ESTIMATION OF TESTOSTERONE IN AND ISOLATION OF PROGESTERONE FROM HUMAN URINE

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INTRODUCTION

In the Edwin Smith surgical papyrus (1600 B.C.) and the Ebers papyrus (1550 B.C.), some of the oldest medical books in the world, ancient Egyptians were the first to report information about the male and female gonads. In men, the effect of impotence, circumcision and testicular tumors were also described (see Major, 1953; Ghaliongui, 1963).

Aristotle, in his Historica animalium described the effect of castration in birds and man. He showed that castration of immature male birds resulted in failure of development of secondary sex characteristics, such as colouring of the crest, attraction to the female and the characteristic call of the male. If castration was performed in the adult, the sexual characteristics were only modified. Similar findings were also described in human subjects. Aristotle reported that castration of the male before puberty caused the high pitched voice of childhood to persist and noted that the sexual hair failed to grow. Thus

the relationship between the testes and virility has been known since ancient times.

The testicular tissue was also used as an aphrodisiac by the Indians in 800 A.D. Thus it seems that such a relationship was recognized, but without any understanding of the mechanism involved.

At the end of 1800 A.D., John Hunter was able to show by his transplantation experiments of the gonads that the status of the accessory organs, i.e. the seminal vesicles and prostate, depended on the presence or absence of the testes, and this demonstrates directly the big role of the testes in the development of these accessory organs.

However, the secretion of humors into the blood by various organs and glands was only recognized in the middle of the 19th century. Berthold, in 1849 A.D. may have performed the first successful physiological experiment to show the relationship between an endocrine gland, the testis, and the development of secondary or accessory sexual organs. He showed conclusively that the cock's comb undergoes atrophy after castration. However, when testicular tissue was transplanted into the abdominal cavity, such atrophy did not occur. Moreover, he showed that the comb remained atrophic and that resumption of growth only occurred after implantation of testicular tissue.

Shortly after Berthold's experiments, Claude Bernard, in 1855, demonstrated that the liver secretes a substance "glucose" directly into the blood. Starling in 1905, stimulated the secretion of pancreatic juice through intravenous injection of an acidic extract of the duodenal mucosa. These observations led to the concept of a substance later called a "Hormone" which was produced by a specific gland, secreted directly into the blood and transported to various organs and tissues on which it exerted its effect. The term hormone was derived from the Greek root meaning to "excite" or to "arouse".

Pézard, in 1911, was able to show that a simple extract of the testis containing no living cells has active material which promotes comb growth in the capon. This indicated that the effects of the testes were due to some specific chemical constituent. The nature of this substance was studied by many workers. Loisel (1903) suggested that it was lipid in nature. McGee (1927) was able to achieve a reasonable degree of purification and concentration of this material by extracting the testes with fat solvents. Moreover, he showed that marked growth of cockerel comb was obtained following injection of this extract. However, the amount of the hormone present in the testes is extremely small, and the early attempts of several investigators to obtain highly purified material from

testicular tissue met with failure. Systematic investigation of other sources like urine by Butenandt (1931) eventually led to the first isolation of a crystalline androgen. In this work 15,000 litres of urine yielded only 15 mg. of the steroid. The structure of this compound was found by Butenandt (1932) to be 3αhydroxy-5α androstan-17-one, and it is now known as androsterone. The quantitative differential bioassays conducted by Dingemanse, Freud & Laqueur (1935) revealed differences in biological activity of androsterone and the testicular hormone. It soon became evident that both compounds were also chemically different. In 1935, David, Dingemanse, Freud & Laqueur reported the isolation of a testicular hormone with a melting point of 154° to which he gave the name testosterone. Using about 100 kg. of steer testicular tissue, 10 mg. of testosterone was isolated in a pure form. The biological activity of pure hormone was shown to be about six times more than that of androsterone in promoting comb growth, and the latter was correctly suspected to be a reduction product of testosterone. The chemical formula was established soon after. Testosterone has the absorption spectrum of an α, β unsaturated ketone and it yields androst-4-ene-3,-17-dione* androstenedione) on oxidation. This suggested that testosterone could be a reduction product (at C-17) of androstenedione.

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* In this thesis the systematic names of the compounds are followed by their simpler trivial names; thereafter, the trivial names are generally used.

The chemical structure of testosterone was established as 17 β -hydroxyandrost-4-en-3-one by comparison of its physico-chemical and biological properties with those of the synthetic product. (Butenandt & Hanisch, 1935 and Ruzicka & Wettstein, 1935). However, it should be noted that isolation of the hormone from testicular tissue does not imply that it is secreted by the testes. West, Hollander, Kritchevsky & Dobriner (1952) and Lucas, Whitmore and West (1957) provided evidence that the hormone is a true secretory product of the human testes, when they isolated and identified it in spermatic venous plasma and showed that it was not present in the arterial blood.

Estimation of total androgens in urine

Following the study of Pézard in 1911 on the capon's comb, the first attempt to measure androgenic potencies of urinary extracts was by the use of bioassays. However, the description of a colour reaction for 17-ketosteroids by Zimmermann (1935) and subsequent study by Callow, Callow & Emmens (1938) showed a significant degree of correlation between the colorimetric assay expressed in chromogenic equivalents of androsterone per litre and the previous bioassay procedures. Thus it was concluded that colorimetric assay could replace the bioassay. Since

then this colour reaction has been widely used by many workers. However, such determinations have not proved of great value in the assessment of the androgenic stimulation to which the body is subjected. This is mainly due to the fact that the majority of the weakly androgenic urinary 17-ketosteroids in men and women arise from weakly androgenic precursors secreted by the adrenal cortex rather than by the testes (see p. **83**).

Testosterone in blood

Another attempt to measure androgenic function in intact individuals was based on the fact that testosterone is a more potent androgen than any other compound normally present in blood (Dorfman & Shipley, 1957). Thus plasma levels of testosterone can be assumed to be a measure of the androgenic stimulation to which the body is exposed.

Hollander & Hollander (1958) used an isotopic dilution method which made possible the estimation of testosterone in spermatic vein blood. Levels from 50-160μg./100 ml. of plasma in young men and from 3-10μg./100 ml. in older subjects were found. However, these authors were unable to measure levels in peripheral blood. Oertel & Eik Nes (1959) have estimated levels of testosterone in peripheral plasma after stimulation with human chorionic gonadotrophin

(HCG) and have reported levels of 1·4µg./100 ml. plasma. However, the volume of plasma needed for such a determination was large, i.e. 400 ml.; Migeon (1960) could not demonstrate testosterone in a pool of 2·5 litres of human peripheral plasma. Oertel & Kaiser (1961) did demonstrate and measure testosterone in the peripheral plasma of young men and found levels ranging from 0·13 - 0·26µg./100 ml. using volumes of 200 ml.

At this time it was generally accepted that testosterone existed as free steroid in plasma. However, testosterone glucuronide was reported to be present in female sockeye salmon by Grajcer & Idler (1961), levels up to 7·5µg./100 ml. being encountered.

The conversion of testosterone to oestrogens by placental enzymes (Ryan, 1959) was the basis of a method described by Finkelstein, Forchielli and Dorfman (1961). The oestrogens can be estimated by a very sensitive fluorescence reaction. The figures obtained when this technique was applied to measure testosterone in peripheral plasma by Forchielli, Sorcini, Nightingale, Brust, Dorfman, Perloff and Jacobson (1963) ranged from 0·1 to 0·98µg./100 ml. in men and from 0·02 to 0·26µg./100 ml. in women. The volume of plasma required for a single estimation by this method was 50·0 ml.

More recently, several isotopic methods have been

developed for the estimation of the hormone in blood. These overcome the problem of inadequate sensitivity, amounts of 0·01μg. of steroid being within the sensitivity of the procedures (Tait & Tait, 1962). The formation of labelled derivatives and combination with radioactive reagents has made it possible to estimate low levels of testosterone in peripheral plasma. Hudson, Coghlan, Dulmanis, Wintour & Ekkel (1963) used a double isotope dilution derivative procedure. Following the addition of ^{14}C-testosterone as an internal standard to plasma, the tritium labelled acetyl derivative was formed. These workers reported a mean value of plasma testosterone of 0·74μg./100 ml. (range 0·49 - 0·98) and 0·11μg./100 ml. (range 0·05 - 0·31) for men and women respectively. Later, Burger, Kent & Kellie (1964) modified the method of Hudson, et al. (1963) and replaced the somewhat difficult initial paper chromatographic steps by thin-layer chromatography. Another technique of labelling was also described by Riondel, Tait, Gut, Tait, Joachim & Little (1963) in which ^{35}S-thiosemicarbazide was used for the formation of the derivative and ^{3}H-labelled testosterone was employed as a marker steroid. The level found for a group of 11 normal men was 0·8 ± 0·25 μg./100 ml. plasma and for two normal women was 0·059 and 0·079μg./100 ml. plasma; Lim & Brooks (1965) reported a modification of the method described by Riondel et al.

(1963), and increased the recovery from levels of 7% up to 22%; he also improved sensitivity, specificity and practicability. Levels for normal men and women were similar to those previously described.

Following the observation that certain organic vapours may capture electrons and the application of this principle to the detection of effluents from gas-liquid chromatography columns, suggestions were made that this technique would be suitable for the estimation of small amounts of steroids provided adequate purity of the substances could be obtained before gas-liquid chromatography. Moreover, the formation of a steroid derivative such as the haloacetate or heptafluorobutyrate would increase the sensitivity in the electron capture detector. Brownie, Vander Molen, Nishizawa & Eik Nes (1964) developed a method for estimating the hormone in peripheral plasma using thin-layer chromatography and an electron capture detector after gas-liquid chromatography of testosterone chloroacetate. The sensitivity of this technique was 1×10^{-9} gm.; levels for men were 0·24 µg./100 ml. and for women was 0·06µg./100 ml. Recently a preliminary account was given by Exley & Feder (1967) in which a method for the ultramicrodetermination of plasma testosterone by gas-liquid chromatography was described. The use of a highly sensitive electron capture detector together with the formation of the heptafluorobutyrate derivative of

testosterone has enabled as little as 1 x 10^{-10} gm. of testosterone to be detected. About a hundred times this amount is to be expected in 1 ml. of male plasma.

Generally, there was a reasonable correlation between the plasma levels reported by these methods and the physiological state of the subjects. However, such techniques, being of a laborious nature, are of limited usefulness, particularly if serial estimations are required.

Testosterone in urine

The isolation of testosterone from human urine following administration of large doses of the hormone was reported by Lieberman, Fukushima & Dobriner (1950). Edwards & Kellie (1956) reported a similar finding and showed that testosterone is excreted in the urine as a glucosiduronate (glucuronide) and that the 17-hydroxy group is linked to the hemi-acetal carbon of glucosiduronic acid (glucuronic acid). However, it was not until 1960 that Schubert & Wehrberger demonstrated the isolation of testosterone from normal human urine. There is evidence that testosterone is present in the urine mainly as glucuronide (Schubert & Wehrberger, 1960; Camacho & Migeon, 1963) but small amounts of the free compound (Hudson, Coghlan, Dulmanis & Wintour, 1964) and of testosterone sulphate (Tamm, Volkwein & Voigt, 1967) have also been detected. Recently, there has been considerable

interest in the estimation of urinary testosterone, as a possible means of assessment of gonadal function in the male and of androgen production in females. Many workers using labelled steroids *in vivo* were able to demonstrate clearly that a good correlation exists in the male between the appearance of testosterone glucuronide in urine and the secretion of testosterone by the testes (see P.**83**). However, they showed that in women more than 50% of urinary testosterone glucuronide was probably produced in the liver from precursors other than plasma testosterone.

Since 1963, several methods for measuring testosterone in urine have been described. Futterweit, McNiven, Narcus, Lantos, Drosdowsky & Dorfman (1963), Vermeulen & Verplancke (1963), Ibayashi, Nokamura, Murakawa, Uchikawa, Tanioka & Nakao (1964), Brooks (1964), Panicucci, Savi & Coli (1964), Sparagana (1965) and Wegienka *et al.*, (1967) developed methods basically dependent on separation by thin-layer chromatography and detection by gas-liquid chromatography or by a colour reaction. Acetylation or oxidation was used to increase specificity and to achieve satisfactory separation. In addition, Camacho & Migeon (1963), Dulmanis, Coghlan, Wintour & Hudson (1964), Voigt, Volkwein & Tamm (1964), Schubert & Frankenberg (1964), Sandberg, Ahmad, Cleveland & Savard (1964) and Rosner, Conte, Briggs, Chao, Sudman & Forsham (1965) have developed methods depending on multiple chromatography on

paper, thin-layer and columns in an attempt to achieve greater purification and separation and thereby to increase specificity. Another method depending on gradient elution from alumina and celite was described by Zurbrügg, Jacobs & Gardner (1965). Lim & Dingman (1965) described a method involving impregnated glass fiber paper for chromatography. Horn, Statter & Finkelstein (1966) developed another urinary method depending basically on the procedure of Finkelstein et al. (1961) in which testosterone is converted to oestrogens by placental enzymes, and the oestrogens are estimated quantitatively using a sensitive fluorescence reaction. A new method which involves acid hydrolysis with simultaneous benzene extraction and the use of adsorption chromatography of the trimethyl silyl ether of testosterone an alumina column before gas-chromatographic analysis was recently described by Vestergaard, Raabo & Vedso (1966).

The majority of the published methods for the estimation of testosterone in urine have disadvantages. Some do not completely separate testosterone from its 17α epimer, 17α-hydroxyandrost-4-en-3-one (epitestosterone), which is not derived from testosterone (Brooks & Giuliani, 1964). Others require large volumes of urine. Many of these methods involve numerous and complicated chromatographic separations which make them unsuitable for clinical application. In addition, some of the procedures have such low sensitivity that the low concentrations

found in body fluids of females are below the limit of estimation. Nevertheless, the results obtained by the majority of these methods showed no overlap between the levels of the hormone excretion in men and women - this finding strongly suggests that such techniques may be of great value in the assessment of testicular function in man.

The purpose of the present research was to develop a new method for the estimation of testosterone which would be suitable for clinical application and to avoid many of the disadvantages of previously described techniques. The developed method has been applied to a study of the hormone excretion in normal and in pathological conditions.

The present thesis describes:-

1. A new and practicable method for estimation of urinary testosterone.
2. Application of the method
   (a) To a study of excretion of the hormone in normal males.
   (b) To an investigation of the pattern of testosterone excretion in women during the normal menstrual cycle.
3. Isolation of progesterone from human pregnancy urine.

A METHOD FOR THE ESTIMATION OF URINARY TESTOSTERONE

The present section describes the development of a new method for the estimation of urinary testosterone. The fundamental operations of the method are as follows: (1) Hydrolysis of the testosterone conjugates, (2) extraction of the liberated testosterone (and other steroids) into ether, (3) separation of the steroids possessing a "conjugated ketone" function (testosterone) from other steroidal alcohols and steroidal saturated ketones, by means of a modified Girard reaction, (4) purification of the "conjugated ketone" fraction by alumina column chromatography, (5) purification of the extract containing testosterone by paper chromatography, (6) quantitative analysis of the testosterone in the final extract by gas chromatography as testosterone itself or as androstenedione after its oxidation with chromium trioxide. The great degree of purification achieved by using the modified Girard reaction permits the use of hot acid hydrolysis despite of the fact that this

method initially yields more impurities than enzyme hydrolysis. Further purification of the "conjugated ketone" fraction by alumina column chromatography and by paper chromatography yields testosterone in extracts that can be conveniently analysed by gas-liquid chromatography. The reliability of this analytical procedure has been studied.

MATERIALS AND METHODS.

Reagents and solvents

The solvents and reagents used were of analytical grade quality in all cases in which this grade was avail-able. The solvents were redistilled before use. Girard reagent T was washed with ethanol. The cation exchange resin Amberlite IRC-50 (H) (British Drug Houses, Rohm and Haas Co.) was washed three times in 10-20 g. batches with about 10% warm aqueous sulphuric acid (v/v); the resin was then washed with distilled water until the washings were neutral. It was stored with a small quantity of water. Aqueous formaldehyde must be kept at a temperature above 25° to avoid polymerization. All steroid standards were purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks. England). Their purity was checked by thin-layer, and by paper chromatography on appropriate systems and using suitable methods of detection.

Paper and thin-layer chromatography

Paper chromatography was performed on Whatman No. 42 paper in the system toluene-light petroleum

(b.p. 60°- 80°) - methanol - water (33:66:80:20,by vol.). Paper chromatograms were run automatically overnight after at least 4 hours of equilibration. An alarm clock was modified to turn a tap which filled the trough with solvent. Chromatograms were inspected under ultraviolet light (wavelength approx. 250mμ) and were then treated either with m-dinitrobenzene in alkali (Zimmermann reagent), or with 8% (w/v) phosphomolybdic acid in ethanol, or with a saturated ethanolic solution of 2,4-dinitrophenylhydrazine acidified with a few drops of concentrated hydrochloric acid (see Bush, 1961). The development of the colour was achieved by heating the paper chromatograms gently on a hot plate. Elution of steroids from paper chromatograms was performed after location of the standard with the aid of ultraviolet light. The corresponding area was marked off lightly in pencil, and cut into small strips not more than 7mm square, which were then shaken with methanol for a period of 30 minutes. The eluate was then filtered through sintered glass and an aliquot was taken for further investigation.

Thin-layer chromatography was performed on silica gel GF 254 for detection of ultraviolet absorbing compounds, on silica gel G, and on aluminium oxide G. (E. Merk. A.G. Darmstadt.). These were spread in sheets

approximately 250 μ thick by the standard technique. After spreading , the silica gel coated plates were heated at 100° for 30 minutes. Plates coated with aluminium oxide G were heated at 150° for 120 minutes. The plates were then allowed to cool and stored in a desiccator until required. The systems chloroform-ethyl acetate (2:1, v/v) and benzene-ethyl acetate (3:2 and 3:1, v/v) were employed for silica gel thin-layer chromatography. The system dichloromethane-ether (100:15, v/v) was employed for chromatography on aluminium oxide. The R_f values of testosterone and epitestosterone in these systems are shown in table 1. In addition to the methods of detection described above for paper chromatography, thin-layer plates were in some instances developed with 0.5% (w/v) vanillin in ethanolic sulphuric acid (1:4, v/v).

Alumina column Chromatography

Column chromatography was carried out in glass tubes with an internal diameter of 0.5 cm. and with taps at the lower ends. The adsorbent was supported on a small plug of cotton wool placed at the lower end of the tubes. The alumina (Hopkins and Williams 100 - 240 mesh alkaline) had a Brockman activity of 1 ; this was reduced by the addition of about 10% (v/w) of water which was thoroughly mixed in. The activity of the

TABLE 1

R_f values of testosterone and epitestosterone in thin-layer chromatography using various systems

(Values represent a mean of 4 experiments)

Stationary phase	Mobile phase	R_f of testosterone	R_f of epitestosterone
Aluminium oxide G	Dichloromethane-Ether (100:15, v/v)	0·67	0·52
Silica Gel G and GF 254	Chloroform-Ethyl acetate (2:1, v/v)	0·41	0·40
Silica Gel G and GF 254	Benzene-Ethyl acetate (3:2, v/v)	0·55	0·55
Silica Gel G and GF 254	Benzene-Ethyl acetate (3:1, v/v)	0·29	0·24

alumina was adjusted by step-wise addition of water so that when testosterone was applied in 5 ml. of benzene to a 6·0 x 0·5 cm. column of alumina equilibrated with benzene and the column was eluted with 10 ml. of 0·15% (v/v) ethanol in benzene followed by 15 ml. of 0·3% ethanol in benzene, testosterone appeared in the 4-11 ml. portion of the second eluate.

Gas-liquid Chromatography

This was performed in a Pye Panchromatograph fitted with a hydrogen flame ionisation detector. All glass columns 150 x 0·4 cm. were used. Analytical samples were deposited into metallic gauzes and introduced into the chromatographic columns in the solid state by the technique described by Menini and Norymberski (1965). The columns were packed with silicone-treated Gas-Chrom P (an acid and alcoholic base washed diatomaceous support - 100-120 mesh) coated with 1% (w/w) of the stationary phases SE-30 (methyl silicone rubber gum) or QF-1 (fluoroalkyl silicone polymer) by the filtration technique of Horning, Moscatelli and Sweeley (1959) as described by Brooks and Hanainah (1963). The flow of the mobile phase (nitrogen) was at a rate of 30 ml./min; column temperature was 220° for SE-30, and 205° for QF-1. The evaporation chamber was kept at least 20° above the temperature at which the column was operated. The flame ionisation detector was used at the optimum conditions

determined by Fowlis, Maggs and Scott (1964). Testosterone or androstenedione in the final extract was measured by gas-liquid chromatography on a column containing 1% QF-1. The quantity of steroid was calculated from the response (peak height) given by a known amount of the appropriate reference compound (external standard) run at the beginning and at the end of a series of analyses. The external standard was also used to establish the retention time of the compound under analysis.

~~reference compound~~.

Collection of gas-liquid chromatographic fractions was carried out in a Perkin-Elmer, Model 801 apparatus fitted with a stream splitter giving a ratio of effluent of 1:4, and with a hydrogen flame ionisation detector. The chromatographic column contained Gas Chrom P (100-200 mesh) coated with 1% of QF-1 and was operated at 220° . The flow of nitrogen was 25 ml./min. When the peak with the retention time expected for testosterone appeared on the recorder chart, the major portion of the effluent was compelled to pass through a glass tube (4 mm. i.d. and 20 cm. long) that had two constrictions near the end. The glass tube was placed at one exit of the chromatographic oven and was kept at room temperature. The eluted material condensed on the inner

walls of the glass tube from where it was recovered by a washing with chloroform. The efficiency of the collection was of the order of 60% for testosterone.

Formation of derivatives for gas-liquid chromatography, namely the acetate and trimethylsilyl ether of the trapped component for further gas chromatographic analysis were performed using techniques described by Norymberski and Riondel (1967) and Menini, Orr, Gibb and Engel (1967) respectively. In these techniques, derivatives were prepared in the gas phase. For the formation of the acetate, the trapped component was transferred onto a metallic gauze which was placed in a vessel saturated with pyridine and acetic anhydride and the reaction was allowed to proceed at 40° overnight. Testosterone was quantitatively converted to testosterone acetate under these conditions. For the formation of trimethylsilyl ether derivatives, bis-trimethylsilyl acetamide (and phosphorous pentaoxide to maintain anhydrous condition) was used in a similar way to that described for acetate formation. Authentic testosterone was also subjected to the same procedures for subsequent comparison using gas-liquid chromatography.

The Determination of Radioactivity

Radioactivity due to ^{14}C was measured by liquid scintillation counting using the method described by

Harkness and Fotherby (1963). The equipment used was the assembly NE 8301 (Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh 11). The residue containing radioactive material was dissolved in a few drops of absolute ethanol and 3 ml. NE 213 scintillator was then added. The solution was then transferred to a quartz counting vessel. The counting vessel was then placed into the counting head for at least 3 minutes before the counting started. Samples were counted for sufficient time to maintain the error less than 5%.

Spectroscopy

The absorption spectra in the visible and ultraviolet region were recorded in a Unicam SP-800 spectrophotometer. Quartz cells with a light path of one cm. were used. Infrared spectra (2·5 to 16·0 μ) were taken in a Perkin-Elmer 237 spectrophotometer. Specimens from paper chromatograms were dissolved in carbon disulphide (for spectroscopy; British drug Houses Ltd.) and placed in a cell with a 0·5 mm. light path. For components trapped from gas-chromatographic effluents, a Unicam SP-200 with a beam condenser was used. Samples

dissolved in chloroform were deposited as a spot on a micro-disc of KBr by a technique developed by Sykes & Kelly (personal communication) and the spectrum was then recorded.

Gas-liquid Chromatography combined with Mass-Spectrometery

This technique was used in order to gain additional information on the specificity of the present analytical technique. The instrument used was the LKB Model 9000 Gas Chromatograph Mass Spectrometer (LKB Produkter AB, Stockholm, Sweden) equipped with the molecular separator described by Ryhage (1964) , made available by the courtesy of Dr. C.J.W.Brooks (Chemistry Department, University of Glasgow). Conditions for gas-chromatography were as described in p.**20** except that the length of the columns were 10 feet. The mass spectra of the compound tentatively identified as testosterone during the gas chromatographic analysis (on columns containing QF-1 and XE-60) of urinary extracts obtained by the present analytical procedure were compared with the mass spectra of authentic testosterone subjected to gas chromatography and mass-spectrometry under identical conditions. In all occasions mass spectra have been taken at different points in the ascending, at the maximum and the descending

parts of the gas-chromatographic peak, to check the homogeneity of the peaks.

Preparation of Urine Extracts

Complete 24 hour samples were collected and specimens of less than 1200 ml. were made up to that volume with distilled water. Urine samples were stored at 4^{o} and were analysed within two weeks of collection. Hydrolysis of testosterone conjugates was then performed using either the enzyme β-glucuronidase or by a technique involving hot acid. The source of β-glucuronidase was the common limpet Patella Vulgata; enzyme preparations were obtained by the method described by Fotherby and Love (1960) and had activities of $1.5x10^{6}$ - $3.0x10^{6}$ Fishman units/g. of dry powder. Enzyme hydrolysis was performed by the method described by Fotherby and Love (1960). Urine was adjusted to pH 4.7 and 0.1 of its volume of molar acetate buffer of pH 4.7 , and the required amount of enzyme powder were added (see p.**32**). The mixture was well shaken and incubated at 37^{o} for the required time. Acid hydrolysis was carried out by boiling the urine under reflux with 15 ml. concentrated hydrochloric acid added to 100 ml. of urine. Hydrolysis for one hour gave the best results (see p.**35**). Following hydrolysis , the urine was extracted with ether and the extract was evaporated to dryness.

Modified Girard separation

The residue was dissolved in 1.0 ml. of 97% ethanol and 150-200 mg. of Amberlite IRC-50 (H), and 300-500 mg. of Girard's reagent T were added. The mixture was then refluxed for one hour, cooled and mixed with 10 ml. of distilled water. The solution was decanted and was extracted once with one volume and twice with 0.5 volumes of ethyl acetate. Following each extraction the upper phase was removed by suction. An aliquot of 9.5 ml. was then taken from the aqueous phase and 2.0 ml. of 36% aqueous formaldehyde (w/v) were added. The solution was allowed to stand on the bench overnight and was extracted three times with 12 ml. of ethyl acetate to obtain the formaldehyde labile "conjugated ketone" fraction containing testosterone (see p.**42**). The combined ethyl acetate extracts were washed once with 5 ml. N. sodium hydroxide saturated with sodium chloride and 5 times with 5 ml. of distilled water and were dried with anhydrous sodium sulphate. An aliquot of 32 ml. was evaporated to dryness under reduced pressure in a water bath at 70°. The last traces of formaldehyde were removed with a jet of air.

Oxidation of testosterone to androstenedione

Optimal conditions for the oxidation of testosterone using chromium trioxide in glacial acetic acid were

investigated (see p.**56**). The reaction was carried out by dissolving the residue containing testosterone in 0.2 ml. of glacial acetic acid and adding 0.2 ml. of freshly prepared aqueous solution of 15% (w/v) chromium trioxide. The solution was allowed to stand in the dark at 25° for 15 minutes. At the end of this period 3 ml. of 8% (w/v) aqueous sodium citrate were added. The oxidation products were extracted with 10 ml. of chloroform which was washed once with 1 ml. of 0.1 N aqueous sodium hydroxide and twice with 1 ml. of distilled water. The extract was then dried with anhydrous sodium sulphate and an aliquot was evaporated to dryness and used for analysis by gas-liquid chromatography or by the micro-Zimmermann reaction.

Micro-Zimmermann reaction

Determination of androstenedione by the micro-Zimmermann reaction was carried out according to Corker, Norymberski & Thow,(1962). The residue was dissolved in 0.1 ml. of freshly prepared 0.5% (w/v) m-dinitrobenzene in ethanol and 0.1 ml. of a 20% (w/v) aqueous solution of tetra-ethyl ammonium hydroxide was added. The solution was kept at 0-4° for 3 hours. The reaction mixture was then diluted with 0.5 ml. ethanol. Optical

densities were measured at 432, 512 and 592 mμ against a reagent blank treated similarly and the corrected extinction was calculated by the method of Allen (1950).

RELATIONSHIP BETWEEN THE MASS OF TESTOSTERONE AND THE CORRESPONDING PEAK HEIGHT.

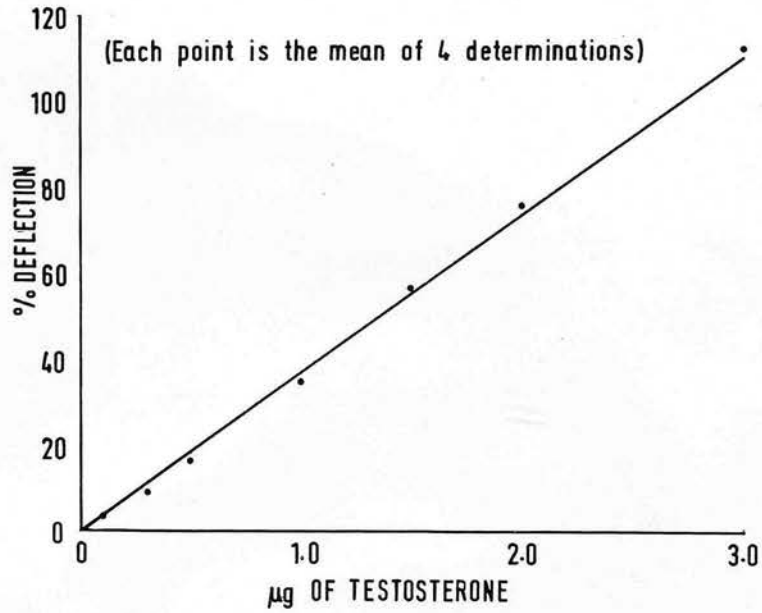


Fig. 1 .

EXPERIMENTAL

GAS-LIQUID CHROMATOGRAPHY

Samples for gas-chromatographic analysis were prepared according to the technique described by Menini and Norymberski (1965). The residue was dissolved in 0·3 ml. of chloroform and transferred onto a Dixon gauze ring deposited in a polytetrafluoroethylene plate (PTFE). On evaporation of chloroform the sample was deposited quantitatively on the metallic gauze.

The quantity of steroid under investigation in each sample was estimated in terms of the response given in the same day by the external standard (testosterone or androstenedione). This technique can be used if a linear relationship exists between the mass of steroid and the corresponding peak height. Such linear relationship was demonstrated by plotting different quantities of standard testosterone versus the means of their corresponding peak height. A plot of the results obtained over a range varying from 0·1 to 3·0 μg. showed a straight line passing through the origin (Figure 1).

The reproducibility of external standard for quantitative estimations is illustrated in four typical runs, performed over a period of 10 days. In each run, four testosterone standards each 1·1 μg. were used, two of which were chromatographed at the beginning of a series of analysis and two at the end. The results obtained are shown in Table 2. Considerable variations were

TABLE 2

Reproducibility of peak heights and retention time of the external standards (testosterone) used in gas chromatographic analysis

Four standards each 1·1 μg. were used in each experiment on the same day. The four experiments were performed over a period of 10 days. The column used was 1% (w/w) QF-1 at 205°.

Experiment	% Deflection				Mean ± S.D.	Retention Time (min)			
	1	2	3	4		1	2	3	4
A	63·0	62·0	60·0	57·0	60·5 ± 2·64	11·1	10·8	10·5	11·1
B	45·0	43·5	40·0	39·0	41·9 ± 2·82	10·4	10·8	10·6	10·8
C	41·5	42·0	49·0	45·0	44·4 ± 3·42	10·8	10·8	11·1	11·3
D	43·0	52·0	55·0	54·0	51·0 ± 5·47	10·6	10·8	10·8	10·8

observed between the height of the peaks obtained in different days, and therefore, a series of external standards have to be run in every analysis. Testosterone in the urinary extract was tentatively identified by its retention time in relation to that of the external standard.

HYDROLYSIS

(1) Effect of enzyme concentration and period of incubation on the yield of testosterone: Previous methods for the determination of testosterone in urine have used enzymic hydrolysis with various β-glucuronidase preparations (see reference in Table 10). The majority of workers have incubated the urine at 37° for 48 hours with different concentrations of this enzyme. In the present work, an investigation was made of the effect of different enzyme concentrations and of different periods of incubations on the yield of testosterone from the urine. In a series of nine experiments three equal portions from each urine specimen were incubated at 37° with different amounts of enzyme and for different periods of time and were then analysed by the procedure described in page 60 . The results of the experiments are shown in Table 3. It was found that incubation at 37° for 48 hours with a concentration of 1,000 units per milliliter of urine of β-glucuronidase gave virtually the same results as incubation for 72 hours with a concentration of 1,500 units/ml. The majority of the testosterone was released by incubation for 18 hours with a concentration of only 500 units/ml. From the above experiments it appears that the conditions of choice are incubation for 48 hours at 37° with a concentration of 1,000 units/ml. of β-glucuronidase.

TABLE 3

Testosterone yield (µg./24 hr.) after hydrolysis with different concentrations of β-glucuronidase and different times

No. of Expt.	Incubation for 18 hrs with a concentration of 500 units/ml.	Incubation for 48 hrs with a concentration of 1,000 units per ml.	Incubation for 72 hrs with a concentration of 1,500 units/ ml.
1	41.5	41.0	43.1
2	37.0	39.0	42.3
3	49.0	48.0	55.1
4	55.0	61.5	60.5
5	47.5	53.0	55.5
6	49.0	50.0	47.4
7	55.0	60.0	64.0
8	10.0	13.0	12.6
9	7.0	8.0	8.8

(2) Effect of hot acid hydrolysis on the stability of testosterone: Hydrolysis of urinary steroid conjugates by hot mineral acid is a rapid and simple procedure which is widely used in many methods of steroid analysis. However, it is well known that such procedures have deleterious effects on the molecules of many steroids which may be destroyed or altered giving rise to artifacts. Substitution, dehydration and rearrangements are the most common reactions leading to artifact formation (Dorfman and Ungar, 1965). Destruction of the steroid molecule or formation of artifacts during an analytical process is obviously undesirable as it often gives rise to underestimation or over-estimation of the compound under investigation depending on the final method of analysis. Therefore, before attempting to establish the optimal conditions for the hydrolysis of the urinary conjugates of testosterone the stability of free testosterone under the conditions of hot acid hydrolysis was investigated. Five micrograms of authentic testosterone were added to 200 ml. of distilled water and boiled for one hour with 0·15 vol. concentrated hydrochloric acid, which was subsequently extracted with two volumes of ether. The ether extract was washed with 1/10 N sodium hydroxide and water, dried with anhydrous sodium sulphate and an aliquot was taken to dryness. The residue was chromatographed on thin-layer chromatography into the system benzene - ethyl acetate (3:1, v/v) and the areas

corresponding to testosterone were eluted and estimated by gas-liquid chromatography. The eluted compound behaved as authentic testosterone. In six experiments the mean amount of testosterone recovered by this procedure was 96·8% ± 2·0 (Mean ± S.D.). This figure may represent a slight overestimation of the actual recovery due to the fact that ether was used in the experiment and some evaporation might have occurred before the aliquot was taken.

In connection with the stability of testosterone to the conditions of hot acid hydrolysis, it is of interest to note that when epitestosterone was subjected to the above conditions, almost complete destruction occurred. This finding has been recently reported by Ismail, Davidson, Faro and Loraine (1967).

(3) Effect of time on hydrolysis of testosterone using 15% concentrated hydrochloric acid at 100°: Three equal portions from nine urine samples obtained from men and women were subjected to hot acid hydrolysis using 15% concentrated hydrochloric acid and boiling for 15, 30 and 60 minutes. The amounts of the hormone recovered from samples of the same urine after different times of boiling and using the method described in p.60, are shown in table 4. It was also found that when the urine was boiled for one hour much less contamination was observed in the final extract, and the amount of testosterone

TABLE 4.

Testosterone yield (µg./24 hr) after hydrolysis with 15% concentrated hydrochloric acid (100°) and different times.

Specimen No.	Time of hydrolysis			
	15 mins.	30 mins.	60 mins.	120 mins.
1	31.5	43.0	47.1	-
2	29.9	40.0	50.0	-
3	40.2	49.5	50.3	-
4	44.0	51.6	63.3	-
5	53.2	57.9	55.9	-
6	29.5	39.9	47.9	-
7	5.8	7.8	8.5	-
8	9.9	10.3	10.8	-
9	10.8	13.3	18.0	-
10	-	-	53.4	39.5
11	-	-	47.5	49.6
12	-	-	46.6	44.5

recovered was also greater than when the urine was boiled for 15 or 30 minutes.

In a series of four experiments on 200 ml. of urine from normal males, following hot acid hydrolysis for one hour and ether extraction, the urine samples were subjected further for one hour hydrolysis, and the assay of testosterone was repeated again. Negligible amounts could only be detected by gas-chromatographic analysis. In another experiment performed on 3 equal portions obtained from a pool of urine from normal males, hydrolysis for one and two hours showed similar results (see table 4).

(4) Comparisons between the yields of testosterone from urine subjected to enzyme hydrolysis and to acid hydrolysis: The amounts of testosterone found in the same urine specimens after acid and enzymic hydrolysis are shown in Table 5. In an attempt to ensure complete hydrolysis with the enzyme preparation, a higher concentration and a longer period of incubation were used. The difference in the yields of testosterone obtained by the two methods of hydrolysis were tested for statistical significance using 'students'' 't' test. The mean difference between the two systems of hydrolysis carried out on 22 urine specimens (Table 5) was 2·4 μg./24 hours, and was barely significant (<·05 = P = >·02). If the male and female samples were analysed separately the increase in yield from acid hydrolysis in male specimens

TABLE 5.

Comparison of Hot Acid and Enzymic Hydrolysis.
(urinary testosterone in µg./24 hours).

Urine Specimen	Hydrolysis with 0·15 vol. conc. HCl at 100° for one hour	Limpet β-glucuronidase 1500 units / ml. of urine: incubation at 37° for 72 hours.
Male		
1	48.1	43.1
2	50.0	42.3
3	60.3	55.1
4	63.3	48.6
5	64.4	60.5
6	47.9	44.8
7	46.2	46.5
8	44.1	36.2
9	60.9	47.8
10	55.7	55.5
11	49.0	47.4
12	45.2	47.3
13	63.5	64.0
14	62.2	57.1
15	87.1	96.5
16	102.9	105.4
Mean	59.4	56.9
Female		
17	9.5	8.8
18	12.8	11.6
19	18.0	16.5
20	7.7	7.0
21	9.1	8.6
22	12.5	12.0
Mean	11.6	10.8

was 3·6 µg./24 hours (<·05 = P = >·02) while the female specimen in which much smaller amounts of testosterone were detectable, showed a difference of 0·85 µg./24 hours in favour of acid hydrolysis. The urine specimens numbered 4, 8 and 9 showed much lower yields of testosterone after enzymic hydrolysis when compared with acid hydrolysis. The differences being 14·7, 7·9 and 13·1 µg./24 hours respectively. Such low yields (Table 5) may have arisen from the presence of an endogenous inhibitor of β-glucuronidase in these urine specimens. An attempt was made therefore to destroy the inhibitor by alkalinisation (Harkness and Fotherby, 1963; Marsh, 1963) and enzymic hydrolysis was repeated. The estimates of urinary testosterone in µg./24 hours were 61·2, 36·8 and 45·0 respectively. Although the yield was increased in sample 4 only (+ 12·6), it was possible therefore that one cause of the differences in effectiveness between acid and enzyme hydrolysis might be the endogenous inhibitor of β-glucuronidase in urine, glucaro- 1, 4-lactone (Marsh, 1963). Clearly however, there may be other reasons that might account for the higher yields of testosterone when acid hydrolysis is used.

Since the effect of alkalinization on the endogenous inhibitor improved the yield of testosterone in urine sample 4, the new value (61·2 µg./24 hours) was substituted and the statistical analysis repeated. In male urine samples the higher mean yield of 2·51 µg./24 hours

was not significant (<·1 = P = >·05), although yields by acid hydrolysis tended to be higher (2·01 μg./24 hours).

There is no significant difference in testosterone yield of female urine subjected to acid and enzyme hydrolysis. Also, much greater statistical variation was encountered with the higher level of testosterone in male urine and in the two highest values recorded (specimens 15 and 16). Enzymic hydrolysis tended to give higher yields of the hormone.

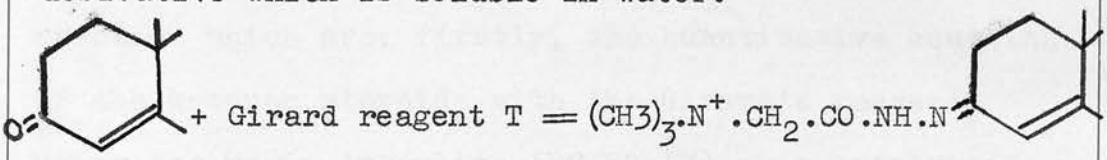
The slightly higher yields of testosterone obtained from the acid hydrolysis procedure together with the possibility that β-glucuronidase inhibitors might be present in some urine specimens would in some instances (specimen 4 in Table 5) affect the determination of the hormone, favoured the selection of acid hydrolysis as the method of choice. In addition the technique of acid hydrolysis is simple to carry out and more economic. However, acid hydrolysis produced small amounts of compound which were not detected in gas liquid chromatograms when enzyme hydrolysis was used (Figs. 4 and 5). The major peak with long retention time in tracing A and B correspond to 0·1 μg. and to about 3·0 μg. of androstenedione in Figs. 4 and 5 respectively.

It should be noted that the experiments performed to investigate the problems of hydrolysis did not rule out the possibilities of either incomplete hydrolysis of

testosterone conjugates or of interactions between these conjugates and other urine constituents. In an attempt to study these possibilities, it is planned to conduct recovery experiments following the addition to urine of testosterone conjugates labelled with ^{14}C on the steroid moiety.

GIRARD SEPARATION

The Girard reagent T (trimethylaminoacetohydrazide chloride)($(CH_3)_3N^+.CH_2.CO.NH.NH_2$) Cl^- originally described by Girard and Sandulesco (1936) is frequently used to separate ketonic compounds from non-ketonic compounds. Such separation depends on the combination of the hydrazino grouping ($-NH-NH_2$) of the reagent with carbonyl group in the steroid molecule to give a Girard derivative which is soluble in water.



The separation of this derivative depends basically on differences in solubility in water of the ionized Girard derivatives and the non-ionized steroidal alcohols which remain unaffected by this procedure. After the separation, the ketonic compounds are regenerated by hydrolysis of the Girard derivatives with strong mineral acid.

Although this reaction was of great value in the separation of ketonic steroids from those lacking such group, the relatively harsh conditions utilized in the reaction cause destruction of labile steroids and acetylation of others. More recently, a modified form of Girard separation was described by Teitelbaum (1958) in which the conditions used were mild and suitable for labile steroid compounds. In this modified

reaction, resin Amberlite IRC-50 (H) was used as a catalyst for the coupling reaction instead of glacial acetic acid and aqueous formaldehyde was the hydrolysing agent replacing the hydrolysis with strong mineral acids in the original procedure. Taylor (1958) applied the modified procedure to some authentic steroids. His findings were extended and confirmed by Lindner (1960). These workers showed three important features of this reaction which are: firstly, the quantitative coupling of the ketonic steroids with the Girard's reagent using the resin Amberlite IRC-50 (H) as a catalyst; only small amounts not exceeding 2% were recovered into the non-ketonic fraction. Secondly, the reaction conditions were mild and produced no artefacts of the labile steroids. Thirdly the conjugated ketones (α,β -unsaturated ketones) could be separated from the saturated ketones by means of differential hydrolysis as these derivatives of the former are easily split by aqueous formaldehyde while the saturated ketones are only released in relatively smaller amounts which did not exceed 13%. In order to release saturated ketones, acid hydrolysis is still required. Thus the major advantages showed by the modified reaction in addition to the mild conditions which avoid destruction of labile steroids and acetylation of others was the further separation of the ketonic fraction into a formaldehyde

labile fraction which contains mainly steroids having Δ^4 -3 ketone group and only small amounts of other steroids.

When the modifications of the Girard reaction described by Taylor (1958) and by Lindner (1960) were used (see p.**26**) with authentic steroids, satisfactory results were obtained (see Table 6). However, when the technique was applied to urinary extracts, low recoveries of testosterone were obtained. It was found that when the amounts of Girard reagent T and Amberlite IRC-50 (H) were increased and the latter was activated by washing with dilute acid (Astle 1957), high and reproducible recoveries were achieved. These excess reagents are required in the presence of small amounts of steroids in a relatively large amount of residue.

Behaviour of various steroids towards the modified Girard separation: Experiments were carried out to investigate and check the behaviour of some steroids towards the modified form of Girard reaction. Thus, 20·0 µg. of testosterone, androsterone, etiocholanolone and androstenedione were subjected to the modified

procedure described in p.**26** . The non-ketonic and the formaldehyde labile fractions were extracted with ethyl acetate as previously described. The aqueous layer was then further acidified by adding 0.5 ml. of concentrated acid (HCl) and the solution was allowed to stand on the bench overnight and was extracted with 2 × 10 ml. with ethyl acetate for the acid labile fraction. Following extraction of each fraction, the combined ethyl acetate extract was washed with water, dried with anhydrous sodium sulphate and an aliquot was subjected to thin-layer chromatography into the system benzene-ethyl acetate (3:2, v/v). Authentic standards were chromatographed in parallel. Testosterone and androstene-dione were located by their absorption of ultra-violet light and androsterone and etiocholanolone by the phosphomolybdic acid reagent. The area corresponding to each compound was then eluted and subjected to gas-liquid chromatography for quantitative estimation. The percentage recovery of various steroids in each fraction is shown in Table 6. The present results are in agreement with those of Lindner (1960) who demonstrated that the bulk of $\alpha\beta$-unsaturated-3-oxosteroids were released mainly in the formaldehyde labile fraction, while the bulk of 17-oxosteroids could only be recovered by acid hydrolysis.

TABLE 6

Recovery (Percentage ± S.D.) of different steroids (20·0 μg.) after fractionation by the modified Girard reaction

Compound	No. of experiments	Non-ketonic fraction	Formaldehyde labile fraction	Acid labile fraction
Testosterone	12	3·0 ± 1·2	93·0 ± 2·3	2·1 ± 1·0
Androsterone	6	3·2 ± 0·96	5·2 ± 1·2	58·6 ± 4·5
Aetiocholanolone	6	2·8 ± 1·7	4·0 ± 2·1	65·7 ± 3·9
Androstenedione	6	1·9 ± 1·1	15·5 ± 3·7	60·4 ± 6·6

A number of organic solvents namely ethyl acetate, benzene, ether and petroleum ether were used to extract the fraction containing the "conjugated ketones". The results (percentage $\pm$ S.D) obtained using the above mentioned solvents from six experiments not corrected for losses were 94.0 $\pm$ 1.2, 89.0 $\pm$ 2.8, 83.0 $\pm$ 1.4 and 81.0 $\pm$ 2.1 respectively. The best results were achieved with ethyl acetate.

Use of aqueous sodium hydroxide to wash the ethyl acetate probably facilitates the removal of traces of formaldehyde. This is possibly due to the reaction of sodium hydroxide and formaldehyde with the production of sodium formate and methyl alcohol in accordance with the equation

$$2\ HCHO + NaOH = CH_3OH + HCOONa$$

Sodium formate can then be easily removed by successive water washings (Vogel, 1964).

CHROMATOGRAPHY

(1) Alumina column chromatography.

The modified Girard reaction was found to separate a great deal of pigments and steroids from the fraction containing the urinary testosterone. Nevertheless, the residue was still bulky in the majority of samples and too large for direct application to paper (or to thin-layer plates). However, chromatography of the residue on an alumina column removed the majority of coloured materials and gave in all cases a testosterone fraction suitable for application to paper.

Recoveries of testosterone from alumina column was performed by applying to columns 10.0 mμc of (4-^{14}C) testosterone (specific activity 29.4mc/mM purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England), together with 20.0 μg. of authentic testosterone. The preparation of the column and elution procedure was as described in p.**18** .In 12 experiments the recoveries of radioactive testosterone in the 0.3% (v/v) ethanol in benzene eluate were 99.4% ± 0.94. Another aliquot was taken from the same fraction to calculate the recovery using gas-liquid chromatography. The result of the 12 experiments estimated by this technique was 98.3% ± 3.9.

(2) Paper and thin-layer chromatography.

When the testosterone fraction from the alumina column was chromatographed on thin-layers of silica gel in the three different systems already described, at least five spots could be detected with the reagent vanillin in ethanolic/sulphuric acid. Testosterone standard was not clearly separated from epitestosterone. Both isomers, however, could be separated on alumina thin-layer using the system described in p.16 in which testosterone and epitestosterone had R_f values equal to 0.67 and 0.52 respectively.

Paper chromatography in the system light petroleum - toluene - methanol - water (66:33:80:20, by vol) separated testosterone from epitestosterone. The R_f values of testosterone and epitestosterone in this system are 0.38 and 0.46 respectively. Extracts of urine from twenty different males and females were examined in this paper chromatographic system using one of the methods of detection described in p.16 Simultaneous inspection of the lanes containing testosterone and epitestosterone standards and of the lanes corresponding to the urinary extracts indicated good separation of testosterone from epitestosterone and from the major urinary contaminants.

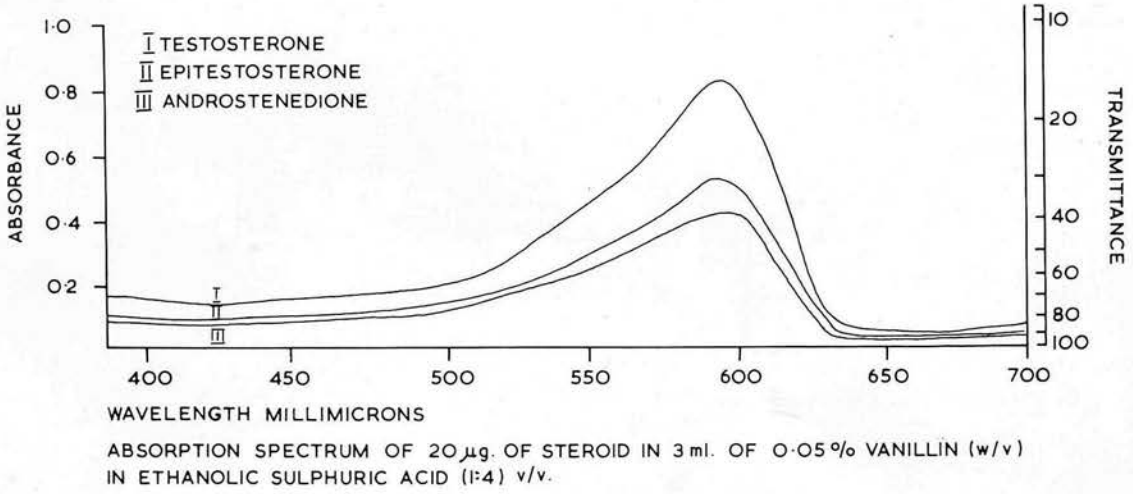
The recoveries of testosterone from paper chromatograms by the method described in p.16 was

checked by the recovery of 10.0 mμc of (4-^{14}C) testosterone and 50 μg of authentic standard which was subsequently estimated by gas-liquid chromatography. The recoveries obtained in 6 experiments were 100.2% ± 0.69 and 98.3% ± 3.1 by counting of radioactivety and by gas-chromatographic analysis.

The convenience of running the paper overnight together with the simplicity, reproducibility and quantitative recovery of the hormone in addition to the satisfactory separation achieved in all samples investigated as judged by gas-chromatographic analysis and other criteria favoured the selection of paper chromatography as the method of choice in the present work.

METHODS OF DETECTION

(1) A colour reaction for testosterone: In the present work, it was necessary to measure small quantities of testosterone accurately. For this a sensitive and simple method was needed. The absorption of ultraviolet light by testosterone in ethanol and sulphuric acid was affected by impurities and the Koenig colour reaction (1941) was laborious. In the present study, a colour reaction was developed which is somewhat similar to those described by Koenig (1941) and by others. A solution of 0·5% vanillin in ethanolic sulphuric acid (1:4, v/v) is a highly sensitive reagent for the detection of steroids on thin-layer chromatograms. The intense yellow colour of 1 ml. of this reagent in solution only showed a slight change when 10 μg. of testosterone were added and the solution was heated at 25°, 70°, and reducing the concentration of vanillin to 0·05% (w/v) did not reduce the colour in the blank reagent and there was little change in the colour after the addition of testosterone under the above conditions. However, after incubation for 17 hours at 25°, dilution of the reaction mixture with two volumes of distilled water caused the colour of the reagent blank to disappear and a bluish-violet colour developed in the presence of testosterone, epitestosterone,



ABSORPTION SPECTRUM OF 20 µg. OF STEROID IN 3 ml. OF 0·05 % VANILLIN (w/v) IN ETHANOLIC SULPHURIC ACID (1:4) v/v.

Fig. 2.

and androstenedione. Also development of the colour at high temperature produces high blank values. The colour obtained with testosterone was approximately twice that of epitestosterone and androstenedione. The absorption of visible light was maximal at 595 mμ; no other maxima were found between 400 and 700 mμ. (Fig. 2). No appreciable colour was produced with 20 μg. of 3α-hydroxy-5α-androstan-17-one (androsterone), 3α-hydroxy-5β-androstan-17-one (eticholanolone) 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone) (DHA), pregn-4-ene-3, 20-dione (Progesterone), 3β-hydroxy-pregn-5-en-20-one (pregnenolone), 5β-pregnane-3α-20α-diol (pregnanediol), 5β-pregnane-3α, 17α, 20α-triol(pregnanetriol), 11β, 17α, 21-trihydroxypregn-4-on-3, 20-dione(cortisol) and 17α, 21-dihydroxypregn-4-en-3, 11, 20-trione(cortisone). Testosterone 0·3 μg. in a total volume of 0·5 ml. of reagent diluted in this way gave an optical density of 0·060 when corrected by the method of Allen (1950) using readings at 510, 595 and 680 mμ. This colour reaction was employed for the standardisation of the alumina used in column chromatography when radioactive testosterone was not available. In addition, the procedure was employed in the estimation of testosterone in a pool of male urine.

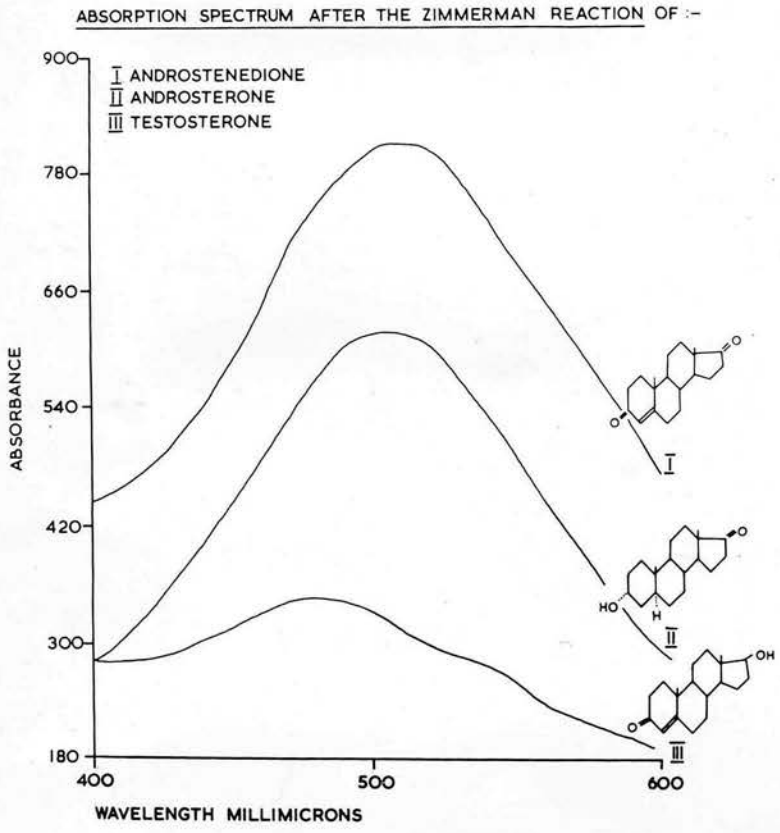

ABSORPTION SPECTRUM AFTER THE ZIMMERMAN REACTION OF :-
I ANDROSTENEDIONE
II ANDROSTERONE
III TESTOSTERONE
900
780
660
540
420
300
180
ABSORBANCE
400
500
600
WAVELENGTH MILLIMICRONS
HO
H
OH
I
II
III

Fig. 3.

2. Micro-Zimmermann reaction. Attempts to use the sensitive micro-Zimmermann reaction to estimate the oxidation product of testosterone, androstenedione, were carried out. The reaction was performed by the method described in p.**27.**

The chromogenicity of three compounds, namely, testosterone, androsterone and androstenedione in the micro-Zimmermann reaction were studied to investigate the relative contribution of the keto group at positions 3 and 17 to the final colour. Thus 3.4 µg. of authentic steroids were subjected to the described reaction conditions and the absorption spectrum of each compound was recorded. It was found that the chromogenicity of androstenedione in this reaction was approximately equal to the sum of the colours produced by androsterone and testosterone (Fig. **3**). The effect of base and temperature on the blank value of the reaction products were investigated in a series of 6 experiments. The base benzyl trimethyl ammonium hydroxide and tetraethyl ammonium hydroxide were examined in parallel for their blank values at 25° for one hour. The blank readings corrected by the method of Allen (1950) were 0.054± 13 and 0.024± 11 for the two bases respectively. The corresponding reading given by 0.5 µg of authentic androstenedione was approximately 0.045± 5. Extraction of the reaction

products with ether or dichloromethane did not improve the results. However, lower blank values equal to -0·003 ± 5 were achieved by developing the reaction with tetraethyl ammonium hydroxide at a temperature 0-4° for 3 hours rather than at 25° for 1 hour.

In the present work, the micro-Zimmermann was performed using the base tetraethyl ammonium hydroxide and the colour allowed to develop at 0-4° for 3 hours.

(3) Gas-liquid Chromatography: Initially a column containing 1% SE-30 was used for the analysis of the final urinary extracts. However, impurities from solvents and urine have retention times similar to those of the steroids under consideration. In an attempt to decrease amounts of impurities from paper chromatograms, elution of testosterone using chloroform saturated with water and benzene was used. In six experiments the recovery of testosterone was only 60-70%. Moreover, this procedure did not decrease the amount of impurities. A column containing 1% (w/w) QF-1 provided much better separations of testosterone from the accompanying impurities. The impurities from urinary extracts, solvents and reagents had shorter retention times than that of testosterone and did not interfere with its determination. The use of a column containing QF-1 produced a smooth base line from which the height of

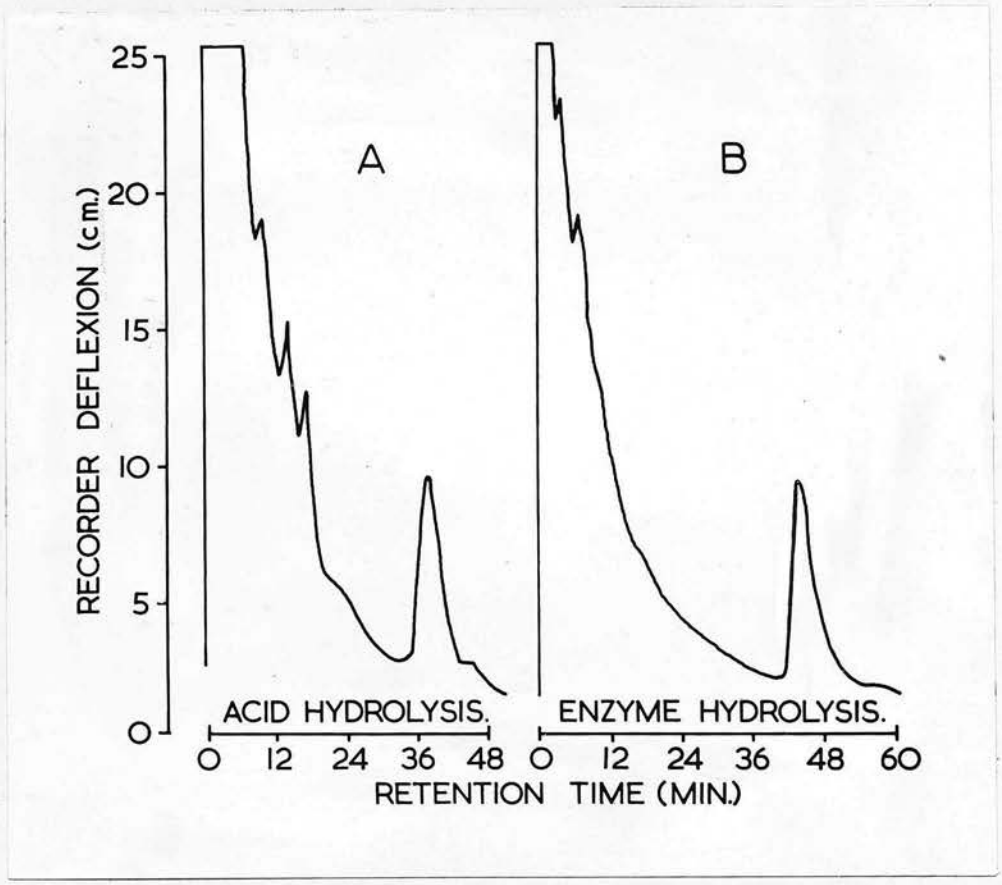


Fig. 4 .

Gas-liquid chromatogram on a column containing 1%w/w) QF-1 of an extract obtained by the present method from a female urine, after oxidation of testosterone to androstenedione

(a) An extract equivalent to 1/20th of a 24 hour urine specimen after acid hydrolysis. Peak corresponds to androstenedione content of 0.14 µg.

(b) An extract equivalent to 1/20th of the same urine specimen after enzyme hydrolysis. Peak corresponds to androstenedione content of 0.11 µg.

The two extract were analysed on separate days which explain difference in retention time.

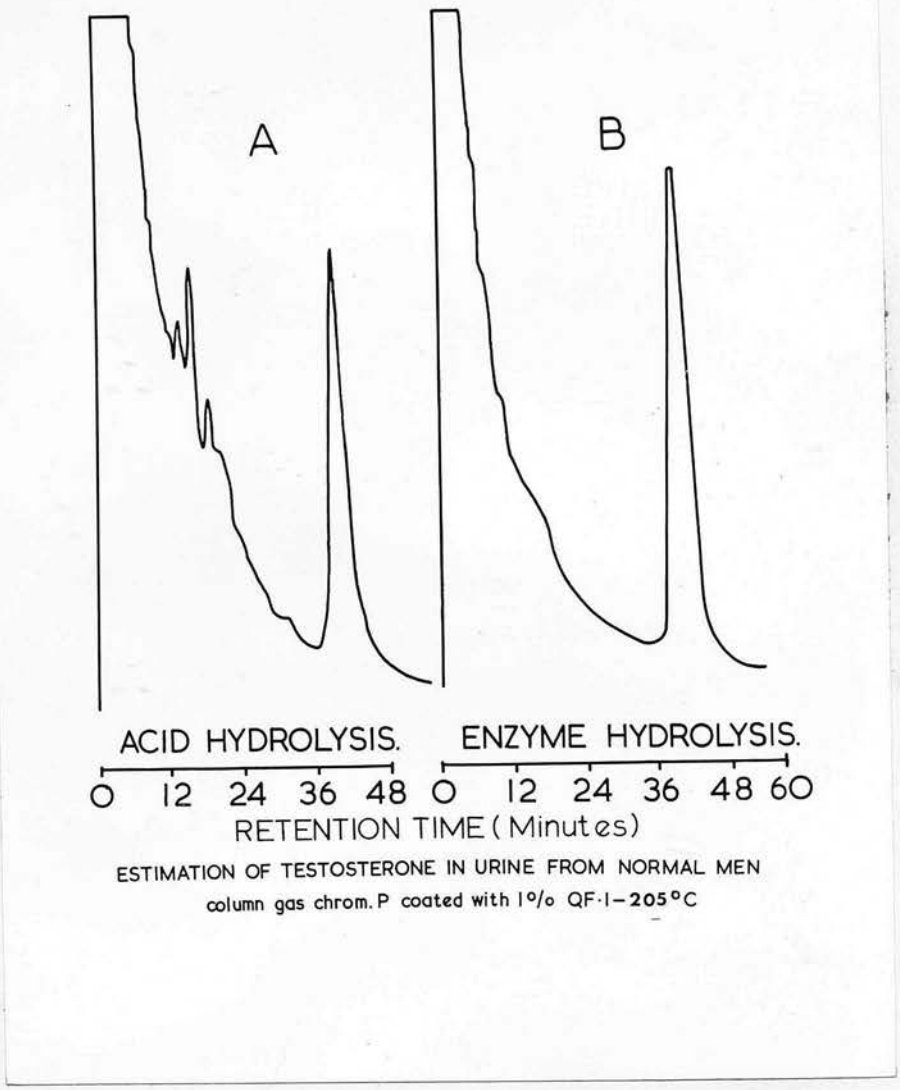
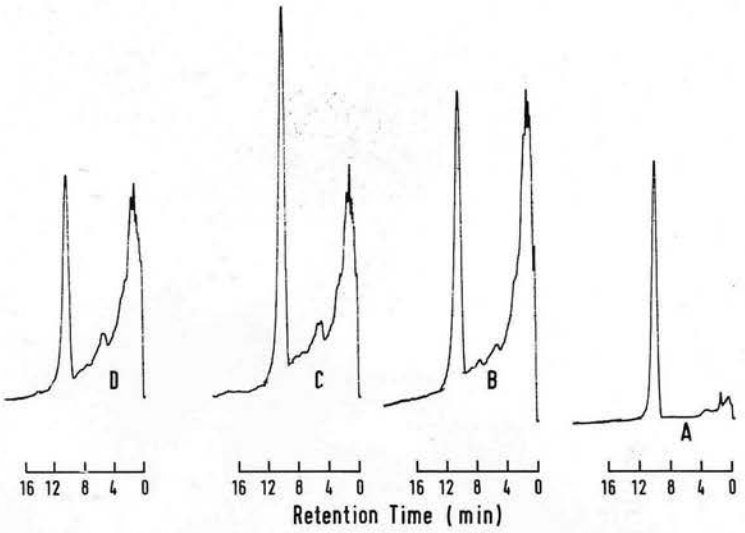


Fig. 5 .

Gas-liquid chromatogram obtained by the present method from a male urine, after oxidation of testosterone to androstenedione

(a) An extract equivalent to 1/34th of a 24 hour specimen after acid hydrolysis. Peak corresponds to androstenedione content of 2.7 µg.

(b) An extract equivalent to 1/26th of the same urine specimen after enzyme hydrolysis. Peak corresponds to androstenedione content of 3.2 µg.



GAS CHROMATOGRAPHIC TRACING OF TESTOSTERONE OBTAINED FROM ACID HYDROLYSED MALE URINE ON A QF-1 COLUMN AT 205°

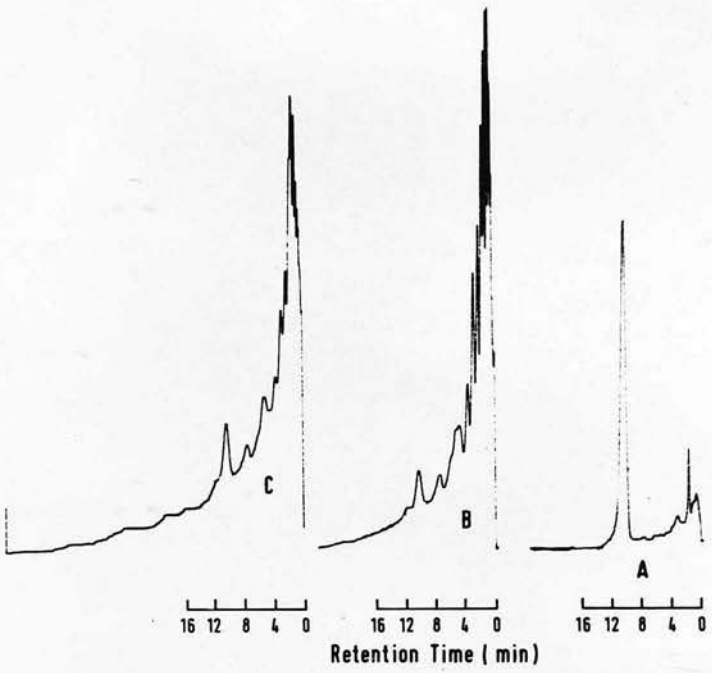
A. 2·2 µg standard testosterone

B. Urinary extract equivalent to 1/20th of a 24 hour specimen. Peak corresponds to testosterone content of 2·4 µg.

C. Urinary extract equivalent to 1/24th of a 24 hour specimen. Peak corresponds to testosterone content of 3·1 µg.

D. Urinary extract equivalent to 1/24th of a 24 hour specimen. Peak corresponds to testosterone content of 1·7 µg.

Fig. 6 .



GAS CHROMATOGRAPHIC TRACING OF TESTOSTERONE OBTAINED FROM ACID HYDROLYSED FEMALE URINE ON A QF-1 COLUMN AT 205°

A. 1·0 µg standard testosterone

B. Urinary extract equivalent to 1/22nd of a 24 hour specimen. Peak corresponds to testosterone content of 0·13 µg.

C. Urinary extract equivalent to 1/26th of a 24 hour specimen. Peak corresponds to testosterone content of 0·19 µg.

Fig. 7 .

the peaks due to testosterone and androstenedione could be measured. The extracts used to obtain the tracings shown in Fig. **4**. represent 1/20th of a 24 hour speciman from a female which contained a large number of contaminants and little testosterone. The majority of fresh urine specimens from both men and women contained much less interfering materials (Fig. **7**.). Also, it should be pointed out that impurities produced by acid hydrolysis were only slightly greater than those following enzyme hydrolysis (Fig. **5**.). Generally, in routine analysis of fresh urine specimens from both men and women, it is possible to perform gas-chromatographic analysis of the hormone testosterone directly on a QF-1 column without oxidation to androstenedione. Examples of gas-chromatographic tracings are shown in Fig, **6** & **7** for male and female respectively.

(4) Oxidation of testosterone to androstenedione: The oxidation procedure which employs tert-butyl chromate described by Menini and Norymberski (1962) required two hours for completion. In the present study, oxidation of testosterone was performed using chromium trioxide in aqueous acetic acid. The effect of various concentrations of chromium trioxide, temperature and time on the reaction have been investigated. Five micrograms of authentic testosterone were subjected to the oxidation procedure described in p. **26** , using various conditions. The recoveries of androstenedione in 12 experiments as estimated by gas-liquid chromatography are shown in Table 7. The conditions which gave the optimum yield of androstenedione were 7·5% (w/v) CrO_3 in 50% aqueous acetic acid for 15 minutes. It should be pointed out that in some cases of low titre female urines, oxidation of testosterone to androstenedione ensured that the small peak of androstenedione is well separated from the relatively large amounts of impurities which have shorter retention times on QF-1 column and thus the steroid peak appeared on a reasonably flat base line (see Figs. 4 and 5). A further advantage was the large difference in retention time between testosterone and androstenedione. Thus, oxidation and further chromatography provided a check on the specificity of the determination. Generally, extracts from male and female urine were chromatographed as testosterone (Figs. 6 and 7).

TABLE 7

Effect of Time, Temperature and Various Concentrations of Chromium Trioxide on Oxidation of Testosterone to Androstenedione

No. of Expt.	Using 50% (v/v) aqueous acetic acid + 7·5% CrO_3 at 25° for 15 mins.	Using 50% (v/v) aqueous acetic acid + 5.0% (w/v) CrO_3 at 25° for 15 mins.	Using 50% (v/v) aqueous acetic acid + 7·5% (w/v) CrO_3 at room temp. for 15 mins.	Using 50% (v/v) aqueous acetic acid + 7·5% (w/v) CrO_3 at 25° for 5 mins.	Using 50% (v/v) aqueous acetic acid + 7·5% (w/v) CrO_3 at 25° for 10 mins.	Using 50% (v/v) aqueous acetic acid + 7·5% (w/v) CrO_3 at 25° for 20 mins.
1	90·1	91·1	79·1	91·4	93·6	91·6
2	95·3	85·6	84·9	80·3	79·9	92·3
3	98·1	88·9	93·4	76·6	94·7	94·0
4	88·6	79·5	94·1	94·6	92·5	89·1
5	92·3	93·7	87·4	71·6	82·5	87·0
6	93·1	73·8	89·1	92·0	93·6	90·2
7	83·6	94·7	75·9	86·6	91·8	88·0
8	97·7	89·3	84·6	79·1	93·2	93·3
9	91·4	77·7	93·2	81·1	85·5	95·6
10	93·6	97·1	91·4	88·8	89·3	89·7
11	89·9	78·6	89·6	93·7	92·0	93·5
12	90·3	-	88·7	89·0	87·0	90·0
Mean	92·0	86·4	87·6	85·4	89·6	87·4
S.D.	±4·03	±7·85	±5·2	±7·24	±4·13	±3·43

(5) Assessment of various methods of detection: The amount of testosterone in the final extracts from urine were estimated using various techniques of detection. Thus six urine samples from males were assayed by the procedure described in p. **60** , and the final eluates from paper were divided into two equal aliquots which were evaporated to dryness. The amount of testosterone in one aliquot was estimated by the vanillin-ethanolic sulphuric acid colour reagent and by gas-liquid chromatography in the other. The mean testosterone value of six determinations using the vanillin colour was 49·7 µg./24 hour. The standard deviation was ± 8·0 µg./24 hours. The mean of the six determinations by gas-liquid chromatography was 56·5 µg./24 hour, and the standard deviation was ± 3·6 µg./24 hour. This latter standard deviation is on the borderline of being significantly lower than that for the colorimetric procedure. The mean values obtained by this procedure are not significantly different.

Estimation of testosterone by oxidation to androstenedione and subsequent micro-Zimmermann showed results which are also more variable and about 20% lower than those obtained using gas-chromatographic analysis (see Table **8**). A possible reason may be due to impurities from paper chromatograms which interfered in the coloured reaction.

On the basis of these experiments, gas-liquid

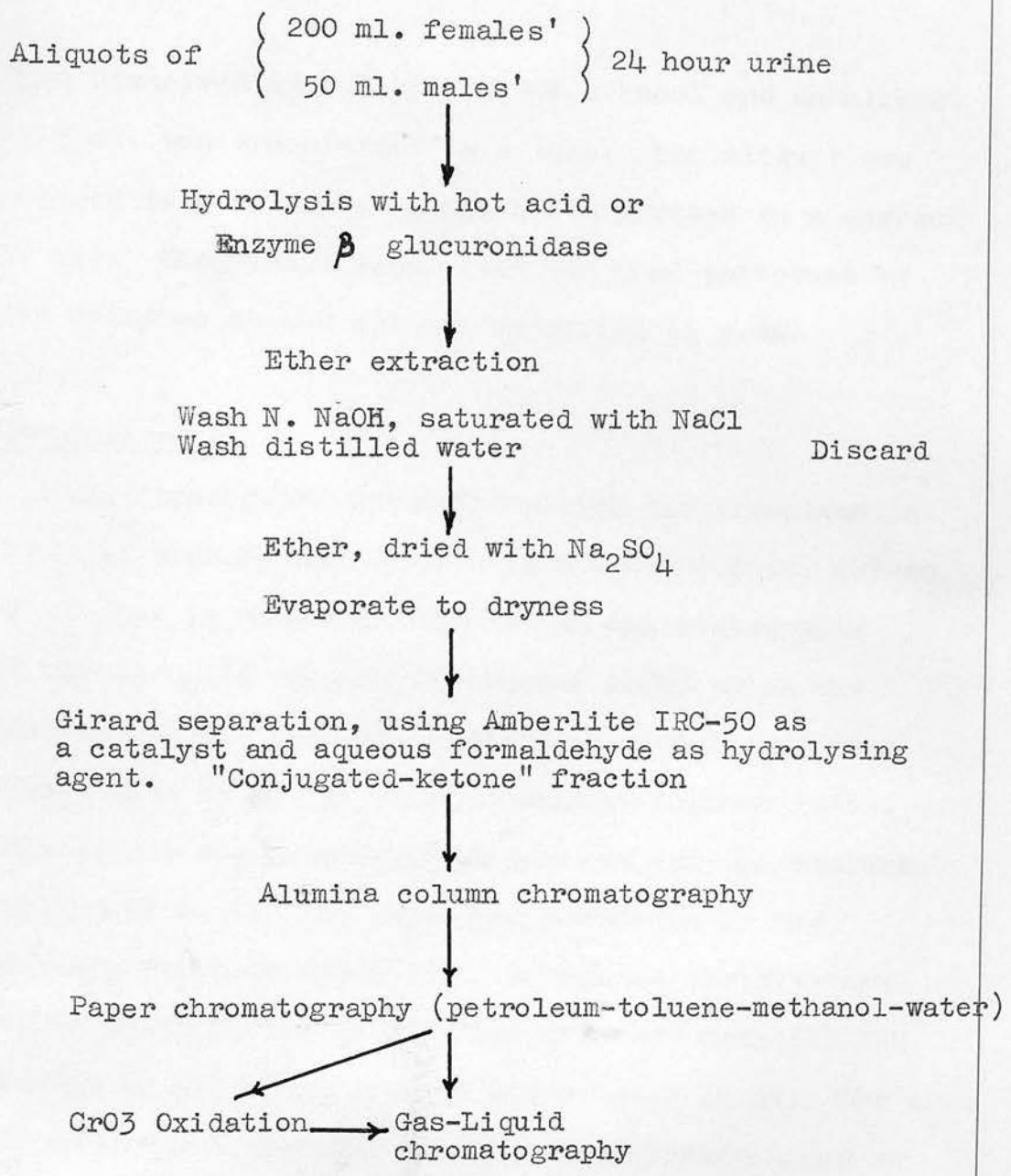
chromatography using a column containing 1% QF-1 was selected as the method for the estimation of testosterone or androstenedione in the final urinary extracts.

STANDARD ANALYTICAL PROCEDURE.

The results obtained from the previous experiments were the basis for a standard procedure which is applied routinely for clinical investigations. A flow sheet for the present method is shown in scheme 1.

Hydrolysis and extraction.

Aliquots of 50 ml. of male urine and 200 ml. of female urine were used for each determination. The method of choice for routine assays was hydrolysis by boiling for one hour with 0.15 volume of concentrated hydrochloric acid. Following hydrolysis the urine was cooled and extracted twice with one volume of ether. The combined ether extracts were washed twice with 0.05 volume normal sodium hydroxide saturated with sodium chloride. These were usually sufficient to remove the yellow pigments. Occasionally a third wash was necessary. The extract was then washed three times with 0.05 volume of distilled water. The ether extract was dried with anhydrous sodium sulphate and evaporated to dryness on a water bath. The residue was



Scheme I. Outline of the present method for measurement of testosterone in urine.

then dissolved in 5.5 ml. of 97% ethanol and an aliquot of 5 ml. was transferred to a tube. The aliquot was reduced to a volume of 1 ml. or to dryness in a current of air. The Girard separation was then performed by the modified method already described in p.**26.**

Chromatography

The "conjugated ketone" fraction was dissolved in 5 ml. of benzene and applied to a 6.0 x 0.5 cm. column of alumina in benzene. The column was eluted with 10 ml. of 0.15% ethanol in benzene (v/v) which was discarded. The fraction containing testosterone was eluted with 15 ml. of 0.3% ethanol in benzene (v/v). This eluate was evaporated to dryness and the residue applied to paper. The paper was developed in the system previously described. Authentic testosterone was chromatographed in parallel with the extracts and located by its absorption of ultraviolet light. The area of the chromatograms of the extracts corresponding to testosterone were then eluted with methanol. The eluate was then filtered through sintered glass and an aliquot was evaporated to dryness. The residue was then subjected to gas-liquid chromatography. In some low titre female urine and in pregnancy urine and other cases obtained from patients under treatment with drugs,

unsatisfactory gas chromatographic tracings were obtained due to the presence of impurity peaks with retention time close to that of testosterone or due to large amounts of impurities which gave rise to steep base line. In these cases the further oxidation of testosterone to androstenedione could produce extracts much cleaner for gas chromatographic analysis in addition to the large retention time for androstenedione which ensured that the peak appeared on a reasonably flat base line. Oxidation was carried out as described in p.26 After oxidation an aliquot was evaporated to dryness and gas-liquid chromatography was then carried out. This was performed on a column coated with the stationary phase "QF-1". External standards of the relevant steroids were used to estimate retention times and peak heights for a given weight of a steroid. The results were corrected for the aliquots taken during the procedure and for the overall recovery.

RELIABILITY CRITERIA OF THE METHOD

Accuracy: This was estimated on the basis of recovery experiments. Twenty micrograms of unlabelled testosterone were added to 200 ml. of distilled water and 0·01 μg. of (4-^{14}C) testosterone was added to 400 ml. of female urine. These samples were then submitted to the operations of the present procedure p. **60** . The recoveries obtained using different methods of detection are shown in Table 8. It will be noted that estimates of about 70 - 80% were obtained using gas-liquid chromatography and by counting the radioactive testosterone. However, the recoveries obtained using the micro-Zimmermann colour reaction were only about 50 - 60%. Recently, more variable and about 20% lower recoveries were obtained when various batches of resin Amberlite IRC-50 (H) were used. In these circumstances, it was decided to add (4-^{14}C) testosterone as an internal standard following hydrolysis to correct for losses throughout the entire procedure.

Precision: The precision of a method may be estimated from the difference between duplicate assays using the formula

$$\sigma = \sqrt{\frac{S(d^2)}{2N}}$$

where d is equal to difference between duplicates and N is the number of pairs of duplicates (Snedecor, 1952). From male urine, the standard deviation from 28 pairs of

Table 8

Recoveries of Testosterone

Method of Detection	Amount of testosterone added in μg.	Mean percentage recovery ± S.D. (number of observations in brackets)
Radioactivity due to [4 ^{14}-C] - Testosterone	0·01	81·4 ± 5·2 (12)
GLC as Testosterone	20·0	79·5 ± 4·5 (12)
GLC as Androstenedione	20·0	72·3 ± 4·2 (11)
Micro-Zimmermann after oxidation to Androstenedione	20·0	57·2 ± 11·6 (10)

estimates was ± 3·2 µg. for a mean of 50·0 µg./24 hours (range 40·0 to 64·0). In 29 pairs of estimates from female urine the standard deviation was ± 0·75 µg. for a mean of 9·4 µg./24 hours (range 2·1 - 14·7). The coefficient of variation was 6·4% and 8% for male and female urine respectively, indicating that the present method is reasonably precise.

Specificity: The gas chromatographic instruments in current use incorporate ionization detection systems (hydrogen flame or argon) that are essentially non-specific. They respond to a greater or lesser extent, to any kind of organic vapour emerging from the chromatographic columns, and therefore cannot be used to establish the nature of any gas chromatographic peak. The appearance on a chromatogram of a single peak with the retention time of the expected compound in itself is not proof of specificity as this peak could contain one or more compounds with identical or similar chromatographic behaviour.

Analytical procedures for complex mixtures of product employing gas chromatography as the final method of analysis necessarily depend for their success (a) on a number of chemical reactions and operations which reduce the complexity of the original sample to a simple mixture of products of similar physicochemical properties and which can be resolved into individual components in gas chromatographic columns, and (b) on the positive

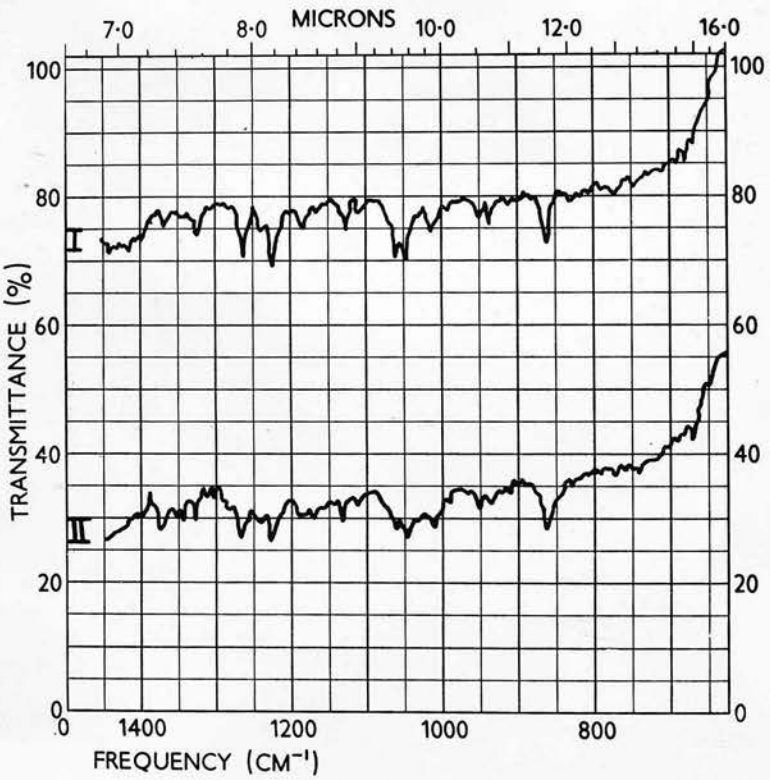
identification of the compound having the expected retention time by independent means.

In the present procedure for the estimation of testosterone, urinary extracts were purified by means of a modified Girard separation, alumina column chromatography, and paper chromatography. The modified Girard separation (p.42) yielded a fraction containing the "conjugated ketones" (testosterone) and alumina chromatography reduced the bulk of the extract to a residue that could be conveniently chromatographed on paper. Chromatography on the system previously described in p.16 and subsequent elution of the zone corresponding to testosterone, yielded an extract in which the latter steroid was the main constituent. When eluates from paper chromatograms corresponding to 60 different samples of male urine expected to contain 2 to 3 μg. of steroid were submitted to thin-layer chromatography on silica gel in various systems described in p.16 , only one compound with the mobility of testosterone could be detected. Oxidation (see p.26) of 30 residues obtained from different paper chromatograms produced a compound with the properties of androstenedione in thin-layer chromatography and in gas-liquid chromatography (on QF-1 and SE-30 columns). In 12 experiments, the eluates from paper chromatograms, corresponding to testosterone were submitted to acetylation and the reaction products were analysed by thin-layer chromatography and by gas-liquid chromatography on

columns containing 1% (w/w) SE-30 and 1% (w/w) QF-1. In all cases a compound behaving as testosterone acetate was detected.

Epitestosterone is not expected in urinary extracts obtained after acid hydrolysis as this steroid is largely destroyed by this treatment (Ismail, Davidson, Faro and Loraine, 1967); however, small amounts of surviving epitestosterone would be adequately separated from testosterone by the paper chromatographic system included in the present method (Rf. of testosterone and epitestosterone are 0·38 and 0·46 respectively).

Twelve extracts corresponding to male urines, after the paper chromatographic purification were analysed by thin-layer chromatography on aluminium oxide in a system capable of separating testosterone from its 17α-epimer epitestosterone (Table 1). When the plates were examined under ultraviolet light (250 mμ) and sprayed with the reagents, phosphomolybdic acid in ethanol or vanillin-ethanol-sulphuric acid reagent, only one spot with the mobility of testosterone could be detected. An attempt was also made to identify the compound by infrared spectroscopy in extracts obtained from 72 hour pool of male urine, subjected to the purification procedure of the present method. After the paper chromatography the material in the zone corresponding to testosterone was eluted and subjected to infrared analysis (see p.23). Unfortunately a poor spectrum was obtained (Figure 8),



INFRA-RED SPECTROSCOPY OF

I. Standard testosterone 100 μg.

II. Testosterone separated from adult male urine, after paper chromatographic separation, using this method.

Fig. 8.

which was not dissimilar to that of authentic testosterone. When this compound was subjected to the reaction with sulphuric acid and vanillin in ethanolic sulphuric acid reagent described in p.51 the spectrum of the urinary component was similar to that of authentic testosterone with maxima at 298 and 595 mμ respectively. Only negligible amounts of testosterone could be detected in 12 urine samples from a woman subjected to bilateral adrenalectomy and ovariectomy (Fig. 9), and accordingly it was concluded that neither the non-steroidal materials present in the patient's urine nor impurities from the solvents or reagents used, interfere with the present method.

The experiments described above provide cumulative evidence that the extract which was obtained after the paper chromatography contained mainly testosterone.

Evidence that material eluted from the gas chromatographic columns with the retention time of testosterone was indeed this substance was obtained in the following experiments.

(a) Two extracts corresponding to urines submitted to the reactions and operations of the present procedure were analysed by the technique of combined gas chromatography and mass spectrometry (see p. 24). One extract was analysed on a column containing 1% (w/w) XE-60 and the other on a column containing 1% (w/w) QF-1. In order to check the homogeneity of the gas chromatographic peaks, mass spectra were taken at the maximum of

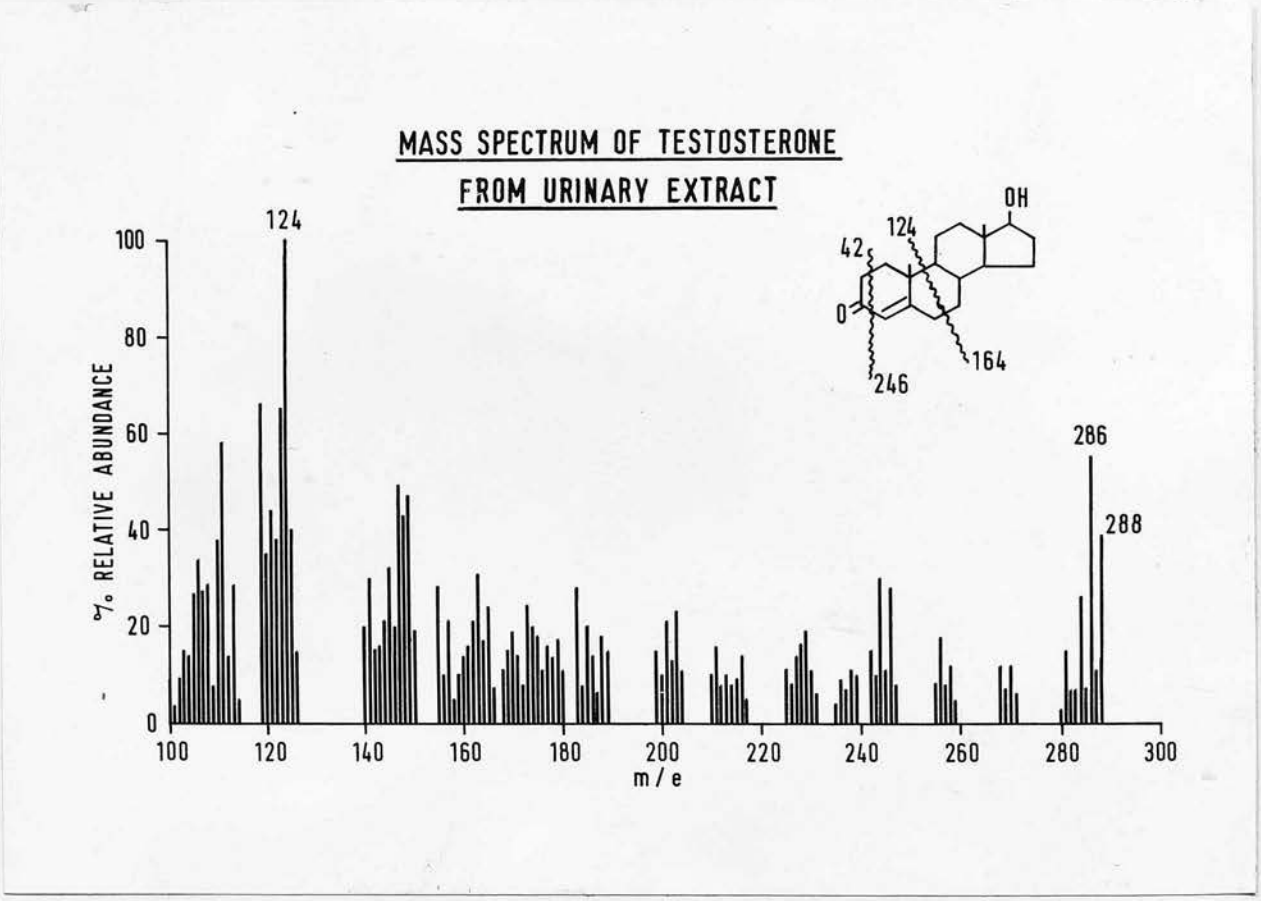

MASS SPECTRUM OF TESTOSTERONE
FROM URINARY EXTRACT
% RELATIVE ABUNDANCE
m / e
124
286
288
OH
O
42
124
164
246

Fig. 10 .

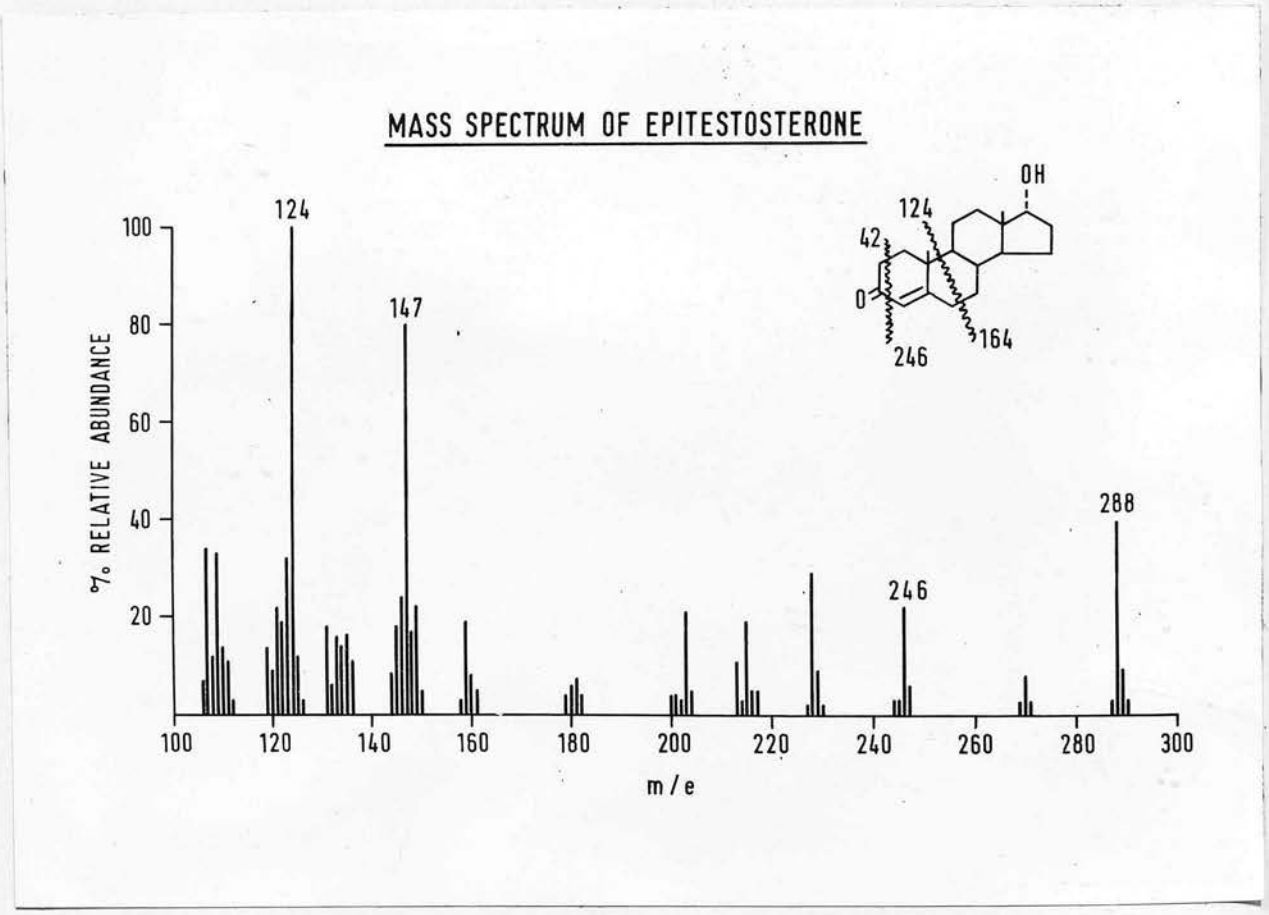

MASS SPECTRUM OF EPITESTOSTERONE
% RELATIVE ABUNDANCE
100
80
60
40
20
124
147
246
288
100
120
140
160
180
200
220
240
260
280
300
m / e
OH
124
42
O
246
164

Fig. 11 .

the peak, and on its ascending and descending portions. The three mass spectra showed no appreciable differences. After chromatography on the two phases XE-60 and QF-1 the mass spectra of the urinary compound were indistinguishable (Fig. 10) with the mass spectrum of authentic testosterone. Apart from the molar peak at m/e 288, the diagnostically most significant and mechanistically most interesting peak for steroids having the Δ^4-3-ketone grouping at m/e 124 (Shapiro and Djerassi, 1964) was also present. As a matter of interest, the mass spectrum of authentic epitestosterone after gas chromatography on a QF-1 column, was also recorded (Fig. 11). Differences between the spectra of the two epimers exist. The mass spectrum of testosterone shows a prominent peak at two mass units below the molar peak, which is absent in the spectrum of epitestosterone. On the other hand, the spectrum of epitestosterone has a very prominent peak at m/e 147 which is much less intense in the spectrum of testosterone.

(b) Further proof of the identity of the peak tentatively identified as testosterone in gas-chromatographic analysis was achieved by trapping the peak corresponding to testosterone as described in p.**21** The acetate and trimethyl silyl ether derivatives were then prepared in the gas phase as described by Norymberski and Riondel (1967) and Menini, Orr, Gibb and Engel (1967) respectively (p.**22**). Further chromato-

graphy of the trapped component on the stationary phases SE-30 and QF-1 as free and as derivatives showed peaks with virtually identical retention times to those of the corresponding compounds (Table 8A). From the above experiments it was concluded that the present technique is specific for estimating the hormone testosterone in urine.

Sensitivity: About 0·5 µg. of androstenedione could be measured in the final residue with fair accuracy using the micro-Zimmermann test and 0·1 µg. employing gas-liquid chromatography (Figs. 4 and 7). The sensitivity of this technique calculated from estimations of peak height in the gas-chromatographic tracings with a fair degree of accuracy versus the background was found to be about 2·0 µg./24 hours. Below this value the method still can detect testosterone but the result will only be of limited quantitative significance.

Practicability: It has been found that six determinations can be performed by a technician in two working days. Thus the method is suitable for clinical application.

TABLE 8A.

Further gas chromatographic analysis of the trapped component, corresponding to testosterone in various stationary phases

Compound	Retention time in minutes			
	1% QF-1		1% SE-30	
	Standard	Urinary component	Standard	Urinary component
Testosterone	9.6	9.6	16.6	16.7
Epitestosterone	8.9	-	16.5	-
Testosterone acetate	14.8	14.7	25.5	25.6
Epitestosterone acetate	13.6	-	21.2	-
Testosterone trimethyl silyl ether	6.6	6.6	18.4	18.3
Epitestosterone trimethyl silyl ether	5.4	-	15.7	-

DISCUSSION

The evidence obtained during the development of the present method shows that testosterone is the major steroidal component in the final extract. The more efficient separation of testosterone from contaminants and closely related steroids on the selective polar phase QF-1 was also shown by Sandberg, Ahmad, Cleveland and Savard (1964). The main reason for the more efficient separation was that the majority of contaminants from the materials used in the method described herein had very short retention times on a column coated with QF-1.

Two other steroids which may be present in biological material, namely androst-4-en-3,11,17-trione (adrenosterone) and 17α-hydroxypregn-4-en-3,20-dione (17α-hydroxyprogesterone), behave chromatographically on paper in the same way as testosterone (Cathro, Cameron and Birchall, 1965). Adrenosterone was clearly separated from testosterone and androstenedione by gas-liquid chromatography using a column coated with the phase QF-1. When 17α-hydroxyprogesterone was subjected to gas-liquid chromatography under the conditions used in the present investigation no compound could be detected in the effluent.

This steroid, if present in urine would probably be destroyed by acid hydrolysis (Dorfman & Shipley, 1957). However, it should be noted that the compound would be oxidized with chromium trioxide to androstenedione. It is also of interest to note that almost complete destruction of epitestosterone occurs using the technique of hot acid hydrolysis described in the present method (Ismail, Davidson, Faro & Loraine, 1967). Testosterone could be furthur separated from the surviving epi-testosterone, if any by paper chromatography in the system described, and can be estimated by subsequent gas-liquid chromatography of the extract of the appropriate area of the final paper chromatogram.

There is considerable evidence to show that the present method is specific for testosterone. The compound estimated as testosterone in the technique possesses a "conjugated ketone" group and an oxidizable hydroxyl group. The compound produced by oxidation has the absorption spectrum of a 17-oxosteroid in the Zimmermann reaction. The behaviour of the compound in three different types of chromatographic procedure, is identical to that of authentic testosterone. Chromatographic and spectroscopic evidence has shown that testosterone is the main

compound in the final eluate from paper. In the method described herein the homogeneity of the peak tentatively identified as testosterone in gas-liquid chromatography was checked by recording the mass spectra of this peak at different parts. In addition, the mass spectra of both standard testosterone and the compound identified from the urine were virtually indistinguishable. Further proof of the specificity of this method was achieved by trapping the compound which gave peaks in gas-chromatographic analysis corresponding to testosterone; subsequent formation of derivatives such as the acetate and the trimethyl silyl ether gave compounds having identical retention times to those of authentic standards on gas-liquid chromatography using columns containing the stationary phases QF-1 and SE-30. Therefore, on the basis of this cumulative evidence, it may be concluded that the method is specific.

In the present work, the results of experiments on enzymic hydrolysis have agreed with previous reports. Thus the amount of testosterone recovered was increased slightly by prolonged incubation, by adding increased quantities of enzyme and by abolishing the effect of the endogenous inhibitor of β-glucuronidase known to be present in urine (Marsh, 1963). In the present procedure,

simple acid hydrolysis was the method of choice for routine analysis. All other published methods have employed enzymic hydrolysis except that of Vestergaard, Raabo and Vedso (1966). The capacity of the present method to separate testosterone from large amounts of impurities is also shown by its ability to estimate testosterone in pregnancy urine. The similarity of the results obtained with acid and with enzyme hydrolysis using a β-glucuronidase preparation suggests but does not prove that testosterone conjugate in human urine is mainly the glucuronide. This is in agreement with the finding of Camacho and Migeon (1964), who found that approximately 0.03% of radioactive testosterone dose was recovered from the solvolysable fraction of urine. Furthur work by Dessypris, Drosdowski, McNiven & Dorfman (1966) and by Tamm, Volkwein and Voigt (1967) showed the presence of amounts up to 17.0 µg./24 hours of testosterone sulphate in male's urine. It should be pointed out that the enzyme preparations used in the present work are a crude form of β-glucuronidase and it may also contain some sulphatase and or phosphatase . However, it still remain to be shown whether or not testosterone conjugates other than glucuronide are affected by the presence of these small amounts of activities in

the crude preparation.

The accuracy and precision of the method was satisfactory and it was not necessary to use an internal standard. However, further work using different batches of resin Amberlite IRC-50 (H) showed variable and about 20% lower recoveries than those initially obtained. Accordingly, it was found that addition of radioactive testosterone as an internal standard is necessary to achieve a high degree of accuracy and precision. It is also of interest that the estimates obtained by gas-liquid chromatography could be corrected by the recoveries obtained from added internal standard. The results of the present investigation also show that the recoveries obtained with one method of detection may not agree with those obtained using another. Thus, from the results of the present investigation, it would not be justifiable to correct the estimates obtained with the micro-Zimmermann reaction for losses observed with added radioactive testosterone as in the method of Rosner et al (1965). The fact that the estimates obtained by gas-liquid chromatography on replicates of material used for determinations by the Zimmermann reaction were always higher suggests that impurities from paper may have interfered in this reaction. Attempts to use

the reagent vanillin in ethanolic sulphuric acid gave also lower and variable results than those obtained using gas-liquid chromatography for final detection, thus in the present work gas-liquid chromatography is the procedure of choice. A cause of the higher results obtained with some other methods (Table 10) may be the incomplete separation of testosterone from other closely related compounds such as epitestosterone.

The colour reaction using vanillin in ethanolic sulphuric acid developed in the present investigation differs from that of Allen, Hayward and Pinto (1950) in that little or no colour is obtained with dehydroepiandrosterone. The latter reagent is only a simple mixture of ethanol and sulphuric acid. Several methods for urinary testosterone have used the colour reaction described by Allen et al (1950) with some modifications (Zurbrugg, Jacobs & Gardner, 1965; Lim & Dingman, 1965). The difference in the colour produced with dehydroepiandrosterone may,in part, be due to the presence of an aldehyde since Ittrich (1965) has obtained similar results using formaldehyde in place of vanillin.

The modifications of the Girard reaction presented herein are based on the procedure of Teitelbaum (1958) for unstable aldehydes and ketones;

it was adapted to steroids by Taylor (1958) whose findings were confirmed and extended by Lindner (1960). The present modification of the Girard separation avoids the harsh conditions of the original method which may destroy some labile steroids and acetylate others. The derivatives of the saturated ketones in the original method are hydrolysed by dilute acid; the Girard derivatives of the "conjugated ketones" require considerably higher concentrations of acid for hydrolysis (Fieser & Fieser, 1959). It is of interst that, in the modified Girard separation, the derivatives of the "conjugated Ketones" are easily split by aqueous formaldehyde. In order to release the saturated ketones, acid hydrolysis is still required.

In the present investigation, a considerable increase in the amounts of the Girard reagent T and in the catalyst, resin Amberlite IRC-50 (H), was necessary to achieve quantitative results with crude urinary extracts; the resin had to be activated by washing with acid. The separation of the "conjugated ketone" fraction from the non-ketonic fraction and the major bulk of the saturated ketonic compounds simplifies the subsequent separation procedures necessary to obtain a pure compound. In the present modification of the Girard

separation it was found that testosterone could be extracted with benzene almost as effectively as with ethyl acetate. This evidence suggests that the corticosteroids which can be obtained in the "conjugated ketone" fraction, could be extracted with chloroform or ethyl acetate after the removal of testosterone and compounds of similar polarity. It is therefore seems likely that procedures similar to that described herein for testosterone estimations could be used to simplify the estimation of many hormonally active neutral steroids. Such a procedure has already been used in the isolation of progesterone from human pregnancy urine.

SUMMARY

1. A method has been developed for the estimation of testosterone in human urine. This has the following steps:

a - Acid hydrolysis

b - Separation of a 'conjugated ketone' fraction from the urinary extract by a modified form of the Girard reaction. In this step, the resin Amberlite IRC-50 (H) is used as a catalyst and formaldehyde as a hydrolysing agent.

c - Chromatography on alumina and on paper.

d - Final determination using gas-liquid chromatography.

2. Comparison of methods of estimation of testosterone in the final fraction shows that estimation by gas-liquid chromatography is more reproducible than by colorimetric methods applied to the same eluates from the paper chromatogram.

3. The mean recovery of testosterone by gas-liquid chromatography is 79·5%. The coefficients of variation were 6·4% and 8% for male and female urines respectively, and the method appears to be specific for the hormone.

4. The procedure is relatively rapid, and six determinations can be performed by one worker in two days.

APPLICATIONS OF THE PRESENT METHOD

A. URINARY TESTOSTERONE EXCRETION BY NORMAL MEN

INTRODUCTION

In the investigation of gonadal function in men, urinary 17-oxosteroid determinations have not proved of great value because the testes contribute only about one third of the total urinary 17-oxosteroids excretion. The remaining two thirds arise from precursors secreted by the adrenal cortex (Vande Wiele, McDonald, Gurpide & Lieberman, 1963). The wide range of variation in 17-oxosteroids excretion in normal males, from 5 to 15 mg./24 hr., mainly reflects variations in the secretion of precursors by the adrenal cortex. However, it has been shown that the estimation of testosterone in urine provides a satisfactory index of testosterone production which in the normal male is largely due to the testicular secretion of the hormone (Vande Wiele et al. 1963; Kirschner, Lipsett & Collins,1965,

and Horton, Shinsako & Forsham, 1965; Rosner & Conte, 1966; Horten & Tait, 1966). Only small quantities of urinary testosterone arise from precursors secreted from other sources (Vande Wiele et al., 1963; Camacho & Migeon, 1963). However such variations have not been sufficient to explain the large range of urinary testosterone levels found by previous workers (see Table 10).

The purpose of the present study was to investigate the variations in urinary testosterone excretion levels over a short period of time as those previously reported by other workers and to study the day to day variations, in serial 24 hr. assays performed over longer periods of time than those in early investigations. An attempt has also been made to assess possible causes of variation in testosterone excretion such as the effect of age and sexual activity have been investigated.

MATERIALS AND METHODS

Subjects: Urine samples from 20 normal men aged between 21-63 years and 5 normal males aged between 16-20 years were investigated for testosterone excretion over a period of 3 successive days. Five normal men ranging in age from 20-34 years were studied over long periods of time. Each subject was normally active and in good health, and in none was there a previous history of endocrine disorders. All collected complete 24 hr. urine samples continuously throughout the period of investigation.

The effect of sexual activity on testosterone excretion was investigated in two subjects, E and NC. Both were married, with one child, and the wives were away from Edinburgh; thus no sexual activity took place before the investigation started for a period of 18 and 7 weeks for subjects E and NC respectively. Sexual activity restarted during the period of investigation. Urines were analysed as single 24 hr.

samples in order to detect any rapid changes in hormone output.

Methods of Assay: For the estimation of 17-hydroxy-corticosteroids and total 17-oxosteroids, modifications of the techniques described by Appleby, Gibson, Norymberski & Stubbs (1955) and Vestergaard (1951) were employed. Urinary testosterone excretion was estimated by the present method. The studies on subjects H and J were performed after the addition of [4-^{14}C] testosterone as an internal standard to correct for losses during the procedure. All samples were assayed after a period of storage at 4^{o} of less than 2 weeks.

RESULTS

In general, levels of testosterone were higher in young men than in the older age group. In 80 determinations from twenty normal males aged between 21-63 years, the mean excretion of testosterone was 51·7 µg./24 hr. (range 40·0 - 64·5). In 15 determinations from five young men aged between 16-20 years, the mean excretion was 78·0 µg./24 hr. and the range was from 60·0 - 103·0 µg./24 hr. The results of such determinations are shown in Table 9. Urinary testosterone levels measured by different procedures are shown in Table 10. The present method gave levels which are in agreement with the results of some of these previous techniques. However, the upper limits of the results obtained with the present method were generally lower than those of some of the previous methods.

Day to day variations in testosterone excretion in normal men.

This was studied in 5 subjects for periods of 13 to 29 days. Marked alterations were found with

TABLE 9

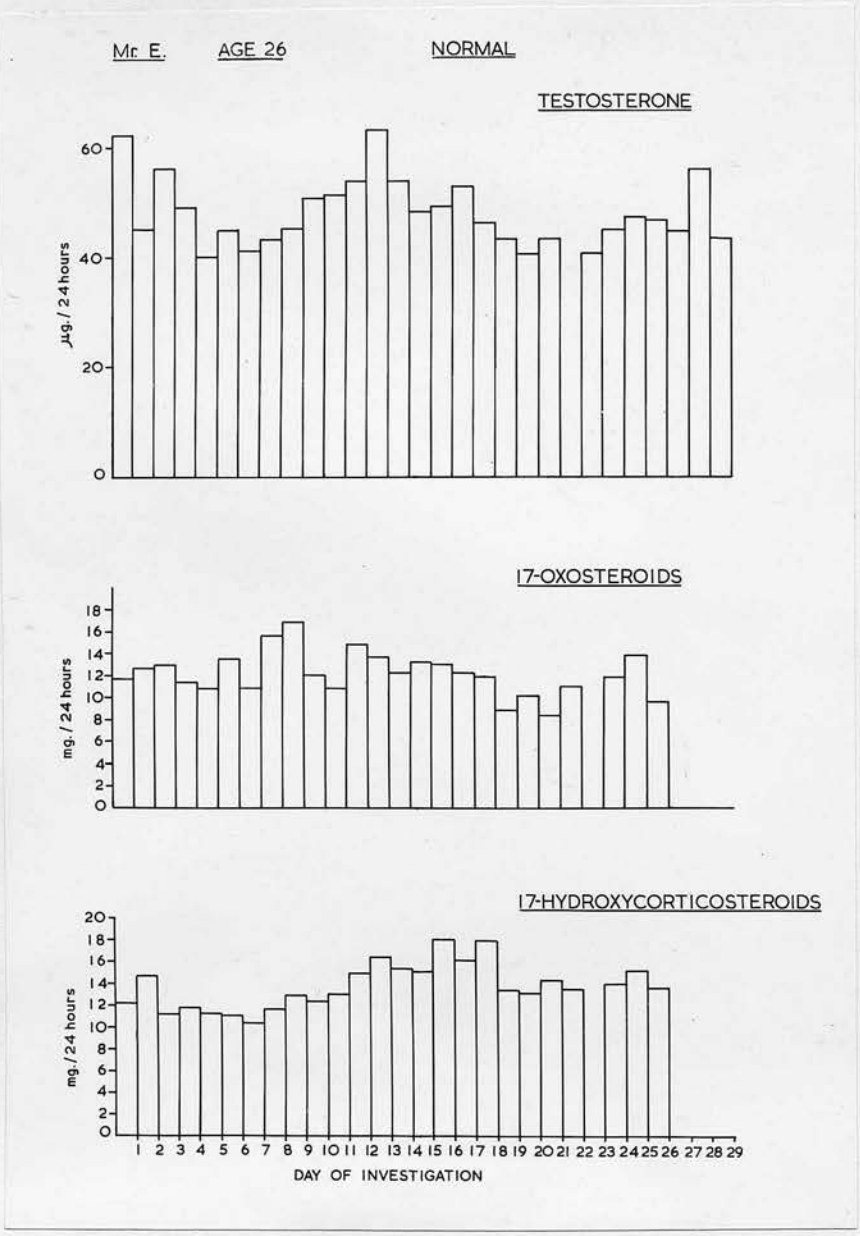
Urinary testosterone excretion values in normal men estimated by the present method

Subject's number	Age	µg./24 hours
1	16	71.3
2	16	60.0
3	17	87.9
4	20	67.9
5	20	103.0
1	21	61.9
2	21	47.5
3	26	40.0
4	27	43.0
5	30	63.1
6	33	57.1
7	33	45.8
8	34	52.9
9	34	59.3
10	36	64.4
11	37	48.3
12	39	64.5
13	39	53.5
14	40	40.6
15	44	46.6
16	46	64.1
17	55	51.6
18	57	44.4
19	60	43.3
20	63	42.0

TABLE 10

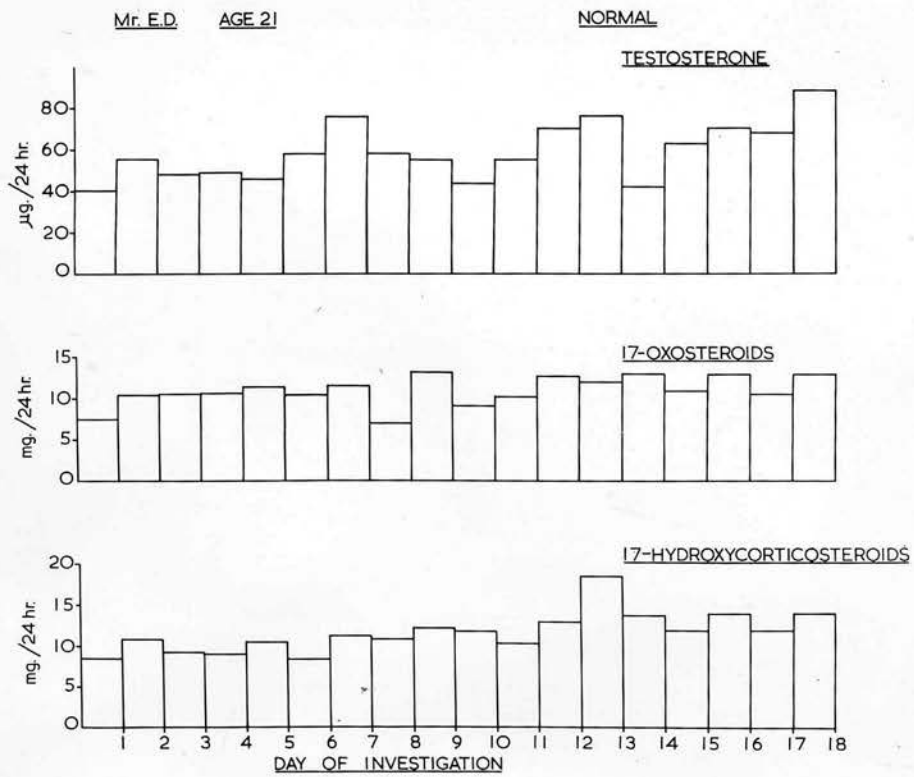
Urinary testosterone excretion by normal men as estimated by different methods

Age in years	Number of subjects	Urinary Testosterone µg./24 hours Mean	Range	Reference
23-34	5	87·0	(46·1-106·0)	Camacho & Migeon(1963)
23-60	4	29·5	(16·0-51·0)	Vermeulen & Verplancke (1963)
19-55	21	113·8	(38·0-291·0)	Futterweit et al.(1963)
29-56	4	71·5	(33·0-120·0)	Brooks (1964)
18-22	2	163·0	(126-200)	Ibayashi et al.(1964)
26-62	14	95·3	(14-189)	
27-33	12	82·5	(47-140)	Sandberg, Ahmad, Cleveland & Savard (1964)
18-35	5	54	(32·0-69·0)	Voigt, Volkwein & Tamm (1964)
18-44	9	34	(10-75)	Schubert & Frankenberg (1964)
21-63	14	182·5	(15·1-523-3)	Dulmanis, Coghlan, Wintour & Hudson (1964)
18-60	10	73·0	(16·0-120·0)	Panicucci, Savi & Coli (1964)
30	3	64·3	(42·0-79·0)	Zurbrügg, Jacobs & Gardner (1965)
30-40	20	88	(28-143)	Rosner et al. (1965)
17-24	5	151	(113-193)	Lim & Dingman (1965)
18-65	27	49·7	(30·2-86·3)	
20-48	9	148	(59·0-202·0)	Sparagana (1965)
22-40	8	-	(50·0-77·0)	Horn, Statter & Finkelstein (1966)
18-47	15	37·0	(20·0-65·0)	Vestergaard, Raabo & Vedso (1966)
22-38	10	91·5	(33·4-144·5)	Wegienka et al.(1967)
21-63	20	51·7	(40·0-64·5)	The present method
16-20	5	78·0	(60·0-103·0)	



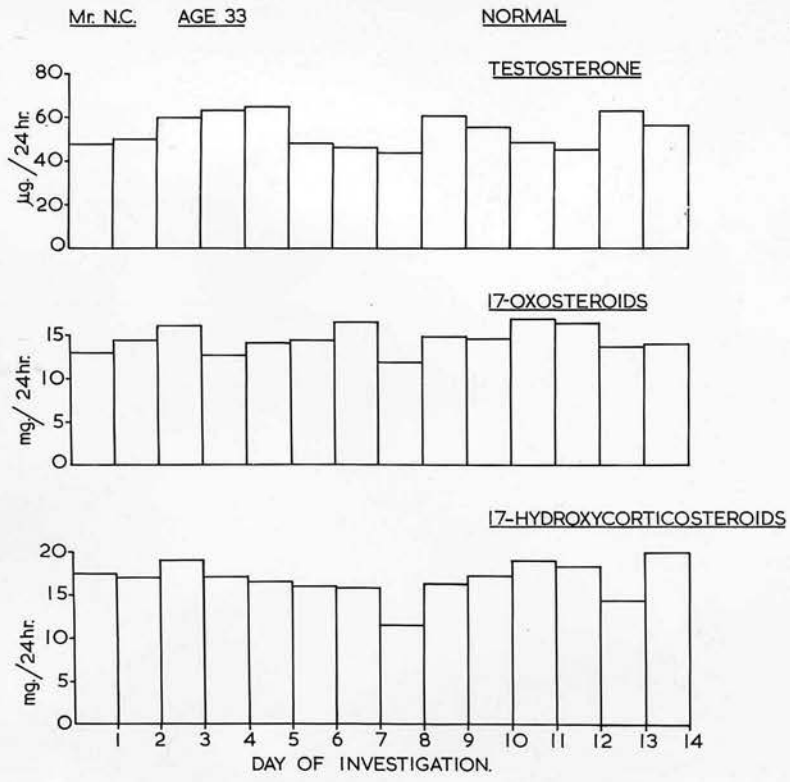
Steroids excretion in normal male subject

Fig. **12**



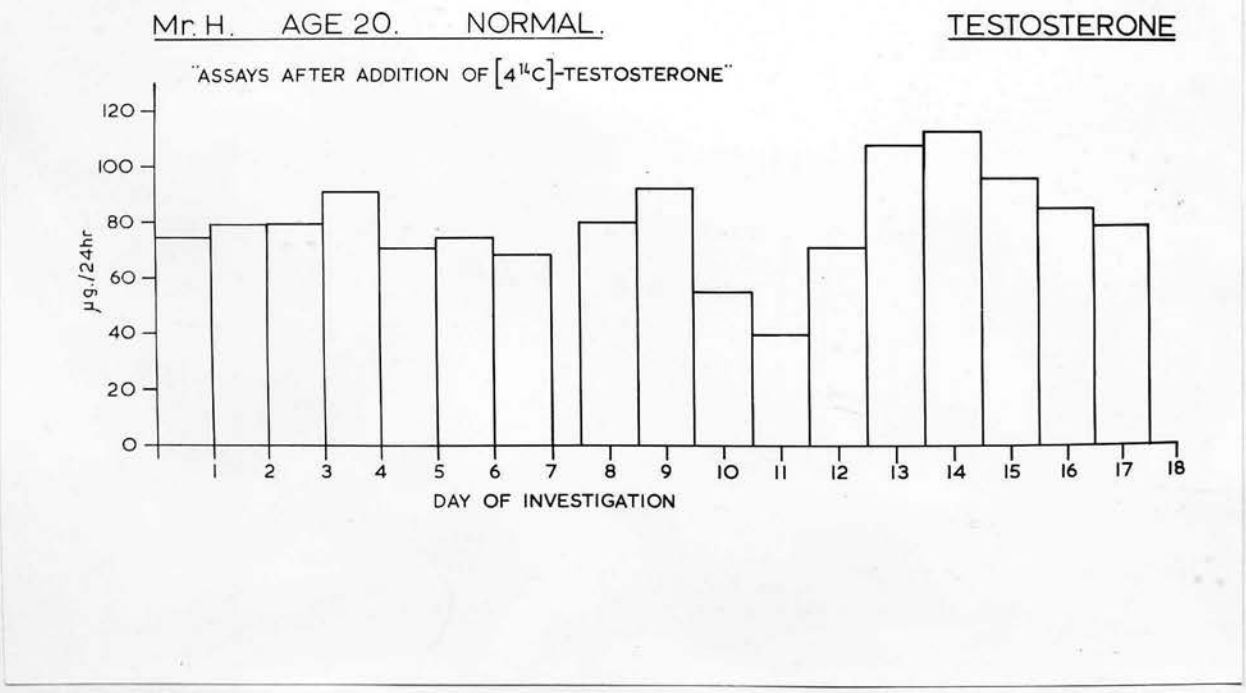
Steroids excretion in normal male subject

Fig. 13



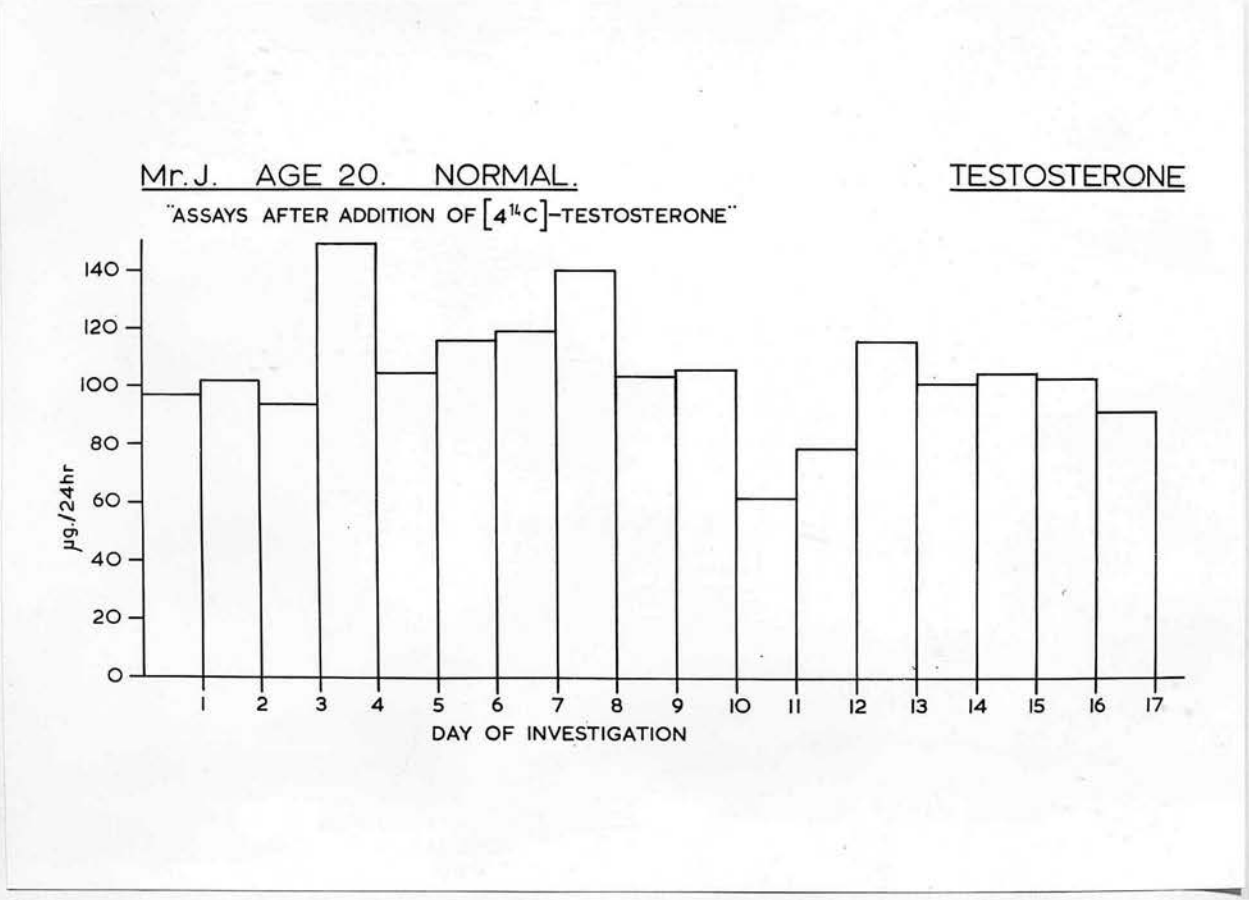
Steroids excretion in normal male subject

Fig. 14



Testosterone excretion in normal male subject

Fig. 15



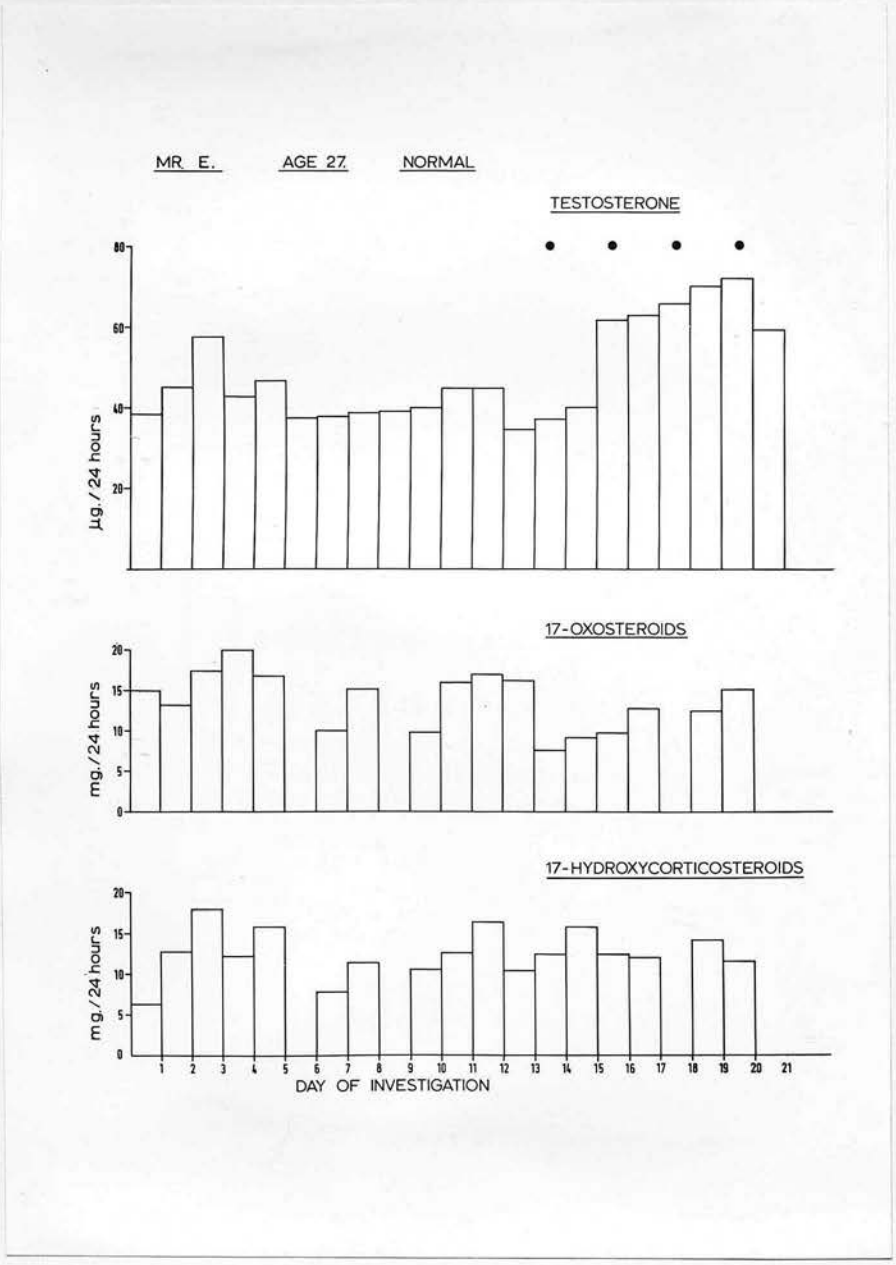
Testosterone excretion in normal male subject

Fig. 16

peaks of excretion occurring at regular intervals.

The results in subject E showed marked variations in the levels of urinary testosterone (Fig.12). Moreover, these variations appeared to be cyclic with peaks at about 12 day intervals. There is no marked correlation of the testosterone levels with those of 17-oxosteroids or 17-hydroxycorticosteroids. The second study on subject ED showed a similar variation in testosterone excretion from 40·3 to 90·5 µg./24 hr. with peaks at about 6 day intervals (Fig.13). Again there is no definite correlation with 17-oxosteroid or 17-hydroxycorticosteroid output. However, a 17-hydroxycorticosteroid peak does coincide with a testosterone peak on day 13 of this study. A third investigation in subject NC also showed considerable variations in testosterone excretion, figures varying from 44·1 to 63·5 µg./24 hr. (Fig.14); peaks of excretion occurred at intervals of about 5 days. Again no correlation between the urinary testosterone levels and that of 17-oxosteroids or 17-hydroxycorticosteroids could be observed.

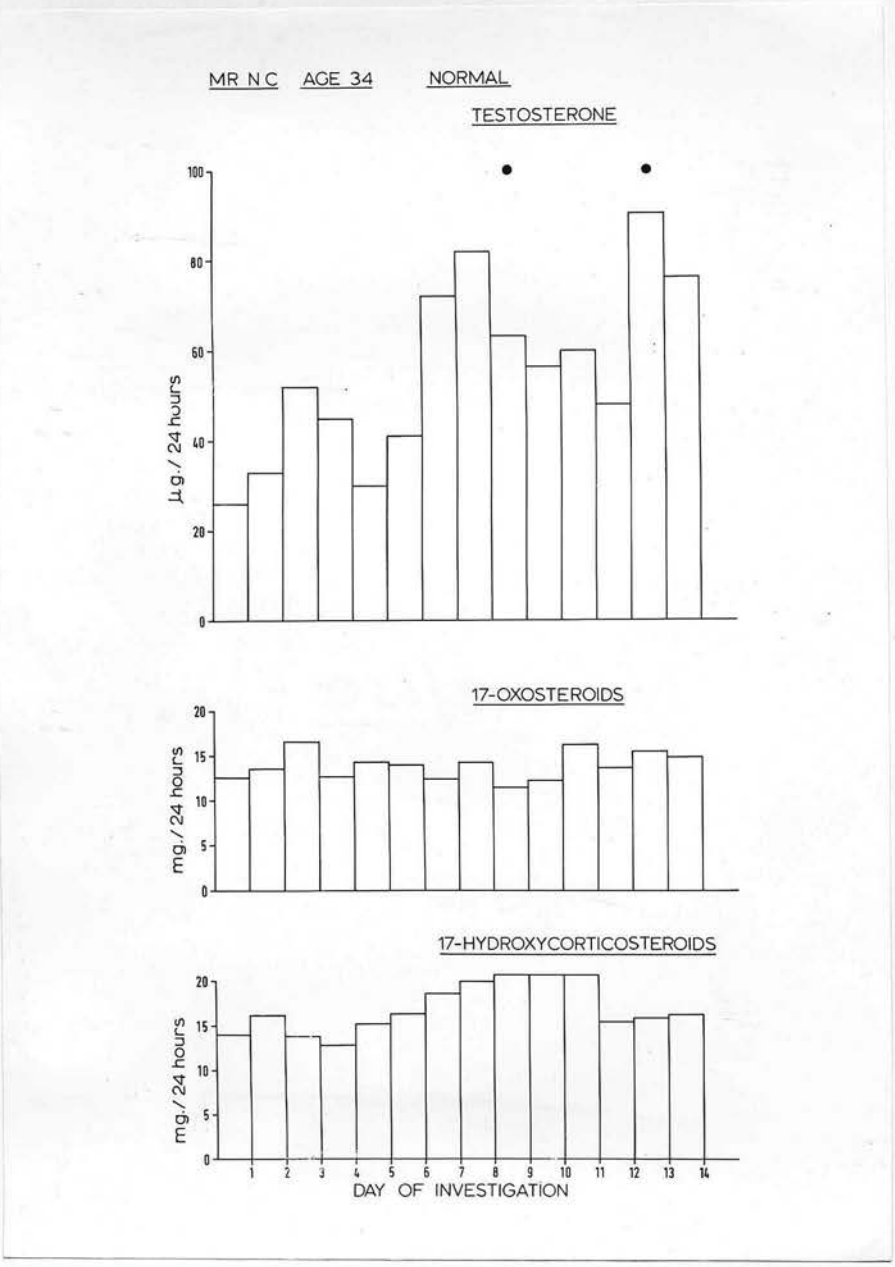
Testosterone levels in two other subjects both aged 20, showed variations which in subject H was from 39·2 to 113·3 µg./24 hr. with peaks at 5-6 day intervals (Fig.15). The variations in subject J were from 61·4 to 149·5 µg./24 hr. with peaks at 4-6 day intervals (Fig.16). Both subjects were studied for a



The effect of sexual intercourse on hormone excretion

Black dots indicate sexual intercourse

Fig. 17



The effect of sexual intercourse on hormone excretion

Black dots indicate sexual intercourse

Fig. **18**

period of 17 days and the assays were corrected by the recoveries obtained using [4-^{14}C-] testosterone as an internal standard. In this way the possibility of any cyclic variations in recoveries was eliminated.

Other factors affecting testosterone excretion

The effect of sexual intercourse on testosterone excretion was studied on subjects E and NC. There were marked variations in urinary testosterone excretion of both subjects, with regular peaks at about 8 days interval in subject E (Fig. 17) and at 5 day intervals in subject NC (Fig.18) throughout the period of investigation. However, the levels when these men were not sexually active were generally lower than those when the sexual activity was resumed. It should be noted that there is a reasonable correlation between 17-oxosteroids excretion, in subject E, with that of urinary testosterone. The mean levels of urinary testosterone when subjects E and NC were not sexually active were 42 and 37 µg./24 hr. respectively: when normal sexual activity was resumed the mean levels rose to 55·0 and 66.0µg./24 hr. respectively.

The effect of age on urinary testosterone levels is a problem which is at present under investigation. Testosterone excretion in men aged between 21 and 63 ranged from 40 to 64.5µg./24 hr. with

a mean of 52 μg./24 hr. Men over 50 years tended to show somewhat lower levels. The levels in normal young men aged 16-20 years ranged from 60 to 103 μg./24 hr.

DISCUSSION

Testosterone is the most potent androgen known to be present in human blood. Its measurement thus provides an estimate of the androgenic stimulation to which the body is exposed. In males, the plasma production rates and the urinary production rates are similar (Horton et al. 1965; Rivarola, Saez, Mayer, Jenkins & Migeon, 1966; Horton & Tait, 1966). Thus the urinary levels of the hormone provide an index of the testicular secretion of androgen. The observation of regular peaks of testosterone excretion in the present study suggests, but certainly does not prove, that there may be a cyclic variation in the secretion of the hormone by the testes. This is analogous to the findings of Exley & Corker (1966) who in normal male subjects demonstrated a cyclic pattern in 17-oxosteroids and oestrone output which was not associated with a similar change in urinary 17-hydroxycorticosteroids. A detailed mathematical analysis of changes in 17-oxosteroids excretion has been reported by Halberg, Engeli, Hamburger & Hillman (1965) who demonstrated a weekly

cycle in 17-oxosteroid levels. However, in the present investigation variations in testosterone excretion have not generally been correlated with alterations in 17-oxosteroid output the pattern of excretion of which tended to follow that of the urinary 17-hydroxycorticosteroids arising from the adrenal cortex. However, there may be some correlation between all 3 parameters in the studies shown in Figs. **13** and **17** .

The dependance of sexual activity on the gonadal hormones has long been recognised (Young, 1961). In the present study, the relationship between the sexual activity and hormone excretions was investigated in two cases. The results suggest that sexual activity had no effect on the occurence of regular peaks of testosterone excretion. However, an increase in testosterone output was found which paralleled sexual activity. The results in Figs. **17** and **18** are in agreement with findings in bulls of Branton, D'Arensbourg & Johnston (1952) (See also Mann, 1964). The former workers found that excitation prior to service has a stimulating effect on the fructose level in bull semen. Similar studies have not yet been demonstrated in human subjects. However, previous studies by Lindner & Mann (1960) have shown that the levels of fructose in semen are maintained by testosterone and that there is a positive correlation between the levels of fructose

and citric acid in the seminal vesicles and that of circulating testosterone. Thus the present findings are in agreement with those of previous investigators using other techniques. Moreover, the maturation of spermatozoa is indirectly dependent upon the presence of testosterone. The sperm formed in the testes are not functionally mature, and structural and biochemical changes occur during their transport through the male genital tract (Bishop, 1961). The epididymis and epididymal fluids which have been shown to be androgen dependent (Dawson, Mann & White, 1957; Dawson & Rowlands, 1959) are essential for the changes occurring in sperm after leaving the testes (Maraud & Stoll, 1958). Therefore, the dependence of the male accessory organs and their secretions upon testicular testosterone is well established; it has been found that these secretions are necessary for the normal development of spermatozoa. Thus from the results of the present investigations, the emptying of the genital tract as a result of sexual activity may be associated with a stimulation of testicular secretion. Such an increase in hormone levels might stimulate spermatogenesis and increase secretion in the male genital tract and thus accelerate the refilling of the system.

In general, the results obtained by studying the hormone excretion in the urine of men over long

periods of time suggest that there is a considerable variation from day to day in the secretion of the precursors of urinary testosterone by the testes. It may, therefore, be necessary to determine urinary testosterone for about 10 days in men, in order to detect abnormalities in the excretion of the hormone.

SUMMARY

1. In a limited number of studies testosterone excretion in normal young men aged from 16 to 20 years was generally higher than that of men aged from 21 to 63 years.

2. Serial testosterone assays in 5 adults aged from 20 to 33 years over a period of 14 to 26 days revealed large variations, figures ranging from 40·0 - 149·0 µg./24 hr. Peaks of testosterone excretions were noted at approximately 12 day intervals in one volunteer and from 4 to 6 days in the remaining four subjects. The rises in testosterone excretion were generally not associated with marked alterations in urinary 17-oxosteroids or 17-hydroxycorticosteroids output.

3. In the two cases studied an increase in testosterone output was encountered in association with sexual activity. However, sexual intercourse did not affect the presence of testosterone excretion peaks.

4. It may be necessary to determine urinary testosterone serially for about 10 days in male subjects in order to demonstrate abnormalities of testosterone excretion.

B. TESTOSTERONE EXCRETION DURING THE NORMAL MENSTRUAL CYCLE

INTRODUCTION

In recent years a number of investigators have studied steroid excretion patterns during the normal menstrual cycle (see Loraine & Bell, 1966, for references). The pattern of urinary oestriol, oestrone, oestradiol-17β, pregnanediol and pregnanetriol is now well established. However, urinary testosterone excretion has not been adequately investigated. Hudson, Coghlan, Dulmanis & Wintour (1964) reported the presence of three peaks of testosterone output during a menstrual cycle, while Apostolakis, Starcevic & Voigt (1965) and Horn, Statter & Finkelstein (1966) described two such peaks.

The purpose of the present investigation was to provide further information regarding the pattern of urinary testosterone excretion in normally menstruating women, and to compare the results obtained with those already reported in the literature.

MATERIALS AND METHODS

Subjects: Complete 24 hour urine samples were collected continuously throughout one menstrual cycle in 4 subjects. All the women continued normal activities during the period of investigation and in none was there a previous history of menstrual abnormalities. Clinical details were as follows:

Subject T.Y. (Fig. 19) aged 34 years, para 0., regular 24 day cycles with bleeding lasting 4 days.

Subject G. (Fig. 20) aged 31 years, para 0, regular 31 day cycles with bleeding lasting 4 days.

Subject A.U. (Fig. 21) aged 33 years, para 0., regular 28 day cycles with bleeding lasting 4 days.

Subject N.C. (Fig. 22) aged 22 years, para 0., regular 29 day cycles with bleeding lasting 3 days.

Method of Assay: Estimations were generally conducted on 48 hour urine pools, except in the case of subject N.C. in whom assays were performed on 24 hour

specimens and following the addition of (4-C^{14}) testosterone as an internal standard. All the results were expressed per 24 hour sample.

In addition to urinary testosterone assays, pregnanediol estimations by the technique of Klopper, Michie & Brown (1955) were performed on specimens from subjects T.Y., A.U., and N.C. In subject T.Y. oestrogens were also determined using the method of Brown (1955).

RESULTS

Urinary testosterone levels in 15 apparently normal women determined in a 72 hr. pools, and irrespective of the phase of the cycle is shown in Table 11. A comparison of urinary testosterone levels in female subjects measured by different methods is shown in Table 12.

Subject T.Y. (Fig. 19)

Ovulation as judged by urinary oestrogen excretion probably occurred on days 8-10 of this 24 day cycle. The pattern of pregnanediol excretion was normal, levels of the steroid rising in the luteal phase, thus confirming that ovulation had occurred. The pattern of urinary testosterone output showed a marked rise in the luteal phase; levels at about the time of ovulation were somewhat higher than in the follicular phase. It is possible that the very small size of this volunteer may be the cause of the rather low levels observed.

Subject G. (Fig. 20).

Previous studies on oestrogen excretion in this subject showed that ovulation occurred regularly on about day 15-16 of the cycle. In the present investigation a luteal phase peak of testosterone excretion was observed together with a marked rise in the follicular phase. An ovulatory peak was not noted.

TABLE 11

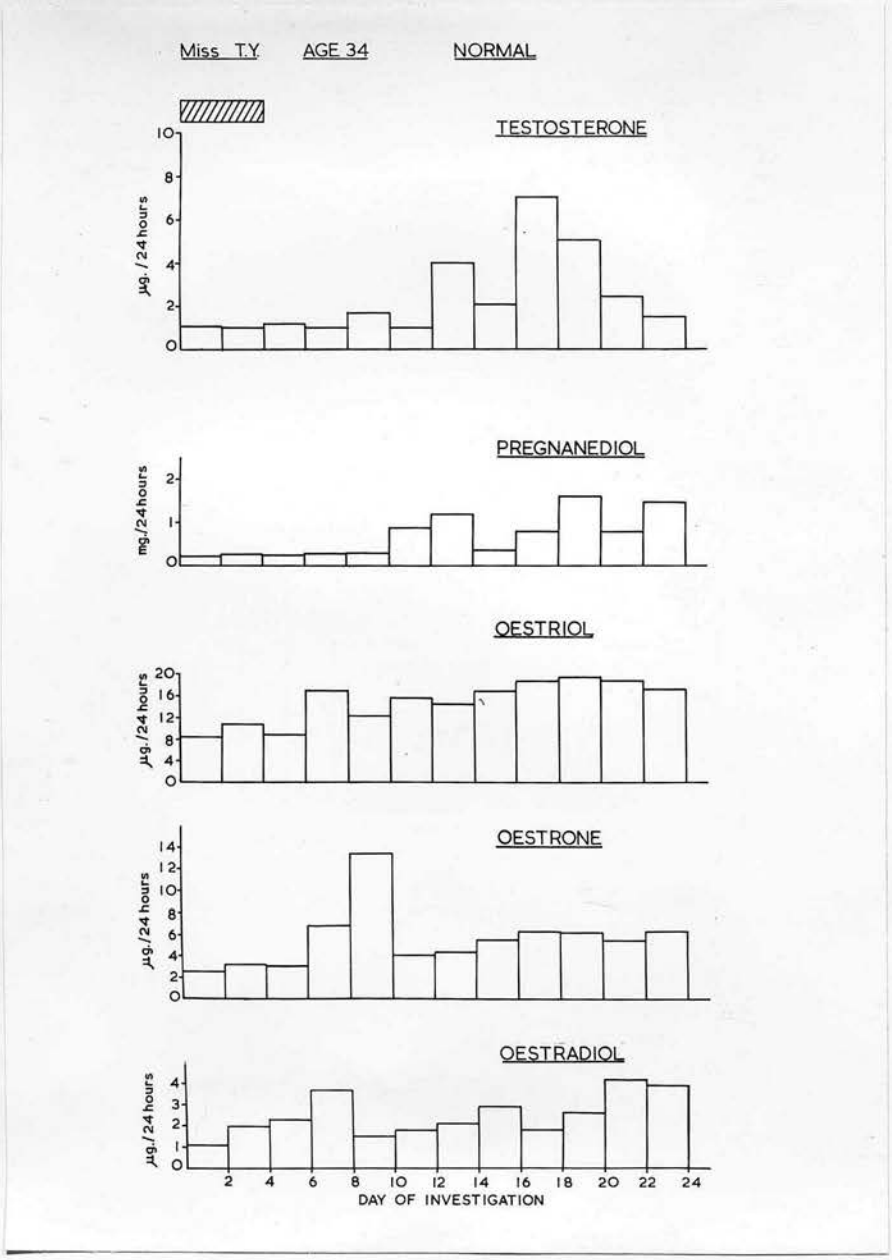
Urinary testosterone excretion values in women estimated by the present method

Subject's number	Age	µg./24 hours
1	20	6.5
2	20	4.7
3	21	4.7
4	25	6.5
5	26	5.7
6	31	7.1
7	33	9.5
8	33	10.7
9	33	4.5
10	34	2.1
11	34	2.4
12	36	10.5
13	47	8.7
14	48	7.9
15	55	5.9

TABLE 12

Urinary testosterone excretion by normal women as estimated by different methods

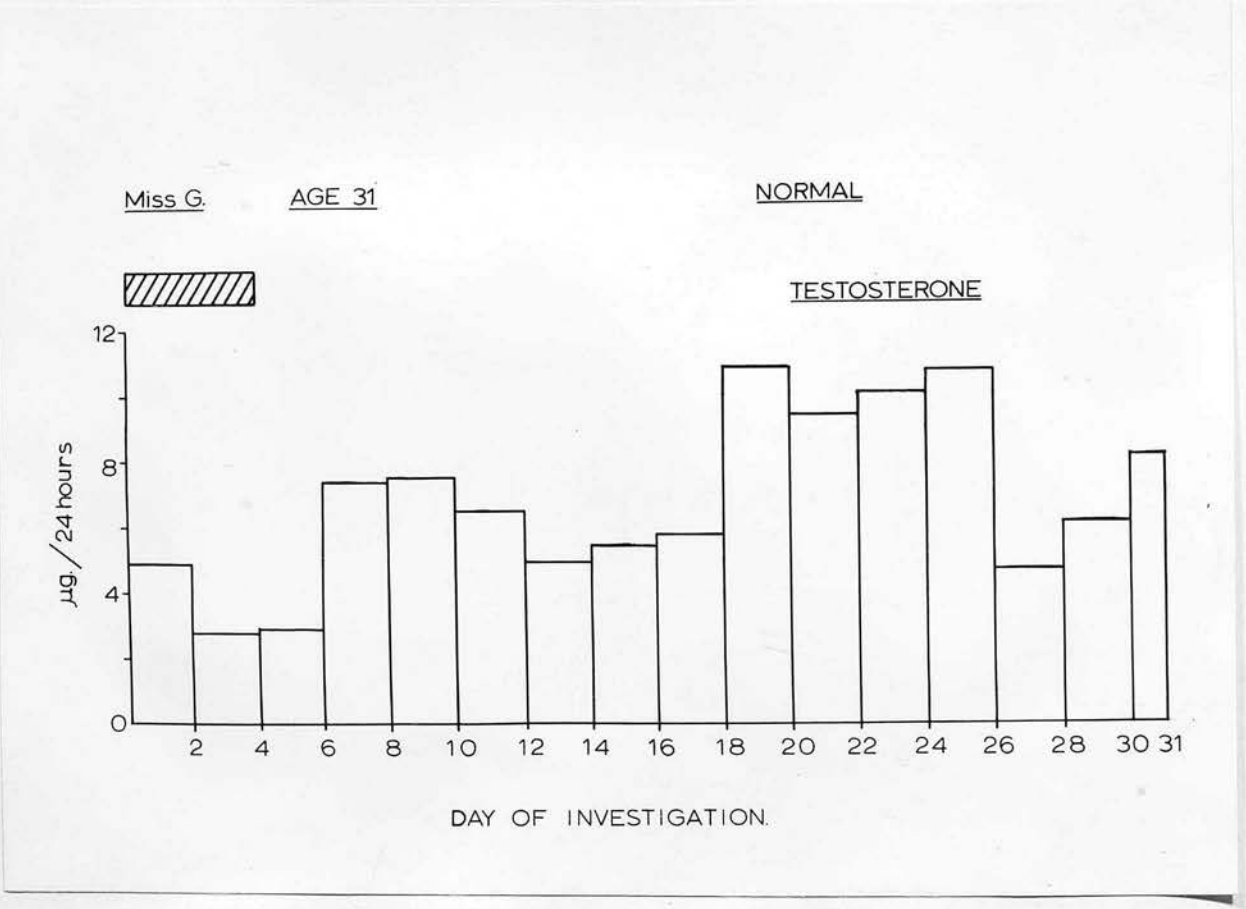
Age in years	Number of subjects	Urinary Testosterone µg./24 hours Mean	Range	Reference
19-23	4	5·9	(3·5-7·5)	Camacho & Migeon (1963)
23-37	6	6·1	(2·8-8·0)	Futterweit et al. (1963)
18-63	4	8·3	(5·0-12·0)	Vermeulen & Verplanke (1963)
21-32	4	12	(7·0-18·0)	Brooks (1964)
20-72	10	<5·3	(<5·0-8·0)	Ibayashi et al. (1964)
22-35	4	10	(3·0-18·0)	Voigt, Volkwein, Tamm (1964)
21-68	18	48·9	(2·9-196·7)	Dulmanis, Coghlan, Wintour & Hudson (1964)
20-52	6	6·0	(3·0-8·0)	Panicucci, Savi & Coli (1964)
29-35	2	4·8	(4·1-5·6)	Zurbrügg, Jacobs & Gardner (1965)
20-40	20	19·0	(6-57)	Rosner et al. (1965)
22-59	16	7·1	(4·3-10·4)	Lim & Dingman (1965)
18-30	7	-	(<1·0-3·8)	Horn, Statter & Finkelstein (1966)
17-53	10	7·7	(3·0-14·0)	Vestergaard, Raabo & Vedso (1966)
20-32	10	6·1	(2·8-10·0)	Wegienka et al. (1967)
20-55	15	6·5	(2·1-10·7)	The present method



Urinary hormone excretion pattern in a normally menstruating woman

Lined block area indicate periods of menstrual bleeding

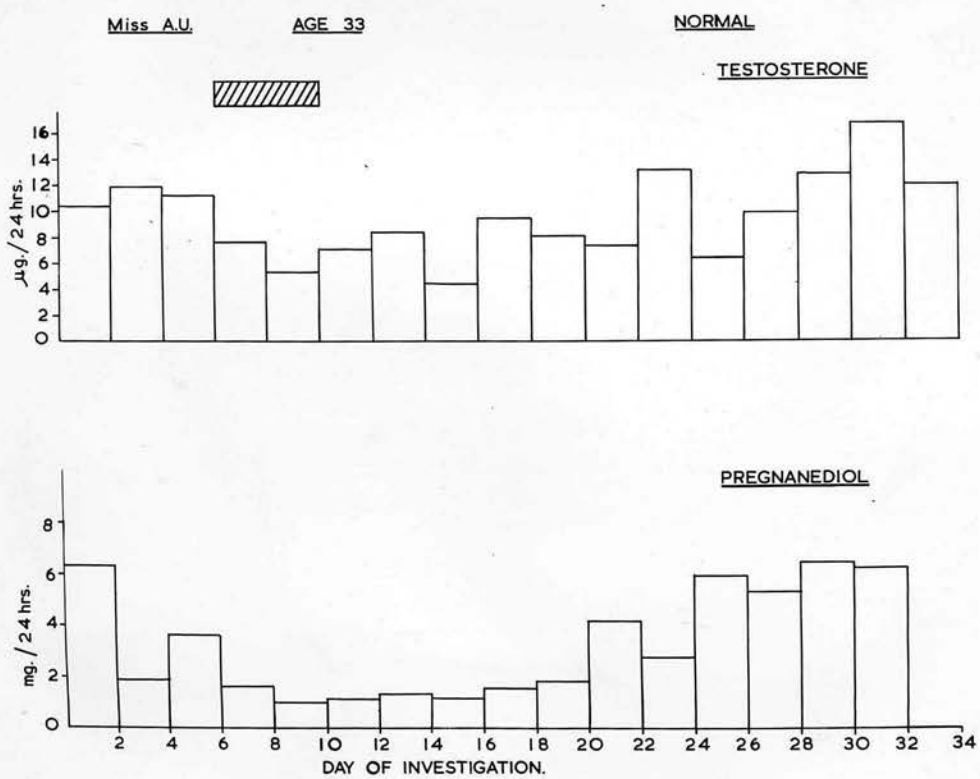
Fig. 19



Urinary testosterone excretion pattern in a normally menstruating woman

Lined blocked area indicate periods of menstrual bleeding

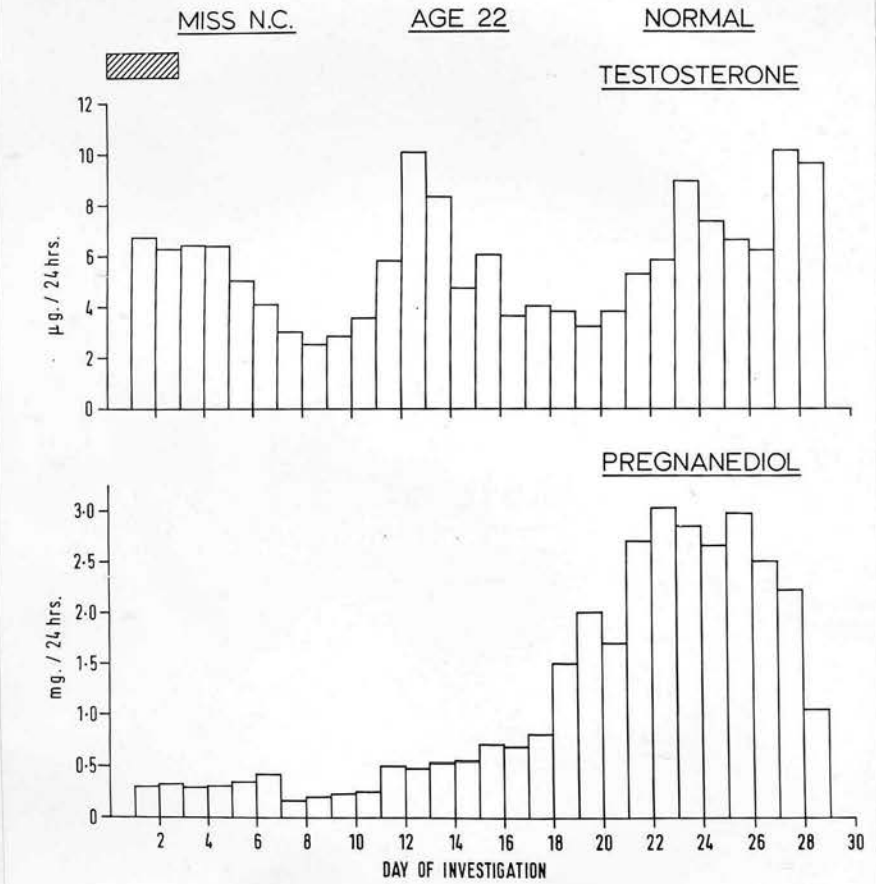
Fig. 20



Urinary hormone excretion pattern in a normally menstruating woman

Lined block area indicate periods of menstrual bleeding

Fig. 21



Urinary hormone excretion pattern in a normally menstruating woman

Lined blocked area indicate periods of menstrual bleeding

Fig. **22**

Subject A.U. (Fig. **21**).

In this woman the rise in pregnanediol output confirmed that ovulation had occurred and that a functional corpus luteum had been formed. Urinary testosterone excretion increased in the luteal phase; there was also an increase at or about the time of ovulation, and a small rise in the follicular phase.

Subject N.C. (Fig. **22**).

Pregnanediol values started to rise on day 18 reaching their luteal phase maximum from day 21 to 28. Urinary testosterone excretion showed a definite peak at or about the time of ovulation and there was a second peak during the luteal phase. A third peak was also observed early in the follicular phase of this cycle.

DISCUSSION

The subjects included in this study showed a steroid excretion pattern consistent with ovulation and with formation of a functioning corpus luteum. It is generally agreed that peaks of oestrogen excretion occur at or about midcycle and that in the luteal phase of the cycle there is generally a second oestrogen peak associated with a rise in pregnanediol output.

The occurrence of cyclical fluctuations in testosterone production during the menstrual cycle has been observed by Hudson et al. (1964), Apostolakis et al. (1965) and more recently by Horn et al. (1966). In one cycle Hudson et al. (1964) showed that three peaks of testosterone excretion could be detected, the highest occurring about eight days prior to ovulation, another at ovulation, and a third smaller and broader peak during the luteal phase. Apostolakis et al. (1965) demonstrated two testosterone excretion peaks in two cycles, the first occurring at the time of ovulation and the second towards the end of the luteal phase. In one menstrual cycle investigated by Horn et al. (1966), the lowest level was found during the few days following menstruation. Two peaks were observed, one at midcycle and the second during the luteal phase. The present investigation has shown

that peaks of testosterone excretion are consistently associated with the presence of a functioning corpus luteum. An increase at midcycle was noted in three subjects while a rise in the follicular phase occurred in only two cycles. Thus, the results described herein are in agreement with those of other workers using different assay methods. However, the magnitude of rises observed in this study is rather less marked than those previously reported by Hudson et al. (1964). In addition, the mean testosterone excretion during the cycle is slightly lower and much lower than the results reported by Apostolakis et al. (1965) and Hudson et al. (1964) respectively. On the other hand, the urinary testosterone levels reported by Horn et al.(1966) were much lower than in the present investigation. The reason for these differing mean values is not entirely clear, but may well be due to methodological factors.

Studies of plasma testosterone levels during the menstrual cycle have in general shown changes similar to those reported herein in the urine. Thus Lobotsky, Wyss, Segre and Lloyd (1964) using a sensitive isotopic procedure found high levels at midcycle and in the luteal phase and low readings during menstruation. On the other hand, in the investigation of Hudson et al. (1964) and Burger, Kent & Kellie (1964) testosterone levels were relatively constant throughout various phases of the cycle. It is probable that in the later studies the precision of the

assay method used was not adequate to detect minor changes which may have taken place.

It is generally agreed that in females the adrenal is a major source of androgen production (Riondel et al. 1963; Horton, Romanoff & Walker, 1966; Horton & Tait, 1966). The fluctuating testosterone levels during the normal menstrual cycle suggests, but does not prove, that the ovaries may also constitute another potential site for the elaboration of these hormones, the rise in testosterone output in association with the functional corpus luteum being especially indicative of this fact. The precise cause of these fluctuating levels is indeed very obscure and remains to be determined, but it appears reasonable to suppose that they are associated with alterations in the biosynthesis of steroids by the ovary and/or by the adrenal cortex since testosterone and other closely related steroids are precursors of the oestrogens. It is less likely that changes in urinary testosterone levels are due to alteration in the peripheral interconversion of the steroids such as androstenedione and dehydroepiandrosterone to testosterone. Also the capacity of the ovary to secrete C-19 steroids such as testosterone, androstenedione and dehydroepiandrosterone has been demonstrated in the normal ovary (Kase, Forchielli and Dorfman, 1961). Lamb, Dignam, Pion & Simmer (1964) noted a rapid fall in plasma testosterone levels following ovariectomy. Mahesh, Greenblatt, Aydar & Roy (1962) described the finding of substantial amounts of androstenedione and

dehydroepiandrosterone in ovarian venous blood obtained from patients with polycystic ovaries or from women receiving pituitary FSH. In this connection Horton et al. (1966) reported that androstenedione is present in ovarian venous plasma in a concentration 26 times higher than in peripheral venous plasma. Higher levels of testosterone were also found in ovarian venous blood. It is also of interest that neither Lamb et al. (1964) nor Korenman, Krishner and Lipsett (1965) could demonstrate an increase in plasma testosterone levels or testosterone production rates following the administration of HCG to normal women. HCG alone was similarly ineffective in increasing oestrogen excretion (Gemzell, Diczfalusy and Tillinger, 1958). However, the last-mentioned workers showed that pituitary FSH could increase oestrogen excretion and this would presumably result in increased synthesis of biosynthetic precursors such as dehydroepiandrosterone, androstenedione and testosterone. Moreover, when HCG was given following FSH, a further increase in oestrogen excretion occurred. Gemzell et al. (1958) concluded that the difference in responsiveness to HCG may be due to a "priming" effect of FSH. In two women Korenman et al. (1965) found that the use of FSH and HCG increased the testosterone production rate, while Lobotsky et al. (1964) demonstrated a marked rise in plasma testosterone levels in a woman receiving FSH and HCG.

In view of these facts it appears reasonable to think that the fluctuations in urinary testosterone levels found during the menstrual cycle may represent ovarian rather than adrenal activity.

SUMMARY

The excretion of urinary testosterone has been investigated on a serial basis in each of four normally menstruating women throughout one cycle.

Ovulation occurred in all four cycles as judged either by urinary pregnanediol or oestrogen assays.

In all subjects there was a rise in testosterone output in the luteal phase and in three there was an increase at about the time of ovulation. A slight increase was also noted during the follicular phase in two of the cycles studied. In general, the pattern described herein was similar to that previously reported by other investigators.

The present study provides evidence in favour of the concept that the ovaries secrete steroids which are precursors of urinary testosterone.

ISOLATION OF PROGESTERONE FROM HUMAN PREGNANCY URINE

INTRODUCTION

The isolation of progesterone from corpora lutea was first reported over 30 years ago by four groups of workers (Butenandt, Westphal & Hohlweg, 1934; Hartmann & Wettstein, 1934; Wintersteiner & Allen, 1934; Slotta, Ruschig & Fels, 1934a,b). In 1938 the hormone was found in ox adrenals (Beall, 1938) and later in human placental tissue (Pearlman & Cerceo, 1952; Salhanick, Noall, Zarrow & Samuels, 1952); the presence of progesterone has also been demonstrated in human pregnancy plasma (Salhanick, Jones, Merrill & Neal, 1954; Zander, 1954; Zander & Simmer, 1954).

On the other hand, successive attempts to detect progesterone in urine have previously been unsuccessful. In 1937, Marker, Kamm & McGrew extracted 10,000 gallons of human pregnancy urine but failed to demonstrate the presence of the hormone, while more recently, Drosdowsky, Dessypris, McNiven, Dorfman & Carlos Gual (1965) reported yet another

unsuccessful attempt. The further development of a modification of the Girard reaction in which a fraction containing a 'conjugated ketone' could be separated from both the non-ketonic fraction and the major bulk of the saturated ketones, made it possible to re-investigate this problem.

MATERIALS AND METHODS

Subject: The subject was a normal woman, in good health and in the last three months of pregnancy. Complete urine collections were made for approximately five weeks; urine was stored at 4° for no longer than one week before extraction.

Paper and Thin-layer Chromatography: Paper chromatography was performed on Whatman No.42 paper in the Bush A system (1952) light petroleum (b.p. 60-80°)-methanol-water (100:80:20 by vol.). Details of the preparation of thin-layer plates and methods of detection have been described on page 16 The systems used for developing thin-layer plates were n-hexane-ethyl acetate (5:1·5, v/v) and benzene-ethyl acetate (4:1, v/v).

Gas-Liquid Chromatography: A description of the apparatus and operating details have already been given on page 20 The columns used for the isolation and identification of progesterone were treated Gas-Chrom P

(100 - 120 mesh) coated with 3% SE-30, 1% SE-30 and 1% QF-1.

ISOLATION PROCEDURE AND RESULTS

1. Extraction: Approximately 50 litres of unhydrolysed urine was extracted in batches of 10 litres twice with one volume of ether. The combined ether extract was washed twice with 0·05 volumes of normal sodium hydroxide saturated with sodium chloride; this was usually sufficient to remove the yellow pigments. The extract was then washed three times with 0·05 volumes of distilled water. The ether extract was dried with anhydrous sodium sulphate and evaporated to dryness in a water-bath.

2. Girard separation: The residue was dissolved in a suitable volume of 97% aqueous ethanol and transferred to a tube. The solution was reduced to a volume of 1·0 ml. or to dryness in a current of air. The Girard separation was then performed by the modified method already described on page.26.

3. Paper chromatography: The 'conjugated ketone' fraction obtained was chromatographed on paper in the system light petroleum (b.p.60-80°)-Methanol-Water(100:80:20 by vol.) Authentic progesterone was chromatographed in parallel

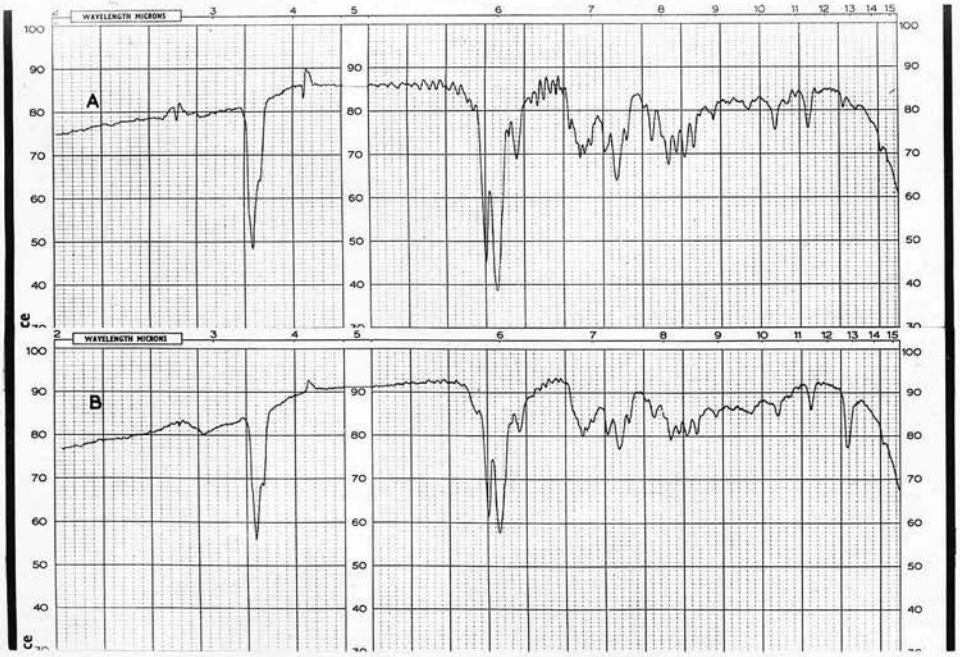
with the extracts and located by its absorption of ultra-violet light and by its reaction with a saturated solution of dinitrophenylhydrazine in ethanol. The area of the chromatograms of the extracts corresponding to progesterone was eluted with methanol. The eluate was then filtered through sintered glass funnels and the filtrate was evaporated to dryness.

4. Thin-layer chromatography: The residue from paper was then chromatographed on a thin-layer of silica gel G in the system n-hexane-ethylacetate (5:1·5 v/v). The plates were removed from the tank, dried in air for 15 minutes and then rechromatographed in the same system. Progesterone has a low R_f value equal to 0·15 in this system, and "over-running" increased the separation from accompanying materials considerably. The progesterone area was located by spraying with dinitrophenylhydrazine in ethanol. The area from the extract corresponding to progesterone was eluted with methanol, filtered through a sintered glass funnel and evaporated to dryness.

5. Gas-liquid chromatography: An aliquot of the residue was subjected to gas-liquid chromatography in the Pye panchromatograph. The stationary phases 1% SE-30 (w/w) and 1% QF-1 (w/w) were used. Three components were generally found (Fig. **23**), the last of which had a retention time of 31·0 and 43·0 minutes on the two phases

Gas–Liquid Chromatogram of Urinary Extract on 1% (w/w) SE-30 at 200°C.

Fig. 23

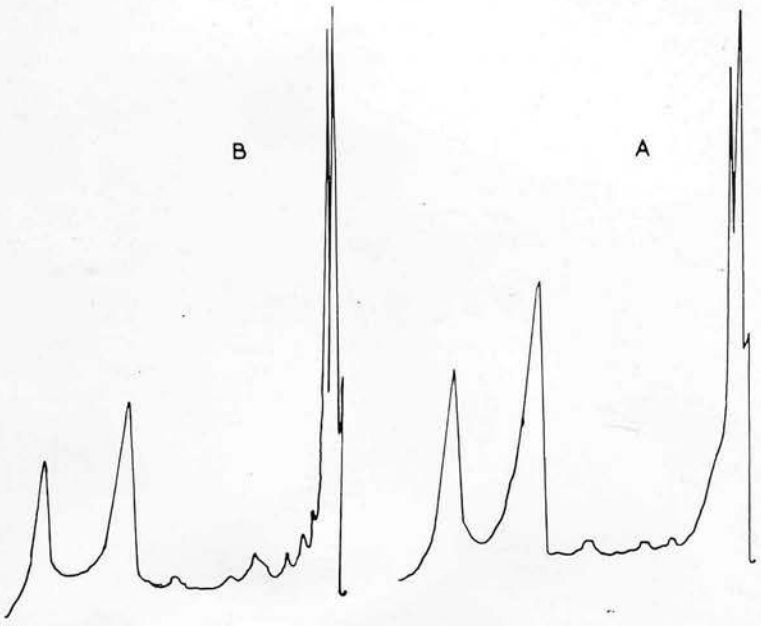


INFRARED ABSORPTION SPECTRUM.

A. 100 µg. Progesterone.

B. Progesterone Fraction from Urinary Extract.

Fig. 24



Products of Reduction, with KBH_4, of Progesterone (A) and Urinary Extract (B) – on 1% (%w) SE-30 at 200°C.

Fig. 25

respectively, which was virtually identical with that of authentic progesterone. Attempts to separate these components from progesterone using repeated chromatography on thin-layer were unsuccessful. Furthermore, neither mild oxidation with 2·5% chromium trioxide (w/v) in 50% aqueous acetic acid (v/v) nor acetylation markedly altered the chromatographic mobilities of the other components travelling with progesterone.

In order to obtain a pure progesterone fraction, gas-liquid chromatography was used and the component corresponding to progesterone was trapped.

6. Spectroscopy and chemical reactions: The infrared absorption spectrum of the trapped component shown in Figure **24** is virtually identical with that of authentic progesterone, the only significant differences at 13 μ being due to a small amount of chloroform trapped in the spot. It should be noted that authentic progesterone was recovered unchanged when it was subjected to this procedure. The ultra-violet absorption spectrum in ethanol was also recorded as previously described, and the spectrum was identical with that of authentic progesterone. Further gas-liquid chromatography of the trapped component on SE-30 and QF-1 phases confirmed that it behaved as a single compound corresponding to progesterone. In addition, its chromatographic behaviour was unaltered by mild chromium trioxide oxidation and by acetylation. When the trapped compound was reduced with potassium

borohydride in methanol at 4° for 3 hr. (Norymberski & Woods, 1954)* and the products of the reaction were analysed by gas chromatography on an SE-30 column, two products were detected, one having the retention time of progesterone (Rt 31·0 minutes) (unreacted material) and another one with the retention time of 20β-hydroxypregn-4-en-3-one (Rt. 41·0 minutes). Similar results were obtained when a sample of authentic progesterone was submitted to the same treatment (Fig. 25). Analysis of the reaction products by thin-layer chromatography into the system benzene-ethyl acetate (4:1 v/v) again gave two compounds with the mobility of progesterone (Rf 0·41) and 20β-hydroxypregn-4-en-3-one (Rf. 0·18).

On the basis of this cumulative evidence it was concluded that the component obtained from the urine was indeed progesterone. It was estimated from the gas-chromatographic peak heights that the pooled urine residues contained approximately 250 µg. of progesterone, but as losses during the estimation procedure were not estimated, this value has no quantitative meaning.

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* Under these conditions 20-oxosteroids are reduced to the corresponding 20β-hydroxy compounds and the 4-en-3-oxo grouping is unaffected as the double bond reduces its activity.

DISCUSSION

Although Drosdowsky et al. (1965) could not demonstrate progesterone in urine they showed that approximately 0·01% of an intravenous dose of the radioactive hormone could be recovered from the urine as a component which behaved chromatographically like progesterone. A similar result was reported by Harkness (1962). If 0·01% of the secreted progesterone is excreted unchanged in the urine, it is then possible to calculate the amount of the hormone which might be expected to be present in the urine during pregnancy. It has been estimated by Zander & Münstermann (1956) that towards the end of the gestation period approximately 200 mg. of progesterone are secreted in 24 hours. If 0·01% of this is excreted in the urine unchanged then approximately 20 µg. of the hormone should be found in a 24 hour sample, and it should be possible to obtain enough material for identification by physical methods.

The modified Girard procedure described herein

separates progesterone from cholesterol and related compounds; these contaminents have similar properties to progesterone on paper, thin-layer and gas-liquid chromatography (see Drosdowsky et al. 1965). In the present study, subsequent chromatography on paper and thin-layer gave a fraction containing progesterone together with three other components. Neither repeated chromatography on thin-layer using different systems, nor mild oxidation and acetylation markedly improved the separation. However, gas-liquid chromatography using columns coated with 1% SE-30 (w/w), 3% SE-30 (w/w) or 1% QF 1 (w/w) gave a complete separation of the progesterone peak from those of other compounds.

The present investigation has shown that progesterone is present in small amounts in urine. The evidence from paper, thin-layer and gas-liquid chromatography, together with the physical confirmation by spectroscopy and chemical reactions, has established the identity of the compound.

The isolation of testosterone from urine by Schubert & Wehrberger (1960) and the subsequent measurement of the small amounts present in human urine has proved of value in the studies of patients with endocrine disorders. Such measurements do provide a reasonably satisfactory index of androgen production in man, (see page 83); this is due to the

fact that a good correlation exists between the amount of the hormone produced in the body and that excreted in the urine. If a relatively constant quantity of the progesterone secreted was excreted in the urine, and if other steroids produced in the body were not converted in significant amounts into this steroid, the estimation of progesterone in urine should provide an index of the secretion of the hormone by the placenta, corpus luteum and to a lesser extent the adrenal cortex. The value of the isolation reported herein would therefore appear to be the possibility it holds out for more direct measurement of the secretion of progesterone in human subjects. At present the only practical means of assessing progesterone production in human subjects on a serial basis is to measure the urinary excretion of its principal metabolite, 5β-pregnane-3α, 20α-diol (pregnanediol), and the limitations of this approach to the problem have been frequently emphasised (see Loraine & Bell, 1966). The work reported herein suggests that the measurement of the parent hormone progesterone in urine is feasible and may also be useful.

SUMMARY

Progesterone was isolated from the free fraction of approximately 50 litres of pregnancy urine. Chemical and physical properties showed that the compound isolated was indeed progesterone.

The isolation procedure involved a modified form of the Girard reaction in which a 'conjugated ketone' fraction containing progesterone was separated from both the non-ketonic fraction and from the major bulk of the saturated ketones. This was followed by paper and thin-layer chromatography. The final separation was achieved using gas-liquid chromatography with a stream splitter; the progesterone fraction was collected from the larger stream which had not passed through the detector.

It was estimated from the gas-chromatographic peak heights that the pooled urine residues contained approximately 250 μg. of progesterone.

FINAL SUMMARY AND CONCLUSION

1. A method for the estimation of urinary testosterone is described. This depends on acid hydrolysis, a modified form of the Girard separation and chromatography on an alumina column and on paper. The final method of detection is by gas-liquid chromatography.

2. The reliability criteria of the method have been investigated. The recovery of added testosterone was approximately 80%. The method is reasonably precise, the coefficients of variation being 6·4% and 8·0% for male and female urine respectively. The chromatographic, chemical and physical behaviour of the compound estimated in the final fraction is similar to that of authentic testosterone. Accordingly, the method which is also practicable, appears to be specific.

3. When testosterone assays were performed in normal men and in normally menstruating women, considerable variations were found from one subject to another. However, levels in males were consistently higher than those in females, there being no overlap between the two groups.

4. Serial assays of urinary testosterone in normal male subjects showed the presence of peaks of excretion at fairly regular intervals. In two cases studied sexual intercourse had no effect on the occurrence of such peaks but caused an overall rise in testosterone output.

5. Testosterone excretion values in normal young men aged from 16 to 20 were generally higher than those of the elder group of subjects aged from 21 to 63 years.

6. Serial assays of urinary testosterone in normally menstruating women have shown an elevation of levels during the luteal phase probably associated with the presence of a functioning corpus luteum. A second rise was also noted at the time of ovulation, and a slight increase was observed during the follicular phase in two of the cycles studied.

7. The assay method for urinary testosterone is now being applied to conditions such as acne vulgaris, sex chromosome abnormalities, athersclerosis in males and hirsutism in females.

8. Progesterone has been isolated from the urine of a woman in late pregnancy. A modification of the Girard reaction was used by which it was possible to separate the 'conjugated ketone' fraction both from non-ketonic fraction and from the bulk of the saturated ketones. The final separation was made using gas-liquid

chromatography with a stream splitter.

9. Work is proceeding on the use of the modified Girard separation technique to estimate plasma steroids with a Δ^4-3-ketone structure. By this method the majority of the biologically active neutral steroid can be estimated simultaneously in one sample.

ACKNOWLEDGEMENTS

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PUBLICATIONS FROM THE CONTENTS OF THE THESIS

1. The isolation of progesterone from human pregnancy urine. (1967). Acta endocr.Kbh. 56, 272.
2. The urinary excretion of testosterone by normal men and women. (1966). J, Endocrin. 34, xvii.
3. A method for the estimation of urinary testosterone. Biochem.J. 99, 717.
4. Factors associated with alterations in urinary testosterone levels. (1966). J.Endocrin. 35, xx-xxi.

Dr. R.A.Harkness was co-author in all these publications.

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Medical Research Council Clinical Endocrinology Research Unit,
University of Edinburgh

THE ISOLATION OF PROGESTERONE FROM HUMAN PREGNANCY URINE

By

*A. A. A. Ismail and R. A. Harkness**

ABSTRACT

Progesterone was isolated from the »freely extractable« fraction of approximately 50 litres of pregnancy urine. Chemical and physical properties showed that the compound isolated was indeed progesterone.

The isolation procedure involved a modified form of the Girard reaction in which a »conjugated ketone« fraction containing progesterone was separated from both the non-ketonic components and the major bulk of the saturated ketones. This was followed by paper and thin-layer chromatography. The final separation was achieved using preparative gas-liquid chromatography.

It was estimated from the gas-chromatographic peak heights that there was no more than 250 μg of progesterone in the pooled urine residues.

The isolation of progesterone (pregn-4-ene-3,20-dione) from corpora lutea was first reported over 30 years ago by four groups of workers (*Butenandt et al.* 1934; *Hartmann & Wettstein* 1934; *Wintersteiner & Allen* 1934; *Slotta et al.* 1934 *a, b*). In 1938 the hormone was found in ox adrenals (*Beall* 1938) and later in human placental tissue (*Pearlman & Cerceo* 1952; *Salhanick et al.* 1952); the presence of progesterone has also been demonstrated in human pregnancy plasma (*Salhanick et al.* 1954; *Zander* 1954; *Zander & Simmer* 1954).

On the other hand, successive attempts to isolate and identify progesterone in urine have previously been unsuccessful. *Marker et al.* (1937) extracted

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10 000 gallons of human pregnancy urine but failed to demonstrate the presence of the hormone. More recently, *Drosdowsky et al.* (1965) showed that approximately 0.01 % of a dose of radioactive progesterone appeared in the urine as a fraction which behaved chromatographically like progesterone; this was in agreement with the results of *Harkness* (1962). However, no definite evidence of identity was obtained in both these studies. If 0.01 % of the secreted hormone was present in urine then it should have been possible to obtain sufficient material for identification by physical methods. The further development of a modification of the Girard reaction in which a fraction containing »conjugated ketones« could be separated from both non-ketonic fraction and the major bulk of the saturated ketones, made it possible to re-investigate this problem. The aim of the present communication, a preliminary account of which has been given elsewhere (*Ismail & Harkness* 1966 *a*), is to report the isolation and identification of progesterone from the »freely extractable« fraction of human pregnancy urine.

MATERIALS AND METHODS

Subject

The subject was a normal woman, in good health and in the last three months of pregnancy. Complete urine collections were made for approximately five weeks; urine was stored at 4° C for no longer than one week before extraction.

Reagents and Solvents

The solvents and reagents used were of analytical grade quality in all cases in which this grade was available. The solvents were re-distilled before use. The Girard reagent T and cation exchange resin Amberlite IRC-50 (H) were treated as described by *Ismail & Harkness* (1966 *b*). Consistently good recoveries of progesterone were obtained by using resin of particle size about 0.5 mm diameter. Fine particle resin was found to give incomplete coupling under these conditions.

Paper and Thin-layer Chromatography

Paper chromatography was performed on Whatman No. 42 paper in the system light petroleum (b. p. 60–80° C) -methanol-water (100:80:20 by volume). The systems used for developing thin-layer plates were hexane-ethyl acetate (5:1.5, v/v); benzene-ethyl acetate (4:1, v/v) and chloroform. Details of the preparation and methods of detection have been described previously.

Gas-liquid Chromatography

A Pye panchromatograph with a flame ionization detector was used (see *Ismail & Harkness* 1966 *b*). In addition, separation of the components in the final extract was performed using a Perkin-Elmer model number 801 with a stream splitter of ratio 4:1. The column used in the latter apparatus was packed with silicone treated Gas-Chrom P (100–120 mesh) coated with 3 % SE-30. Nitrogen flow was 25 ml/min, and column temperature 240° C. The larger stream was passed directly to the exterior and the

peak corresponding to the progesterone was trapped in a glass tube at room temperature. The glass tube had a diameter of 4 mm, was 20 cm in length, and had two constrictions near the end.

Spectroscopy

The visable and ultraviolet absorption spectrum was recorded in a Unicam SP. 800 spectrophotometer with 1 cm light-path. For infrared spectroscopy a Unicam SP. 200 was used, and a micro-technique (*Sykes & Kelly*, personal communication) was employed. The residue was dissolved in chloroform and deposited as a spot on a KBr disc. The latter was then placed in a beam condenser and the infrared spectrum recorded.

ISOLATION PROCEDURE AND RESULTS

1. *Extraction*

The unhydrolysed urine was extracted in batches of 10 litres with two volumes of ether. The combined ether extract was washed twice with 0.05 volume N sodium hydroxide saturated with sodium chloride; this was usually sufficient to remove the yellow pigments. The extract was then washed three times with 0.05 volume of distilled water. The ether extract was dried with anhydrous sodium sulphate and evaporated to dryness in a waterbath.

2. *Girard separation*

The residue was dissolved in a suitable volume of 97 % aqueous ethanol and transferred to a tube. The solution was reduced to a volume of 1.0 ml or to dryness in a current of air. The Girard separation was then performed by the modified method of *Ismail & Harkness* (1966 *b*).

3. *Paper chromatography*

The »conjugated ketone« fraction obtained was chromatographed on paper in the system previously described. Authentic progesterone was chromatographed in parallel with the extracts, and located by its absorption of ultraviolet light and by its reaction with a saturated solution of dinitrophenylhydrazine in ethanol. The area of the chromatograms of the extracts corresponding to progesterone was eluted with methanol. The eluate was then filtered through sintered glass funnels and the filtrate was evaporated to dryness.

4. *Thin-layer chromatography*

The residue from paper was then chromatographed on a thin-layer of silica gel G in the system hexane-ethyl acetate (5:1.5 v/v). The plates were removed from the tank, dried in the air for 15 minutes and then rechromatographed in

the same system. Progesterone has a low R_F value in this system and this »over-running« procedure increased the separation considerably. The progesterone area was located by spraying with dinitrophenylhydrazine in ethanol. The area from the extract corresponding to progesterone was eluted with methanol, filtered through a sintered glass funnel and evaporated to dryness.

5. *Gas-liquid chromatography*

An aliquot of the residue was subjected to gas-liquid chromatography in the Pye panchromatograph. The stationary phases 1 % SE-30 (w/w) and 1 % QF-1 (w/w) were used. Three components were generally found, the last of which had the same retention time as progesterone. Attempts to separate these components from progesterone using repeated chromatography on thin-layers of silica gel were unsuccessful. Furthermore, neither mild oxidation with 2.5 % chromium trioxide (w/v) in 50 % aqueous acetic acid (v/v) nor acetylation altered markedly the chromatographic mobilities of the other components travelling with progesterone.

In order to obtain a pure progesterone fraction, gas-liquid chromatography was used and the component corresponding to progesterone was trapped as previously described.

6. *Spectroscopy and chemical reactions*

The infrared absorption spectrum of the trapped component shown in Fig. 1 is virtually identical with that of authentic progesterone; the only significant differences at 13 μ being due to a small amount of chloroform trapped in the spot. It should be noted that authentic progesterone was recovered unchanged when it was subjected to this procedure. The ultraviolet absorption spectrum in ethanol was also recorded as previously described and the spectrum was identical with that of authentic progesterone. Further gas-liquid chromatography of the trapped component on SE-30 and QF-1 phases confirmed that it behaved as a single compound corresponding to progesterone. In addition, its chromatographic behaviour was unaltered by mild chromium trioxide oxidation and by acetylation. Reduction with potassium borohydride as described by *Bush* (1961) gave products behaving similarly to those obtained from authentic progesterone on gas-liquid chromatography and in thin-layer chromatography. In addition, when the trapped component was reduced with potassium borohydride and the reaction products were acetylated with acetic anhydride and pyridine the thin-layer and gas-liquid chromatographic patterns of the reaction products were identical with those of authentic progesterone. On the basis of this cumulative evidence it was concluded that the component obtained from the urine was indeed progesterone. It was estimated from the gas chromatographic peak heights that the pooled urine (approx. 50 litres) residue contained approximately 250 μg of progesterone.

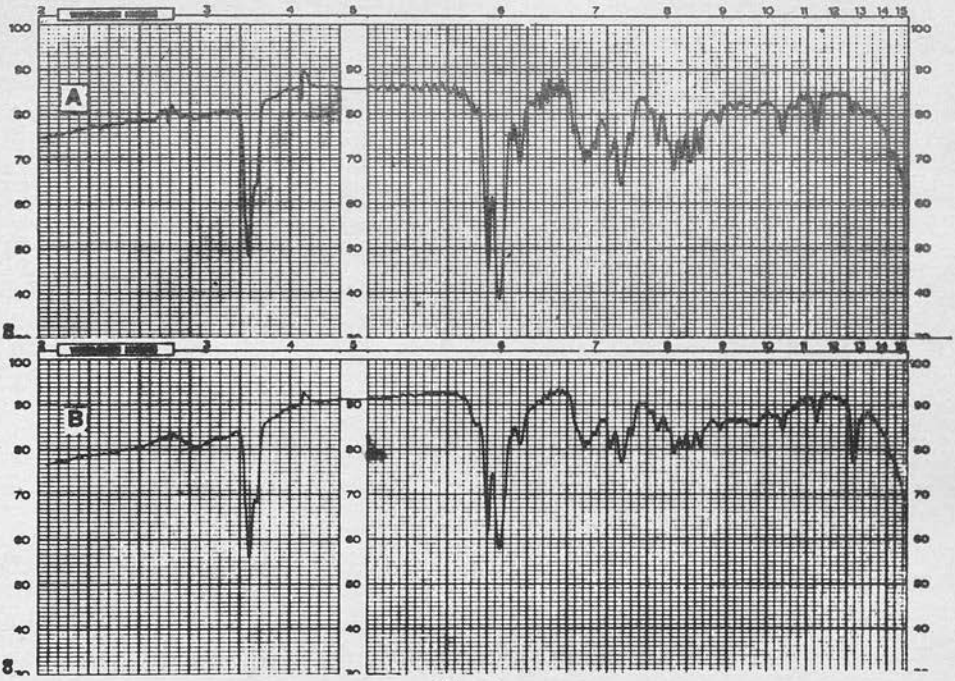

Fig. 1.
Infrared absorption spectrum.
A. 100 μg Progesterone.
B. Progesterone fraction from the urinary extract.

DISCUSSION

The modified Girard procedure described by *Ismail & Harkness* (1966 *b*) for the separation of a »conjugated ketone« fraction, separates progesterone from cholesterol and related compounds, these contaminants have similar properties to progesterone on paper, thin-layer and gas-liquid chromatography (see *Drosdowsky et al.* 1965). In the present study, subsequent chromatography on paper and thin-layer gave a fraction containing progesterone together with two other components. Neither repeated chromatography on thin-layer using different systems, nor mild oxidation and acetylation markedly improved the separation. However, gas-liquid chromatography using columns coated with 1 % SE-30 (w/w), 3 % SE-30 (w/w) or 1 % QF-1 (w/w) showed complete separation of the progesterone peak from other compounds.

The present investigation has shown that progesterone is present in small amounts in urine. The evidence from paper, thin-layer and gas-liquid chromatography, together with the confirmation by spectroscopy and chemical behaviour, has established the identity of the compound.

The isolation of testosterone from urine by *Schubert & Wehrberger* (1960) and the subsequent measurement of the small amounts present in human urine has proved of value in the study of patients with endocrine disorders. Such measurements do provide a reasonably satisfactory index of androgen production in men (*Vander Wiele et al.* 1963; *Horton et al.* 1965); this is due to the fact that a good correlation exists between the amount of the hormone produced in the body and that excreted in the urine. If a relatively constant quantity of the progesterone secreted were converted to urinary progesterone, and if other steroids produced in the body were not converted in significant amounts into this steroid, the estimation of progesterone in urine should provide an index of the secretion of the hormone by the placenta, corpus luteum and to a lesser extent the adrenal cortex. The value of the isolation reported herein would therefore appear to be the possibility it holds out for a more direct serial urinary measurement related to the secretion of progesterone in human subjects.

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The urinary excretion of testosterone by normal men and women. By A. A. A. Ismail and R. A. Harkness. *Medical Research Council Clinical Endocrinology Research Unit, University of Edinburgh*

Serial urinary testosterone assays by the method of Ismail & Harkness (1965) have been used to study the variations of levels in the urine of men and women. The testosterone excretion of three normal women was studied during one menstrual cycle. In all three subjects there was an approximately two- to threefold rise in the luteal phase of the normal menstrual cycle from levels of about 2–6 μg./24 hr. at the beginning of the cycle. In one subject there was a less marked rise at about the time of ovulation, and in another there was a rise during the follicular phase of the cycle. The present pattern of urinary testosterone during the menstrual cycle is similar to that described by Hudson, Coghlan, Dulmanis & Wintour (1965); luteal and ovulatory phase rises were also observed by Apostolakis, Starcevic & Voigt (1965). However the levels in the present study were somewhat lower than those described by the above workers. Urinary testosterone levels in amenorrhoeic and in postmenopausal women were similar to those at the beginning of the menstrual cycle. The administration of ACTH to an amenorrhoeic woman doubled her urinary excretion of testosterone.

Urinary testosterone was measured daily for a period of 14–26 days in three normal men aged 21–33 years; simultaneous assays of 17-oxosteroids and 17-hydroxycorticosteroids were also performed. The levels of testosterone varied from 40 to 90 μg./24 hr. in an approximately regular fashion. Peaks of testosterone excretion were noted at approximately 5- and 6-day intervals in two of the subjects and at 12-day intervals in the third subject. The rises were generally not associated with marked alterations in urinary 17-oxosteroids or 17-hydroxycorticosteroids. The urinary excretion of an adrenalectomized man was 38 μg./24 hr. over a 24-day period. The levels in a castrate man were 3·6 to 8·9 μg./24hr. No testosterone was detected in the urine of an adrenalectomized ovariectomized woman.

The results of the present investigations suggest that there is a considerable variation in the secretion of the precursors of urinary testosterone by the testes as well as by the ovaries and probably by the adrenal cortex. It may be necessary therefore, to determine urinary testosterone throughout the menstrual cycle in women and for about 10 days in men in order to detect the majority of abnormalities of testosterone excretion.

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A Method for the Estimation of Urinary Testosterone

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1. A method has been developed for the estimation of testosterone in human urine by using acid hydrolysis followed by a quantitative form of a modified Girard reaction that separates a 'conjugated-ketone' fraction from a urine extract; this is followed by column chromatography on alumina and paper chromatography. 2. Comparison of methods of estimation of testosterone in the final fraction shows that estimation by gas–liquid chromatography is more reproducible than by colorimetric methods applied to the same eluates from the paper chromatogram. 3. The mean recovery of testosterone by gas–liquid chromatography is 79·5%, and this method appears to be specific for testosterone. 4. The procedure is relatively rapid. Six determinations can be performed by one worker in 2 days. 5. Results of determinations on human urine are briefly presented. In general, they are similar to earlier estimates, but the maximal values are lower.

Testosterone (17β-hydroxyandrost-4-en-3-one) is the most potent androgen that has been isolated from natural sources. Although it is metabolized to 17-oxo steroids, the estimation of these compounds in the urine is rarely useful in the assessment of androgen production because the principal precursors of the 17-oxo steroids are only weakly androgenic.

Schubert & Wehrberger (1960) isolated testosterone from a large number of pooled urine samples from normal men. Since then several methods have been described for the estimation of this hormone in urine and such methods do provide a reasonably satisfactory index of androgen production (Vander Wiele, McDonald, Gurpide & Lieberman, 1963; Horton, Shinsako & Forsham, 1965).

The published methods (see the references in Table 3) for the estimation of testosterone in urine have disadvantages. Some methods require the use of radioactive testosterone either for the location of the testosterone fraction in a chromatographic procedure or to calculate the recovery. Some procedures do not completely separate testosterone and its 17α-epimer, epitestosterone, which is not derived from the hormone (Brooks & Giuliani, 1964). Others require large volumes of urine. In addition, many of these methods involve numerous or complicated chromatographic separations.

The present paper describes a method that avoids many of the disadvantages associated with previously described procedures. A preliminary account has been published (Ismail & Harkness, 1965). After hydrolysis of the urine and subsequent extraction of the free steroids, a 'conjugated-ketone' fraction containing testosterone was separated from this extract by using a modified Girard reaction. This purification step allows acid hydrolysis to be employed and also simplifies the subsequent procedures necessary to obtain a pure fraction containing the hormone. Further simple chromatography on alumina and on paper provides a reasonably pure sample of testosterone. The compound may then be determined by a colour reaction or by gas–liquid chromatography. The reliability criteria of the method are assessed. Some preliminary results obtained in normal subjects are briefly presented.

MATERIALS AND METHODS

Reagents and solvents. The solvents and reagents used were of analytical-grade quality in all cases in which this grade was available. The solvents were redistilled before use. Girard reagent T was washed with ethanol. The cation-exchange resin Amberlite IRC-50 (H^+ form) (British Drug Houses, Ltd., Poole, Dorset, and Rohm and Haas Co., Philadelphia, Pa., U.S.A.) was washed three times in 10–20g. batches with warm aq. 5% (v/v) H_2SO_4; the resin was then washed with water until the washings were neutral. It was stored with a small quantity of water.

Paper and thin-layer chromatography. Paper chromatography was performed on Whatman no. 42 paper in the system toluene–light petroleum (b.p. 60–80°)–methanol–water (33:66:80:20, by vol.). Chromatograms were inspected under ultraviolet light (wavelength approx. 250mμ) and then treated with either Zimmermann reagent, 8% (w/v) phosphomolybdic acid in ethanol, or a saturated ethanolic solution of dinitrophenylhydrazine acidified with a few drops of conc. HCl. Paper chromatograms were run automatically overnight after at least 4hr. equilibration. An alarm clock was modified to turn a tap that filled the trough with solvent. Thin-layer chromatography was performed on silica gel G spread in sheets approx. 250μ thick.

After spreading, the coated plates were heated at 100° for 30 min. and then stored in a desiccator until required. The systems chloroform–ethyl acetate (2:1, v/v) and benzene–ethyl acetate (3:2 and 3:1, v/v) were employed. In addition to the methods of detection described above for paper chromatograms, thin-layer plates were treated with 0·5% (w/v) vanillin in ethanol–conc. H_2SO_4 (1:4, v/v).

Alumina column chromatography. Column chromatography was carried out in glass tubes with an internal diameter of 0·5 cm. and with taps at the lower ends. The packing was supported on a small plug of cotton wool placed at the lower end of the tubes. The alumina used (100–240 mesh, alkaline; Hopkin and Williams Ltd., Chadwell Heath, Essex) had a Brockmann activity of I; this was reduced by the addition of about 10% (v/w) of water which was then thoroughly mixed with the alumina. The activity of the alumina was adjusted by the stepwise addition of more water so that, when testosterone was applied to a 6·0 cm. × 0·5 cm. column of alumina in benzene in 5 ml. of the same solvent followed by 10 ml. of 0·15% (v/v) ethanol in benzene, the steroid began to be eluted after approx. 4 ml. of 0·3% (v/v) ethanol in benzene had been passed through the column. All the testosterone should appear in the next 7 ml. of eluate.

Gas–liquid chromatography. Gas–liquid chromatography was performed in a Pye Panchromatograph with a flame ionization detector. All-glass columns 150 cm. × 0·4 cm. were used. The apparatus for the introduction of the sample into the column and its mode of operation were those described by Menini & Norymberski (1965). The columns were packed with silicone-treated Gas Chrom P (100–120 mesh) coated with 1% (w/w) SE-30 or QF-1 as described by Brooks & Hanaineh (1963). Nitrogen flow was 30 ml./min., and the column temperature was 220° for SE-30 and 205° for QF-1. The evaporation chamber was kept at least 20° above the column operating temperature. The flame ionization detector was used at the optimum conditions determined by Fowlis, Maggs & Scott (1964). The quantity of steroid in the extract was estimated from the peak height by using a corresponding external standard.

Determination of radioactivity and spectroscopy. Radioactivity due to ^{14}C was measured by liquid-scintillation counting by using the method described by Harkness & Fotherby (1963). Absorption spectra were recorded in a Unicam SP.800 spectrophotometer for the visible and ultraviolet region and in a Perkin–Elmer 237 spectrophotometer for the infrared region of the spectrum from wavelengths 2·5 to 16·0 μ. For infrared spectroscopy specimens were dissolved in carbon disulphide (for spectroscopy; British Drug Houses Ltd.) and placed in a cell with a 0·5 mm. light-path. In the visible and ultraviolet region a 1 cm. light-path was used.

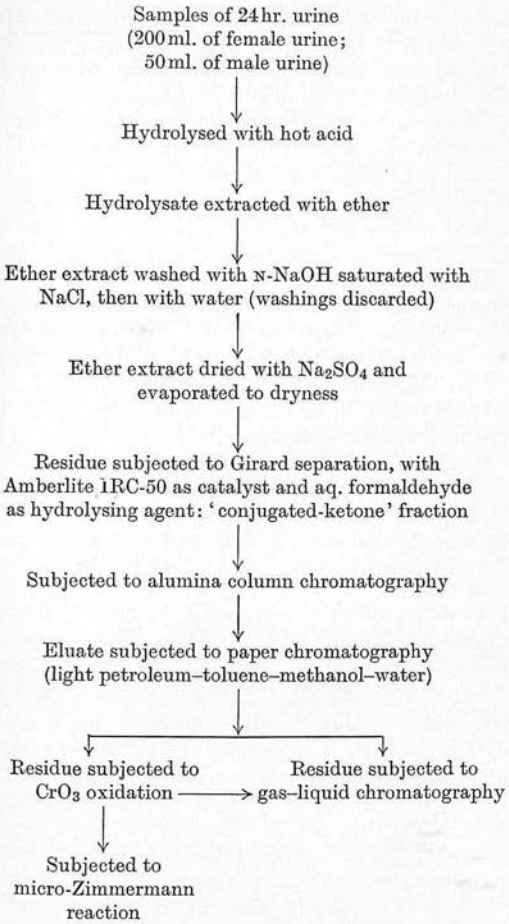
Preparation of urine extracts. Complete 24 hr. collections of urine were made and stored at 4°. Specimens of volume less than 1200 ml. were made up to that volume with water. Enzymic hydrolysis was performed by the method described by Harkness & Fotherby (1963). The conditions chosen were a concentration of 1000 units of β-glucuronidase/ml. The urine was incubated at 37° for 48 hr. Acid hydrolysis was performed by boiling the urine under reflux condensers after the addition of acid. After hydrolysis, the urine was extracted with ether. The extract was evaporated to dryness. Although benzene might have been expected to yield cleaner extracts, it led to considerable emulsion formation.

Modified Girard separation. The residue was dissolved in 1·0 ml. of 97% (v/v) ethanol, and 150–200 mg. of Amberlite IRC-50 and 300–500 mg. of Girard reagent T were added. The mixture was then refluxed for 1 hr., cooled and mixed with 10 ml. of water. The solution was decanted and was extracted once with 1 vol. and twice with 0·5 vol. of ethyl acetate. After each extraction, the upper phase was removed by suction. A 9·5 ml. sample was then taken from the aqueous phase and 2·0 ml. of aq. 36% (w/v) formaldehyde was added. The solution was left on the bench overnight and was extracted three times with 12 ml. of ethyl acetate to obtain the formaldehyde-labile 'conjugated-ketone' fraction containing testosterone. The combined ethyl acetate extracts were washed six times with 5 ml. of water and were dried with anhydrous Na_2SO_4. A 32 ml. sample was evaporated to dryness under reduced pressure in a water bath at 70°. The last traces of formaldehyde were removed with a jet of air.

Standard analytical procedure. A flow sheet for the present method is shown in Scheme 1.

(*a*) Hydrolysis and extraction. Samples of volume 50 ml. of male urine and of volume 200 ml. of female urine were used for each determination. The method of choice for routine assays was hydrolysis by boiling for 1 hr. with 0·15 vol. of conc. HCl. After hydrolysis, the urine was cooled and extracted twice with 1 vol. of ether. The combined ether extracts were washed twice with 0·05 vol. of N-NaOH saturated with NaCl. This was usually sufficient to remove the yellow pigments. Occasionally a third wash was necessary. The extract was then washed three times with 0·05 vol. of water. The ether extract was dried with anhydrous Na_2SO_4 and evaporated to dryness on a water bath. The residue was then dissolved in 5·5 ml. of 97% (v/v) ethanol and a 5·0 ml. sample was transferred to a B14 tube. The sample was concentrated to a volume of 1·0 ml. or to dryness in a current of air. The Girard separation was then performed by the modified method described above.

(*b*) Chromatography. The 'conjugated-ketone' fraction was dissolved in 5 ml. of benzene and applied to a 6·0 cm. × 0·5 cm. column of alumina in benzene. The column was eluted with 10 ml. of 0·15% (v/v) ethanol in benzene, which was discarded. The fraction containing testosterone was eluted with 15 ml. of 0·3% (v/v) ethanol in benzene. This eluate was evaporated to dryness and the residue applied to paper. The paper was chromatographed in the system described above. Authentic testosterone was chromatographed in parallel with the extracts and located by its absorption of ultraviolet light. The areas of the chromatograms of the extracts corresponding to testosterone were then eluted with ethanol. The eluate was then filtered through sintered glass and a sample was evaporated to dryness. The residue was then subjected to gas–liquid chromatography or to oxidation with CrO_3. Oxidation was carried out by dissolving the residue in 0·2 ml. of acetic acid and adding 0·2 ml. of freshly prepared aq. 15% (w/v) CrO_3. The solution was kept in the dark at 25° for 15 min. Then 3 ml. of aq. 8% (w/v) sodium citrate was added. The oxidation products were extracted with 10 ml. of chloroform, which was washed once with 1 ml. of aq. 0·1 N-NaOH and twice with 1 ml. of water. The extract was then dried with anhydrous Na_2SO_4 and a sample was evaporated to dryness. Gas–liquid chromatography or the micro-Zimmermann reaction was then performed. The latter reaction was carried out by dissolving the residue in 0·1 ml. of freshly



Scheme 1. Outline of the present method for the measurement of testosterone in urine.

prepared 0·5% (w/v) *m*-dinitrobenzene in ethanol and adding 0·1ml. of aq. 20% (w/v) tetraethylammonium hydroxide. The solution was kept at 0–4° for 3hr. The reaction mixture was then diluted with 0·5ml. of ethanol and with a similarly treated reagent blank the extinctions were measured at 432, 512 and 592mμ. The results were corrected by the method of Allen (1950). Gas–liquid chromatography was carried out on a column coated with QF–1. External standards of the relevant steroid were used to estimate retention times and peak heights for a given weight of steroid. The results were corrected for the samples taken during the procedure and for the overall recovery.

RESULTS

Hydrolysis

Previous methods for the determination of testosterone in urine have used enzymic hydrolysis with various β-glucuronidase preparations (see the references in Table 3). Most workers have incubated at 37° for 48hr. with different concentrations of this enzyme. In the present work, incubation at 37° for 48hr. with a concentration of 1000 units of β-glucuronidase activity/ml. was found to give reproducible results. With longer periods of incubation or with higher concentrations of enzyme activity the increase in the amount of testosterone released was only 5–10%. Most of the testosterone was released by incubation for 18hr. with a concentration of only 500 units/ml.

In the present study, it was possible to use acid hydrolysis; the amounts of testosterone recovered from the same urine specimen after acid and after enzymic hydrolysis were similar (Table 1). In an attempt to ensure complete hydrolysis with the enzyme preparation, a higher concentration and longer period of incubation were used in the comparison with acid hydrolysis. However, the urine specimens numbered 4, 8 and 9 showed lower yields of testosterone after enzymic hydrolysis; the urinary excretion of testosterone was 48·6, 36·2 and

Table 1. *Comparison of hot-acid and enzymic hydrolysis used for the release of urinary testosterone*

Experimental details are given in the text.

Urine specimen	Urinary testosterone (μg./24hr.): Hydrolysis with 0·15 vol. of conc. HCl at 100° for 1hr.	Urinary testosterone (μg./24hr.): Limpet β-glucuronidase (1500 units/ml. of urine) incubated at 37° for 72hr.
Male		
1	48·1	43·1
2	50·0	42·3
3	60·3	55·1
4	63·3	48·6
5	64·4	60·5
6	47·9	44·8
7	46·2	46·5
8	44·1	36·2
9	60·9	47·8
10	55·7	55·5
11	49·0	47·4
12	45·2	47·3
13	63·5	64·0
14	62·2	57·1
15	87·1	96·5
16	102·9	105·4
Mean	59·4	56·9
Female		
17	9·5	8·8
18	12·8	11·6
19	18·0	16·5
20	7·7	7·0
21	9·1	8·6
22	12·5	12·0
Mean	11·6	10·8

47·8 μg./24hr. respectively. The endogenous inhibitor of β-glucuronidase in these urine specimens was destroyed by treatment with alkali (Harkness & Fotherby, 1963; Marsh, 1963) and enzymic hydrolysis was repeated. The repeat estimates of urinary testosterone were 61·2, 36·8 and 45·0 μg./24hr. respectively. From the results on urine specimen 4 it is possible that one cause of the difference in effectiveness between acid and enzyme hydrolysis is the endogenous inhibitor of β-glucuronidase in urine, glucaro-(1→4)-lactone (Marsh, 1963). Conditions for hot-acid hydrolysis were studied. When the urine was boiled for 1hr. much less contamination was observed in the final extract and the amount of testosterone recovered was also greater than when the urine was boiled for 15 or 30min. The stability of testosterone under the conditions of acid hydrolysis was confirmed. A 5 μg. sample of authentic testosterone was added to 200ml. of water and boiled for 1hr. with 0·15 vol. of concentrated hydrochloric acid. In six experiments the mean amount of testosterone recovered by ether extraction, as estimated by gas–liquid chromatography, was 96·8 ± 2·0% (S.D.). In these experiments no artifacts from testosterone were detected. However, acid hydrolysis of urine produced small amounts of compounds that were not detected in gas–liquid chromatograms when enzyme hydrolysis was used (Fig. 1).

Girard separation

When the modifications of the Girard reaction described by Taylor (1958) and by Lindner (1960) were used with urine extracts, low recoveries of testosterone were obtained. In the present study, increased amounts of Girard reagent T and of Amberlite IRC-50 were found to be necessary to achieve high and reproducible recoveries with small amounts of steroids in relatively large amounts of residue. It was also necessary to activate the Amberlite IRC-50 by washing with dilute acid (Astle, 1957). In a series of 12 experiments, 20 μg. of testosterone was subjected to the present modification and 93 ± 2·3% (mean ± S.D.) was recovered in the formaldehyde-labile fraction.

Chromatography

Alumina column chromatography. The Girard separation as modified in the present study was found to remove a great many contaminants from the urine extracts. Nevertheless, the residue was still bulky in most samples after acid hydrolysis; the residues were generally large for direct application to paper or to thin-layer plates. Chromatography of the residue on an alumina column was found to give, in all cases, a testosterone fraction suitable for application to paper. However, after enzymic hydrolysis of the urine of normal men, the 'conjugated-ketone' fraction could be applied easily to paper or to thin-layer plates.

Paper and thin-layer chromatography. When the testosterone fraction from the alumina column was chromatographed on thin layers of silica gel in the three different systems described above, five spots could be detected with vanillin in ethanolic sulphuric acid. Testosterone was not clearly separated from other closely related compounds, especially epitestosterone. However, paper chromatography in the system described above clearly separated testosterone from epitestosterone and other closely related compounds in the extract. Extracts from 20 different male and female urines were examined in this paper-chromatographic system by using all the methods of detection described above. Only five spots could be detected and these were well separated. The separations achieved in the chromatographic systems A and B3 (Bush, 1952) were less satisfactory.

Methods of detection and oxidation

Colour reaction for testosterone. In the present work, it was necessary to measure small quantities of testosterone accurately. For this a sensitive and simple method was needed. The absorption of ultraviolet light by testosterone in ethanol and in sulphuric acid was affected by impurities, and the Koenig colour reaction (Koenig, Melzer, Szego & Samuels, 1941) was laborious. A colour reaction was developed that is somewhat similar to those described by Koenig *et al.* (1941) and others. A solution of 0·5% vanillin in ethanol–sulphuric acid (1:4, v/v) is a highly sensitive reagent for the detection of steroids on thin-layer chromatograms. The intense yellow colour of this reagent in solution only showed a slight change when 10 μg. of testosterone was added and the solution was heated. Decreasing the concentration of vanillin to 0·05% (w/v) did not diminish the colour in the reagent blank and there was little change in the colour after the addition of testosterone. However, after incubation for 17hr. at 25°, dilution of the reaction mixture with 2 vol. of water caused the colour of the reagent blank to disappear and a bluish-violet colour developed in the presence of testosterone, epitestosterone and androstenedione (androst-4-ene-3,17-dione). The colour obtained with testosterone was approximately twice that from epitestosterone and androstenedione. The absorption of visible light was maximal at 595mμ; no other maxima were found between 400 and 700 mμ. No appreciable colour was produced with 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one, 3β-hydroxyandrost-5-en-17-one, pregn-4-ene-

3,20 - dione, 3β - hydroxypregn - 5 - en - 20 - one, 5β-pregnane-3α,20α-diol, 5β-pregnane-3α,17α,20α-triol, 11β,17α,21 - trihydroxypregn - 4 - ene - 3,20-dione and 17α,21-dihydroxypregn-4-ene-3,11,20-trione. Further, 0·3μg. of testosterone in a total volume of 0·5ml. of reagent diluted in this way gave an extinction value of 0·060 when corrected by the method of Allen (1950) by using readings at 510, 595 and 680mμ. This colour reaction was employed for the standardization of the alumina used in column chromatography. In addition, this procedure was employed in the estimation of testosterone in a sample of pooled male urine. The mean of six replicates was 49·7μg./24hr. The standard deviation was $\pm$8·0μg./24hr. The mean of six determinations by gas–liquid chromatography was 56·5μg./24hr., and the standard deviation was $\pm$3·6μg./24hr. This latter standard deviation is on the borderline of being significantly lower than that for the colorimetric procedure. The mean values obtained by this procedure are not significantly different. The oxidation of testosterone to androstenedione allowed the sensitive micro-Zimmermann reaction to be used. In the Zimmermann reaction the chromogenicity of androstenedione was approximately equal to the sum of the colours produced by 3α-hydroxy-5α-androstan-17-one and testosterone. Low blank values were obtained by developing the colour at 0–4° and using tetraethylammonium hydroxide as an organic base. The development of the colour at higher temperatures or with other bases produced higher blank values. Extraction of the Zimmermann colour did not improve the results.

Conditions for the oxidation of testosterone with chromium trioxide were adjusted to give the optimum yield of androstenedione without appreciable formation of artifacts; different times, temperatures and concentrations of chromium trioxide were investigated. The procedure described by Menini & Norymberski (1962) was also found to be satisfactory but required 2hr. instead of 15min. for oxidation. In a series of 30 experiments with the conditions described above the mean recovery ($\pm$S.D.) of androstenedione from 5μg. of testosterone was 92$\pm$3·0%.

Gas–liquid chromatography. Initially a column coated with 1% SE–30 was used. However, impurities from solvents and urine have retention times similar to those of the steroids under consideration. A column coated with 1% QF–1 provided much better separations. The impurities from urine extracts, solvents and reagents had shorter retention times than those of the steroids and thus did not interfere with their determination. The use of a column coated with QF–1 produced a smooth base line from which the height of the peaks due to testosterone and androstenedione could be measured. Extracts from male urine could always be chromatographed as testosterone. However, in low-titre female urine oxidation of testosterone to androstenedione ensured that the peak appeared on a reasonably flat base line. A further advantage was the large difference in retention time between testosterone and androstenedione; oxidation of the extract and further gas–liquid chromatography provided a check on the specificity of the determination. The extracts used to obtain the tracings shown in Fig. 1 contained a large amount of contaminants and little testosterone; most fresh urine specimens from both men and women contained much less interfering material. It should be noted that the impurities produced by acid hydrolysis were only slightly greater than those after enzymic hydrolysis.

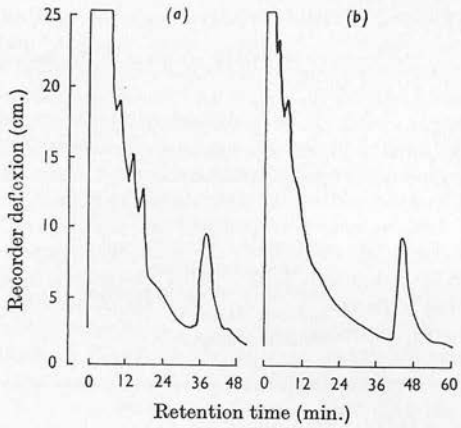


Fig. 1. Gas–liquid chromatogram on a column coated with 1% (w/w) QF–1 of an extract obtained by the present method from a female urine, after oxidation of the testosterone to 0·1μg. of androstenedione: (*a*) after acid hydrolysis; (*b*) after enzyme hydrolysis.

Reliability criteria of the method

Accuracy. This was estimated on the basis of recovery experiments. A 20μg. sample of unlabelled testosterone was added to 200ml. of water and 0·01μg. of [4-^{14}C]testosterone was added to 400ml. of female urine. The recoveries obtained with different methods of detection are shown in Table 2. Estimates of about 70–80% were obtained by using gas–liquid chromatography and by counting the radioactive testosterone. However, the recoveries obtained by using the micro-Zimmermann colour reaction were only about 50–60%.

Precision. The precision of a method may be

Table 2. *Accuracy of the method used for determining urinary testosterone*

Experimental details are given in the text. The numbers of observations are given in parentheses.

Method of detection	Amount of testosterone added after hydrolysis (μg.)	Mean percentage recovery $\pm$ S.D.
Radioactivity due to [4-^{14}C]testosterone	0·01	81·4 $\pm$ 5·2 (12)
Gas–liquid chromatography as testosterone	20·0	79·5 $\pm$ 4·5 (12)
Gas–liquid chromatography as androstenedione	20·0	72·3 $\pm$ 4·2 (11)
Micro-Zimmermann reaction after oxidation to androstenedione	20·0	57·2 $\pm$ 11·6 (10)

estimated from the difference between duplicate assays (see Snedecor, 1952). From male urine, the standard deviation from 28 pairs of estimates was $\pm 3{\cdot}2\,\mu$g. for a mean of 50·0 μg./24hr. In 29 pairs of estimates from female urine the standard deviation was $\pm 0{\cdot}75\,\mu$g. for a mean of 9·4 μg./24hr. The coefficients of variation were 6·4% and 8% for male and female urine respectively, indicating that the present method is reasonably precise.

Specificity. The identity of the compound estimated in the present method was examined in several different ways. Thin-layer chromatography of the eluate from the area of paper chromatograms corresponding to testosterone showed only one component when the plates were sprayed with vanillin in ethanolic sulphuric acid or with phosphomolybdic acid in ethanol. In 60 chromatograms from different samples of male and female urine only one spot could be detected, corresponding in colour and position to testosterone. Oxidation of 30 residues obtained from different paper chromatograms produced a compound identical with androstenedione both in thin-layer chromatograms and in gas–liquid chromatography on columns coated with QF–1 and SE–30. In 12 experiments, formation of the acetate of the compound in the final eluate from paper gave a substance of the same mobility as authentic testosterone acetate in thin-layer chromatography and the same retention time in gas–liquid chromatography on columns coated with SE–30 and QF–1. The latter system is capable of partially separating testosterone acetate from epitestosterone acetate (Brooks, 1964). Spectroscopic investigations were carried out on the final extracts from male urine. The infrared spectrum of the substance obtained after the paper-chromatographic procedure in the present method was similar to that of authentic testosterone. There was some interference in the 'carbonyl stretching zone', which was greatly decreased by further thin-layer chromatography. The absorption spectrum of the eluate from paper in sulphuric acid and the colour reaction with vanillin in ethanolic sulphuric acid described above were identical with those of authentic testosterone. Testosterone could not be detected in 12 urine samples from a woman subjected to bilateral adrenalectomy and ovariectomy and accordingly it was concluded that the non-steroidal materials present in the patient's urine did not interfere with the method.

Sensitivity. About 0·5 μg. of androstenedione could be measured in the final residue with fair accuracy by using the micro-Zimmermann reaction and 0·1 μg. by gas–liquid chromatography. The sensitivity calculated according to the method employed by Brown, Bulbrook & Greenwood (1957) was 0·5 μg. in a 24hr. collection of urine (P 0·05).

Practicability. It has been found that six determinations can be performed by a technician in 2 working days. The present method is therefore similar in length to that of Brown (1955) for urinary oestrogens.

Urinary excretion of testosterone in normal subjects

In general the urinary excretion of testosterone was higher in young men than in the older age group. In 80 determinations from 20 normal males aged 21–63 years, the mean excretion of testosterone was 51·7 μg./24hr. (range 40·0–64·5 μg./24hr.). In 15 determinations from five young men aged 16–20 years, the mean excretion was 78·0 μg./24hr. (range 60·0–103·0 μg./24hr.).

The mean excretion in 45 determinations from 15 women aged 20–55 years was 6·5 μg./24hr. (range 2·1–10·7 μg./24hr.). Thus there was a marked difference between the amounts excreted in male and female urine. Tables 3 and 4 show the urinary excretion of testosterone measured by different methods. The present method gave values that are in agreement with the results of some of these previous methods. However, the upper limits of the results obtained with the present method were

generally lower than those of some of the previous methods.

DISCUSSION

The evidence obtained by gas–liquid chromatography (Fig. 1) shows that testosterone is the major steroid component in the final extract. The more efficient separation of testosterone from contaminants and closely related steroids on the selective polar phase QF–1 was also shown by Sandberg *et al.* (1964). The main reason for the more efficient separation was that most contaminants from the materials used in the method had very short retention times on a column coated with QF–1.

Two other steroids that may be present in biological material, namely androst-4-ene-3,11,17-trione (adrenosterone) and 17α-hydroxypregn-4-ene-3,20-dione (17α-hydroxyprogesterone), behave chromatographically on paper in the same way as testosterone (Cathro, Cameron & Birchall, 1965). Adrenosterone was clearly separated from testosterone and androstenedione by gas–liquid chromatography on a column coated with the phase QF–1. When 17α-hydroxyprogesterone was subjected to gas–liquid chromatography under the conditions used in the present investigation no compound could be detected in the effluent. This steroid, if present in urine, would probably be destroyed by acid hydrolysis (Dorfman & Shipley, 1956). However, this compound would be oxidized with chromium trioxide to androstenedione. The 17α-epimer, epitestosterone, is not completely separated from

Table 3. *Urinary testosterone excretion by normal men as estimated by different methods*

Age (years)	No. of subjects	Urinary testosterone (μg./24hr.) Mean	Range	Reference
23–34	5	87·0	46·1–106·0	Camacho & Migeon (1963)
23–60	4	29·5	16·0–51·0	Vermeulen & Verplancke (1963)
19–55	21	113·8	38·0–291·0	Futterweit *et al.* (1963)
29–56	4	71·5	33·0–120·0	Brooks (1964)
18–22	2	163·0	126–200	Ibayashi *et al.* (1964)
26–62	14	95·3	14–189	
27–33	12	82·5	47–140	Sandberg *et al.* (1964)
18–35	5	54	32·0–69·0	Voigt *et al.* (1964)
18–44	9	34	10–75	Schubert & Frankenberg (1964)
21–63	14	182·5	15·1–523·3	Dulmanis *et al.* (1964)
30	3	64·3	42·0–79·0	Zurbrügg *et al.* (1965)
30–40	20	88	28–143	Rosner *et al.* (1965)
17–24	5	151	113–193	Lim & Dingman (1965)
18–65	27	49·7	30·2–86·3	
20–48	9	148	59·0–202·0	Sparagana (1965)
21–63	20	51·7	40·0–64·5	Present method
16–20	5	78·0	60·0–103·0	

Table 4. *Urinary testosterone excretion by normal women as estimated by different methods*

Age (years)	No. of subjects	Urinary testosterone (μg./24hr.) Mean	Range	Reference
19–23	4	5·9	3·5–7·5	Camacho & Migeon (1963)
23–37	6	6·1	2·8–8·0	Futterweit *et al.* (1963)
18–63	4	8·3	5·0–12·0	Vermeulen & Verplancke (1963)
21–32	4	12	7·0–18·0	Brooks (1964)
20–72	10	$<5·3$	$<5·0$–8·0	Ibayashi *et al.* (1964)
22–35	4	10	3·0–18·0	Voigt *et al.* (1964)
21–68	18	48·9	2·9–196·7	Dulmanis *et al.* (1964)
29–35	2	4·8	4·1–5·6	Zurbrügg *et al.* (1965)
20–40	20	19·0	6–57	Rosner *et al.* (1965)
22–59	16	7·1	4·3–10·4	Lim & Dingman (1965)
20–55	15	6·5	2·1–10·7	Present method

testosterone by thin-layer chromatography or gas–liquid chromatography, but it can be separated on paper, and can be estimated by subsequent gas–liquid chromatography of the extract of the appropriate area of the final paper chromatogram.

There is considerable evidence to show that the present method is specific for testosterone. The compound estimated as testosterone in the present method possesses a 'conjugated-ketone' group and an oxidizable hydroxyl group. The compound produced by oxidation has the absorption spectrum of a 17-oxo steroid in the Zimmermann reaction. The behaviour of the compound in three different types of chromatographic procedure is similar to that of authentic testosterone. Further chromatographic and spectroscopic evidence has shown that testosterone is the main compound in the final eluate from paper. In the present method a further gas–liquid-chromatographic step is generally used after paper chromatography and therefore it may be concluded that the method is reasonably specific.

In the present work, the results of experiments on enzymic hydrolysis have agreed with previous findings; the amount of testosterone recovered was increased slightly by prolonged incubation, by increased quantities of enzyme and by abolishing the effect of the endogenous inhibitor of β-glucuronidase known to be present in urine (Marsh, 1963). In the present procedure, simple acid hydrolysis was the method of choice for routine analysis. All other published methods have employed enzymic hydrolysis. The capacity of the present method to separate testosterone from large amounts of impurities is also shown by its ability to estimate testosterone in pregnancy urine. The similarity of the results obtained with acid and with enzyme hydrolysis by using a β-glucuronidase preparation suggests that the testosterone conjugate in human urine is mainly the glucuronide. This is in agreement with the findings of Camacho & Migeon (1964) and others.

The accuracy and precision of the present method make the estimation of recoveries in all determinations by added internal standards unnecessary. However, the estimates obtained by gas–liquid chromatography could be corrected by the recoveries obtained from added internal radioactive standards. The results of the present investigation also show that the recoveries obtained with one method of detection may not agree with those obtained with another. Thus from the results of the present investigation it would not be justifiable to correct the estimates obtained with the micro-Zimmermann reaction for the losses observed with added radioactive testosterone as in the method of Rosner *et al.* (1965). The fact that the estimates obtained by gas–liquid chromatography on replicates of material used for determinations by the Zimmermann reaction were always higher suggests that impurities from the paper may have interfered in the Zimmermann reaction. Thus gas–liquid chromatography is probably the procedure of choice. A cause of the higher results obtained with some other methods (Tables 3 and 4) may be due to the incomplete separation of testosterone from other closely related compounds such as epitestosterone.

The colour reaction with vanillin in ethanolic sulphuric acid developed in the present investigation differs from that of Allen, Hayward & Pinto (1950) in that little or no colour is obtained with 3β-hydroxyandrost-5-en-17-one. Several methods for urinary testosterone have used the colour reaction described by Allen *et al.* (1950) with some modifications (Zurbrügg *et al.* 1965; Lim & Dingman, 1965). This difference in the colour produced with 3β-hydroxyandrost-5-en-17-one may, in part, be due to the presence of an aldehyde, since G. Ittrich (personal communication) has obtained similar results with formaldehyde in place of vanillin.

The modification of the Girard reaction used in the present work is based on the procedure of Teitelbaum (1958) for unstable aldehydes and ketones that was adapted to steroids by Taylor (1958), whose findings were confirmed and extended by Lindner (1960). The present modification of the Girard separation avoids the harsh conditions of the original method, which may destroy some labile steroids and acetylate others. The derivatives of the saturated ketones in the original method are hydrolysed by dilute acid; the Girard complexes of the 'conjugated ketones' require considerably higher concentrations of acid for hydrolysis (Fieser & Fieser, 1959). However, in the modified Girard separation the derivatives of the 'conjugated ketones' are easily split by aqueous formaldehyde. To release the saturated ketones, acid hydrolysis is still required.

In the present investigation, a considerable increase in the quantity of Girard reagent T and also in the quantity of the catalyst, Amberlite IRC-50, was necessary to achieve quantitative results with crude urinary extracts; the resin also required activation by washing with acid. The separation of the 'conjugated-ketone' fraction from the non-ketonic fraction and the bulk of the saturated ketonic components simplifies the subsequent separation procedures necessary to obtain a pure compound. In the present modification of the Girard separation it was found that testosterone could be extracted with benzene or light petroleum (b.p. 60–80°) almost as effectively as with ethyl acetate. This evidence suggests that the corticosteroids that can be obtained in the 'conjugated-ketone' fraction could be extracted with chloroform or ethyl acetate after the removal of testosterone

and compounds of similar polarity. It seems likely therefore that procedures similar to that described above for testosterone estimation could be used to simplify the estimation of many hormonally active neutral steroids. Such a procedure has already been used by Ismail & Harkness (1966) in the first isolation of progesterone from human pregnancy urine.

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Factors associated with alterations in urinary testosterone levels. By A. A. A. Ismail and R. A. Harkness. *Medical Research Council Clinical Endocrinology Research Unit, University of Edinburgh*

The purpose of the present study was to assess the effect of some possible causes of variations in testosterone excretion. Urinary testosterone was measured by the method of Ismail & Harkness (1966). The effect of sexual activity on the levels was investigated in two normal men in whom no sexual activity took place for a period of 18 and 7 weeks respectively before the investigation started. Higher urinary testosterone levels were observed at 8-day intervals in one subject and at 5-day intervals in the other, irrespective of sexual activity. However, when sexual activities restarted the mean level of urinary testosterone in one subject rose from 42 to 55 μg./24 hr. and in the other subject from 37 to 66 μg./24 hr.

The effect of undernutrition associated with psychological and physical illness was investigated. Three young men, 17–20 years of age, with severe undernutrition associated with psychological disturbances, had low urinary testosterone levels of 6·2, 11·2 and 31·2 μg./24 hr. when they were measured in 7- to 10-day urine pools. A low mean level of urinary testosterone of 6·5 μg./24 hr. was found over an 11-day period in a man aged 40 who had severe gastrointestinal disease associated with marked undernutrition. This reduction in testosterone levels is similar to changes which have been shown to occur in cases of undernutrition in animals (see Mann, 1964).

The results of the present investigations suggest that sexual activity may be a factor affecting urinary testosterone levels and that undernutrition is associated with a marked reduction in testosterone excretion.

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