated at 37° for 16h and extracted with hexane:ether (4:1, 5 ml). 2 ml of this extract was purified by paper (Bush B3) chromatography and the amount of androstenedione estimated by radioimmunoassay.

The good correlation ($R=0.9$) obtained (Fig. 2.12) indicated that the method using hexane:ether could also be used for the estimation of the total amount of androstenedione in urine.

2.7 Androstenedione excretion in normal subjects and subjects with pathological disorders

Urinary androstenedione excretion values have been reported by other workers, mainly in normal subjects, although some workers have measured androstenedione in patients with hirsutism and cases of adrenal dysfunction. The following work gives values for urinary androstenedione in normal subjects as well as subjects with pathological conditions.

(i) Normal subjects

Urinary excretion of androstenedione has been measured for 12 normal men (aged 22-38) and 11 normal women (aged 21-37). For the men the mean excretion was 198 nmol/24h with a range of 81-327 and for

Table 2.3 Normal male and female ranges for
androstenedione in urine. (nmol/24h)

	MALE	FEMALE
Longhino et al. (1968)	46 - 213 (n=10)	11 - 82 (n=10)
Schubert et al. (1964)	49 - 297 (n=9)	45 - 108 (n=15)
Schollberg et al. (1969)	-	17 - 185 (n=2)
Tajic (1965)	21 - 64 (n=10)	21 - 39 (n=8)
This study	81 - 327 (n=12)	45 - 108 (n=11)

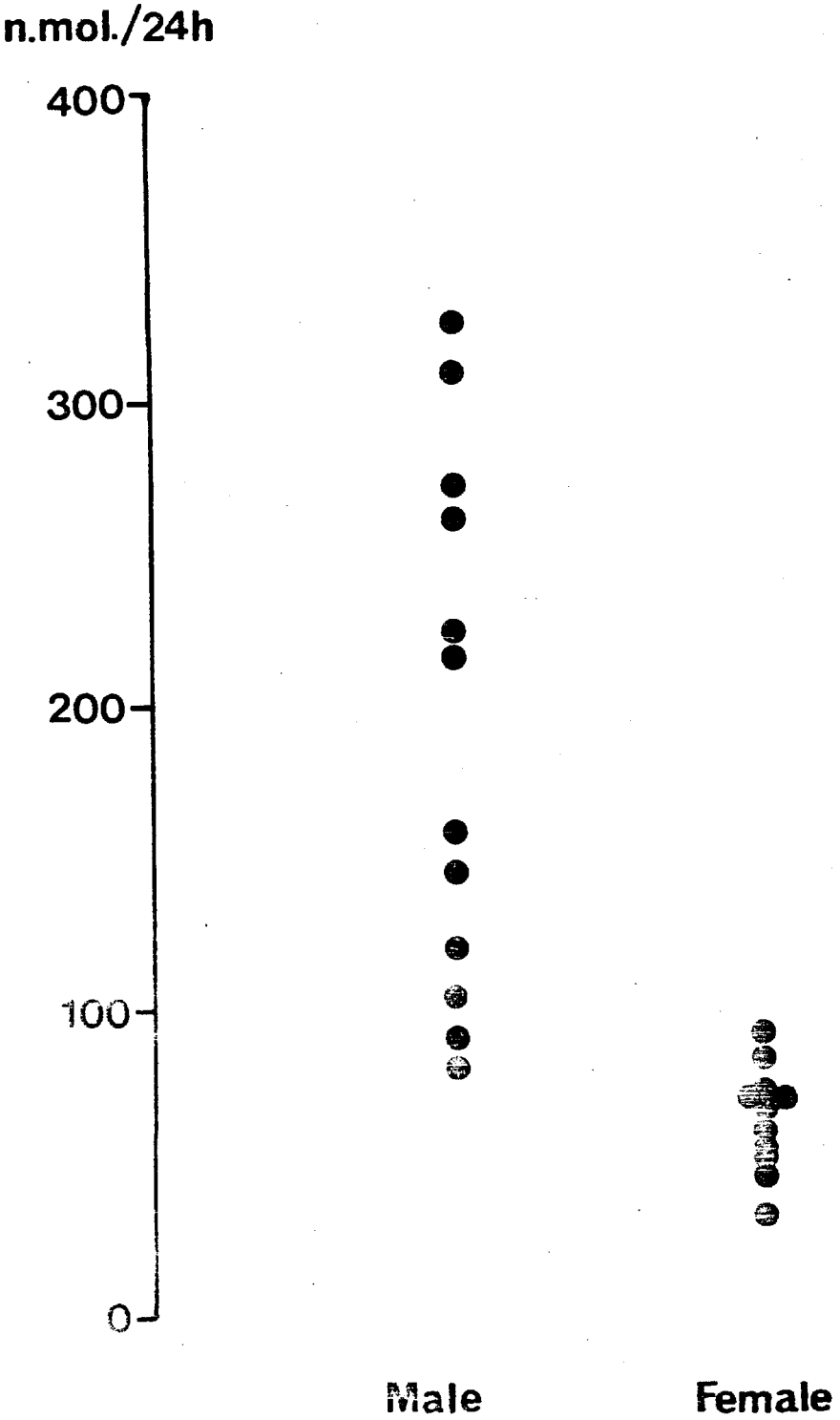


Fig2.13 Normal ranges

the women, the mean was 77 nmol/24h with a range of 45-108 (Fig. 2.13). These ranges, together with those obtained by other workers, are shown in Table 2.3. Tajic found values that are lower than the values obtained by other workers but this may be due to the extensive purification procedures which were used. Longhino et al. (1968) also found lower values. These values were estimated after incubation with β -glucuronidase and acid hydrolysis. The results were not corrected for the recovery of androstenedione which in the mean of 5 experiments was found to be 83%. The acid hydrolysis procedure used, was boiling with concentrated hydrochloric acid for 1h. These are fairly harsh conditions which could lead to the formation of artefacts resulting in lower values.

The different ranges for values of urinary androstenedione for the sexes found in this study and by other workers is unexpected, because the levels of androstenedione in plasma from male and female subjects are similar (Friedrich et al., 1975) and the metabolic clearance rate of androstenedione is also of the same order in both sexes (Horton and Tait, 1966). This difference in urinary excretion may, therefore, reflect some difference in metabolism of androstenedione.

(ii) Effect of administration of dexamethasone to a normal female subject

The administration of dexamethasone causes inhibition of the secretion of ACTH and hence secretion of androstenedione and cortisol by the adrenal cortex.

Urinary androstenedione excretion has been measured on 5 consecutive days for a normal female subject (aged 25) (Fig. 2.14). Day 1, prior to the administration of dexamethasone, Days 2, 3 and 4 dexamethasone (1.5 mg a day) was administered each evening at the

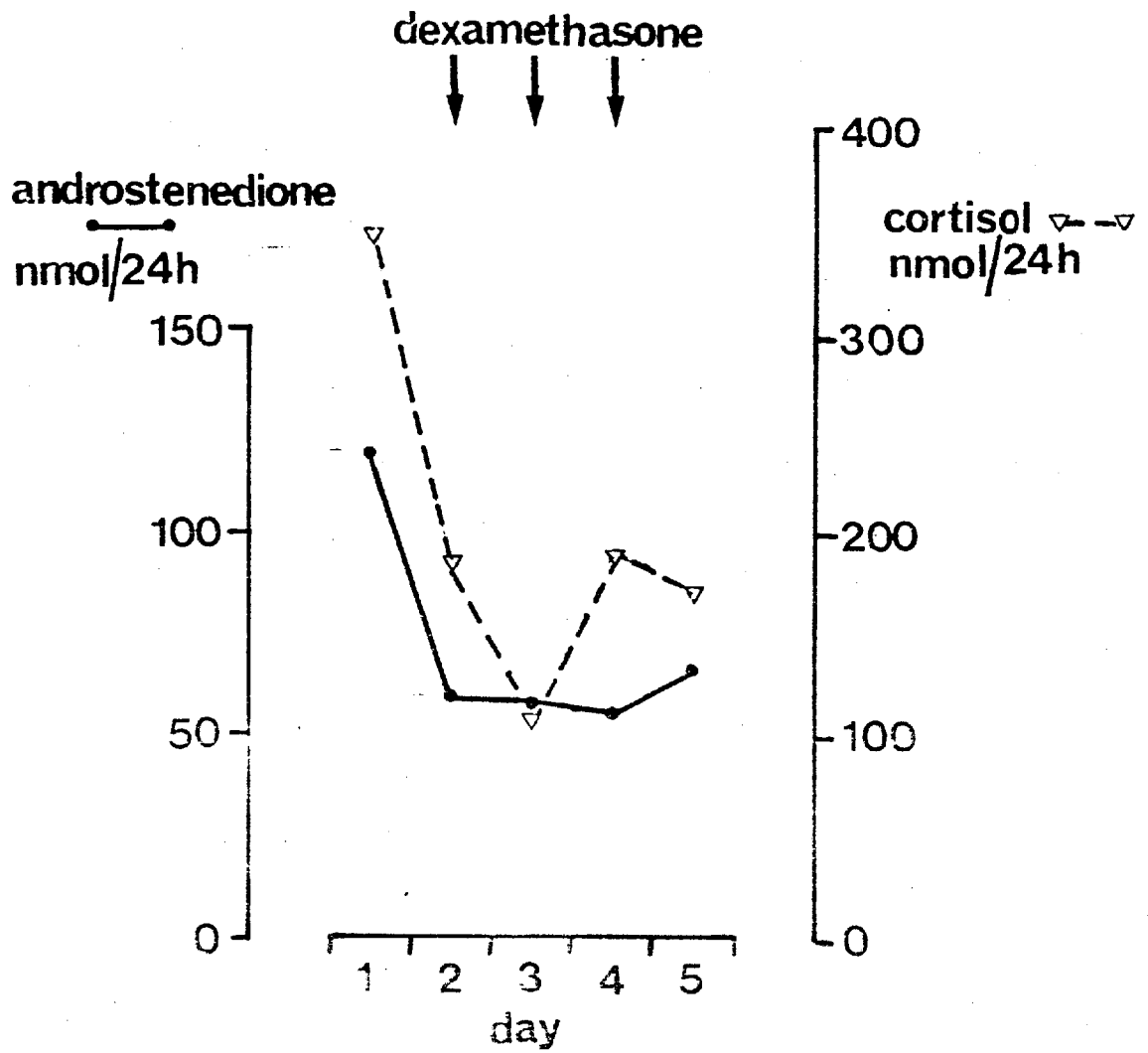


Fig 2.14 Effect of administration of dexamethasone to a normal subject

start of the urine collection and Day 5 no dexamethasone was taken. Urinary free cortisol was measured on the specimens to assess whether the suppression of adrenal secretion had occurred. The urinary excretion of androstenedione falls on the first day of dexamethasone administration and there is a slight decrease on the following 2 days, with an increase on Day 5. The urinary free cortisol measurements show the same pattern, but on Days 2, 3, 4, 5 the values are below the limit of sensitivity of the method.

(iii) Variations during the menstrual cycle

Both the ovary and the adrenal cortex secrete androstenedione. Ovarian secretion of androstenedione is thought by some workers to vary during the menstrual cycle. Several groups of workers have measured androstenedione in plasma samples, taken at various times during the menstrual cycle and some have shown an increase in values at midcycle. Lobotsky and Lloyd (1973) measured androstenedione in the peripheral plasma of women at 9 a.m. on Day 2 of the cycle and at midcycle, and found that the values at midcycle were higher than those obtained on Day 2 of the cycle but these values obtained are slightly higher than those usually found in normal subjects. Abrahams (1974) studied plasma androstenedione levels during normal cycles and also in cycles where dexamethasone was administered to the subjects. He concluded that the adrenal contribution to androstenedione production was relatively constant during the menstrual cycle but that the ovarian contribution reaches a maximum at midcycle. In a detailed study, Judd and Yen (1973) found a progressive increase in androstenedione levels in plasma during the follicular phase in 3 out of 6 subjects, and that the mean androstenedione level during the middle third of the menstrual cycle was significantly higher than

that during the earlier or later parts of the cycle. Guerrero et al. (1976) found changes in plasma androstenedione values during the cycle, but their data showed a significant increase in values during the luteal phase and although there was a peak at midcycle the increase was not significant. Frölich, Brand and Van Hall (1976) did find, however, an increase at the time of ovulation, but more recently, Kader, McConway, Macnaughton and Coutts (1977) found no significant increase associated with ovulation, though the levels in the luteal and the later follicular phase were not significantly different from each other but were significantly higher than those just before and after menses. These results appear to contradict one another. This may be because androstenedione was being assessed in plasma samples and as a result of fluctuations of androstenedione levels due to diurnal rhythm and stress, a single sample may not be a good reflection of the production during the day. Abrahams (1974), Guerrero et al. (1976) and Frölich et al. (1976) only showed a mean and standard deviation for all their subjects combined, whereas in their paper, Judd and Yen (1973) showed the subjects' values individually and these results showed a large subject to subject variation.

There are only 3 reports of the measurement of androstenedione in urine during the cycle, 2 by Longhino's group (Longhino et al., 1968; Longhino et al., 1972) and the other by Schollberg et al. (1969). Longhino et al. (1968) measured urinary androstenedione excretion during complete cycles from 2 subjects. The values were much higher for one subject than the other, although the values for both subjects showed a decrease from Day 12 to 16 and an increase during the luteal phase reaching maximum levels between Days 20-25 in one subject and 20-24 in the other. In the other paper, Longhino et al. (1972) estimated the amounts of androstenedione excreted in the free and

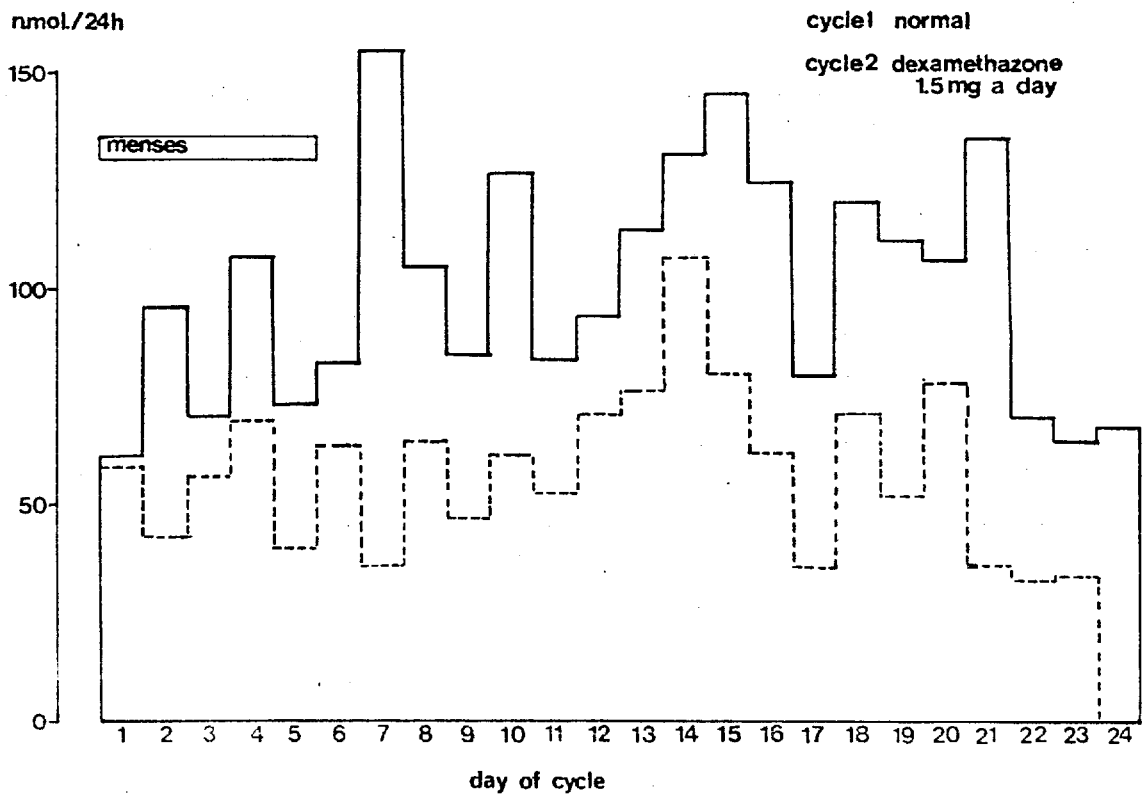


Fig2.15 Variations during the menstrual cycle

conjugated forms. Schollberg et al. (1969) measured androstenedione excretions during the menstrual cycle of 2 subjects. The first subject's cycle was normal, the androstenedione values were low at midcycle and rose after ovulation. In the other patient with oligomenorrhoea, there was no clearly defined pattern to the levels.

Urinary androstenedione has been measured in 2 cycles from a normal female, although both cycles were anovulatory as indicated by the measurement of urinary pregnanediol in the first cycle and plasma progesterone in the second cycle. Menstruation started in a normal way on Days 23 and 24 of the respective cycles. During the second cycle dexamethasone (1.5 mg) was taken by the subject each evening. The results (Fig. 2.15) for the normal cycle (Cycle 1) show a fairly consistent excretion of androstenedione during the cycle, although there are fluctuations from day to day with the amounts excreted being lower just before and after menstruation. Urinary free cortisol has been measured on all these days, and the ratio of total androstenedione to urinary free cortisol has been calculated (Table 2.4), in an attempt to assess the relationship of adrenal to ovarian production of androstenedione, as cortisol is produced only by the adrenal cortex but androstenedione is secreted by the ovary and the adrenal cortex. These results do not show any obvious trend.

The values for urinary excretion of androstenedione during Cycle 2 in which dexamethasone was taken are also shown in Fig. 2.15. A similar pattern was obtained for this cycle. In Cycle 2 there is an increase in the excretion towards Day 14 and a decline in values prior to the onset of menstruation. The mean value for Cycle 1 was 96.8 nmol/24h and for Cycle 2, 57.5 nmol/24h, indicating that the ovary is accounting for about 50% of the production of androstenedione. This assumes that the administration of dexamethasone does

Table 2.4 Urinary androstenedione and urinary free cortisol during a normal menstrual cycle

Day of cycle	Urinary androstenedione (A) nmol/24h	Urinary free cortisol (F) nmol/24h	A/F
1	61.5	248.0	0.25
2	95.8	121.4	0.79
3	70.3	273.0	0.26
4	115.7	500.4	0.23
5	77.2	228.0	0.33
6	112.9	284.8	0.4
7	122.4	409.6	0.3
8	110.1	265.0	0.42
9	84.6	231.8	0.36
10	127.9	271.6	0.47
11	83.6	351.9	0.24
12	93.0	351.3	0.26
13	113.6	311.0	0.36
14	131.1	387.5	0.34
15	145.1	304.1	0.48
16	124.8	570.6	0.24
17	79.4	304.7	0.26
18	120.0	353.5	0.34
19	110.8	278.2	0.4
20	106.3	236.8	0.45
21	134.6	357.7	0.38
22	69.9	167.8	0.42
23	64.6	370.9	0.17
24	68.2	397.4	0.17

not affect ovarian production. Kirschner and Jacobs (1971) found that in hirsute subjects, dexamethasone may reduce ovarian production of androstenedione as well as adrenal production.

The results obtained for the values during these cycles are similar to those obtained by other workers in that no peak was obtained at mid cycle. In fact, other workers (Longhino et al., 1968) found a fall prior to ovulation which is opposite to that claimed from results obtained in plasma.

(iv) Effect of administration of dexamethasone to a postmenopausal subject

The state of the ovary, with respect to the secretion of androstenedione, after the menopause is a matter of dispute. Judd, Judd, Lucas and Yen (1974) concluded from the measurement of androstenedione in the peripheral and ovarian vein samples from postmenopausal subjects undergoing bilateral oophorectomy that the postmenopausal ovary continues to secrete a moderate amount of androstenedione. This was also concluded by Vermeulen (1976a and b). Hutton, Jacobs, James, Murray and Rippon (1977a and b) reported that the major glandular source of steroid hormones after the menopause was the adrenal cortex. Crilly, Marshall and Nordin (1979) have studied the effect of age on plasma androstenedione concentration of oophorectomized subjects, and their data showed that a significant reduction occurs in the plasma androstenedione concentration between the ages of 50 and 55 and suggested that an 'adrenopause' may occur. In this short communication though, no values for plasma cortisol or any indication as to the time of day the samples were taken, was given.

For 1 postmenopausal subject (aged 51) it has been possible to compare plasma values of androstenedione and the amount of androstenedione in 24h urine collections before and after administration

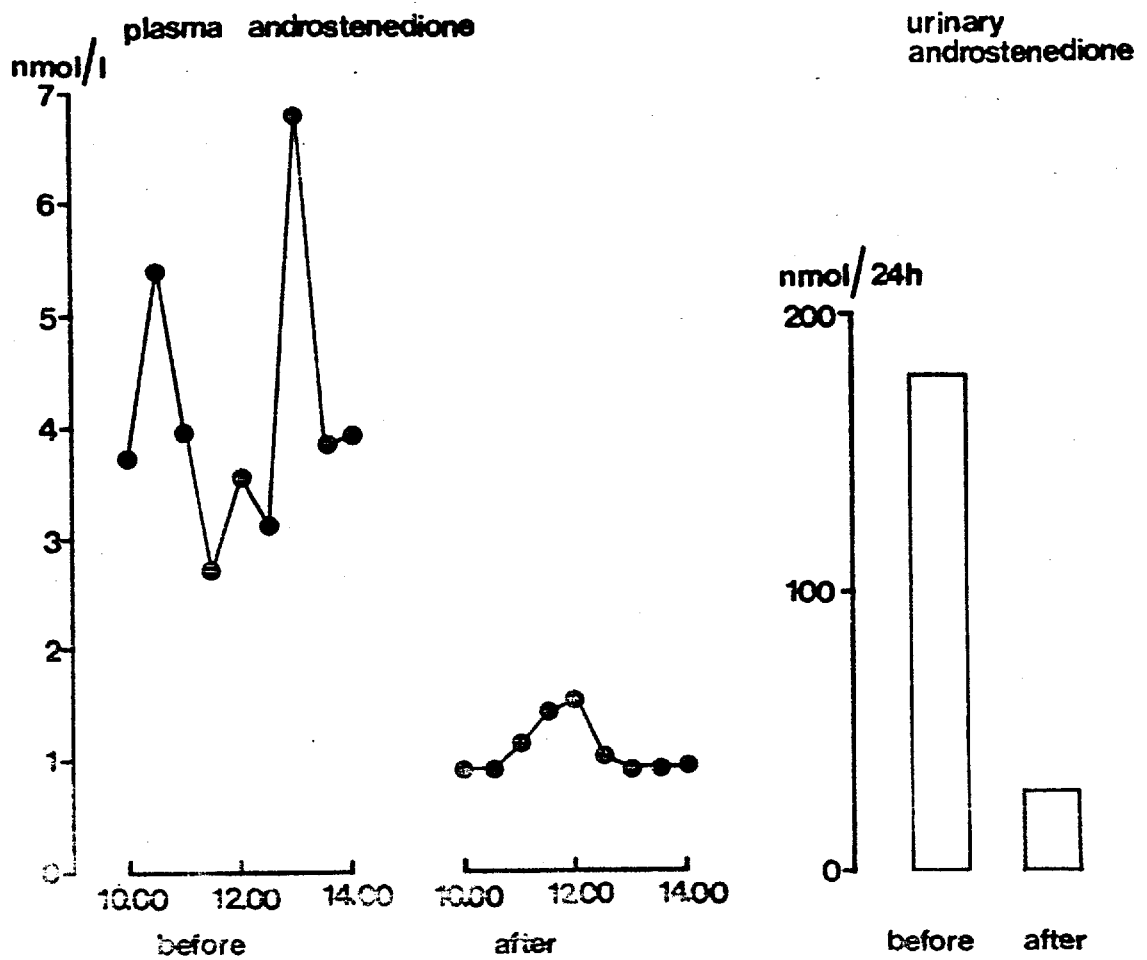


Fig2.16 Effect of administration of dexamethasone to a postmenopausal subject

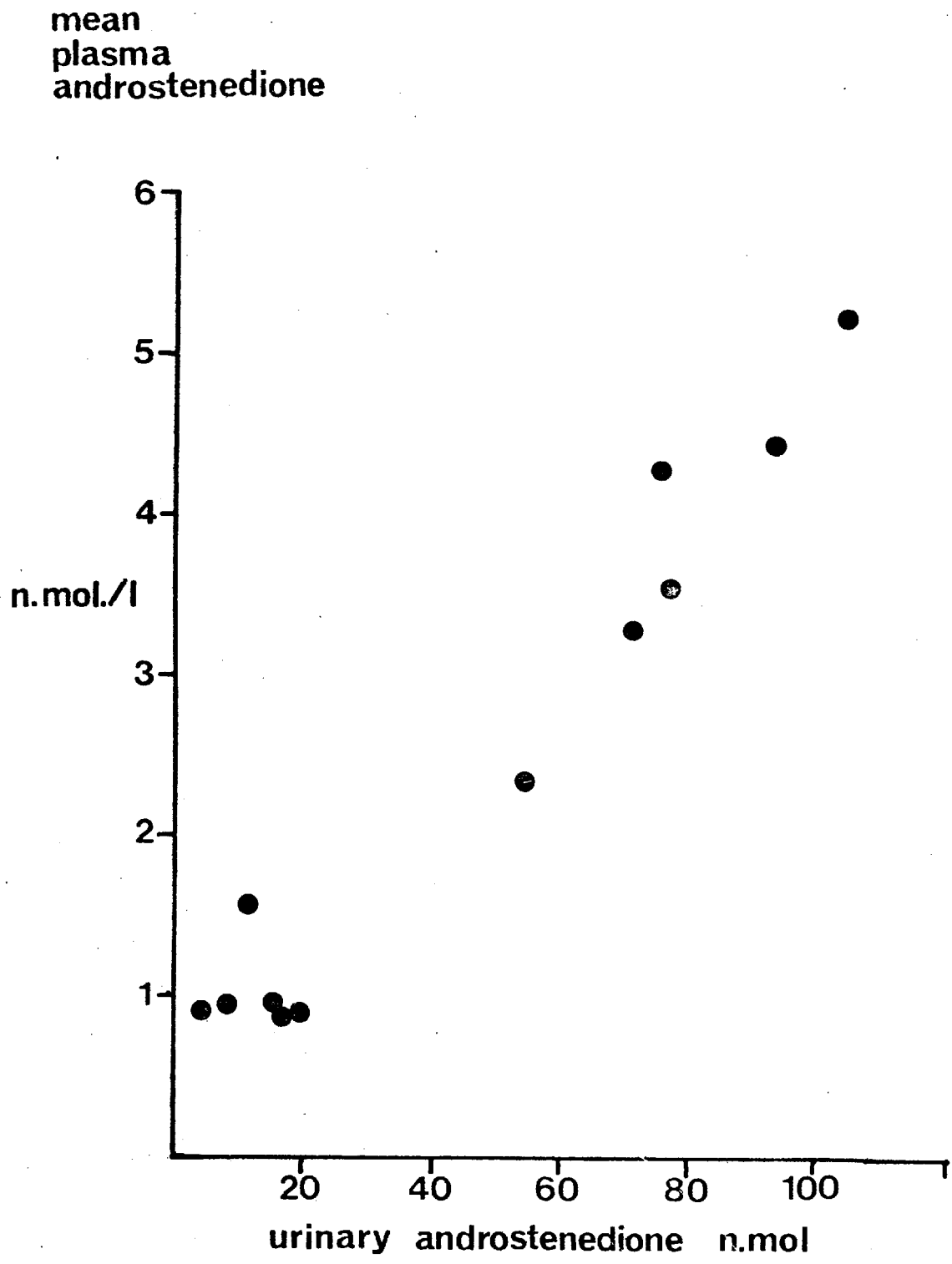


Fig 2.17 Correlation of plasma and urinary androstenedione values

of dexamethasone (1.5 mg a day for 7 days). Plasma samples were taken at $\frac{1}{2}$ h intervals for 4h prior to the start of treatment with dexamethasone and again at the end of this treatment. 24h urine collections were made on the same 2 days as the blood samples were taken. As Fig. 2.16 shows, androstenedione values in plasma and androstenedione excretion are reduced after the administration of dexamethasone, and indicate that the majority of androstenedione production after the menopause results from secretion by the adrenal cortex.

(v) Correlation between androstenedione excretion and plasma androstenedione values for a group of postmenopausal subjects

For 2 groups of postmenopausal subjects the mean plasma value of androstenedione over a given number of hours has been correlated with the total amount of androstenedione excreted in the urine, over the same period. The aim of this experiment was to determine whether urinary androstenedione excretion was a useful index of androstenedione production.

The subjects had sequential blood samples taken every 30 minutes, during various studies. The average number of plasma samples obtained was 25, with a minimum of 17 and a maximum of 47. A good correlation (correlation coefficient of 0.97) has been obtained between these 2 parameters (Fig. 2.17) and these results indicate that the urinary excretion of androstenedione was a good index of plasma androstenedione levels.

(vi) Hirsutism

Hirsutism has been defined by Abrahams and Manlimos (1978) as a form of hyperandrogenization characterized by the appearance of terminal hair on face, chest, upper back and abdomen. Androstenedione is

an important prehormone of testosterone and dihydrotestosterone. Hence, the measurement of androstenedione values in hirsutism is of some importance. 50-60% of the total testosterone production in women arises from peripheral metabolism of prehormones, primarily androstenedione which is transformed by several sites including liver, fat and skin and re-enters the circulation as testosterone (Horton and Tait, 1966; Bardin and Lipsett, 1967). Bardin and Lipsett (1967) demonstrated that prehormone contribution is not increased in proportion to the elevated testosterone production rate, and androstenedione metabolism accounts for only 25% of the total testosterone production in these women. Kirchner, Zuker and Jespersen (1976) have attempted to define the origin of the excessive androgens in women with idiopathic hirsutism by measuring the amounts of hormones in adrenal and ovarian veins by direct catheterisation. Their data suggested that the ovary was the major site of androgen overproduction, including women whose androgens were suppressed after exogenous glucocorticoids. They confirmed the previous data of Ito and Horton (1970) which indicated that dihydrotestosterone is formed virtually entirely from androstenedione. Andre and James (1974) found a good correlation of plasma testosterone and androstenedione in normal subjects but not in subjects with hirsutism. They found an overlap of the ranges for normal and hirsute subjects for androstenedione, although the mean of the values for the hirsute subjects was significantly higher, and suggested that the poor correlation of plasma testosterone and androstenedione in these subjects may have been due to the altered binding of testosterone to plasma proteins. More recently, in a group of hirsute subjects plasma androgen variations over short periods of time and the effect on these levels of minimal doses of glucocorticoid has been studied (James, Jacobs and Rippon,

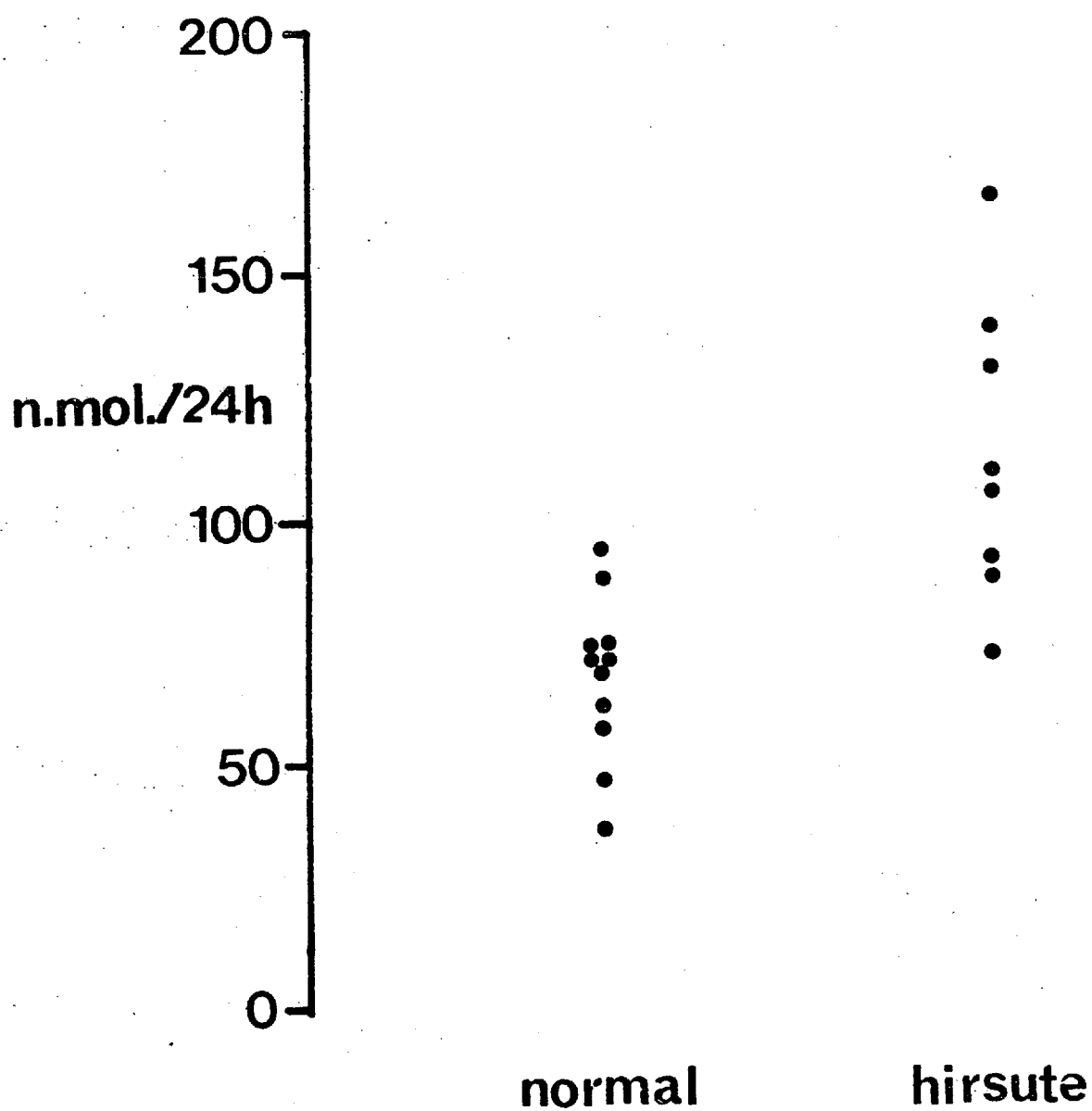


Fig 2.18 Values in hirsute subjects

1976). In hirsute women rapid and well defined changes in testosterone and androstenedione levels occurred and major peaks of androstenedione unrelated to changes in plasma cortisol levels occurred which were not reduced by glucocorticoid administration, suggesting that the ovary was the most probable site of this secretion, although secretion by the adrenal cortex independent of ACTH could not be discounted. These fluctuations also occurred in normal women, although they were more marked in patients with hirsutism. This highlights the problems in determining androstenedione in peripheral plasma samples and catheterisation studies in hirsute subjects, as the secretion of androstenedione occurs episodically from both the ovary and adrenal cortex.

Total urinary androstenedione has been measured in a group of hirsute subjects to determine whether there is an increased excretion of androstenedione.

For 8 hirsute subjects (age 22-55), the mean value for urinary androstenedione was 113 nmol/24h (73-169) compared with the mean for the normal female subjects of 77 nmol/24h (45-108) (Fig. 2.18). Hence, there is an overlap of the 2 populations, just as has been found for plasma androstenedione levels when these are compared in normal female and hirsute subjects (Andre and James, 1974).

Urinary androstenedione has been measured by Tajic (1965) in 2 hirsute subjects, before and after treatment with cortisol (1 subject) and with human chorionic gonadotrophin (1 subject). The levels before treatment were above the normal female range quoted in the paper and decreased on treatment.

Cyproterone acetate combined with ethinyl oestradiol is now commonly used for the treatment of hirsutism (Ismail, Davidson, Souka, Barnes, Irvine, Kilimnik and Van der beeketh, 1974; Hammerstein,

Table 2.5 Effect of administration of ethinyl oestradiol, dexamethasone and cyproterone acetate on plasma androstenedione, and testosterone and urinary androstenedione, in a hirsute subject.

Month	Treatment	Plasma			
		Urinary androstenedione nmol/24h	androstenedione nmol/l	testosterone nmol/l	cortisol nmol/l
1	Ethinyl oestradiol	92.7	-	-	-
2	Ethinyl oestradiol dexamethasone	76.6	2.9	0.9	65
3	Ethinyl oestradiol dexamethasone cyproterone acetate	126.9	7.7	1.3	<30
6	As month 3	26.6	<0.9	<0.3	<30

Meckies, Leo-Rossberg, Moltz and Zielske, 1975). There is disagreement whether this therapy causes suppression of adrenal function.

Girard, Baumann, Zuppinger, Haas, Staub and Wyss (1978) have concluded that cyproterone acetate has a definitive ACTH suppressive effect in addition to its anti-androgenic and anti-gonadotrophic activities, both for children and hirsute women. Smals, Kloppenborg, Goverde and Benraad (1978) have concluded though, that short term (2 weeks and up to 3 months) treatment does not significantly influence pituitary adrenal function in adult women with idiopathic hirsutism.

For 1 subject, urinary androstenedione excretion has been measured before and during treatment for hirsutism. The collections, during therapy, were made on Day 15 of the cycle after the final dose of cyproterone acetate for that cycle. The details of the treatment and results from plasma and urine samples are given in Table 2.5.

Urinary androstenedione excretion during treatment with ethinyl oestradiol was within the normal female range. This level decreased after treatment with dexamethasone, together with plasma androstenedione and testosterone values. The cortisol level indicates suppression of adrenal function. During the first month of cyproterone acetate treatment, there was an increase in the androstenedione excretion and plasma androstenedione and testosterone values, although the cortisol value was below the limit of detection indicating that this was increased ovarian production. After a further 4 months treatment the urinary androstenedione value was below the normal range and the plasma values of androstenedione, testosterone and cortisol were below the limit of detection of the assays. These results would indicate that at the beginning of treatment with cyproterone acetate a different effect on the metabolism of androgens occurs than when prolonged treatment is given. Evidence for this has been found by other

workers. Voigt, Apostolakis and Tamm (1968) found an increase in the excretion of androstenedione in males after treatment with cyproterone, and Tamm and Beischer (1968) concluded from the measurement of testosterone that cyproterone caused altered metabolism and production of testosterone glucuronide. This work has all involved treatment with cyproterone, and the situation may be different when cyproterone acetate treatment is given. More recently, Frolich, Lachinsky and Moolenaar (1977) have studied the influence of combined cyproterone acetate - ethinyl oestradiol therapy on serum levels of androstenedione and other steroids in hirsute women, and found that androstenedione levels were higher than normal females before treatment and decreased markedly during treatment, though the samples were taken 6-8 and 12-14 months after the start of treatment. To my knowledge nobody has measured androstenedione immediately after the start of treatment, which may be why this rise in androstenedione after the start of treatment has not previously been reported. The decrease in values after 4 months of treatment is the same as obtained by other workers.

(vii) Children

Urinary androstenedione excretion has been measured in children by 3 groups of workers (Hubl, Siebert, Buchner and Hering, 1971; Waaler, Thorsen, Støa and Aarskog, 1974; Danzell and Elattar, 1974). Waaler et al. (1974) measured urinary androstenedione in normal male children during puberty. Until the adolescent years excretion remained at less than 17.5 nmol/24h and increased to values of 17.5-139.7 nmol/24h at 16y. These results are lower than those found by Danzell and Elattar (1974) who measured urinary androstenedione by gas chromatography, after hydrolysis with β -glucuronidase in samples

Table 2.6 Urinary and plasma androstenedione in children
with congenital adrenal hyperplasia

Subject	Age	Treatment	Urinary	Plasma	
			androstenedione nmol/24h	Androstenedione nmol/l	Testosterone nmol/l
G.J. 10/77	9	Cortisone acetate	80.4	16.3	13.2
4/78	9	"	82.5	7.4	10.4
D.J. 10/77	13	Cortisone acetate	197.2	25.0	17.9
4/78	13	"	111.9	11.0	12.5
R.R.	6	None	61.5	43.4	-
C.L.	6	Cortisone acetate	<2.5	<0.9	<0.35

from adolescent and preadolescent children. This method though, would not measure any androstenedione present conjugated to sulphuric acid. In children between the ages 6.2 and 13.2y androstenedione was only detected in 15% of the samples (0-13.3 nmol/24h) and values gradually increased at 13.4y, with a significant increase at 14.6y. At full sexual maturation, the range was 12.6-65.6 nmol/24h which is considerably lower than the adult ranges given in this thesis and found by other workers (Schubert et al., 1964; Longhino et al., 1968). These reduced values excreted by children are comparable with the lower values of androstenedione found in plasma from children (e.g. Ducharme, Forest, De Peretti, Sempe, Collu and Bertrand, 1976).

Congenital adrenal hyperplasia is an inherited disorder caused by an enzyme block in the biosynthetic pathway leading from cholesterol to cortisol. Recently Korth-Schutz, Virdis, Saegner, Chow, Levine and New (1978) have shown that serum androstenedione is probably the best androgen for determining whether a subject is receiving the correct treatment. Urinary and plasma androstenedione has been measured in several children who were being treated for congenital adrenal hyperplasia (Table 2.6).

The 2 brothers (D.J. and G.J.) have both had values estimated at 6 month intervals. These brothers have a history of not administering their tablets as instructed. The high urine and plasma values obtained indicate that these subjects were not having the correct treatment. This is in contrast to the subject C.L. who has undetectable plasma and urinary androstenedione, indicating that the correct treatment was being given. Samples from subject R.R. were taken prior to the treatment for congenital adrenal hyperplasia, and hence were high.

These results indicate that the estimation of androstenedione in urine may be a useful parameter to measure in the diagnosis of adrenal disorders in children, when venepuncture is a problem and treatment must be carefully monitored, although care must be taken to see that an accurate 24h urine collection is obtained.

2.8 Estimation of excretion of unconjugated androstenedione and androstenedione conjugated to glucuronic and sulphuric acid

Most authors reporting the measurement of androstenedione in urine, have estimated the combined amounts present after β -glucuronidase and acid hydrolysis. Tajic and Kovacic (1968) estimated the percentages of free, glucuronide and sulphate conjugated androstenedione in urine samples from 3 male and 3 female subjects. The results indicated that the majority of androstenedione in urine is in a form conjugated to sulphuric acid. For 1 female subject androstenedione was only present in a form conjugated to sulphuric acid. More recently, Longhino, Drobnjak, Krk and Vedris (1972) measured the urinary excretion of androstenedione, conjugated and unconjugated, during the menstrual cycle of 1 normal subject. In these samples also the majority of androstenedione present was excreted in a form conjugated to sulphuric acid.

The total hydrolysis method for the measurement of androstenedione in urine has been modified so that the amounts of androstenedione in the conjugated and unconjugated fractions can be assessed.

(i) Method

^3H androstenedione (30,000 c.p.m.) in methanol (0.1 ml) was added to an aliquot of urine (0.5 ml). The mixture was extracted with hexane:ether (4:1, 4 ml) to remove the unconjugated androstenedione present. This organic layer was removed and retained. A further aliquot of tracer was added to the urine, together with phosphate buffer (1 ml) and β -glucuronidase (10 μl). This mixture was

then incubated at 37° for 6h and again extracted with hexane:ether, to remove any androstenedione. After the addition of further tracer, 0.5 ml of the urine-buffer mixture was hydrolysed by the same method as for the measurement of the total amount of androstenedione in urine. The organic extracts from the 3 stages were assayed to estimate the amount of androstenedione present, after paper (Bush B3) chromatographic purification.

(ii) Values in normal subjects and subjects with pathological disorders

(a) Normal subjects

Androstenedione excretion has been measured in 10 male and 9 female subjects (Table 2.7). The range and mean for the male subjects (aged 21-38) in nmol/24h are: unconjugated 6.5-18.5 (mean 12.2), glucuronide 13.8-87.1 (mean 38.0), sulphate 31.2-237.8 (mean 130.1) and for the female subjects (aged 21-37): unconjugated 4.1-18.9 (mean 8.8), glucuronide 8.0-30.1 (mean 20.7), sulphate 26.1-67.8 (mean 45.8). Values of unconjugated androstenedione excreted in urine have been measured by Andino et al. (1976), using a similar method. The normal ranges which were obtained were of the same order.

The amounts of androstenedione in both the glucuronide and sulphate fractions in men are greater than in women, although the ranges overlap. The greatest difference was in the sulphate fraction and it would appear that it was the androstenedione which was conjugated to sulphuric acid, that results in the increased excretion of androstenedione by men. The amounts excreted in the various fractions are in agreement with the evidence obtained from t.l.c. of urine extracts, which also indicate that the largest amount of androstenedione excreted in urine was conjugated to sulphuric acid.

Table 2.7 Amounts of androstenedione excreted in free, glucuronide and sulphate fractions by normal subjects (nmol/24h)

	Free	Glucuronide	Sulphate
Males	9.1	25.5	237.8
	12.6	60.1	235.0
	14.3	38.8	168.2
	13.8	87.1	225.9
	9.8	22.4	148.1
	6.5	43.1	31.2
	13.3	36.9	67.8
	18.5	26.2	55.9
	11.6	26.0	50.7
	12.3	13.8	80.4
Females	15.0	30.1	52.7
	4.3	21.7	28.0
	8.0	25.2	66.4
	7.5	22.0	56.3
	5.8	22.4	42.3
	4.1	11.9	67.8
	18.9	29.8	26.1
	4.0	8.0	22.7
	11.4	15.2	49.7

Table 2.8 Amounts of androstenedione excreted in free, glucuronide and sulphate fractions in hirsute subjects (nmol/24h)

Free	Glucuronide	Sulphate
9.1	11.4	51.7
15.4	8.9	33.3
4.6	9.6	31.9
12.5	6.9	30.5
14.2	5.3	33.7
10.1	9.4	118.5
12.9	14.3	128.0
12.9	8.4	51.0

(b) Hirsutism

For a group of 8 patients with hirsutism, the amounts of androstenedione excreted in the various fractions is shown in Table 2.8. The range for unconjugated was 4.6-15.4 nmol/24h and is similar to the range for normal females. Andino et al. (1976) also measured unconjugated androstenedione in hirsute subjects, 4 of their 8 subjects though, had very elevated values compared with the normal range.

The range for the amounts of androstenedione conjugated to glucuronic and sulphuric acid are different to those for normal subjects. The amounts conjugated to glucuronic acid are lower than those for normal subjects and the amount conjugated to sulphuric acid are higher. These data suggest that there is an altered pattern of metabolism or conjugation in patients suffering from hirsutism.

2.9 Discussion

These results given in this section, have shown by various methods that androstenedione is excreted in human urine, both in conjugated and unconjugated forms.

As androstenedione is secreted episodically, the primary reason for investigating the excretion of androstenedione in urine was an attempt to find either a unique metabolite of androstenedione or an index of androstenedione production, and this value used, rather than taking sequential blood samples, to assess a subject's androstenedione production. As an initial approach to this problem, Andino et al. (1976) measured unconjugated androstenedione excretion, but there are problems with this method because of the large amount of conjugated androstenedione that is also excreted, as spontaneous hydrolysis of this conjugated fraction can occur to give spuriously high results. For this reason, the measurement of the total amount of androstenedione excreted in urine has been explored.

A number of hydrolytic procedures (glucuronidase, sulphatase, solvolysis and acid hydrolysis) have all been shown to cleave androstenedione conjugates present in urine. The optimum conditions for these procedures have been determined, in view of the report by Matsui et al. (1974) that polar artefacts may result from the hydrolysis of androstenedione 3-enol glucuronide and this would cause an underestimate of the amounts of androstenedione present.

Although formal evidence for the presence of these conjugates in urine is still lacking, as the conjugates have not been isolated and physically chemically characterised, prior to hydrolysis, both the sulphate and the glucuronide conjugate have been synthesised (Wotiz et al., 1959; Oertel et al., 1967; Matsui et al., 1974). This evidence, together with the indirect evidence in this section, including t.l.c., Sephadex LH20 column chromatography and hydrolysis studies of the conjugates of androstenedione in urine, would indicate that androstenedione exists in urine in 2 conjugated forms. A larger amount of androstenedione excreted in urine is conjugated to sulphuric acid than glucuronic acid, with only a small proportion of androstenedione excreted in an unconjugated form.

As a result of the enol linkage between the conjugating acid and androstenedione, it has been possible to develop an assay to estimate the total amount of androstenedione excreted in urine, in which the conjugates are hydrolysed by acid treatment. This method has been used to investigate androstenedione excretion in various physiological and pathological conditions. Good agreement has been found for the ranges obtained for normal subjects with those of other workers. The difference in male and female normal ranges is somewhat surprising, in view of the fact that the metabolic clearance rates and plasma levels for androstenedione are similar for both sexes. This finding must,

therefore, reflect some difference in metabolism. For the group of postmenopausal subjects, where urinary excretion of androstenedione was correlated with plasma values of androstenedione, a good correlation was obtained. These results indicate that androstenedione excretion estimated in this way may be a useful measurement in conditions of androgen abnormality.

SECTION 3

Conjugated androstenedione in plasma

3.1 Introduction

Steroid conjugates in blood have not been so widely investigated as conjugated steroids in urine. A major difficulty in studying conjugated steroids, in both blood and urine has been the isolation and identification of the intact steroid conjugate. Hence, most workers have used indirect methods and have estimated the amount of steroid after the conjugating acid has been cleaved from the steroid.

The steroid conjugates in plasma most extensively studied are oestrone sulphate and dehydroepiandrosterone sulphate. The biochemistry of dehydroepiandrosterone sulphate and other 3β -hydroxy-5-ene-steroid sulphates has been reviewed by Roberts and Liebermann (1970). The adrenal secretion of dehydroepiandrosterone sulphate was first recognised by Weiland et al. (1966), although Baulieu (1962) found dehydroepiandrosterone sulphate in adrenal venous blood from a patient with a tumour. Even though the abundance of dehydroepiandrosterone sulphate in plasma is now well recognised, its physiological significance is still unknown.

To my knowledge, there is only one report of the presence of conjugated androstenedione in plasma. Rhamy and Hadd (1973) identified androstenedione 3-enol glucuronide in the plasma of a patient with an ovarian tumour. Oertel, Kaiser and Zimmerman (1963) did not find any evidence for the presence of conjugated androstenedione in the adrenal effluent of a 60y old woman, with carcinoma of the breast, but this may have been because the detection limit of the method of estimation that they were using was 17.5 nmol/l.

As there was some evidence for the possible existence of androstenedione in a conjugated form in plasma, a method has been devised

to determine whether androstenedione in a conjugated form was present in plasma and to study the levels in the plasma of normal subjects. The amount of conjugated androstenedione has been assessed, indirectly, by hydrolysing the conjugated steroid, using either acid or sulphatase (*Helix Pomatia*) and estimating the amount of androstenedione by radio-immunoassay. This method has been used to estimate conjugated androstenedione in peripheral plasma of subjects who have been administered dexamethasone, tetracosactrin or androstenedione in an attempt to determine the source of this conjugated steroid. Conjugated androstenedione has also been estimated in adrenal venous blood.

3.2 Development of an acid hydrolysis method for the estimation of conjugated androstenedione

In initial experiments, the amount of conjugated androstenedione in plasma was assessed using the method for the estimation of the total amount of androstenedione in urine (Section 2.5). With this method, though, the values obtained when distilled water was put through the hydrolysis and chromatographic purification procedure were greater than was acceptable. Hence, a different method has been used as it was thought that this problem was caused by the ethyl acetate used for the hydrolysis stage. In the second method, the pH of the plasma was adjusted to 1, using M sulphuric acid. As addition of the acid caused precipitation of the proteins in the plasma, resulting in the plasma becoming gelatinous when incubated at 37° for 16h and a lower recovery of the androstenedione, the plasma was, therefore, diluted with distilled water. The ratio of water (1 ml) to plasma (0.5 ml) was found to be the optimum dilution at which the majority of the androstenedione was recovered from the plasma-water mixture. Attempts to precipitate the proteins present in the plasma with ethanol and remove them so that the plasma did not need to be diluted were unsuccessful.

with chromatography

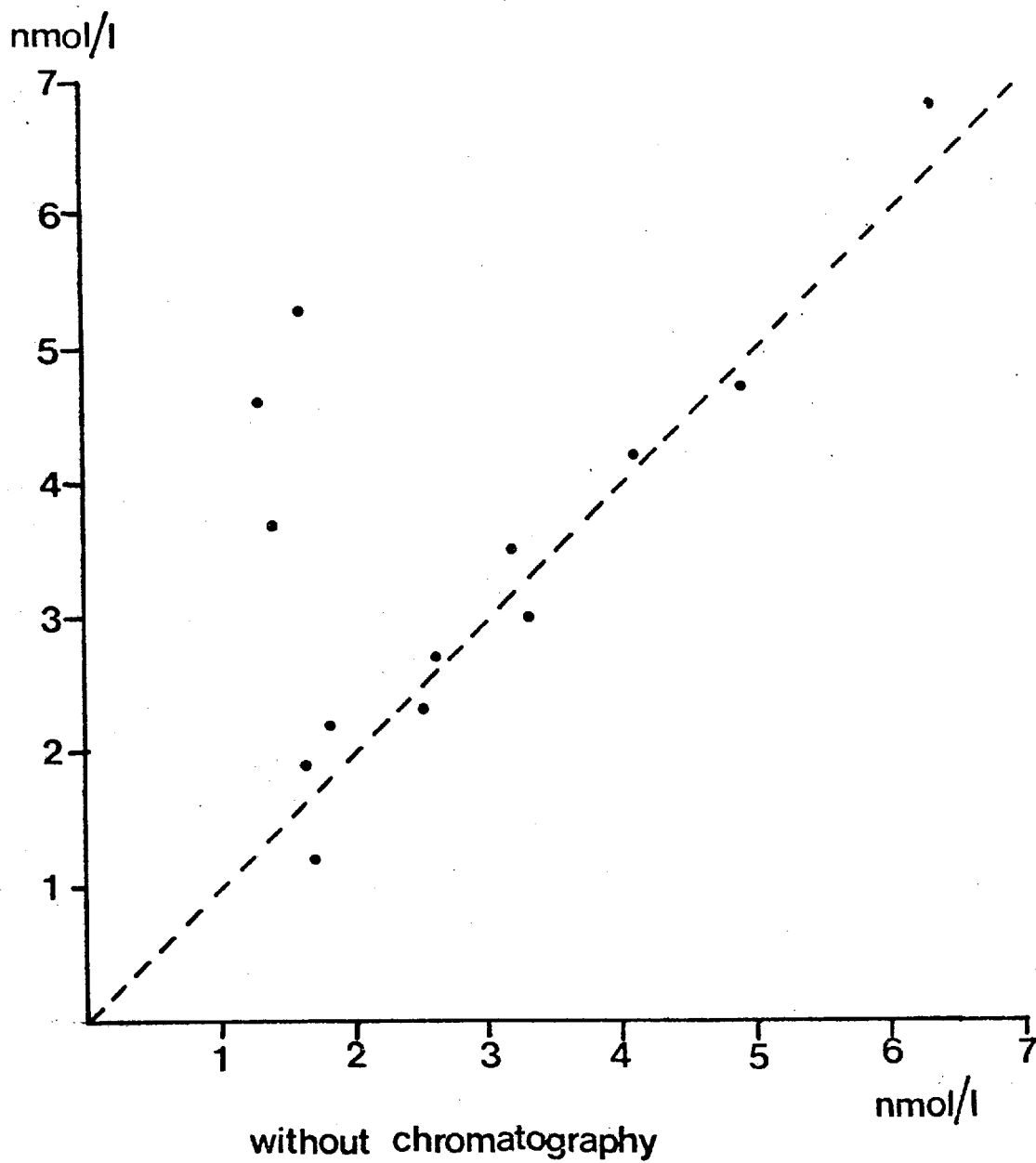


Fig 3.1 Comparison of values for conjugated androstenedione with and without paper chromatography

(i) Comparison of the amounts of conjugated androstenedione present, with and without a chromatographic purification stage

In order to determine whether chromatographic purification of the organic extracts of the plasma after hydrolysis was necessary, the amount of conjugated androstenedione was estimated in the same samples by methods, with and without a chromatographic purification stage.

Aliquots of plasma 2 x 0.3 ml for the method without chromatography and 2 x 0.5 ml for the method with chromatography were extracted with hexane:ether (4:1, 4 ml) and the organic extract removed. To the plasmas, for hydrolysis, followed by a chromatographic purification stage, phosphate buffer (1 ml), ^3H androstenedione (30,000 c.p.m.) in phosphate buffer (0.1 ml) and M sulphuric acid (3 drops) were added. The mixtures were incubated at 37° for 16h and extracted with hexane:ether (4:1, 4 ml). The organic extract was separated from the plasma and evaporated under nitrogen. The residue was purified by paper (Bush A) chromatography and the amount of androstenedione estimated by radioimmunoassay. To the plasma aliquots for which the amount of androstenedione was to be estimated without chromatographic purification, phosphate buffer (1 ml) and M sulphuric acid (3 drops) were added. The mixtures were incubated at 37° for 16h and extracted with hexane:ether (4:1, 4 ml). The amounts of androstenedione in 2 x 0.5 ml aliquots of the organic extract were estimated by radioimmunoassay.

The results (Fig. 3.1) show a good correlation between the values of androstenedione, with and without chromatographic purification. For 3 samples, the values obtained with chromatography are higher than the values estimated without chromatographic purification. This is a little surprising and may be due to methodological problems with the paper chromatography stage. For one of these samples the recovery from the paper chromatography stage was low but for the other 2 samples, it may have been due to non specific interference from the paper used for the chromatographic stage, although there was no detectable value in a water sample put through the procedure at the same time as the plasma samples.

These results indicate that for peripheral plasma samples, it is not necessary to purify the residue before radioimmunoassay to estimate the amount of androstenedione present.

(ii) Optimization of the pH for acid hydrolysis

The initial attempts to optimize the pH for the hydrolysis of conjugated androstenedione in plasma showed no relationship between the amount of androstenedione cleaved and pH. Poor duplicates were obtained for samples hydrolysed at the same pH. In an attempt to overcome this problem, the pH was adjusted in aliquots of diluted plasma which were then divided into 3 aliquots.

Plasma (1.2 ml) was extracted with hexane:ether (4:1, 5 ml) to remove the unconjugated androstenedione in the sample. Phosphate buffer (5 ml) and ^3H androstenedione (2000 c.p.m.) in phosphate buffer (0.1 ml) were added to the extracted plasma. The pH of the different aliquots were adjusted to values between 6.4 and 1.9. Aliquots (3 x 1 ml) of each of these solutions were incubated at 37° for 16h and extracted with hexane:ether (4:1, 4 ml) and the amount of androstenedione was estimated in this organic extract by radioimmunoassay.

This experiment was carried out on 2 different plasma pools. Fig. 3.2 shows the results for one of these pools. The reproducibility was better than that obtained in previous experiments. The optimum pH is between 3 and 4, although once the pH of the solution is greater than 4, hydrolysis occurs at greater rate. This is not surprising as an enol linkage would not be stable at acid pH. The replicates, in the samples between pH 3 and 4, were not as good as at higher or lower pH.

(iii) Optimization of time for acid hydrolysis

Aliquots of plasma (0.5 ml) were extracted with hexane:ether (4:1, 4 ml) to remove the unconjugated androstenedione. Phosphate buffer (1 ml), ^3H androstenedione (5000 c.p.m.) in phosphate buffer (0.1 ml) and M sulphuric acid (3 drops) were added and the mixtures were

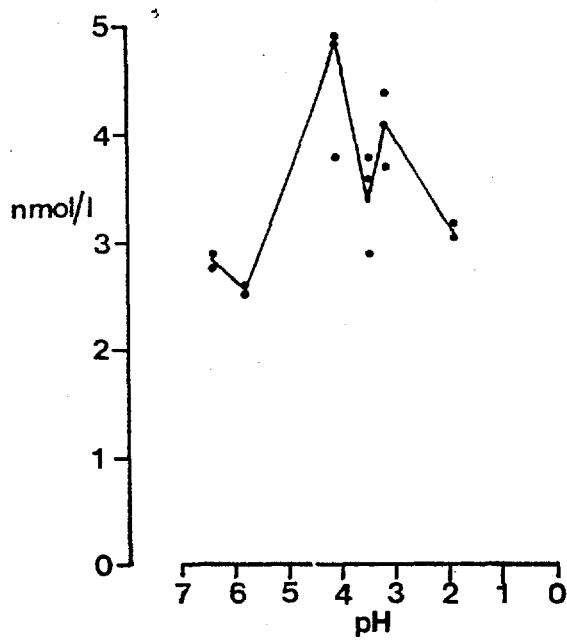


Fig3.2 Hydrolysis at varying pH

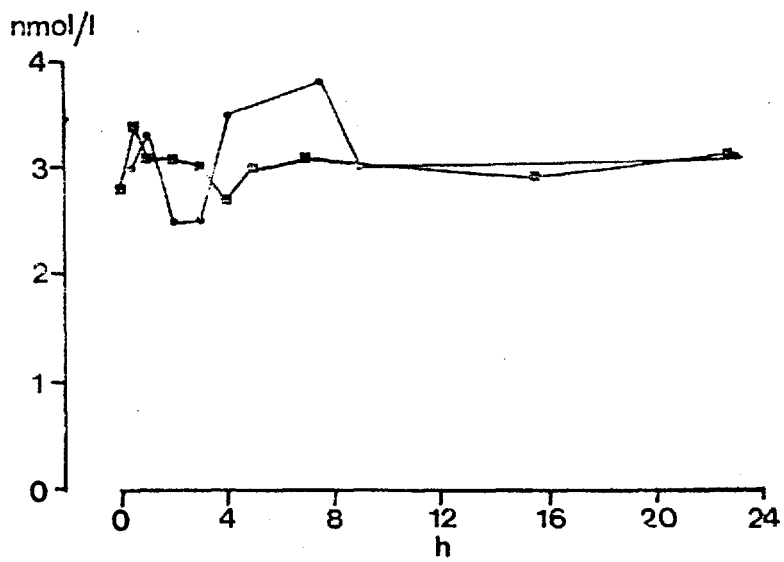


Fig3.3 Incubation of plasma with acid for various times

incubated at 37° for times between 0.5 and 23h after extraction with hexane:ether (4:1, 4 ml). The amount of androstenedione was estimated in aliquots of the extract (2 x 0.5 ml) by radioimmunoassay. 1 ml of the extract was transferred to a plastic counting vial to assess recovery.

The results obtained for 2 different pools are shown in Fig. 3.3. These values indicate that there appears to be little change in the amount of androstenedione that was cleaved with prolonged incubation at 37° . This may be a result of the enolic conjugation of the steroid molecule which is unstable under acidic conditions. The results show no decrease in the amount of androstenedione released with time, indicating that even for prolonged incubation no modification of the androstenedione present in solution occurs.

(iv) Carryover from the unconjugated to the conjugated fraction

When the method for the measurement of unconjugated androstenedione in plasma was developed, recovery experiments for the extraction of the unconjugated androstenedione from the plasma indicated that all the unconjugated androstenedione was extracted into the hexane:ether organic layer (Goodall, Rippon and James, 1979). The problem, though, with measuring the conjugated fraction is different, as although total extraction from the plasma into the organic phase occurs, this extract then has to be completely removed. This can be done in two ways.

(i) By removal of the hexane:ether extract by pasteur pipette, in which case a small amount of the hexane:ether extract is left above the plasma as it is impossible to remove the organic phase completely without removing some of the plasma. The hexane:ether extract may also retain some of the aqueous phase which could lead to a reduction in the amount of androstenedione obtained after hydrolysis. (ii) By freezing the plasma-buffer mixture in dry ice-ethanol bath and decanting the organic extract. This has the advantage that any water

trapped in the hexane:ether extract is frozen out but the hexane:ether can be trapped in the frozen plasma, as shown by a small amount of hexane:ether which was present above the plasma-buffer mixture when it had been thawed.

Attempts to quantify this problem have been made by several experiments, estimating the different amounts of conjugated androstenedione cleaved, after either 1 or 2 extracts of the unconjugated androstenedione, and also estimating the unconjugated androstenedione in both of these extracts.

The results obtained from these experiments indicated that the problem was more complicated and that it would be impossible to determine conclusively the carryover between the 2 fractions until labelled conjugated androstenedione is available. In order to determine that androstenedione is present in a conjugated form in plasma, it is important to ensure that no unconjugated androstenedione is retained by the plasma prior to hydrolysis of any conjugated androstenedione present. For this reason, all plasma samples have been hydrolysed after they have been extracted twice with hexane:ether. This could lead to a reduction of the values after hydrolysis due to aqueous phase being retained in the organic phase. Hence, values obtained are likely to be an underestimate rather than an overestimate.

(v) Effect of temperature on the amount of unconjugated androstenedione

The effect of leaving plasma or serum samples at room temperature on the amount of unconjugated androstenedione present in the samples is important for determining the best conditions for transport. This is especially important in view of the fact that androstenedione is present in a conjugated form, as by analogy with the amount of unconjugated androstenedione present in urine an increase might occur.

unconjugated
androstenedione
nmol/l

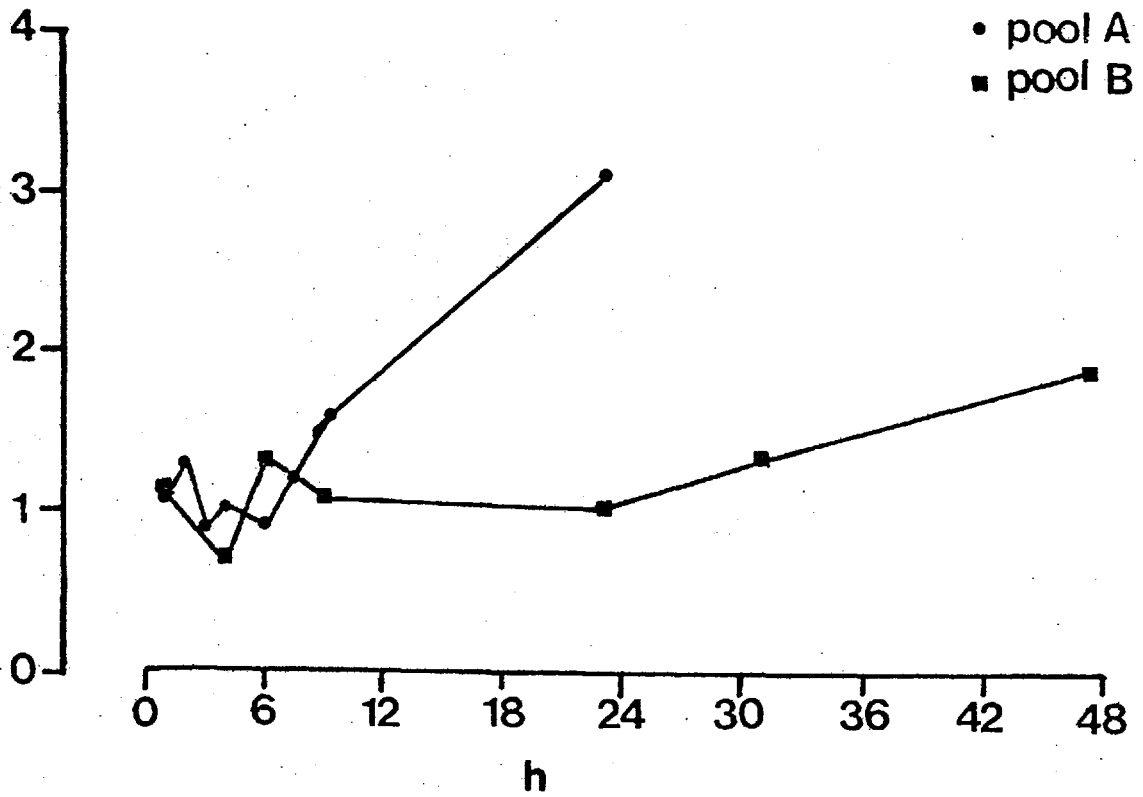


Fig3.4 Effect of incubating plasma at 37°

Wickings and Nieschlag (1976) have studied the stability of androstenedione in plasma under various conditions. In pooled blood samples left unseparated, at room temperature for varying time periods up to 3 days before being centrifuged, androstenedione values showed only small insignificant variations. In plasma from pooled blood samples left at room temperature for up to 24h, there was no significant change in the amount of androstenedione or in the amount present after repeated freezing and thawing of the samples. As many samples may take longer than 24h to reach a laboratory, especially if they are being sent through the post, this problem has been investigated.

Aliquots of plasma from 2 different pools (0.3 ml for first experiment and 0.5 ml for the second), after removal of any unconjugated androstenedione present, were incubated at 37° for varying times and extracted with hexane:ether (4:1, 4 ml). The amount of androstenedione in the extract (2 x 0.5 ml) was estimated by radioimmunoassay.

For Pool A for the first 6h, the values were near the limit of detectability but there was an increase from 8 to 23h (Fig. 3.4). In Pool B, again there was no increase in the values until 10h after the start of the experiment, after which an increase occurred.

Although the results from these experiments are not very conclusive, they would indicate that hydrolysis of the conjugated fractions could occur to give increased unconjugated values under very extreme conditions, and hence, caution should be observed in the handling of samples whenever possible

3.3 Use of sulphatase (Helix Pomatia) for the hydrolysis of conjugated androstenedione

As this sulphatase preparation hydrolyses conjugated androstenedione in urine, its possible use for the hydrolysis of conjugates in plasma has been investigated. This sulphatase is a fairly crude

Table 3.1 Amount of androstenedione (nmol/l) released by hydrolysis from plasma with sulphatase (Helix Pomatia)

Sample	Hydrolysis		Unconjugated Androstenedione
	Incubation with sulphatase	4 ^o Overnight (no enzyme added)	
1	1.8 } 2.0 2.3 {	0.5 } 0.4 0.4 {	7.2
2	1.3 } 1.1 1.0 {	0.4 } 0.4 0.4 {	3.7
3	2.0	0.4	8.7
4	1.7 } 1.6 1.5 {	<0.3 } <0.3 <0.3 {	6.2
5	2.1	0.6	10.9

preparation from *Helix Pomatia*, which also contains β -glucuronidase, and hydrolyses preferentially $3\beta, \Delta^5$ and $3\beta, 5\alpha$ configurations rather than $3\alpha, 5\alpha$ and $3\alpha, 5\beta$ configurations (Bradlow, 1970; Roy, 1970).

An initial experiment in which aliquots of plasma were incubated with sulphatase (20 I.U.) after the unconjugated androstenedione had been extracted, indicated that the conjugated steroid was cleaved by sulphatase. The results (Table 3.1) indicate that this cleavage was as a result of the presence of the sulphatase, as in samples which were incubated under the same condition but to which no sulphatase had been added, the amounts of androstenedione present were just above the limit of detection for the assay.

(i) Optimization of incubation time for hydrolysis

Aliquots of pooled plasma samples (2 x 0.5 ml) were extracted with hexane:ether (4:1, 2 x 4 ml). Sulphatase (*Helix Pomatia*, 20 I.U.) in acetate buffer (0.5 ml) was added and the sample incubated at 37° for various times up to 23h. The mixture was extracted with hexane:ether (4:1, 4 ml) and in 2 x 1 ml of the extract the amount of androstenedione was estimated by radioimmunoassay.

The results (Pool A, Fig. 3.5) indicate that increased amounts of conjugate were hydrolysed with increasing time, but that maximum release had not been obtained at 23h. The experiment was, therefore, repeated, incubating the plasma with sulphatase for a longer time (Pool B, Fig. 3.5). This experiment indicated that after 25h there was no significant increase in the amount of conjugated androstenedione cleaved. These results indicate that a minimum of 24h incubation time is required for the hydrolysis of conjugated androstenedione in plasma using sulphatase.

(ii) Optimization of the amount of sulphatase for hydrolysis

Aliquots of pooled plasma (0.5 ml) were extracted with hexane:ether (4:1, 2 x 4 ml). Aliquots of sulphatase (20 I.U.) in acetate

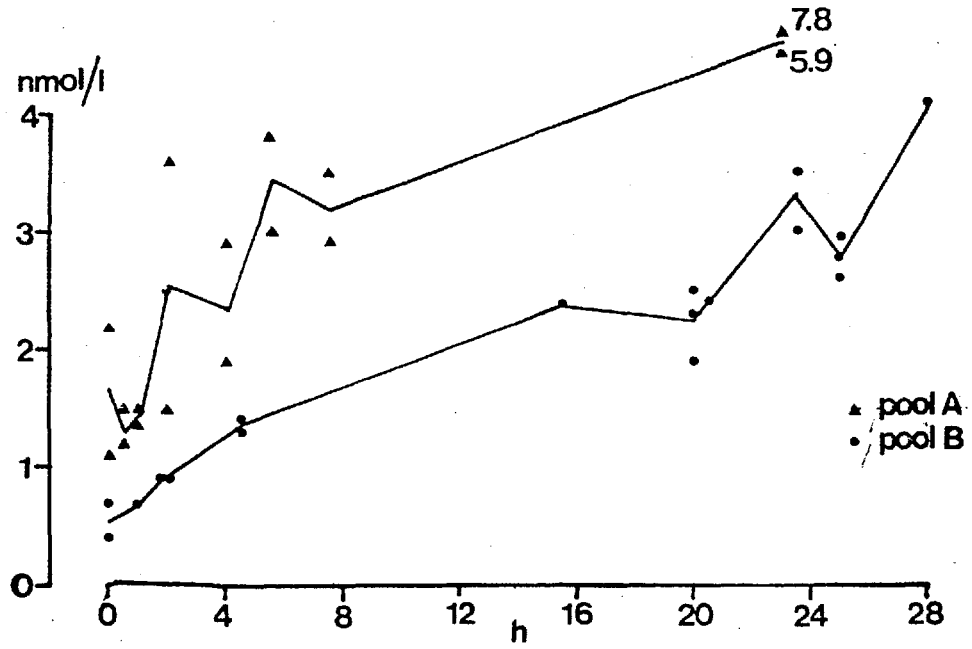


Fig3.5 Incubation of plasma with sulphatase for various times

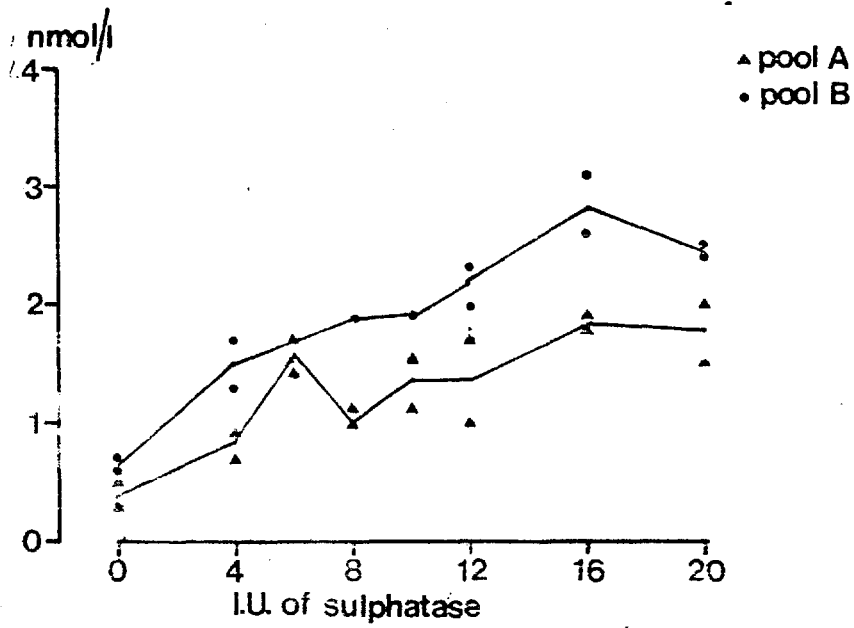


Fig3.6 Incubation of plasma with different amounts of sulphatase

buffer (0.5 ml) were added, such that the units of sulphatase between 0 and 20 were added to aliquots of plasma. The total volume of acetate buffer added to each sample was made up to 0.5 ml. The samples were incubated at 37° for 16h and extracted with hexane:ether (4:1, 4 ml). The amount of androstenedione was estimated by radioimmunoassay in 2 x 1 ml of this extract.

The results for 2 different pools (Fig. 3.6) indicate that the maximum amount of androstenedione was released when between 16 and 20 units of sulphatase were added.

(iii) Comparison of amounts cleaved by acid and sulphatase hydrolysis methods

The amounts of conjugated androstenedione in 10 plasma samples have been estimated using both acid and sulphatase hydrolysis methods. The amounts of androstenedione after cleavage with sulphatase have been estimated, with and without chromatographic purification of the residue, before radioimmunoassay.

The aliquots of plasma (0.5 ml) after removal of the unconjugated androstenedione were hydrolysed by sulphatase (Helix Pomatia, 20 I.U.), by incubating in acetate buffer for 24h at 37° . The plasma-buffer mixture was extracted with hexane:ether (4:1, 4 ml). The extracts, for chromatography, were purified, using the t.l.c. method given for adrenal vein samples (Section 3.4, ii). For the samples for estimation of the amount of androstenedione present after sulphatase hydrolysis without chromatography, 2 x 1 ml of the extracts were assayed by the method for the estimation of androstenedione in plasma. The samples hydrolysed using acid, were assayed by the method for the estimation of the amount of conjugated androstenedione in peripheral blood samples (Section 3.4, i).

The results from the comparison of these methods are shown in Table 3.2, together with the amounts of unconjugated androstenedione in the same samples. These results indicate that the amount of androstenedione present after hydrolysis with acid, was 50% of that after hydrolysis with sulphatase, the mean for values by acid

Table 3.2 Comparison of the amounts of conjugated androstenedione
(nmol/l) cleaved by acid and sulphatase hydrolysis methods

Unconjugated	Conjugated		
	Sulphatase		Acid
	Without chromatography	With chromatography	
9.4	2.0 } 2.4 2.9 {	2.1 } 2.3 2.5 {	2.5 } 1.9 1.3 {
7.7	2.1 } 2.2 2.3 {	2.4 } 2.1 1.9 {	1.4 } 1.4 1.4 {
7.1	2.4 } 2.4 2.4 {	2.3 } 2.4 2.5 {	1.3 } 1.4 1.6 {
5.6	2.9 } 2.6 2.4 {	1.9 } 1.9 2.0 {	1.4 } 1.2 1.1 {
9.1	9.3 } 9.4 9.5 {	7.3 } 7.0 6.7 {	1.4 } 1.3 1.3 {
5.6	1.8 } 1.7 1.6 {	1.9 } 2.1 1.3 {	1.0 } 0.9 0.9 {
4.3	1.5 } 1.5 1.6 {	2.0 } 2.2 2.5 {	0.7 } 0.7 0.8 {
5.0	1.9 } 1.8 1.7 {	2.3	0.8 } 0.7 0.7 {
5.8	1.7 } 1.5 1.3 {	1.7 } 1.9 2.2 {	0.9 } 0.9 1.0 {
25.8	3.7 } 4.6 5.5 {	2.0 } 2.1 2.2 {	2.6 } 2.6 2.7 {

hydrolysis being 1.3 nmol/l and using sulphatase, without chromatography of the residue 3.0 nmol/l and with chromatography 2.6 nmol/l. The values obtained with chromatography are similar to those obtained without chromatography except for samples which had high values. This difference in the amounts of conjugate cleaved to give androstenedione, may be a result of the formation of polar artefacts during the cleavage with acid. This formation of artefacts probably occurs during the removal of the conjugating acid, as previous experiments showed that prolonged incubation did not decrease the amount of androstenedione present. Until radiolabelled conjugates are available to estimate the amount of conversion of conjugated to free androstenedione, using different hydrolysis methods, the reason for the discrepancy cannot be determined absolutely.

3.4 Values in normal subjects and subjects with pathological disorders

(i) Peripheral samples

The method used for this preliminary investigation into the amount of conjugated androstenedione present in plasma, was the acid hydrolysis method. More recent work, though, has indicated that a greater amount of conjugated androstenedione present in plasma is hydrolysed to androstenedione when sulphatase is used. Hence, the values given in this section are possibly an underestimate.

Aliquots of plasma (0.5 ml) and distilled water (1 ml) were dispensed on the Hamilton diluter, and extracted with hexane:ether (4:1, 2 x 4 ml) to remove the unconjugated androstenedione in the sample. The first extract was usually retained for the estimation of the amount of unconjugated androstenedione by the usual plasma method. M sulphuric acid (3 drops) was added to the plasma-buffer mixture and after mixing this was incubated at 37° for 16h. The mixture was extracted with hexane:ether (4:1, 4 ml) and 2 x 1 ml of the extract was assayed by radioimmunoassay, using the method for the estimation of amount of unconjugated androstenedione present in plasma.

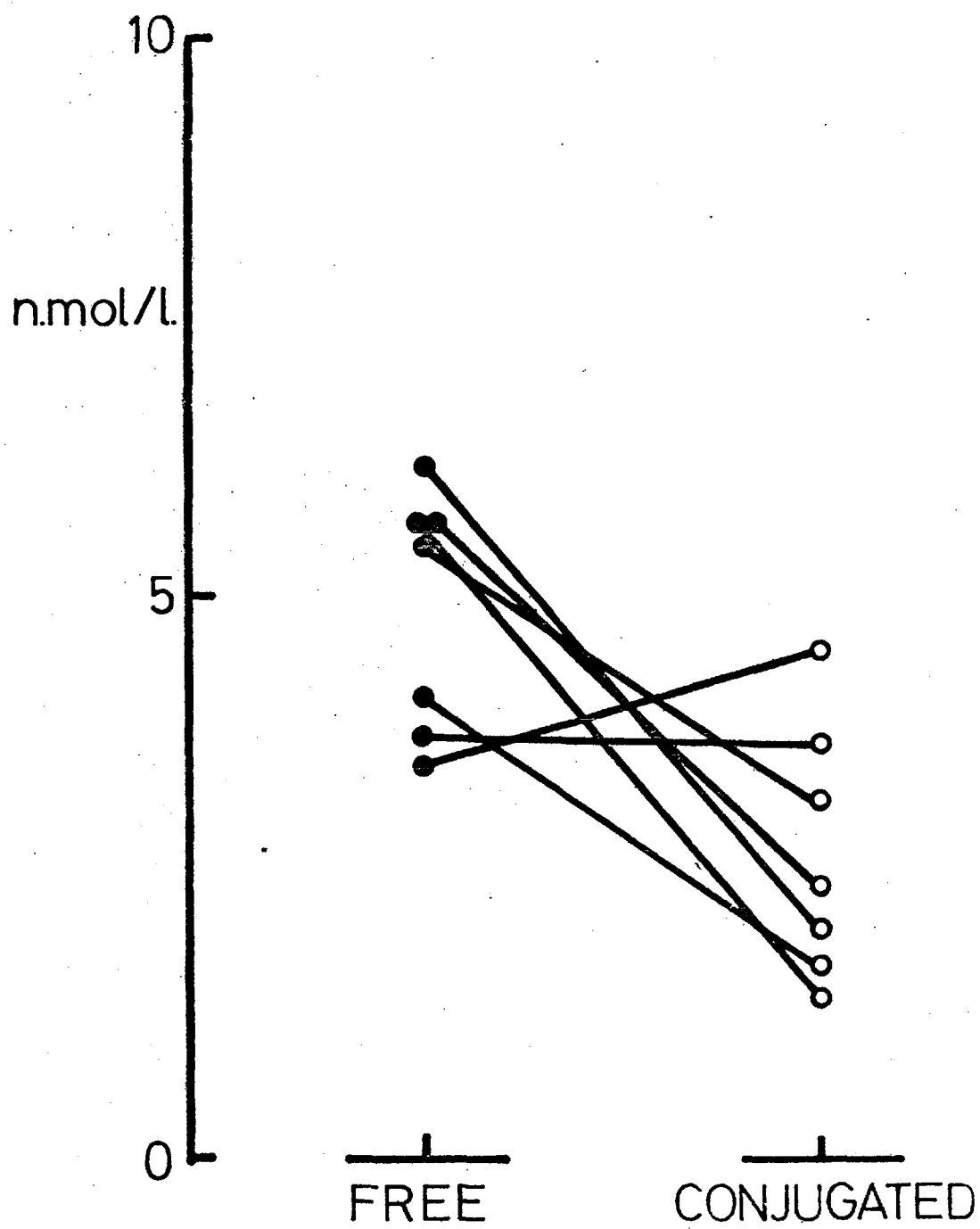


Fig3.7 Free and conjugated androstenedione levels in normal subjects

This method has been used to determine the values of conjugated androstenedione in the plasma from various subjects, in an attempt to determine the source of this conjugated steroid.

Normal subjects

The amount of conjugated androstenedione has been measured in 7 normal subjects (1 female and 6 male, aged 22-39). The results are shown in Fig. 3.7, together with the values of unconjugated androstenedione in the same subjects. These samples were all single samples taken at 14.30h. The unconjugated androstenedione values range from 3.5 nmol/l to 6.2 nmol/l and are within the normal range. The values of conjugated androstenedione in the plasma samples are of the same order as the values of unconjugated androstenedione, although in all but 1 subject, the values are lower than those for the amount of unconjugated androstenedione (3.7 nmol/l to 1.2 nmol/l). These values are approximately 45% of the unconjugated androstenedione values

Profiles from 2 postmenopausal subjects

For 2 postmenopausal subjects, conjugated and unconjugated androstenedione have been measured in plasma samples taken at $\frac{1}{2}$ h intervals from 04.00 to 08.30h (Fig. 3.8). For both these subjects, the values for unconjugated androstenedione show a typical rise in the early morning. A similar rise is seen in the values for conjugated androstenedione, the increase in values being greater for subject B than A.

These results indicate that either conjugated androstenedione is secreted under the same stimulus as unconjugated androstenedione, or that conjugated androstenedione is a metabolite of unconjugated androstenedione.

Profile from a subject being investigated for hyperprolactinaemia

Plasma samples were taken at $\frac{1}{2}$ h intervals from this subject from 05.00 to 12.00h, tetracosactrin (250 μ g, intravenously) was given at

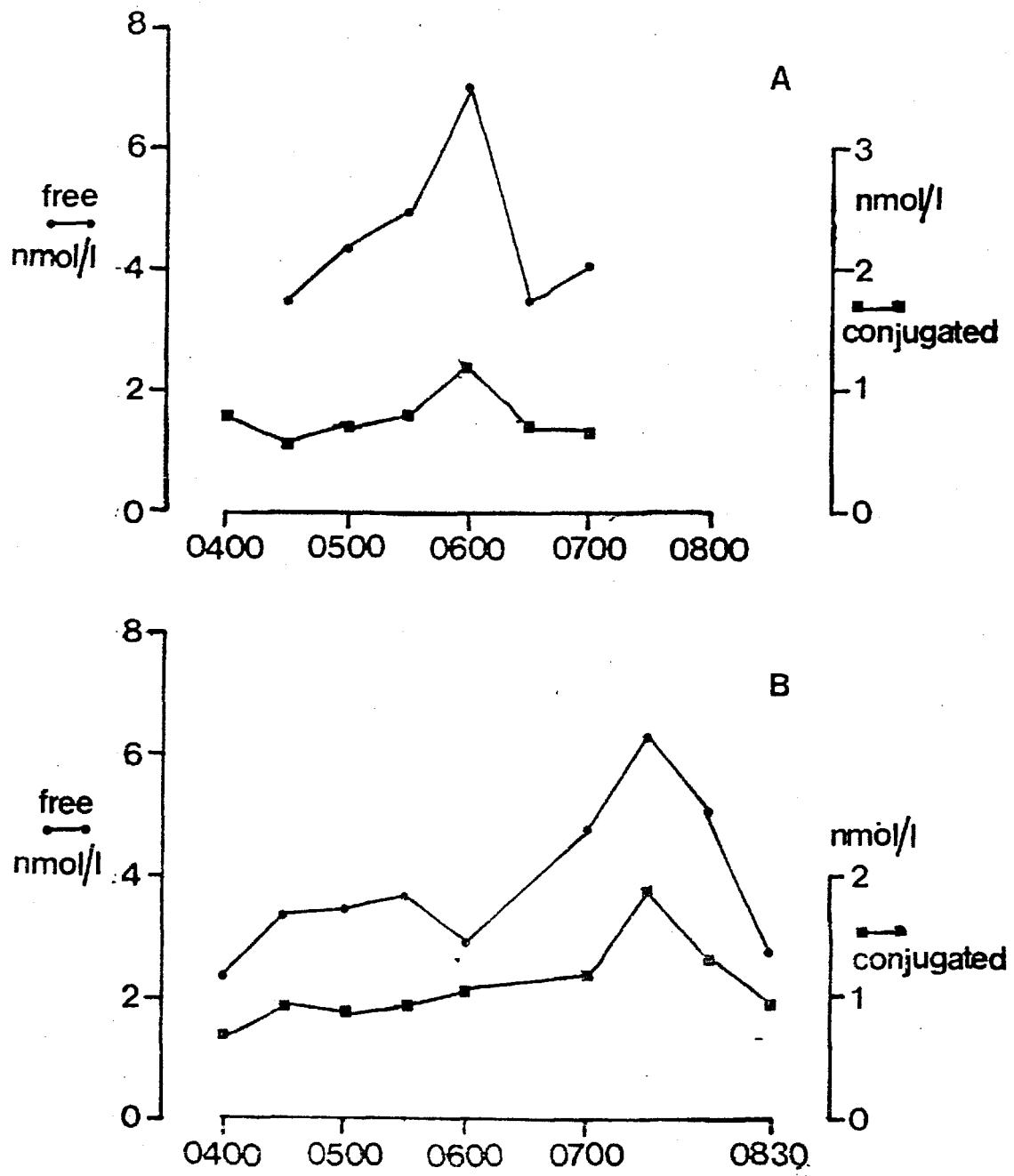


Fig3.8 Free and conjugated androstenedione in plasma from 2 postmenopausal subjects

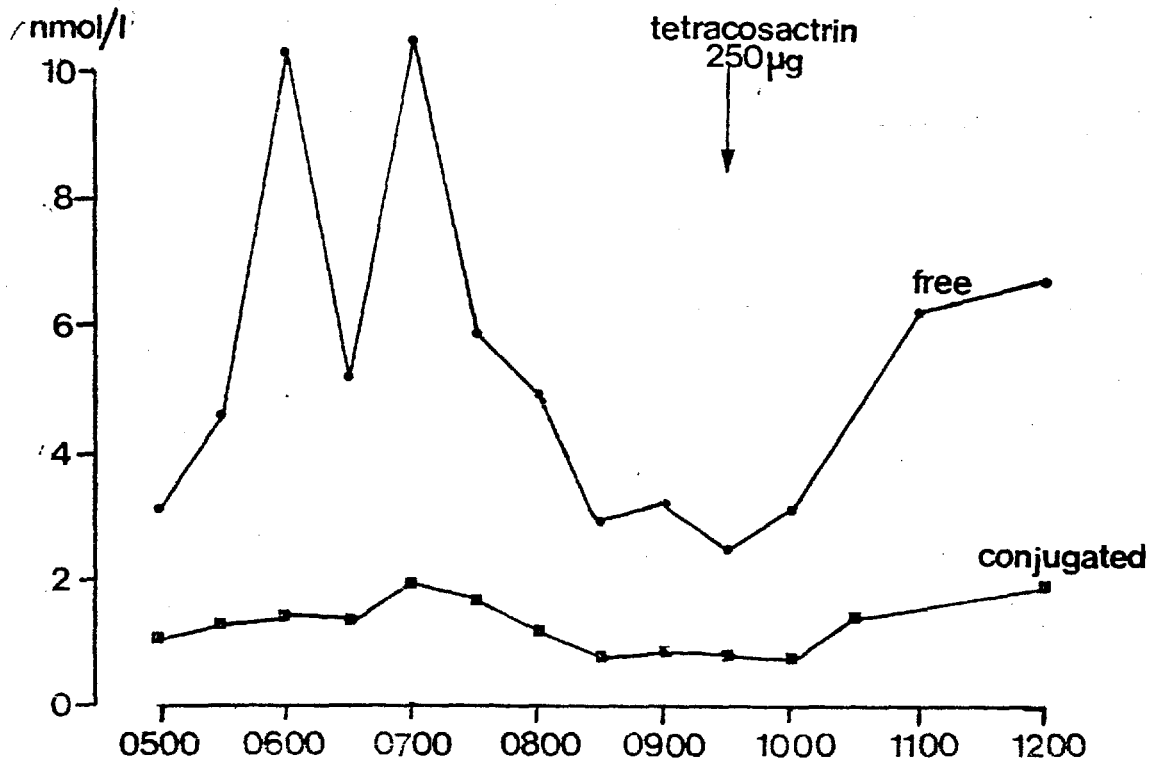


Fig3.9 Free and conjugated androstenedione levels in plasma

09.30h. Conjugated and free androstenedione values have been estimated in these samples (Fig. 3.9). The values for conjugated androstenedione are in the range 1.1-1.7 nmol/l. The increase of unconjugated androstenedione in this subject is again mirrored by an increase in conjugated androstenedione values, and both values also rise after the administration of tetracosactrin.

Profile from a subject who was infused with tetracosactrin

As a result of the findings for the conjugated androstenedione values in the subject, who had hyperprolactinaemia, conjugated androstenedione has been measured in a subject who has been infused with tetracosactrin for a period of 6h after the suppression of adrenal function with dexamethasone. This subject was a 42y old postmenopausal lady who was 4y postovariectomy. At 24.00h the subject was given dexamethasone (2 mg) and a further 0.5 mg at 8.00h and 16.00h. Tetracosactrin was infused at a rate of 3 µg/h for 6h, from 08.30h to 14.30h. The results for conjugated and unconjugated androstenedione are shown in Fig. 3.10.

After the start of the infusion the unconjugated androstenedione values rise rapidly, as would be expected, and reach a peak at 14.00h, although the values do not form a plateau. After the infusion had ceased the values fell rapidly. The increase in the values for conjugated androstenedione is more gradual and smoothly reach a maximum value at 14.00h. After the end of the infusion, they decline at a slower rate than the values for unconjugated androstenedione. This may be a reflection of the different rates of clearance of conjugated and unconjugated steroids from the peripheral circulation, and may possibly be due to the binding of the conjugated steroid to plasma proteins, as has been found for other conjugated steroids (Fehér and Halmy, 1974).

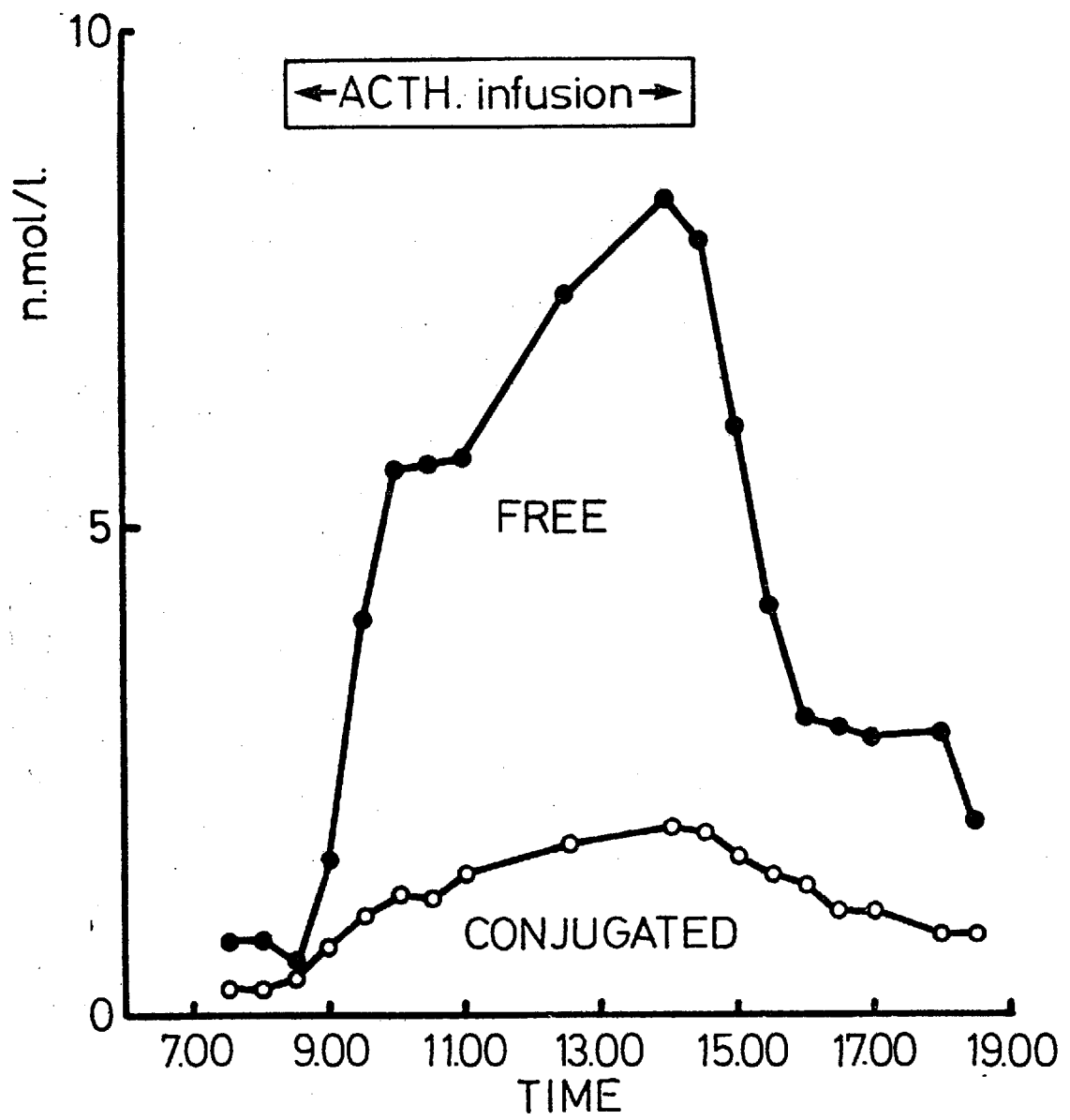


Fig 3.10 Free and conjugated androstenedione levels
in a subect during the infusion with ACTH

Profile of subjects who had been infused with androstenedione

The increase in conjugated androstenedione which mirrors the increase in unconjugated androstenedione, could be because conjugated androstenedione is a metabolite of free androstenedione.

In an attempt to assess how much interconversion occurs between the conjugated and the unconjugated fractions, their values have been measured in peripheral plasma samples from 2 subjects who were infused with androstenedione.

The first subject was a 47y old women, who had been ovariectomised 11y previously. This subject was given dexamethasone (1.5 mg) daily for 1 week and then infused with androstenedione at a rate of 250 µg/h for 6h. The results are shown in Fig. 3.11. After the start of the infusion there is a marked increase in the amount of circulating unconjugated androstenedione, as would be expected, but there is only a very slight increase in the conjugated androstenedione in the same samples, indicating that little conversion is occurring between unconjugated and conjugated androstenedione. As dexamethasone had been given for 1 week, this may have resulted in depletion of the body's stores of both conjugated and unconjugated androstenedione, and larger amounts of androstenedione may be needed to replenish these stores, before conversion can be demonstrated, than was given.

The plasma conjugated and unconjugated levels have, therefore, been measured in a subject who had been infused with androstenedione but to whom dexamethasone was not given. The results shown in Fig. 3.12, indicated that again no increase occurred in the conjugated plasma androstenedione values, even though a marked increase occurs in the unconjugated levels. The plasma cortisol values in the same samples are also shown in Fig. 3.12, and during most of the period the changes in cortisol levels are mirrored by those of conjugated andro-

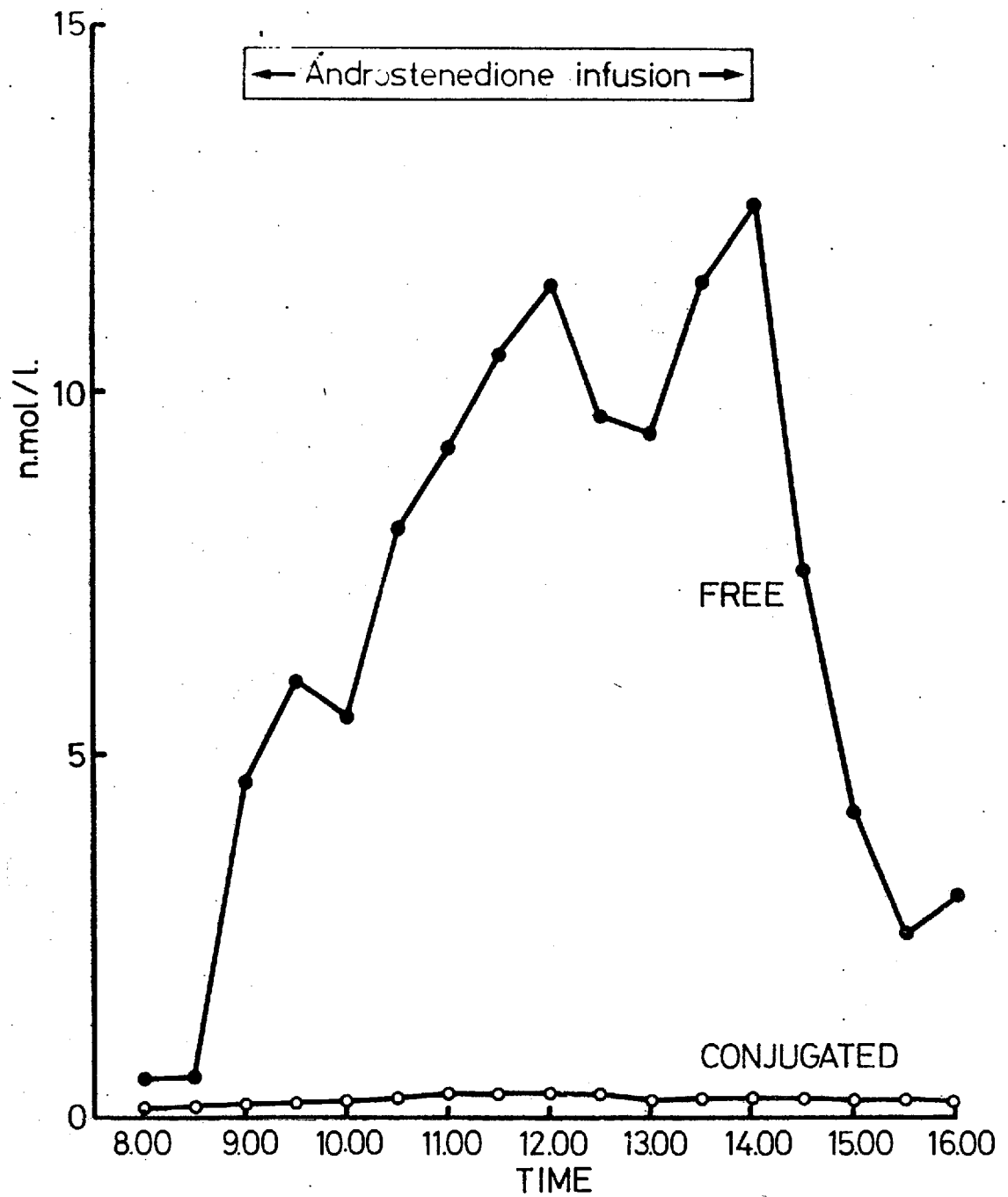


Fig 3.11 Levels , during the infusion of androstenedione, of free and conjugated androstenedione

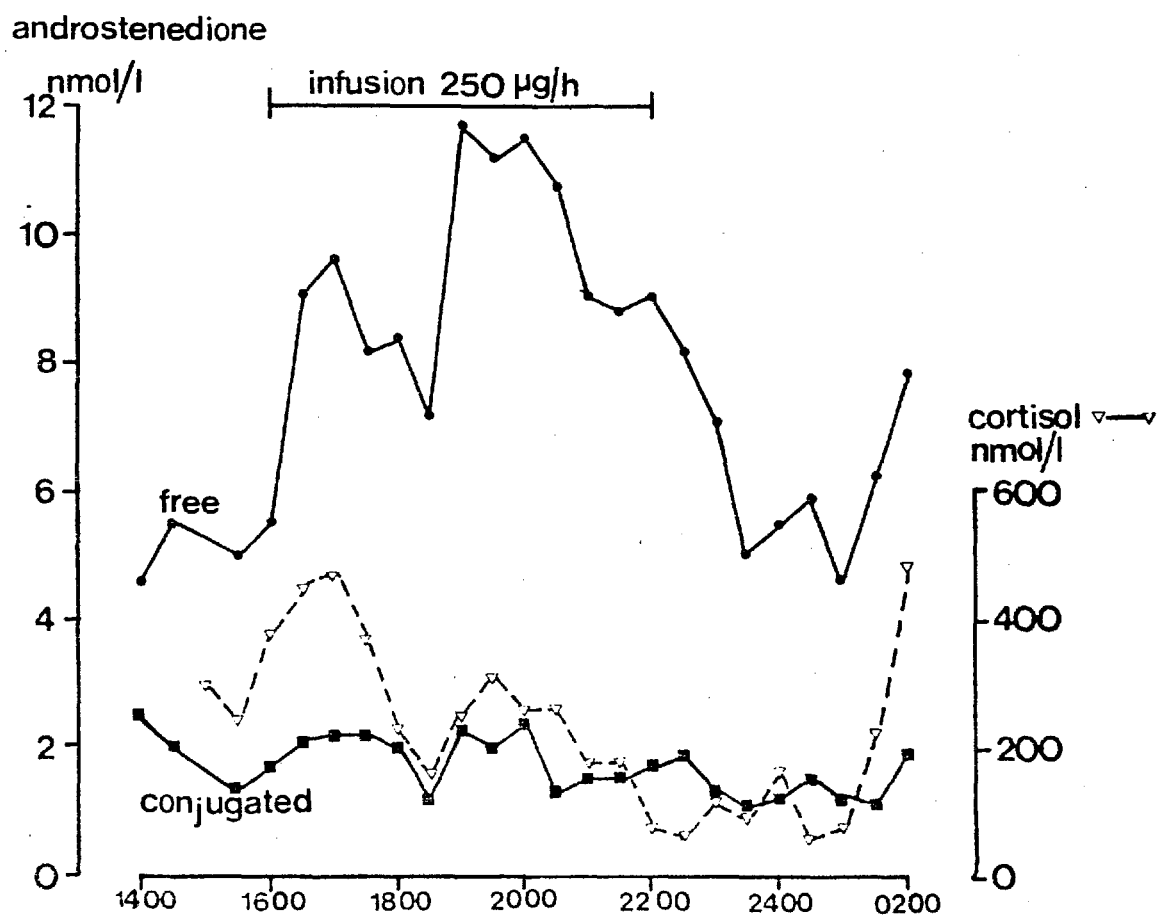


Fig 3.12 Androstenedione and cortisol levels during the infusion of androstenedione

stenedione. These results would appear to indicate that conjugated androstenedione is a direct secretion product of the adrenal gland and is not a metabolite of androstenedione produced by conjugation in the periphery.

(ii) Adrenal vein samples

In order to determine that conjugated androstenedione is being secreted by the adrenal gland, a difference in values must be shown between blood samples taken from the adrenal vein and samples from the periphery at the same time. As the blood from the adrenal vein is likely to contain other steroids in larger amounts than in the periphery, which might crossreact with the antibody in the radioimmunoassay, the amount of conjugated androstenedione has been estimated in adrenal vein samples after chromatographic purification of the extract from plasma.

For the samples from the first subject, a Bush A paper chromatographic purification system was used, but there were methodological problems with this system which caused the value, when water was put through the procedure, to be detectable in the radioimmunoassay. Hence, for all subsequent sets of samples the amount of androstenedione was estimated after purification by t.l.c. using the method given below.

The amount of conjugated androstenedione has been estimated in adrenal vein blood samples from 5 subjects, who had been catheterised for various reasons, including Cushing's syndrome, Conn's syndrome and adrenal tumours

Unconjugated androstenedione has been measured using the method described previously (Goodall et al., 1979). Plasma cortisol and aldosterone have been measured by specific radioimmunoassay methods (see Appendix).

Method for the measurement of conjugated androstenedione in plasma with t.l.c. purification

Various t.l.c. systems were tried for the separation of androstenedione from androsterone and aetiocholanolone, as they are structurally the most similar steroids likely to be present. The systems which were tested, by running standard on aluminium backed, silica gel thin layer plates, were toluene:acetone, 1:1; toluene:acetone, 1:2; chloroform:methanol, 9:1; ethyl acetate:acetone, 9:1; ether:acetone, 9:1 and ether. Of these systems, ether:acetone, 9:1, gave the best separation and was used for purification of the adrenal vein samples.

For the first few sets of adrenal vein and peripheral samples that were assayed, using a t.l.c. purification stage, extracts from hydrolysed plasma were assayed with and without a purification stage.

Aliquots of plasma (0.1, 0.3 or 0.5 ml, in quadruplet, 2 samples for assay after chromatography and 2 samples for assay without chromatography) were dispensed using the Hamilton diluter, with distilled water (1 ml). The aliquots were extracted with hexane:ether (4:1, 2 x 4 ml). ^3H androstenedione tracer (2,200 c.p.m.) in distilled water (0.1 ml) and M sulphuric acid (3 drops) were added to all samples and after mixing they were incubated at 37° overnight (16h). The samples were extracted with hexane:ether (4:1, 4 ml).

Samples for assay without chromatography

Hexane:ether extract (2 x 1 ml) were transferred into binding tubes for estimation of the amount of androstenedione by the plasma androstenedione method. 1 ml of the extract was also taken into a plastic scintillation vial to estimate the recovery.

Samples for assay with chromatography

The hexane:ether extract was separated and evaporated under nitrogen. The residue was redissolved in the dichloromethane:methanol (1:1, 3 drops) and applied to a silica t.l.c. plate. The residues were chromatographed in an ether:acetone system (9:1),

Table 3.3 Conjugated and unconjugated androstenedione
(nmol/l) and cortisol (nmol/l) for subject F.S.

Sample	Androstenedione				Cortisol		
	Conjugated		Unconjugated				
	With chromatography	Without chromatography					
Left adrenal	59.0 } 40.7 {	49.9	16.4 } 29.7 {	23.1	300.7 } 240.5 {	270.6	2730
Left adrenal	145.9 } 28.6 { 66.9 {	80.4	12.3 } 14.0 {	13.1	244.8 } 251.7 {	248.3	3035
Peripheral at time of left adrenal	-		2.7 } 2.4 {	2.5	21.9 } 26.6 {	24.2	880
Right adrenal	13.0 } 9.9 {	11.5	5.5 } 7.3 {	6.4	67.1 } 60.1 {	63.6	1300
Peripheral at time of right adrenal	1.9 } 1.2 {	1.6	2.8 } 2.8 {	2.8	24.2 } 23.8 {	24.0	800

together with an ^3H androstenedione marker in a separate lane of the plate, until the solvent front reached the top of the plate (ca. 1.5h). The areas of activity were located using the β -graph scanner, cut out and extracted with ether (6 ml) by shaking, leaving at 4° overnight and shaking again. This extract (2 x 1 ml) was transferred to glass binding tube for radioimmunoassay by the usual plasma method. 1 ml was taken to assess recovery.

Subject F.S.

This 22y old female subject was diagnosed as having adrenal hyperplasia. Conjugated and unconjugated androstenedione have been measured in adrenal and peripheral vein samples as well as cortisol. The values are given in Table 3.3. The amounts of conjugated androstenedione were estimated with and without paper (Bush A) chromatographic purification. The results obtained after chromatography were higher than those obtained without chromatography, which was somewhat surprising, although the values still indicate that there was a gradient between peripheral and adrenal vein samples. It was because of these surprising results that the chromatographic purification stage was changed from paper to t.l.c. The duplicate or triplicate values obtained for these samples are not very acceptable, and this may be the reason why values are higher after chromatography.

Subject L.G.

This subject was a hirsute postmenopausal lady who was being investigated for an adrenal tumour. Conjugated androstenedione has been determined with and without a t.l.c. chromatographic purification stage. There is a gradient between the 2 peripheral samples and the low and high inferior vena cava samples (Table 3.4) and the samples obtained from the adrenal veins, indicating that androstenedione is secreted in a conjugated as well as an unconjugated form by the

Table 3.4 Conjugated and unconjugated androstenedione (nmol/l)
in a subject (L.G.) with a left adrenal tumour

Sample	Conjugated		Unconjugated	
	With chromatography	Without chromatography	With chromatography	Without chromatography
Low I.V.C.	2.8 } 1.8 { 2.3	2.4 } 2.3 { 2.4	5.7	
High I.V.C.	2.6 } 2.0 { 2.3	2.7 } 2.2 { 2.5	6.8	
Peripheral at time of left adrenal	2.0 } 3.7 { 2.8	1.1 } 1.1 { 1.1	9.9	
Left adrenal	11.0 } 13.6 { 12.3	22.9 } 21.5 { 22.2	447.6	
Peripheral at time of right adrenal	2.5 } 4.1 { 3.3	2.0 } 2.0 { 2.0	8.5	
Right adrenal	10.7 } 10.9 { 10.8	13.2 } 13.5 { 13.3	279.7	

Table 3.5 Conjugated and unconjugated androstenedione,
cortisol and aldosterone in a subject (M.P.)
with suspected Conn's syndrome

	Androstenedione (nmol/l)		Cortisol (nmol/l)	Aldosterone (pmol/l)
	Conjugated	Unconjugated		
Lower I.V.C.	0.8	9.0	153	220
Hepatic	1.0	1.9	70	85
R. Renal	0.7	9.5	107	45
L. Suprarenal	2.1	286.7	1072	410
L. Renal	1.4	93.7	755	150

Table 3.6 Conjugated and unconjugated androstenedione,
cortisol and aldosterone in a subject (M.G.)
with suspected Conn's syndrome

Sample	Androstenedione (nmol/l)		Cortisol (nmol/l)	Aldosterone (pmol/l)	
	Conjugated	Unconjugated			
Left adrenal vein (11.30)	1.6 } 2.8 {	2.2	47.5	307	620
Left adrenal vein (11.40)	6.9 } 6.5 {	6.7	447.5	3025	3086
I.V.C. near right suprarenal	4.5 } 2.6 {	3.5	86.7	735	3780
I.V.C. near right suprarenal	2.5 } 2.6 {	2.6	26.3	538	1900
High I.V.C.	4.6 1.7		15.7	313	840
Low I.V.C.	1.2 } 1.5 {	1.4	2.2	174	270

adrenal gland. The left adrenal vein had increased amounts of conjugated and unconjugated androstenedione. Again, in these results, the amount of conjugated androstenedione in the peripheral samples was higher with chromatography than without, but the duplicates obtained for these samples with chromatography are poor compared with the duplicates obtained for the other samples.

Subject M.P.

This female subject (51y old) had hypertension and hypokalcaemia. The values were determined using t.l.c. chromatographic purification. The values for conjugated and unconjugated androstenedione, cortisol and aldosterone are shown in Table 3.5. There was again a gradient between the amount of conjugated androstenedione in the peripheral and adrenal veins. The values for conjugated androstenedione are elevated in the same samples as the value for cortisol and androstenedione, which would seem to indicate that conjugated androstenedione is secreted in response to the same stimulus as cortisol and unconjugated androstenedione, that is, in response to ACTH.

Subject M.G.

The values of conjugated androstenedione in the adrenal and renal vein samples were determined, after t.l.c. chromatography, for this female subject (Table 3.6). There was a gradient between the left adrenal vein sample, taken at 11.40h, and the lower I.V.C. sample. Both values obtained for the amount of androstenedione in the higher I.V.C. are shown. The value which was probably more correct is 1.7 nmol/l and the other value was probably high due to interference in the radioimmunoassay. The pattern of high and low values for the samples are the same for conjugated and unconjugated androstenedione, again indicating that secretion of conjugated androstenedione is ACTH dependent like that of cortisol and unconjugated androstenedione.

Table 3.7 Conjugated and unconjugated androstenedione
(nmol/l) for subject V.S.

	Conjugated	Unconjugated
Lover I.V.C.	<2.5 <1.8	20.1
High I.V.C.	<0.72 <0.82	22.7
L. adrenal	9.2 1.2	258.7
R. adrenal	2.1 2.2	363.6

Subject V.S.

The values for the amounts of conjugated and unconjugated androstenedione for this 33y old female subject are shown in Table 3.7. The adrenal vein values were again elevated above those from the periphery. The duplicate values obtained for the left adrenal samples are poor. The values obtained for the lower I.V.C. samples are most likely a lot less than the values quoted, and more of the order of the high I.V.C. samples. This was because a poor recovery of the ^3H androstenedione added as a recovery correction was obtained in one of the duplicates for these samples.

3.5 Discussion

As the first preliminary experiments indicated the presence of androstenedione in a conjugated form in plasma, the source of this conjugated steroid has been investigated. As an initial approach to the measurement of conjugated androstenedione in plasma, an acid hydrolysis method was used to cleave the conjugate and the amount of free steroid estimated by radioimmunoassay. The hydrolysis method was developed after the optimum conditions had been determined, and this method used to estimate the amounts of conjugated androstenedione in plasma samples.

In more recent work, the enzyme sulphatase (*Helix Pomatia*) has been used to hydrolyse the conjugated androstenedione present in plasma. This was a fairly crude enzyme preparation which also contained β -glucuronidase. Hence, using this enzyme does not determine whether the androstenedione is conjugated to glucuronic or sulphuric acid, and also non-enzymic hydrolysis could occur as the optimum pH used for hydrolysis with this enzyme is 4.7. A comparison of values of conjugated androstenedione cleaved by the enzyme with and without chromatographic purification and acid hydrolysis, indicated that

values for hydrolysis using enzyme were higher than those with acid for the same plasma samples. Values for hydrolysis with enzyme were lower for the same samples after chromatographic purification, although these values were still higher than for hydrolysis with acid. These results would indicate that because of the harsher conditions, when acid is used for the hydrolysis, the conjugated androstenedione is being converted to polar artefacts rather than to androstenedione. It would appear that this conversion to polar artefacts is occurring during the cleavage of the conjugated acid, as reduced levels of androstenedione are not obtained when the incubation time is prolonged. Until it is possible to carry out recovery experiments using radio-labelled androstenedione conjugates, it will not be possible to determine the precise reason for the different values obtained using differing hydrolysis methods.

The values for conjugated androstenedione in peripheral and adrenal vein samples, given in this section, have all been measured using the acid hydrolysis method. This means that these values may be an underestimate of the true amounts that are present, but they still give an indication of the source of this conjugated steroid, as the values have all been measured using the same method and each series of results have been estimated in the same assay.

The values of conjugated androstenedione in 7 normal subjects were 1.2-3.7 nmol/l, which was approximately 45% of the unconjugated androstenedione values in the same subjects. Measurement of conjugated androstenedione in plasma samples from 3 subjects taken during the early morning, indicated that conjugated androstenedione levels fluctuate in the same way as the unconjugated levels changed. These findings, together with the increase of conjugated androstenedione plasma levels during the infusion of tetracosactrin, suggested that

either conjugated androstenedione is secreted by the adrenal gland in response to the same stimulus as free androstenedione, or that conjugated androstenedione was a metabolite of androstenedione. In view of the fact that plasma conjugated androstenedione levels did not increase when androstenedione was infused into 2 subjects, these results would indicate that conjugated androstenedione is a direct secretion product of the adrenal gland.

In order to confirm this finding, conjugated androstenedione has been estimated in samples taken from the adrenal vein. The patients were being investigated for possible adrenal dysfunction; Cushing's syndrome (1), Conn's syndrome (2), virilization (2). The values for these samples from the adrenal vein when compared with samples taken from periphery showed between a 3:1 to 20:1 adrenal to peripheral vein ratio for conjugated androstenedione. The subject in which the highest ratio for conjugated androstenedione was found was subject F.S., who had adrenal hyperplasia.

Hence, these studies indicate that androstenedione exists in a conjugated form in plasma and that one source of this steroid is the adrenal gland.

SECTION 4

Preparation and studies of androstenedione 3-enol sulphate

4.1 Introduction

In order to confirm the existence of androstenedione in a conjugated form in biological fluids, it is necessary to synthesise these conjugates, characterise them using physicochemical methods and compare their properties with those substances isolated from plasma or urine. These synthetic compounds, if they are radiolabelled, enable the stability and methodological losses of the conjugates during assay procedures to be assessed.

After the possible existence of androstenedione 3-enol glucuronide was suggested by Schubert (1958) several successful attempts to prepare this compound and determine its physical characteristics were reported (Wotiz, Smakula, Lichtin and Leftin, 1959). Smakula, Leftin and Wotiz (1959) studied its infra-red characteristics and Matsui et al. (1974) studied the stability of ^{14}C androstenedione 3-enol glucuronide under varying conditions of hydrolysis including β -glucuronidase and acid hydrolysis. They found that polar artefacts could be formed and that conditions for the hydrolysis of these enol conjugates should, therefore, be chosen carefully.

The 3,5-dienol sulphate of androstenedione was prepared by Oertel et al. (1967) and the aromatization of this sulphate by placental microsomes was studied. In this short communication, only very brief details of the method of preparation were given, although the conjugate was purified by several chromatographic methods and physically characterised. These details, though, were not sufficiently explicit to allow the preparation to be easily repeated. There are no reports in the literature, to my knowledge, of studies of the stability or the

best conditions for the isolation of this conjugate from biological fluids.

Dr. Matsui kindly sent a few milligrams of androstenedione 3-enol glucuronide which could be used for comparison with products isolated from urine or plasma in chromatography systems, therefore it was not so essential to synthesise this compound. Hence, only androstenedione 3-enol sulphate has been synthesised and studies made of its stability and recovery during hydrolysis procedures.

These steroid conjugates differ from the conjugates of other steroids because during the formation of these enol conjugates a chemical modification occurs of the steroid nucleus, whereas in the formation of conjugates of other steroids, for example, oestrone sulphate and dehydroepiandrosterone sulphate, the sulphuric or glucuronic acid is linked to the steroid through a hydroxyl already present in the steroid. This change in structure of the steroid has the advantage that the physical characteristics of the conjugates formed from androstenedione will be different to those of the parent compound, whereas with conjugates formed from other steroids the only change is loss of a proton from the hydroxyl grouping.

4.2 Attempted preparation using trimethylamine-sulphur trioxide complex

Various methods have been used for the preparation of sulphuric acid conjugates of steroids (Bernstein, Dusza and Joseph, 1970), mostly by linking sulphuric acid to the steroid through an hydroxyl group already present in the steroid.

For the preparation of the 3-enol sulphate of androstenedione, Oertel et al. (1967) used pyridine-sulphur trioxide complex. Since then, other amine complexes which are sulphating agents have become commercially available. Initial attempts were made to prepare the sulphate conjugate of androstenedione using trimethylamine-sulphur

trioxide, as the reagent has the advantage that it is more stable than pyridine-sulphur trioxide complex and anhydrous conditions are not necessary.

Attempts to react this complex with androstenedione under various conditions, e.g. refluxing in benzene or pyridine, refluxing with benzene in the presence of p-toluene sulphonic acid and using trimethyl-sulphur trioxide complex recrystallised from dichloromethane, were tried but they were all unsuccessful.

4.3 Preparation of pyridine-sulphur trioxide complex

Oertel et al. (1967) used the method of Roy (1956) for the preparation of androstenedione enol sulphate. In an attempt to repeat this pyridine-sulphur trioxide complex was prepared by the reaction of a molar excess of pyridine with chlorosulphonic acid, using either anhydrous dichloromethane or pyridine as a solvent. Androstenedione dissolved in a minimum of dichloromethane was added to the prepared complex and refluxed. Several variations were tried in an attempt to force the reaction to completion. The amount of product formed seemed to be independent of the amount of pyridine-sulphur trioxide complex present or the time and temperature of the reaction.

This method was not very successful for several reasons, (i) the reaction of the androstenedione with pyridine-sulphur trioxide did not go to completion. (ii) The suggested method of Roy (1956) for separating the product from the reaction mixture was to allow the mixture to cool for 1h at 0°C, after diluting with petroleum ether (b.p. 60-80°). This should precipitate the salt of the steroid conjugate which was filtered, washed with light petroleum and extracted with hot boiling chloroform, in order to separate the pyridinium salt from the excess of pyridine-sulphur trioxide complex.

This procedure did not work as suggested as the reaction mixture on cooling after reflux was very oily and did not crystallise. Further purification using Sephadex LH20 column chromatography by the method used for separating conjugates in urine extracts (Section 2.4, ii) gave a flaky crystalline solid with a melting point $>320^{\circ}$ and the ultra violet spectrum that indicated that an aromatic group was present, indicating that the product was probably a pyridinium complex.

(iii) During the addition of the androstenedione in either pyridine or dichloromethane to the complex, side reactions occurred causing a brown discolouration.

Due to these problems a different method of preparation of the pyridine-sulphur trioxide complex was used. The most successful method was that given in a review by Jenkins and Sandberg (1969). Chlorosulphonic acid (0.2 ml, 3 mmol) was added dropwise to pyridine (1.44 ml, 18 mmol, A.R. stored over molecular sieve) contained in a stoppered flask and cooled in an ice bath. Violent reaction occurred on addition of the chlorosulphonic acid forming a white precipitate which was liquified by heating at 50° - 60° , for about 20 minutes. This method has been used for the preparation of the pyridine-sulphur trioxide for reaction with androstenedione.

4.4 Preparation and characterisation of androstenedione 3-enol sulphate

Initial experiments to prepare the 3-enol sulphate of androstenedione gave a very low yield of product. Several attempts have been made to improve this low yield by altering the reagents and conditions of the reaction. In these experiments, ^3H androstenedione was used to determine the yield. The method used for the preparation of 3-enol sulphate of androstenedione is given below followed by an outline of the experiments to improve the yield and the characterisation of the product obtained.

(i) Method

Androstenedione (0.572 g, 2 mmol) dissolved in pyridine (2 ml), was added to pyridine-sulphur trioxide complex, prepared from pyridine (1.44 ml, 18 mmol) and chlorosulphonic acid (0.2 ml, 3 mmol), and refluxed for 6.5h. After cooling, water (10 ml) was added to the reaction mixture, the pH was adjusted to 9 using 2M aqueous sodium hydroxide and left to stand at room temperature overnight.

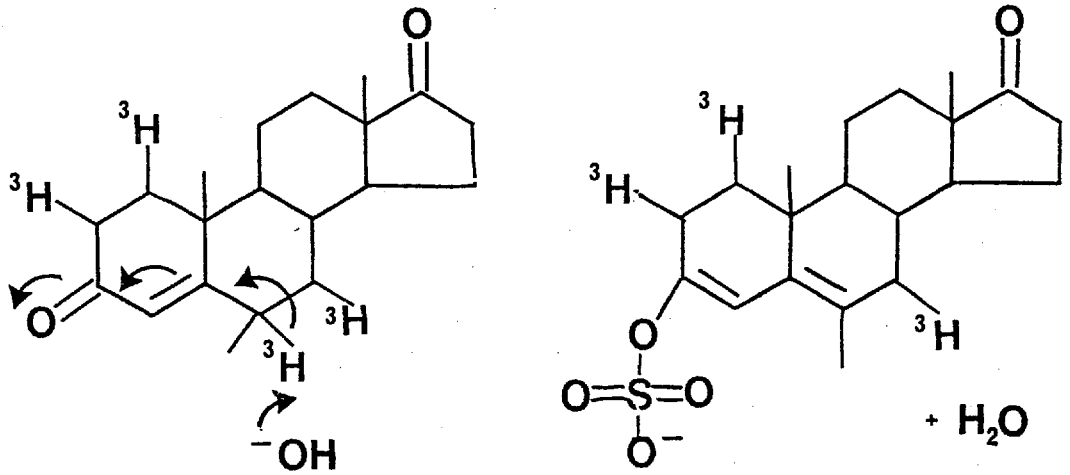
The mixture was pre-extracted with hexane:ether (4:1, 50, 30 and 20 ml) and then with n-butanol (2 x 30 ml, 20 ml). The combined butanol extracts were dried over sodium sulphate and evaporated under reduced pressure. T.l.c. of a small amount of the butanol extract in dichloromethane:methanol:water (70:30:2) indicated one major ultra violet visible product with an R_f of 0.5. T.l.c. in ether:acetone (9:1) indicated that the residue also contained a small amount of unconjugated androstenedione.

The residue of the butanol extracts was chromatographed on Sephadex LH20 eluting with chloroform:methanol (1:1), containing 0.01 mol/l of sodium chloride. 5 ml fractions were collected and a small amount of each fraction was chromatographed in dichloromethane:methanol:water system (70:30:2) to determine which fractions contained the product. These fractions were combined and the product crystallised out of the solution after 2 weeks. The needle shaped crystals were filtered off and washed with a small amount of chloroform:methanol (1:1), yield 23 mg, m.p. 215-220° (uncorr.).

(ii) Attempts to improve method

Initially, [1,2 6,7-³H] androstenedione was used as the radioactive tracer. In the process of enolization, either acid or base catalysed, a proton at position 6 is removed (Fig. 4.1). This reduces the theoretical yield by 13.5% and the different size of the atom could also influence the reaction, hence, [7(n)-³H] androstenedione was used. In order to convert the 3-enol conjugate of androstenedione from the pyridinium salt formed in the reaction mixture to the sodium salt, aqueous sodium hydroxide is added. It was found, though, that if the reaction mixture was not left with alkali overnight, complete conversion of the pyridinium salt to the sodium salt did not occur.

Basic conditions



Acidic conditions

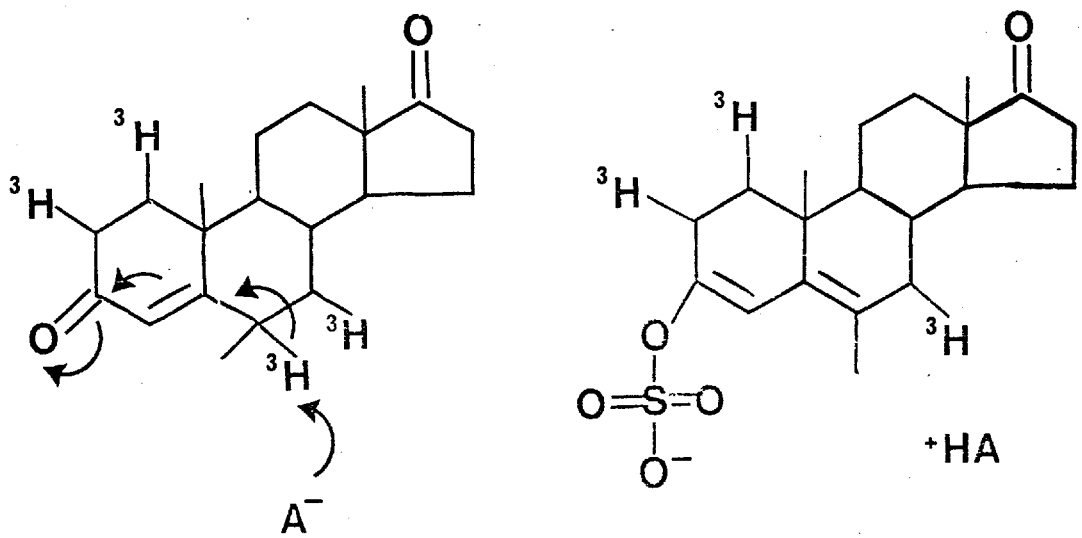


Fig4.1 Mechanism of enolization

In an attempt to determine the optimum conditions for the formation of the 3-enol sulphate of androstenedione, the reaction time and amount of complex to be added have been optimized. The reaction yield was estimated at room temperature, 60° overnight and reflux for 6h, and with different amounts of pyridine-sulphur trioxide complex. Raising the temperature increased the reaction yield slightly, and the highest yield was obtained when the reaction mixture was refluxed, although it was never above 20%. The amount of pyridine-sulphur trioxide complex added did not alter the yield. Hence, these results indicated that it would be better to develop separation methods to purify the required conjugate than to attempt to improve the yield.

Silica gel t.l.c. plates were used initially for the purification of the conjugate. When the purified conjugate was hydrolysed by acid, and the product of the hydrolysis extracted and chromatographed (paper, Bush B3) it was apparent that polar artefacts were being formed at some stage of the experiment. This problem may have been caused by the silica gel as it tends to be acidic, though the sulphate fraction extracted from urine was stable during silica gel t.l.c. (Section 2.4, i). The formation of polar artefacts could also be linked to the time which the conjugate steroid is left in a dry state on the t.l.c. plate. The radiolabelled conjugate, though, may be unstable under these conditions, when the unlabelled conjugate is stable. Matsui et al. (1974) found this problem with the preparation of ^{14}C androstenedione 3-enol glucuronide in that this compound decomposed to ^{14}C androstenedione on silica t.l.c. plates, but the unlabelled androstenedione 3-enol glucuronide was stable during the same chromatography procedure.

In order to extract the conjugated steroid from the reaction mixture, a fairly polar solvent n-butanol was required. The problem

with using n-butanol for the extraction of conjugates is that n-butanol retains large amounts of water and a large mass of sodium sulphate was required to remove the water which could trap the conjugate, hence reducing the yield. To attempt to overcome this problem, the aqueous pyridine mixture was extracted with ether:ethanol (3:1). This solvent mixture is used for the extraction of conjugated steroids (Edwards, Kellie and Wade, 1953) from plasma and urine, after saturation with ammonium sulphate. Extraction of the reaction mixture with ether:ethanol (3:1, 2 x 10 ml, 5 ml) and column chromatography on Sephadex LH20 of the residue of the extracts, indicated that only the unconjugated androstenedione had been extracted.

Sephadex LH20 column chromatography has been used to separate the starting material from the reaction product in the same way as it was used to separate the conjugated and unconjugated steroids in urine extracts (Section 2.4, ii). In the methods for analysis of androstenedione hexane:ether (4:1) is used to remove the unconjugated androstenedione from plasma or urine. This has proved to be a useful method for separating the reaction product from starting material. After the pH of the reaction mixture has been adjusted to 9 and then left at room temperature overnight, the mixture was extracted with hexane:ether (4:1, 3 x 10 ml) and then with n-butanol (2 x 10 ml, 5 ml). The extracts were washed with water (10 ml), dried over sodium sulphate and evaporated to dryness under reduced pressure. Methanol (1 ml) was added to redissolve the residue from the extract and 2 x 50 μ l taken for counting. Results indicated that 28.7% of the total counts were in the residue of the n-butanol extract. Sephadex LH20 column chromatography of the residues from the hexane:ether (4:1) extraction and the n-butanol extraction indicated that a good separation of the conjugated from the unconjugated steroid was obtained.

(iii) Characterisation of androstenedione 3-enol sulphate

In order to obtain enough of the compound to determine its physical characteristics, the products of several reactions were combined and purified by Sephadex LH20 column chromatography. Both t.l.c. and column chromatography of the product indicated that it was a monosulphated steroid conjugate. In the t.l.c. system dichloromethane:methanol:water (70:30:2) used for separating sulphates (Klein and Giroud, 1965) the monosulphated steroids have an R_f of 0.5. Using Sephadex column chromatography, the product was eluted from the column using chloroform:methanol (1:1) within the first 50 fractions, whereas disulphated steroid would not be eluted in these fractions (Sjovall, Sjovall and Vihko, 1968). The product obtained also gave a positive reaction with methylene blue reagent (Roy, 1956) indicating that there was a sulphate group present.

Ultra violet absorption

The diene structure of the compound absorbs in the ultra violet spectrum, and this has been compared with that of androstenedione using methanol as a solvent. The $\lambda_{\max}$ for the product was 237 nm (ϵ 22,000) compared with that for androstenedione of 240 nm (ϵ 237,900). This can be compared with the $\lambda_{\max}$ of 238 found by Oertel et al. (1967) using methanol as a solvent. No value for ϵ was quoted.

Infra red spectrum

The infra red spectrum of the product has been compared with that of androstenedione in a potassium bromide disc. In both the spectrum for androstenedione and its 3-enol sulphate, there was an absorption band at 1740 cm^{-1} due to the five ring ketone in the D ring of the steroid. In androstenedione there was an absorption at 1618 cm^{-1} due to the ketone at the 3 position conjugated to the double

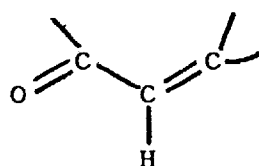
band. This was not present in the 3-enol sulphate, neither was the absorption at 1660 cm^{-1} but they have been replaced by an absorption in the 3-enol sulphate at 1650 cm^{-1} which may be due to the dienol stretching, although Oertel et al. (1967) quote this absorption maximum as being 1638 cm^{-1} .

In the 3-enol sulphate, there were also absorptions due to the stretching of the sulphur oxygen bands, at 1220 cm^{-1} due to SO-O , at 1135 cm^{-1} due to $>\text{SO}$ and 1040 cm^{-1} due to $>\text{SO}=\text{O}$ (reference values Williams and Fleming, 1966a). Oertel et al. (1967) found asymmetric and symmetric S-O vibrations at 1245 cm^{-1} and 1048 cm^{-1} .

Nuclear magnetic resonance spectrum

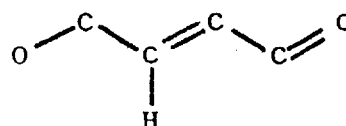
This spectrum of the product has been measured in deuterated dimethylsulphoxide, as the 3-enol sulphate was not soluble in deuterated chloroform, and compared to the spectrum of androstenedione in the same solvent. Trimethyl silane was used as a reference standard. In both compounds, there were resonances due to the 18 and 19 methyl groups, at 9.17τ and 8.77τ respectively. In the spectrum of androstenedione, there was a well defined 5 proton multiplet, at 7.5τ due to the resonance of the protons in the D ring, but with the 3-enol sulphate this multiplet was not as well defined. The major difference between the spectrum of androstenedione and its 3-enol sulphate was in the resonance of the proton at position 4 in the A ring. The expected shift for the protons in the differing environments are shown below (Williams and Fleming, 1966b).

(i)



$4.2 - 3.3\tau$

(ii)



$3.5 - 2.2\tau$

The shift for this proton in androstenedione was 4.4 τ which is similar to (i) and the shift for this proton in androstenedione 3-enol sulphate was 3.5 τ which is similar to structure (ii).

Mass spectrometry

An attempt to determine the molecular ion for androstenedione, 3-enol sulphate was made using the mass spectrometry service of the University of London at the London School of Pharmacy. The spectrum showed a molecular ion with a m/e ratio of 286, the molecular weight of androstenedione. The abundance of this peak increased the longer the sample was in the ionization chamber indicating that there was a compound present which was disintegrating to androstenedione. This probably occurred because the 3-enol sulphate of androstenedione was unstable under the conditions of the ionization chamber.

Purification of the product by paper chromatography

A Bush B23-9 system was used initially but in this system androstenedione only moved a small distance from the origin (Table 4.1). This was somewhat surprising as androstenedione 3-enol sulphate would be expected to have a similar type of planar structure as oestrone sulphate. To overcome this problem a modified Bush B23-9 system has been used.

4.5 Preparation and studies of radiolabelled androstenedione

3-enol sulphate

(i) Preparation

For this preparation [7(n)- ^3H] androstenedione has been used. Initial attempts to prepare labelled 3-enol sulphate, of fairly high specific activity, were not very successful. The pyridine-sulphur trioxide complex (200 μl) was added to ^3H androstenedione (3 μCi) dissolved in pyridine (1 ml) and refluxed for 6h. The product was separated from unreacted starting material but only a small proportion

Table 4.1 Chromatographic mobility of steroid conjugates
in Bush B23-9 and modified Bush B23-9 systems

	Distance (cm)			
	Bush B23-9		This study	Modified Bush B23-9
	Baulieu (1962)	Mckenna & Rippon (1965)		
Running time	36h	24h	60h	47h
Androstenedione sulphate	-	-	3	10
Oestrone sulphate	-	8	38	28
Dehydroepiandrosterone sulphate	34	15.8	>44	41

of the counts were eluted in the first 50 fractions from Sephadex LH20 column chromatography. The solvent was changed to methanol and the remaining activity was eluted in fractions 68-78, indicating that a disulphated steroid had been formed (Sjovall et al., 1968). Disulphation could be occurring due to the vast molar excess of sulphating reagent present compared to the amount of androstenedione, the other possible position of sulphation being the 17 position as a result of enolization to 16-en-17-ol steroid.

To overcome this problem unlabelled androstenedione (0.05 g) was added to the reaction mixture. Column chromatography of the product obtained indicated that although the reaction had not gone to completion, the only product was monosulphated steroid conjugate.

(ii) Hydrolysis studies

One of the most important criterion for any assay in which the conjugate is estimated indirectly, by assaying the amount of cleaved steroid, is that the yield of the unconjugated steroid from the conjugated steroid is high, and that artefacts are not formed during the hydrolysis procedure.

In order to assess this for the androstenedione 3-enol sulphate, the radiolabelled conjugate has been hydrolysed using various conditions. 3 different methods have been used to cleave the labelled conjugate, (i) hydrolysis using the same method as was used to estimate the total amount of androstenedione in urine, (ii) hydrolysis in water (0.5 ml) with M sulphuric acid (3 drops) added, incubation at 37° for 16h, and extraction with hexane:ether (4:1, 4 ml). (iii) Sulphatase (Helix Pomatia, 20 I.U.) was added to the conjugate in a solution of acetate buffer (0.5 ml) and the mixture incubated at 37° for 16h and extracted with hexane:ether (4:1, 4 ml). The extracts from the 3 methods of hydrolysis were chromatographed in a Bush A

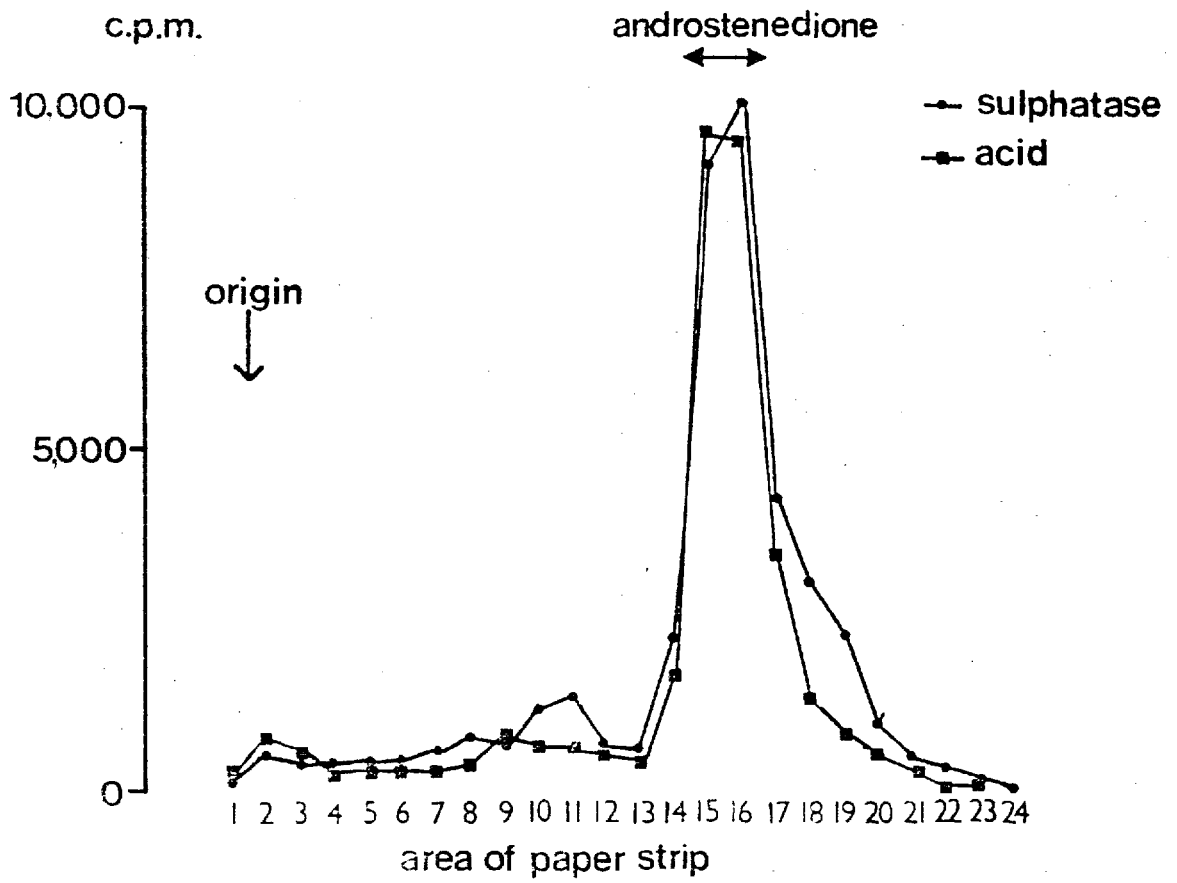


Fig 4.2BushA paper chromatography of hydrolysis products

paper system to determine whether the conjugate had been hydrolysed to androstenedione.

The products of several experiments to prepare the radiolabelled conjugate have been hydrolysed using these conditions. As the activity of the radiolabelled conjugate was very low, it was often impossible to detect the activity using the Panax or β -graph scanner. Hence, the areas of activity on the paper strips were detected by dividing the paper strip into 1.5 cm lengths, which were placed in the bottom of large plastic counting vials. Scintillator (10 ml) was added, and after shaking for 10 minutes, the activity was estimated in a liquid scintillation counter.

Fig. 4.2 shows the c.p.m. in each length of the paper strip. Results from hydrolysis of the radiolabelled conjugate with acid and sulphatase are shown. The radiolabelled conjugate used in this experiment had been freshly prepared and purified by Sephadex LH20 column chromatography. With hydrolysis using sulphatase, a larger proportion of the activity (34%) was not extracted using hexane:ether (4:1), but remained in the aqueous phase, compared to when the conjugate from the same experiment was hydrolysed using acid, when 28% of the activity remained in the aqueous phase. These results indicate that cleavage of the conjugated androstenedione can occur cleanly without the formation of artefacts. The problem with the hydrolysis of the radiolabelled conjugate has been that it has not been possible to obtain consistent results. Radiolabelled conjugate, prepared in other experiments using exactly the same method as that for the conjugate prepared above, gave a product which could not be cleaved cleanly to give androstenedione but gave more polar artefacts. This may have been for several reasons, including length of time of storage after synthesis of the conjugate, the conditions under which the product

from the column was evaporated or some differing condition or reagent used in the hydrolysis.

4.6 Discussion

Although it has proved fairly difficult to repeat the preparation of androstenedione 3-enol sulphate, several milligrams of the compound have now been prepared. The physicochemical characteristics of this conjugate are similar to those of the conjugate prepared by Oertel et al. (1967). The attempts to improve the yield for the reaction by varying the reagents and conditions were all unsuccessful and the poor yield has been accepted for the present, as it was concluded that it was best to prepare and characterise at least a small amount of the conjugate. The needle shaped crystals of androstenedione 3-enol sulphate had the physicochemical characteristics from infra red and ultra violet analysis, indicating that enolisation of the 4-ene-3-ketone system had occurred and that sulphur had been incorporated into the molecule as indicated by the sulphur oxygen stretchings in the infra red spectrum of the compound.

The preparation of radiolabelled androstenedione 3-enol sulphate and studies of hydrolysis of the conjugate have proved more difficult. The major problem was the inconsistency of the results of hydrolysis obtained from the radiolabelled conjugate prepared from different experiments, even though much care was taken to see that the conditions of reaction and hydrolysis were carefully controlled during these experiments. This problem was most obvious from the hydrolysis of conjugate in that the product from one experiment could be cleaved to give mainly androstenedione, whereas the hydrolysis of the product from a subsequent reaction would give polar artefacts and only small quantities of androstenedione. This problem of hydrolysis of the conjugated steroid to give the unconjugated steroid has also been

shown to be very dependent on the conditions of hydrolysis for the 3-enol glucuronide of androstenedione (Matsui et al., 1974). These workers concluded that if the biochemistry of steroid 3-enol glucuronide conjugates is to be investigated, caution must be shown with regard to the chemical stability of these conjugates. This could equally well apply to steroid 3-enol sulphates. The instability of this type of conjugate is not very surprising considering the type of bonding between the conjugating acid and the steroid. It would seem that at present no way of storing this radiolabelled conjugate of androstenedione successfully has been found, and the stability of the conjugate may also be affected by some stage of the purification procedure. Matsui et al. (1974) found this for the 3-enol glucuronide of androstenedione; when purification of the ^{14}C conjugate was attempted on silica t.l.c. plates, decomposition of the conjugate occurred, yet this did not happen when the unlabelled conjugate was purified in this way.

More recent experiments have shown that radiolabelled androstenedione 3-enol sulphate can be prepared with $[4-^{14}\text{C}]$ androstenedione. With these preliminary experiments, it has not been necessary to add androstenedione to prevent the formation of disulphated steroid, which means that the preparation of high specific activity labelled conjugate would be possible. Hydrolysis studies of this radiolabelled conjugate have shown the cleavage of the conjugate to androstenedione, but again the results have not been consistent.

Hence, these studies indicate that androstenedione can exist in a form conjugated to sulphuric acid and that this conjugate, under the right conditions, can be cleaved to give androstenedione in reasonable yield.

SECTION 5

Conclusions

The work presented in this thesis has involved the development of methods for the estimation of conjugates of androstenedione in urine and plasma. Using these methods, the source of this conjugated steroid has been investigated.

Evidence for the presence of conjugated androstenedione in urine was first presented by Schubert (1958), who suggested that the conjugating acid was linked to the steroid by an enolic structure. After this report, several groups of workers (Tajic, 1964; Longhino et al., 1968; Schollberg et al., 1968) estimated the amounts of androstenedione in urine after β -glucuronidase and acid hydrolysis of the conjugated androstenedione. A method for the estimation of the total amount of androstenedione in urine after acid hydrolysis, using a sensitive radioimmunoassay to estimate the amounts cleaved, has been developed and this method used to determine whether androstenedione excretion is a good index of androstenedione production.

Unconjugated androstenedione in urine had previously been found to be a useful reflection of androstenedione secretion (Andino et al., 1976), but as spontaneous hydrolysis of conjugated androstenedione occurs under some conditions, with the risk of spuriously high values occurring, the method for the measurement of total amount of androstenedione in urine was developed.

As the conjugating acid is linked to androstenedione via an enol linkage, both the glucuronic and sulphuric acid conjugates will be cleaved under acidic conditions. Hence, hydrolysis with β -glucuronidase is not necessary and a method was developed which was an adaptation of that of Burnstein and Lieberman (1958). This has proved a reliable method which has now been used to estimate the total amount

of androstenedione in 200 urine samples. Interestingly, the range for normal female subjects, obtained using this method, is identical to that obtained by Schubert et al. (1964), although the range for male subjects is slightly different (Table 2.3). The ranges found for normal subjects by most groups of workers are of the same order, except for those of Tajic (1964) whose values are much lower, but this may be because of the extensive purification procedures which were used. The mean excretion of androstenedione in women was lower than for men, although the ranges overlap. This difference in ranges, which has also been found by other workers, is unexpected because plasma levels (Friedrich et al., 1975) and metabolic clearance rates (Horton and Tait, 1966) in men and women are similar. Hence, this difference in ranges would seem to indicate that the metabolism of the androstenedione rather than the production must differ between the sexes.

Previously, no data had been available to relate urinary excretion to plasma levels of androstenedione. The good correlation obtained between the excretion of total androstenedione in urine and the mean plasma level measured over periods up to 24h, for a group of postmenopausal subjects, has indicated that urinary excretion is indeed a good index of plasma levels. In addition, the study of urinary androstenedione excretion in normal and postmenopausal subjects, when adrenocortical function was depressed by the administration of dexamethasone and also in subjects with various pathological disorders, illustrates the relationship between blood and urine levels. Hence, as androstenedione secretion is episodic, it would appear that urinary excretion is a better index of androstenedione production than can be obtained from random plasma levels, and the estimation of urinary androstenedione may be a useful means of investigating normal and abnormal aspects of androgen production.

Androstenedione in urine is conjugated to at least 2 different acids. Other workers have used β -glucuronidase to cleave androstenedione and Longhino's group in Yugoslavia have estimated the amounts of androstenedione in the free, glucuronide and sulphate fractions excreted in the urine from one woman on all the days of the menstrual cycle (Longhino et al., 1972) and in pregnant subjects (Longhino et al., 1976). Thin layer and Sephadex LH20 column chromatography of urine extracts, as well as the estimation of the amounts of androstenedione extractable after enzymic and acid hydrolysis, indicated that the majority of androstenedione present in urine is in a form conjugated to sulphuric acid, probably as the 3-enol sulphate conjugate. Androstenedione conjugated to glucuronic acid is present in greater amounts than unconjugated androstenedione but less than the sulphate conjugate. The difference in the normal male and female ranges for androstenedione excretion, seems to be mainly accounted for by the androstenedione conjugated to sulphuric acid.

Previously, there has been only one report of the presence of androstenedione in a conjugated form in plasma (Rhamy and Hadd, 1973), although Oertel, using the theory of direct metabolism, considered that androstenedione conjugates could exist as intermediates in the biochemical pathway between dehydroepiandrosterone sulphate and oestrone sulphate. As initial experiments indicate that conjugated androstenedione existed in plasma in similar amounts to unconjugated androstenedione, a method has been developed to estimate this fraction. Comparison of the values for conjugated androstenedione in plasma, assayed in samples which had been purified by paper (Bush A) chromatography, with those which had not, indicated that this chromatographic purification stage was not necessary. Hence, for samples, except for those from adrenal and ovarian veins, conjugated androstenedione was

estimated without chromatographic purification of the residue, prior to radioimmunoassay.

The source of this conjugated androstenedione in plasma has been investigated using sequential plasma samples. From the amounts of conjugated and unconjugated androstenedione in these samples, it was clear that increases in unconjugated androstenedione were mirrored by increases in conjugated androstenedione. Plasma conjugated androstenedione also increased when subjects were infused with tetracosactrin. One possible conclusion to be drawn from these results was that conjugated androstenedione was being secreted by the adrenal gland, and that this secretion was ACTH dependent, as with cortisol and unconjugated androstenedione. This evidence, though, could not be regarded as conclusive of adrenal secretion of conjugated androstenedione as it is possible that androstenedione was being rapidly conjugated either extrahepatically or in the liver. This theory of conjugation external to the adrenal gland was not supported by evidence that when androstenedione was infused into the periphery, the resulting increase in unconjugated androstenedione in the periphery was not matched by an increase in conjugated androstenedione.

In view of these results, it would appear that conjugated androstenedione is secreted in response to ACTH, directly by the adrenal gland. To produce conclusive evidence for this, conjugated androstenedione has been measured in adrenal vein samples from 5 patients and compared with peripheral samples taken at the same time. A gradient between the adrenal vein and peripheral samples was found in all these subjects varying from 1:20 to 1:3 (peripheral:adrenal) and this is positive evidence for the direct secretion of conjugated androstenedione by the adrenal glands.

These values for conjugated androstenedione in plasma have been estimated after cleavage of the conjugated steroid with acid. More recently, further work has shown that when sulphatase was used to cleave conjugated androstenedione in plasma, greater amounts are present than when acid was used. This may be the result of the milder conditions that are used for hydrolysis with sulphatase, which results in a greater conversion of the conjugated androstenedione to androstenedione rather than polar artefacts.

These results highlight one of the major problems with the methods for estimating the amounts of conjugated androstenedione present in plasma and urine, which has been the lack of radiolabelled conjugates that could be used to assess the recovery of androstenedione from the conjugate. This means that the results are likely to be an underestimate rather than an overestimate, due to possible conversion of androstenedione conjugates to polar artefacts rather than androstenedione. This is the problem that is encountered because conjugates are usually estimated by indirect methods, although radioimmunoassay methods have recently been developed for the estimation of some steroid conjugates directly (Catteno, Forti, Fiorelli, Barbieri and Serio, 1975; Tresguerres, Volkwein and Tamm, 1977).

Both the glucuronide and sulphate conjugate of androstenedione have been prepared synthetically (Wotiz et al., 1959; Oertel et al., 1967; Matsui et al., 1974). ^{14}C androstenedione 3-enol glucuronide was synthesised by Matsui et al. (1974) and the hydrolysis of this conjugate under various conditions studied. These workers found that side reactions occurred during the cleavage of the conjugating acid to give artefacts more polar than androstenedione, possibly 6-oxo and 6-hydroxy androstenedione. Though, when a method for hydrolysis of androstenedione glucuronide was used which was similar

to that for the hydrolysis of androstenedione conjugates in urine, used in this work, 87% of ^{14}C conjugate was hydrolysed to give androstenedione.

In the literature, there is only one report of the synthesis of androstenedione 3-enol sulphate (Oertel et al., 1967). This preparation has been repeated and attempts made to prepare the radiolabelled conjugate so that the hydrolysis of the conjugate under various conditions could be studied. This work proved more difficult to repeat than was anticipated. Although the compound has been well characterized, no methodological details of its preparation were given. When the synthesis was attempted low reaction yields were obtained and after several unsuccessful attempts to improve the yield, it was accepted that it would be better to prepare a small amount of the compound. The crystalline androstenedione sulphate showed the physical characteristics indicating that androstenedione was conjugated to sulphuric acid through an enol linkage. Attempts to prepare radiolabelled androstenedione sulphate were partially successful. Experiments to cleave the conjugate did not give consistent results. The hydrolysis of the radiolabelled conjugate from some preparations gave mainly androstenedione, but the hydrolysis of the product from other experiments synthesised in the same way gave mainly polar artefacts. The reason for this inconsistency at the moment is not apparent, but may be related to the product not being purified carefully enough or the length of time between synthesis and hydrolysis. Until the cause of this inconsistency is determined, it will not be possible to use the radiolabelled conjugate for the assessment of recovery of androstenedione from androstenedione 3-enol sulphate in plasma or urine.

As yet, neither of the conjugates of androstenedione have been isolated from plasma or urine in a conjugated form. The evidence for their existence in biological fluids has come from indirect methods. Although indirect evidence does not prove conclusively their existence, evidence has now been shown by many different methods; for example, hydrolysis with enzymes and acid, the chromatography of the extracts from urine and the synthetic preparation of the conjugates.

The type of conjugation between androstenedione and the conjugating acid is unusual because of the enol linkage. The physico-chemical analysis of the synthetically prepared compounds indicate that this type of bonding is present in the molecule. The acid lability of these conjugates is also indicative of this type of bonding. Steroid glucuronides are not usually hydrolysed by acid, and a β -glucuronidase enzyme preparation is normally used to cleave these conjugates, but androstenedione glucuronide is cleaved by acid in the same way as steroid sulphates are hydrolysed, as well as by β -glucuronidase (Matsui et al., 1974).

The enol bonding for steroids to conjugating acids could possibly exist for steroids other than androstenedione. Recently, Kjeld, Puah and Joplin (1978, 1979) suggested this type of bonding for conjugates of testosterone, as they observed the presence in urine of an acid labile conjugate of testosterone which was neither testosterone 17-sulphate or testosterone 17-glucuronide. This work, together with the evidence for the existence of conjugates of androstenedione, suggests that this type of conjugation could exist for any steroid, with a double bond conjugated to a ketone, in their structure.

The existence of conjugated androstenedione in plasma and its apparent direct secretion by the adrenal gland was unexpected but parallels the now well accepted observation of the direct adrenal

secretion of dehydroepiandrosterone sulphate. The importance of conjugated androstenedione and other circulating conjugated steroids, other than a method of excretion of unconjugated steroids from the body, has yet to be defined and is a subject for further investigation. The important question with regard to conjugated androstenedione, is whether it is a precursor of other steroid conjugates, e.g. oestrone sulphate, in the same way as unconjugated androstenedione is an important precursor for oestrone, especially in the postmenopausal female, and whether this conversion of the steroid part of the molecule can occur without the loss of the acid conjugated to it. This type of biochemical pathway for conjugates of steroids has been called direct metabolism, and to determine whether this type of conversion occurs, further in vitro and in vivo studies will have to be made. In view of the fact that in vitro studies have shown a low conversion of androstenedione to oestrone (Schindler, Ebert and Friedrich, 1972; Nimrod and Ryan, 1975) and yet androstenedione is a major source of oestrogen in the postmenopausal female, the possibility that androstenedione 3-enol sulphate could be regarded as a precursor for oestrone sulphate is a subject that is open to investigation. This theory is supported by the work of Oertel et al. (1967), who found that androstenedione 3-enol sulphate was metabolised by placental microsomes to oestrone sulphate. The other possibility, although less likely, is that dehydroepiandrosterone sulphate may be a precursor of androstenedione sulphate. This theory has been put forward again by Oertel's group (Knapstein et al., 1967a, b) after infusing patients with conjugates of dehydroepiandrosterone.

In summary, there is evidence for the existence of at least 2 conjugates of androstenedione, the 3-enol sulphate and the 3-enol glucuronide. The estimation of the total amount of androstenedione

in urine has been shown to be a useful index of the production of androstenedione. In plasma, androstenedione has been shown to exist in a conjugated form as well as an unconjugated form. The types of conjugate in plasma have not, as yet, been fully elucidated, although this conjugated fraction is directly secreted by the adrenal gland and secretion is ACTH dependent.

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APPENDIX

Methods used for the estimation of steroids in plasma and urine

(i) Radioimmunoassay of testosterone in plasma

The radioimmunoassay for testosterone in plasma is similar to that for androstenedione in plasma and will only be outlined briefly. Aliquots of plasma (100 μ l for female and 10 μ l for male samples) in duplicate were extracted by vortexing for 4 minutes with diethyl-ether (1.5 ml). The plasma was frozen in a dry ice ethanol bath and the ether extract decanted into assay tubes. Aliquots of standards (10, 20, 40, 80, 160, 320 pg) from working solutions in methanol (0.1 ml) were also pipetted into assay tubes. The solvent was evaporated from all tubes at 32° under vacuum.

The working antiserum solution was prepared in borate buffer at 1:75,000 dilution containing 0.1% ($^w/v$) bovine serum albumin, 0.05% ($^w/v$) human γ -globulin and [1, 2, 6, 7- 3 H] testosterone (approximately 24,000 c.p.m. per ml) and 0.25 ml was added to all tubes, including 2 tubes containing zero antigen, except 2 tubes for assessment of non specific binding to which buffer solution containing all the constituents except antiserum was added. After rinsing the walls of the assay tubes, the mixture was incubated at room temperature for 2h followed by $\frac{1}{2}$ h in an ice-water bath.

The separation of the antibody bound and free fractions was carried out using a solution of polyethylene glycol in the same way as for the assay of androstenedione in plasma.

Normal range: male 10.4 - 38.2 nmol/l
female 0.5 - 2.1 nmol/l

(ii) Measurement of urinary 11-hydroxycorticosteroids

The method used for the determination of urinary 11-hydroxycorticosteroids, was a method of Mathingly, Pennis, Pearson and Cope (1964). This method though, is not totally specific for cortisol and

interference may occur due to non specific fluorescence.

Normal range: 220 - 1100 nmol/24h

(iii) Measurement of cortisol in plasma

Cortisol was measured in plasma either by radioimmunoassay or by the fluorimetric method (Townsend and James, 1968). Both methods have been shown to give similar values.

Normal range: 140 - 690 nmol/l

(iv) Radioimmunoassay of androstenedione in plasma

Overleaf is a copy of the paper by Goodall, Rippon and James (1979) giving the method for the measurement of androstenedione in plasma.

Normal range: 1.4 - 7.0 nmol/l

(v) Radioimmunoassay of aldosterone in plasma

Plasma aldosterone has been determined using the method of James and Wilson (1976).

Normal range: (Recumbent/normal diet)
100 - 500 pmol/l

A SIMPLE NON-CHROMATOGRAPHIC RADIOIMMUNOASSAY FOR PLASMA ANDROSTENEDIONE

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SUMMARY

A simple non-chromatographic radioimmunoassay method for the measurement of androstenedione in plasma is described. Polyethylene glycol is employed to separate the bound fraction, which is counted directly by adding scintillator to the precipitate in the assay tube. This simplifies the assay and improves the precision at low steroid concentrations. The method gives results which show a good correlation with a previously validated method.

INTRODUCTION

Methods for measuring androstenedione in plasma have usually employed a chromatographic purification stage to achieve adequate specificity. Various procedures have been used including alumina column chromatography [1,2], Sephadex LH20 column chromatography [3,4], celite column chromatography [5], and thin layer chromatography [6]. The inclusion of a chromatographic purification stage, prior to radioimmunoassay of the extract makes it more difficult and tedious to handle a large number of samples. The aim of the present method was to develop an assay for androstenedione, in which no chromatographic purification stage was necessary, but which still retained the specificity required to obtain accurate and reliable results.

The method described below involves a simple extraction stage with hexane:ether, followed by evaporation and incubation of the residue with the anti-serum, separation of the bound and free fractions with polyethylene glycol and finally counting the bound fraction in a liquid scintillation counter.

MATERIALS

Androstenedione was supplied by Sternaloids Ltd, Croydon, England. [1,2,6,7, (n) ^3H]-androstenedione (104 Ci/mmol) was obtained from the Radiochemical Center Ltd, Amersham, England. All chemicals, except where stated were supplied by Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire. Human gamma globulin, HG 11, from Sigma London Chemical Company Ltd., Poole, Dorset. Bovine plasma albumin from Armour Pharmaceutical Co., Eastbourne, Sussex. 0.05M Borate buffer pH 8.0 was prepared from boric acid, 6.2 g in distilled water made up to 2 l. containing 2% methanol and the pH adjusted. Scintillation solution was prepared by dissolving 6 g *p*-terphenyl and 0.08 g dimethyl POPOP in 2 l. of sulphur free toluene containing 2% meth-

anol. Extraction tubes manufactured by Quickfit & Quartz Ltd., Stone, Staffordshire, type MF 24/0/4 with stoppers type SB 10. Glass assay tubes, 50 mm $\times$ 10 mm with plastic closures, controlled neck shell vial No. 3/B/D/08/015/01/A manufactured and supplied by F. B. G. Trident Ltd, Temple Cloud, Bristol, were rinsed with methanol before use. Racks for holding assay tubes during decanting stage are Analmatic racks type 401/2113 fitted with adaptors type 401/2113/12 supplied by Searle Instrumentation, P.O. Box 53, Lane End Road, High Wycombe, Buckinghamshire. Constriction pipettes manufactured by H. E. Pedersen, 7 Sommerstedgade, DK1718, Copenhagen V, Denmark. Compu-Pet dispenser, dual micro model from William R. Warner Ltd., Eastleigh, SO5, 3ZQ, England.

ASSAY METHOD

An aliquot of plasma (0.3 ml), previously mixed on a vortex mixer was pipetted into an extraction tube using a constriction pipette. After adding 4 ml of *n*-hexane-diethyl ether (4:1, v/v) the tubes are stoppered and shaken horizontally on a mechanical shaker for 10 min. The two phases are allowed to separate and 2 $\times$ 0.5 ml aliquots of the extract transferred to assay tubes using a constriction pipette. 0.1 ml of 10% v/v propylene glycol in methanol was added to all tubes and the contents of the tubes evaporated at 45°C under vacuum until only the propylene glycol residue remained.

Standard quantities of androstenedione 10, 20, 40, 80, 160, 320 pg in 0.1 ml of methanol were pipetted into assay tubes from individual working solutions. Into these tubes, as well as two tubes for the estimation of non-specific binding and two zero antigen tubes was added 0.1 ml of 10% propylene glycol in methanol. The contents of the tubes were evaporated as above.

The working solution of antiserum was prepared in borate buffer at 1:30,000 dilution containing 0.2%

(v/v) bovine plasma albumin; 0.1% (v/v) human γ -globulin and tritiated androstenedione (approximately 40,000 d.p.m. per ml). After mixing gently, 0.25 ml was added to all tubes, using the Compu-Pet automatic dispenser. The amount of radioactivity added to each tube was determined by dispensing the same volume of antiserum solution into two extra tubes and counting after the addition of scintillator (1.2 ml).

To the two non specific binding tubes 0.25 ml of buffer solution was added containing all the constituents with the exception of the antiserum.

The walls of the assay tubes were rinsed with antiserum by gently mixing on a vortex mixer for 1 or 2 s. All tubes were incubated overnight at 4°C or for half an hour at room temperature followed by one hour at 4°C.

After incubation, the tubes were stood in an ice/water bath during the addition by displacement pipette of an equal volume (0.25 ml) of 30% polyethylene glycol in borate buffer.

Polyethylene glycol may be added more conveniently to large numbers of assay tubes using the Compu-Pet. Due to the very high viscosity of the reagent, and because the Compu-Pet is normally calibrated to dispense aqueous solutions, adjustments have to be made to the Compu-Pet settings and concentration of the polyethylene glycol solution such that the final concentration of polyethylene glycol in the assay tube is 15%. This may best be achieved by leaving the instrument calibrated for aqueous solutions but depressing the buttons to deliver 0.4 ml and reducing the concentration of the working solution of polyethylene glycol to 27%. This arrangement has proved quite satisfactory and was arrived at by using a solution of polyethylene glycol in buffer containing a radioactive marker (e.g. tritiated testosterone), in order to compare the volumes dispensed automatically against those dispensed manually.

After the addition of the polyethylene glycol, the contents of each tube were mixed thoroughly on a vortex mixer for several seconds, left to stand for 10 min in the ice/water bath and finally centrifuged at 1500 *g* for 30 min at 4°C.

The tubes, after replacing in the racks, were inverted to decant the supernatant (free) to waste and left to drain, inverted on several layers of absorbent paper for 10 min. Scintillator (1.5 ml) was added and the stoppered tubes shaken horizontally on a mechanical shaker, for 10 min. All the tubes were then placed in standard glass counting vials whose caps had been drilled out such that the assay tubes were suspended centrally and vertically by the rim of their stoppers. They were then counted in a liquid scintillation counter at ambient temperature for 10 min or to an error of 2%.

CALCULATION

The bound c.p.m. (B) of the duplicate standards were calculated as a percentage of the mean bound

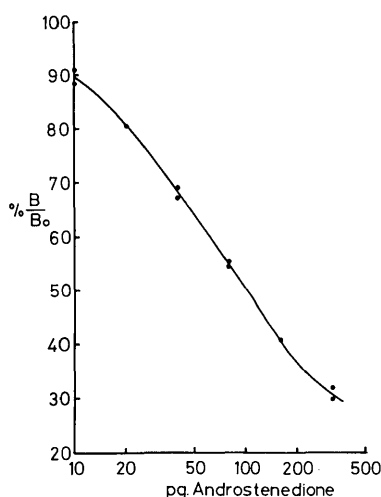


Fig. 1. A typical standard curve. The bound c.p.m. are expressed as a percentage of the bound c.p.m. in the zero tube, plotted against the mass of androstenedione on a log. scale.

c.p.m. (B_0) of the zero tubes. These percentages B/B_0 were plotted against the mass in pg of androstenedione on a logarithmic scale to give the standard curve as shown in Fig. 1. Similarly the mean bound c.p.m. for the plasma aliquots were calculated and the relative mass of androstenedione found from the standard curve.

VALIDATION

The precision of the assay was assessed from within batch analyses of duplicate plasma samples. This gave a within assay variation with coefficient of variation (C.V.) of 8.8% ($N = 24$). The between assay variation was assessed by assaying aliquots of two plasma pools in successive batches. This gave a C.V. of 10% ($N = 23$) with a mean value of 4.2 nmol/l and a C.V. of 9.8% ($N = 12$) with a mean value of 1.9 nmol/l.

When the following volumes 0.1, 0.2, 0.3, 0.4 and 0.5 ml of one plasma sample were assayed good linear relationship was obtained as shown in Fig. 2.

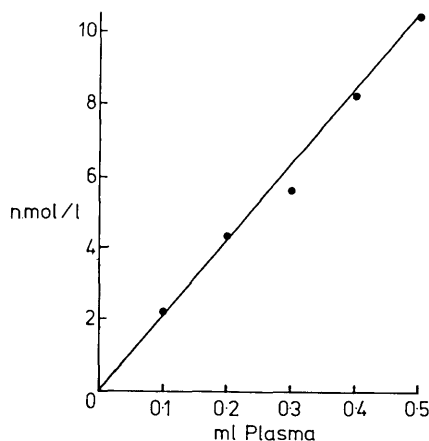


Fig. 2. The linearity achieved by plotting the values obtained from the assay of increasing volumes of the same plasma specimen.

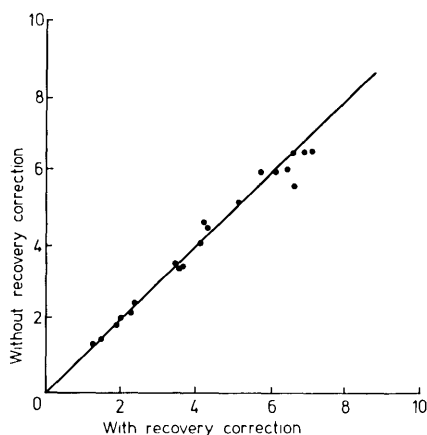


Fig. 3. The results obtained with and without making a correction for methodological losses. Values expressed as n.mol./l.

The recovery from plasma was estimated by adding 0.5 ng and 1.0 ng of unlabelled androstenedione to a previously assayed plasma sample and reassaying. In three experiments, where 0.5 ng was added, recoveries of 96, 101 and 104% were obtained. In four experiments where 1.0 ng was added, a mean recovery of 101% was obtained with a range of 93–106%.

In addition, confirmation that the extraction step was quantitative was obtained from an experiment in which radioactive androstenedione, added to plasma samples, was extracted and counted. This gave a mean recovery of 96.6% and a C.V. of 4.1% ($N = 28$). Furthermore, when 10 plasma samples were assayed in duplicate and the value corrected for methodological losses by measuring the recovery of an added amount of radiolabelled androstenedione, originally added to the plasma sample, the comparison of these results with those obtained when this correction was not applied, gave very good agreement, as shown in Fig. 3. The C.V. for these duplicates with the correction was 9.0% and without the correction 9.5%. Thus, it was considered justifiable to omit a correction for recovery from the method.

Ten plasma samples were assayed using the method described above and also by the method described previously [1] which used ammonium sulphate precipitation for the separation of the bound and free fractions and required an initial purification of the plasma extracts on an alumina column. As Fig. 4 shows, a good correlation ($r = 1.0$) has been obtained between these two methods.

The non specific blank of the radioimmunoassay over 15 batches gave a mean value of 8.7 with a range of 6.4–12.5 as a percentage of the total counts. The blank value of the method was assessed by substituting distilled water into the assay procedure. This gave a value which was determined to be 3 pg from nine estimations. The sensitivity of the assay was estimated as being 5 pg, by calculating two standard deviations from a series of 10 zero tube determinations and esti-

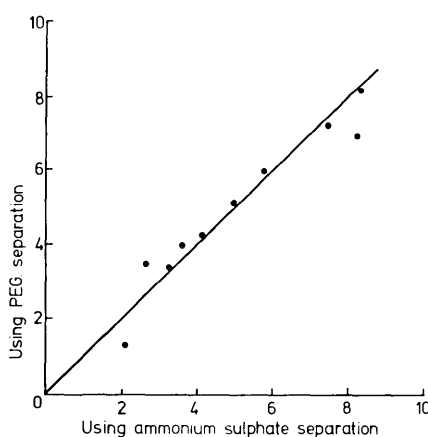


Fig. 4. The values obtained when 10 plasma specimens were assayed by the method using polyethylene glycol separation and that using ammonium sulphate precipitation after purification of the plasma extracts by alumina column chromatography. Values expressed in nmol/l.

imating this value from a standard curve drawn on a linear graph paper.

DISCUSSION

The method described here has a number of advantages over other published radioimmunoassays for androstenedione in plasma. It has sufficient specificity to eliminate the need for a chromatographic stage. This is partly due to the specificity of the antiserum employed, but it depends in part, as has been found for other methods [13, 14] on the choice of the separation technique. In the method previously described [1], in which ammonium sulphate precipitation of the bound fraction was employed with the same antiserum, it was still essential to carry out further purification of the hexane-ether extract of the plasma by alumina column chromatography in order to achieve sufficient specificity. By using polyethylene glycol separation, this chromatographic step can be dispensed with, without sacrificing the specificity as shown by the close similarity of the results obtained when plasma samples were analysed by both methods.

Similar results have been obtained in our radioimmunoassay for testosterone [7]. An aliquot of plasma is extracted with ether and after evaporation, the residue is incubated with a specific antiserum. The bound fraction is precipitated using polyethylene glycol, as for the androstenedione plasma assay, and then counted in the incubation tube after the addition of scintillator.

The use of polyethylene glycol to separate bound and free fractions was first described by Desbuquois and Aurbach [8] in peptide hormone assays. Since then several authors have employed the technique and its use has been extended to steroid radioimmunoassays (e.g. testosterone [1, 9], oestriol [10] and progesterone [11]). Tresguerres *et al.* [12] also

employed this reagent in their assay for testosterone glucosiduronate.

By counting the precipitated bound fraction directly in the incubation tube, the use of expensive counting vials is eliminated, and the volume of scintillator needed is reduced to 1.5 ml. Disposal costs of the used scintillator are also minimised. As with other methods where the bound fraction is counted, in preference to the free, samples containing a low mass of steroid have high count rates facilitating improved counting conditions. Although there are several advantages to the technique in this form, the only other report, to our knowledge, of a method in which scintillator is added directly to the incubation tube is that by Hennam *et al.*[4]. The disadvantages of the method are the need for a large centrifugal force and the longer centrifuge times to separate the precipitate. However, this ensures firm packing of the precipitate to the tube and ensures that it is not easily disturbed. The supernatant fluid may then easily be removed by decanting, which enables large batches of samples to be handled.

This method has now been in use in our laboratory for over a year and has proved to be robust and reliable, features which are highly desirable when large numbers of samples have to be processed. The use of polyethylene glycol and the direct counting of the precipitate as described has been applied to other steroid assays and offers major advantages over other separation techniques of separation and counting.

Acknowledgement—We are grateful to Dr. W. Schopman for his gift of antiserum.

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Publications which include work from this thesis

Goodall A.B. and James V.H.T. (1977) Conjugated androstenedione in urine. J. Endocr 75 27p.

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Conjugated androstenedione in urine. By Ann Goodall and V. H. T. James. *Department of Chemical Pathology, St Mary's Hospital Medical School, London, W2 1PG*

Androstenedione is present in human urine both as the free steroid (Andino, James, Parker & Rippon, 1976) and also in a conjugated form (Schubert, 1958; Longhino, Tajic, Vedris, Jankovic & Drobnjak, 1968). A preliminary study of the types of conjugates and the total amount of androstenedione present has been made using hydrolysis and thin-layer chromatographic studies.

In hydrolysis studies, urine to which [^3H]androstenedione had been added as a tracer was extracted with hexane: ether to remove unconjugated androstenedione. After the addition of further tracer, the urine was incubated with β -glucuronidase, extracted, and then any remaining androstenedione was released by solvolysis (method of Burstein & Lieberman, 1958). The amounts of androstenedione in the extracts were estimated after paper (Bush B3) and silica gel column chromatography by radioimmunoassay. The amount of androstenedione that was released by solvolysis was larger than that released by β -glucuronidase and only a small proportion of androstenedione in urine is in the unconjugated form. For one normal male subject the amount of androstenedione in the solvolysis fraction was 42 $\mu\text{g}/24\text{ h}$, in the β -glucuronidase fraction 6 $\mu\text{g}/24\text{ h}$ and in the free fraction 2.8 $\mu\text{g}/24\text{ h}$.

To examine further the nature of the conjugates, a total urine extract, prepared by adsorption on an Amberlite XAD-2 resin column and elution with ethanol, was chromatographed on a silica thin-layer plate. The silica plate was divided into areas and the androstenedione estimated after solvolysis and chromatography as above. These studies indicate that at least two conjugates of androstenedione exist in urine, possibly the 3-enol glucuronide and the 3-enol sulphate.

Total urinary values of androstenedione have been estimated by employing solvolysis (Burstein & Lieberman, 1958) at pH 1 in 20% saline solution with ethyl acetate, and the androstenedione was estimated by radioimmunoassay after paper (Bush B3) and silica gel column chromatography. The glucuronides would be hydrolysed by this method due to the acid lability of their enol linkage. For nine normal women (age 21-37 years) excretion levels of androstenedione in $\mu\text{g}/24\text{ h}$ were: mean 23 (range 13-31) and for ten normal men (age 22-37 years) mean 57 (range 25-93). The adrenocortical function of a post-menopausal woman was suppressed with dexamethasone and plasma androstenedione levels were sampled at half-hourly intervals for 4 h before and after suppression. The fall in the plasma level is reflected by the fall in total urinary androstenedione measured in 24 h urine collections taken on the same 2 days.

These data show that the majority of the androstenedione present in urine is in the conjugated form and that men excrete larger amounts of androstenedione in a conjugated form than do women. There is evidence for at least two conjugates of androstenedione.

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Measurement of androstenedione in urine and correlation with levels in plasma. By Ann B. Goodall and V. H. T. James. *Department of Chemical Pathology, St Mary's Hospital Medical School, London, W2 1PG*

Androstenedione secretion by the adrenal cortex is episodic since it is responsive to adrenocorticotrophin, in the same manner as cortisol. A single plasma determination is clearly an unreliable reflection of the adrenal secretory pattern throughout the 24 h. A method has been developed for the measurement of androstenedione in urine (Goodall & James, 1977) and this has been used to compare urinary excretion values with mean plasma levels.

For a group of 11 postmenopausal subjects, urine specimens were collected for the same period of time as blood samples were taken, at half hourly intervals. A good correlation (correlation coefficient of 0.97) between the mean of the plasma estimations and the total amount of androstenedione in the urine for the same period was obtained.

In eight patients with hirsutism, the total amount of androstenedione in the urine has been estimated. The range in nmol/24 h was 72-166 (mean 112) compared with 44-109 (mean 78) for 11 normal female subjects.

In cases of adrenal dysfunction, hypersecretion of androstenedione often occurs. In two such patients with adrenal hyperplasia, urinary androstenedione has been measured during the administration of dexamethasone and metyrapone (Metopirone). In the first subject the androstenedione in the urine fell after dexamethasone (1.5 mg/day) from 252 nmol/24 h before treatment to 66 nmol/24 h on the third day. After Metopirone treatment (4 g in a divided dose six times a day) there was an increase from 211 nmol/24 h to 1254 nmol/24 h. Similar results were obtained for the second subject.

Ovarian secretion of androstenedione accounts for about 50% of androstenedione production, but other workers have reported varying contributions of the ovary at different times of the menstrual cycle from values determined in urine (Longhino, Tajic, Vedris, Jankovic & Drobnjak, 1968) and in plasma (for example, Judd & Yen, 1973; Kadar, McConway, Macnaughton & Coutts, 1977). For a normal subject androstenedione has been measured in two cycles in the second of which (cycle 2) dexamethasone (1.5 mg) was taken each evening. Both cycles were anovulatory. The mean for cycle 1 was 102 nmol/24 h and for cycle 2 it was 57 nmol/24 h, indicating that the ovarian contribution was around 50%. There was no major trend in the values except a fall in values for the 3 days before the onset of menstruation.

Hence, the correlation obtained between the mean of the plasma samples and urinary androstenedione indicates that total urinary androstenedione is a good reflection of integrated androstenedione values and may be a useful measurement in cases of adrenal and ovarian dysfunction.

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