STUDIES ON THE MEASUREMENT AND SIGNIFICANCE OF ANDROGENS IN SALIVA

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A thesis submitted as part requirement for the degree of Doctor of Philosophy of the University of London

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September 1982

ABSTRACT

Sensitive radioimmunoassays have been developed to enable the measurement of testosterone, androstenedione, dihydrotestosterone and cortisol in human saliva. The relationships between the concentration of salivary steroid and the unbound and total plasma concentrations in normal subjects and patients with endocrine disorders have been examined, and the factors affecting androgen concentrations in saliva have been investigated. The applications of salivary androgen measurements to physiological and pathological studies have been evaluated.

There were highly significant correlations between the concentration of steroid in saliva and the concentration of unbound hormone in matched samples collected from normal women, women with clinical signs of hyperandrogenism and antiandrogen treated women. A good correlation was observed between the concentration of testosterone in saliva and the concentration not bound to sex hormone binding globulin in plasma.

Salivary steroid concentrations were independent of salivary flow rate at basal and stimulated secretion rates, but when salivary flow was diminished, the androgen concentration increased. Consequently salivary androgen levels are not a good indication of unbound plasma androgen levels at very low salivary flow rates.

Salivary and plasma androgen concentrations in patients with polycystic ovaries and idiopathic hirsutism were significantly higher than in normal subjects. After treatment with cyproterone acetate and ethinyl oestradiol, concentrations of testosterone and dihydrotestosterone in plasma remained elevated. However, a sharp decline was observed in salivary concentrations in association with a decrease in the unbound androgen concentration. The decrease in salivary androstenedione was similar to the fall in plasma levels, reflecting the

decreased production of androstenedione and not an alteration in plasma protein binding.

Although salivary measurements allow no greater discrimination between normal and hyperandrogenic women, they are a useful alternative to plasma assays and may give a better indication of the biologically available androgen than do total plasma assays.

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ACKNOWLEDGEMENTS

I am deeply grateful to Professor V.H.T. James for his expert advice and supervision during the course of this work.

I would also like to thank all the members of the steroid laboratory in the Department of Chemical Pathology, St Mary's Hospital Medical School, in particular Dr M.J. Reed and Mr A.E. Rippon for their help and cooperation. I am grateful to Dr H.S. Jacobs and the staff of the Endocrine Clinic at the Sammaritan Hospital for Women for their help in collecting samples and allowing me to study their patients.

I would also like to express my gratitude to Mrs R. Baxendale for her rapid and competent preparation of the manuscript and to Mrs J. Baxendale for her support and encouragement throughout.

ABBREVIATIONS AND TRIVIAL NAMES

Abbreviations commonly used in the text are listed below:
ACTH, corticotrophin; BSA, bovine serum albumin; CBG, corticosteroid binding globulin; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; FSH, follicle stimulating hormone; LH, luteinizing hormone; PCO, polycystic ovarian disease; REM, rapid eye movement; SHBG, sex hormone binding globulin; TLC, thin layer chromatography.

The following trivial names have been used:
Androstenedione, 4-androstene-3,17dione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten -17-one; dihydrotestosterone, 17β-hydroxy-5α-androstan -3-one; testosterone, 17β-hydroxy-4-androsten -3-one.

SECTION 1

INTRODUCTION

Since the introduction of saturation techniques (Ekins, 1960; Yalow and Berson, 1960) there have been few methodological advances in the field of clinical endocrinology. Recently, however, the measurement of steroid concentrations in saliva has attracted some attention. It offers two major advantages over conventional techniques. Firstly, since sampling is simple and non-invasive, it avoids the difficulties and possible stressful effects of venepuncture; furthermore there is no requirement for skilled personnel to take samples. Secondly, salivary concentrations appear to reflect the non protein bound steroid concentration in blood (Katz and Shannon, 1969), and since protein binding is an important factor in determining steroid availability to the tissues, this may be a more useful measurement than that of the total plasma steroid concentration. Although there have been few published data to document this, the measurement of steroids in saliva may prove to be an important contribution in the assessment of endocrine function in both adults and children.

Saliva is a readily accessible fluid; a 3 ml sample may be collected in 5 minutes. It is produced in three main pairs of glands, the submaxillary, the parotid and the sublingual, and from smaller glands in the oral mucosa. Each gland contains a rich supply of nerves, from both sympathetic and parasympathetic systems, but their role in the control of secretion is not well understood. Considering the ease of collection of saliva samples, they have rarely been used for diagnostic purposes. However, the measurement of drug concentrations in saliva is being increasingly used for monitoring anticonvulsant therapy (Reynolds et al., 1976; Horning et al., 1977).

The presence of corticosteroids in saliva was first observed in 1959 by Shannon et al., (1959), but it has not been until the last few years that with the development of sensitive assay techniques it has been possible to measure other steroids. Numerous steroids have now been measured in human saliva, including cortisol (Greaves and West, 1963; Walker et al., 1978; Umeda et al., 1981), cortisone (Katz and Shannon, 1969), aldosterone (McVie et al., 1979), 17-hydroxyprogesterone (Walker et al., 1979) and progesterone (Walker et al., 1978). The possibility of measuring oestrogens in saliva as a means of monitoring fetal development during pregnancy was explored by Heap and Broad in 1974. Since then, Fischer-Rasmussen et al., (1981), and Robinson et al., (1981), using specific assays for oestriol, have shown saliva to be a suitable alternative fluid since concentrations of oestriol in saliva paralleled both unconjugated and total oestriol in serum. Likewise, oestrone (Luisi et al., 1980) and oestradiol (Evans et al., 1980) concentrations in saliva show a close relationship with plasma levels in men and women.

A number of investigators have now measured testosterone concentrations in saliva of normal subjects (Landman et al, 1976; Gaskell et al., 1980; Luisi et al., 1980; Turkes et al., 1980; Walker et al., 1980; Wang et al., 1981) and in pathological conditions (Smith et al., 1979; Luisi et al., 1980). Although concentrations have been shown to be a good reflection of either the unbound or total steroid level in plasma, the values reported in both men and women vary widely. This may be due to the lack of sensitivity and specificity of radioimmunoassays described by some authors, which are not suitable for the measurement of low levels of steroid in saliva.

Circulating androgens in plasma derive from the testes, ovary or adrenal cortex, either by direct glandular secretion or by conversion from less active precursors (Fig. 1.1). The concentration of individual

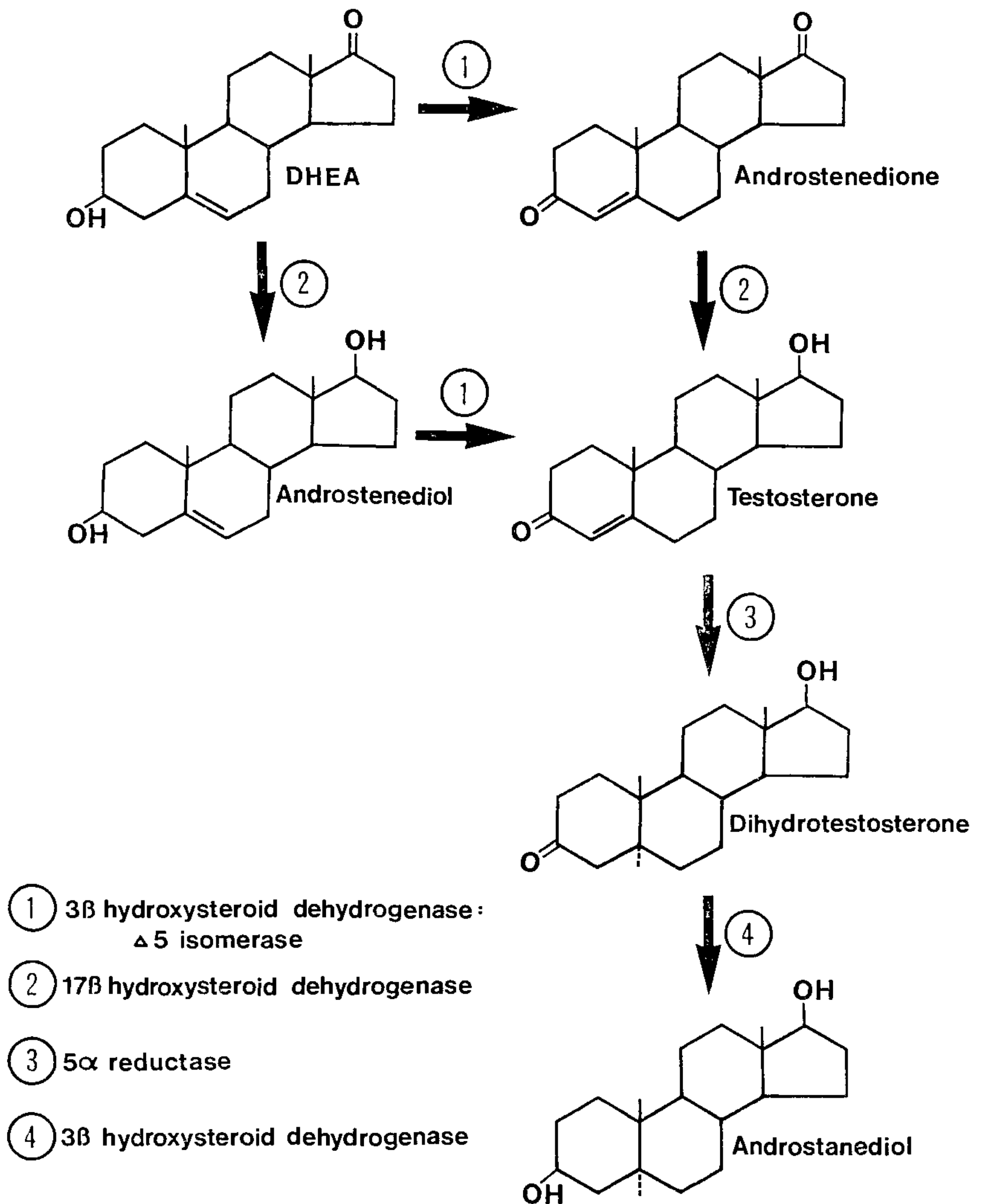

Fig 1.1 Major pathways of androgen synthesis

steroids in plasma is determined by the relative glandular secretion, the interconversion of prehormones and the metabolic clearance rate. Measurement of these parameters is difficult and therefore has little value for routine assessment of androgen status. Free and conjugated androgens have been measured in urine (Andino et al., 1976; Goodall and James, 1979), but the collection of 24 hour samples can be unreliable and inconvenient for the patient. Methodological advances over the last few years haveenabled the accurate measurement of low plasma levels, consequently this has become the method of choice for most investigators.

Quantitatively, the most important androgens in adults are dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) which arise from the adrenal cortex. The main biological significance of these androgens is to serve as prehormones for the more active metabolites. Androstenedione derives from both the adrenals and gonads. In women, equal amounts are secreted from both glands, depending on the stage of the menstrual cycle (Abraham, 1974), and about 10% arises from precursors. In men, up to 30% is derived from the testes (Judd et al., 1973). Testosterone in male plasma is almost exclusively formed by direct secretion from the testes (Lipsett, 1974), whereas in women, 25% is secreted by the ovaries, 25% by the adrenals and the remainder formed from the peripheral conversion of androstenedione (Kirschner and Bardin, 1972). Although a small amount of the potent androgen dihydrotestosterone may be secreted by the gonads, the majority is formed from the conversion of testosterone in men and androstenedione and testosterone in women (Ito and Horton, 1971). Conversion to dihydrotestosterone prior to binding to the specific receptor is required for androgen action in certain tissues, including the skin. However, reported levels of dihydrotestosterone in plasma of women with idiopathic hirsutism are variable and disappointingly low (Tulchinsky and Chopra, 1974; Rosenfield, 1975; Meikle et al., 1979), probably

because the circulating level of this steroid is a poor reflection of the intracellular concentration. Recently, it has been suggested that the determination of plasma androstanediol may represent a useful parameter to reveal abnormal peripheral androgen metabolism, since intracellular dihydrotestosterone is converted to androstanediol in tissues, which then passes into the peripheral circulation without undergoing further metabolic transformation (Toscano et al., 1982).

In plasma androgens are largely bound to proteins. Androstenedione, DHEA and DHEAS are almost entirely bound to high capacity but low affinity albumin. In addition, testosterone, dihydrotestosterone, androstanediol and androstenediol are bound to the high affinity, low capacity sex hormone binding globulin (SHBG). Testosterone and possibly other androgens are also non specifically associated with corticosteroid binding globulin (CBG) and α1-acid glycoprotein. It is generally believed that only the unbound steroid fraction is biologically active (Mowszowicz et al., 1970; Laznitzki and Franklin, 1972; Anderson, 1974), although there is increasing evidence to suggest that albumin bound steroid is also available for metabolism in certain tissues (Baird et al., 1969; Pardridge and Meitus, 1979a).

The physiological significance of steroid binding plasma proteins has not been clearly defined. A number of functions have been suggested such as transportation, protection and regulation. Certain steroids are secreted in a pulsatile manner (James et al., 1978). It is likely that proteins may serve as a buffer so as to minimise any rapid oscillations of steroid in the tissues, and also to serve as a store for immediately accessible hormone for use in the event of urgent demand.

It is generally believed that lipid soluble steroids freely diffuse from plasma into saliva, and that it is only the fraction not associated with protein that is able to pass through the salivary gland membranes, hence salivary steroid concentrations should reflect the unbound hormone

level in plasma. Salivary steroid measurements may thus be particularly helpful in the investigation of altered androgen metabolism, for example in some patients with hirsutism or with polycystic ovarian disease (PCO). It is well documented that the free testosterone concentration in plasma is elevated in many of these cases in association with diminished levels of SHBG (Anderson, 1974; Motohashi et al., 1979; Yen, 1980), a finding which renders difficult the interpretation of total plasma testosterone levels. Plasma SHBG has also been shown to be decreased in patients with hypothyroidism, acromegaly and after androgen administration. Increased concentrations of SHBG have also been demonstrated in a number of conditions, including pregnancy, hyperthyroidism, hypogonadism, gynecomastia, cirrhosis of the liver and after oestrogen administration (Anderson, 1974). Therefore, measurement of unbound steroid has been a more rational approach, on the basis that it is this fraction which is biologically active, in determining androgen exposure in such patients. But since the measurement of unbound hormone is complicated and time consuming, hence unsuitable for routine analysis, salivary assays may be a useful alternative, and give a better indication of the biologically available androgen than do total plasma levels.

The purpose of this study was: 1) to develop and evaluate sensitive radioimmunoassays to enable the measurement of low levels of testosterone, androstenedione, dihydrotestosterone and cortisol in human saliva, 2) to examine the relationship between the concentrations of androgens in saliva and the unbound and total plasma levels in normal men and women and those with endocrine disorders, 3) to investigate the factors which affect androgen concentrations in saliva, 4) to evaluate the application of salivary androgen measurements to physiological and pathological studies, for example, in women with hyperandrogenism and men with hypogonadism.

SECTION 2

MATERIALS AND METHODS

2.1 Materials

a) Chemicals

All chemicals except where stated were supplied by Fisons Scientific Apparatus Ltd., Loughborough, England. 5_{α} androstane $3_{\alpha},17_{\beta}$ diol and androstene $3_{\beta},17_{\beta}$ diol were supplied by Sigma London Chemical Company Ltd., Poole, Dorset, England. Testosterone, dihydrotestosterone, androstenedione, cortisol and all other crystalline steroids were obtained from Steraloids Ltd., Croydon, England.

$[1,2,6,7,(n)^{3}H]$testosterone (94 Ci/mmol), $[1,2,6,7,(n)^{3}H]$androstenedione (91 Ci/mmol), $[1,2,6,7,(n)^{3}H]$cortisol (92 Ci/mmol) and $[1,2,(n)^{3}H]$dihydrotestosterone (66 Ci/mmol) were supplied by Amersham International, Amersham, England. $[1,2,6,7,16,17,(n)^{3}H]$testosterone (152 Ci/mmol), $[1,2,4,5,6,7,(n)^{3}H]$dihydrotestosterone (131 Ci/mmol), $[1,2,(n)^{3}H]5_{\alpha}$ androstane $3_{\alpha},17_{\beta}$ diol (40 Ci/mmol) and $[1,2,(n)^{3}H]$androstene $3_{\beta},17_{\beta}$ diol (59 Ci/mmol) were obtained from New England Nuclear Chemicals, GmbH, Dreieich, West Germany. Radiolabelled steroids were purified at regular intervals (1 - 2 months) by paper chromatography. Androgens were run in Bush A or B3 systems and cortisol in a Bush D tank.

Acid washed charcoal (Norit A), globulin free human albumin and mucin from rat submandibulary glands were purchased from Sigma, Poole, Dorset, England. Dextran T70 was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

Ethanol (A.R.) grade was obtained from James Burroughs Ltd., London, England and toluene from May and Baker, Dagenham, London, England. Solid carbon dioxide was supplied by the Distillers Company Ltd., London, England. Phosphomolybdic acid was purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex, England.

b) Reagents

Phosphate buffer (0.1M) was prepared from disodium hydrogen phosphate (22g), sodium dihydrogen phosphate (5.9g), sodium chloride (9.0g), sodium azide (1.0g) and gelatine (1.0g). The volume was made up to one litre with deionised water, and the pH adjusted to 7.0 with 2M sodium hydroxide.

Scintillation solution was prepared by dissolving p-terphenyl (15g) and dimethyl POPOP (0.20g) in 5L sulphur-free toluene containing 2% methanol.

Ethanol, methanol and ethyl acetate were all distilled prior to use. Dichloromethane and dichloroethane (A.R.) were distilled immediately prior to chromatography.

Solvent systems for paper chromatography were based on those described by Bush, (1961).

c) Apparatus

Glass assay and extraction tubes, 4 in x $^5/8$ in, 3 in x $\frac{1}{2}$ in, 3 in x $^3/8$ in were supplied by Glass Wholesale Supplies, Gable Street, London E.1. Stoppered tubes were manufactured by Quickfit and Quartz, Stone, Staffordshire, Type MF24/1/5. Glass tubes 50 mm x 10 mm controlled neck shell vial No3/G/D/08/015/02/A were obtained from F.B.G. Trident Ltd., Temple Cloud, Bristol, England.

Precoated aluminium-backed silica gel thin layer chromatography plates (Merck 5553) were supplied by B.D.H. Ltd., Poole, Dorset, England. Chromatography paper No. 2 was supplied by Whatman Lab Sales Ltd., Springfield Mill, Maidstone, Kent, England. Radiochromatograph imaging system and strip scanner were manufactured by Panax Equipment Ltd., Bournemouth, Dorset, England.

Equilibrium dialysis machine with 1 ml capacity teflon cells was purchased from Dianorm, Diachema AG, Switzerland. Visking tubing, size 36/32 in, was supplied by Medicell International Ltd., Liverpool Road,

London N 1. Ultrafiltration filters, Type CF 50, were obtained from Amicon Ltd., High Wycombe, Buckinghamshire.

Plastic scintillation vials were manufactured by Griffiths and Nielson, Billingshurst, Sussex, England. Multivortex shaker was supplied by Denley Instruments Ltd., Billingshurst, Sussex, England. Mistral 4L and 6L centrifuges were supplied by M.S.E. Ltd., Crawley, Sussex, England.

Compu-Pet dispenser, duel micromodel, was obtained from William R. Warner Ltd., Eastleigh, England. Digital dilutor/dispenser was manufactured by Hamilton Bonaduz AG, CH-7402 Bonaduz, Switzerland. Constriction pipettes were supplied by H. E. Pederson, 7 Sommerstedgade DK 1718, Copenhagen V, Denmark.

Liquid Scintillation Counters used were Beckman LS 7500 and Searle Delta 300. Statistical analysis was performed on a Hewlett-Packard desk top calculator, model 9810A.

2.2 Separation of androgens

In recent years great improvements have been made in the production of specific antisera for certain steroids. Nevertheless, most antisera show a certain affinity to compounds of similar structure to the antigen. Chromatography has been the traditional approach to separate possible assay cross reactants, and although this is becoming less necessary, it is still important for the validation of non-chromatographic radioimmunoassays. Chromatographic procedures also allow the measurement of more than one steroid in a single sample. Thin layer chromatography (TLC) and paper chromatographic systems for the separation of unconjugated androgens have been evaluated.

Tritiated androgen (50,000 dpm) or authentic steroid (100 μg) were applied to TLC plates and run in chambers containing dichloromethane : ethyl acetate (60 : 40, 70 : 30 and 80 : 20 v/v), chloroform : dioxan

(94 : 6), dichloromethane : dioxan (90 : 10, 94 : 6 and 97 : 3) and cyclohexane : ethyl acetate (1 : 1), or in Bush A (heptane : methanol : water, 500 : 400 : 100) and Bush B3 (petroleum ether : toluene : methanol : water, 375 : 125 : 400 : 100) paper chromatographic systems.

Tritiated steroids were located using a radiochromatogram imaging system. Authentic steroids were located by spraying with 20% phosphomolybdic acid in ethanol and heating at 60°C for 5 minutes. Rf values are shown in Table 2.1.

2.3 Radioimmunoassay of testosterone

The introduction of radioimmunoassays and related techniques (Ekins, 1960; Yalow and Berson, 1960) has added a new dimension to the study of endocrinology. Radioimmunoassays enable the accurate and specific measurement of low levels of hormones in blood and urine. Since the concentrations of steroids in saliva are believed to be much lower than those in plasma, very sensitive assays must be developed if the collection of large saliva samples is to be avoided. Radioimmunoassay sensitivity is primarily determined by the antibody's affinity for the antigen, but there are a number of ways whereby the assay sensitivity may be further increased. The usual approach is to reduce the mass of labelled steroid or to use a label with a high specific activity. As the mass of tracer is decreased, the slope of the standard curve will increase to a point where a further reduction in label has no more effect (Chard, 1978). Decreasing the concentration of antiserum has also been shown to increase the sensitivity of an assay, although at very low antibody titres the resultant increased sensitivity is negated by a decrease in precision (Ekins, 1974). The manipulation of other assay conditions, including temperature, incubation time, extraction procedures and the order of reagent addition may also affect the shape of a standard curve.

Steroid	Dichloromethane:Ethyl Acetate				Chloroform: Dioxan	Dichloromethane:Dioxan			Cyclohexane: Ethyl Acetate	Bush A	Bush B3
	60:40%	70:30%	80:20%	90:10%	94.6 %	90:10%	94:6%	97:3%	1:1 %		
Dehydroepiandrosterone	0.63	0.61	0.40		0.55	0.61	0.40				C.59
Androstenedione	0.72	0.71	0.59	0.24	0.81	0.90	0.60	0.26		0.41	C.64
Dihydrotestosterone	0.66	0.59	0.48	0.20	0.65	0.74	0.44	0.19	0.59	0.27	C.58
Testosterone	0.45	0.38	0.28	0.11	0.45	0.55	0.29	0.11	0.42	0.15	C.46
Androstenediol	0.37	0.35	0.22	0.10	0.33	0.43	0.22				C.23
5α androstane 3α,17β diol	0.39	0.34	0.16	0.03	0.36	0.40	0.17			0.09	C.36
5α androstane 3β,17β diol	0.38		0.20			0.39	0.22				
5β androstane 3α,17β diol	0.14		0.05			0.10	0.04				

Table 2.1 Chromatographic separation of the androgens

Expressed as Rf. values.

Three antisera have been evaluated for the radioimmunoassay of testosterone in saliva. As only one of the antisera tested was sufficiently sensitive to allow the accurate measurement of low testosterone levels in saliva, the optimisation procedures described in this thesis will be restricted to this antiserum only.

Antiserum Cl, which was kindly donated by Professor K. Griffiths, (Tenovus Institute, Cardiff) was raised in rabbits against a testosterone 3-(O-carboxy-methyl)-oxime/bovine serum albumin conjugate.

a) Antibody dilution curve

Antiserum dilutions in phosphate buffer (100 µl) ranging from 1 : 16,000 to 1 : 512,000 final titre were incubated with phosphate buffer (500 µl) and 10 pg of testosterone in phosphate buffer (500 µl) for 30 minutes at room temperature. 1,2,6,7,16,17^{3}H-testosterone in buffer (100 µl) containing 20,000 d.p.m. (17 pg) was added, mixed and incubated for 30 minutes at room temperature and for a further 15 minutes in an ice water bath. Separation of bound and free steroid was performed by addition of a cold dextran coated charcoal suspension (200 µl). Tubes were quickly mixed, allowed to stand for 15 minutes and centrifuged at 1500 g for 5 minutes at 4°C. Supernatants were rapidly decanted into counting vials containing p-terphenyl scintillator and capped. Vials were shaken mechanically for 10 minutes and the radioactivity determined in a liquid scintillation counter.

Curves showing the effect of antibody titre on the initial binding to testosterone, and binding in the presence of 10 pg authentic testosterone are shown in Fig. 2.1. The optimum dilution of antiserum to give maximum displacement of 10 pg testosterone was between 1 : 128,000 and 1 : 256,000.

b) Effect of antiserum dilution on testosterone standard curves

Testosterone standards (1.2 - 40 pg) in phosphate buffer (500 µl)

100
80
60
% BOUND
40
20
0
Initial binding
Binding in the presence of 10pg T
Displacement
50
40
DISPLACEMENT
30
$\frac{B}{B_O}$
20
10
10^{-4}
10^{-5}
10^{-6}
ANTIBODY DILUTION

Fig 2.1 The effect of antiserum dilution on the initial binding to ^3H testosterone, and the inhibition of 10pg authentic testosterone.

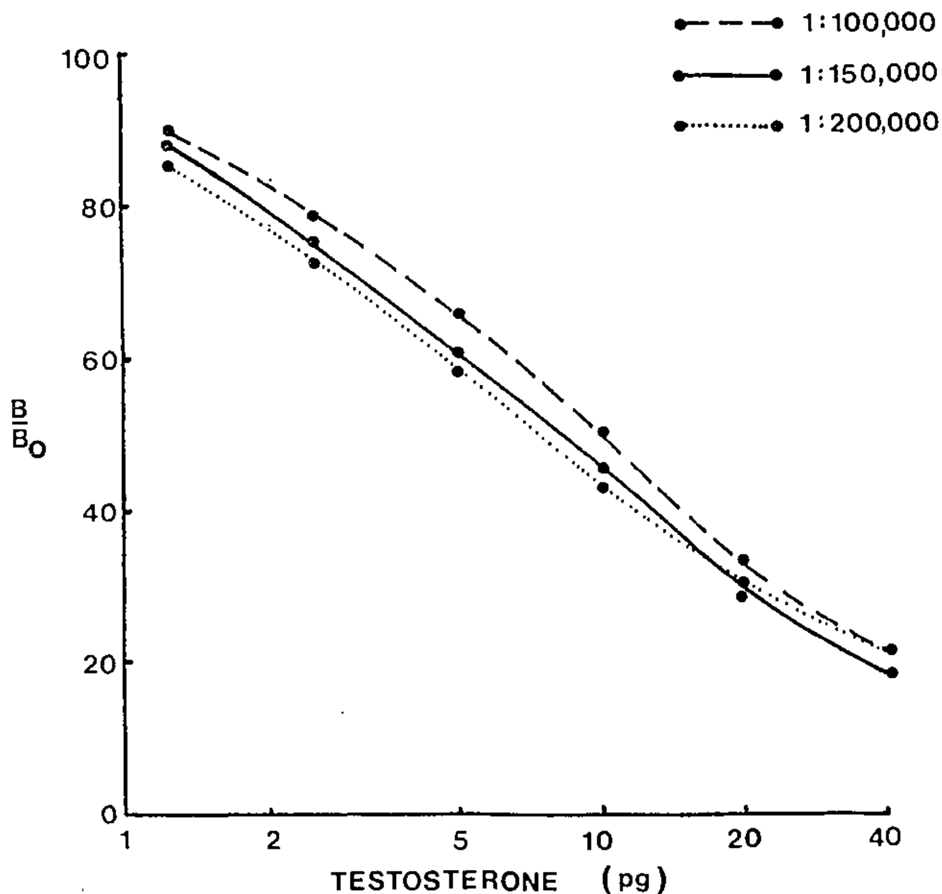

Fig 2.2 The influence of antiserum dilution on the shape of the standard curve.

were incubated with C1 antiserum (100 μl) at dilutions of 1 : 100,000, 1 : 150,000 and 1 : 200,000 for 30 minutes, and for a further 30 minutes with 20,000 d.p.m. 1,2,6,7,16,17 ^{3}H-testosterone. Dextran coated charcoal was used to separate bound from free antigen as previously described. The greatest displacement of 10 pg testosterone was obtained using a final antibody dilution of 1 : 150,000 (Fig. 2.2).

c) Influence of antibody incubation time on standard curves

Standard curves (0.6 - 40 pg) were compared when the incubation time and order of reagent addition were varied. Antiserum and ^{3}H-testosterone were added simultaneously to one set of standards, whereas other standards were preincubated with antiserum only for 15, 30 and 45 minutes before addition of label. The total incubation time was 1 hour at room temperature and 15 minutes in ice in all experiments. The curve sensitivity was increased when antiserum was added before tracer. For convenience, future assays were incubated for 30 minutes with antiserum before the addition of labelled testosterone. There was no difference between the shape of the standard curve when incubating overnight at 4°C and for 1 hour at room temperature.

The intraassay variation of six consecutive standard curves, using a final antibody dilution of 1 : 150,000 is shown in Fig. 2.3. The initial binding was 28% and the non-specific binding 2.5%.

d) Validation

(i) Cross-reactions

The only significant cross-reactions, calculated by the 50% displacement method (Abraham, 1969) were 5α dihydrotestosterone 17.2%, 5α androstane-3α,17β-diol 7.9%, androstene-3β,17β-diol 0.8% and 4-androstene-3,17 - diol 0.4%.

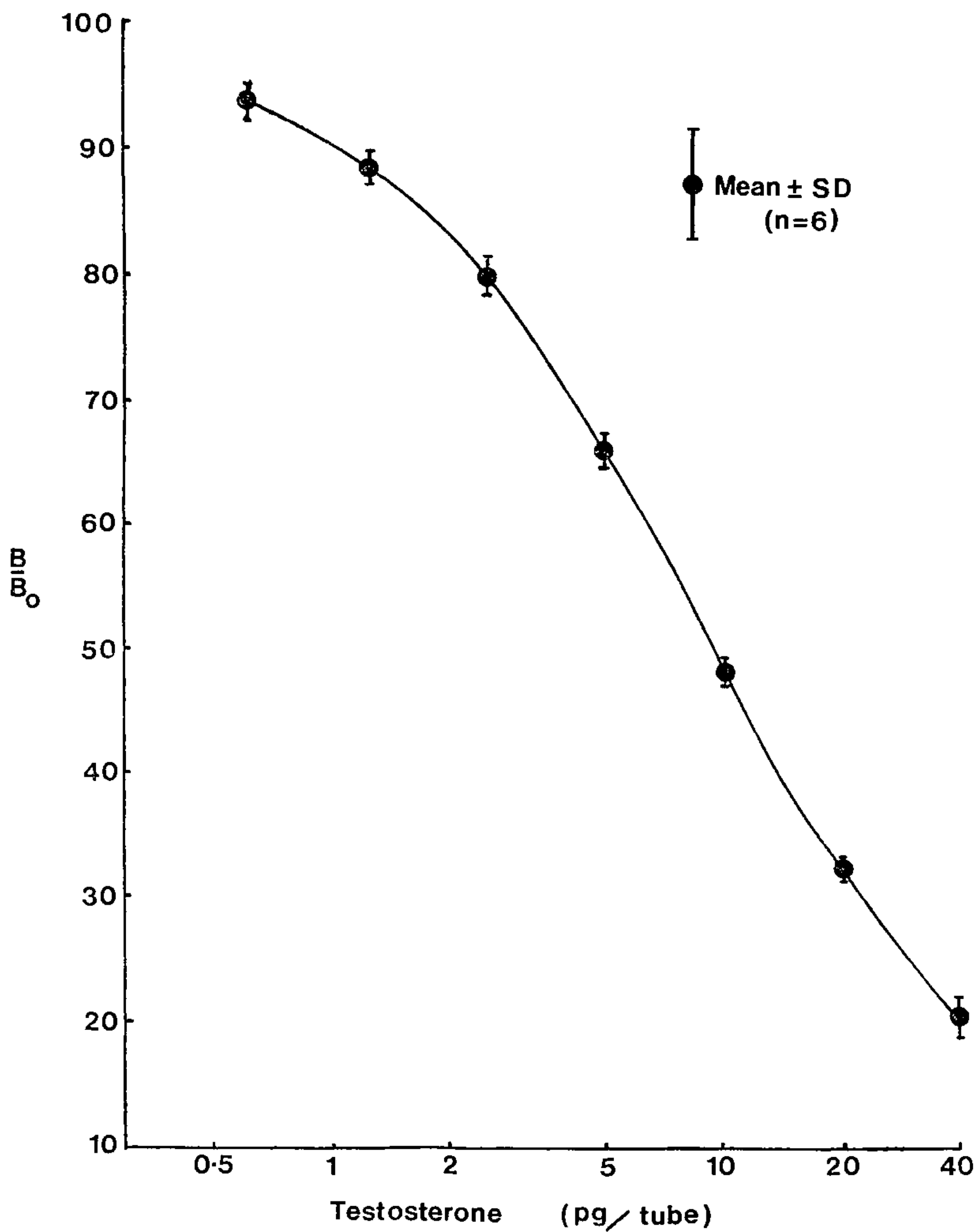

Fig 2.3 A typical standard curve for testosterone using an antibody dilution of 1:150,000.

(ii) Comparison of testosterone values in male and female saliva samples, measured with and without chromatography

In order to test the specificity of the assay, testosterone concentrations in male and female saliva and plasma samples were measured before and after a TLC step.

1500 d.p.m. 1,2,6,7,16,17 ^{3}H-testosterone was added to glass extraction tubes (4 x $^5/8$ in) containing saliva (0.2 ml male, 1.0 ml female) or plasma (20 µl 1:5 dilution male, 50 µl female) and to 3 scintillation vials in order to monitor methodological losses. After adding diethyl ether (5.0 ml) from a freshly opened bottle, the tubes were mixed in a multivortex shaker for 5 minutes.

Tubes were placed into a dry ice - ethanol mixture in order to freeze the aqueous layer, and the ether was decanted into glass tubes (3 x ½ in) and dried under a steady stream of nitrogen at 30°C. The residue was dissolved in phosphate buffer (0.75 ml), or for the chromatographic procedure, in ether (100 µl). The ether extracts were applied to TLC plates and run in a chamber containing dichloromethane : dioxan (94 : 6 v/v). Two markers containing a sufficient amount of radioactive testosterone to be detected on a radiochromatogram imaging system were run on each plate. After chromatography, radioactive areas were located on the TLC plates, and the corresponding sample area cut out and placed into extraction tubes containing diethyl ether (3.0 ml). Samples were eluted for at least 1 hour, then decanted into glass tubes. After evaporation of the ether with nitrogen, the purified sample was reconstituted in phosphate buffer (0.75 ml), vortex mixed, and allowed to stand for 30 minutes. From each solution, 150 µl was placed into scintillation vials in order to calculate the recovery.

Antiserum (100 µl, 1 : 150,000 dilution) was added to glass incubation tubes (3 x $^3/8$ in) containing reconstituted sample (500 µl) or duplicate aliquots of testosterone standard (0.6, 1.2, 2.5, 5, 10, 20

and 40 pg) made up to 500 μl with phosphate buffer. Tubes were mixed and incubated for 30 minutes at room temperature. 1,2,6,7,16,17 ^{3}H-testosterone (20,000 d.p.m.) in 100 μl phosphate buffer was added to all tubes, mixed and incubated for a further 30 minutes at room temperature followed by 15 minutes in an ice water bath. Bound and free testosterone were separated with dextran coated charcoal.

The results (Table 2.2) indicated that the antibody was specific for testosterone in male but not female saliva samples. Levels after TLC were roughly half those without chromatography ($y = 0.47x + 0.07$). Likewise there was no significant difference between concentrations of testosterone in plasma from male subjects before and after chromatography, whereas female plasma levels were lower after TLC. Consequently a preassay purification step was included in all female saliva and plasma measurements. Method flow charts are shown in Figs. 2.4 and 2.5.

The mean extraction of testosterone from male saliva by ether was 97% (n = 38). Furthermore, when 12 saliva samples were analysed in duplicate and the value corrected for methodological losses, the comparison of these results with those obtained without recovery calculation gave very good agreement. Thus it was considered justifiable to omit a correction for recovery in male saliva and plasma measurements. The mean recovery of testosterone in female saliva was 72% (n = 22) after T.L.C.

Since the TLC system used in the female method does not provide a good separation of testosterone from androstenediol, 160 pg androstenediol was added to a previously assayed sample and reassayed. No difference was observed between the two values.

The influence of internal recovery standard was assessed in two ways. The addition of an equivalent concentration of recovery standard to authentic testosterone standards failed to produce a change in the shape of the curve. Also there was no significant difference between sample concentration after correcting for the increased mass and activity

Male

Without TLC	With TLC
163	151
207	190
47	58
53	47
57	59
128	106
68	66
96	103
72	65
99 ± 56	94 ± 49

Female

Without TLC	With TLC
10.2	5.9
19.4	7.8
16.2	7.5
20.6	7.9
20.5	8.8
25.7	14.2
18.7 ± 5.2	8.7 ± 2.9

All results expressed as pg/ml

1 pg/ml = 3.47 pmol/l

Table 2.2 Salivary testosterone concentrations in male and female subjects measured with and without chromatography.

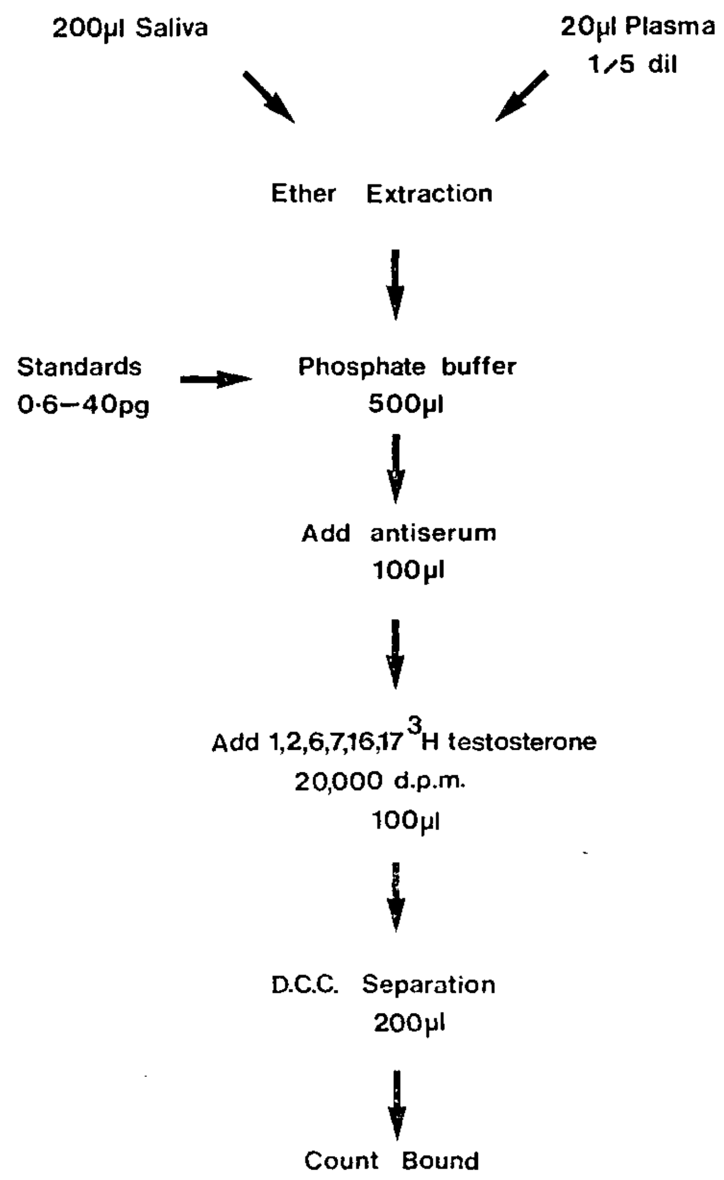

Fig 2.4 Flow chart for the measurement of testosterone in male saliva and plasma samples.

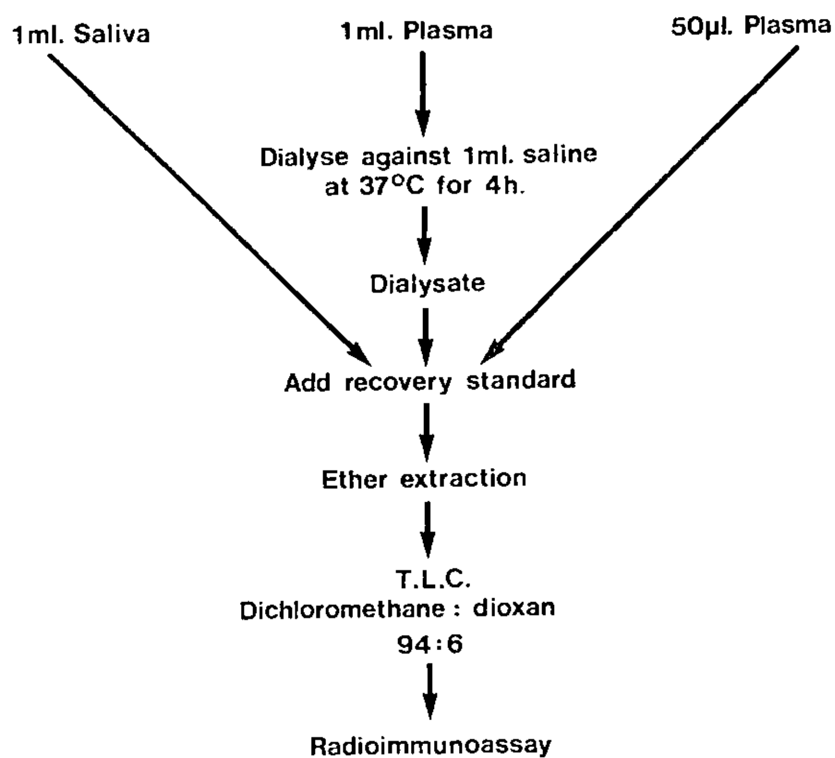

Fig 2.5 Flow chart for the measurement of salivary, unbound and total plasma testosterone in women.

of recovery standard as described by Cekan, (1975).

The precision of salivary and plasma measurements was assessed from within-batch analyses of duplicate samples. The coefficients of variation (C.V.) for samples from male subjects were 7.8% (n = 9) in the range between 38 and 127 pg/ml (132 and 441 pmol/l) for saliva, and 7.1% (n = 18) in the range between 1260 and 8590 pg/ml (4.4 and 29.8 nmol/l) for plasma. The intra-assay C.V.'s were 8.6% (n = 28) in the range 2.8-26.3 pg/ml (9.7 - 91.2 pmol/l) for saliva, and 6.3% (n = 31) in the range 48 - 745 pg/ml (0.2 - 2.6 nmol/l) for plasma in female subjects.

The between batch C.V.'s, which were assessed by assaying duplicate aliquots of pooled samples in successive batches, were 8.9% (n = 25) for saliva and 10.2% (n = 11) for plasma in males and 9.9% (n = 20) for saliva and 13.4% (n = 13) for plasma in females.

When 0.25, 0.5, 1.0 and 1.5 ml of a pooled female saliva sample were assayed, a good linear relationship was obtained as shown in Fig. 2.6. A similar relationship was observed when 50, 100, 150, 200 and 250 µl of male saliva was assayed.

The recovery from saliva was assessed by adding 5, 10 and 20 pg of authentic testosterone to a previously assayed saliva sample and re-assaying. The mean recovery was 96% (Fig. 2.7).

The assay sensitivity, defined as the smallest concentration significantly different from zero at the 5% level and using duplicate assays was 3.0 pg/ml (10 pmol/l) for saliva and 150 pg/ml (0.5 nmol/l) for plasma testosterone in male subjects, and 0.7 pg/ml (3 pmol/l) for saliva and 15 pg/ml (52 pmol/l) for plasma in female subjects.

e) Experiment to investigate the nature of interfering compounds in female saliva

The disparity between the apparent concentration of testosterone in saliva before and after TLC could not be explained solely on the basis of the antisera cross reaction, hence the nature of the interfering

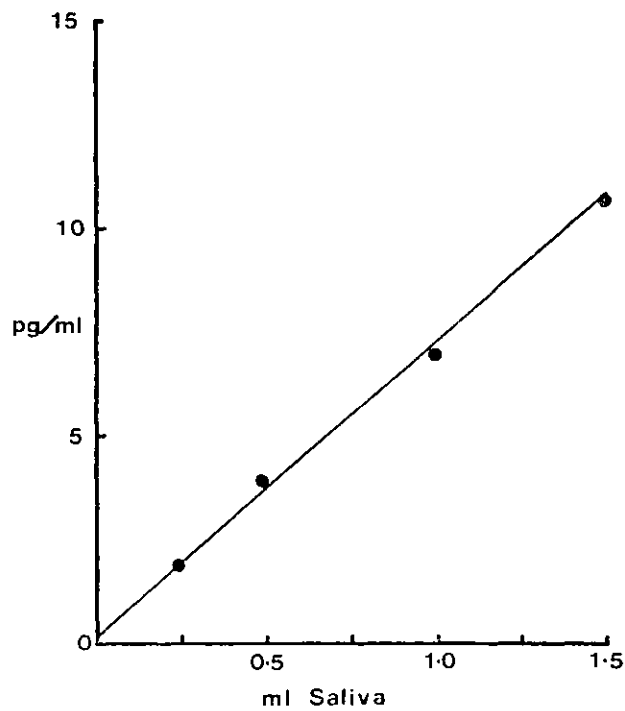

Fig 2.6 Linearity of testosterone values obtained from the assay of increasing volumes of the same saliva sample.

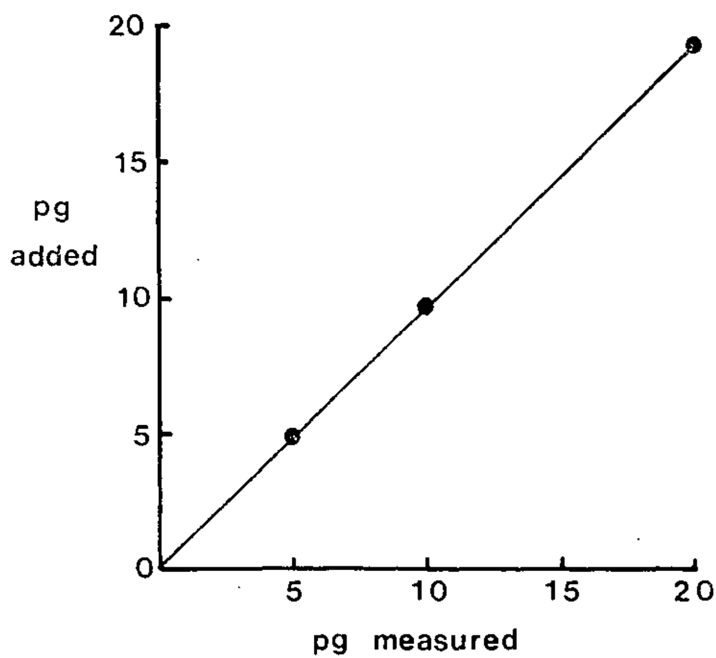

Fig 2.7 Recovery of 5,10 and 20pg of authentic testosterone added to saliva.

material was investigated. Female saliva was extracted with ether, applied to a TLC plate and run in a dichloromethane : dioxan (94 : 6 v/v) system. The plate was dried and divided horizontally into 2 cm^2 sections. These areas were cut out, eluted in ether and assayed. The salivary testosterone concentrations with and without TLC were 9.0 pg/ml (31 pmol/l) and 18.9 pg/ml (66 pmol/l) respectively. The major contaminant was a more polar compound than testosterone which did not migrate from the origin, (Fig 2.8). A significant amount of non-polar impurity was also observed which corresponded to the position of dihydrotestosterone.

No difference was observed in testosterone values in female saliva when ether was substituted by hexane, hexane : ether (4 : 1) or petroleum ether (40-60 B.P.) as extraction solvent.

f) Comparison of testosterone levels measured by a direct radioimmunoassay with those after ether extraction in male subjects

Saliva (125 µl) from 10 male subjects was made up to 500 µl with phosphate buffer, incubated with antiserum and ^{3}H-testosterone. Bound and free ligand was separated with dextran coated charcoal.

In 9 subjects, levels measured by the direct assay (53 ± 17 pg/ml, 184 ± 59 pmol/l) compared very well ($r = 0.96$, $p < 0.001$) with those assayed after ether extraction (62 ± 16 pg/ml, 215 ± 55 pmol/l). But in one normal male, the concentration measured by the direct assay was less than a third of that measured after ether extraction. When the assay was repeated with another sample from the same subject the same phenomenom was observed. Since it has been impossible to obtain more saliva from this subject (due to emigration), the reasons for this discrepancy have not been fully investigated. It would appear to be unlikely that salivary pH would have this effect, since decreased testosterone-antiserum binding caused by pH extremes would result in a higher estimate by the non-extracted method. Consequently, ether

Apparent Testosterone Concentration pg/ml		
<1·2		
<1·2		
<1·2		
<1·2		
2·4		← Area corresponding to D.H.T.
1·3		
9·0		← Area corresponding to testosterone
1·8		
3·7		
		← Origin

Fig 2.8 Division of TLC plate showing the mobility of cross-reacting compounds.
1pg/ml = 3.47pmol/l.

extraction has been performed in all testosterone measurements in this thesis.

Testosterone binding in saliva is discussed in more detail in Section 3.13.

2.4 Radioimmunoassay of androstenedione and dihydrotestosterone

It was necessary to introduce a preassay purification step for the determination of androstenedione in saliva. Levels after chromatography were reduced in both male and female samples (n = 9) from 263 $\pm$ 41 pg/ml (917 $\pm$ 143 pmol/l) to 90 $\pm$ 22 pg/ml (315 $\pm$ 77 pmol/l). Plasma androstenedione concentrations were also lower after TLC, 843 $\pm$ 133 pg/ml (3.0 $\pm$ 0.5 nmol/l) compared to 1132 $\pm$ 212 pg/ml (4.0 $\pm$ 0.7 nmol/l). Since the dichloromethane : dioxan (94 : 6 v/v) TLC system gave a consistently good separation of androstenedione, testosterone and dihydrotestosterone, it was possible to measure these 3 androgens in the same sample.

1500 d.p.m. 1,2,6,7^{3}H-androstenedione and 1,2,4,5,6,7^{3}H-dihydrotestosterone were added to glass extraction tubes containing saliva (2.0 ml) or plasma (400 µl) and to 3 scintillation vials. After adding diethyl ether (5.0 ml), tubes containing plasma were mixed in a multi-vortex shaker whilst saliva samples were shaken horizontally in stoppered tubes for 5 minutes. Ether extracts were separated from the aqueous layer, applied to TLC plates and run in a dichloromethane : dioxan (94 : 6 v/v) system. After chromatography, purified steroids were eluted with ether (3.0 ml) and reconstituted in phosphate buffer (0.75 ml). Tubes were allowed to stand for at least 30 minutes before taking 150 µl for recovery calculation. Aliquots of reconstituted sample and standards (5-320 pg androstenedione and 1.2-80 pg dihydrotestosterone) in duplicate were made up to 500 µl with phosphate buffer, incubated for 30 minutes at room temperature with the respective antiserum in buffer (100 µl), and

for a further 30 minutes after addition of 20,000 d.p.m. 1,2,6,7^{3}H-androstenedione or 1,2,4,5,6,7^{3}H-dihydrotestosterone in buffer (100 μl). Tubes were transferred to an ice water bath for 15 minutes before separating bound and free steroid with dextran coated charcoal.

The antiserum for androstenedione, which was kindly donated by Dr. W. Schopman (Rotterdam), was raised in rabbits immunised with androstenedione 11-hemisuccinate conjugated to bovine serum albumin. The final antiserum dilution was 1 : 256,000. The antiserum to dihydrotestosterone was also raised in rabbits, against a 3-(O-carboxy-methyl)-oxime/BSA conjugate. This antiserum was provided by Professor K. Griffiths, and was used at 1 : 300,000 dilution.

Intra-assay C.V.'s for androstenedione measurements were 6.5% (n = 24) in the range between 17 and 130 pg/ml (60 and 455 pmol/l) for saliva and 4.4% (n = 26) in the range between 490 and 1580 pg/ml (1.7 and 5.5 nmol/l) for plasma. Intra-assay C.V.'s for dihydrotestosterone measurements were 12.7% (n = 12) in the range between 2.9 and 11.7 pg/ml (10.0 and 40.2 pmol/l) for salivary and 5.4% (n = 27) in the range between 47 and 327 pg/ml (0.2 and 1.1 nmol/l) for plasma measurements. Inter-assay C.V.'s for androstenedione were 10.4% (n = 16) for saliva and 12.8% (n = 13) for plasma measurements. For dihydrotestosterone, inter-assay C.V.'s were 14.6% (n = 15) in saliva and 12.3% (n = 11) in plasma samples.

Assay sensitivities were 5 pg/ml (18 pmol/l) and 35 pg/ml (123 pmol/l) for salivary and plasma androstenedione, and 0.8 pg/ml (3 pmol/l) and 10 pg/ml (35 pmol/l) for salivary and plasma dihydrotestosterone. Recovery from saliva was assessed by adding increasing amounts of authentic steroid to saliva and reassaying. The percentage of steroid recovered ranged from 90 - 101%.

2.5 Radioimmunoassay for cortisol

Saliva (50 µl) and cortisol standards (50 - 1000 pg) were made up to 500 µl with phosphate buffer. Plasma (1 : 20 dilution) was heated at 60°C for 30 minutes. Denatured plasma (50 µl) was also made up to 500 µl with buffer. Antiserum (100 µl, 1 : 21,000 dilution) raised in rabbits against a cortisol 21-hemisuccinate/bovine serum albumin conjugate was incubatedfor 30 minutes at room temperature. 1,2,6,7^3H-cortisol (20,000 d.p.m.) in phosphate buffer (100 µl) was added to all tubes, mixed and incubated for a further 30 minutes followed by 15 minutes in an ice water bath. Dextran-coated charcoal was used to separate antibody-bound from free cortisol.

Concentrations of cortisol in saliva measured by this direct assay (18.9 ± 3.7 nmol/l) compared well (r = 0.99) with levels obtained after dichloroethane extraction followed by TLC (dichloroethane : methanol : water, 150 : 20 : 1 v/v) (17.4 ± 4.4 nmol/l). Inter- and intra-assay C.V.'s for salivary cortisol measurements were 7.1% (n = 14) and 3.6% (n = 18). The assay sensitivity was 0.9 nmol/l.

2.6 Radioimmunoassay of 5_α androstane 3_α 17_β diol

Initial studies showed that the measurement of 5_α androstane 3_α, 17_β diol concentrations in saliva samples was impractical due to the low sensitivity of available assay systems. Nevertheless, it was felt that the measurement of androstanediol in plasma samples would complement salivary and plasma testosterone, androstenedione and dihydrotestosterone data in women with androgen imbalance.

1500 d.p.m. 1,2^3H-androstanediol was added to glass extraction tubes containing 2 ml of plasma and to 3 scintillation vials in order to monitor methodological losses. Stoppered tubes were shaken horizontally for 5 minutes with diethyl ether (5.0 ml) twice. Ether extracts were separated from the aqueous layer, applied to TLC plates

and run in a dichloromethane : dioxan (90 : 10 v/v) system. After chromatography, samples were eluted with ether (3.0 ml), extracts dried with nitrogen and reconstituted with phosphate buffer (750 µl). Tubes were mixed and allowed to stand for 30 minutes before taking 500 µl aliquots for assay and 150 µl for recovery calculation. Standards (10 - 320 pg) in duplicate, containing an equivalent mass of radiolabelled androstanediol as in sample tubes, were made up to 500 µl with buffer. Samples and standards were incubated for 30 minutes with antiserum (100 µl) and for a further 30 minutes after the addition of 1,2 ^{3}H-androstanediol in buffer (100 µl). Tubes were transferred to an ice water bath for 15 minutes before separating bound from free steroid with dextran coated charcoal.

The antiserum, which was obtained from Serono Biodata Diagnostics, was raised in rabbits against a 5αandrostane 3α,17β diol -15 carboxymethyl oxime / BSA conjugate and was used at a final titre of 1 : 3000. Thin layer chromatography separated androstanediol from all important steroids except androstene 3β,17βdiol and 5αandrostane 3β,17βdiol. The respective cross reactions of these androgens were 0.03% and 1.0%. The intra-assay C.V. was 9.6% (n = 15) in the range between 22 and 144 pg/ml (75 and 492 pmol/l). The inter-assay C.V. was 15.2% (n = 7). The assay sensitivity was 12 pg/ml (41 pmol/l). Recovery from plasma, which was 107%, was assessed by adding increasing amounts of authentic steroid to plasma and reassaying.

2.7 Measurement of unbound steroids

Numerous methods have been applied to the study of steroid- protein interactions. The general requirement is for a system whereby protein and steroid can be distinguished from each other. This usually requires a separation step which results in a disturbance to the thermodynamic equilibrium. Although in vitro methods do not necessarily represent the steroid-protein interactions in plasma, they are thought to be a

good reflection of <u>in vivo</u> conditions (Forest et al., 1968). The most frequently used techniques of measuring unbound steroid levels are equilibrium dialysis (Haarmann et al., 1940; Schellman et al., 1954; Vermeulen and Verdonck, 1968; Moll and Rosenfield, 1979), ultrafiltration (Toribara, 1953; Forest et al., 1968; Hammond et al., 1980) and gel filtration (Vermeulen and Verdonck, 1968; Fisher et al., 1974).

a) <u>Ultrafiltration</u>

The advantage of the ultrafiltration technique is the relatively short time required to separate bound from free steroid. This reduces possible errors due to protein denaturing or decomposition of labelled hormone, but there is a constant change to the equilibrium during centrifugation as a result of the increased concentration of protein in plasma. Westphal, (1971) suggested that this error was negligible, as long as the filtrate remained less than 10% of the filtrans.

Plasma (7.0 ml) which had been equilibrated overnight with 1,2,6,7,16,17^{3}H-testosterone (50,000 d.p.m.) was centrifuged in Amicon membrane cones (Type CF 50) at 900 g for 3 minutes at 37°C. The percent free steroid was calculated as:-

$$\frac{\text{d.p.m./200 µl filtrate}}{\text{d.p.m./200 µl plasma}} \times 100$$

The concentration of testosterone was also measured directly in the filtrate by radioimmunoassay following centrifugation of plasma (7.0 ml) from a male subject.

Levels of unbound testosterone measured by both these techniques (0.2 - 1.3%, n = 5) were considerably lower than those reported in the literature for normal males (Vermeulen et al., 1971; Moll and Rosenfield, 1979) and those obtained by equilibrium dialysis in the same samples. The reason for this discrepancy was shown to be due to adsorption of testosterone to the Amicon cones. The mean adsorption, calculated by centrifuging saline containing ^{3}H-testosterone through the cones, was 43%.

The concentration of testosterone in the filtrate after centrifuging plasma through a visking membrane was found to be very inconsistant. Levels ranged from 0.6 - 19.6% of the total plasma concentration. This technique was time-consuming and impractical for routine usage. The possibility of plasma leakage through knots in the membrane could not be ruled out.

b) Equilibrium dialysis

The principal merit of equilibrium dialysis is its thermodynamic validity, since unbound steroid may freely move through the membrane, but equilibrium dialysis always involves a dilution of plasma, which can be as much as 20% of the starting volume (Moll and Rosenfield, 1978). Both diluted and undiluted plasma have been used in the measurement of unbound hormone at 4 and 37°C. This variation in experimental conditions has made the comparison of results from different laboratories difficult. Some investigators have failed to make any correction for dilution, whilst Chopra et al, (1972), applied a constant correction factor to the value obtained in diluted plasma. This may give rise to erroneous levels since the weight increase of undiluted plasma has been shown to vary between patient (Kley et al., 1977). The introduction of dialysis cells with a large surface area / volume ratio (Weder et al., 1971) has now made it possible to reach equilibrium quickly using small plasma volumes.

(i) The influence of incubation time on the binding of testosterone to plasma proteins

Undiluted pooled female plasma (1.0 ml) was equilibrated against 0.9% saline (1.0 ml) containing 40,000 d.p.m. 1,2,6,7,16,17^{3}H-testosterone in a dialysis machine at 37°C. The contents of each half cell were emptied 0.5, 1, 2, 3, 4 and 19 hours later. Aliquots of dialysate and plasma (500 µl) were counted for radioactivity. The percentage unbound, calculated according to Antoniades, (1960), is shown in Fig2.9.

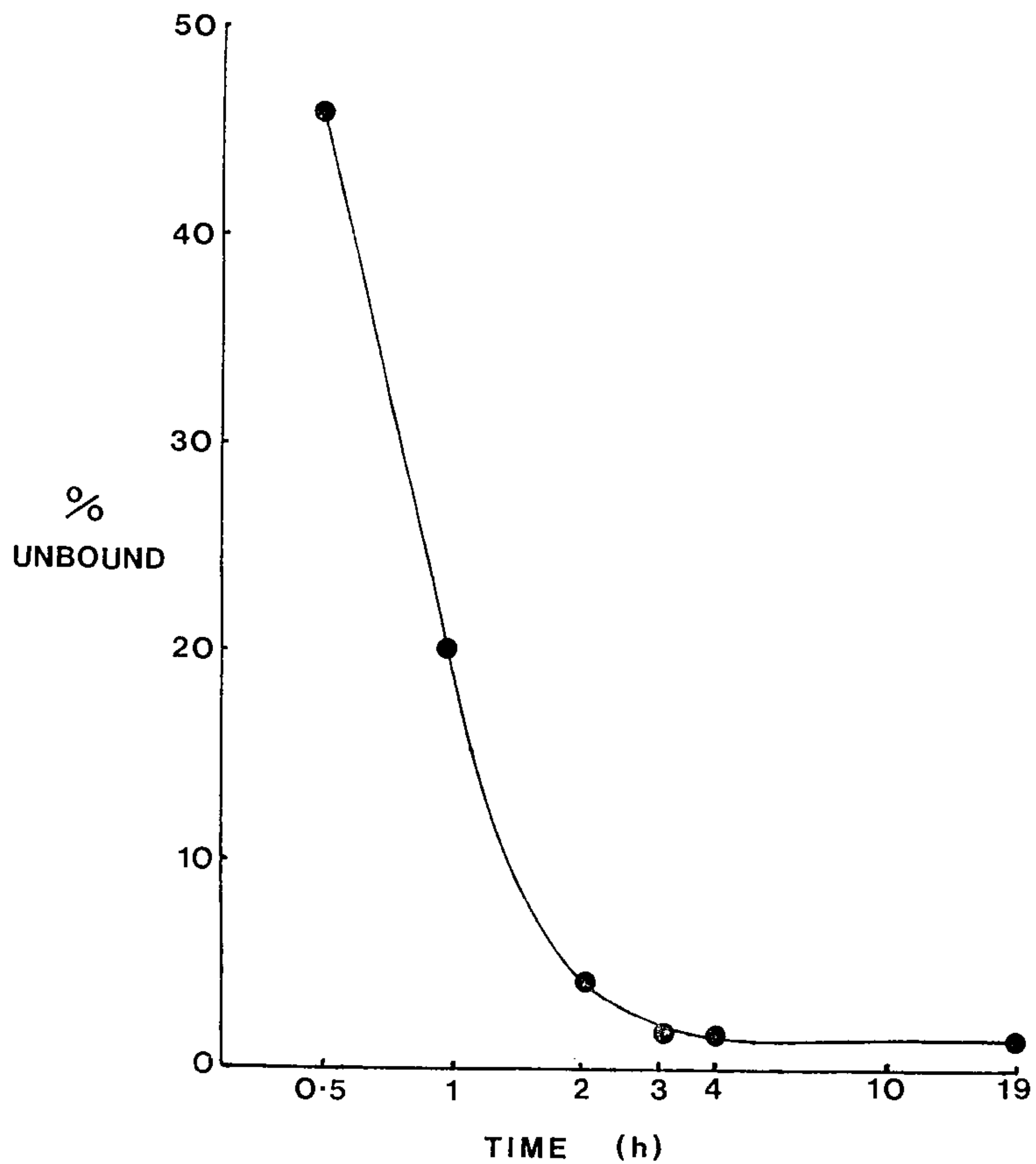

Fig 2.9 The effect of duration of dialysis at 37°C on the percentage binding of testosterone in plasma.

A 4 hour incubation was used in all future dialyses.

(ii) Measurement of unbound steroid by radioimmunoassay following equilibrium dialysis

Presoaked visking tubing was cut into squares 4 x 4 cm and placed between the teflon half cells of an equilibrium dialysis machine. Undiluted plasma (1,0 ml) was equilibrated for 4 hours at 37°C whilst rotating at 12 r.p.m. against 0.9% saline (1.0 ml). The dialysate was collected and the steroid concentration measured by radioimmunoassay. Using an equation similar to that described for free plasma cortisol (Clerico et al., 1978), the unbound plasma steroid concentration was calculated.

$$FS = \frac{Sd \cdot Vi}{(TS \cdot Vp) - (Sd \cdot Vo)} TS$$

Where FS = unbound plasma steroid concentration (pg/ml)

TS = total plasma steroid concentration (pg/ml)

Sd = concentration of steroid in the dialysate measured by RIA (pg/ml)

Vi = final plasma volume following dialysis (ml)

Vp = initial volume of dialysis plasma (ml)

Vo = volume of dialysate

This technique was used to measure unbound plasma levels of testosterone, androstenedione and cortisol. Unbound dihydrotestosterone was measured by the tracer method, since the dihydrotestosterone radioimmunoassay was not sufficiently sensitive to allow an accurate measurement using less than 2 ml of dialysate. Undiluted plasma (1.0 ml) was equilibrated against saline containing 20,000 d.p.m. 1,2^{3}H-dihydrotestosterone for 4 hours at 37°C. The percentage unbound dihydrotestosterone was calculated according to Antoniades et al., (1960). Unbound plasma dihydrotestosterone was the product of percent unbound and total plasma level.

The between batch variation for unbound plasma testosterone samples was assessed from duplicate pooled samples assayed after equilibrium dialysis. The C.V. was 12.5% (n = 12). Frozen plasma could be stored for at least 6 months with no change in the measured concentration of free testosterone.

There are no previous reports in the literature describing the direct determination of unbound androgens in plasma. Levels are similar to those using isotopically labelled steroid (Vermeulen et al., 1971; Moll and Rosenfield, 1979). The present technique has two important advantages over methods requiring radiolabelled isotope:- the mass of labelled steroid introduced into the system may shift the equilibrium, and more importantly, frequent chromatographic purification of the label is required. Even a small amount of non-binding impurity produces an overestimate of the free testosterone measured.

2.8 Measurement of testosterone not bound to sex hormone binding globulin

Non sex hormone binding globulin (SHBG) bound plasma testosterone was measured by a modification to the technique of O'Connor et al., (1973). Plasma (200 µl) was incubated with saline (300 µl) containing 20,000 d.p.m. 1,2,6,7,16,17^{3}H-testosterone for 1 hour at room temperature followed by 15 minutes in an ice water bath. SHBG was precipitated by addition of cold saturated ammonium sulphate (500 µl) whilst vortex mixing the sample tubes. Saline (500 µl) was added to control tubes containing plasma and ^{3}H-testosterone. All tubes were left for 15 minutes in ice before centrifuging at 1500g for 30 minutes at 4°C. 500 µl of the supernatant was taken for radioactivity measurement.

$$\text{Percent Non SHBG bound testosterone} = \frac{\text{d.p.m. / sample supernatant}}{\text{d.p.m. / control supernatant}}$$

$$\text{Non SHBG bound testosterone (pg/ml)} = \text{Percent non SHBG bound testosterone} \times \text{total plasma testosterone (pg/ml)}$$

In order to test whether any albumin-bound testosterone was precipitated with ammonium sulphate, a globulin free albumin solution (46g/l) was assayed. No precipitate was formed.

This technique used for measuring non-SHBG bound testosterone levels is based on the precipitation of ß globulins by a 50% solution of ammonium sulphate. Although protein dilution and low temperature are obligatory, this method permits the rapid and reproducable determination of that proportion of testosterone not bound to SHBG.

Levels of non specifically bound plasma testosterone (102 $\pm$ 95 pg /ml, 356 $\pm$ 330 pmol/l) compared well ($r = 0.98$) with concentrations measured after dialysing plasma (1.0 ml) against albumin (1.0 ml) at a concentration similar to the plasma sample, (129 $\pm$ 96 pg/ml, 448 $\pm$ 333 pmol/l). Although this latter technique is physiologically more sound, it is time consuming, very expensive to perform and not applicable to the analysis of multiple plasma samples.

The intra-assay coefficient of variation was 4.1% ($n = 29$) in the range between 9 and 60%, and the between batch C.V. was 2.3% ($n = 8$).

SECTION 3

SALIVARY STEROID SECRETION IN NORMAL SUBJECTS

3.1 Introduction

Saliva originates from 3 main pairs of glands. The submaxillary glands, which produce on average between 60 and 70% of the total saliva secretion, the parotid glands, which contribute between 25 and 35%, and the sublingual glands, which make up about 5%. A further 5% is derived from smaller glands of the oral mucosa (buccal, labial, lingual and palatal) and the gingival pockets.

The relative contribution from each gland to the total salivary pool varies with the time of day, the rate of flow and the type and duration of stimulus. Also, the concentration of individual glandular components may vary with these same factors. Although saliva is an easily obtainable fluid, it has rarely been used for diagnostic purposes, since little is known about the factors which determine the relative secretion rates from each source, and controlled conditions are difficult to provide for standard comparison. There are a number of good publications giving detailed descriptions of important features such as pH, flow rate, electrolyte, carbohydrate and protein concentrations in mixed and glandular secretions (Dawes, 1972; Mason and Chisholm, 1975; Jenkins, 1978; Ferguson and Botchway, 1979). Some compounds are produced more in one gland than another. The majority of glycoprotein, for instance, which is the major organic constituent of human saliva, is produced in the submaxillary and sublingual glands, hence some investigators have analysed the secretion of steroids in parotid saliva because it is less viscous and easier to pipette.

The mechanism of passage of plasma constituents into saliva is also poorly understood. Active transport mechanisms have been shown to exist for sodium, potassium and bicarbonate (Shannon, 1958; Burgen, 1961),

levels of potassium and bicarbonate being higher in saliva than plasma.

Transfer of lipid-insoluble non-electrolytes is dependent on the molecular size (Burgen, 1961). The saliva : plasma ratios of the compounds glycerol (MW = 90), creatinine (MW = 113), mannitol (MW = 192) and sucrose (MW = 346) were 0.40, 0.12, 0.032 and 0.005 respectively. This is consistent with transfer through pores that are restrictive of the transport of molecules whose size approaches the pore diameter.

The concentration of drugs in saliva has been used extensively for monitoring anticonvulsant therapy (Reynolds et al., 1976; Horning et al., 1977). Recently it has been shown that salivary drug concentrations are a good reflection of the unbound plasma levels of drugs, but only if ionisation constants lie outside the range of physiologically encountered salivary pH values, (McAucliffe et al., 1977; Mucklow et al., 1978). In the case of phenobarbital for example (pKa 7.2), salivary concentrations were as much as 44% below unbound plasma levels at pH values less than 6.5, whereas at values above pH 7.5, salivary concentrations were 18% or more higher than unbound plasma levels.

The mechanisms of transfer of steroids into saliva have not been studied in any detail, but it is thought likely that the permeation of lipid-soluble steroids is a passive process occuring through all cell membranes of the glands, and is independent of the rate at which saliva is secreted (Burgen, 1961). This may not be the case for water soluble steroid conjugates though. Some of the factors affecting salivary steroid secretion, and androgen levels in saliva from normal subjects have been investigated in this section.

3.2 Composition of steroids in mixed and parotid saliva

Since the composition of saliva differs in each gland, it was necessary to compare the levels of steroid in individual glandular secretions with those in mixed saliva. Parotid fluid was collected

from 2 normal male subjects using a Lashley cannula. The concentrations of testosterone and cortisol in the parotid secretion were found to be similar to those in mixed saliva obtained immediately after removing the collecting cup. This supports the findings of Walker et al. (1978), who showed no difference between cortisol levels in parotid fluid and mixed saliva at various times during the day. This has also been observed for 17α hydroxyprogesterone (Walker et al., 1979).

The location of individual glands and the attachment of sampling cups is not easy, which makes sample collection by patients themselves difficult to perform. Hence it was decided to use whole mixed salivary collections for all steroid measurements. Submaxillary fluid has rarely been used due to the inaccessibility of these glands (Katz and Shannon, 1969b).

3.3 Effect of centrifugation on concentrations of steroids in saliva

Mixed saliva contains a large amount of suspended matter, consisting mainly of squamous epithelial cells, denatured protein, leucocytes and bacteria. A number of investigators have separated this debris by centrifugation before analysing samples (McVie et al., 1979; Smith et al., 1979; Fischer-Rasmussen et al., 1981; Odlind and Johansson, 1981; Umeda et al., 1981).

Whole saliva was collected from 6 normal male volunteers between 0900 and 1000. Each sample was divided into 2 aliquots. One set of samples was centrifuged at 800 g for 10 minutes and the clear supernatant retained for analysis. The concentrations of testosterone (89 ± 32 pg/ml, 309 ± 111 pmol/l) and cortisol (13.0 ± 4.1 nmol/l) were not significantly different from levels in whole saliva (83 ± 28 pg/ml, 288 ± 97 pmol/l for testosterone and 12.5 ± 4.3 nmol/l for cortisol). Therefore it was considered unnecessary to include a centrifugation step prior to assaying samples.

3.4 Influence of flow rate and sample volume on salivary steroid concentrations

The mean flow rate of unstimulated saliva is 0.5 ml/min. Consequently, a 3 ml sample can usually be collected in little over 5 minutes. If the occasion arises whereby a larger volume must be collected, the flow rate may be increased as much as five times after chemical (Walker et al., 1978; McVie et al., 1979) or mechanical (Greaves and West, 1963; Wang et al., 1981) stimulation.

The concentration of lipid-soluble compounds in saliva is thought to be independent of the rate of salivary secretion (Burgen, 1961), but the reported influence of flow rate on steroid concentrations is contradictory. In order to clarify this fundamentally important point, saliva was collected before and after stimulation in 2 normal male subjects. The flow rate was increased from 0.5 ml/min to 1.5 ml/min by applying one drop of lemon juice to the tip of the tongue. Concentrations of cortisol and testosterone remained unaltered after increasing the salivary flow rate. This is in agreement with Walker et al. (1978) and McVie et al. (1979), who demonstrated that cortisol and aldosterone concentrations were independent of an increased flow rate. Katz and Shannon (1969), on the other hand, found that with increasing flow rate, parotid fluid cortisol and cortisone concentrations diminished, and said that the amount of corticosteroid excreted per minute remained constant at different flow rates.

A further view proposed by Riad Fahmy et al. (1980), was that steroid concentrations in saliva are independent of flow rate as long as the sample collected is limited to 4 ml, after which the equilibrium in the gland is disturbed resulting in decreased concentration of steroid. In order to test this theory, 3 samples of unstimulated whole saliva were collected from a normal male volunteer between 1500 and 1515. The collection volumes were 0.3 ml, 10.0 ml and 0.5 ml respectively.

Concentrations of testosterone and cortisol remained constant throughout the 15 minute period.

Atropine inhibits the response to stimulation of post-ganglionic cholinergic nerves, particularly those of the parasympathetic system which innervates exocrine glands and smooth muscle. When administered at low doses the only prominent effect is to decrease the rate of salivary secretion (Cullumbine et al., 1955). Intramuscular injections of atropine sulphate (0.6 mg) were given to a normal male volunteer at 10.00 and 12.30. The only side effects at this dose were a dry mouth and slight light-headedness. The time of occurrence of these symptoms was 25 minutes after the initial injections and lasted for about 90 minutes before the repeat dose was administered. Flow rates and concentrations of testosterone and cortisol are shown in Fig. 3.1. An increase in testosterone concentrations (mean 45%) was observed after each injection. Levels rapidly decreased as the flow rate returned to normal, during which time total and unbound plasma testosterone concentrations remained constant. It is difficult to assess the influence atropine had on cortisol concentrations in saliva. There was a mean increase of 10% in salivary cortisol levels following atropine injection. This data suggests that at low salivary flow rates the concentration of testosterone in saliva is not a good indication of total or unbound plasma testosterone levels, and that steroid levels are dependent on saliva flow at low secretion rates. These results also contradict the findings of Katz and Shannon, (1969), since the steroid secreted per minute was not constant at different flow rates.

3.5 The stability of steroid hormones in saliva

The presence of bacteria in saliva inevitably results in changes in the composition of saliva on standing. It was therefore necessary to investigate the stability of steroid hormones in saliva samples. Saliva

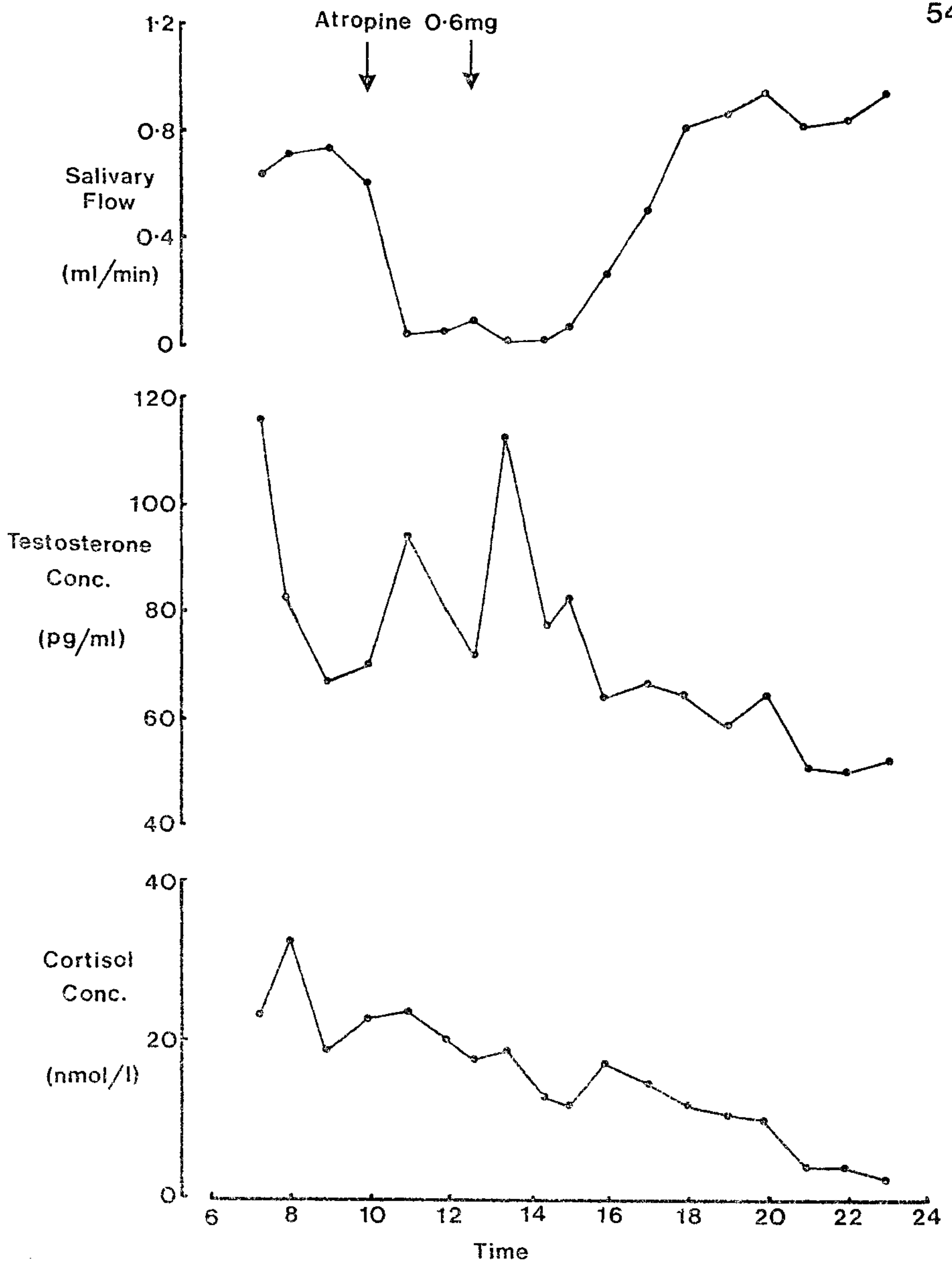

Fig 3.1 The effect of atropine sulphate administration on the flow rate and concentration of testosterone and cortisol in saliva.
Testosterone 1pg/ml = 3.47pmol/l.

samples collected from 5 males were pooled, half being stored at room temperature and half at 4°C. Aliquots were taken from each at periods of 3, 6, 13 and 20 days, and frozen before analysis. When stored at 4°C, testosterone in male saliva was stable for at least 6 days, thereafter levels increased slightly (Table 3.1), but storage at room temperature resulted in increased levels after as little as 3 days. After 20 days storage at room temperature, the pooled sample had changed to a dark brown colour.

Table 3.2 shows the effect of 4 days storage of saliva on concentrations of testosterone, dihydrotestosterone and androstenedione in 3 women. Clearly the length of time between collection and analysis is an important consideration in salivary steroid measurements. Whenever possible samples were immediately frozen after collection. Results from samples analysed 2 days or more after collection were deemed unreliable.

3.6 The relationship between salivary testosterone and cortisol with unbound and total levels in plasma of normal men

Matched saliva and plasma samples were collected from normal male subjects between 0900 and 1600. Whole unstimulated saliva (1.0 ml) was collected directly into sterile plastic tubes. Blood was taken by venepuncture into heparinized tubes; plasma and whole saliva were frozen and stored at -20°C prior to assay.

As shown in Fig. 3.2, there was a highly significant correlation ($r = 0.81$, $p < 0.001$) between the concentration of testosterone in saliva (79 $\pm$ 21 pg/ml, 274 $\pm$ 73 pmol/l, $n = 24$) and the unbound concentration of testosterone in plasma (75 $\pm$ 18 pg/ml, 260 $\pm$ 62 pmol/l, $n = 24$). The unbound testosterone in plasma as measured by this direct method corresponded to 1.3 - 2.6% of the total plasma testosterone concentration, and is therefore similar to values obtained by indirect methods

Days	4° C	Room Temperature
0	61	61
3	62	67
6	62	72
13	66	37
20	65	79

All results expressed as pg/ml

1pg/ml=3.47 pmol/l

Table 3.1 The influence of storage time and temperature on testosterone levels in male saliva

Subject	Testosterone		Dihydrotestosterone		Androstenedione	
	Pre	Post	Pre	Post	Pre	Post
K	4.0	6.2	4.1	5.9	58	71
N.J.	5.6	4.2	3.2	5.5	34	66
E.F.	4.8	9.6	5.3	3.5	48	38
Mean	4.8	6.7	4.2	5.0	47	58

All results expressed as pg/ml

Testosterone 1 pg/ml = 3.47 pmol/l

Dihydrotestosterone 1 pg/ml = 3.44 pmol/l

Androstenedione 1 pg/ml = 3.50 pmol/l

Table 3.2 The effect of storage for 4 days at room temperature on androgen concentrations in female saliva.

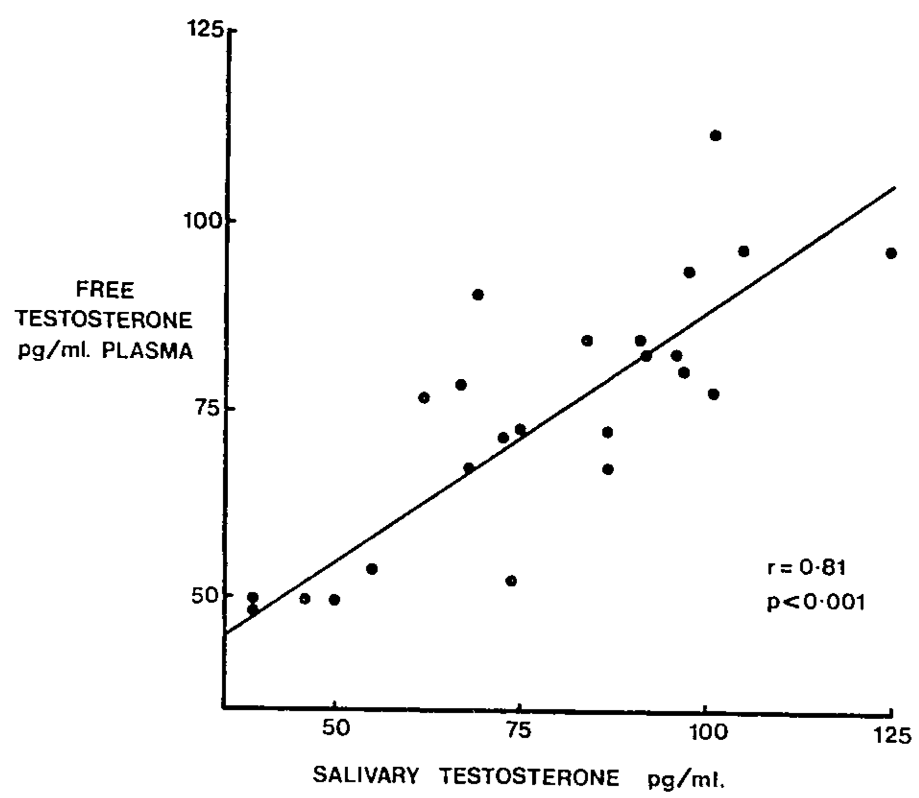

Fig 3.2 Comparison of salivary testosterone with unbound plasma testosterone in normal men.
1 pg/ml = 3.47pmol/l.

involving the use of isotopically labelled steroids.

The correlation ($r = 0.59$, $p < 0.005$) between salivary testosterone and total plasma testosterone levels (5262 ± 1852 pg/ml, 18.3 ± 6.4 nmol/l) in the same group was similar to that between unbound and total plasma testosterone levels ($r = 0.56$, $p < 0.005$).

A good relationship ($r = 0.86$, $p < 0.001$) was also observed between the concentration of cortisol in saliva (17.2 ± 5.7 nmol/l) and the unbound plasma level (24.0 ± 10.4 nmol/l) of 12 normal subjects (Fig3.3). Similarly, an excellent correlation ($r = 0.94$, $p < 0.001$) was observed between salivary cortisol levels (17.7 ± 5.9 nmol/l) and the total plasma cortisol concentration (359 ± 132 nmol/l) in 19 subjects (fig. 3.4). The unbound plasma cortisol concentrations were 5.8 - 8.8% of the total plasma levels, which compares well with reported levels in the literature using other techniques (Baumann et al., 1975; Robin et al., 1978).

Since it is generally believed that only the unbound steroid fraction is biologically active (Mowszowicz et al., 1970; Laznitzki and Franklin, 1972; Anderson, 1974), salivary concentrations should give a better indication of the testosterone and cortisol concentrations available for metabolism in the tissues in patients with altered plasma protein-binding. Although the concentrations of testosterone in saliva and the non protein bound plasma fractions are in good agreement, the levels of salivary cortisol are lower than unbound plasma levels. This phenomenon has also been observed by Umeda et al., (1981), who suggested that this may be as a result of cortisol binding to oral cell debris or reabsorption of the molecule by oral epithelial cells. The former of these possibilities which is studied in detail in Section 3.13 has been investigated. No binding of cortisol to either oral debris or soluble proteins was detected. A further explanation could be steroid metabolism in salivary glands or the oral mucosa. The concentration of cortisone in saliva has been shown to exceed cortisol, whereas in plasma the

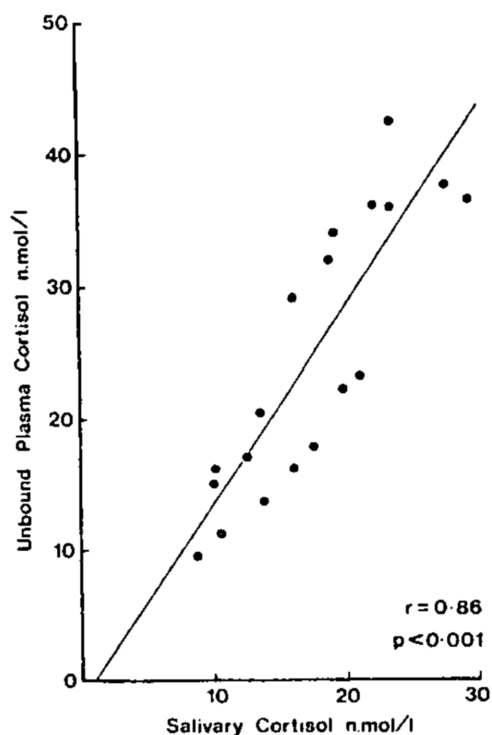

Fig 3.3 The relationship between salivary cortisol and unbound plasma cortisol concentrations in normal men.

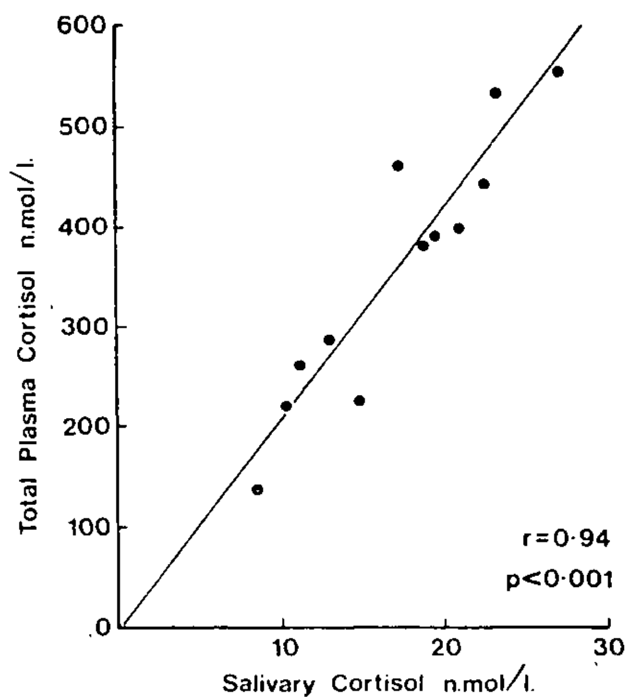

Fig 3.4 Comparison of salivary cortisol with total plasma

reverse is true (Katz and Shannon, 1969). Administration of ACTH increases both of these corticosteroids but with a resultant excess of cortisol in saliva. The authors postulated that in the basal state cortisol was being oxidised in the salivary glands to cortisone and that the sudden increase in cortisol presented to the glands after ACTH injection caused a saturation of the enzyme system resulting in the decreased percentage conversion. This mechanism, if correct, suggests the existence of a most efficient enzyme system considering the rapid passage of labelled cortisol into saliva (Hiramatsu, 1981).

3.7 Diurnal changes in salivary and plasma androgen concentrations

a) Males

Salivary testosterone concentrations were measured through the day (0700 - 2300) in 7 normal subjects (Fig. 3.5). The level of testosterone in saliva fell throughout the day, which confirms previously reported data (Landman et al., 1976; Walker et al., 1980) that morning levels (0800 - 0900) of testosterone in saliva are significantly higher than evening (2200 - 2300) concentrations.

Numerous studies have been performed to examine circadian fluctuations of testosterone in male plasma, but there has not been any uniform agreement in the findings of these investigators, possibly due to the differing experimental conditions in which these studies were carried out. Testosterone production occurs in a pulsatile manner, and some investigators (Fairman and Winter, 1971; Rose et al., 1972; Piro et al., 1973), but not all (Boon et al., 1972; Serio et al., 1974), have described the existence of regular diurnal rhythms in male plasma.

A diurnal rhythm of testosterone in plasma was observed in each of 5 male subjects studied. A typical example is shown in Fig. 3.6. For this study, matched blood and saliva samples were collected hourly for 25 hours from 1000 to 1100 during which time the subject continued

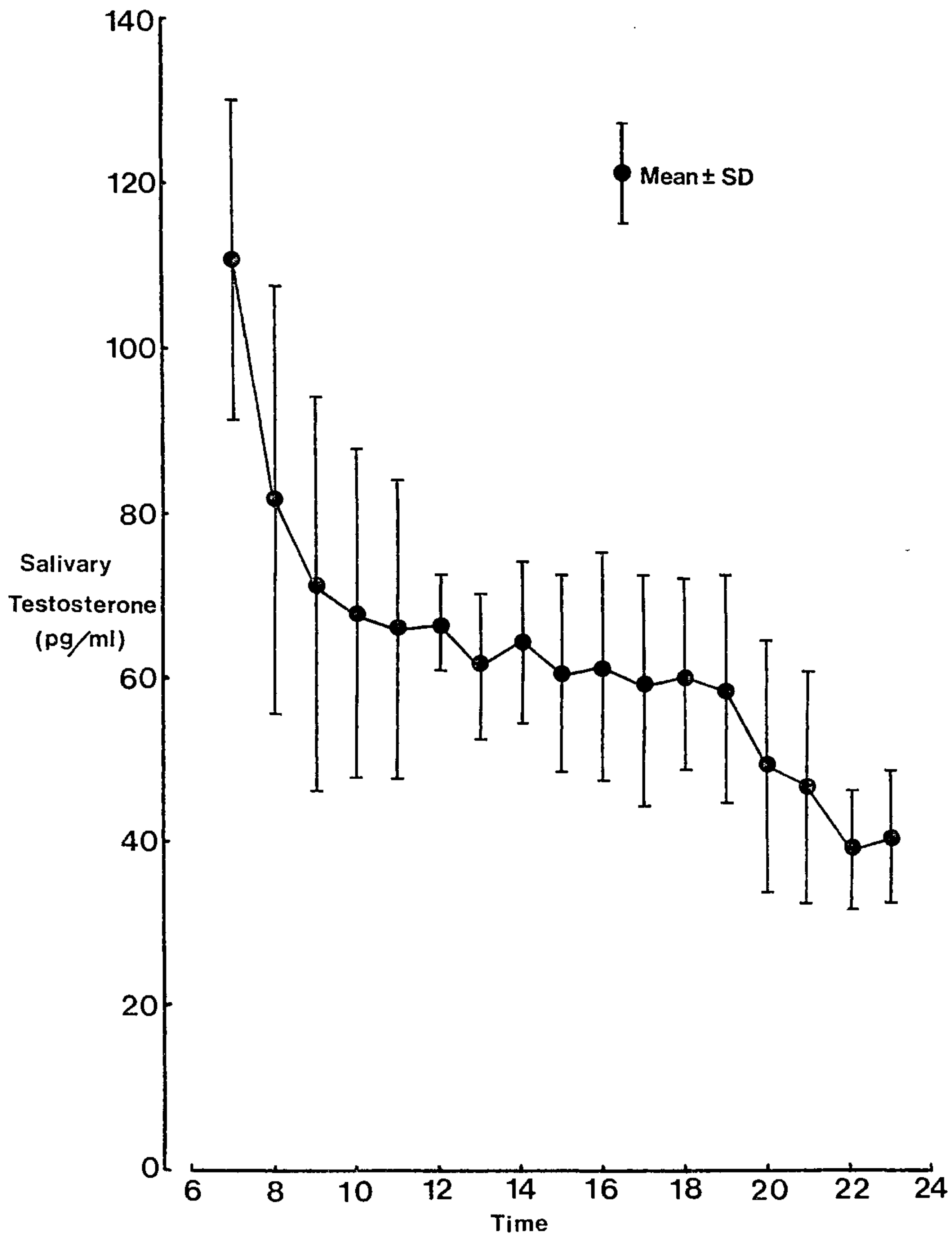

Fig 3.5 Salivary testosterone levels through the day in 7 normal male subjects.
1 pg/ml = 3.47 pmol/l.

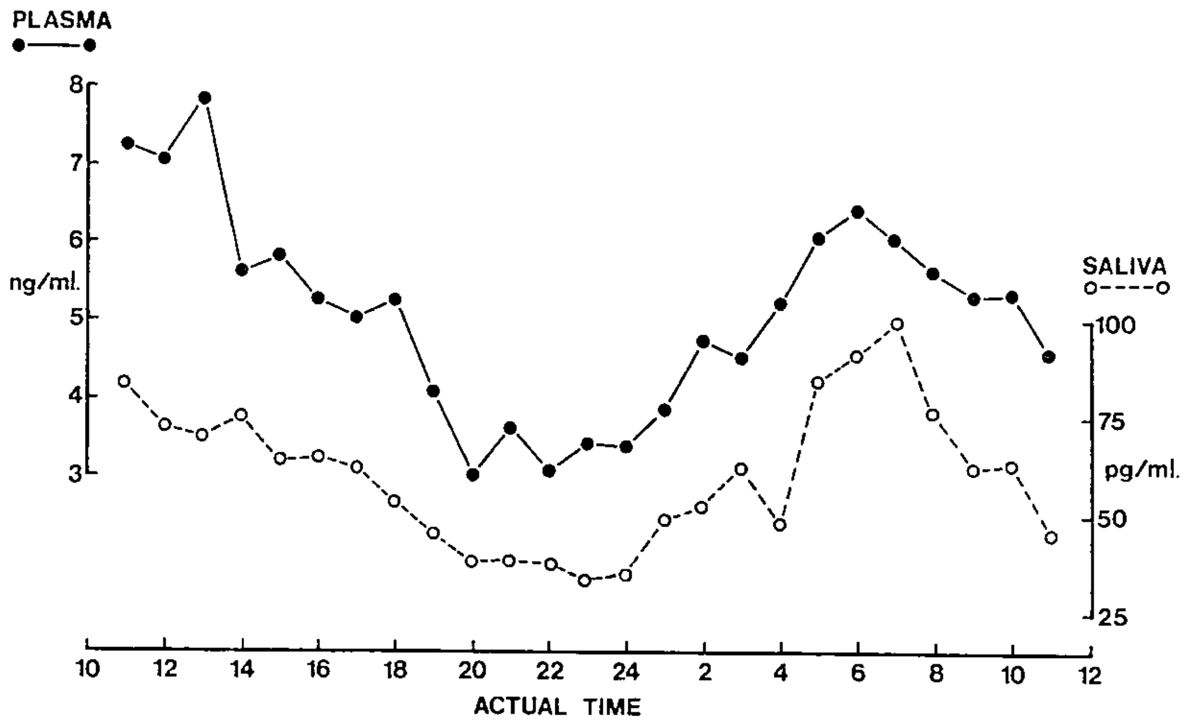

Fig 3.6 Diurnal rhythm of testosterone in matched saliva and plasma samples in a normal male subject.
1 pg/ml = 3.47 pmol/l.

his normal daily routine as much as possible. The subject slept between 2300 and 0700, awaking hourly to deliver saliva samples (300µl) and withdraw blood through an indwelling cannula.

A good relationship also exists between salivary and unbound plasma testosterone at different times throughout the day (Table 3.3). Matched samples were collected at 3-hourly intervals from a normal male between 0900 and 2100. Throughout this period the percentage levels of testosterone in saliva or the unbound fraction in plasma ranged between 1.2 and 1.5% of the total plasma concentration.

Saliva samples were collected from another male volunteer when the times of waking and arising were delayed to 0700, 0800 and 1000 on 3 consecutive days. Salivary testosterone levels were found to decrease rapidly upon waking on each day (Fig. 3.7), levels being significantly lower 1 hour after waking and arising. In order to investigate the influence of posture on salivary testosterone, concentrations were measured through the day in a subject who returned to a recumbent position between 1300 and 1700. This change in posture was not accompanied by an increase in salivary testosterone levels (Fig 3.8). When a recumbent position was maintained upon awakening, salivary testosterone levels still decreased rapidly (Fig. 3.9). It would therefore appear unlikely that the rapid decrease in salivary testosterone levels was due to posture, but was dependent upon the time of waking and arising.

A number of investigators have attempted to elucidate the nocturnal rise of testosterone in plasma. This increase is believed to reflect a change in testicular secretion, since administration of dexamethasone blocked the nightly increase in cortisol, androstenedione and dehydroepiandrosterone but had no effect on testosterone levels (Judd et al., 1973), and the clearance from blood does not vary with the time of day (Southren et al., 1967). As the amplitude and frequency of LH peaks has

Time	Saliva	Unbound Plasma	Total Plasma
0900	82 (1.4)	75 (1.3)	5760
1200	72 (1.3)	79 (1.4)	5640
1500	64 (1.4)	68 (1.5)	4644
1800	53 (1.3)	51 (1.2)	4142
2100	38 (1.3)	41 (1.4)	3025

All results expressed as pg/ml

1 pg/ml = 3.47 pmol/l

Figures in brackets represent a percentage of the total plasma concentration.

Table 3.3 Testosterone concentrations at different times during the day in a normal male subject.

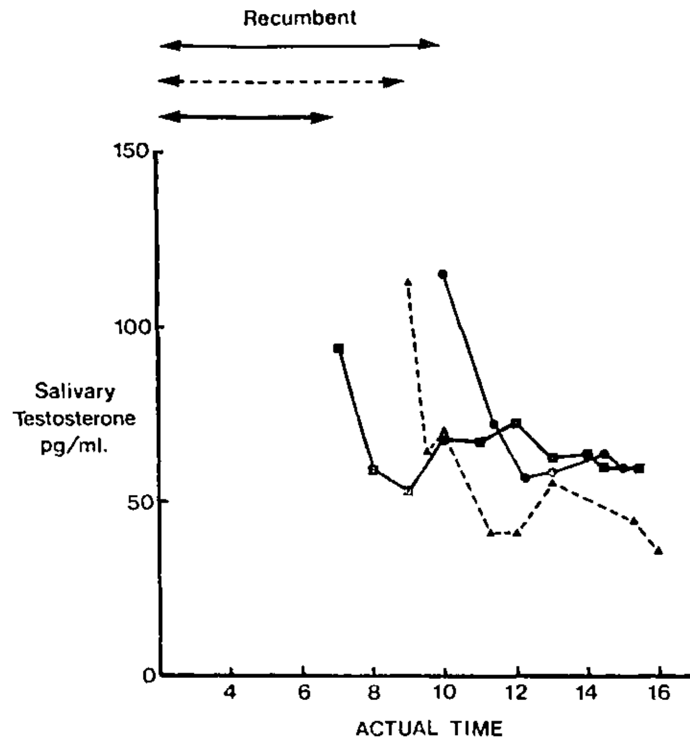

Fig 3.7 The influence of time of waking and arising on salivary testosterone levels.
1 pg/ml = 3.47 pmol/l.

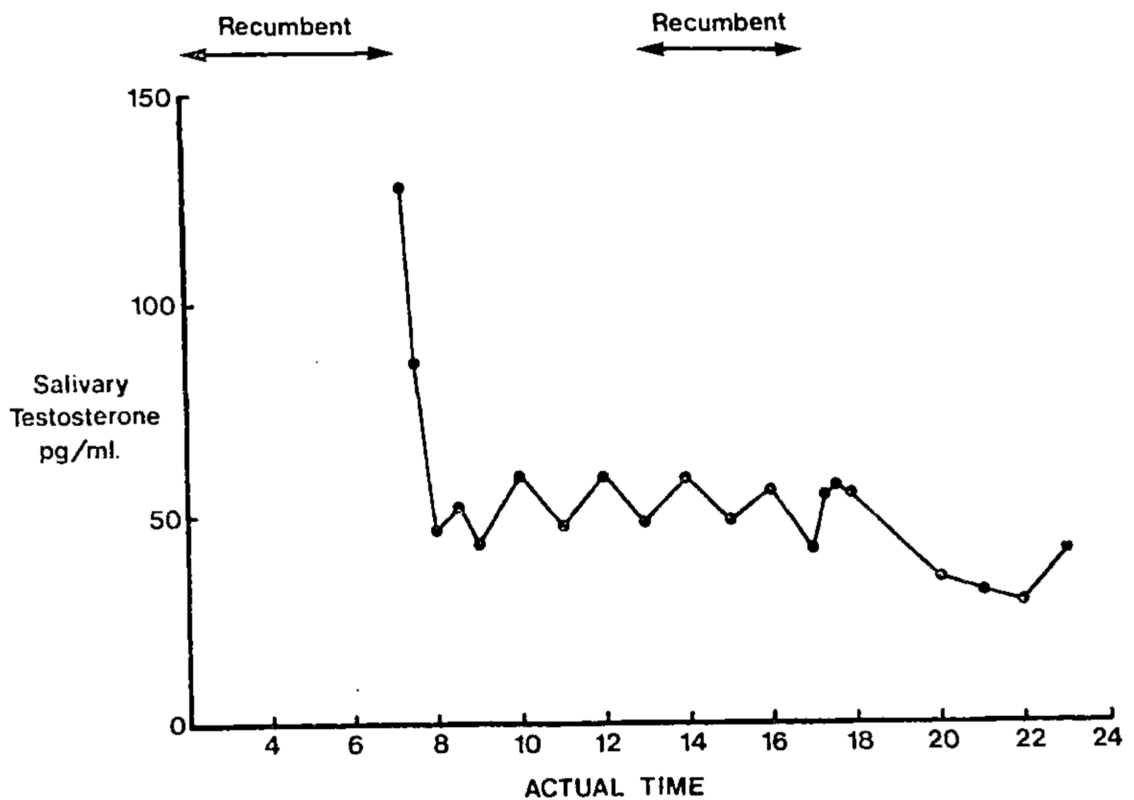

Fig 3.8 The effect of postural change on salivary testosterone concentrations.
1 pg/ml = 3.47 pmol/l.

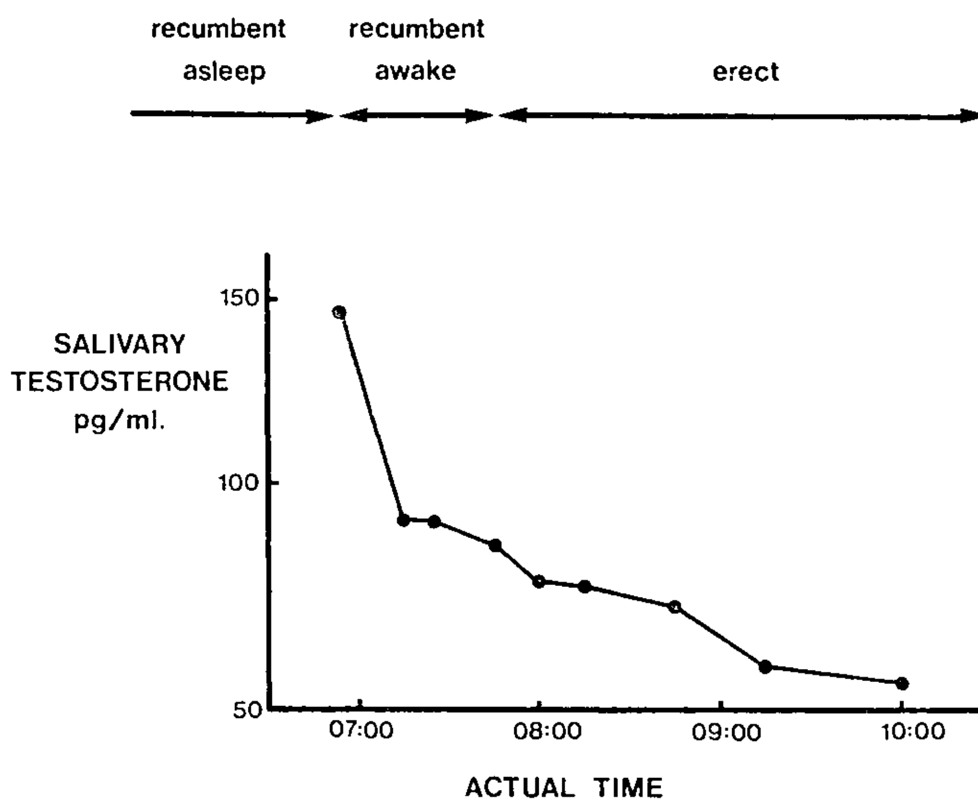

Fig 3.9 The influence of posture on early morning concentrations of testosterone in saliva.
1 pg/ml = 3.47 pmol/l.

not been shown to vary through the day (Faiman and Winter, 1971; Krieger et al., 1972), it has been postulated that the rise in testosterone may be related to the variability of the Leydig cell response to LH at different times during the day. Indeed, it has recently been reported that the response to HCG is greater at 0900 than 1700 (Nankin et al., 1980), which may account for the high testosterone levels in the morning.

Prolactin and Growth Hormone have also been shown to influence the leydig cell response to gonadotrophins in laboratory animals (Zipf et al., 1978). It is well known that prolactin concentrations increase overnight during sleep (Sassin et al., 1972; Parker et al., 1974), and when administered to hypophysectomised male rats, an increase in plasma testosterone was observed (Hafiez et al., 1972). These investigators also demonstrated that the response of testosterone to LH was increased 3 fold when administered with prolactin. Further support for the importance of prolactin in maintaining Leydig cell function in man has come from experiments with dopamine antagonists. Haloperidol has been shown to increase testosterone secretion (Rubin et al., 1976) and Ambrosi et al., 1976, demonstrated an enhanced response of testosterone to HCG in patients given sulpiride.

Rapid sampling techniques, however, have failed to relate the nocturnal increase in plasma testosterone concentration with any change in prolactin levels (Judd et al., 1973; Miyatake et al., 1980), although Rubin et al. (1975), when analysing the results of a number of hormone levels, found that the best correlation was obtained between plasma testosterone and the prolactin level 1 hour earlier.

A number of investigators have examined the direct effect of sleep and sleep stages on plasma testosterone concentrations. Evans et al., 1971, found that fluctuations in testosterone levels were related to sleep in 3 different ways:- 1) On the onset of REM sleep periods an

increase in plasma testosterone was observed. 2) Sometimes a rise preceded the period of REM sleep. 3) Arousal during the night was often associated with increased testosterone concentrations. This study also showed that the usual diurnal trend was reversed when a subject slept during the day on 3 occasions. The majority of investigators on the other hand, have failed to find any relationship between REM sleep and plasma testosterone concentrations (Schiavi et al., 1974; Miyatake et al., 1980). Judd et al., (1973), undertook an extensive study on hormone levels during the night and following daytime sleeping. Subjects slept during the first night, but remained awake during the subsequent 2 nights, and slept during the second and third study days. During the first night, the normal nocturnal increase in testosterone was observed, which was less pronounced on the second night. By the final study night the testosterone rises were of a small magnitude or not present.

The association of sleep, posture, LH, FSH, prolactin, growth hormone and cortisol concentrations have been examined in great detail by a number of groups, yet no definitive mechanism for the nocturnal increase in plasma testosterone has been accepted. This may be due to the differing experimental conditions between groups of workers. Analysis of results is further complicated by episodic fluctuations by different hormones and it may be necessary to increase the sample size further before any meaningful results can be obtained.

There are no previously published data on androgen concentrations taken upon waking and the following 2 hours. Our own studies in the early morning period have shown that salivary testosterone levels do not always match those in plasma and hence saliva samples have not been collected within 2 hours of awakening in any of the studies in this thesis.

b) Females

Testosterone concentrations in saliva samples collected upon waking and through the day on 2 separate occasions from a normal woman are shown in Fig. 3.10. There was an initial and substantial fall in salivary testosterone concentrations commencing at 0700 when the subject awoke, but after 2 hours the levels had reached a plateau and declined little thereafter. This rapid fall in levels in the morning supports the findings of Turkes et al., (1980), and shows a similar pattern to that observed in men. It is therefore clear that for purposes of further study and investigation, it is necessary to standardise the time of day at which samples are taken and in general early afternoon samples have been taken, a time at which salivary levels appear to be on a plateau.

Daily fluctuations of testosterone in female plasma have been studied by a number of investigators in great detail. The majority of workers have failed to demonstrate any regular diurnal pattern in unbound or total plasma samples (Valette et al., 1975; Aedo et al., 1977; Strickler et al., 1981), whereas other investigators (Rosenfield and Helke, 1974; Melis et al, 1978; Yen, 1978) have shown levels to be higher in the morning. Even so, the decrease in unbound and total plasma testosterone is by no means as sharp as that observed in salivary testosterone in the early morning, and so at this time of day saliva may not accurately reflect the unbound plasma concentration.

Cortisol, androstenedione, dihydrotestosterone and testosterone levels in saliva samples collected upon waking and throughout the day from a normal female subject are shown in Fig. 3.11. Concentrations of androstenedione, testosterone and dihydrotestosterone decreased upon waking, whereas the maximum cortisol concentration was not observed until 45 minutes after awakening. It is well documented that cortisol and androstenedione are responsive to the same stimulus, ACTH, and

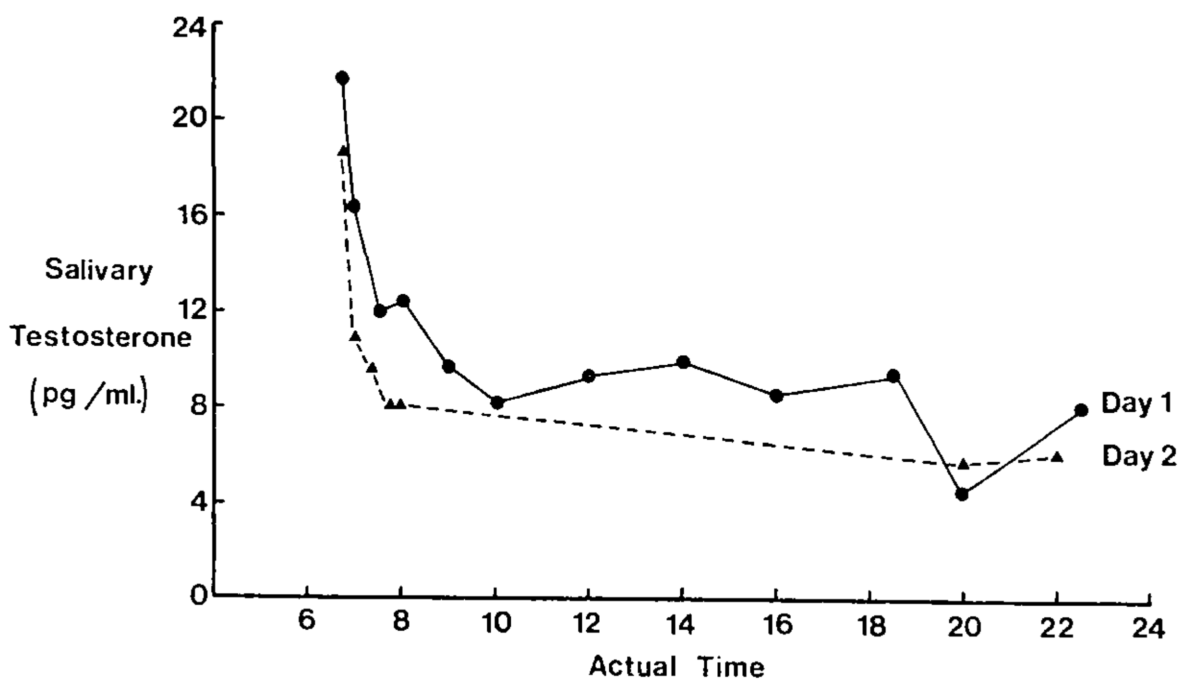

Fig 3.10 Concentrations of testosterone in saliva collected from a normal female subject on two consecutive days.
1 pg/ml = 3.47 pmol/1.

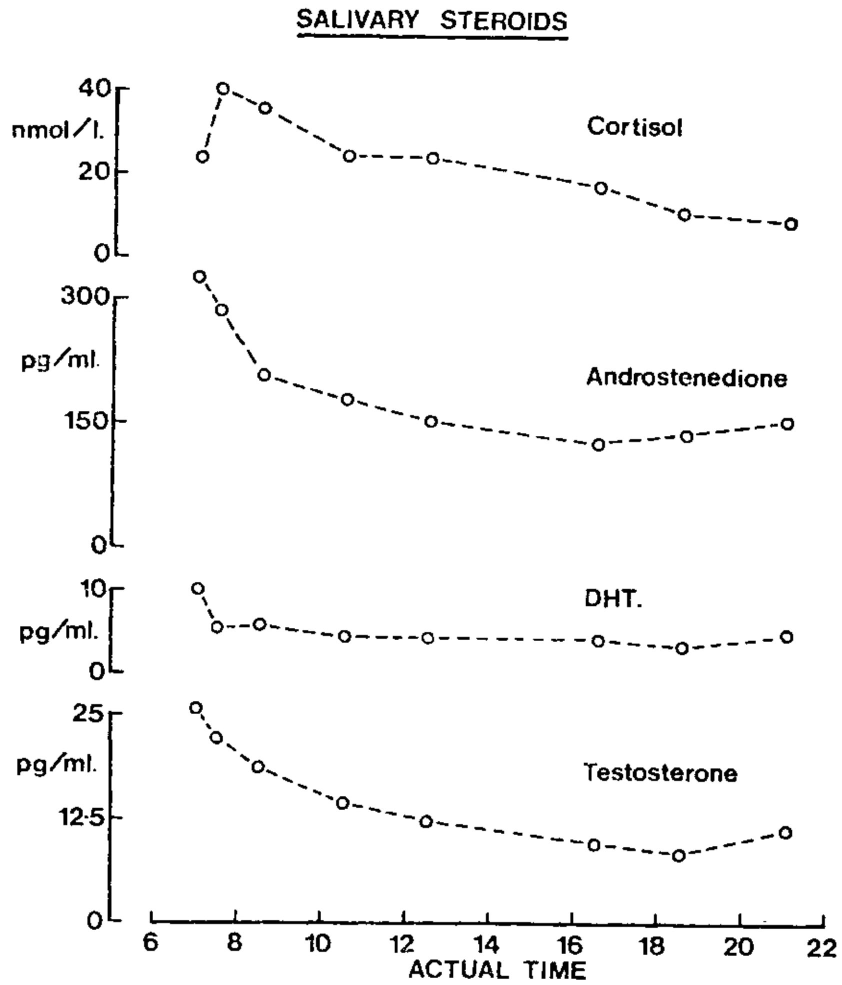

Fig. 3.11 Salivary cortisol, androstenedione, dihydrotestosterone and testosterone levels through the day in a normal woman.

A 1 pg/ml = 3.50 pmol/l.

DHT 1 pg/ml = 3.44 pmol/l.

T 1 pg/ml = 3.47 pmol/l.

exhibit the same circadian rhythm in plasma (Tunbridge et al., 1973; James et al., 1978), but in all subjects studied no correlation was found between cortisol and androstenedione in early morning saliva samples; instead the pattern of androstenedione was similar to testosterone. A decrease was also observed in salivary dihydrotestosterone concentrations upon waking, thereafter levels stayed constant throughout the day. A diurnal rhythm of dihydrotestosterone has not been demonstrated in plasma (Aedo et al., 1977).

3.8 Levels of salivary and plasma androgens in normal subjects

a) Salivary steroids

All samples for androgen measurements were obtained between 0900 and 1400. Unstimulated whole saliva was collected directly into sterile plastic tubes and stored at -20°C prior to analysis. Levels of testosterone, androstenedione and dihydrotestosterone in normal male and female subjects are shown in Table 3.4. Concentrations of testosterone in male saliva show very good agreement with the majority of those previously reported (Turkes et al., 1979; Gaskell et al., 1980; Walker et al., 1980; Wang et al, 1981), but are much lower than those described by Landman et al., (1976), and Luisi et al., (1980). These latter 2 authors used commercial kits designed for the measurement of testosterone in plasma, which do not have the necessary sensitivity required to measure low levels of testosterone in saliva. Likewise, the range of reported values for testosterone in normal female saliva by different authors is great (Table 3.5). Again, the lack of assay sensitivity may account for some of these discrepancies. For example, Luisi et al., (1980), used a sample volume of 0.5 ml and a standard curve sensitivity of 8 pg/tube. Using such an assay system the minimum possible sensitivity assuming 100% recovery would be 16 pg/ml, which is above the upper limit of normal reported in this study. Concentrations of testosterone in saliva as reported by other investigators are

		Males	Females
Testosterone	pg/ml	78 ± 23	7.7 ± 2.6
	pmol/l	271 ± 80	27.0 ± 9.0
	No. of subjects	24	34
Androstenedione	pg/ml	69 ± 22	70 ± 30
	pmol/l	242 ± 77	245 ± 105
	No. of subjects	15	18
Dihydrotestosterone	pg/ml	6.7 ± 2.0	4.5 ± 1.3
	pmol/l	23.0 ± 7.0	15.5 ± 4.5
	No. of subjects	13	17

Table 3.4 Normal salivary androgen concentrations (mean ± SD)

		Males	Females
Testosterone	pg/ml	4050 ± 1450	196 ± 68
	nmol/l	14.1 ± 5.0	0.7 ± 0.3
	No. of subjects	12	36
Androstenedione	pg/ml	631 ± 160	787 ± 355
	nmol/l	2.2 ± 0.6	2.8 ± 1.2
	No. of subjects	13	18
Dihydrotestosterone	pg/ml	373 ± 107	90 ± 44
	nmol/l	1.3 ± 0.4	0.3 ± 0.2
	No. of subjects	13	16

Table 3.5 Normal plasma androgen concentrations (mean ± SD)

shown in Table 3.6. When extracting saliva volumes of greater than 0.5 ml (as is the case for female samples), it was necessary to separate testosterone from more polar artifacts by chromatography. This separation procedure was not necessary when assaying male samples, when the volume is 0.2 ml. This discrepancy can be explained by the relatively higher concentration of interfering compounds compared to testosterone in female saliva samples. A pre-assay purification procedure has not been performed by other workers so may account for their high values.

Concentrations of androstenedione and dihydrotestosterone in male or female saliva have not previously been reported. There was no significant difference between levels of androstenedione in saliva from normal males or females, but as expected, levels of dihydrotestosterone in male samples were higher than those found in female saliva ($p < 0.001$).

Salivary cortisol concentrations are similar to those reported in the literature (Walker et al., 1978; Umeda et al., 1981).

b) Plasma androgens

Levels of androgens in male plasma are in good agreement with levels reported in the literature (West et al., 1973; Fiorelli et al., 1976; Harman and Tsitouras, 1980). However, reported concentrations of testosterone in female plasma are generally higher than those described in this thesis. But levels in the present study compare well with samples measured by other investigators after chromatography (Parker et al., 1975; Yen, 1978). De Gomez et al., (1982), using 3 different antibodies, demonstrated that chromatographic separation lowered the apparent level of testosterone in female plasma samples by as much as 200%, whereas levels in male plasma showed no difference. The antibody cross-reactions could not solely account for the disparity between measurements, thus it is clear that pre-assay chromatographic purification is necessary in female plasma if accurate results are to be achieved.

INVESTIGATOR	MALE SALIVA	FEMALE SALIVA
Landman et al., 1976	295 ± 36	195 ± 25
Smith et al., 1978		4 - 24
Turkes et al., 1979	32 - 90	
Gaskell et al., 1980	70 - 105	
Luisi et al., 1980	104 - 360	34 - 108
Turkes et al., 1980		am 50 pm 9.5
Walker et al., 1980	am 106 ± 48; pm 67 ± 38	
Wang et al., 1981	84 ± 35	
Tames and Swift, 1982	am 118 ± 44; pm 56 ± 39	am 54 ± 32; pm 24 ± 21
Present study	78 ± 23	7.7 ± 2.6

All levels expressed as pg/ml 1 pg/ml = 3.47 pmol/l

Table 3.6 Reported concentrations of salivary testosterone.

3.9 Salivary testosterone concentrations through the menstrual cycle

Saliva samples were collected at 1400 from 2 women aged 26 and 35 years with normal menstrual histories. This limited investigation did not reveal any obvious pattern of testosterone values, thus there did not appear to be any reason for preferring one particular part of the cycle at which samples should be taken. Testosterone concentrations through the cycle are shown in Fig. 3.12.

There are some reports in the literature which suggest that plasma testosterone levels vary through the menstrual cycle, with a tendency to peak at the time of ovulation and possibly in the luteal phase. (Judd and Yen, 1973; Abraham, 1974; Dawood and Saxena, 1976; Guerrero et al., 1976; Kim et al., 1976; Vermeulen and Verdonck, 1976; Motohashi et al., 1979). Abraham, (1974), using selective endocrine suppression procedures showed that the adrenal contribution to peripheral testosterone, dihydrotestosterone, androstenedione, DHEA and DHEAS was relatively constant throughout the cycle, and that it was the increased ovarian contribution that was accountable for the high plasma androgen concentration at mid cycle. Some investigators have failed to find any difference in levels through the cycle (Tyler et al., 1975; Valette et al., 1975). This discrepancy may be explained by the random variations in plasma androgen levels observed by all investigators and the differing experimental conditions and analysis of results by these authors.

3.10 Effect of ACTH administration on salivary and plasma androgen and cortisol concentrations

Synacthen (250 μg) was administered intravenously to a normal male volunteer over a period of 1 hour starting at 1130. Matched saliva and blood samples were collected at intervals of 15 minutes before and during the injections, and a further 3 matched samples in a period of 75 minutes following the last injection. Saliva and plasma levels of

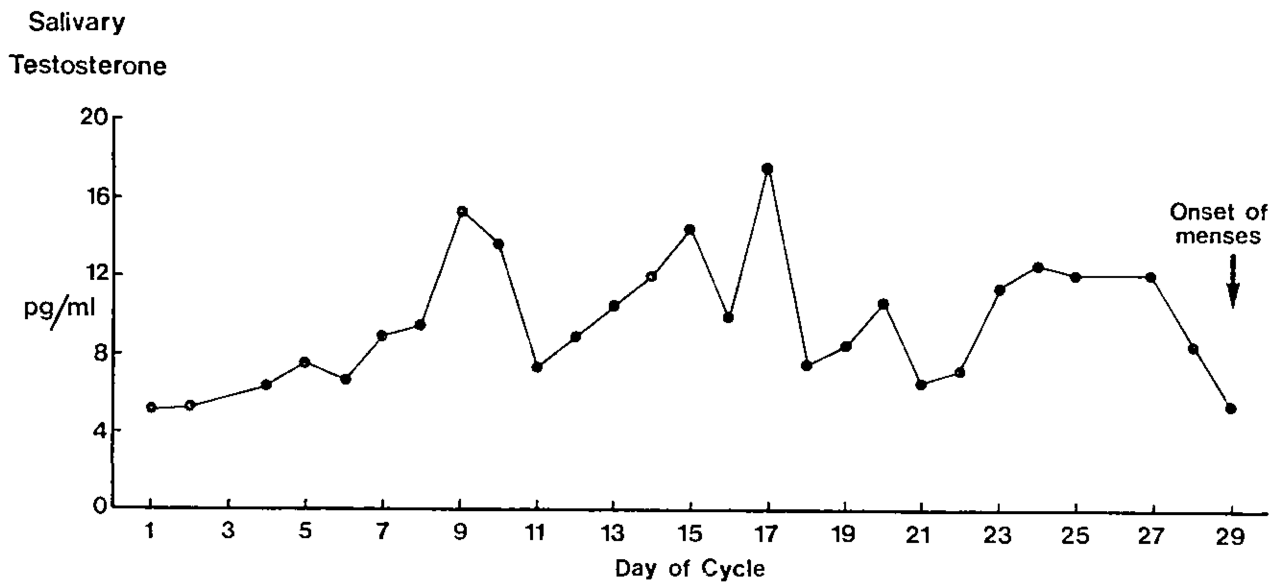

Fig 3.12 Salivary testosterone levels through the menstrual cycle. 1pg/ml = 3.47pmol/1.

cortisol, androstenedione, dihydrotestosterone and testosterone are shown in Fig. 3.13.

As expected, there was a good response of cortisol and androstenedione concentrations in both saliva and plasma after ACTH stimulation. Cortisol is secreted exclusively in the adrenal, androstenedione, on the other hand, is derived from both the adrenal glands and the testes in men, the relative contribution from each source depending upon the time of day (Judd and Yen, 1973). The rise in both salivary and plasma steroids was evident 30 minutes after the first injection, and after 60 minutes salivary androstenedione levels had increased by 350%. A similar rise was observed for plasma concentrations. Mean levels of androstenedione in saliva represented 8% of the total plasma concentration. This is similar to reported levels of unbound androstenedione in plasma (Forest et al., 1968; Dunn et al., 1981).

The increment of the cortisol response to ACTH after 60 minutes was higher in saliva (590%) than plasma samples (340%) , and after 2 hours this discrimination was even greater. This can be explained by the change in distribution of cortisol amongst the binding proteins in plasma. The binding capacity of corticosteroid binding globulin (CBG) is limited and as the total plasma concentration approaches the binding capacity, there is a disproportionately higher level of unbound and albumin bound cortisol. This further supports the observation that salivary cortisol concentrations are a good reflection of the unbound level of cortisol in plasma.

In the normal male, testosterone and dihydrotestosterone are almost exclusively derived from the testes. Consequently no response to ACTH was observed in either saliva or plasma samples of these androgens. This is in contrast to the findings of Vermeulen and Verdonck, (1976), who found a mean decrease in plasma testosterone of 19%, 60 minutes after ACTH injection in 10 subjects. Smals et al., (1974), attributed

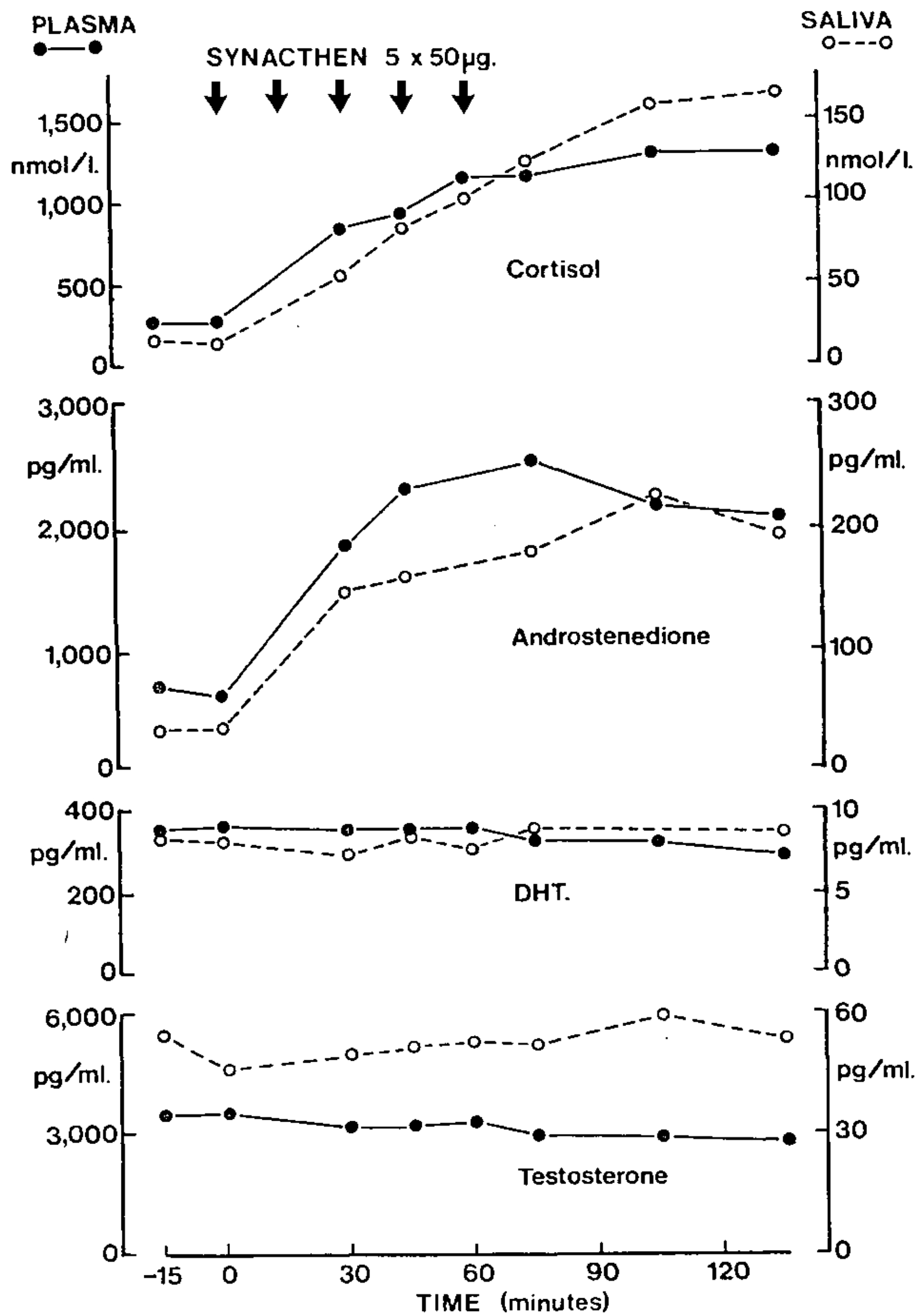

Fig 3.13 The response of salivary cortisol, androstenedione, dihydrotestosterone and testosterone levels to ACTH administration in a normal male subject.

A 1 pg/ml = 3.50 pmol/l.

DHT 1 pg/ml = 3.44 pmol/l.

T 1 pg/ml = 3.47 pmol/l.

this decrease purely as the normal diurnal rhythm. Although the acute response to ACTH is controversial, it is generally accepted that chronic ACTH administration (1 - 4 days) results in a marked decrease in plasma testosterone concentrations in normal men (Irvine et al., 1974; Forest et al., 1978). It is believed that ACTH exhibits its effect on the hypothalamic-pituitary-testicular axis at the periphery, since no effect on pituitary release of gonadotrophins has been demonstrated. Nevertheless, it is not known whether ACTH acts upon the Leydig cell itself or whether this action is mediated through an adrenal secretory product.

3.11 The effect of exercise on salivary and plasma androgens in men

Matched saliva and plasma samples were collected from a normal male subject at 15 minute intervals for 3 hours. During this period the subject remained in a recumbent position for 90 minutes, allowed to gently walk around for the following 30 minutes before exercising for 10 minutes. The exercise consisted of continuous stool stepping which resulted in an increase in pulse rate from 72 to 180 pulses/minute. During the recovery period, the subject was allowed to walk around freely.

Salivary cortisol and androstenedione levels closely matched each other and their corresponding plasma concentrations (Fig. 3.14). The usual diurnal decrease was observed for the first half of the study, but levels rose slightly when the subject stood up and walked around. It is not clear from this data whether this alteration resulted from the change in posture or the increased stress associated with movement. Certainly after intense exercise, there was a marked increase in both salivary and plasma cortisol concentrations, and a similar but smaller rise in androstenedione levels which fell off 30 minutes post exercise. The response of the testicular androgens was far less obvious; only a slight increase of testosterone was observed in both saliva and plasma, whilst dihydrotestosterone levels remained constant.

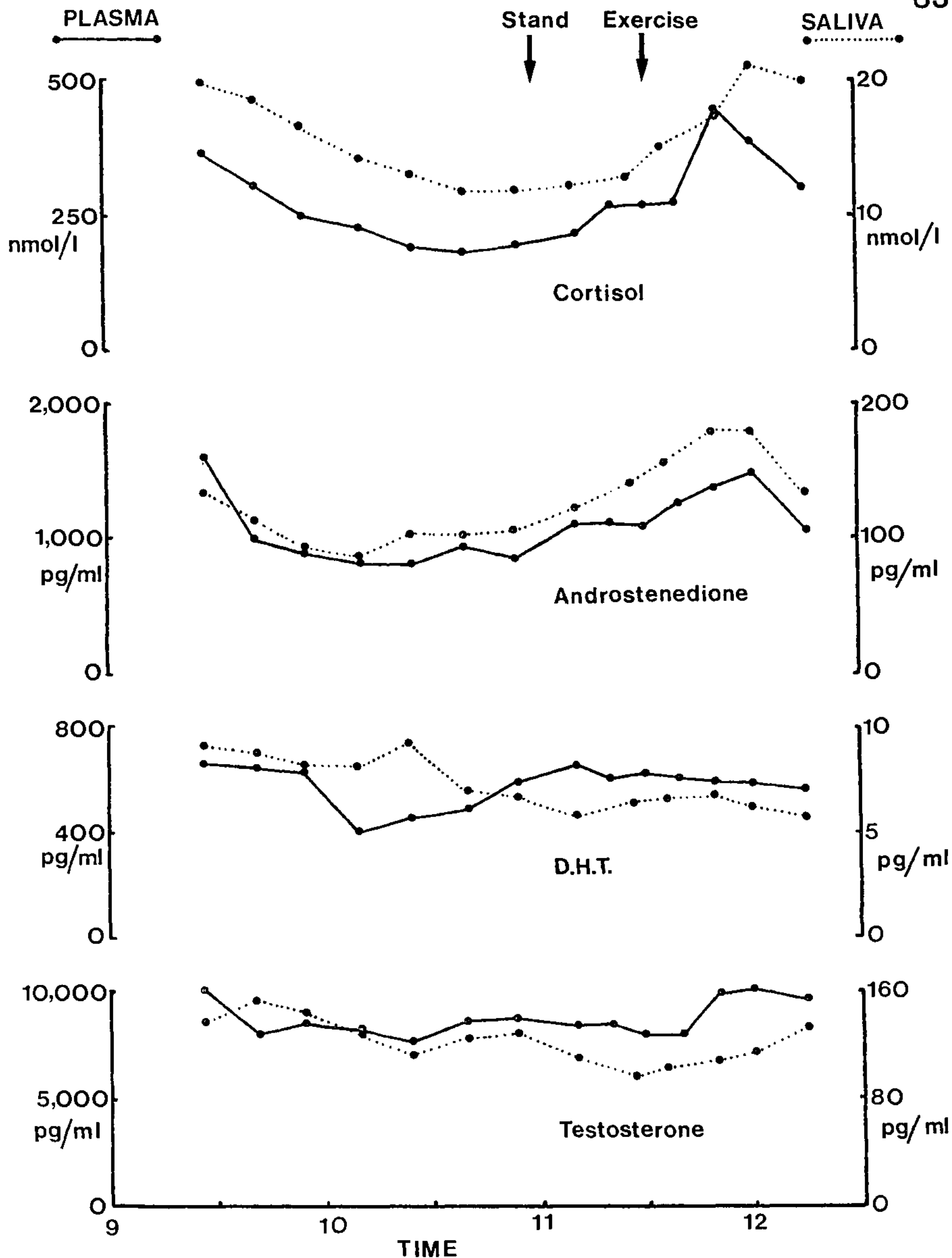

Fig 3.14 The effect of short intense exercise on salivary and plasma androgen and cortisol concentrations in a normal male subject.

A 1 pg/ml = 3.50 pmol/l.

DHT 1 pg/ml = 3.44 pmol/l.

T 1 pg/ml = 3.47 pmol/l.

The effect of exercise on corticosteroids is well documented (Few, 1974; Cashmore et al., 1977), and has been shown to influence plasma testosterone concentrations, the pattern depending upon the type of exercise (Berchthold et aL, 1978). Short intense exercise increases levels whereas long endurance exercise, such as marathon running, suppresses levels significantly, (Morville et aL, 1979).

This experiment further demonstrates the usefulness of salivary measurements in physiological studies. Steroid levels in saliva are a good monitor of plasma concentrations in normal subjects. Saliva sampling is particularly suited to the investigation of steroid hormone metabolism and exercise, since samples may be collected with the minimal disruption to the subject's endocrine balance or study protocol.

3.12 Salivary androgen and cortisol concentrations during pregnancy

Saliva samples were collected from a normal woman at intervals of 2 weeks during the second and third trimesters of pregnancy, and 1 week post partum. All samples were collected at 1400. It is generally accepted that the hepatic production of SHBG increases during pregnancy. Thus increased concentrations of plasma testosterone during pregnancy result in only modest rises in unbound plasma testosterone, if any at all (Rivarola et al., 1968). As shown in Fig 3.15, levels of testosterone and dihydrotestosterone in saliva remain in the normal non - pregnant range throughout pregnancy, thus substantiating the view that salivary steroid levels are a good monitor of unbound plasma concentrations. Accordingly, levels of testosterone and dihydrotestosterone remained fairly constant after delivery, whereas total plasma concentrations would be expected to decline sharply (Pearlman et aL, 1967).

Concentrations of androstenedione in saliva show a slight tendency to decrease through pregnancy. Since androstenedione shows little

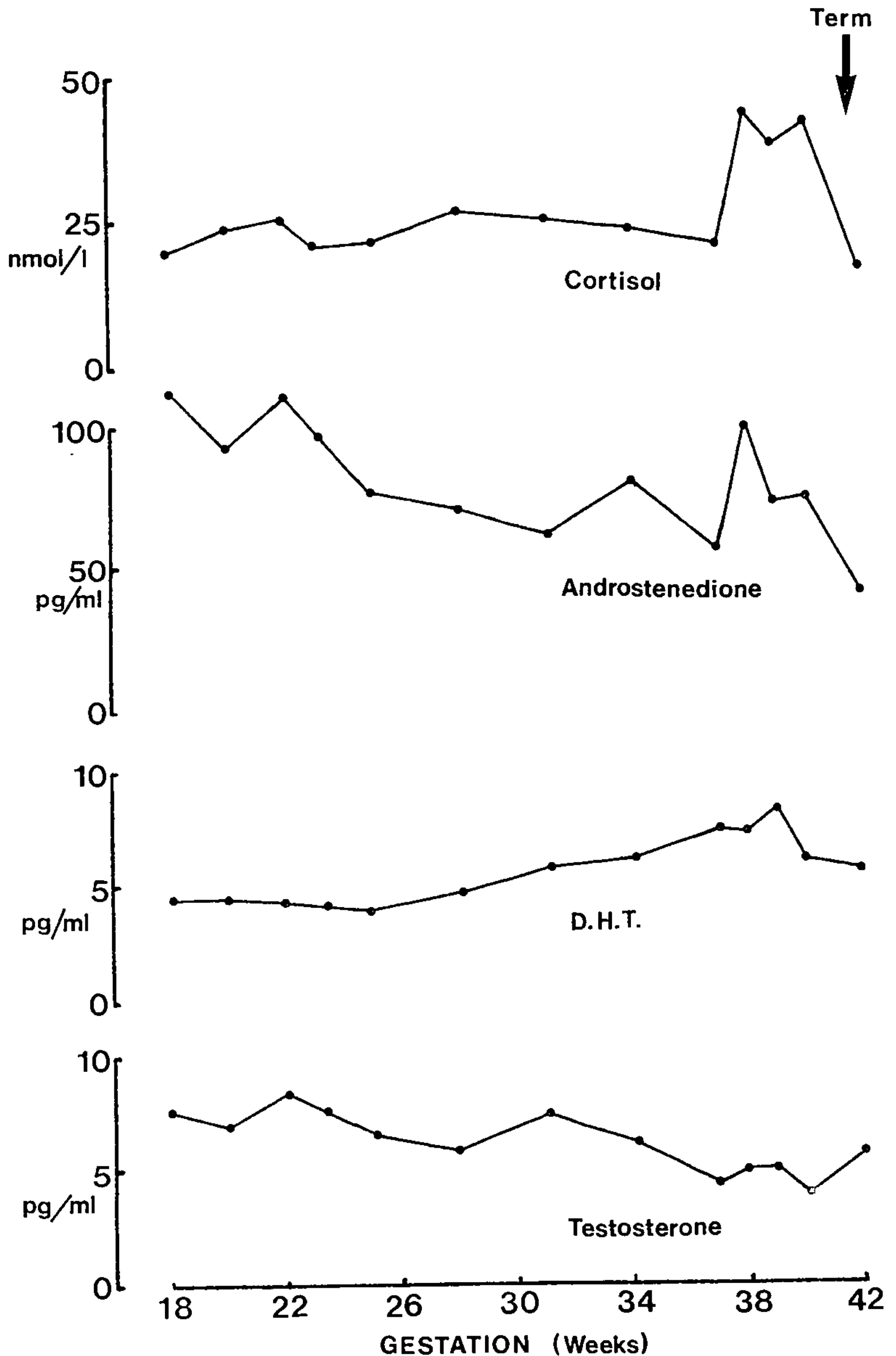

Fig 3.15 Salivary androgen and cortisol concentrations through the 2nd and 3rd trimesters of pregnancy.

A 1 pg/ml = 3.50 pmol/l.

DHT 1 pg/ml = 3.44 pmol/l.

T 1 pg/ml = 3.47 pmol/l.

affinity to SHBG, the percentage binding of androstenedione to plasma proteins in pregnant women is no different from non-pregnant subjects. Hence this decrease in salivary levels represents a decrease in androstenedione production and not any alteration in the binding.

Many investigators have studied the metabolism of cortisol in pregnancy. Total plasma cortisol and CBG progressively rise during gestation and a smaller but significant rise in unbound plasma cortisol has also been demonstrated, (O'Connell and Welsh, 1969; Burke and Rowlett, 1970; Westphal, 1971). This rise in unbound cortisol may be responsible for symptoms of hypercortisolism in late pregnancy. An interesting explanation for this increase in unbound cortisol is the release of a corticotrophin-like hormone from the placenta which may be non-suppressible by unbound plasma cortisol (Genazzani et al., 1975; Rees et al., 1975).

Salivary cortisol levels in the subject studied were higher than the majority of levels in non pregnant women at 1400 and remained constant until week 38. Thereafter a sharp increase in cortisol levels, synchronous with androstenedione, was observed. The only previous report of salivary cortisol concentrations in pregnant women showed levels in the third trimester of pregnancy to be twice those in non pregnant women (Greaves and West, 1963).

3.13 Binding of steroids to salivary constituents

Fresh whole saliva (1.0 ml) was dialysed against sterile saline containing 20,000 dpm 1,2,6,7,16,17 ^{3}H-testosterone, 1,2,6,7 ^{3}H-androstenedione, 1,2,6,7 ^{3}H-cortisol and 1,2,4,5,6,7 ^{3}H-dihydrotestosterone for 4 hours at 37 $^{\circ}$C. The radioactivity on either side of the membrane was determined and the percentage steroid bound calculated according to Antoniades et al., (1960).

The percentage of testosterone bound to salivary constituents in

9 normal subjects was 15.6 ± 7.4%. This binding which showed no sex difference was similar to that observed in parotid fluid in the same subjects. Likewise no difference was found between the binding of testosterone in whole saliva and saliva which had been centrifuged prior to dialysis.

It is known that salivary albumin originates from plasma (Hattingh, 1979), although the significance of albumin in saliva is unknown. The present consensus of opinion is that the molecule passively diffuses into the glandular secretion from plasma or there may be significant extra-glandular transfer, or the mechanism may involve both processes. The concentration of albumin in saliva is thought to be about 1% of that in plasma, nevertheless transfer of small amounts of testosterone bound plasma proteins may account for the higher levels of hormone in female saliva compared to the unbound plasma fraction. This is described in more detail in the next section. The difference between the testosterone level in saliva and the unbound plasma testosterone concentration was not observed in normal men. Perhaps the high androgen concentrations mask any protein bound transfer. Wang et al., (1981), showed that testosterone did not bind to SHBG in the saliva of males with normal plasma SHBG levels.

The major organic constituent of human saliva is mucin. This is a glycoprotein with a molecular weight of around one million and a concentration in mixed saliva of about 2.5 g/l. The percentage binding of testosterone to 0.25% mucin, obtained from mouse submaxillary glands, was 14%, which is similar to that observed in saliva. The amount of dihydrotestosterone bound to saliva from 5 normal females was 32 ± 13%, which also compared well with the binding to mucin (31%). Whereas androstenedione binding to mucin (7%) could not solely account for the association in saliva (15 ± 9%). No affinity was demonstrated between cortisol and mixed or parotid saliva which is in agreement with previous

reports (Greaves and West, 1963; Katz and Shannon, 1969). Likewise, cortisol was not shown to bind to mucin.

3.14 Discussion

This study confirms the early work of Katz and Shannon, (1969), and more recent investigators (McVie et al., 1973; Smith et al., 1979; Umeda et al., 1981) that salivary concentrations of certain steroids reflect the non protein bound concentration in blood. Since the measurement of unbound plasma levels is complicated and time consuming, salivary steroid measurements are a useful alternative, and may give a better indication of the biologically available steroid than do total plasma levels, especially in conditions of altered plasma protein binding.

The technique of sampling is simple and non-invasive and allows the collection of multiple samples with little inconvenience to the patient. Two physiological studies have been described when salivary collection is preferable to taking blood samples. Salivary measurements are particularly useful in the investigation of steroid metabolism in exercise, since catheterisation or painful venepuncture would hinder the athletes performance. It is well known that steroid hormones are secreted in an episodic manner, hence a single plasma determination may not necessarily be representative of the mean hormone level over a prolonged period of time. Plasma proteins are thought to act as a store and buffer for steroids so that large fluctuations in secreted steroid do not result in varying tissue exposure. Salivary and unbound concentrations should, therefore, be a more stable indication of biologically active hormone than total plasma levels.

It is clear that the time of day at which salivary samples should be taken must be standardised. Wherever possible, samples should be collected at a time where levels are most stable. High salivary

testosterone levels in the morning have been reported by a number of investigators, but the reasons have not been investigated. This study has demonstrated a steady posture independent increase in salivary testosterone through sleep, which fell rapidly upon waking with only minor changes then throughout the rest of the day. A number of explanations can be postulated for these high androgen levels in early morning saliva. Unstimulated whole saliva shows a significant circadian rhythm in flow rate, levels at 1500 being roughly twice those at 0500 in subjects without sleep (Dawes, 1972). However, during sleep, the flow rate is extremely low (< 50 µl/min), in fact much lower than when samples are collected from subjects wakened in the middle of the night (Schneyer, 1965). Possible evaporation of water from saliva during sleep would be expected to increase concentrations of salivary constituents, which upon waking would rapidly return to normal. In order to test this theory, concentrations of steroids were measured in a normal male immediately upon waking and 10 minutes after a water mouthwash. Although testosterone concentrations were slightly lower after the mouthwash, they were no lower than was expected for 10 minutes following waking. In addition, if these high early morning androgen levels were caused by water evaporation, then salivary cortisol concentrations in the same samples would also be expected to fall. On the contrary, both salivary and plasma cortisol concentrations increased upon waking in all subjects studied.

As previously described in this section, the atropine induced reduction of salivary flow resulted in increased concentrations of salivary testosterone. This would be similar to the flow rate during sleep, therefore it seems that the low salivary flow rate overnight is responsible for the high concentrations of androgens in the morning. The effect of decreased flow rate on cortisol concentrations is less well defined.

A possible explanation for the discrepancies between testosterone concentrations in saliva and the unbound plasma fraction at low flow rates is the possible overnight metabolism of steroids in salivary glands. As the flow rate increases, steroid metabolites would be washed out, and the relationship between salivary and unbound steroids restored. Both oxidative and reductive metabolism of androgens has been demonstrated in animals. Baldi et al., (1972), showed that incubation of ^{14}C testosterone with minced rat submaxillary gland for 2 hours at $37^{o}C$ resulted in the formation of ^{14}C androstenedione, which accounted for 90% of the incubation product, and to a lesser extent androstanedione and androsterone. Likewise, oestradiol was shown to be formed from oestrone. The 17β hydroxysteroid dehydrogenase activity was shown to be higher in female adult animals and decreased with age. Weiner et al., (1970), also showed that physiological amounts of ^{14}C testosterone infused for 10 minutes into the submaxillary gland of adult dogs resulted in 80% of the radioactivity being incorporated into androstenedione.

In contrast to these studies labelled androstenedione has been converted to androsterone, testosterone, dihydrotestosterone and androstanediol in the presence of NADPH in rat submaxillary gland <u>in vitro</u> (Coffey, 1973; Coffey et al., 1979), indicating the presence of 5α reductase and 3β hydroxysteroid dehydrogenase activity. Likewise metabolism of androstenedione in human submaxillary gland was shown to be mainly reductive (Elattar, 1974). The presence of 5α reductase activity has also been demonstrated in rat oral mucosa (Vittek et al., 1974), which was increased with administration of medroxyprogesterone acetate.

The passage of steroid hormones into saliva secretions may not be as simple as was first thought. Testosterone levels are dependent on salivary flow at low flow rates. Although the presence of low affinity androgen binding proteins have been demonstrated in salivary secretions,

good relationships between salivary testosterone levels and the unbound plasma concentration still exist. This relationship though has not been examined in patients with salivary gland disorders, so the assessment of endocrine function by salivary measurements must be performed with caution in these patients.

SECTION 4

SALIVARY AND PLASMA ANDROGENS IN PATHOLOGICAL CONDITIONS

4.1 Introduction

There have been a substantial number of studies in women with hyperandrogenism and men with hypogonadism, in which various steroid levels have been determined. Many of these studies have been undertaken with the objective of defining altered hormone patterns, and attempting to relate these to the clinical status of the patient. Initially it was anticipated that when it became practical to measure circulating plasma testosterone levels in women, these might reflect the supposed increase in production of androgen which was assumed to be the basis of the hyperandrogenism observed. However, in the case of testosterone the effect of binding proteins made the interpretation of plasma testosterone levels in biological terms extremely difficult.

In the previous section salivary testosterone and cortisol concentrations have been shown to be a good reflection of the unbound steroid in normal males. It is generally believed that only this unbound plasma fraction of steroid is biologically active (Mowszowicz et al., 1970; Laznitzki and Franklin, 1972; Anderson 1974), hence salivary assays may give an indication of the steroid available for metabolism in the tissues without the need to employ the complicated techniques required to measure unbound plasma levels. This measurement is particularly helpful in conditions of altered plasma protein binding. However there is evidence to suggest that albumin bound steroid is also available for metabolism in certain tissues (Baird et al., 1969; Pardridge and Meitus, 1979b). The biological significance of albumin bound steroid will be discussed in more detail in Section 5.

It is well documented that the free testosterone concentration in

plasma is elevated in many cases of polycystic ovarian disease (PCO) and idiopathic hirsutism, in association with decreased levels of SHBG (Anderson, 1974; Motohashi et al, 1979; Yen, 1980), a finding which renders difficult the interpretation of total plasma testosterone levels. Hence salivary testosterone concentrations would permit greater biochemical discrimination between normal and hyperandrogenic women than total plasma testosterone levels.

The production of androstenedione, which is an important precursor for the more potent androgens testosterone and dihydrotestosterone, has been shown to be increased in nearly all cases of PCO and hirsutism (Bardin and Lipsett, 1967; Kirshner and Jacobs, 1971). The conversion of testosterone to dihydrotestosterone prior to binding to the intracellular receptor is an important mechanism for androgen action in skin. Nevertheless the reported incidence of elevated plasma androgen levels in patients with hirsutism is variable and disappointingly low (Tulchinsky and Chopra, 1974; Abraham et al., 1976; Meikle et al., 1979). Consequently a more discriminating test would be helpful in the diagnosis and treatment of female hyperandrogenism.

The purpose of this study was to examine the relationship between salivary, unbound and total plasma testosterone, androstenedione and dihydrotestosterone in normal subjects and women with hyperandrogenism, and to investigate the effect of antiandrogen treatment on these levels. The relationship between unbound and non-SHBG bound and salivary testosterone have also been investigated in these patients.

Another application of salivary androgen measurements is in the study of men with infertility and hypogonadism. A large proportion of men with hypogonadism have elevated plasma SHBG concentrations, resulting in disproportionately lower levels of unbound testosterone and dihydrotestosterone than total plasma concentrations compared with normal men. Salivary and plasma androgen concentrations have been measured in men

with hypogonadotrophic hypogonadism, Klinefelters syndrome and gynecomastia.

4.2 Relationship between salivary, unbound and total plasma androgen concentrations in normal and hyperandrogenic women

a) Testosterone

Blood and saliva were collected from 10 normal women aged between 18 and 38 who were not receiving any form of hormonal contraception and who had regular menstrual cycles. Matched samples were also collected from 39 women with clinical signs of hyperandrogenism (23 with idiopathic hirsutism, 12 with polycystic ovaries and 4 with acne) at their first visit to the clinic, and from 10 cases following 3 months antiandrogen treatment. This consisted of the administration of cyproterone acetate (CA) and ethinyl oestradiol (EE) in a reverse sequential regimen (Hammerstein and Cupceanu, 1969) for 3 months. CA (100 mg) daily was taken from days 5 - 15 of the menstrual cycle and EE (50 μg) from days 5 - 26. The diagnosis of PCO was based on the presence of amenorrhoea or oligomenorrhoea with elevated plasma LH levels. Laparoscopy in 7 patients confirmed this diagnosis. All patients with idiopathic hirsutism had normal cycles. Abnormal LH concentrations were observed in 2 cases.

There was a highly significant correlation ($r = 0.79$, $p < 0.001$) between the concentration of testosterone in saliva (12.3 ± 7.8 pg/ml, 42.7 ± 27.1 pmol/l) and the unbound concentration of testosterone in plasma (5.2 ± 3.1 pg/ml, 18.0 ± 10.8 pmol/l) in matched samples collected from normal, hyperandrogenic and CA and EE treated women (fig 4.1). Likewise the correlation between saliva and total plasma was 0.77 in the same group of patients.

These results are in good agreement with the findings of Smith et al.,(1979), who demonstrated a good relationship between salivary and plasma

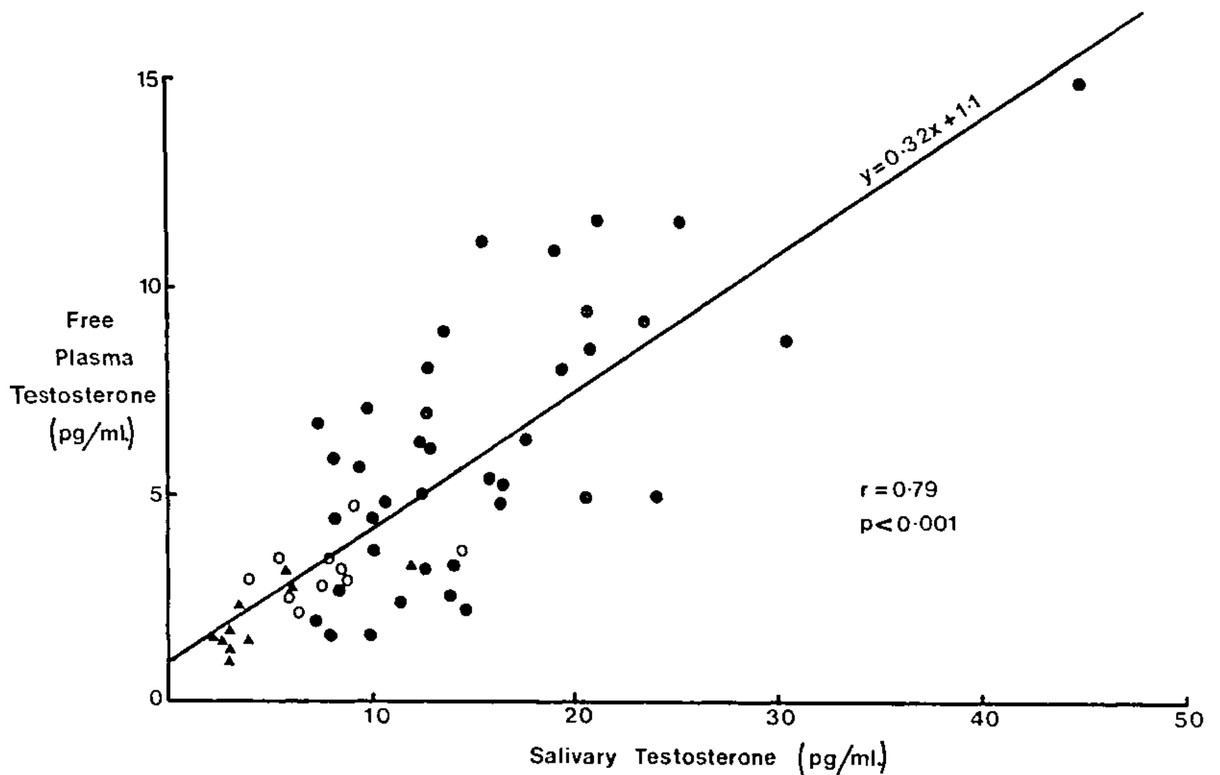

Fig 4.1 Comparison of salivary testosterone with unbound plasma concentrations in patients with PCO, idiopathic hirsutism or acne before treatment (●) after treatment (△) and normal subjects (○).
1 pg/ml = 3.47 pmol/l.

free testosterone in normal women and women with PCO. The concentration of testosterone in saliva was significantly higher than the unbound fraction in plasma. This anomaly has been discussed in detail in Section 3.13, and probably results from the presence of non specific binding proteins in saliva.

A highly significant correlation ($r = 0.75$, $p < 0.001$) was also found between the concentration of testosterone in saliva (11.1 ± 7.3 pg/ml, 38.5 ± 25.3 pmol/l) and the non SHBG bound concentration of testosterone in plasma (94 ± 79 pg/ml, 326 ± 274 pmol/l) in 66 matched samples collected from normal, hyperandrogenic and CA and EE treated women, (Fig 4.2). This data further supports the view that salivary measurements are a good reflection of the biologically active androgen.

An excellent relationship existed ($r = 0.84$, $p < 0.001$) between the unbound plasma testosterone concentration (5.2 ± 4.2 pg/ml, 18.0 ± 14.5 pmol/l) and the non SHBG bound plasma testosterone concentration (106 ± 95 pg/ml, 368 ± 330 pmol/l) in a group of 36 women, comprising of normal subjects, hyperandrogenic and antiandrogen treated women (Fig 4.3). This agrees with the observation that the metabolic clearance rate of testosterone is linearly related to both the free and non-specifically bound testosterone fractions (Vermeulen and Ando, 1979).

b) Androstenedione

In 29 female subjects (17 controls and 12 hyperandrogenic patients) plasma free androstenedione levels and salivary androstenedione concentrations were measured. As shown in Fig 4.4, a significant correlation was found ($r = 0.92$, $p < 0.001$). The mean plasma free level was 118 ± 63 pg/ml (413 ± 217 pmol/l) and the mean salivary concentration was 95 ± 49 pg/ml (326 ± 169 pmol/l). The free androstenedione in plasma ranged from 6.0 to 10.4% of the total concentrations. There was also an excellent correlation ($r = 0.96$, $p < 0.001$) between the total plasma andro-

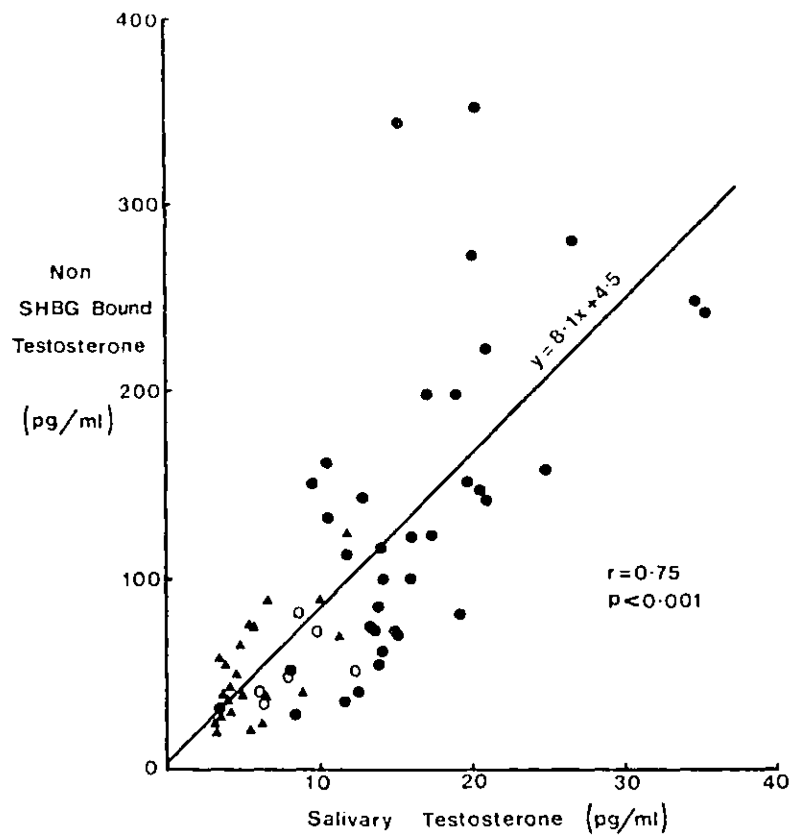

Fig 4.2 Comparison of salivary testosterone with non SHBG bound plasma concentrations in patients with PCO, idiopathic hirsutism or acne before treatment (●), after treatment (▲) and normal subjects (○). 1 pg/ml = 3.47 pmol/l.

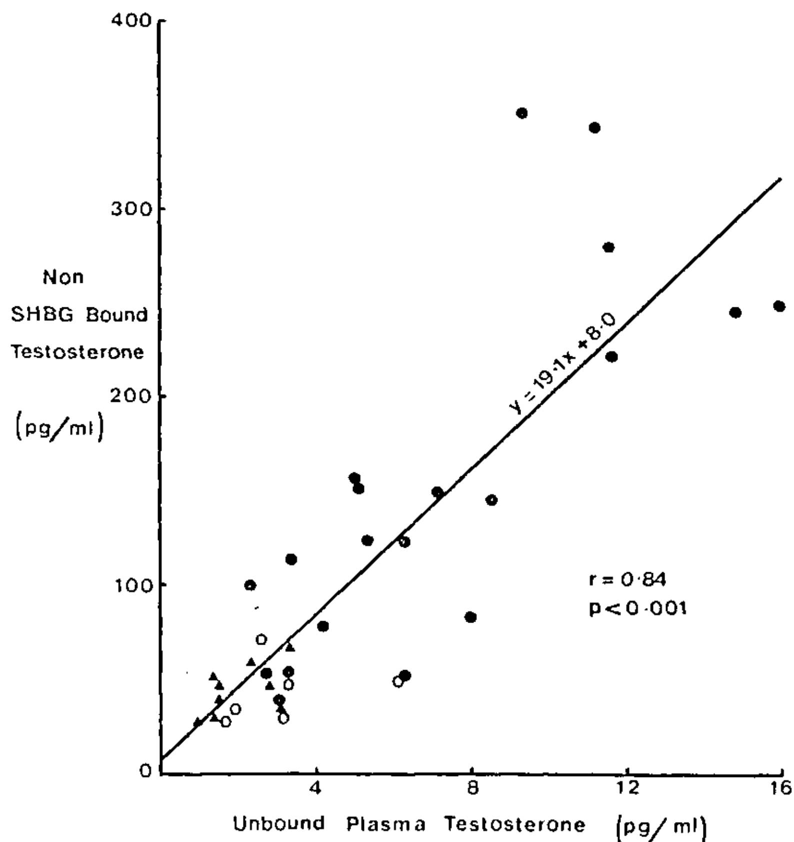

Fig 4.3 The relationship between unbound and non SHBG bound plasma testosterone in hyperandrogenic (●), treated (▲) and normal women (○).
1 pg/ml = 3.47 pmol/l.

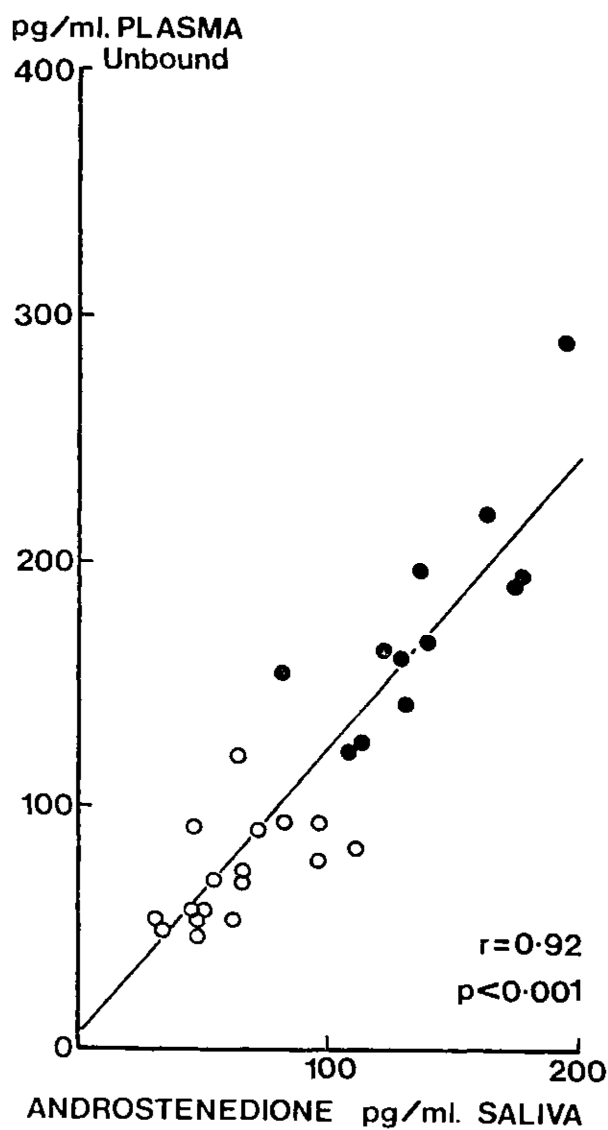

Fig 4.4 Comparison of salivary and unbound plasma androstenedione in hyperandrogenic (●) and normal women (○).
1 pg/ml = 3.50 pmol/l.

stenedione and salivary androstenedione concentrations.

c) Dihydrotestosterone

Salivary, unbound and total plasma dihydrotestosterone levels were measured in matched samples collected from normal, hyperandrogenic and CA and EE treated women. There was a highly significant correlation ($r = 0.82$, $p < 0.001$) between the concentration of dihydrotestosterone in saliva (6.9 ± 2.7 pg/ml, 23.7 ± 9.3 pmol/l) and the unbound plasma concentration (0.93 ± 0.35 pg/ml, 3.2 ± 1.2 pmol/l) in 15 women (Fig 4.5). A similar relationship ($r = 0.82$, $p < 0.001$) was observed between salivary and total plasma concentrations.

4.3 Salivary and plasma androgens in hyperandrogenic and antiandrogen treated women

Hirsutism has been defined by Maroulis, (1981), as excessive growth of androgen dependent sexual hair, such as that of the upper lip, the tip of the nose, the chin, sideburns and earlobe, the upper pubic triangle, the chest, the abdomen as well as the trunk and limbs. Many classifications have been used in an attempt to quantitate the degree of hirsutism. The rate of growth of hair may be assessed by counting or weighing terminal hair, but this is not practical for routine purposes. Other investigators have graded hirsutism generally as mild, moderate or severe, the mild form being diagnosed by the presence of fine pigmented hair over one or a combination of the chin, upper lip and sideburns but excluding complete beard. The hirsutism was classed as moderate if there was coarse pigmented hair on the face, chest and lower abdomen, and severe as typical male distribution of hair, including coarse pigmented hair in the whole beard area often accompanied by other forms of virilisation.

Since the severity of hirsutism at different sites may vary between individuals, Hatch et al., (1981), developed a more complex grading system.

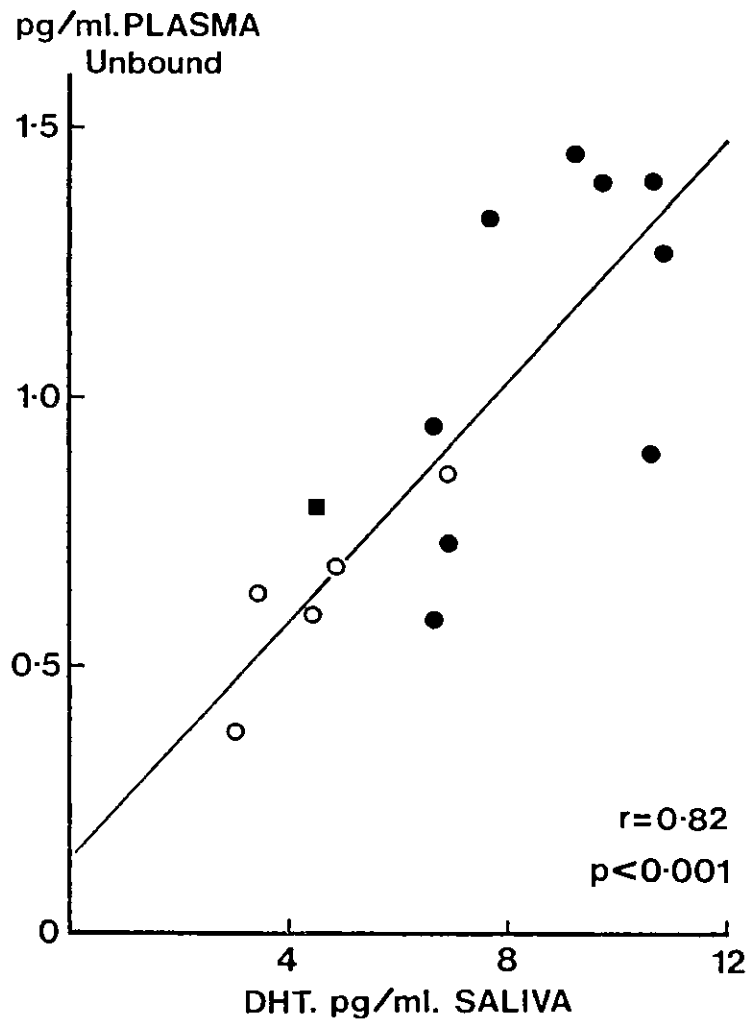

Fig 4.5 Comparison of salivary and unbound plasma dihydrotestosterone in normal (○), hyperandrogenic (●) and treated women (■). 1 pg/ml = 3.44 pmol/l.

The body was divided into 9 areas each scoring in the range 1 to 4 depending on the severity of hair growth. A total score exceeding 8 indicates hirsutism. The problem of grading hirsutism as such is that the amount of sexual hair growth varies with age, race, genetic and ethnic group, and is subject to the clinician's own interpretation. Nevertheless such systems are helpful in the assessment of treatment.

The association between bilateral polycystic ovaries and a clinical syndrome of amenorrhoea, hirsutism and obesity was first demonstrated by Stein and Leventhal, (1935). Since then, a number of similar endocrine disorders but with diverse aetiology have been described. As bilaterally enlarged multicystic ovaries was a prerequisite for the diagnosis, the broader term of polycystic ovarian disease was introduced to cover the range of these syndromes. Hirsutism has been found in 69% of these cases and 41% of patients with PCO are obese (Goldzieher, 1981). Chronic anovulation and elevated LH levels compared with FSH are fundamental features of PCO, but whether these elevated LH concentrations constitute the primary pathology in PCO or result from increased oestrogens is not known.

The relationships between steroid hormone levels and the gonadotrophins in PCO is conflicting. Some authors have shown direct whilst others inverse correlations between LH and testosterone. Likewise, the reported relationships between oestrogens and LH levels are contradictory.

A number of investigators have shown that certain adrenal disorders are associated with PCO. Numerous enzyme defects have been observed in these women, including 11 hydroxylase, 21 hydroxylase and 3β hydroxysteroid dehydrogenase, with a consequent increase in adrenal androgen levels in plasma.

The exact aetiology of PCO is unknown but it has been proposed that the disorder results from the non ACTH mediated increase in adrenal androgen secretion at the time of the adrenarche. This results in the

increased peripheral formation of oestrogen which stimulates LH secretion through a positive feedback on the hypothalamic-pituitary axis. The elevated gonadotrophin drives ovarian stroma cells to produce more androgens which in turn cause more oestrogen to be formed, and a vicious circle is initiated. In this way the androgenic basis of this syndrome is slowly shifted from the adrenals to the ovary (Yen, 1980).

a) Testosterone

Testosterone concentrations in saliva and plasma samples from women with infertility arising from PCO, idiopathic hirsutism, normal subjects and hyperandrogenic women following 3 months treatment with CA and EE are shown in Fig. 4.6. Mean salivary levels were 7.7 ± 2.6 pg/ml (27 ± 9 pmol/l, n = 34) for normal women, 20.6 ± 8.5 pg/ml (71 ± 31 pmol /l, n = 14) for patients with PCO, 13.9 ± 5.6 pg/ml (48 ± 19 pmol/l, n = 30) for women with idiopathic hirsutism and 6.1 ± 4.2 pg/ml (21 ± 14 pmol/l, n = 18) for treated women. The corresponding total plasma testosterone levels were 196 ± 68 pg/ml (0.7 ± 0.2 nmol/l, n = 36) for normal women, 626 ± 187 pg/ml (2.2 ± 0.6 nmol/l, n = 14) for patients with PCO, 421 ± 170 pg/ml (1.5 ± 0.6 nmol/l, n = 30) for women with idiopathic hirsutism and 279 ± 77 pg/ml (1.0 ± 0.3 nmol/l, n = 18) for treated women. Concentrations of LH and FSH in plasma from women with PCO were 30.8 ± 21.2U/L and 6.1 ± 3.5U/L respectively, whereas levels in women with idiopathic hirsutism were significantly lower (8.4 ± 6.2U/L and 3.8 ± 1.7U/L).

Both total plasma and salivary testosterone concentrations in women with PCO and idiopathic hirsutism were significantly higher ($p < 0.001$) than in normal subjects. Salivary levels of testosterone in patients treated with CA and EE were not significantly different from normals, whereas total plasma levels in treated women remained elevated ($p < 0.001$).

This result is further illustrated in Fig 4.7 which shows the effect of 3 months treatment with CA and EE on plasma total and salivary testosterone concentrations in 15 hyperandrogenic women. There was a mean

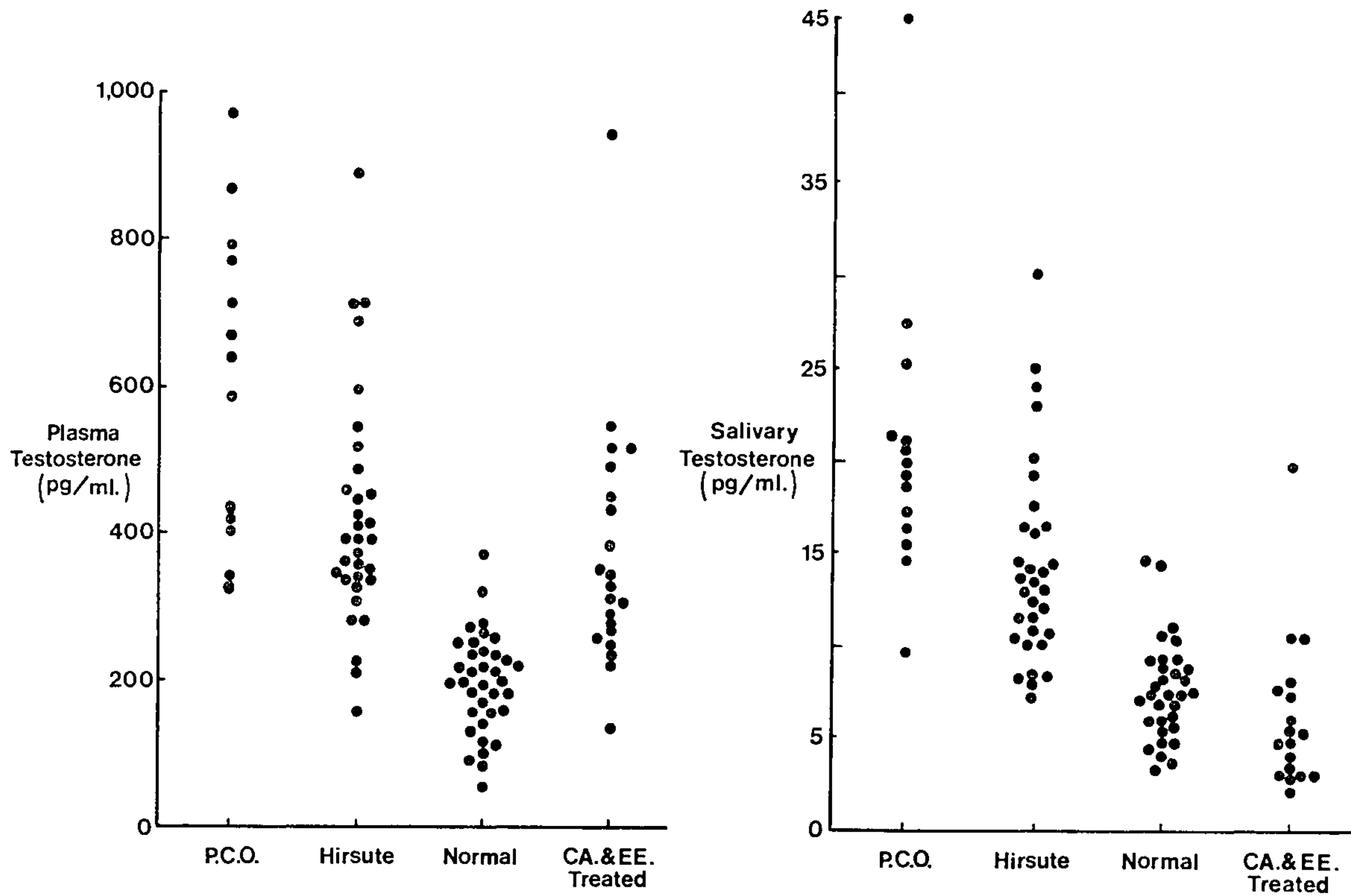

Fig 4.6 Salivary and plasma testosterone concentrations in normal and hyperandrogenic women before and after treatment.

1 pg/ml = 3.47 pmol/l.

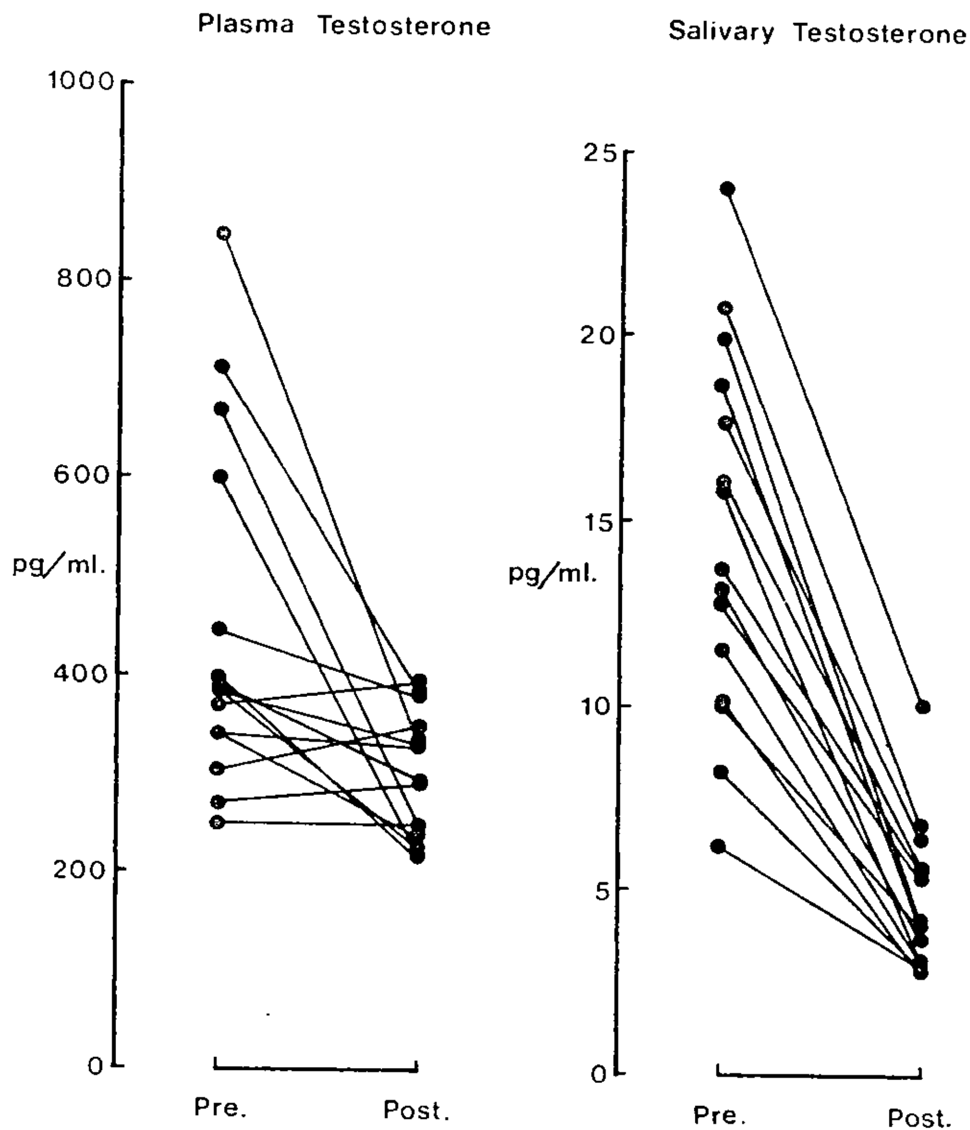

Fig 4.7 Response of salivary and plasma testosterone concentrations in 15 hyperandrogenic women after 3 months treatment with CA and EE.
1 pg/ml = 3.47 pmol/l.

decrease in salivary concentrations of 68%, but a drop in plasma levels was not observed in all patients, the mean decrease being 34%. Concentrations of testosterone in saliva and plasma from 6 women treated with CA and EE for one year were no different from levels after 3 months.

Salivary testosterone levels from patients with PCO, idiopathic hirsutism and normal women correlate well with plasma concentrations. These levels are lower than those reported in the literature, probably due to the inclusion in this study of a preassay purification step, and the use of a very sensitive assay. The most significant finding was seen in patients treated with CA and EE. As reported by Frölich et al., (1977), and Peereboom-Wynia and Boekhorst, (1980), plasma testosterone concentrations decreased in some but not all patients studied. A sharper decline was however observed in all salivary testosterone levels in these patients, in association with a decrease in the plasma free testosterone concentration.

Cyproterone acetate has been used successfully in the treatment of hirsutism (Ismail et al., 1974; Barnes et al., 1975; Hammerstein et al., 1975). It is usually prescribed in combination with ethinyl oestradiol to ensure cyclical withdrawal bleedings and prevent conception. CA is a potent antiandrogen and acts in several ways including inhibition of gonadotrophin secretion (Barnes et al., 1975), by interfering with steroid action on target organs, by competing for receptors (Giorgi, 1976) and affecting steroid synthesis (Panesar and Stitch, 1976). This study demonstrated a decrease in unbound plasma testosterone concentrations on combined CA and EE therapy. It is known that treatment with oestrogen increases production of SHBG in the liver and therefore results in the decrease in the free testosterone fraction (Anderson, 1974). CA has little effect on SHBG alone (Frölich et al., 1980) although it has been shown to enhance the hepatic response to oestrogen (Sawers et al., 1980).

The mean percentage free testosterone levels measured by the direct

method were 1.8% for patients with PCO, 1.5% in patients with idiopathic hirsutism, 1.3% in normal subjects and 0.7% in patients treated with CA and EE. These levels are similar to those obtained using isotopically labelled steroid (Vermeulen et al., 1971; Moll and Rosenfield, 1979).

The concentration of non specifically bound plasma testosterone in women with PCO, idiopathic hirsutism, normal subjects and hyperandrogenic women following 3 months antiandrogen treatment are shown in Fig. 4.8. Mean levels were 56 ± 21 pg/ml (194 ± 73 pmol/1, n = 23) for normal women, 175 ± 87 pg/ml (607 ± 253 pmol/1, n = 15) for patients with PCO, 117 ± 70 pg/ml (406 ± 243 pmol/1, n = 23) for women with idiopathic hirsutism and 48 ± 22 pg/ml (166 ± 76 pmol/1, n = 26) for antiandrogen treated women.

These results are in good agreement with those of other investigators using other methods (Rosenfield, 1971; Vermeulen et al., 1971). The mean percent non SHBG bound testosterone levels were 30% for patients with PCO, 26% in women with idiopathic hirsutism, 22% in normal subjects and 17% in patients treated with CA and EE. Non specifically bound plasma testosterone concentrations were elevated in 87% of patients with PCO and 56% with idiopathic hirsutism. This is similar to the incidence of increased unbound levels in the same group of patients, 83% with PCO and 58% with idiopathic hirsutism had raised values.

The effect of 3 months antiandrogen treatment on non specifically bound plasma testosterone is shown in Fig. 4.9. Levels declined in all subjects, the mean decrease being 60%, which is similar to that observed in saliva. Although the biological significance of albumin bound testosterone is still controversial, it is generally believed that steroid bound to SHBG is not available for metabolism in the tissues. Hence this data provides further support that salivary testosterone measurements give a useful indication of non specifically bound testosterone. Although salivary measurements allow no greater discrimination between

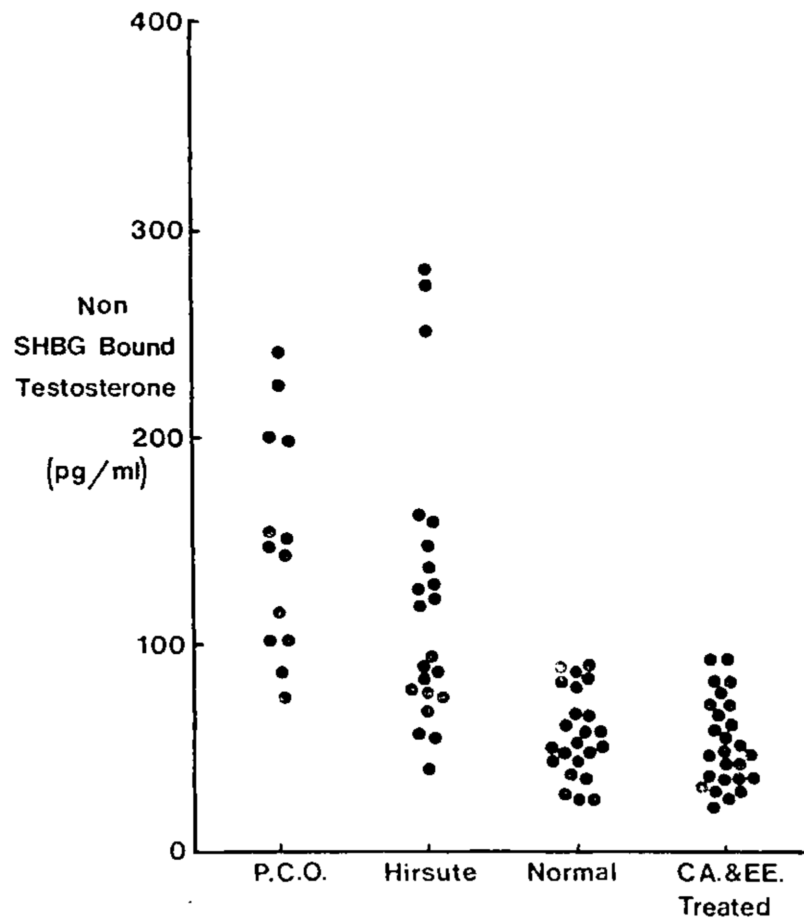

Fig 4.8 Non SHBG bound plasma testosterone concentrations in normal and hyperandrogenic women before and after treatment with CA and EE for 3 months.
1 pg/ml = 3.47 pmol/1.

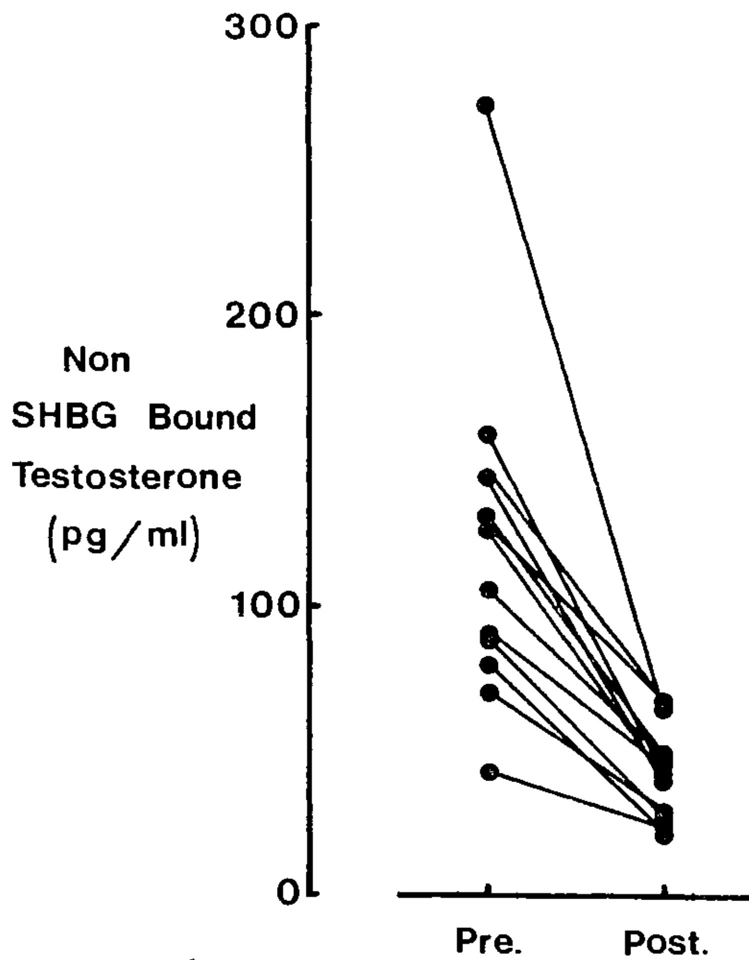

Fig 4.9 The effect of 3 months treatment with CA and EE on non SHBG bound plasma testosterone concentrations in 13 hyperandrogenic women.
1 pg/ml = 3.47 pmol/l.

normal and hyperandrogenic women than do plasma estimations, they are a useful alternative to plasma assays, and may give a better indication of the biologically available androgen in hyperandrogenic women than do plasma assays before and during antiandrogen therapy.

The association of androgens with the aetiology of acne vulgaris has been known for some time. It is believed that the skin of patients with acne is hypersensitive to potent androgens, since acne bearing skin has 2 to 20 times the 5α reductase activity than normal skin from corresponding areas (Sansone and Ressner, 1971). Acne lesions result from the inflammation of sebaceous follicles due to the growth and toxic by-products of two main species of bacteria, Staphylococcus epidermides and Proprionibacterium acnes. Testosterone and dihydrotestosterone are thought to stimulate sebaceous gland secretion which may irritate the lining of the follicular duct causing an obstruction, and a suitable environment for these bacteria to thrive. Hence acne vulgaris is a hormone influenced skin disease, not an endocrine disease.

Despite numerous reports, no truly conclusive evidence has been presented to show that patients whose only pathological symptom is acne vulgaris have abnormal circulating levels of plasma androgens. Recent studies though have demonstrated increased levels of unbound plasma testosterone as a result of reduced SHBG (Lawrence et al., 1981; Odling et al., 1982), whereas total plasma testosterone concentrations in the same patients were normal or only slightly raised.

Concentrations of testosterone in saliva and plasma have been measured in 12 women whose only clinical symptom was acne. All patients had normal menstrual cycles, and none were receiving any form of hormonal contraception. Mean levels of testosterone in saliva (11.8 $\pm$ 5.6 pg/ml, 40.9 $\pm$ 19.4 pmol/l) and plasma (341 $\pm$ 99 pg/ml, 1.2 $\pm$ 0.3 nmol/l) were slightly higher ($p < 0.001$) than in normal subjects, although there was a large overlap between groups. Only 27% of salivary values and 25% of

plasma levels were elevated.

Salivary and plasma testosterone levels were also measured in patients with alopecia. Although mean levels of testosterone in saliva (10.3 ± 1.0 pg/ml, 35.7 ± 3.5 pmol/l) from 5 women with alopecia alone were higher than normal subjects, no patients showed values outside the normal range. The term alopecia signifies hair loss and is only applied when 25% of the terminal hairs are lost. In women it often results in severe psychological disturbance. There are a number of possible causes of hair loss including X-radiation, genetic predisposition, contact dermatitis, malignant disease, thyrotoxicosis and hypothyroidism may cause baldness of the scalp which in some cases is associated with hirsutism. Androgen concentrations have been shown to be elevated in women with androgenic alopecia, but in view of these findings it seems that if the condition is androgen dependent there must be an increased local conversion of prehormones to dihydrotestosterone in the affected hair follicles.

b) <u>Androstenedione</u>

The androstenedione concentration in saliva and plasma samples from women with PCO, idiopathic hirsutism, normal subjects and hyperandrogenic women following 3 months treatment with CA and EE are shown in Fig. 4.10. Mean salivary levels were 78 ± 30 pg/ml (273 ± 105 pmol/l, n = 18) for normal women, 185 ± 72 pg/ml (648 ± 252 pmol/l, n = 11) for women with PCO, 151 ± 110 pg/ml (529 ± 385 pmol/l, n = 25) for women with idiopathic hirsutism and 66 ± 15 pg/ml (231 ± 52 pmol/l, n = 21) for treated women. The corresponding total plasma androstenedione levels were 787 ± 355 pg/ml (2.8 ± 1.2 nmol/l, n = 18) for normal women, 3262 ± 814 pg/ml, (11.4 ± 2.8 nmol/l, n = 12) for patients with PCO, 2177 ± 1096 pg/ml (7.6 ± 3.8 nmol/l, n = 25) for women with idiopathic hirsutism and 1112 ± 456 pg/ml (3.9 ± 1.6 nmol/l, n = 21) for CA and EE treated women. Both salivary and plasma androstenedione levels were significantly higher ($p < 0.001$)

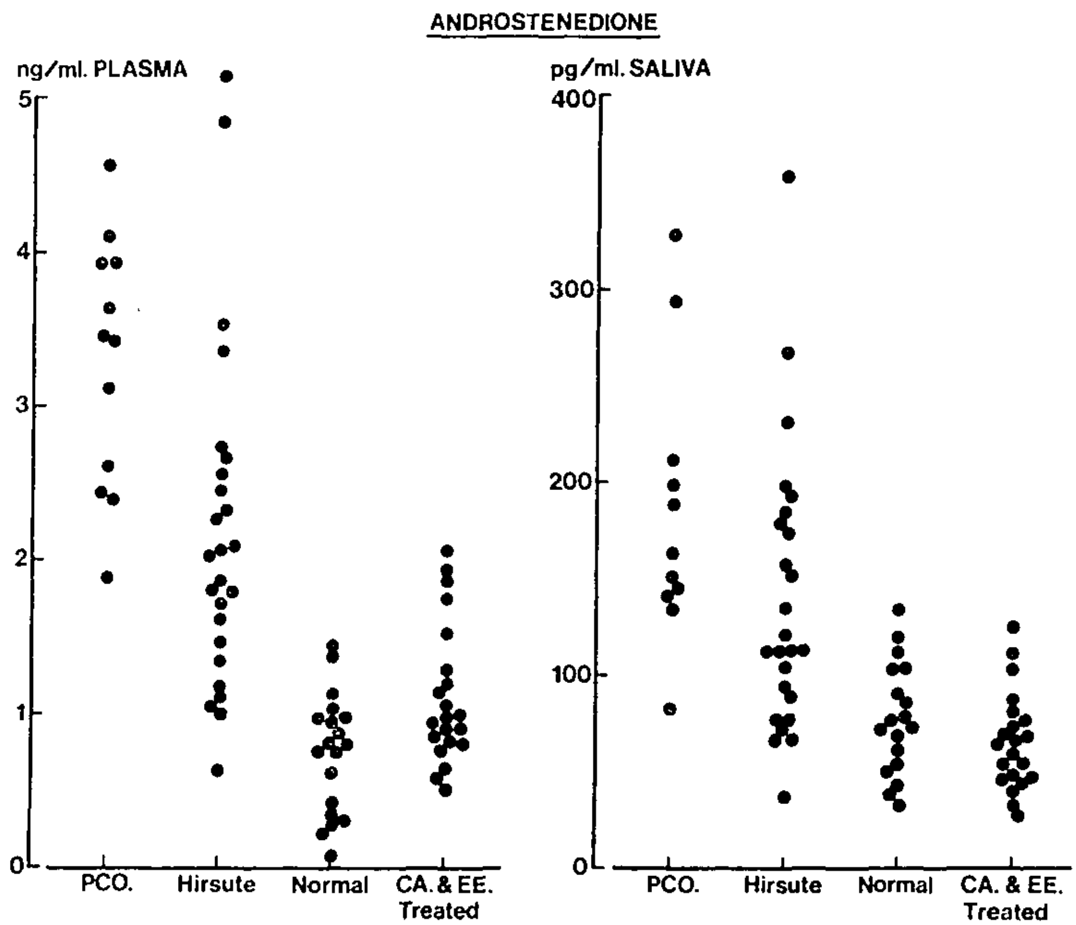

Fig 4.10 Salivary and plasma androstenedione concentrations in normal, hyperandrogenic and treated women.
1 pg/ml = 3.50 pmol/l.

in women with PCO and idiopathic hirsutism than in normal subjects.

Salivary androstenedione levels showed a mean decrease of 55% in 12 patients after 3 months treatment with CA and EE (Fig 4.11). A similar fall (54%) was observed in plasma concentrations (n = 15). This decline in plasma levels is in agreement with most authors (Frölich et al., 1977; Kutten et al., 1980; Sawers et al., 1980) although Peerboon-Wynia and Boekhorst, (1980), and Lunnel et al., (1982), failed to detect any change in androstenedione, testosterone or DHEA with this treatment.

Kirshner, (1970), demonstrated that oestrogen therapy did not alter the metabolic clearance rate for androstenedione, furthermore, androstenedione shows little affinity for SHBG. Of the total plasma concentration, 85% is loosely bound to albumin and only 6% to SHBG (Dunn et al., 1981). Hence the decrease in salivary androstenedione after treatment with CA and EE reflects the decreased production of androstenedione and not an alteration in plasma protein binding. The unbound fraction of androstenedione in plasma ranged from 6.0 - 10.4% of the total concentration, and was similar in hyperandrogenic, normal and treated women. These levels agree with published data on the binding characteristics of androstenedione in undiluted plasma (Forest et al., 1968; Dunn et al., 1981).

Androstenedione concentrations in saliva (121 ± 59 pg/ml, 423 ± 207 pmol/l) and plasma (1640 ± 581 pg/ml, 5.7 ± 2.0 nmol/l) from 10 women with acne were significantly higher than normal. 44% of patients had increased levels in saliva whereas 60% had raised plasma androstenedione concentrations. In 4 patients with alopecia, none had levels of salivary androstenedione outside the normal range, but one patient had an elevated level in plasma.

c) Dihydrotestosterone

Levels of dihydrotestosterone in saliva and plasma samples from normal, hyperandrogenic and treated women are shown in Fig. 4.12. Mean

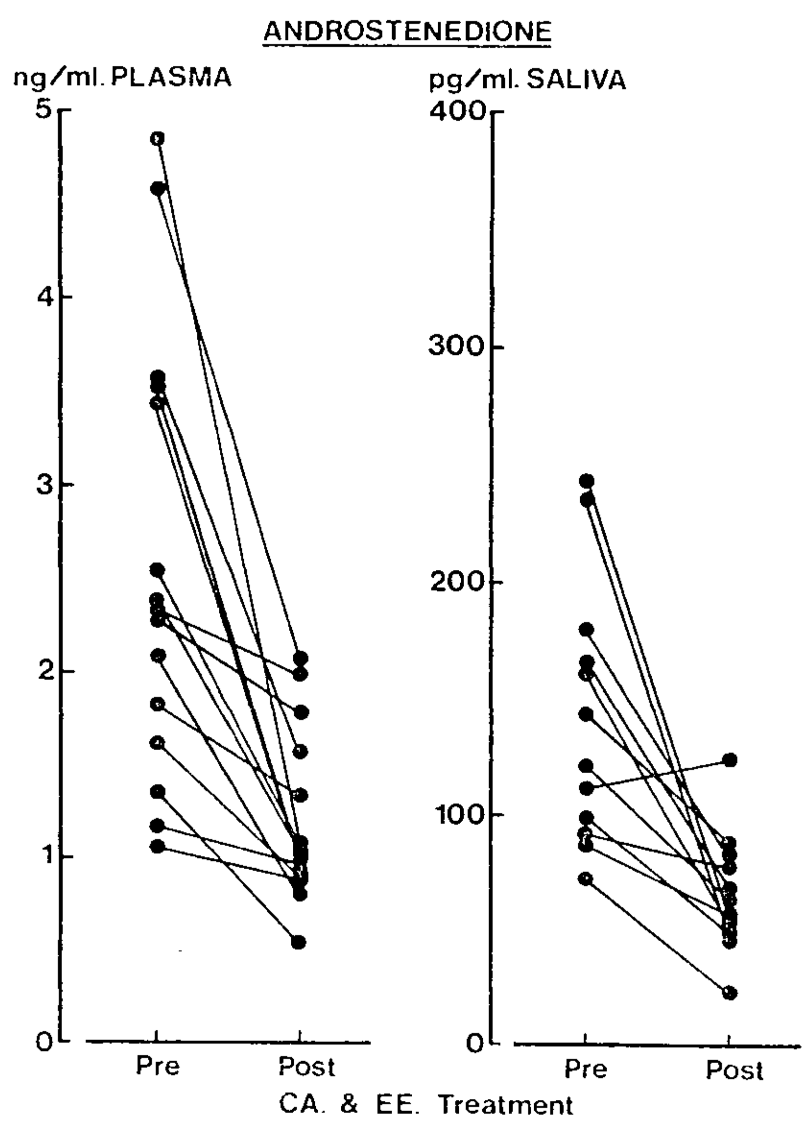

Fig 4.11 The response of salivary and plasma androstenedione levels in hyperandrogenic women to 3 months treatment with CA and EE. 1 pg/ml = 3.50 pmol/l.

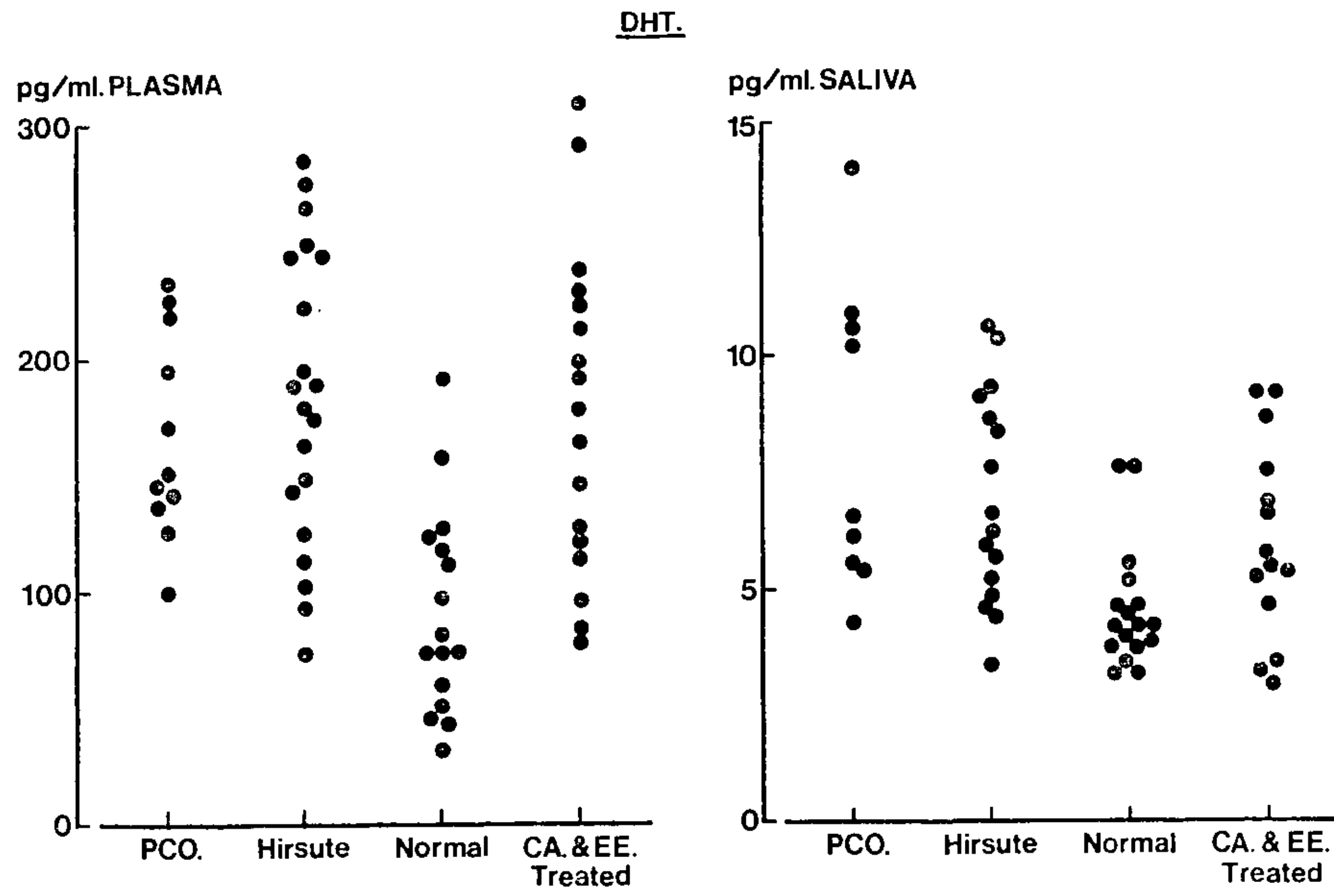

Fig 4.12 Salivary and plasma dihydrotestosterone concentrations in normal, hyperandrogenic and treated women.
1 pg/ml =3.44 pmol/l.

salivary levels were 4.5 ± 1.3 pg/ml (15.5 ± 4.5 pmol/l, n = 17) for normal women, 8.2 ± 3.3 pg/ml (28.2 ± 11.3 pmol/l, n = 9) for women with infertility arising from PCO, 6.9 ± 2.3 pg/ml (23.7 ± 7.9 pmol/l, n = 16) for women with idiopathic hirsutism and 6.0 ± 2.1 pg/ml (20.6 ± 7.2 pmol/l n = 14) for women treated with CA and EE for 3 months. The corresponding total plasma dihydrotestosterone levels were 90 ± 44 pg/ml (0.31 ± 0.15 nmol/l, n = 16) for normal women, 167 ± 45 pg/ml (0.57 ± 0.15 nmol/l, n = 11) for women with PCO, 183 ± 64 pg/ml (0.63 ± 0.22 nmol/l, n = 20) for women with idiopathic hirsutism and 176 ± 69 pg/ml (0.61 ± 0.24 nmol/l n = 17) for treated women. Although there is a large overlap between groups, both salivary and plasma dihydrotestosterone concentrations in patients with PCO and idiopathic hirsutism were higher than normal subjects ($p < 0.001$). The mean percentage free dihydrotestosterone in plasma was 0.78% in women with hirsutism, 0.61% in normal subjects and 0.40% in patients treated with CA and EE.

Only 36% of patients with PCO and 40% with idiopathic hirsutism had elevated plasma dihydrotestosterone concentrations, which is similar to the findings of Ito and Horton, (1970), and Meikle et al., (1979), whereas 45% of patients with PCO and 38% with idiopathic hirsutism had elevated salivary dihydrotestosterone concentrations. After antiandrogen therapy 47% still had raised plasma dihydrotestosterone, but only 3 salivary measurements were elevated. Matched samples from hyperandrogenic women before and after treatment (Fig. 4.13) revealed a similar decrease in salivary and plasma concentrations. Levels in saliva fell by a mean 18% (n = 7) and plasma concentrations decreased by 17% (n = 15). This is surprising since dihydrotestosterone shows a high affinity for SHBG, and any oestrogen induced increase in SHBG production would be expected to decrease the free fraction in plasma and the concentration in saliva as has been shown for testosterone. Perhaps further investigation will prove this to be the case for dihydrotestosterone also.

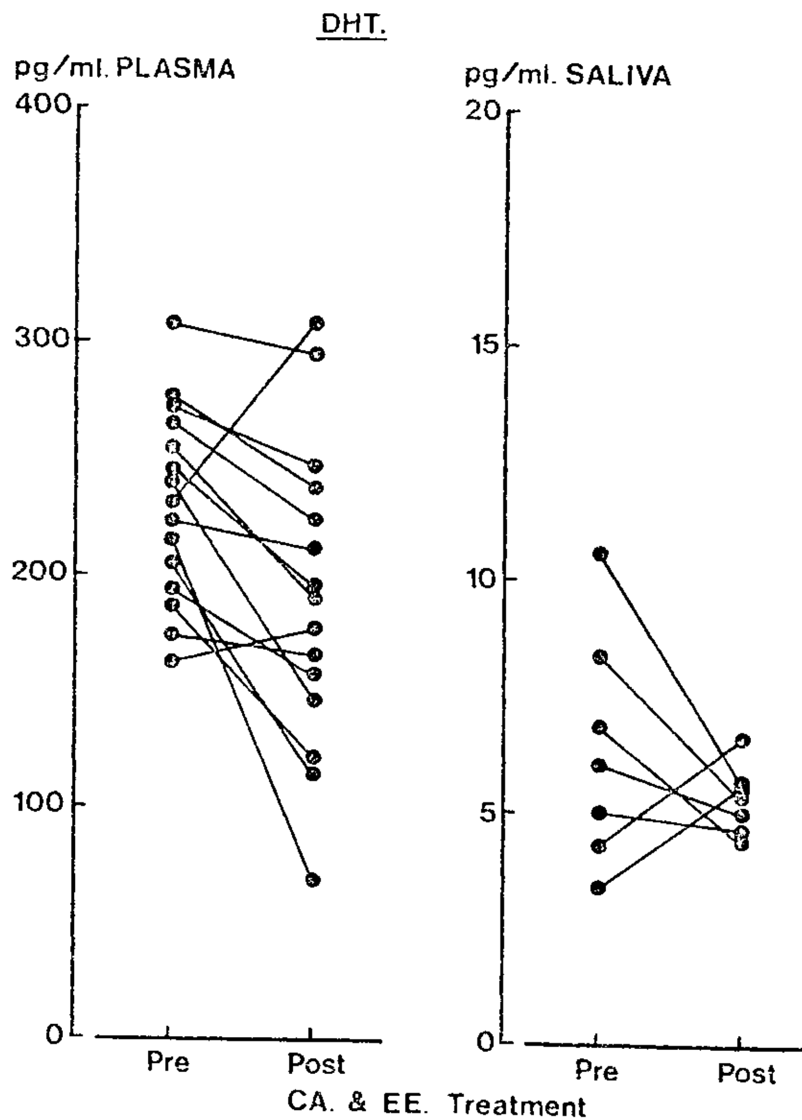

Fig 4.13 The response of salivary and plasma dihydrotestosterone levels in hyperandrogenic women to 3 months treatment with CA and EE.
1 pg/ml = 3.44 pmol/l.

The mean level of dihydrotestosterone in saliva from 8 women with acne (7.3 ± 2.9 pg/ml, 25.1 ± 10.0 pmol/l) was almost twice that of normal subjects. Also plasma levels were significantly raised (175 ± 61 pg/ml, 0.60 ± 0.21 nmol/l). The data for dihydrotestosterone in patients with alopecia is limited, but salivary concentrations were elevated in one of the two patients studied and dihydrotestosterone in plasma raised in 2 out of 4 patients investigated.

It would appear from this data that the measurement of dihydrotestosterone in saliva offers no particular advantage over the measurement of total plasma concentrations in the initial investigation of patients with hirsutism. It may be that the free fraction in plasma and the concentration in saliva do not reflect the amount of steroid available to the tissues, or that the tissue exposure is of less importance than the metabolic activity of the tissues themselves.

d) Androstanediol

Concentrations of androstanediol in plasma from women with PCO, idiopathic hirsutism, normal women and antiandrogen treated women are shown in Fig. 4.14. Mean plasma levels were 55 ± 15 pg/ml (188 ± 51 pmol/l, n = 12) in normal subjects, 95 ± 37 pg/ml (325 ± 126 pmol/l, n = 8) in women with PCO, 102 ± 42 pg/ml (349 ± 143 pmol/l, n = 17) in women with idiopathic hirsutism and 55 ± 15 pg/ml (188 ± 51 pmol/l, n = 13) in women treated with CA and EE for 3 months.

The variation of plasma androstanediol values in the literature is very large. Kinouchi and Horton, (1974), reported concentrations in normal women to be 20 ± 6 pg/ml (68 ± 21 pmol/l) whereas Strickland and Apland, (1977), found levels of 240 ± 150 pg/ml (821 ± 513 pmol/l). Concentrations of androstanediol in this study are similar to those reported by Meikle et al, (1979).

The majority of patients with idiopathic hirsutism (71%) had elevated plasma androstanediol concentrations, also androstanediol was

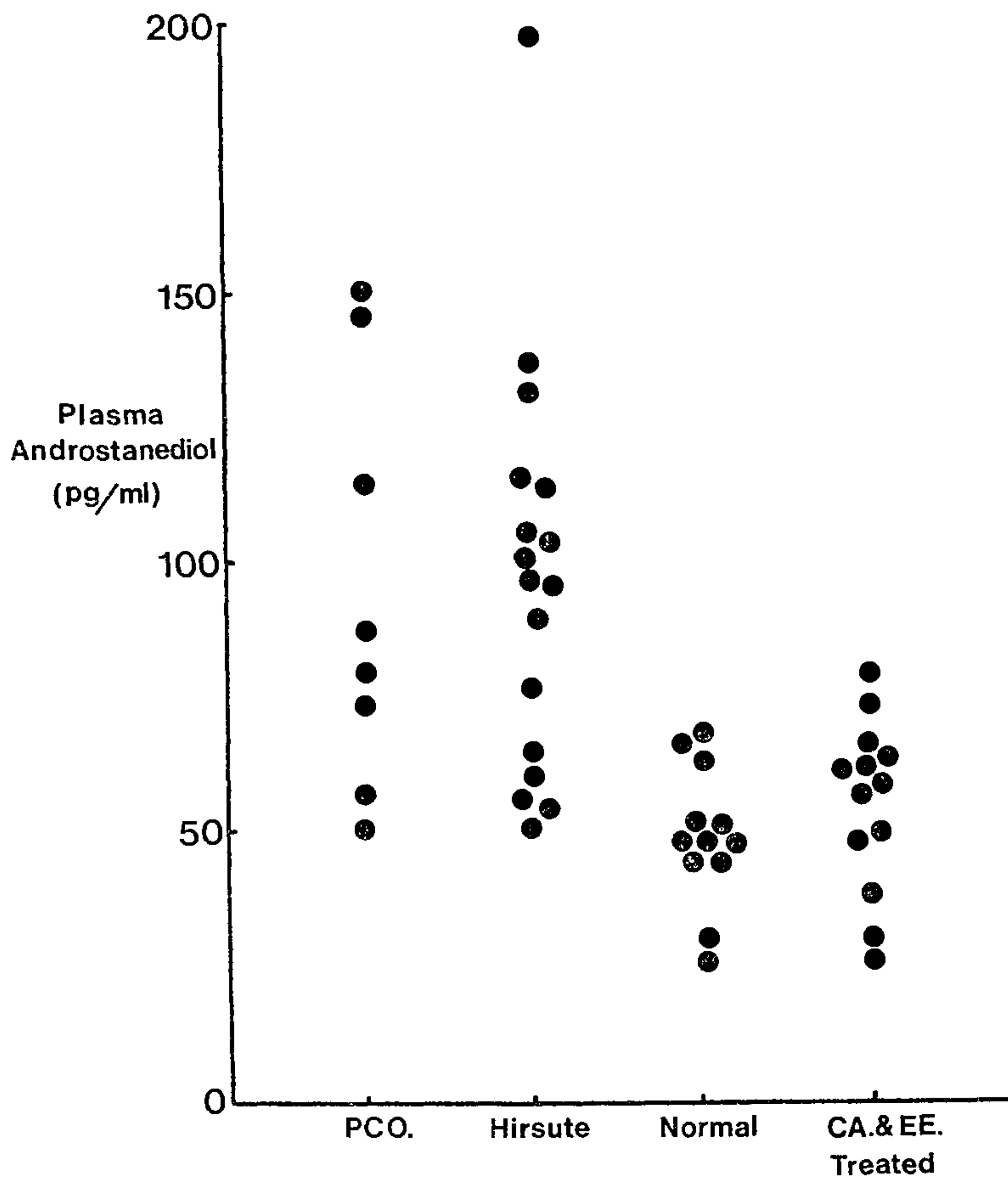

Fig 4.14 Plasma androstanediol concentrations in women with polycystic ovaries, idiopathic hirsutism, normal women and hyperandrogenic women following treatment with CA and EE for 3 months.
1 pg/ml = 3.42 pmol/l.

raised in 75% of cases of polycystic ovarian disease. All five hirsute patients studied who had normal androstanediol concentrations also had normal testosterone, dihydrotestosterone and androstenedione concentrations. It may be that these patients have an altered plasma protein binding, and that the unbound concentration of androstanediol may prove to be raised. Of the 2 patients with PCO who had raised androstanediol levels, neither suffered any sign of hirsutism. This is the first study to report concentrations of androstanediol in plasma following CA and EE therapy. Levels in treated patients were no different from normal subjects.

e) Cortisol

Plasma cortisol concentrations measured in the same series of hyperandrogenic patients showed a mean increase of 145% after treatment with CA and EE for 3 months. However, salivary cortisol concentrations increased in some and decreased in other patients. The mean rise was 17%. Although progestogens have no effect on corticosteroid binding globulin (CBG) (Brien, 1981), it is well accepted that oestrogens increase plasma cortisol concentrations due to increased production of CBG (Taliafero et al, 1956; Sandberg and Slaunwhite, 1959). This data further supports the view that salivary steroid concentrations are a good reflection of the unbound level in plasma.

4.4 The effects of ovarian wedge resection on salivary and plasma androgen concentrations in a patient with polycystic ovarian disease

Matched saliva and plasma samples were collected from a woman with infertility arising from polycystic ovarian disease 1 day before and 3 days after ovarian wedge resection. This patient had failed to ovulate after clomiphene citrate. Samples were taken between 1400 and 1600 on each occasion. Concentrations of testosterone, dihydrotestosterone and androstenedione are shown in Table 4.1. A sharp fall was observed for

ANDROGEN	SAMPLE	PRE-OP	POST-OP
TESTOSTERONE	Saliva	15.4	3.4
	Plasma	779	138
ANDROSTENEDIONE	Saliva	141	44
	Plasma	3632	939
DIHYDROTESTOSTERONE	Saliva	5.6	3.5
	Plasma	145	77

All levels expressed as pg/ml

Testosterone 1 pg/ml = 3.47 pmol/l

Androstenedione 1 pg/ml = 3.50 pmol/l

Dihydrotestosterone 1 pg/ml = 3.44 pmol/l

Table 4.1 The effect of ovarian wedge resection on salivary and plasma androgen concentrations in a woman with polycystic ovaries.

all androgens following surgery as previously reported for plasma androgens (Judd et al., 1976; Laatikainen et al, 1980).

Unbound plasma testosterone levels declined from 11.2 pg/ml (38.8 pmol/l) to 2.4 pg/ml (8.3 pmol/l) which represents a decrease of 78%. Since similar falls were observed for saliva (77%) and total plasma concentrations (82%), it may be assumed that the alteration in plasma testosterone concentrations following surgery reflects changes in hormonal production. This is in agreement with the findings of Judd et al., (1976), who showed no significant difference in SHBG concentrations following ovarian wedge resection.

Restoration of ovulation in women following surgery is variable. In an extensive review of the literature, Goldzieher, (1981), found that the incidence of success was between 13 and 89%. The mechanism by which wedge resection induces ovulation and menses is not clear. Three possible mechanisms have been postulated by Yen, (1980). 1) An increased local intraovarian blood flow, secondary to the healing process, would permit increased delivery of gonadotrophins to the follicles. 2) An acute localised reduction of androgen would decrease the inhibitory effect of androgens on follicular maturation. 3) A reduction in ovarian inhibin would permit an increased FSH secretion, and thus restore the appropriate LH/FSH ratio required for follicular maturation.

4.5 Salivary and plasma androgens in hyperprolactinaemic women

a) Baseline levels

Several investigators have attempted to elucidate the association between prolactin and androgen secretion. Prolactin concentrations have been shown to be raised in some women with hirsutism (Seppälä et al., 1975) but biochemical investigations have produced conflicting results. Elevated concentrations of plasma DHEA and DHEAS have been found in women with hyperprolactinaemia, also the administration of

sulpiride, a dopamine antagonist, caused an increase in DHEAS (Vermeulen et al., 1977). Nevertheless there is evidence to the contrary since Belisle and Menard, (1980), failed to show abnormal levels of DHEAS, DHEA, androstenedione, testosterone or cortisol in women with pituitary prolactinomas. In an attempt to clarify these relationships, testosterone, androstenedione and dihydrotestosterone concentrations have been measured in saliva and plasma samples collected from women with hyperprolactinaemia. All patients had amenorrhoea without any sign of hirsutism or virilisation. Prolactin levels in all cases exceeded 700 mU/L. Samples were collected between 1000 and 1400.

Concentrations of androgens in saliva of hyperprolactinaemic women are shown in Fig. 4.15. Levels of testosterone (11.3 $\pm$ 6.6 pg/ml, 39.2 $\pm$ 22.9 pmol/l, n = 20) and dihydrotestosterone (6.2 $\pm$ 2.0 pg/ml, 21.2 $\pm$ 6.9 pmol/l, n = 8) were higher than normal ($p < 0.05$), whereas androstenedione concentrations (85 $\pm$ 46 pg/ml, 298 $\pm$ 161 pmol/l, n = 19) were no different from normal subjects.

The corresponding androgen concentrations in plasma (Fig. 4.16) show a similar pattern. Only 4 out of 21 patients had raised plasma testosterone concentrations whereas 3 out of 19 had raised dihydrotestosterone and 3 out of 21 had androstenedione concentrations outside the normal range. Gonadotrophin levels in these patients with elevated androgens were all at the upper end of the normal range. No correlation was found between prolactin concentrations and any of these androgens, although Jones et al., (1980), showed a good relationship to exist between prolactin and both DHEA and DHEAS.

Reported values of testosterone, androstenedione and dihydrotestosterone in women with hyperprolactinaemia are sparse, but the majority of those studies have found these steroids to be normal. A recent report however showed that although total plasma testosterone concentrations were normal in women with prolactinomas, free plasma testosterone

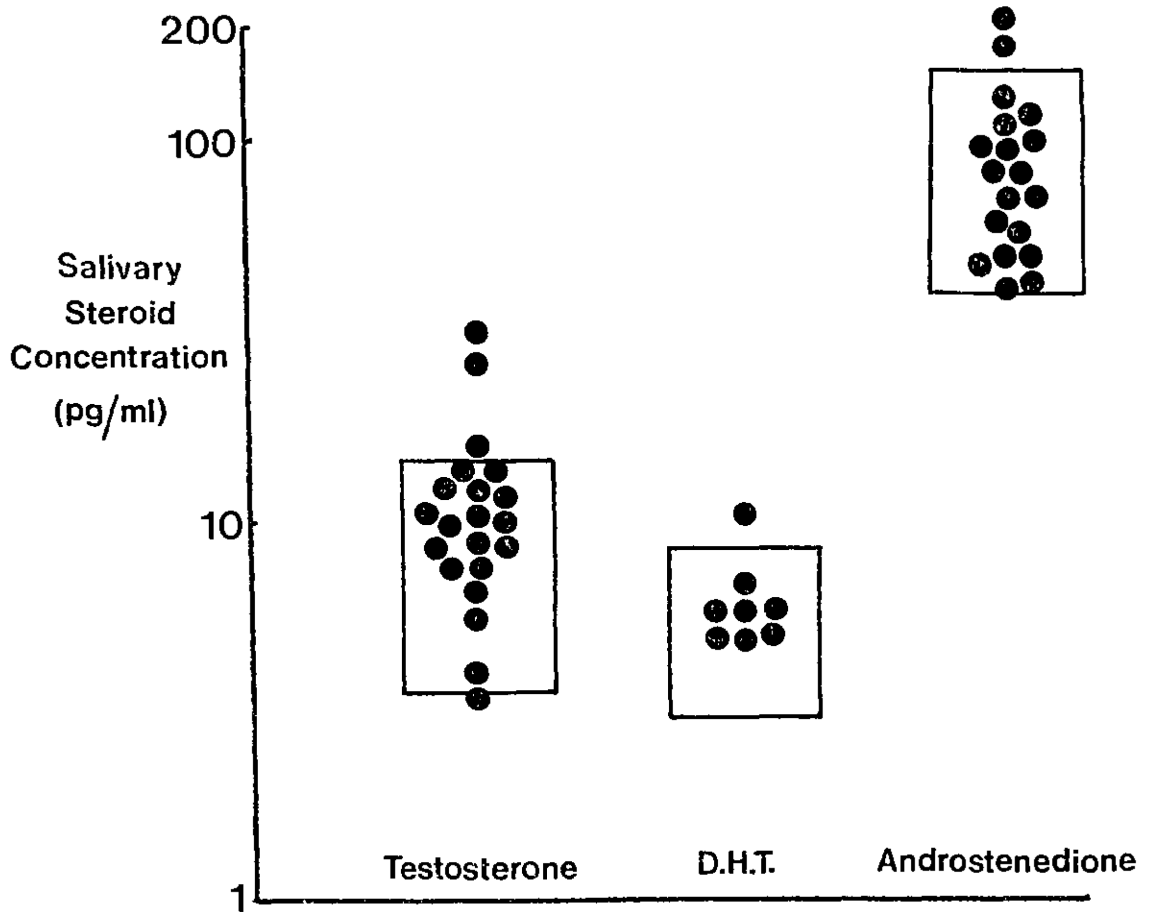

Fig 4.15 Salivary androgen concentrations in hyperprolactinaemic women.
Boxed areas represent normal range.
T 1pg/ml=3.47pmol/l. DHT 1pg/ml=3.44pmol/l. A 1pg/ml=3.50pmol/l.

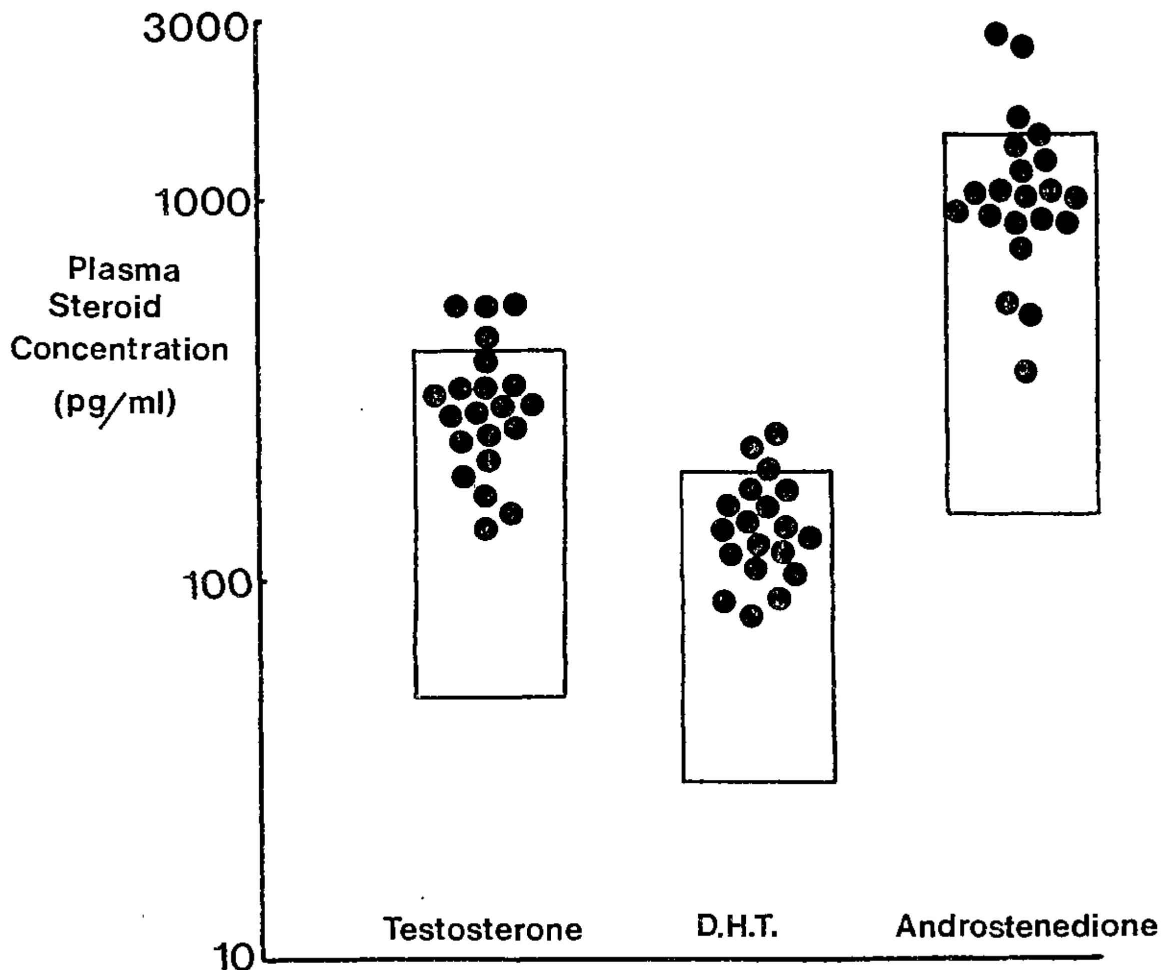

Fig 4.16 Plasma androgen concentrations in hyperprolactinaemic women.

concentrations were elevated as a result of diminished SHBG concentrations (Vermeulen et al., 1982). The metabolic clearance rate of testosterone and dihydrotestosterone, but not androstenedione, in these patients returned to normal after bromocriptine therapy.

b) Effect of bromocriptine treatment

Bromocriptine (2.5 mg) was taken daily by 5 women with hyperprolactinaemic amenorrhoea. A further patient who had grossly elevated prolactin levels took 5 mg daily. No difference was observed in concentrations of testosterone, dihydrotestosterone or androstenedione after treatment for 4 months in either saliva or plasma samples (Table 4.2). This is in agreement with Seppälä et al., (1976) and Carter et al., (1977), although Vermeulen et al., (1982), demonstrated a reduction in unbound plasma testosterone concentrations in patients with prolactinomas whilst total plasma levels remained stable after bromocriptine treatment.

Bromocriptine is a dopamine agonist, and is effective in lowering plasma prolactin concentrations by binding to specific dopaminergic receptors on prolactin secreting cells in the pituitary (Flückiger, 1981). In a large proportion of women, menses and ovulation are resumed. After 4 months treatment, prolactin concentrations had decreased in all patients studied by a mean of 50%, although concentrations were still above normal in 2 subjects. The mean decrease was 2192 $\pm$ 1525 mU/L to 1078 $\pm$ 867 mU/L.

In view of this data it seems that the concentration of the androgens testosterone, androstenedione and dihydrotestosterone are independent of prolactin secretion in patients with hyperprolactinaemia, unlike the predominantly adrenal hormones DHEAS and DHEA which have been shown to decrease after bromocriptine treatment (Carter et al., 1977). Since there is no evidence to suggest that bromocriptine acts directly on the adrenal gland or ovaries, it is likely that prolactin acts on the adrenal possibly by suppressing 3β hydroxysteroid dehydrogenase activity,

ANDROGEN	SAMPLE	PRE TREATMENT	DURING TREATMENT
TESTOSTERONE	Saliva	13.8 ± 6.5	14.7 ± 7.0
	Plasma	331 ± 8.7	380 ± 162
ANDROSTENEDIONE	Saliva	6.9 ± 2.2	7.7 ± 2.5
	Plasma	131 ± 16	160 ± 19
DIHYDROTESTOSTERONE	Saliva	121 ± 50	125 ± 59
	Plasma	1497 ± 620	1525 ± 774

All values expressed as pg/ml

Testosterone 1 pg/ml = 3.47 pmol/l

Androstenedione 1 pg/ml = 3.50 pmol/l

Dihydrotestosterone 1 pg/ml = 3.44 pmol/l

Table 4.2 The influence of bromocriptine therapy on salivary and plasma androgen concentrations in 6 hyperprolactinaemic women.

resulting in increased Δ^5 steroid compared to Δ^4 in hyperprolactinaemic women (Bassi et al., 1977; Vermeulen and Ando, 1978).

The most interesting finding from this study is the occurrence of elevated testosterone, androstenedione and dihydrotestosterone in saliva and plasma from 3 women with hyperprolactinaemia without any clinical sign of androgen excess. None of these patients had any signs of hirsutism, 2 were obese and gonadotrophin levels were at the upper end of normal. Hirsutism has been demonstrated in a number of hyperprolactinaemic women; also prolactin concentrations have been shown to be elevated in certain cases of polycystic ovaries, (Thorner, 1974). Reduction of prolactin concentrations in hirsute women after bromocriptine therapy has been shown to lower plasma testosterone concentrations and reduce facial hair growth (Seppälä et al., 1975). Treatment with bromocriptine for 4 months in a patient with high androgen concentrations had little effect on salivary (25.1 - 27.0 pg/ml, 87 - 93 pmol/l) or plasma testosterone concentrations (505 - 627 pg/ml, 1.7 - 2.2 pmol/l). Androstenedione and dihydrotestosterone levels also remained stable.

4.6 5α reductase activity in normal and pathological conditions

a) Baseline levels

The conversion of testosterone to dihydrotestosterone is an important mechanism of androgen action in certain tissues. Patients with a genetic inability to produce dihydrotestosterone have scanty sexual hair growth. Also these cases do not suffer from acne, gynecomastia or temporal hair recession (Imperata - McGinley et al., 1974). Since it is believed that the majority if not all dihydrotestosterone is produced by the peripheral conversion of androstenedione and testosterone rather than direct glandular secretion, the ratio of testosterone : dihydrotestosterone concentrations in plasma should give an approximation of the 5α reductase activity. Two important criteria have to be considered

first though. The metabolic clearance rate of testosterone is about twice that of dihydrotestosterone, hence has a shorter half-life. A sudden decrease in testosterone production therefore would tend to shift the ratio towards lower values for a short time, since the dihydrotestosterone concentration would be slower to respond. The concentration of dihydrotestosterone in plasma is not only controlled by 5α reductase but by the activity of 3β hydroxysteroid dehydrogenase and the formation of androstanediol.

The plasma testosterone : dihydrotestosterone ratio in normal women, women with polycystic ovaries, idiopathic hirsutism and hyperprolactinaemic women have been calculated. The ratio in women with PCO was 4.03 (n = 10), which was significantly higher than in normal subjects. Values for women with idiopathic hirsutism (2.01, n = 20) and hyperprolactinaemic subjects (2.12, n = 15) were no different from normals (1.91, n=16).

b) Treated patients

The testosterone : dihydrotestosterone ratios in plasma from 13 hyperandrogenic women who had been treated with CA and EE for 3 months (1.73, n = 15) was slightly lower than normal subjects, whereas treatment of hyperprolactinaemic women with bromocriptine had no effect.

The relationship between this ratio and the plasma testosterone concentration in normal women, hyperandrogenic women, hyperprolactinaemic women and treated women is shown in Fig. 4.17. As expected, the testosterone : dihydrotestosterone ratio is a function of the plasma testosterone concentration ($r = 0.94$, $p < 0.001$), and increases at high testosterone levels. This is similar to the findings of Vermeulen, (1976), who showed that this ratio was not dependent upon sex but solely on the substrate concentration. At similar testosterone concentrations, the testosterone:dihydrotestosterone ratio was similar in each sex.

5α reductase activity in skin has been shown to be elevated in women with hirsutism and acne (Sansone and Ressner, 1971), and decreased

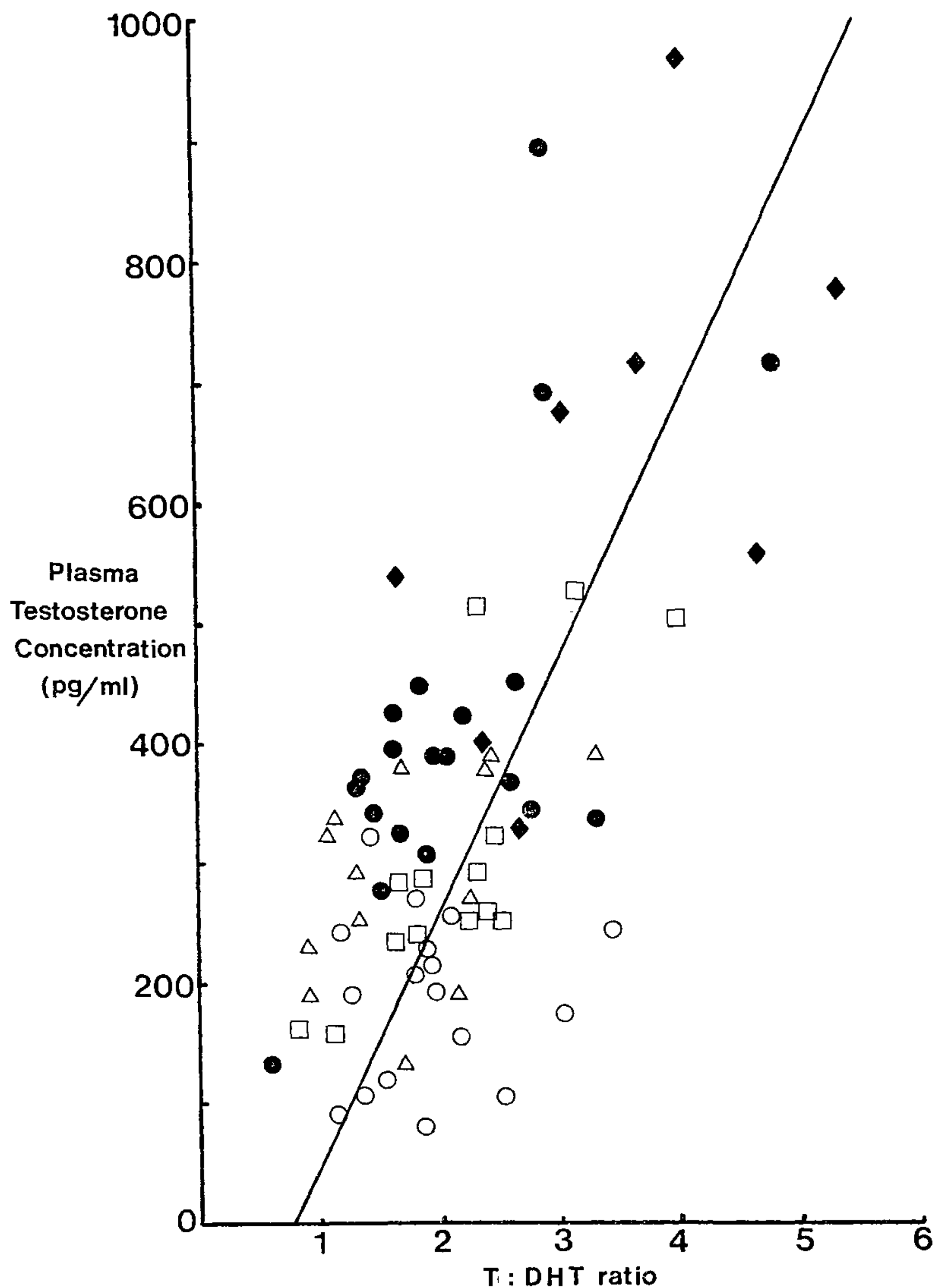

Fig 4.17 The relationship between testosterone and the testosterone : dihydrotestosterone ratio in plasma of normal women (○), women with PCO (◆), idiopathic hirsutism (●), hyperandrogenic women on CA and EE (△) and hyperprolactinaemic women (□). 1 pg/ml = 3.47 pmol/l.

in men with hyperprolactinaemia. This study failed to observe any difference between the testosterone : dihydrotestosterone ratio in normals and women with hirsutism or hyperprolactinaemia, which suggests that the plasma testosterone : dihydrotestosterone ratio is a poor indication of the 5α reductase activity in tissues.

4.7 Salivary and plasma androgens in men with pathological conditions

Matched plasma and saliva samples were collected from patients attending a clinic for male infertility. Samples were collected between 1000 and 1400. Thin layer chromatography was performed on all samples before radioimmunoassay.

a) Hypogonadotrophic hypogonadism

As shown in Table 4.3, concentrations of testosterone in both saliva and plasma samples from 8 patients with hypogonadotrophic hypogonadism (mean age 27 years) were significantly lower than normals. This is not surprising since Leydig cell stimulation by gonadotrophins is deficient in these patients, usually due to a lack in LHRH secretion in the hypothalamus. Dihydrotestosterone levels in both fluids show a similar pattern due to the common source of origin of these two hormones.

Half the patients studied had androstenedione levels outside the normal range. These levels support the findings of Franchimont et al., (1978), who showed that androstenedione levels in 10 patients with hypogonadism (4 with hypogonadotrophic hypogonadism) were lower than normals. Few investigators have studied the origin of androstenedione in males. Judd et al., (1973), treated normal male subjects with 2 mg dexamethasone daily and showed that the diurnal variation of androstenedione was abolished, suggesting that the early morning surge was of an adrenal origin. Morning levels after dexamethasone decreased by 85% and evening levels by about 70%, so assuming dexamethasone has no effect on the testes, the contribution to androstenedione by the testes amounts

SUBJECTS	TESTOSTERONE		ANDROSTENEDIONE		DIHYDROTESTOSTERONE	
	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma
Hypogonadotrophic hypogonadism (n=8)	10.9 ± 9.7	667 ± 502	42 ± 20	505 ± 290	4.1 ± 2.0	118 ± 43
Klinefelters Syndrome (n=3)	18.7 ± 9.1	1896 ± 1580	83 ± 38	1118 ± 368	6.2 ± 1.7	285 ± 34
Gynecomastia (n=3)	43 ± 17	2397 ± 521	85 ± 10	957 ± 490	7.8 ± 0.7	333 ± 70
Normal subjects	78 ± 23	4050 ± 1450	69 ± 22	631 ± 160	6.7 ± 2.0	373 ± 107

All results expressed as pg/ml

Testosterone 1 pg/ml = 3.47 pmol/l

Androstenedione 1 pg/ml = 3.50 pmol/l

Dihydrotestosterone 1 pg/ml = 3.44 pmol/l

Table 4.3 Salivary and plasma androgen concentrations in men with hypogonadism, Klinefelters syndrome and gynecomastia.

to 15% in the morning and 30% in the evening. It is unlikely, therefore, that a decrease of this order would solely account for the low androstenedione levels in hypogonadal males. Also, since plasma cortisol concentrations in all these patients were normal, there is no evidence for impaired ACTH induced adrenal secretion.

b) Klinefelters Syndrome

Klinefelters syndrome comprises the largest group of hypogonadal males with primary testicular abnormalities. Clinical features include small testes, azoospermia and is often accompanied by gynecomastia and mental retardation. The aetiology of the syndrome is the existance of sex chromosome abnormalities. Most patients have an XXY chromosomal composition, although a large variety of karyotypes have been reported.

Concentrations of testosterone and dihydrotestosterone in 3 patients with Klinefelters syndrome (mean age 31) were lower than those in normal subjects and are in good agreement with levels reported by other workers (Wang et al., 1975). Levels of testosterone in plasma were decreased in 2 out of 3 subjects studied, whereas salivary testosterone concentrations were lower than normal in all subjects studied. This is probably due to the increased SHBG concentration in men with Klinefelters syndrome (Weiland et al., 1980), resulting in decreased levels of unbound hormone. Dihydrotestosterone levels in both plasma and saliva were normal in 2 subjects and no patients had decreased levels of androstenedione in either fluids.

c) Gynecomastia

Concentrations of testosterone but not dihydrotestosterone or androstenedione were significantly different from normals in saliva or plasma samples from male subjects whose only clinical symptom was gynecomastia. The mean age was 19. Testosterone concentrations were subnormal in only 1 patient.

4.8 The effect of HCG administration on salivary and plasma androgen concentrations in men with hypogonadotrophic hypogonadism

HCG, 1500 iu i.m., was given to 4 patients with hypogonadotrophic hypogonadism once a week for 2 months. Matched saliva and plasma samples were collected before starting treatment and 5 days after the last injection. The mean response of testosterone, androstenedione and dihydrotestosterone to HCG in saliva and plasma is shown in Table 4.4. This data clearly illustrates that salivary androgen concentrations closely parallel total plasma androgen levels in these patients before and after treatment, and that salivary measurements are a useful alternative method for monitoring Leydig cell function after HCG stimulation. As maximum androgen concentrations after single dosage of HCG are found after 4 days (Martikainen et al., 1980), saliva sampling may relinquish the need for a return visit to the clinic since samples may be posted directly to the laboratory for analysis.

4.9 Discussion

Some applications of salivary androgen measurements to the study of clinical endocrinology have been demonstrated in this section. Salivary steroid measurements may be particularly useful in the investigation of patients with altered plasma protein binding, for example in women with polycystic ovaries and idiopathic hirsutism and in men with hypogonadism.

A good relationship has been shown to exist between salivary and unbound testosterone, androstenedione and dihydrotestosterone concentrations in normal women and patients with endocrine disorders. In patients with altered plasma protein binding, salivary testosterone and dihydrotestosterone are a better reflection of the unbound steroid than total plasma concentrations.

Levels of testosterone in saliva from hyperandrogenic women are slightly lower than those reported for women with PCO (Smith et al., 1979),

	TESTOSTERONE		ANDROSTENEDIONE		DIHYDROTESTOSTERONE	
	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma
PRE	9	923	34	325	4.0	156
POST	54	5200	67	617	9.8	421
% INCREASE	500	460	97	90	145	170

All results expressed as pg/ml

Testosterone 1 pg/ml = 3.47 pmol/l

Androstenedione 1 pg/ml = 3.50 pmol/l

Dihydrotestosterone 1 pg/ml = 3.44 pmol/l

Table 4.4 Mean concentrations of salivary and plasma androgens in response to HCG stimulation in 4 patients with hypogonadotrophic hypogonadism.

and much lower than the values reported by Luisi et al., (1980), for women with idiopathic hirsutism. Both these investigators used commercial kits designed for the measurement of testosterone in plasma, which do not have the necessary sensitivity required to measure low levels of testosterone in saliva. Likewise, levels of testosterone in saliva from men with hypogonadotrophic hypogonadism are lower than the values reported by Luisi et al., (1980).

Concentrations of androstenedione and dihydrotestosterone in saliva have not previously been reported in the literature. Androstenedione concentrations in saliva mirror those in plasma in men and women before and after treatment. On the other hand, testosterone and dihydrotestosterone levels in plasma of some hirsute women remained elevated after treatment with CA and EE, whereas salivary levels decreased in association with the free plasma concentration.

Although salivary androgen measurements allow no greater discrimination between normal and hyperandrogenic women than do plasma measurements, in patients receiving combined therapy with CA and oestrogen measurement of salivary androgen concentrations does appear to give a useful indication of the diminished tissue exposure. Since it is a simple and non-invasive technique for studying some aspects of androgen metabolism, it may be particularly useful in following the effects of therapy.

SECTION 5

CONCLUSIONS

This study clearly demonstrates that salivary steroid measurements are a useful alternative to total plasma assays in clinical endocrinology. Salivary steroid levels accurately reflect the unbound plasma concentration in normal subjects and in patients with altered androgen metabolism and protein binding. A good relationship also exists between salivary testosterone concentrations and non SHBG bound plasma testosterone concentrations.

It is generally accepted that it is the non protein bound fraction of steroid that is available for metabolism in the tissues. However, Baird et al., (1969), showed that the net extraction of steroid hormone passing through the liver exceeds that which is unbound and suggested that steroid loosely bound to albumin dissociates into the free state prior to transport into the liver cells. This implies that at least part of the albumin bound testosterone must be cleared, a view which has recently been supported by Pardridge and Meitus, (1979a), who also found evidence for extra-hepatic clearance of albumin bound steroid, (Pardridge and Meitus, 1979b).

Two distinct models have been postulated regarding hormone availability to the tissues. Tait and Burstein, (1964), suggested that as the hormone passes along the length of the target tissue capillary, free hormone passes into the tissue and that there is very little dissociation of hormone from protein during the transit time. Strongly bound hormone remains unchanged during this passage and the hormone-protein equilibrium is slowly restored as blood passes through the general circulation. On the other hand, Robbins and Rall, (1979), postulated that dissociation of hormone from protein is so rapid that

the free hormone concentration remains at a constant level as it passes through the target tissue capillaries. The discrepancy between these two models can be explained by the different hormone-protein interactions investigated. The original experiments by Tait and Burstein, (1964), were based on the rate of cortisol delivery to the liver, whereas Robbins and Rall, (1979), studied the vascular transport of thyroid hormones, which do indeed dissociate rapidly from protein.

Both of these models are oversimplified and not strictly valid. The amount of hormone delivered to the tissues will depend upon the total concentration in plasma, the concentration of individual binding proteins and the affinity each protein shows for that hormone. Lipid soluble steroids diffuse through the capillary epithelium at rates which vary inversely with the oil : water partition coefficient (Pardridge, 1981). The proportion of hormone in plasma able to pass into individual tissues will also depend upon the capillary transit time through that particular tissue, which is determined by capillary volume, capillary length and plasma velocity. This period is around 5 seconds in adult liver but only 1 second for brain and skeletal muscle (Pardridge, 1981). Since the unidirectional dissociation rate of steroid from albumin is less than 1 second, it might be expected that appreciable amounts of albumin bound steroid is dissociated and transported into the liver and possibly other tissues, thus explaining the early findings of Baird et al., (1969). However, the dissociation rate of steroid from SHBG is much longer - 100 seconds for dihydrotestosterone and 22 seconds for testosterone (Pardridge, 1981). Thus little, if any, SHBG bound steroid would be available for metabolism in most tissues.

Another factor influencing the transfer of steroid is the intracellular processes occuring in the tissues and the binding affinities of the metabolic breakdown products. A further important consideration is the concentration of binding protein in the interstitial fluid and

in the cells themselves, also the leakage of protein from the vascular compartment.

Although the biological significance of albumin bound testosterone is still controversial, it is generally believed that SHBG bound testosterone is not available for metabolism in the tissues. It is clear from the data presented in this thesis that salivary steroid levels give a good indication of unbound and non specifically bound steroid without the difficulties associated with the separation of steroid from protein fractions. Prednisolone appears to be an exception, (Chakraborty et al., 1981). Since these studies show that the amount of steroid in saliva is more closely related to the non- protein bound than the non-SHBG bound fraction, it can be said that in the blood supply to the salivary glands, albumin bound steroid does not freely diffuse into saliva.

Numerous studies have been undertaken in women with hyperandrogenism in an attempt to define the altered hormonal patterns in plasma and relate them to the clinical status of the patient. The literature data showing the mean number of patients in whom increased plasma androgen levels have been detected has been reviewed (Table 5.1). Interpretation of the data is not straightforward because of the problem of definition, since many authors have included in their series patients with PCO as well as those with less obvious causes of hirsutism. Nevertheless, these data arein general agreement with that from other authors, although the incidence of increased androstenedione levels is greater in this study.

It is well documented that SHBG concentrations are diminished with a resultant increase in free testosterone in women with PCO and idiopathic hirsutism, (Anderson, 1974; Motohashi et al, 1979; Yen,1980). Even so, only a proportion of patients studied show a concentration of free testosterone which is in excess of that found in normal women.

Studies of dihydrotestosterone have been made since it is now clear that the mechanism of action of testosterone in some tissues requires conversion of the hormone to dihydrotestosterone prior to binding to a specific intracellular receptor. Nevertheless, the proportion of women with hyperandrogenism showing elevated levels of dihydrotestosterone is not high, probably because the circulating level of this steroid is a poor reflection of the intracellular concentration. In many cases it has been established that the source of increased androgen secretion in these patients is androstenedione deriving from the ovary (Kirschner and Jacobs, 1971; Rosenfield et al., 1972) or less commonly the adrenal, (Horton and Neisler, 1968; Abraham et al., 1976). The measurement of plasma androstenedione is therefore logical in that it is an indication of the secretion rate of this steroid. The survey of the literature, however, reveals that again, only a proportion of patients investigated exhibited increased androstenedione levels.

A few investigators have measured plasma concentrations of DHEA and its sulphate (Abraham et al., 1976; Child et al., 1980), but it is unlikely that extensive investigation of these compounds would prove profitable since they are minor precursors of active androgens and the major usefulness of DHEAS may be only in that it is a marker of adrenal androgen secretion.

The excellent relationship observed between salivary testosterone levels and the unbound plasma testosterone concentration suggested that the measurement of salivary testosterone in patients with hyperandrogenism may prove to be a useful parameter for measurement. As shown in Table 5.1, the results were disappointing from this point of view. Only 35% of the patients with hirsutism had elevated concentrations of testosterone in saliva, although in the patients with PCO, the proportion with elevated levels was identical to that with raised plasma testosterone concentrations. The results for dihydrotestosterone measurements in saliva were similar to those in plasma, and the proportion of patients

Investigator	Percentage of plasma levels elevated in women with PCO and Hirsutism					
	Free T	T	DHT	A	Adiol	DHEA
Abraham et al 1976		52	73	42		41
Andre and James 1974		17		42		
Bardin and Lipsett 1967		69				
Casey 1975	60	48				
Child et al 1980		50				
Clark et al 1975	44					
Duignan et al 1975		54		41		
Easterling et al 1974	80	66				
Ekoe et al 1980		20		54		
Gibson et al 1980		36		57		36
Ito and Horton 1970		60	40			
Mathur et al 1981	56	49		41		31
Mean et al 1977	45	48	26			
Meikle et al 1979		67	43	50	93	
Paulson et al 1977	100	44				
Rosenfield 1971	60	35				
Rosenfield 1975	60		5			
Rosenfield et al 1972	78	44		78		
Szamatowicz & Kulikowski 1979		37	17			
Tamm et al 1980		50	50-55			
Toscano et al 1982						
Tulchinsky and Chopra 1974	75	33	17			
Vermeulen et al 1971	77	54				
Wild et al 1982		60		60		
Wu 1979	43	49				
$\bar{X}$	65	48	34	52		
Present study — Plasma — Hirsutism	58	58	40	72	71	
Present study — Plasma — PCO	83	82	36	100	75	
Present study — Saliva — Hirsutism		35	38	42		
Present study — Saliva — PCO		82	45	82		

Free T - Free Testosterone; T - Testosterone; DHT - Dihydrotestosterone
A - Androstenedione; Adiol - Androstanediol; DHEA - Dehydroepiandrosterone

Table 5.1 The incidence of elevated androgen concentrations in women with polycystic ovaries and hirsutism.

showing abnormal values for androstenedione in saliva was lower than those with elevated plasma levels.

Although the incidence of increased salivary and plasma androgen concentrations in patients with PCO and idiopathic hirsutism was variable and low, 95% of patients with idiopathic hirsutism had at least one out of testosterone, dihydrotestosterone or androstenedione in plasma raised. In the same group of patients, 89% had at least one of these salivary androgen concentrations elevated. Also all patients with PCO had one or more plasma androgen concentration above the normal range, whereas 16% of these cases had normal levels of testosterone, dihydrotestosterone and androstenedione in saliva.

It would appear from these data, therefore, that the measurement of salivary androgen concentrations offers no particular advantage over the measurement of total plasma concentrations in the initial investigation of hyperandrogenism in women. It may be that the free fraction in plasma and the concentration in saliva do not in fact reflect the amount of steroid available to tissues, or that it is the metabolic activity of the tissues themselves that is the major determinant of androgen action. The fact that preliminary reports by Mauvais-Jarvis et al., (1973), and more recent studies (Deslypere et al., 1982; Toscano et al., 1982), suggest that androstanediol is a more consistantly abnormal marker in patients with hirsutism would seem to confirm this latter suggestion.

The measurement of salivary androgen levels has no obvious advantage in the initial investigation of patients with hirsutism, but in patients in whom sex hormone binding globulin levels are altered by therapy, this clearly provides a more useful indicator of the biological changes which are occuring. When patients were treated with cyproterone acetate and ethinyl oestradiol, there was little change in the total concentration of testosterone in plasma. Cyproterone acetate is

known to reduce the production of androgen (Ismail et al., 1974; Barnes et al., 1975), while the effect of ethinyl oestradiol is to increase the concentration of SHBG (Anderson, 1974), and thus the concentration of steroid in plasma no longer reflects the production rate. In contrast, the concentration of testosterone in saliva, which reflects the unbound plasma fraction, is markedly diminished. Hence all patients receiving combined therapy showed a decreased concentration of salivary testosterone. In contrast, concentrations of androstenedione were lowered in both saliva and plasma after treatment, reflecting the decreased production of steroid.

Although salivary androgen measurements allow no greater discrimination between normal and hyperandrogenic women than do plasma measurements, in patients receiving cyproterone acetate and oestrogen, they do appear to give a useful indication of the diminished tissue exposure and may be particularly helpful for following the effects of therapy.

The radioimmunoassays described for testosterone, androstenedione and cortisol utilise a small sample size and allow the direct determination of the unbound steroid level by measuring the steroid concentration in the dialysate after equilibrium dialysis. This technique has two important advantages over methods requiring radiolabelled isotope. Even with frequent chromatographic purification, non binding impurities can produce overestimates of the free steroid level. Also the mass of label introduced into the system will shift the equilibrium. However, as shown in Section 2, this shift is minimised with purified label of high specific activity.

Salivary assays are of particular value where multiple collections are required and for monitoring therapy. Samples may be collected in the patient's own home and posted directly to the laboratory for analysis. The collection of samples is simple and non invasive, hence the apprehension associated with painful venepuncture and the resulting

elevation of adrenal steroids is avoided. Furthermore, there is no requirement for skilled personnel to take samples. Another important application is in paediatric endocrinology where blood sampling techniques are difficult and 24 hour urine collections unreliable.

Two physiological studies have been described where salivary collection is preferable to blood sampling. In the investigation of steroid metabolism in exercise, venepuncture is impractical and indwelling cannulas may affect the athletic performance. Salivary steroid measurements are particularly useful where multiple sampling is necessary, for example in stimulation and suppression tests and for 24 hour profiles.

Due to the low concentration of steroids in saliva, it is necessary to set up very sensitive assays for the accurate determination of steroid levels. This must, at the present time, limit the number of laboratories with sufficient expertise and equipment to perform salivary androgen assays. Testosterone determinations in male saliva are easy to perform and levels closely agree with the unbound plasma concentrations. The accurate measurement of testosterone in female saliva, however, requires the incorporation of a pre-assay purification step. This discrepancy can be explained by the relatively high concentration of interfering compounds compared to testosterone in female saliva. It was also necessary to purify saliva samples prior to measuring androstenedione and dihydrotestosterone in both male and female samples.

Cortisol concentrations in saliva are relatively high, hence salivary cortisol assays are easy to set up. It has recently been shown that with simple modifications to a commercial kit for plasma cortisol, it is possible to directly measure concentrations in saliva (Al-Ansari et al., 1982), thus enabling any laboratory with access to

a γ counter to perform this assay.

The health risk associated with the handling of samples infected with tubercle bacilli has to be considered. The working party under the chairmanship of Sir James Howie recommended that the handling, processing and culturing of sputum or other potentially tuberculus material should be confined to a separate room or at least restricted to an exhaust protection cabinet (Department of Health and Social Security, 1978). Whether these facilities are available or not, protective immunisation and annual chest X-ray should be obligatory to all staff working with such samples.

In summary, salivary androgen measurements are a useful alternative to total and unbound plasma androgen determinations, and may give a better indication of the biologically available steroid than do total plasma levels, especially in conditions of altered plasma protein binding. The technique of sampling is simple and non invasive, and allows the collection of multiple samples with little inconvenience to the patient. With the introduction of more specific and sensitive techniques of analysis, salivary androgen determinations should be increasingly used and become the method of choice by a number of investigators in the study and treatment of gonadal function.

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[REPRINTED FROM THE PROCEEDINGS OF THE SOCIETY FOR ENDOCRINOLOGY, 22–23 MAY 1980]
JOURNAL OF ENDOCRINOLOGY, [1980], 87, 46P–47P
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Testosterone in saliva of normal men and its relationship with unbound and total testosterone levels in plasma. By P. M. Baxendale, M. J. Reed and V. H. T. James. *Department of Chemical Pathology, St Mary's Hospital Medical School, London, W2 1PG*

There are several advantages of measuring hormone concentrations in saliva rather than in plasma. It is a non-invasive technique and frequent samples can be obtained, but the most important advantage of salivary samples is that the steroid concentrations are believed to reflect the unbound (i.e. biologically active) concentration of steroid in plasma. However, this general assumption has so far not been verified in male subjects.

A study therefore was carried out to compare the concentration of testosterone in saliva of male subjects with the concentration of unbound testosterone in plasma. In addition, variations in salivary and plasma testosterone levels throughout the day, and some factors which may influence testosterone levels, have been investigated.

Salivary and plasma testosterone were measured using a specific, sensitive radioimmunoassay (RIA; standard curve 0·6–40 pg/tube). Testosterone was extracted with diethyl ether and dextran-coated charcoal used to separate free from antibody-bound ligand. The antiserum, raised against a testosterone 3-(*O*-carboxylmethyl)-oxime/bovine serum albumin conjugate was kindly donated by Professor K. Griffiths (Tenovus Institute, Cardiff). Thin-layer chromatography showed that the method was specific for testosterone and the sensitivity for saliva and plasma was 3 pg/ml. The intra- and interassay coefficient of variation for salivary measurements were 7·8% ($n = 9$) and 8·9% ($n = 25$) respectively. The unbound plasma testosterone concentration was measured using a technique similar to that described for cortisol (Clerico, Del Chicca, Zucchelli, Mariani & Materazzi, 1978). Using an equilibrium dialysis machine (Dianorm, Diachema AG, Switzerland), 1 ml plasma was equilibrated against 1 ml saline for 4 h at 37 °C. Aliquots of the dialysate (200 μl) were taken for RIA.

There was a highly significant correlation ($r = 0{\cdot}81$, $P < 0{\cdot}001$) between the concentration of testosterone in saliva (79 ± 21 (S.D.) pg/ml, $n = 24$) and unbound concentration of testosterone in plasma (75 ± 18 pg/ml, $n = 24$). The unbound testosterone in plasma as measured by this direct method corresponds to 1·3–2·6% of the total plasma testosterone concentration and is therefore similar to values obtained by an indirect method involving the use of isotopically labelled steroid.

For two normal male subjects, when samples of saliva were obtained at hourly intervals from 08.00 to 24.00 h, the salivary concentration of testosterone was found to decrease rapidly, levels being significantly lower at 10.00 than 09.00 h with minimal levels between 22.00 and 24.00 h. This is consistent with the findings of Landman, Sandford, Howland, Dawes & Pritchard (1976) and Walker, Wilson, Read & Riad-Fahmy (1980). Further studies suggested that this rapid decrease in salivary testosterone levels in the morning did not depend upon the time of day or posture, but upon the time of waking. The relationship between the concentration of testosterone in saliva, the unbound and total plasma testosterone levels was maintained in both early morning and late afternoon samples, suggesting that changes in testosterone production, rather than in the binding of testosterone to plasma proteins, accounts for the circadian rhythm.

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Clinical Endocrinology (1982) **16**, 595–603

SALIVARY TESTOSTERONE: RELATIONSHIP TO UNBOUND PLASMA TESTOSTERONE IN NORMAL AND HYPERANDROGENIC WOMEN

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(*Received 28 October 1981; revised 4 December 1981; accepted 14 December 1981*)

SUMMARY

A sensitive radioimmunoassay (RIA) was used to measure salivary testosterone levels in normal women, in patients with polycystic ovaries (PCO), and in women with hirsutism. There was a highly significant correlation ($r = 0{\cdot}79$, $P < 0{\cdot}001$) between the concentration of testosterone in saliva [$12{\cdot}3 \pm 7{\cdot}8$ (SD) pg/ml] and the concentration of unbound testosterone in plasma ($5{\cdot}2 \pm 3{\cdot}1$ pg/ml) in matched samples collected from 56 women including normals, patients with clinical signs of hyperandrogenism, and women treated with a combination of cyproterone acetate (CA) and ethinyl oestradiol (EE). The unbound plasma testosterone was measured in the dialysate directly using a sensitive RIA. Salivary and plasma testosterone levels in patients with PCO ($20{\cdot}6 \pm 8{\cdot}5$ and 626 ± 187 pg/ml respectively, $n = 14$) and in those with hirsutism ($13{\cdot}9 \pm 5{\cdot}6$ and 421 ± 170 pg/ml, $n = 30$) were significantly higher ($P < 0{\cdot}001$) than levels in normal women ($7{\cdot}7 \pm 2{\cdot}6$ and 196 ± 68 pg/ml, $n = 36$). Treatment for 3 months with CA and EE resulted in a decrease (mean 68%) in salivary testosterone levels in all patients studied ($n = 15$), but the suppression of plasma testosterone (mean 34%) was not observed in all cases. It is concluded that measurement of salivary testosterone gives a useful indication of levels of biologically available androgen in hyperandrogenic women, before and during CA/EE therapy.

The measurement of steroid concentrations in saliva is finding increasing application in clinical endocrinology, and offers two major advantages. Firstly, since the technique of sampling is simple and non-invasive it avoids the difficulties and possible stressful effects of venipuncture; furthermore, there is no requirement for skilled personnel to take samples. Secondly, salivary concentrations appear to reflect the non-protein bound steroid concentration in the blood (Katz & Shannon, 1969; Smith *et al.*, 1979; Baxendale *et al.*, 1980; Umeda *et al.*, 1981), and since protein binding is an important factor in

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determining steroid availability to the tissues, this may be a more useful measurement than that of the total steroid concentration. Salivary steroid measurements may thus be particularly helpful in the investigation of altered androgen metabolism, for example in some patients with hirsutism or with polycystic ovarian disease (PCO). It is well documented that the free testosterone concentration in plasma is elevated in many of these cases in association with diminished levels of sex hormone binding globulin (SHBG) (Anderson, 1974; Motohashi *et al.*, 1979; Yen, 1980), a finding which renders difficult the interpretation of total plasma testosterone levels.

The purpose of this study was to examine the relationship between salivary, unbound and total plasma testosterone concentrations in normal subjects and in women with hirsutism, and to investigate the effect of antiandrogen therapy on these factors. To achieve the necessary sensitivity for measurement in female saliva, it was necessary to develop a sensitive radioimmunoassay for testosterone.

MATERIALS AND METHODS

Reagents

All chemicals were supplied by Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire, except where stated. Testosterone was supplied by Steraloids Ltd, Croydon, England. [1,2,6,7,16,17, (n) ^{3}H]-testosterone (152 Ci/mmol) was obtained from New England Nuclear Chemicals GmbM, Dreieich, West Germany. Pre-coated aluminium backed thin layer chromatography (TLC) plates (Merck 5553) were obtained from BDH Ltd, Poole, Dorset. Phosphate buffer (0·1 M) was prepared from disodium hydrogen phosphate (22 g) sodium dihydrogen phosphate (5·9 g), sodium chloride (9·0 g), sodium azide (1·0 g) and gelatin (1·0 g). The volume was made up to 1 litre with deionized water and the pH adjusted to 7·0 with 2 M sodium hydroxide. Scintillation solution was prepared by dissolving *p*-terphenyl (15 g) and dimethyl POPOP (0·20 g) in 5 l sulphur-free toluene containing 2% methanol.

Equilibrium dialysis was performed using a dialysis machine purchased from Dianorm, Diachema AG, Switzerland. Visking tubing size 36/32 inch, was supplied by Medicell International, Rome. Charcoal (Norit A) was supplied by Sigma, Poole, Dorset and dextran T70 from Pharmacia Fine Chemicals, Uppsala, Sweden. Assay and extraction tubes were purchased from Glass Wholesale Supplies, Gable Street, London, E1, Multivortex shaker from Searle Instrumentation, PO Box 53, Lane End Road, High Wycombe, Buckinghamshire. Tritiated steroids were located using a radiochromatogram imaging system from Panax Equipment Ltd, Bournemouth, Dorset.

Subjects

Blood was collected from thirty-six, and saliva from thirty-two normal women aged between 18 and 38 who were not receiving any form of hormonal contraception and who had regular menstrual cycles. Blood and saliva were obtained from fifty women with clinical signs of hyperandrogenism (thirty-two with idiopathic hirsutism, fourteen with polycystic ovaries, four with acne) at their first visit to the clinic, and from eighteen following 3 months antiandrogen treatment. The diagnosis of PCO was based on the presence of amenorrhoea or oligomenorrhoea with elevated plasma LH levels. Laparoscopy in seven patients confirmed this diagnosis.

Collection of samples

All blood and saliva samples were collected between 09.00 h and 14.00 h. Whole unstimulated saliva (3 ml) was collected directly into sterile plastic tubes. Blood was taken by venipuncture into heparinized tubes; plasma and whole saliva were frozen and stored at −20 C prior to assay.

Antiandrogen treatment

Cyproterone acetate (CA) and ethinyl oestradiol (EE) were given in a reverse sequential regimen (Hammerstein & Cupceanu, 1969) for 3 months. CA (100 mg) daily was taken from days 5–15 of the menstrual cycle and EE (50 μg) from days 5–26.

Measurement of unbound plasma testosterone

Presoaked Visking tubing was cut into squares of 4 × 4 cm and placed between the teflon half cells of an equilibrium dialysis machine. 1 ml of plasma was equilibrated for 4 h at 37 C, whilst rotating at 12 r/min, against 1 ml of sterile saline. The dialysate was collected and the testosterone concentration measured by RIA. Using an equation similar to that described for free plasma cortisol (Clerico *et al.*, 1978), the unbound plasma testosterone concentration was calculated.

$$FT = \frac{Td. \qquad Vi}{(TT.Vp) - (Td.Vo)} . TT$$

where FT = free plasma testosterone concentration (pg/ml)
TT = total plasma testosterone concentration (pg/ml)
Td = concentration of testosterone in the dialysate measured by RIA (pg/ml)
Vi = final plasma volume following dialysis (ml)
Vp = initial volume of dialysed plasma (ml)
Vo = volume of dialysate (ml)

Radioimmunoassay

One thousand five hundred d/min [1,2,6,7,16,17 ^{3}H] testosterone was added to glass extraction tubes (4 × $\frac{5}{8}$ inch) containing saliva (1·0 ml) or plasma 0·5 ml 1/10 dil) or equilibrium dialysate (0·8 ml), and to three scintillation vials in order to monitor methodological losses. After adding diethyl ether (5·0 ml) from a freshly opened bottle, the tubes were mixed in a multivortex shaker for 5 min.

Tubes were placed into a dry ice–ethanol mixture in order to freeze the aqueous layer and the ether extracts were decanted into glass tubes (3 × $\frac{1}{2}$ inch). The ether was dried under a steady stream of nitrogen at 30 C and the residue dissolved in 100 μl of ether. These extracts were applied to TLC plates and run in a chamber containing dichloromethane: 1,4-dioxan (94:6 v/v). (TLC plates had been run in this system before applying the samples). Two markers containing a sufficient amount of radioactive testosterone to be detected by a radiochromatogram imaging system were run on each plate. After chromatography, radioactive areas were located on the TLC plates and the corresponding sample area cut out and placed into extraction tubes containing diethyl ether (3·0 ml). Samples were eluted for at least 1 h, then decanted into glass tubes. After evaporation of the ether with nitrogen, the purified sample was reconstituted in phosphate buffer (0·75 ml), vortex mixed and allowed to stand for 30 min. From each solution, 150 μl was placed into scintillation vials in order to calculate the recovery.

Antiserum, (100 μl, dilution 1/150 000) raised against a testosterone 3-(O-carboxymethyl)-oxime/bovine serum albumin conjugate was added to glass incubation tubes ($3 \times \frac{3}{8}$ inch) containing 500 μl reconstituted sample or duplicate aliquots of testosterone standards (0·6, 1·2, 2·5, 5, 10, 20 and 40 pg) made up to 500 μl with phosphate buffer. Tubes were mixed and incubated for 30 min at room temperature. Twenty thousand d/min [1,2,6,7,16,17^{3}H] testosterone in 100 μl buffer were added to all tubes, mixed and incubated for a further 30 min at room temperature followed by 15 min in an ice water bath. Separation of bound and free steroid was performed by adding cold dextran-coated charcoal suspension (0·2 ml), (0·625% w/v acid washed charcoal and 0·062% w/v dextran T70 in assay buffer). The tubes were quickly mixed, allowed to stand for 15 min and centrifuged at 1500 g for 5 min at 4 C. Supernatants were rapidly decanted into counting vials containing p-terphenyl scintillator, capped, shaken mechanically for 10 min and the radioactivity determined in a liquid scintillation counter.

Validation

Preliminary experiments showed that equilibrium was reached after 4 h of incubation and remained unchanged for up to 19 h. In order to test whether testosterone adsorbed to the Visking membrane, 10 000 d/min [^{3}H] testosterone in saline was dialysed against saline at 37 C. In excess of 98% of the added radioactivity was recovered from the saline and there was no significant difference between the concentrations of radioactivity on either side of the membrane. The unbound testosterone in plasma as measured by this direct method produced similar values to those obtained by an indirect method involving the use of isotopically labelled steroid.

The precision of salivary and plasma measurements was assessed from within-batch analyses of duplicate samples. The coefficients of variation (CV) were 8·6% ($n = 28$) in the range between 2·8 and 26·3 pg/ml for saliva and 6·3% ($n = 31$) in the range between 48 and 745 pg/ml for plasma. The between-batch CV which was assessed by assaying duplicate aliquots of pooled sample in successive batches was 9·9% ($n = 20$) for saliva and 13·4% ($n = 13$) for plasma. The between batch variation for unbound plasma samples was assessed from duplicate pooled plasma samples assayed after equilibrium dialysis over a period of 6 months. The CV was 12·5% ($n = 12$). Frozen plasma could be stored for at least 6 months with no change in the measured concentration of free testosterone.

The assay sensitivity, defined as the smallest concentration significantly different from zero at the 5% level, and using duplicate assays, was 0·7 pg/ml for saliva and 15 pg/ml for total plasma testosterone. Thin layer chromatography showed that the antibody was not specific for testosterone in saliva, levels after TLC being roughly half those without chromatography ($y = 0{\cdot}47\ x + 0{\cdot}07$). Consequently a pre-assay purification step was included in all total, unbound plasma and salivary measurements. In the TLC system used, the R_f for testosterone and dihydrotestosterone were 0·29 and 0·44, ensuring adequate separation.

Recovery from saliva was assessed by adding 5, 10 and 20 pg of authentic testosterone to a previously assayed saliva sample and reassaying. The mean recovery was 96%.

RESULTS

Relationship between salivary and unbound plasma testosterone

There was a highly significant correlation ($r = 0{\cdot}79$, $P < 0{\cdot}001$) between the concentra-

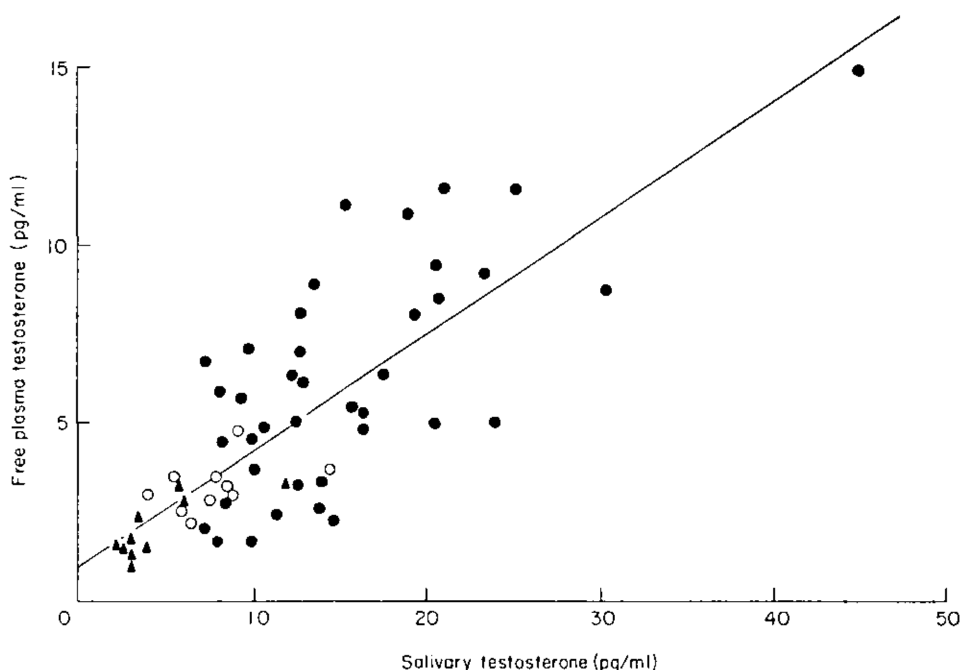

Fig. 1. Comparison of salivary testosterone with unbound plasma testosterone concentrations in matched samples taken from patients with PCO or idiopathic hirsutism before treatment (●), after treatment (▲) and normal subjects (○). $y = 0{\cdot}32\,x + 1{\cdot}1$; $r = 0{\cdot}79$; $P < 0{\cdot}001$.

tion of testosterone in saliva ($12{\cdot}3 \pm 7{\cdot}8$ pg/ml, mean ± SD), and the unbound concentration of testosterone in plasma ($5{\cdot}2 \pm 3{\cdot}1$ pg/ml), in fifty-six matched samples collected from normal, hyperandrogenic and CA/EE treated women (Fig. 1).

Salivary testosterone levels in normal and hyperandrogenic women

Testosterone concentrations in saliva samples collected upon waking and throughout the day on two separate occasions from a normal women (Fig. 2) clearly demonstrate the importance of a controlled time of collection of samples.

Testosterone concentration in saliva and plasma samples from women with infertility arising from PCO and idiopathic hirsutism, normal subjects and hyperandrogenic women following 3 months treatment with CA and EE, are shown in Fig. 3. Mean salivary levels were $7{\cdot}7 \pm 2{\cdot}6$ pg/ml, ($n = 34$) for normal women, $20{\cdot}6 \pm 8{\cdot}5$ pg/ml, ($n = 14$) for patients with PCO, $13{\cdot}9 \pm 5{\cdot}6$ pg/ml ($n = 30$) for women with idiopathic hirsutism, and $6{\cdot}1 \pm 4{\cdot}2$ pg ml ($n = 18$) for treated women. The corresponding total plasma testosterone levels were 196 ± 68 pg/ml ($n = 36$) for normal women, 626 ± 187 pg/ml ($n = 14$) for patients with PCO, 421 ± 170 pg/ml ($n = 30$) for women with idiopathic hirsutism, and 279 ± 99 pg/ml ($n = 18$) for treated women.

Both total plasma and salivary testosterone concentrations in women with PCO and hirsutism were significantly higher ($P < 0{\cdot}001$) than in normal subjects. Salivary levels of testosterone in patients treated with CA and EE were not significantly different from normals, whereas plasma total testosterone levels in treated women remained elevated ($P < 0{\cdot}001$).

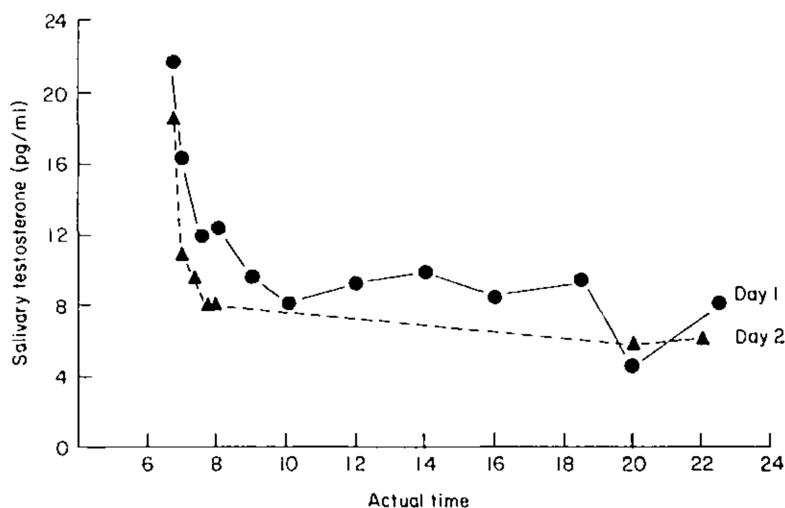

Fig. 2. Concentration of testosterone in saliva samples collected from a normal female on 2 consecutive days. ● Day 1; ▲ day 2.

This result is further illustrated in Fig. 4, which shows the effect of 3 months treatment with CA and EE on plasma total and salivary testosterone concentrations in fifteen hyperandrogenic women. There was a mean decrease in the salivary concentrations of 68%, but a drop in plasma levels was not observed in all patients, the mean decrease being 34%.

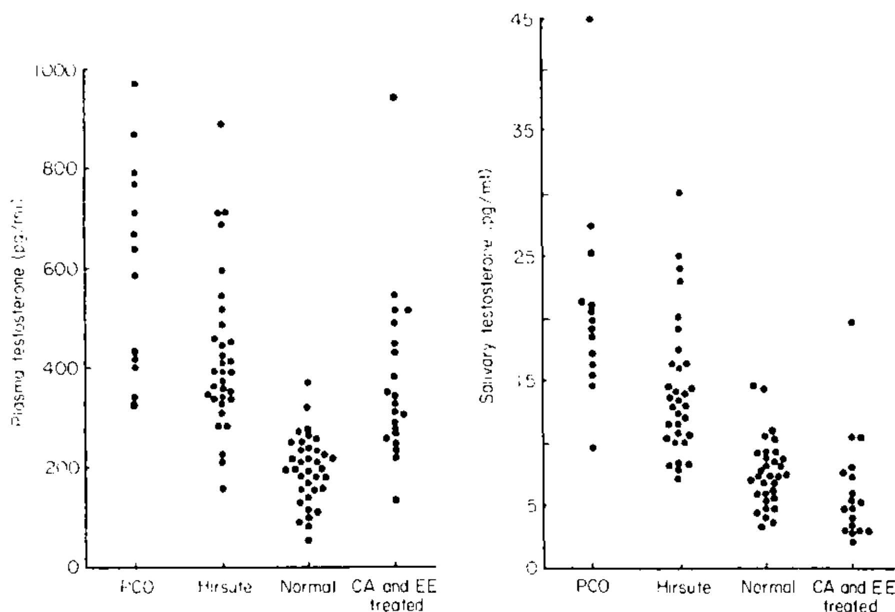

Fig. 3. Comparison of salivary and total plasma testosterone concentrations in patients with PCO, idiopathic hirsutism, normal women and hyperandrogenic women after treatment with CA and EE for 3 months.

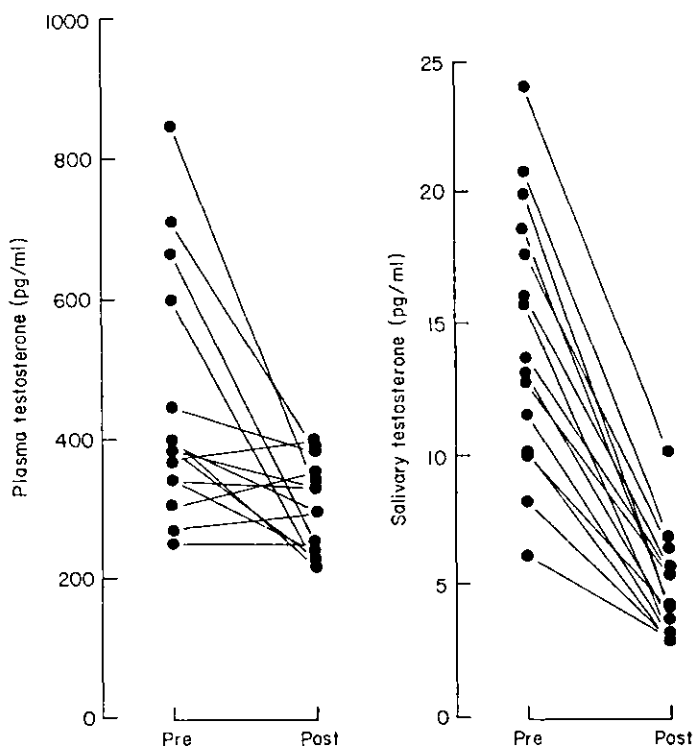

Fig. 4. Response of salivary and total plasma testosterone concentrations in fifteen hyperandrogenic women after 3 months treatment with CA and EE.

DISCUSSION

These studies clearly demonstrate that salivary testosterone levels accurately reflect the unbound plasma concentration in hyperandrogenic and normal women, and that the relationship still exists after antiandrogen therapy. Salivary assays are of particular value when multiple collections are required and for monitoring therapy. Samples can be collected in the patient's own home and posted directly to the laboratory for analysis.

The RIA described utilizes a small sample size and allows the direct determination of unbound plasma testosterone by measuring the testosterone concentration of the dialysate after equilibrium dialysis. The mean percentage free testosterone levels measured by this method were 1·8% for patients with PCO, 1·5% in women with idiopathic hirsutism, 1·3% in normal subjects and 0·7% in patients treated with CA and EE. These levels are similar to those obtained using isotopically labelled steroid (Vermeulen *et al.*, 1971; Moll & Rosenfield, 1979). The present technique has two important advantages over methods requiring radiolabelled isotope: the mass of labelled steroid introduced into the system may shift the equilibrium and, more importantly, frequent chromatographic purification of the label is required. Even a small amount of non-binding impurity produces an overestimate of the free testosterone measured.

Levels of testosterone in saliva of normal and hirsute women reported by other authors (Smith *et al.*, 1979; Luisi *et al.*, 1980) are much higher than our own findings. In the studies quoted, commercially available kits designed for the measurement of testosterone in

plasma were used, but these kits do not have the sensitivity required to measure the low levels of testosterone present in female saliva. The rapid decrease in salivary testosterone in the morning in a normal woman supports the findings of Turkes *et al.*, 1980 and shows a similar pattern to that reported in men (Baxendale *et al.*, 1980; Walker *et al.*, 1980). This sharp fall in the early morning has not been reported for total, or free testosterone levels in plasma (Rosenfield & Helke, 1974) and so at this time of day the saliva may not reflect accurately the unbound plasma concentration.

It is known that salivary albumin originates from plasma, although the exact mechanism has yet to be elucidated (Hattingh, 1979). The concentration of albumin in saliva is thought to be about 1% of that in plasma; nevertheless transfer of small amounts of testosterone bound to plasma proteins may account for the higher levels in saliva compared with the unbound fraction in women. After equilibrium dialysis of fresh whole saliva, the percentage free hormone ranged from 84–89% in three normal females. The differences between the testosterone levels in saliva and the unbound plasma testosterone concentrations were not observed in normal men (Baxendale *et al.*, 1980). Perhaps the high androgen concentrations mask any protein-bound transfer.

Salivary testosterone levels from patients with PCO, idiopathic hirsutism and normal women correlate well with plasma concentrations. Both these levels are slightly lower than most of those reported in the literature, probably due to the inclusion in our study of a preassay purification step. The most significant finding was seen in patients treated with CA and EE. As reported by Frölich *et al.*, 1977, and Peereboom-Wynia & Boekhorst, 1980, plasma testosterone concentrations decreased in some but not all patients studied. A sharper decline was however observed in all salivary testosterone levels in these patients in association with a decrease in the plasma free testosterone concentration. CA and EE have been used by a number of workers in the treatment of hirsutism. CA has been shown to act in several different ways, including the inhibition of gonadotrophin secretion (Barnes *et al.*, 1975) and effects on androgen binding in the tissues (Giorgi, 1976) and steroid synthesis (Panesar & Stitch, 1976). This study demonstrated a decrease in unbound plasma testosterone concentrations on combined CA and EE treatment.

It is well known that treatment with oestrogen increases production of SHBG in the liver and therefore results in a decrease in the free testosterone fraction (Anderson, 1974). CA has little effect on SHBG alone (Frölich *et al.*, 1980), although it has been shown to enhance the hepatic response to oestrogen (Sawers *et al.*, 1980).

The data reported here clearly demonstrate the value of salivary steroid measurements in a clinical situation. Salivary testosterone measurements give useful information about the biologically available androgen without the use of the complicated techniques required to measure unbound plasma levels.

ACKNOWLEDGEMENTS

We are grateful to Professor K. Griffiths (Tenovus Institute, Cardiff) for the kind gift of antiserum and to Schering Chemicals Limited for a grant for equipment.

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