Studies of adrenal androgens

Submitted for the degree of Ph.D. by

David Llewelyn Jones

St. Marys Hospital Medical School,
Paddington,
London W2.

ABSTRACT

The thesis describes the development of specific reproducible radio-
immunoassays for dehydroepiandrosterone (DHA), DHA sulphate (DHAS) and
pregnenolone. Distinct diurnal variations are demonstrated for DHA,
pregnenolone and DHAS, the secretory episodes of DHA and pregnenolone
occurring synchronously with cortisol suggesting control of secretion
by adrenocorticotrophic hormone (ACTH). Three examples of a divergence
between the secretion of cortisol and the adrenal androgens DHA and DHAS
are described, suggesting the presence of a specific adrenal androgen
stimulating hormone (AASH). Prolactin has been proposed as the postulated
AASH and although a good correlation is demonstrated between the plasma
levels of both DHA and DHAS with prolactin, it was concluded that
prolactin is not the postulated AASH.

The adrenal androgens have been implicated in the aetiology of breast
cancer, however, in the patients with breast cancer reported here no
significant difference was found between the plasma levels of either DHA
or DHAS compared to matched control subjects. However, the levels of DHA
in normal breast tissue are significantly lower than those in breast
tumour tissue. Significant correlations are shown between the
concentration of steroid in plasma to that in endometrial tissue and the
role of plasma bound and free steroid in regulating tissue steroid
levels is discussed.

The existence and possible site of origin of novel non-polar conjugates of pregnenolone, DHA and androstenediol is reported. Plasma levels of the non-polar conjugates of pregnenolone, DHA and androstenediol in pre-menopausal women are $4.5 nmol/l$, $1.5 nmol/l$ and $0.9 nmol/l$ respectively. Increased levels of the non-polar conjugate of DHA are seen in hirsute patients ($5.5 nmol/l$) while both the non-polar conjugates of pregnenolone and DHA are elevated in patients with acne. A possible role for the non-polar conjugates in the aetiology of hirsutism and acne is proposed.

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ABBREVIATIONS AND TRIVIAL NAMES

Abbreviations commonly used in the text are listed below:

ACTH, corticotrophin; BPA, bovine plasma albumin; HGG, human gamma globulin; CBG, corticosteroid binding globulin; DHA, dehydro-epiandrosterone; DHAS, dehydroepiandrosterone sulphate; FSH, follicle stimulating hormone; LH, luteinising hormone; SHBG, sex hormone binding globulin; CMO, carboxymethyl oxime; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; AASH, adrenal androgen stimulating hormone.

The following trivial names have been used:

Androstenedione, 4-androstene-3, 17 dione; cortisol, 11β, 17α, 21-tri-hydroxy-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; pregnenolone, 3β-hydroxy-5-pregnen-20-one.

INTRODUCTION

Steroid hormones are generally divided into three main groups depending
on the biological effect elicited in various test bioassay systems.
Of the three groups, mineralocorticoid, glucocorticoid and sex
hormones, it is a subgroup of the latter, the "androgenic steroid"
hormones that will be the topic of investigation in this study.
Encompassed within the group of "androgenic steroids" are many
compounds of differing molecular structure, site of production within
the body, biological activity and physiological importance.

In the human the principle circulating androgens are testosterone,
dihydrotestosterone (DHT), andros-5-ene-3β, 17β diol,
dehydroepiandrosterone (DHA), androstenedione, 5α androstane-3α,
17β diol and DHA sulphate. Testosterone and DHT are the most potent
androgens with DHA and androstenediol only having 16% and 3% of
the potency of testosterone as assessed by the capon comb assay
(Dorfman and Shipley, 1956). The relative degree of potency depends
on the assay system used.

Some androgenic steroids have been studied for many years and their
biological role and significance is quite well understood, e.g.
testosterone and DHT, however, for a number of other androgens no major
biological role has yet been proven e.g. DHA and DHAS.

Circulating androgens in plasma derive from the testis, ovary or adrenal cortex, either by direct glandular secretion or by conversion from less active precursors. The concentration of individual steroids in plasma is determined by the relative glandular secretion, the interconversion of prehormones and the metabolic clearance rate.

In the human 95% of plasma DHAS (Abraham, 1974) and 80% of DHA (Abraham and Chakmajian, 1973; Nishida et al, 1977) is produced by the adrenal cortex, with androstenedione, 50% in women (Abraham, 1974) and 70% in men, also being a major product. As the major site of production is the adrenal these steroids have become known as the adrenal androgens.

The biosynthesis of adrenal androgen by the adrenal cortex is thought to occur quantitatively in the zona reticularis although androgen production by the zona fasciculata and reticularis may be qualitatively very similar (Maroulis and Abraham, 1980). The zona reticularis produces more sulphoconjugated steroid than the zona fasciculata (O'Hare et al, 1980).

The common precursor for steroid synthesis is cholesterol which can be formed by synthesis from acetate by both the gonad and the adrenal cortex. The latter can also obtain cholesterol by uptake from low density lipoprotein (LDL) cholesterol which binds to specific receptors on the adrenal cells (Brown et al, 1979).

The synthesis of pregnenolone from cholesterol by the adrenal is under the control of the pituitary adrenocorticotrophic hormone (ACTH), possibly by affecting the binding of cholesterol to a cytochrome P450 involved in side chain cleavage (Simpson et al, 1971).

Two main pathways exist for the formation of the adrenal androgens, both of which start from pregnenolone, the delta five pathway to DHA and androstenediol (Neher and Wettstein, 1960), and the delta four pathway to androstenedione and testosterone (Slaunwhite and Samuels, 1956), fig. 1.1. Inter-conversion between the two pathways is possible by the action of the delta 3-5 isomerase dehydrogenase enzyme complex.

A third biosynthetic pathway, the so called "sulphate pathway", has been reported from cholesterol sulphate through to DHAS which occurs without the loss of the sulphate moiety (Calvin et al, 1963; Roberts et al, 1964; Baulieu et al, 1965). Knapstein et al, (1967) have suggested that the sulphate pathway can progress to oestrone sulphate but there is little evidence to support this.

Assuming the sulphate pathway has a similar biosynthetic sequence to the unconjugated steroid the production of oestrone sulphate requires the synthesis of androstenedione sulphate. The existence of androstenedione sulphate in plasma is controversial due to the instability of the proposed 3-enol sulphate structure of the steroid A ring. Goodall et al, (1981) have reported the presence of androstenedione-3-enol sulphate in plasma but the work has not been duplicated.

CHOLESTEROL

PREGNENOLONE

PROGESTERONE

17α HYDROXY PREGNENOLONE

17α HYDROXY PROGESTERONE

DHA

ANDROSTENEDIONE

ANDROSTENEDIOL

TESTOSTERONE

Δ^5–Pathway
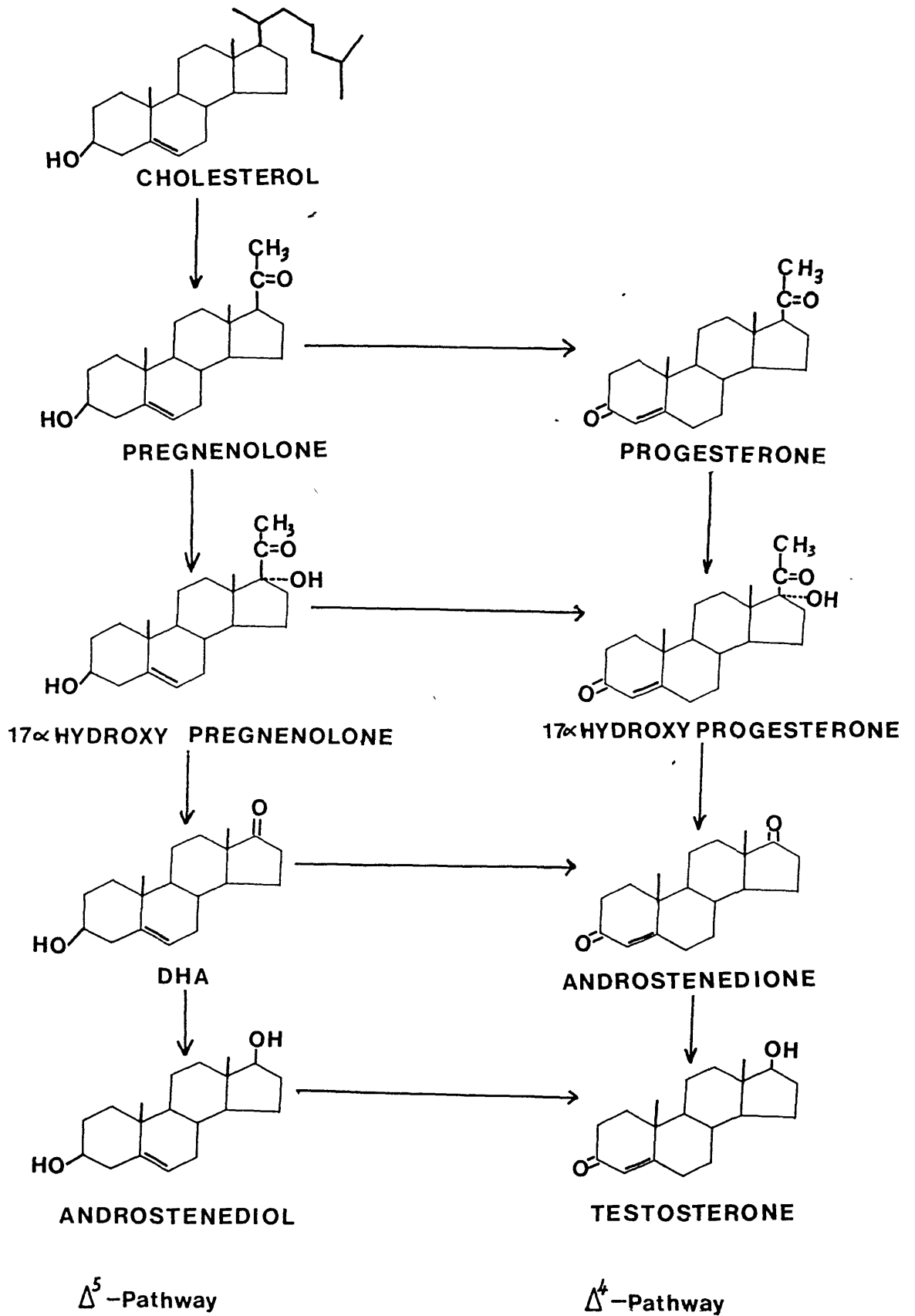
Δ^4–Pathway

Fig. 1.1. Biosynthetic pathways of the delta 4 and delta 5 steroids.

Tissues other than the endocrine tissues also possess the ability to take up steroid from the peripheral circulation and convert a biologically inactive steroid to an active product via the pathways described above prior to releasing the steroid to the circulation or using it intracellularly at the site of production.

The conversion of the adrenal androgens to biologically active metabolites has been demonstrated _in vitro_ for a number of tissues, DHA to DHT in skin (Thomas and Oake, 1975), androstenedione to oestrone in adipose tissue (Siiteri and MacDonald, 1973), DHA and androstenedione to androstenediol and DHT respectively by pulmonary endothelial cells (Milewich _et al_, 1983) and _in vivo_ by muscle tissue of androstenedione to oestrone (Longcope _et al_, 1978).

For a number of steroids peripheral production is the major source of their production. In postmenopausal women proof of the existence of the "sulphate pathway" to oestrone sulphate would be of importance as these women derive the majority of their oestrone via peripheral conversion of androstenedione (Siiteri and MacDonald, 1973). As the unconjugated steroid can be formed from the conjugated steroid the presence of oestrone sulphate formed from DHAS via androstenedione-3-enol sulphate would provide another source of oestrone production, which may lead to excess oestrogen production.

The importance of DHA as a precursor to androstenediol by peripheral conversion is controversial. Bird et al, (1978) claim that all plasma androstenediol can be accounted for by peripheral conversion of DHA, while Demisch et al, (1973), claim that 30% is produced by peripheral conversion of DHA with 60% secreted by the adrenal. The latter information would suggest that despite a low androgenic activity androstenediol should be included with DHA, DHAS and androstenedione as an adrenal androgen.

The association between ACTH secretion and cortisol production by the adrenal cortex has been known for a long time and it has been assumed that as DHA and androstenedione appear in the plasma synchronously with cortisol that ACTH also controlled the secretion of the adrenal androgens. In recent years there have been a number of reports of clinical situations in which the secretion of cortisol and the adrenal androgens is divergent (Parker and Odell, 1980). These findings have led to the suggestion that there is another pituitary hormone that controls the secretion of the adrenal androgens, the so called "adrenal androgen stimulating hormone" (AASH). Proof of the existence and identity of the AASH could be inportant in the understanding of the aetiology of a number of clinical situations of androgen excess.

An increased plasma concentration of a steroid does not necessarily result in an increased "biological effect" as the steroid has to cross the cell membrane before an effect can be elicited.

It is generally believed that the unbound steroid represents the biologically active fraction which crosses the cell membrane (Giorgi, 1980).

In plasma, steroids are bound either to albumin or to a specific binding protein. Of the delta five steroids only androstenediol has the 17β-OH group that facilitates binding to the sex hormone binding globulin (SHBG) (Cunningham et al, 1981). As a result 78.5 of plasma androstenediol is bound to SHBG. Binding to the low affinity high capacity albumin accounts for the majority of bound pregnenolone, 96.5, DHA, 88% (Dunn et al, 1981) and DHAS, greater than 95% (Wang and Bulbrook, 1967). Recently Pardrige, (1981) has suggested that the albumin bound steroid fraction may be capable of crossing the tissue membrane.

To my knowledge there are no reports correlating the plasma level of free steroid to the intracellular level of that steroid. It is possible that in some clinical situations there is an increased intracellular metabolism of a precursor steroid to an active product which results in a biological response with no apparent alteration in the plasma total or free level of the active steroid.

Despite a great deal of investigation no specific biological role for DHA or DHAS has been found other than the role of precursor to the active steroids, e.g. during pregnancy DHAS is of major importance as a precursor to the oestrogens (Gant et al. 1971).

The adrenal androgens, DHA and DHAS, have been implicated in the aetiology of breast cancer in two ways. The first as precursors to androstenediol, a steroid that has been shown to compete with oestradiol for the oestradiol cytoplasmic receptor and to induce biological responses characteristic of oestradiol, Van Doorn et al, (1981), and secondly as a modulator of the enzyme 17 hydroxysteroid dehydrogenase which is responsible for the interconversion of oestrone and oestradiol, (Reed etal, 1982). Bearing these points in mind it was important to investigate the relationship between plasma and tissue levels of DHA and DHAS and to assess the relationship between the levels of DHA and DHAS to tissue androstenediol levels and the activity of tissue 17 hydroxysteroid dehydrogenase activity in patients with breast cancer.

Before any study of the possible alterations in plasma steroid level in patients can be made it is important to have a good data base of the normal variation in steroid levels that can be expected. A great many factors can affect plasma steroid levels among which age, sex, stage of menstrual cycle, time of day or year, weight and drug therapy are the main ones.

To investigate physiological levels and changes in hormone concentration an accurate, specific assay technique is required with sufficient sensitivity to measure the steroid in as small a volume of sample as possible. The latter point is important in the investigation of the steroid through a diurnal profile in which a large number of blood samples are taken.

The development of the immunoassay has fulfilled these requirements for the measurement of most hormones. Simple extraction or direct immunoassays are ideal in that large numbers of samples can be processed quickly, however these methods generally require a highly specific antiserum. Therefore to study the steroids of the delta five pathway it was necessary to obtain specific antisera and develop reliable, sensitive immunoassays using these antisera for the steroids of interest.

As with most areas of study there is always the possibility that new or alternative forms of the compound(s) of interest will be discovered. One such new family of steroids are those conjugated to fatty acids as first described by Hochberg et al, (1977). Until recently these compounds had only been described in bovine tissue, however, I report in this thesis the demonstration of similar conjugates present in human blood. The importance and physiological role of these non-polar derivatives have yet to be elucidated.

The aim of this study was to investigate aspects of adrenal androgen metabolism especially the steroids of the delta five pathway. The aspects investigated include control of adrenal androgen secretion and the study of possible variation and roles of these steroids in normal and diseased conditions. The relationship between plasma and tissue levels of steroid and the metabolism of the steroids of the delta five pathway to non-polar conjugates have also been investigated.

2.1 Materials and Methods

a) Chemicals

All chemicals except where stated were supplied by Fisons Scientific Apparatus Ltd., Loughborough, England. Helix pomatia sulphatase (S9626) was supplied by Sigma London Chemical Company Ltd., Poole, Dorset, England. All unlabelled steroids were obtained from Steraloids Ltd., Croydon, England. Sephadex G25 (coarse) and Dextran T70 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

$[1,2,6,7,(n)^3H]$ testosterone (94 Ci/mmol), $[1,2,6,7,(n)^3H]$ androstenedione (91 Ci/mmol), $[1,2,(n)^3H]$ dihydrotestosterone (66 Ci/mmol), $[7,(n)^3H]$ - pregnenolone (14 Ci/mmol), $[7(a)^3H]$ (14 Ci/mmol) and $[1,2,6,7,(n)^3H]$ DHA (66 Ci/mmol) were obtained from Amersham International, Amersham, England.

$[7(n)^3H]$ DHAS (24 Ci/mmol) and $[1,2,(n)^3H]$ androstene 3β, 17β diol (59 Ci/mmol) were obtained from New England Nuclear Chemicals, GmbH, Dreieich, West Germany. Radiolabelled steroids were tested for purity at regular intervals (3-6 months) by paper chromatography or TLC and purified if necessary.

Acid washed charcoal (Norit A) and human gamma globulin were purchased from Sigma, Poole, Dorset, England. Bovine plasma albumin from Armour Pharmaceuticals, Eastbourne, England.

b) Reagents

Borate buffer (0.05M) was prepared from Boric acid (3.2g) dissolved in 1 litre of distilled water and made to pH 8.0 using 2.1 ml 2M NaOH. Acetate buffer (0.2M) was prepared from sodium acetate (4.1g) dissolved in 250 ml distilled water and made to pH 4.8 with glacial acetic acid. Scintillator was prepared from 12g p-terphenyl, 0.16g dimethyl POPOP and 80 ml methanol made to 4 litres with toluene.

Ethanol, methanol, ethyl acetate and dichloromethane were distilled prior to use.

c) Equipment

Glass extraction tubes, 4 in x $5/8$ in and 3 in x 0.5 in were supplied by Glass Wholesale Supplies, Gable Street, London E.1. Stoppered glass tubes were obtained from Quickfit and Quartz, Stone, Staffordshire. Glass assay tubes 2 in x $7/16$ in controlled neck shell tubes No. 3/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 BDH 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., Bournmouth, Dorset, England.

Plastic 5 ml scintillation vials were obtained from Hughes and Hughes Ltd., Romford, Essex, England. A refridgerated Packard Liquid Scintillation Counter was used.

2.2 Chromatography of steroids

Despite recent improvements in the raising of antisera with high specificity, some cross reaction to closely related steroids is still seen. Chromatography is the traditional approach to isolating the steroid of interest from a plasma sample and is still important for the validation of a non-chromatographic immunoassay. A number of chromatography systems have been investigated using both paper and thin layer supports to purify the steroids under investigation in this thesis. The chromatography systems used and the steroid mobility in these systems are shown below and in table 2.1.

Chromatography systems

Paper systems:

1) Bush B3. Petroleum ether:toluene:methanol:water (660:340:800:200 v/v/v/v).

2) Bush A. Heptane:methanol:water (1000:800:200 v/v/v).

3) Bush B239. Di-isopropyl ether: ligroin:t-butanol:0.88 ammonia:water (5:2:3:1:9 v/v/v/v/v).

Thin layer systems:

1) Dichloromethane:ethyl acetate (93:7 v/v).

2) Dichloromethane:acetone (80:20 v/v).

3) Chloroform:acetone (85:15 v/v).

4) Chloroform:methanol:0.88 ammonia (80:20:02 v/v/v).

5) Isopropanol:chloroform:methanol:water (10:10:5:2 v/v/v/v).

6) Dichloromethane:1,4 dioxan (96:4 v/v).

Table 2.1

FREE STEROID	Paper		TLC				Conjugated Steroid	Paper	TLC	
SYSTEM	(1)	(2)	1.	2.	3.	6.		3	4	5
Cortisol	0	0	-	-	-	-	Testosterone SO_4	7.5	-	-
DHAS	0	0.5	-	-	-	-	Estrone SO_4	10.8	9.0	-
Androstene 3β17β Diol	9.2	1.5	4.4	8.7	7.0	4.0	Estradiol 17-SO_4	11.0	-	-
17α Hydroxy Progesterone	-	4.4	4.8	-	9.8	-	D.H.A. SO_4	21.0	8.2	12.0
D.H.A.	23.0	12.0	8.1	11.1	10.0	7.2	Epiandrosterone SO_4	27.0	-	-
Androstenedione	28.0	16.0	9.9	13.8	13.0	10.8	Pregnenolone SO_4	44	-	-
Androsterone	-	22.5	-	12.0	-	-	D.H.A. Glucuronide	-	-	5.5
Pregnenolone	-	24.5	9.4	-	10.2	-				
Progesterone	-	35.0	12.0	-	14.7	-				
Dihydrotestosterone	22.0	-	8.2	-	11.2	7.9				
Estrone	-		12.6	-	13.5	-				
Estradiol	-		7.2	-	8.5	-				
Testosterone	18.4		4.9	-	8.2	9.9				
Etiocholanolone	-		-	10.2	-	-				
3α5α17β Androstanediol	14.4		-	8.5	-	3.1				
16α Hydroxy D.H.A.	-		2.3	-	-	-				

Relative mobilities of steroids in the systems used (cm)

2.3 Preparation of conjugate

a) Mixed anhydride reaction

The mixed anhydride methodology was as described by Erlanger, et al (1957).

Due to the small amount of the DHA-7-carboxymethylamine (DHA-7-CMO derivative) available to us, an initial reaction was performed using DHA-3-hemisuccinate (DHA-3-HS).

1. DHA-3-HS (5 mg) was placed in a stoppered Quickfit 4" x $\frac{1}{2}$" glass tube attached to a silica gel trap and the steroid dried in a vacuum oven at 45°C for 2-5 hours. Dimethyl formamide (DMF, 500 μl) was added to the dry steroid with constant stirring using a magnetic flea and the temperature taken to 0°C by placing the vessel in an ice bath. To the steroid solution add 50 μl of tri-n-butylamine (TNB, diluted 1:15 with DMF), in 10 μl aliquots followed by 50 μl of isobutyl chloroformate (IBCF, diluted 1:28 in DMF), in 10 μl aliquots, both additions being made with constant stirring. The mixture was allowed to react at 0°C for 35 minutes.

The protein solution was prepared by the dropwise addition of 1.5 ml of DMF to 1.5 ml of distilled water containing 30.1 mg of bovine serum albumin. During the addition of the DMF the temperature of the reaction was taken to -10°C by immersing the reaction vessel into an ethanolic dry ice bath. During all subsequent stages the pH of the reaction mixture was maintained at 8.0 with 0.05M sodium hydroxide.

After dilution of the protein solution the activated steroid was added dropwise with continuous stirring and maintenance of pH and temperature. The reaction was allowed to continue for 3-5 hours.

2. The reaction method was as described above, except the DHA-7-CMO derivative was used. The ratio of reactants was kept the same as for experiment 1, with 3.8 mg of steroid and 22.4 mg of protein.

b) Separation of reaction products

Dialysis was used to separate the products from unreacted material. Viskin tubing ($^{36}/32$" diameter) was soaked for 24 hours in distilled water prior to use. The reaction mixture was sealed into the tubing, and dialysed for 100 hours against a continuous flow of distilled water. After dialysis the tube contents were removed and the conjugate assayed for incorporation of DHA.

c) Estimation of steroid incorporation

Due to the lack of a radioactively labelled or UV absorbing steroid in the conjugate the degree of steroid incorporation was estimated colourimetrically.

d) Measurement of BSA

To estimate the amount of protein present in the conjugate the method of Lowry (1951) was used. Aliquots of the conjugate solution were taken (50-200 µl) and the volume made to 400 µl with distilled water. A standard curve was prepared from a 1 mg/ml aqueous stock solution of BSA to cover the range 5-100 µg/200 ul. To each sample add 2 ml of reagent made from 50 ml of 2% sodium carbonate in 0.1M sodium hydroxide containing 500 µl of 2% aqueous sodium tartrate and 500 ul of 1% aqueous copper sulphate.

The reagents were mixed and allowed to react for 10 minutes prior to the addition of 200 μl 1M Folin Ciocalteau reagent. The solutions were mixed and allowed to stand for 30 minutes prior to reading the extinction at 750nm on an SP 500 spectrophotometer. Inclusion of excess amounts of DHA in the reaction mixture did not result in a false positive reading.

e) Measurement of DHA

The reaction of Zimmerman (1935) was used to determine the incorporation of DHA into the conjugate. Aliquots (100 μl) of the conjugate solution were taken and made to 200 μl with ethanol. A standard curve was prepared to cover the range 5-100 μg/100 μl ethanol, and the volumes made to 200 μl with distilled water. To each tube add 400 μl of freshly prepared Zimmerman reagent, prepared from equal volumes of 1% m-dinitrobenzene in ethanol and 40% aqueous benzyl tri-methyl ammonium hydroxide. The tubes were stoppered, contents mixed and incubated for 1 hour at $25^{\circ}C$ in the dark. The reaction was stopped by the addition of 3 ml of ethanol, and the extinction of the solution read at 440nm, 520nm and 600nm. The mass of DHA present in the solution was calculated from the extinction obtained from the Allen equation ($E_{corr} = E_{520} - 0.5 (E_{446} + E_{600})$), and read from a corrected standard curve. Masses of BSA up to 100 μg per assay tube gave no positive colour reaction.

The degree of incorporation was calculated from the formula:-

$$\text{mol steroid/mol protein} = \frac{\frac{\text{mean mass of steroid}}{\text{mol wt. of steroid}}}{\frac{\text{mean mass of protein}}{\text{mol wt. of protein}}}$$

The molecular weights of DHA and BSA used were 288 and 70,000 respectively.

The degree of DHA incorporation by the methods described above are shown in table 2.2.

Table 2.2 Conjugation ratios and steroid incorporation

Reaction No.	Molar Ratio st:ICBF:TNB:BSA	DHA-derivative	mol steroid/mol protein
1	2 4:20:7.6:1	DHA-3-HS	17.0
2	2 4:20:7.6:1	DHA-7-CMO	9.1

f) Preparation and storage of conjugates

After estimation of the incorporation of steroid onto the protein, aliquots equivalent to 1 mg of conjugate were pipetted and lyopholysed in separate vials prior to sealing and storage at $-20^{\circ}C$.

2.4 Immunogen preparation and immunisation procedure

a) Preparation of immunogen

To 1 mg of DHA-7-CMO-BSA conjugate, 1.7 ml of sterile saline was added followed by 3.4 ml of Freunds complete adjuvant (FCA). The mixture was emulsified by alternately vortex mixing and aspirating through a syringe needle. The preparation remained as an emulsion after leaving overnight at $4^{\circ}C$.

b) Immunisation procedure

Three two year old rabbits were used, one male and one female half lop and one male New Zealand White. The immunisation method of Vaitukaitis et al, (1971) was used. 1.5 ml of the emulsion containing 295 µg of conjugate was injected intradermaly into 30 sites on the shaved back of each rabbit.

Ulceration was present for 4-8 weeks after immunisation but the degree of ulceration did not reflect the development of antibody.

c) <u>Booster injections</u>

Booster injections were given intramuscularly to the hind leg with 160 μg of conjugate emulsified in saline:F.C.A. in the ratio 2:1 with a final volume of 300 μl. Booster injections were given at 4, 8, 22, 34 and 60 weeks after the initial immunisation.

d) <u>Test bleeds</u>

Test bleeds (5 ml) were taken from an ear vein at varying times after each booster injection but never less than 1 week. Large bleeds up to 30 ml were taken by the same method if the test bleed showed the antiserum merited further investigation.

After collection, the blood samples were placed in a $37^{\circ}C$ oven for 2 hours to allow clot formation, followed by 16 hours at $4^{\circ}C$ to allow clot retraction. The blood was centrifuged and the serum aspirated. Antiserum titre was tested prior to freezing the serum and storage at $-20^{\circ}C$.

2.5 <u>Evaluation of antisera</u>

a) <u>Antibody titre</u>

All bleeds were tested for titre within 24 hours of collection, thus enabling a large bleed to be made with the minimum of delay if the result looked promising. Antibody titres are expressed as the final dilution of antibody in the assay tube required to bind 50% of the added label.

Antibody dilution curves were performed with and without the addition of
640 pg of unlabelled DHA to indicate the antibody dilution at which maximal
displacement of labelled DHA was obtained. The unlabelled steroid was added
to the methanol washed, control neck shell tubes (assay tubes), in ethanol,
evaporated to dryness in a vacuum oven at $40^{\circ}C$ prior to the addition of the
reagents.

Doubling dilutions of antiserum were made in 0.05M borate buffer (pH 8.0)
containing 0.125% v/v Human gamma globulin and 0.08% v/v bovine plasma
albumin. ^{3}H-DHA, approximately 4000 cpm (28 pg) in 100 ul of borate buffer
was added to each assay tube followed by 200 μl of antibody dilution. Tube
contents were mixed and incubated for 16 hours at $4^{\circ}C$. All subsequent
operations to sampling the supernatant were performed at $4^{\circ}C$.

Separation of the bound and free fractions was performed using dextran
coated charcoal (DCC, 1 mg of Norit A charcoal and Dextran T70 0.1% w/v in
250 μl of borate buffer) by the addition of 250 μl of DCC solution to each
assay tube, vortex mixing the tube contents and incubating for 10 minutes
prior to centrifugation at 1000 g for 10 minutes. An aliquot (400 μl) of
the supernatant was transferred to a plastic scintillation vial, and 3 ml of
scintillant added. The tube contents were mixed and the bound radioactivity
counted using a refrigerated Packard liquid scintillation counter.

The response in titre to the immunisation protocol is shown in fig. 2.1.
After the initial immunisation two of the three rabbits attained titres of
1:4,500 and 1:5,500. The response to the first booster injection was very
marked with titres reaching a plateau at 9 weeks, at which point a further
booster injection resulted in a decrease in antiserum titre in all animals.
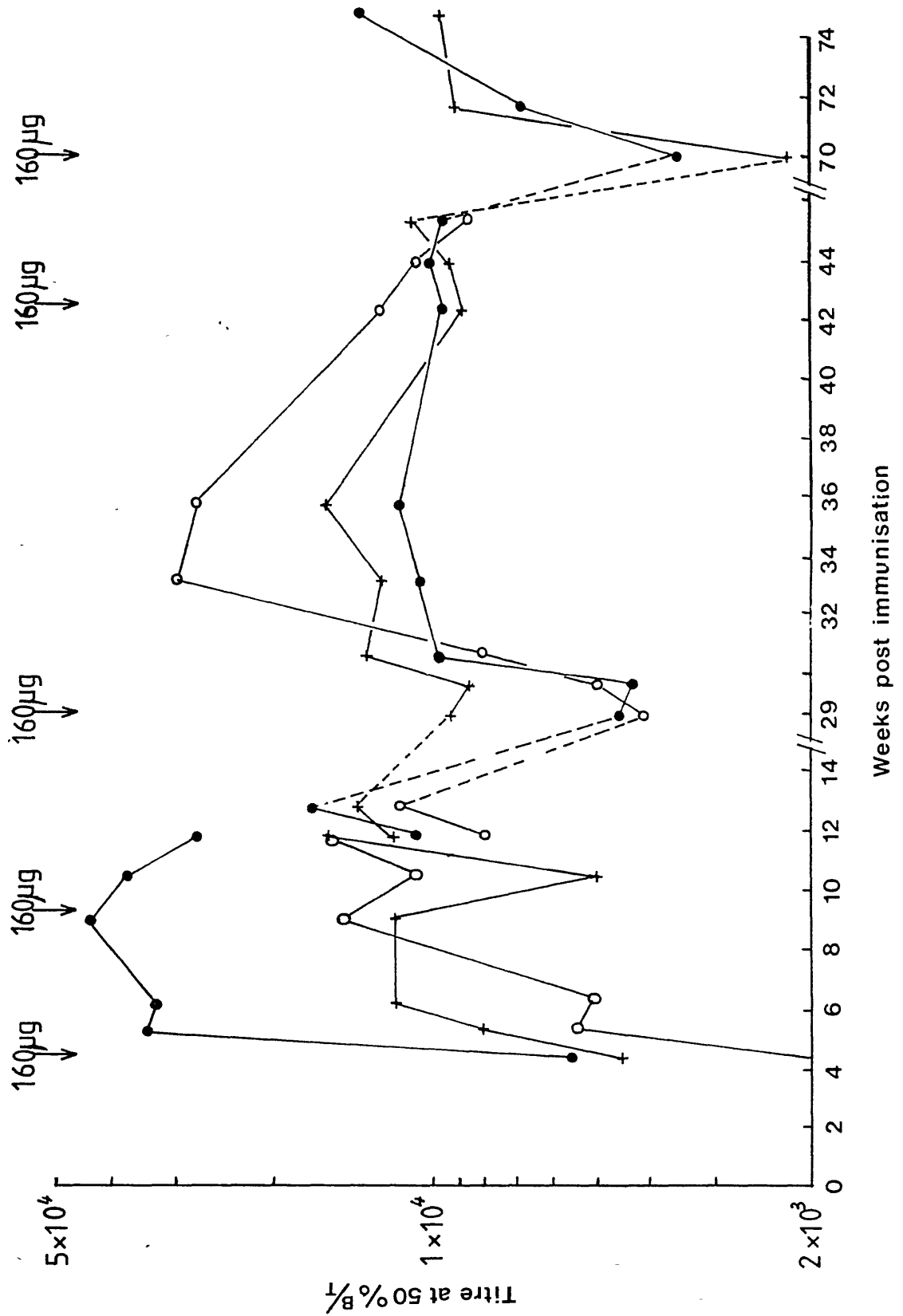
Fig. 2.1. Development of rabbit anti DHA titre with time after initial immunisation in three rabbits. +——+ , DSI; ●——● , DS2; o——o DS3.

Leaving the animals a further 21 weeks caused a 50-70% decrease in the antiserum titres. Response to a booster injection given at this time resulted in a dramatic increase in the titres with rabbit DSI increasing 200 fold. A further booster injection given after a further 12 weeks had very little effect. After a further 31 weeks the antibody titres had fallen to 1:2-3,500 and a booster injection resulted in a 3-700 fold increase in antibody titre within a further 6 weeks. At this time the rabbits were exsanguinated and the blood collected.

There did not appear to be any relationship between the sex or species of rabbit used and the response elicited to the antigen in this study although the numbers are very small.

b) Standard curves

Antisera with high titres and demonstrating good displacement with 640 pg of DHA were tested against standard curves covering the range 10-2560 pg DHA in doubling dilutions. Antiserum was used at a dilution that gave 70% binding of labelled DHA with 0 pg DHA. Standard DHA solutions were prepared in ethanol and 100 μl aliquots pipetted into assay tubes prior to evaporating the solvent in a vacuum oven. The antiserum and labelled DHA were prepared as a single solution in borate buffer containing bovine plasma albumin and human gamma globulin at the same final concentration as before, and 300 μl added to the assay tubes in one pipetting step. Antisera that demonstrated a greater than 50% difference between the % ^{B}/BO at 10 pg and 640 pg were investigated further.

c) <u>Cross reaction studies</u>

Promising antisera were tested for specificity against steroids with a
similar structure to DHA or those present in plasma at very high
concentrations. Dilutions of steroid were prepared covering the range
10 pg to 10 µg per 100 µl ethanol. Aliquots (100 µl) were pipetted into
assay tubes and evaporated to dryness prior to the addition of the antibody
mixture. The assay method was then as described above. Cross reactivity
was defined as that mass of DHA that displaced 50% of bound ^{3}H-DHA divided
by the mass of cross reacting steroid that displaces 50% of labelled DHA
expressed as a percentage.

A general trend of improving specificity, as demonstrated by a decreasing
cross reaction to androstenedione and androstenediol, was seen over the
period of antibody development. Over a period of 5 months the cross
reaction of 3β, 17β androstenediol of the antiserum from rabbit DS3 decreased
from 8.7% to 1.7%. Specificity generally deteriorated for about 10 days
after a booster injection then improved over the next month before
remaining constant.

d) <u>Preparation of antiserum pool</u>

Due to the relatively small volumes of blood obtained during each bleed
an antiserum pool was prepared from rabbits DS1 and DS3. Bleeds with
approximately similar titre and specificity were pooled. The pooled
antiserum had a titre of 1:11,500 at 50% binding and cross reactions as
shown in table 2.3.

Table 2.3 Cross reactions of rabbit anti DHA

Steroid	% cross reaction of pooled antiserum	mean % cross reaction of all bleeds
16αOH DHA	3.4	
3β, 17β androstenediol	1.3	1.5 range (0.9-2.3)
androstenedione	0.7	0.8 range (0.5-1.6)
androsterone	0.04	
DHAS	0.03	
5α, 3α, 17β androstanediol	0.003	
cortisol	<0.0015	

The pooled antiserum gave the same results as the single antiserum bleed which demonstrated the highest titre and specificity when duplicate aliquots of the same plasma extract were assayed simultaneously using each antiserum (fig. 2.2).

2.6 Radioimmunoassay for DHA

Duplicate aliquots of plasma (50 μl), previously mixed on a vortex mixer were pipetted into glass extraction tubes (3" x 0.5") using a constriction pipette. After addition of 1.5 ml of freshly prepared n-hexane:diethyl ether (4:1 v/v) the tubes contents were mixed in a Buchler vortex evaporator. The two phases were separated using an ethanolic dry ice bath and the supernatant decanted into assay tubes.

Standard quantities of DHA (0, 10, 20, 40, 80, 160, 320, 640, 1280 pg) in 100 μl of ethanol were pipetted into assay tubes from individual working solutions. The contents of the standard and sample tubes were evaporated to dryness in a vacuum oven at 40°C.
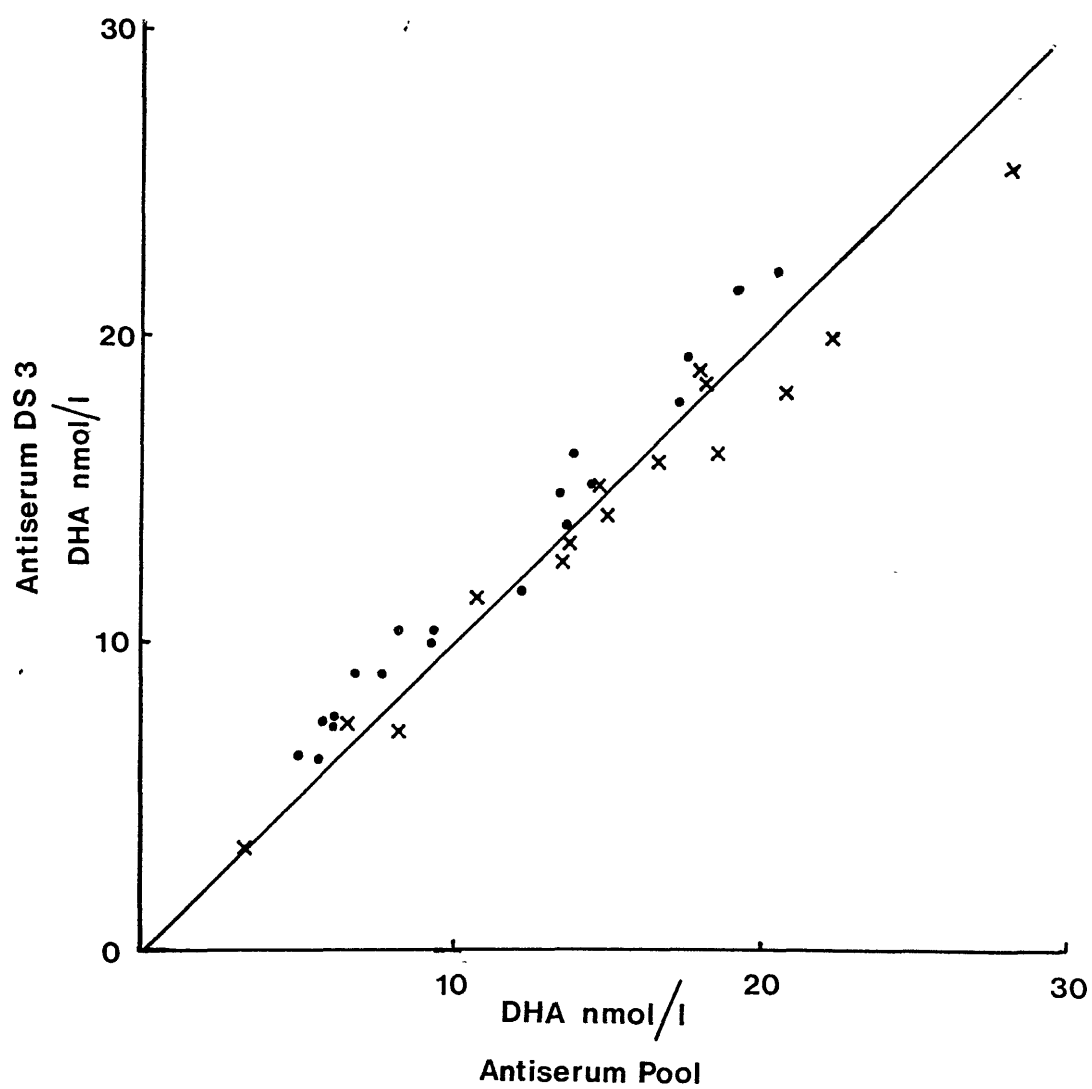
Fig. 2.2. Comparison of plasma DHA results obtained using single and
pooled antiserum bleeds measured in 2 assays (● , assay 1;
✕ , assay 2).

The working solution of antisera was prepared in borate buffer containing 0.125% (v/v) human gamma globulin, 0.08% (v/v) bovine plasma albumin, and ^{3}H-DHA (20,000 dpm/ml). After mixing gently, 300 μl of the mixture was added to each of two assay tubes using a Gilson pipette to determine the total counts and non-specific binding (NSB). Antiserum was added to the remaining volume to give a final dilution of 1:6000 and 300 μl aliquots added to all remaining tubes. All tube contents were gently mixed on a vortex mixer prior to incubation overnight at 4°C or 30 minutes at 37°C followed by 1 hour at 20°C. Both methods ended with 30 minutes incubation in an ice bath. Separation of bound and free fractions was as described in section 2.5.

To calculate the assay results the bound cpm (B) of the duplicate standards were calculated as a percentage of the mean cpm bound for the zero tubes (BO). These percentages (B/BO) were plotted against the mass of DHA in pg on semi-logarithmic graph paper. Similarly the bound cpm for the plasma aliquots were calculated and the mass of DHA extrapolated from the standard curve.

2.7 Validation of DHA radioimmunoassay

Details of assay characteristics are shown in fig. 2.3.

a) Accuracy and sensitivity

To validate an assay, proof of quantitative recovery of unlabelled steroid has to be obtained. Recovery of unlabelled steroid was performed by evaporating aliquots of standard steroid solutions in ethanol to dryness prior to the addition of water or plasma. The amounts of steroid were chosen to be representative of the range of expected plasma values.

Labelled DHA recovery from plasma   = 99% $\pm$ 3%

Sensitivity 9 pg/assay tube   = 0.5 nmol/l

Intra assay C.V. $\bar{x}$ = 16.7 nmol/l   C.V. = 7.4%   n = 83

Inter assay C.V. $\bar{x}$ = 8.6 nmol/l   C.V. = 11.5%   n = 38

             $\bar{x}$ = 14.4 nmol/l   C.V. = 6.1%   n = 21

             $\bar{x}$ = 17.4 nmol/l   C.V. = 8.3%   n = 46

             $\bar{x}$ = 18.5 nmol/l   C.V. = 9.9%   n = 57
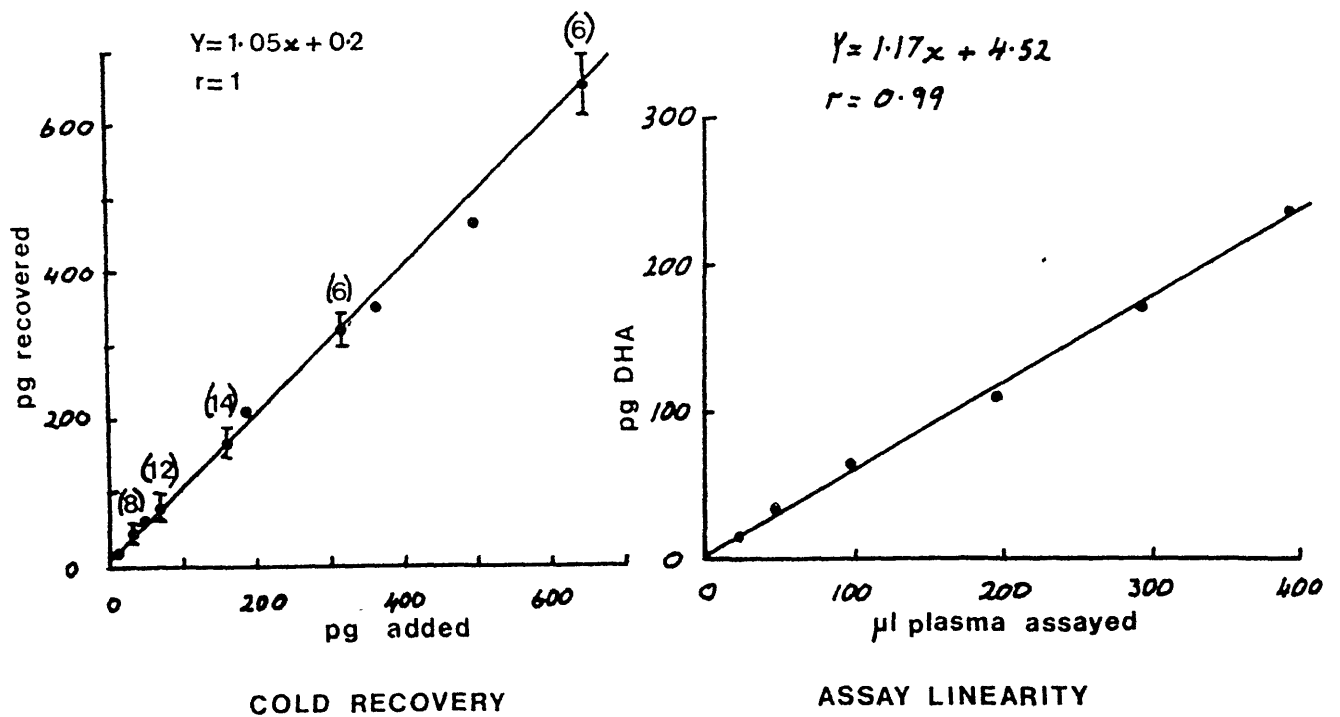
Fig 2.3   Details of DHA assay characteristics.

Recovery experiments were also performed using aqueous standard steroid solutions added to plasma to avoid the effects of evaporation of the ethanolic solution or problems with steroid reconstitution. Samples were allowed to equilibrate overnight at 4°C prior to assay. Quantitative recovery of DHA was obtained for masses of DHA over a range equivalent to plasma levels of 1.39-44.5 nmol/l.

Assay sensitivity was calculated from 10 water samples taken through the assay procedure and the mean and standard deviation of the resulting bound radioactivity calculated. The mean count minus two standard deviations was calculated as a percentage of the zero standard and the mass extrapolated from a linear plot of the standards. This mass is quoted as the sensitivity of the assay.

Water blanks were run in every assay and the assay was rejected if the water blank value was higher than the assay sensitivity.

b) <u>Precision</u>

To estimate intra and inter assay precision the coefficient of variation was calculated using the formula CV = $\left({}^{SD}/mean \right)$ x 100. The intra assay CV was calculated from a random selection of assays taken over the period of study. The inter assay CV figures were calculated from the internal quality controls that were run in each assay.

c) <u>Assay linearity</u>

The effects of varying the volume of sample taken and dilution of sample on the result obtained using the DHA method was investigated. It was found that the extraction of DHA from plasma was not very efficient for volumes over 100 μl using the method described above. However, when the

plasma was extracted with 2 ml of hexane:diethyl ether (4:1) in stoppered glass tubes by horizontal shaking in a mechanical shaker for 10 minutes a linear recovery up to 400 μl of plasma was found. Under these conditions duplicate 500 μl aliquots of the hexane:ether supernatant were taken for assay. This method was only used for plasma samples with a DHA level less than 1.0 nmol/l.

d) <u>Specificity</u>

The specificity of an RIA procedure is determined by the specificity of the antiserum and the method(s) used to isolate the compound under investigation. Upon the initiation of a new study the results obtained from a few test samples using the simple extraction assays were compared to those obtained after chromatography.

The specificity of the plasma assay method was determined using paper chromatography with either the Bush A or Bush B3 solvent system and by thin layer chromatography with dichloromethane:ethyl acetate (93:7 v/v) or dichloromethane:acetone (80:20 v/v). Results from single random plasma samples are shown in fig. 2.4. The results obtained comparing the extraction and chromatography methods on regular timed samples taken from a normal male, a luteal phase and a pregnant female are shown in fig. 2.5.

2.8 <u>Radioimmunoassay for DHAS</u>

a) <u>Hydrolysis of DHAS</u>

Of the hydrolysis methods commonly used the methods of Burstein and Lieberman (1958, sulphuric acid hydrolysis) and Azo <u>et al</u>, (1977, hydrochloric acid hydrolysis) were tried. The acid hydrolysis methods gave high water blank values with erratic results and this method was discontinued.
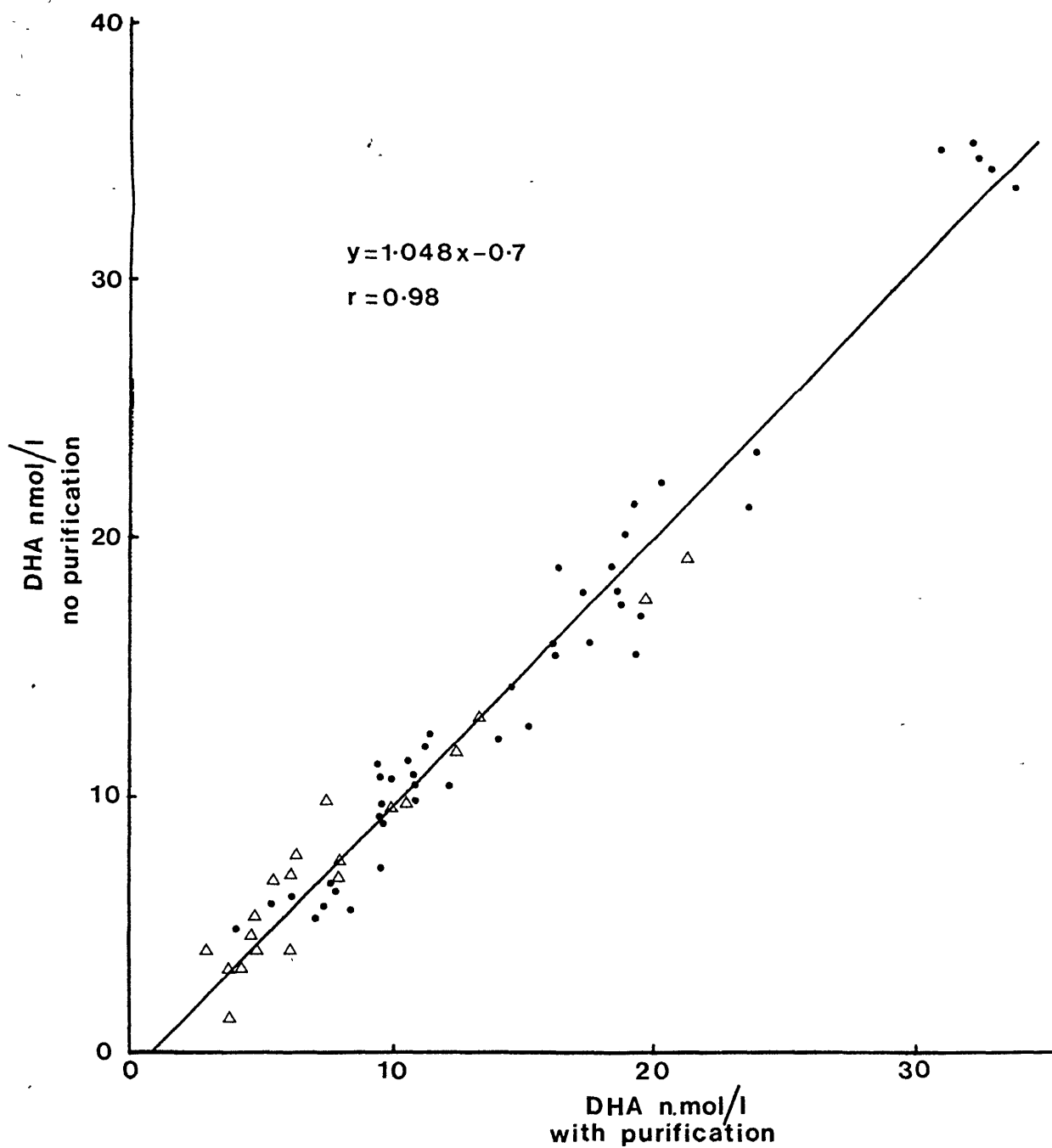
Fig. 2.4. Comparison of plasma DHA levels assayed with and without a chromatographic purification procedure. (● , TLⱼ; Δ , paper chromatography). Samples are from normal subjects and patients taken from each of the studies reported in the thesis.
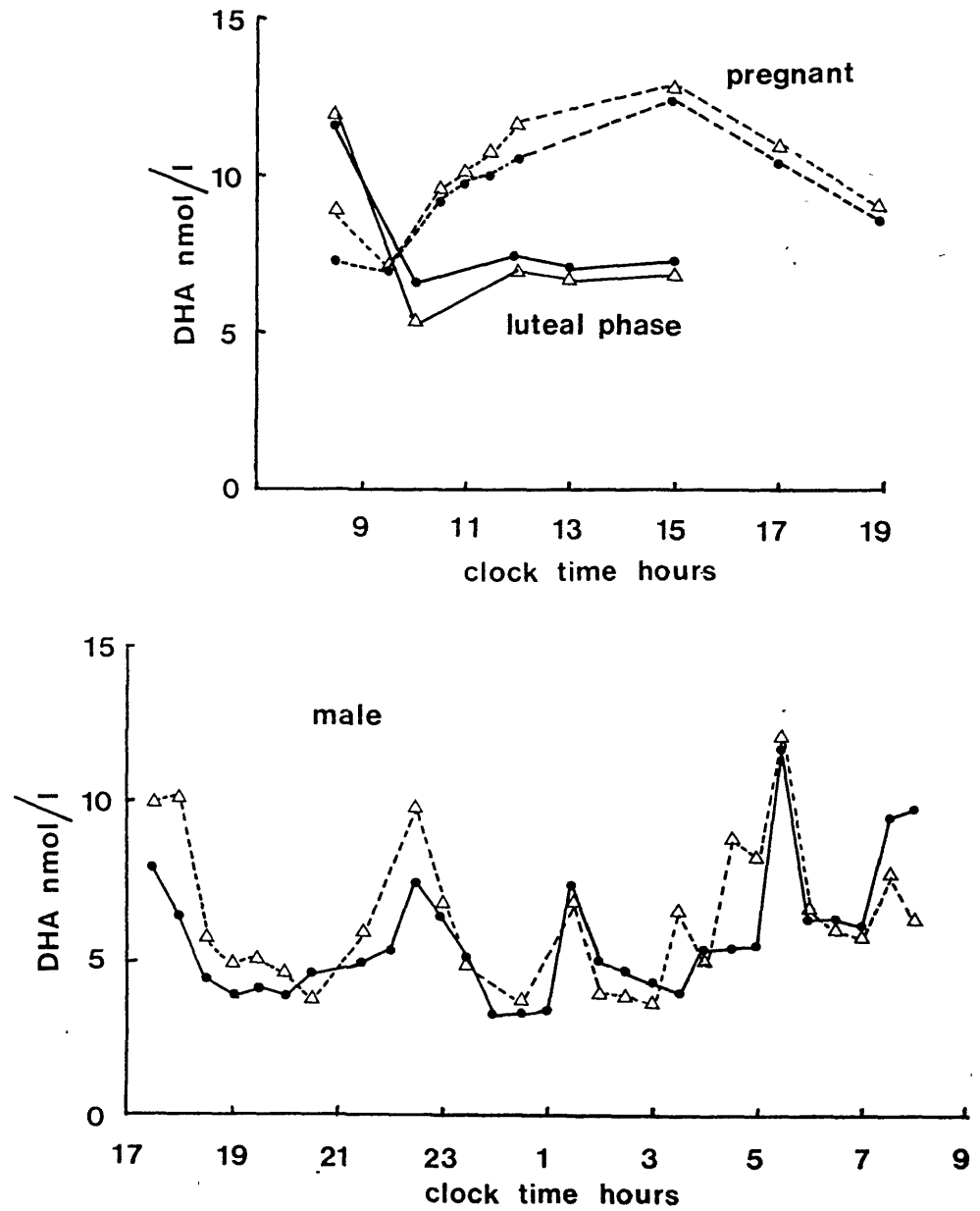
Fig. 2.5. Comparison of plasma DHA levels obtained in sequential samples from normal male and female subjects with (△—△) and without TLC chromatography (●—●) purification.

Hydrolysis using the Helix pomatia sulphatase enzyme proved to be efficient and gave no blank problems. The method also has some degree of specificity for DHAS conferred by the enzyme itself.

Assays were performed using the pooled antiserum raised against the DHA-7-CMO-BSA derivative.

b) Optimisation of the concentration of sulphatase

To 20 μl of plasma diluted with 150 μl of acetate buffer (0.2M, pH 4.8) add 35,000 dpm of ^{3}H-DHAS. After equilibration for 16 hours at 4°C varying volumes of a sulphatase solution (100 units/ml acetate buffer) were added to cover the range 1 to 100 units per tube and the volumes made to 1 ml with acetate buffer. The tube contents were vortex mixed and incubated overnight at 37°C. The hydrolysed DHA was extracted with 5 ml of diethyl ether and the organic phase decanted into five ml plastic scintillation vials for recovery estimation after evaporation of the solvent to dryness and addition of 4 ml scintillator.

Fig. 2.6 shows the recovery obtained and demonstrates that maximal hydrolysis is obtained above 10 units/tube. To ensure maximal recovery 20 units of sulphatase per plasma hydrolysis were used.

c) Effect of time and temperature on hydrolysis

The hydrolysis was performed as described above with 50, 20, 10 and 0 units of sulphatase per tube and the replicate samples incubated for various times (0 to 24 hours) at either 19°C or 37°C.

As shown in fig. 2.7 maximal hydrolysis occurred after 16 hours for the highest sulphatase concentrations at 37°C and 19°C. Incubation at 37°C for 16 hours with 20 units of sulphatase was chosen for the hydrolysis procedure as a compromise between convenience, efficiency and cost.
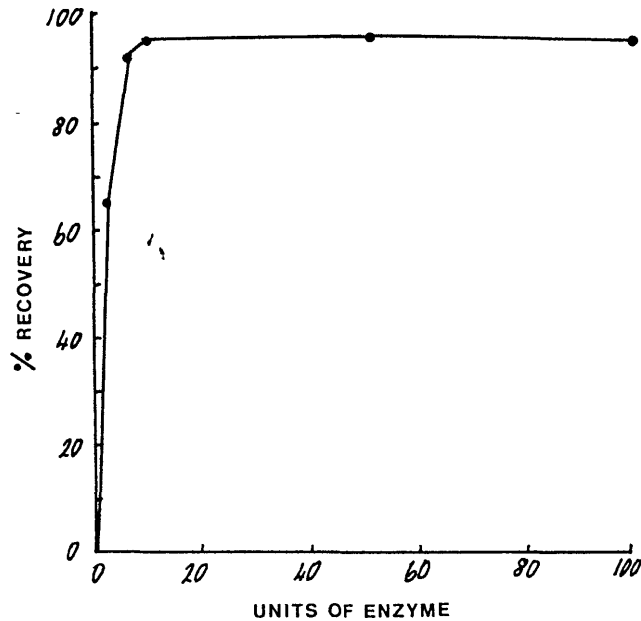
Fig. 2.6. Optimisation of sulphatase concentration for complete hydrolysis of DHAS.
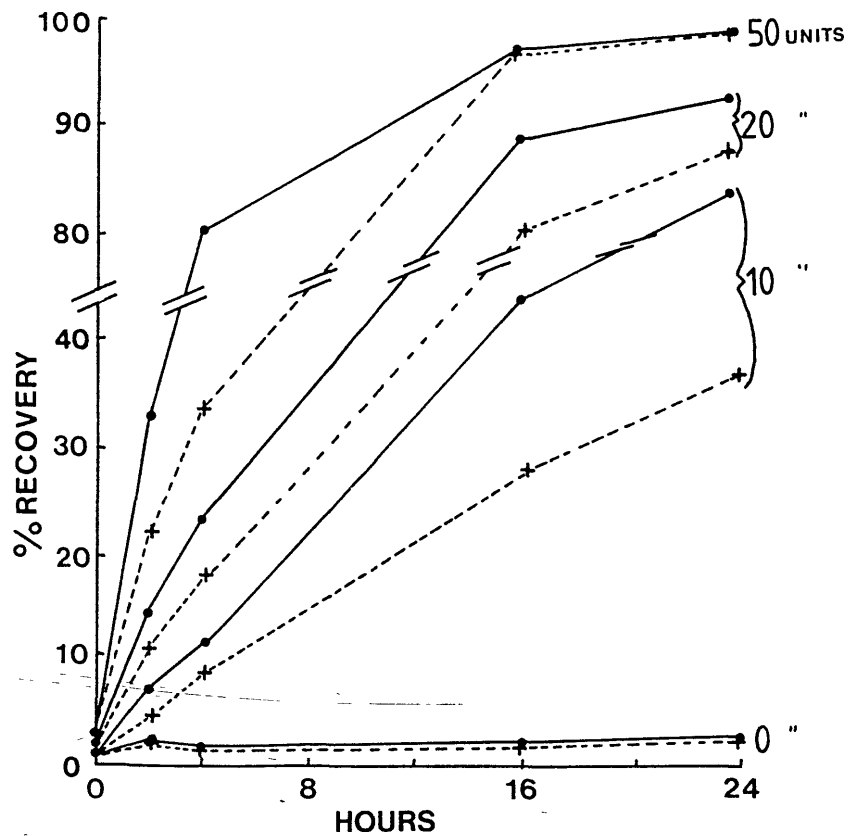
Fig. 2.7. Effects of time, temperature and sulphatase concentration on the hydrolysis of DHAS. (● , 37°C; + , 20°C).

d) Extraction of hydrolysed DHAS

Hydrolysed DHAS was extracted from 100 µl and 200 µl aliquots of hydrolysed plasma with 6 ml of either hexane, hexane:ether (4:1 v/v) or ether. Aliquots (1 ml) of the supernatant were transferred to plastic 5 ml scintillation vials, the contents evaporated to dryness and the residue reconstituted in scintillator. No significant difference was found between the recovery obtained with either solvent. Hexane was chosen for use as the ether has been found to give blank problems when not from a freshly opened bottle, and the hexane does not extract any DHAS that may remain after hydrolysis.

e) DHAS assay method

An aliquot of plasma (0.02 ml) previously mixed on a vortex mixer was pipetted into a glass 3" x 0.5" incubation tube using a Hamilton automatic diluter/dispenser with a 150 µl wash of acetate buffer (pH 4.8, 0.2M). To each tube a recovery tracer of tritiated DHAS (40,000 dpm) in 50 µl of redistilled ethanol was added and the tube contents mixed on a vortex mixer and allowed to equilibrate for at least 1 hr. at room temperature. Three total recovery aliquots were also taken into assay tubes. To each incubation tube 20 units of sulphatase enzyme in 800 µl of acetate buffer was added, the tubes capped and the contents mixed on a vortex mixer prior to incubation at 37°C for 16 hrs.

After incubation the tube contents were mixed on a vortex mixer and an aliquot (100 µl) transferred to a stoppered glass 4" x ½" tube using a Gilson pipette and 6 ml of redistilled n-hexane added. The tubes were stoppered and shaken horizontally on a mechanical shaker for 10 minutes. After extraction the aqueous layer was frozen in an ethanol/dry ice bath and the organic phase decanted into glass 3" x 0.5" tubes.

Three 500 μl aliquots of each sample were transferred to assay tubes and the contents evaporated to dryness in a vacuum oven at 35-45°C. The tubes for total recovery and background estimation were similarly treated. To one tube of each sample, the tubes for total recovery count and the background tubes 1.7 ml of scintillator was added, the tubes were capped and the contents mixed by horizontal shaking for 10 minutes. The tubes were placed in standard glass counting vials and counted for 5 minutes each in a refrigerated Packard scintillation counter. The background count was subtracted from the recovery count for calculation of results.

The standard curve was pipetted using the same standards as for the DHA assay, in addition 100 μl of ethanol containing the mean recovery count of ^{3}H-DHA was also added. The contents of the tubes were evaporated to dryness at 37-45°C under vacuum.

Antibody mixture was prepared as for the DHA assay with the exception that the amount of labelled DHA added per assay tube was corrected for the labelled steroid present as recovery. The remainder of the methodology was identical to the DHA assay.

The calculation of results was as described for DHA except the sample results for DHAS were corrected for methodological losses using the internal recovery and for the molecular weight difference between DHA and DHAS.

2.9 DHAS assay characteristics

Details of the assay characteristics are shown in fig. 2.8. These results were obtained using the same methods as described for DHA. At each stage of the assay a good agreement of results can be obtained by assaying volumes of plasma from 20-200 μl, volumes of hydrolysate from 50-400 μl, and volumes of hexane from 100-500 μl in any combination provided the recovery counts are corrected for.

Specificity was shown by comparing the results obtained after either paper or thin layer chromatography of the hydrolysed DHAS to the results obtained with the above method (fig. 2.9).

2.10 Radioimmunoassay for pregnenolone

The pregnenolone antiserum was obtained from Specific Antisera Ltd, Wilmslow Cheshire, and was raised against pregnenolone-3-HS-BSA in rabbits. Cross Reactions are shown in fig. 2.10.

To 500 μl aliquots of plasma in stoppered (6" x 0.75") glass tubes add 50 μl of labelled pregnenolone (5000 dpm) in ethanol. The tube contents were mixed and allowed to equilibrate at 4°C prior to extraction with 10 ml of diethyl ether from a freshly opened bottle. The aqueous layer was frozen in an ethanolic dry ice bath and the ether decanted and evaporated to dryness at 40°C under a stream of nitrogen.

The residue was applied to silica gel TLC plates using 2 x 100 μl of diethyl ether and the plates developed twice in dichloromethane:ethyl acetate (93:7 v/v). It was found that the dichloromethane had to be freshly redistilled prior to use otherwise high water blanks were obtained.

Labelled DHAS recovery $94.6 \pm 4\%$

Sensitivity 9 pg per assay tube = 0.15 μmol/l

Intra assay C.V. χ = 5.8 μmol/l C.V. = 9.7% n = 66

Inter assay C.V. χ = 0.9 μmol/l C.V. = 13.3% n = 38

χ = 4.4 μmol/l C.V. = 9.9% n = 52

χ = 5.4 μmol/l C.V. = 9.3% n = 46

χ = 7.3 μmol/l C.V. = 7.6% n = 18

COLD RECOVERY

ASSAY LINEARITY
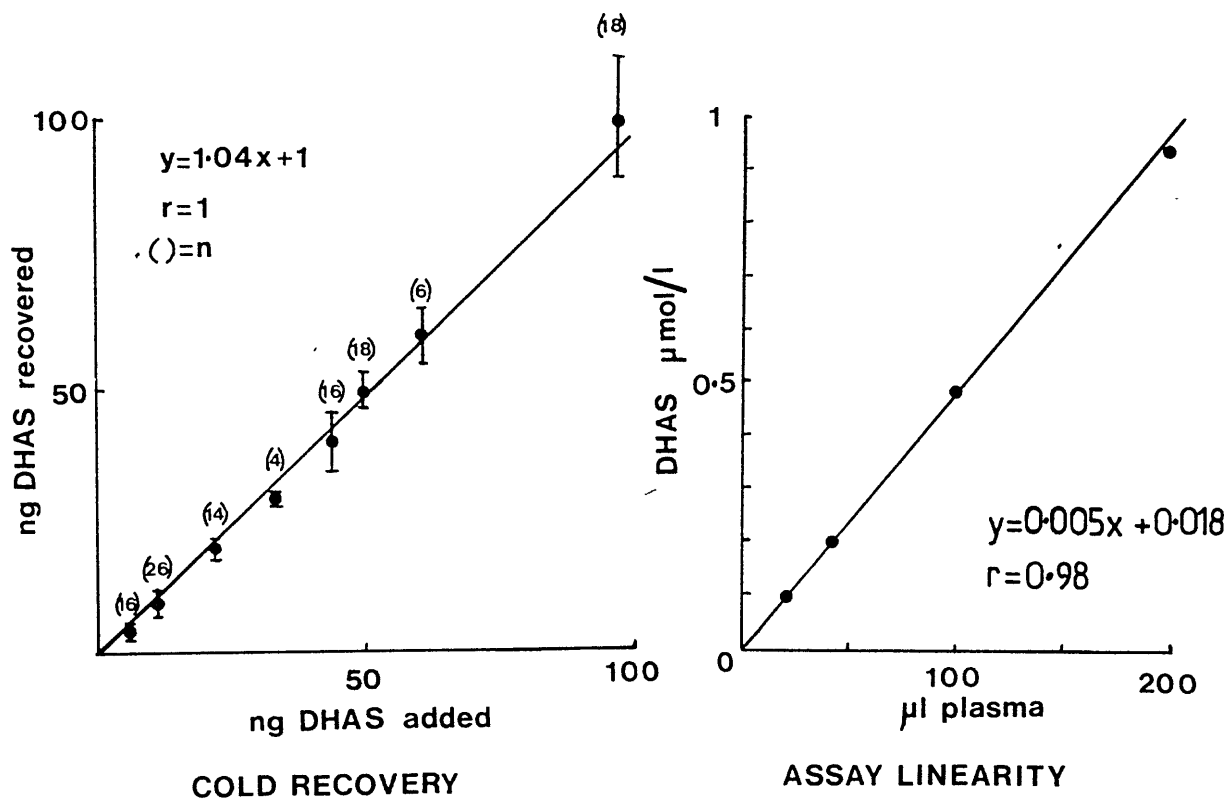
Fig. 2.8   Details of DHAS assay characteristics.
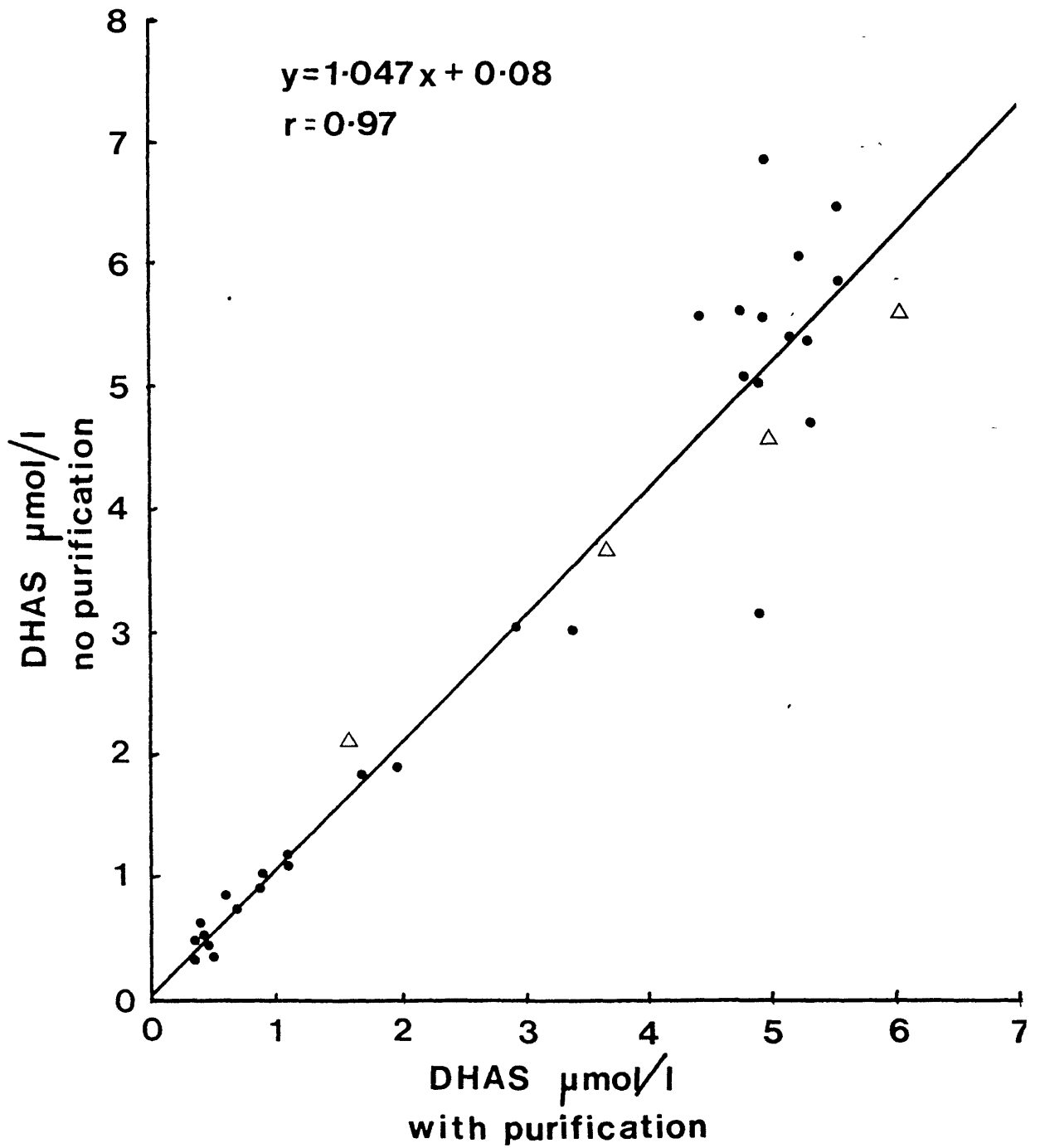
Fig. 2.9. Comparison of plasma DHAS levels assayed with and without a chromatography purification procedure. (●, TLC; Δ , paper chromatography). Samples are selected from subjects studied in the thesis.

The areas of labelled pregnenolone were located on the TLC plates under a Panax beta scanner and the pregnenolone areas excised and eluted in 5 ml of diethyl ether for 2 hours. Longer elution times were found to give assay blank problems. The ether was decanted and evaporated to dryness under nitrogen prior to the addition of 1 ml of ethanol.

Three 200 μl aliquots of the ethanolic samples were taken and transferred to assay tubes prior to drying down in a vacuum oven. One tube was taken for recovery estimation and two for assay. The mean recovery count for the assay was calculated excluding outliers and an ethanolic solution containing this number of counts in 100 μl was prepared. Aliquots (100 μl) of this solution were added to the standard curve tubes and evaporated to dryness. This ensures that the standard curve has an equivalent amount of ^{3}H-pregnenolone as the samples.

The antibody mixture was prepared containing 8,000 dpm of tritiated pregnenolone, human gamma globulin and bovine plasma albumin at a concentration of 0.1% w/v and antiserum at a final dilution of 1:300 in a final volume of 300 μl of borate buffer pH 8.0. 300 μl of the antiserum solution was added to each assay tube and the incubation procedure and separation procedure were as described for the DHA assay.

With repeated counting of the samples the apparent percentage bound increased. This was due to the sample counts increasing while the total counts did not change. It was assumed that this was a reflection of the scintillator gradually extracting more bound labelled pregnenolone from the antiserum. The method described by Kandeel et al, (1976) was used to

overcome this problem. To each plastic scintillation vial add 100 µl
1M HCl prior to the addition of the assay supernatant. This resulted
in stable counting rates for all samples presumably by denaturing the
antiserum thus releasing the steroid.

The details of assay sensitivity, accuracy, linearity and precision are
shown in fig. 2.10.

Comparison of the results obtained by TLC to those using paper chromatography
with the Bush A system are shown in fig. 2.11. The samples were taken from
two normal women, one during the luteal phase and the other 12 weeks
pregnant. As can be seen there is very little difference between the
results obtained using the two methods.

2.11 Radioimmunoassay of DHA and DHAS in tissue

a) Tissue preparation

Tumours were dissected free from non-tumour tissue and portions of both
retained for histological examination. Tissues were transported
immediately to the laboratory and stored at -20°C until required.

Prior to assay the tissue was washed with glass distilled water to remove
any blood. The wet weight of the tissue was recorded and glass
distilled water added to give approximately 100 mg/ml prior to the tissue
being homogenised in a Polytron blender. The breast tissue homogenate was
centrifuged for 10 minutes prior to assay to separate tissue and fat. The
fat was aspirated and discarded.

Labelled pregnenolone recovery $= 80.5 \overset{+}{-} 4.4\%$

Sensitivity   7 pg/assay tube $= 0.2$ nmol/l

Intra assay C.V. $\bar{x} = 4.35$ nmol/l   C.V. $= 10.7\%$ $n = 64$

Inter assay C.V. $\bar{x} = 1.4$ nmol/l   C.V. $= 17.4\%$ $n = 25$

                     $\bar{x} = 5.1$ nmol/l   C.V. $= 8.6\%$ $n = 29$

                     $\bar{x} = 23.4$ nmol/l   C.V. $= 8.8\%$ $n = 32$

Cross Reactions   Progesterone 7%

                     17α OH- Pregnenolone 5%

                     DHA $< 0.1\%$

                     Androstenedione $< 0.1\%$

COLD RECOVERY   **ASSAY LINEARITY**
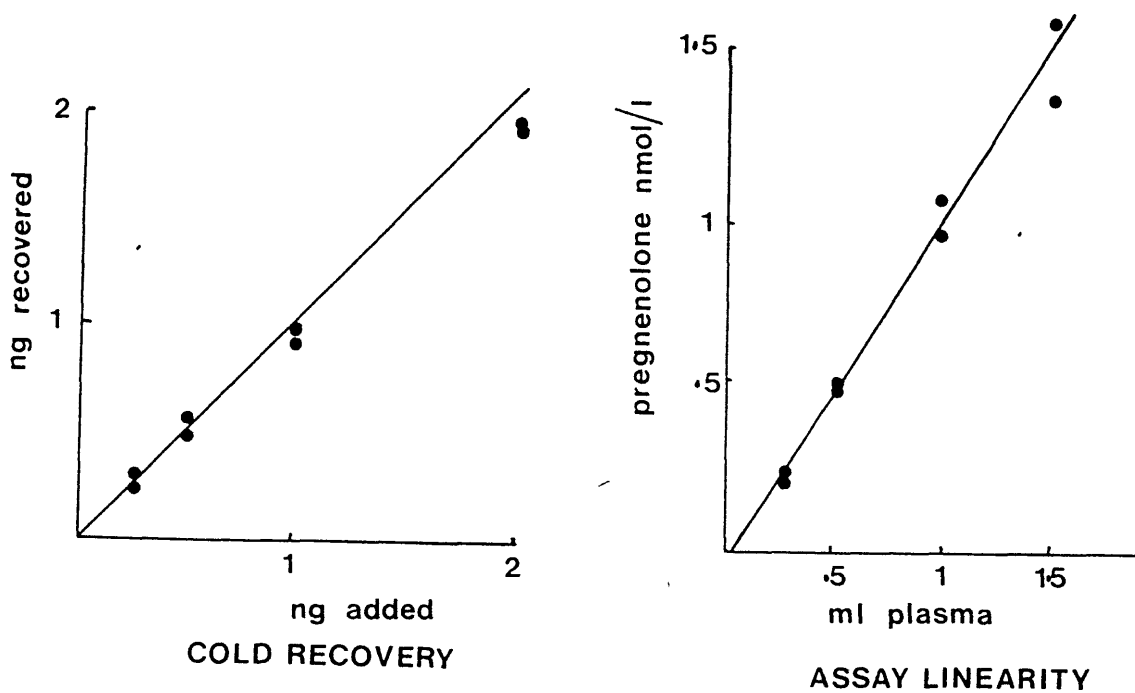
Fig. 2.10   Details of pregnenolone assay characteristics.
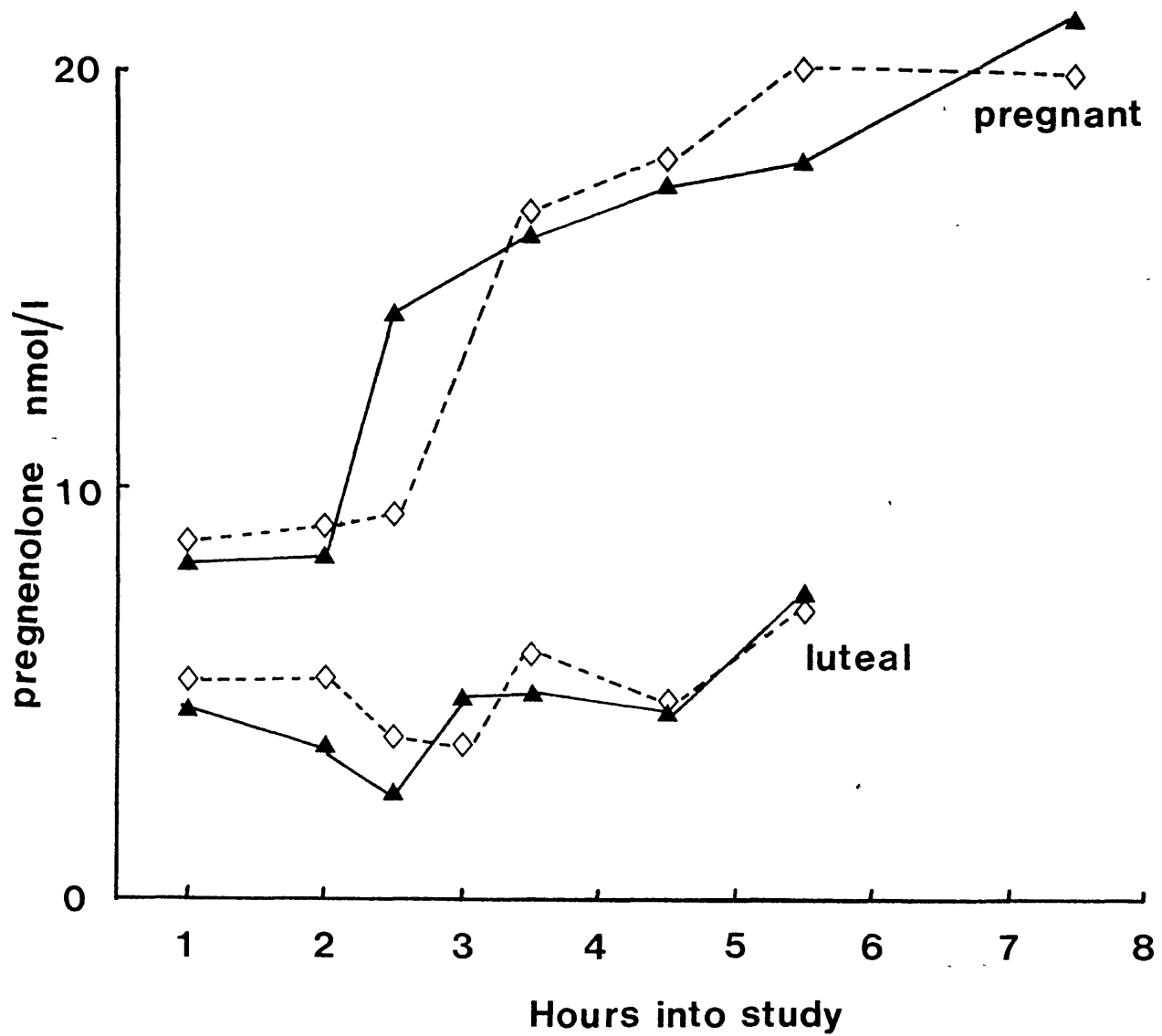
Fig. 2.11. Comparison of plasma pregnenolone levels measured in
sequential samples using thin layer (▲) and paper (◇)
chromatography purification.

b) <u>Tissue DHA measurement</u>

To aliquots, usually 0.8-1.2 ml containing 50-120 mg of tissue homogenate (wet weight), 50 μl of ethanol containing 6,000 dpm of ^{3}H-DHA was added as an internal recovery standard and the sample vortex mixed. The label was allowed to equilibrate for 30 minutes prior to extraction of the free steroids with 5 ml of diethyl ether. The aqueous phase was frozen in an ethanol/dry ice bath and the organic phase decanted. The extraction was repeated and the extracts pooled. The aqueous phase was retained for DHAS estimation.

The residue remaining after evaporation of the ether at 40°C under nitrogen was applied to silica gel TLC plates with 2 x 100 μl of diethyl ether. The TLC plates were developed in dichloromethane:dioxan (94:6 v/v). The areas of ^{3}H-DHA were located under a Panax chromatogram scanner, excised and eluted with 5 ml of diethyl ether. The diethyl ether was decanted, evaporated to dryness under nitrogen and the residue reconstituted in 1 ml ethanol.

Aliquots (3 x 200 ul, one for recovery, two for assay) were taken using an alcohol constriction pipette into methanol washed assay tubes. The methodology was then as described for the plasma DHAS assay. An ethanolic solution was prepared containing the mean assay recovery count of labelled DHA in 100 μl. Aliquots (100 μl) of this solution were added to the standard curve tubes and evaporated to dryness prior to addition of the antibody mixture.

c) <u>Tissue DHAS measurement</u>

^{3}H-DHAS (50 μl containing 10,000 dpm) was added to each of the aqueous phases left after the extraction of free steroid, vortex mixed, and allowed to equilibrate overnight at 4°C. The conjugated-steroids were extracted from the tissue homogenate in stoppered glass tubes by horizontal shaking on a mechanical shaker using 5 ml of ethyl acetate:n-propanol (3:1 v/v). The precipitated proteins were removed by centrifugation at 1000g for 10 minutes.

The decanted supernatant was evaporated to dryness under nitrogen at 45°C and 1 ml of acetate buffer (pH 4.7, 0.2M), containing 50 units of sulphatase added. After vortex mixing and overnight incubation at 37°C the hydrolysed steroid was extracted using 10 ml of diethyl ether. After collection of the organic phase and evaporation to dryness, the residue was applied to silica gel TLC plates with two 100 μl diethyl ether washes, and developed twice in dichloromethane:ethyl acetate (93:7 v/v). The ^{3}H-DHA areas were detected and eluted as described previously, and the residue reconstituted in 2.5 ml of ethanol. Aliquots of 500 μl were taken, one for recovery estimation and two for assay. The assay method was then as described for the plasma DHAS assay.

d) <u>Tissue assay characteristics</u>

The assay accuracy, linearity and precision experiments were performed as described for the plasma DHA and DHAS assays. Assay sensitivity was calculated after measuring 8 water samples as no tissue was available which contained no DHA or DHAS. Details of the tissue DHA and DHAS assay characteristics are shown in table 2.4.

Table 2.4   Details of tissue DHA and DHAS assay characteristics

Mass of steroid added	Percentage recovery			
	DHA	n	DHAS	n
16 pg	90	4	106	4
60 pg	94	4	112	4
256 pg	94	4	100	4

Assay linearity

Steroid	Volume range	Correlation	r-value
Tissue DHA	250-1500 ul	$y = 442.2 x - 2.8$	0.97
Tissue DHAS	250-1500 ul	$y = 0.407 x + 1.46$	1.0

Labelled recovery ($\pm$ SD)

DHA $80 \pm 5\%$ n = 35
DHAS $42 \pm 6\%$ n = 29

Water blanks

< 5 pg per assay tube

Sensitivity

DHA   0.5 ng/g
DHAS   1.12 ng/g

Intra-assay C.V.

DHA   mean = 69.1 ng/g   CV = 10.7%   n = 32
DHAS   mean = 118.4 ng/g   CV = 7.1%   n = 27

2.12 Assay of salivary steroids

a) Radioimmunoassay of salivary DHA

To 2 ml aliquots of saliva add 100 μl of ^{3}H-DHA (20,000 dpm) in borate
buffer. The tube contents were vortex mixed and allowed to equilibrate
at 20°C for at least 30 minutes. Free steroid was extracted with 10 ml
of diethyl ether and the organic phase separated and collected. After
evaporating the ether to dryness the residue was applied to TLC plates
and developed in dichloromethane:ethyl acetate (93:7 v/v) twice. The
areas of labelled steroid were located under a Panax chromatogram scanner,
excised and the steroid eluted with 5 ml of diethyl ether. After decanting
and evaporating the ether the residue was reconstituted in 800 μl of borate
buffer. Aliquots of 200 μl were taken for assay and recovery estimations.
The assay methods were then as described in section 2.6 except the
antibody mixture was made in 100 μl (not 300 μl) and added to the 200 μl
of assay sample.

b) Radioimmunoassay of salivary DHAS

To 500 μl of saliva add 50 μl of ^{3}H-DHAS in ethanol (8,000 dpm) and
equilibrate at 20°C for at least 30 minutes. The unconjugated steroid
was removed by extraction with 5 ml of hexane. Ethanol (3 ml) was added
to the remaining aqueous phase. The tube contents were thoroughly mixed
and centrifuged to remove the precipitated material. After decanting, the
ethanol was evaporated to dryness at 40°C under a stream of nitrogen. To
the residue add 1 ml of acetate buffer (pH 4.8, 0.2M) containing 20 units
of sulphatase. The contents were mixed and incubated overnight at 37°C.
The DHA released was extracted with 10 ml of diethyl ether and subjected
to TLC as described above. The residue containing hydrolysed DHA remaining
after TLC was reconstituted in 2.5 ml of ethanol, and 3 x 500 μl aliquots
taken, two for assay and one for recovery estimation. The assay procedure
was as described in section 2.8.e.

c)   Characteristics of salivary steroid assays

Details of the extraction of labelled steroid from saliva and the linearity
and accuracy of the assays are shown in table 2.5.

2.13   High pressure liquid chromatography (HPLC) of conjugated steroids

a)   Extraction of steroid conjugates

An initial extraction of the steroid conjugate was performed on Sep Pak C18
cartridges.   Prior to use the cartridges were flushed with 5 ml of methanol
and 5 ml of distilled water.   Plasma (3-5 ml) containing ^{3}H-DHAS and ^{3}H-E$_1$S
as markers, was diluted to 5 ml with distilled water and passed through the
cartridge.   The cartridge was washed with 5 ml of water and the steroid
eluted with 3 ml of methanol.   The plasma was extracted again following the
above procedure and the methanol eluates pooled.   The methanol was
evaporated to dryness under nitrogen at 40°C.   The residue was reconstituted
in 40 μl of methanol:water (1:3 v/v).   After centrifugation of the sample
to remove any particulate matter an aliquot (20 μl) was injected onto the
column.

b)   Chromatography system

The apparatus consisted of a Waters model 6000A solvent delivery system
and a Waters 440 UV monitor set at 254 nm.   A stainless steel (250 x 4.6 mm)
hypersil ODS column (particle size 5 μm) from Shandon Southern Products,
Runcorn, Great Britain, was used.   The eluant, deionised water:methanol
(HPLC grade) 3:1 v/v, was pumped at a flow rate of 0.8 ml/min.   Prior to
use the solvents were degassed using an ultrasonic bath.   Aliquots of the
eluate were collected at 72 second intervals.   Testosterone sulphate
was located by UV absorption at 254 nm using the post column detector.

Table 2.5

	Cold recovery				Assay linearity	
	Mass added	Mass recovered	% rec.		vol. assayed	nmol/l
DHA	0	183 pg				
	160 pg	323 pg	87·5			
	640 pg	780 pg	93.3			
	1280 pg	1462 pg	99.9			
DHAS	0	1379 pg			200 μl	6.1
	250 pg	1636 pg	103·2		300 μl	6.6
	500 pg	1817 pg	87·8		400 μl	6.6
	1000 pg	2205 pg	82·7.		500 μl	6.3

Labelled recovery: DHA = 78%

DHAS = 43%

Characteristics of salivary DHA and DHAS assays.

The remaining conjugated steroids were identified in the aliquots as follows: E_2S by absorbtion at 205 and 273 nm, epiandrosterone and pregnenolone sulphate by the methylene blue method of McKenna and Rippon (1965), DHAS and E_1S by radioactive determination. The relative retention times (RT) of the conjugated steroids are shown in table 2.6.

Table 2.6   Retention times of conjugated steroids under HPLC

Steroid	Mean RT (mins)	Inter assay JV	n
E_2S	12	-	1
E_1S	15.9	3.9	4
TS	28.5	7.6	3
DHAS	35.6	6.2	4
Epi.S	44.0	-	1
PS	> 50	-	1

The recovery of DHAS and E_1S from plasma after Sep Pak extraction and HPLC were 72% and 50% respectively. TS = testosterone sulphate, Epi.S = epiandrosterone sulphate, PS = pregnenolone sulphate.

3.1 INTRODUCTION

For the valid comparison of plasma steroid levels in patients and normal subjects an understanding of the inherent variation of plasma steroid levels in normal subjects is required. Such factors as time of day, stage of menstrual cycle, age and sex of the subject all affect the normal plasma steroid levels. The first section of this chapter attempts to outline the normal variation expected for each hormone so that appropriate controls may be chosen for comparison with data obtained from patients with breast or endometial cancer in the second section.

The steroids of the delta five pathway all demonstrate episodic secretion and diurnal rhythm to varying degree (Rosenfeld et al, 1971; Sjoberg et al, 1979; Lachelin et al, 1979). To obtain a good estimate of the mean 24 hour plasma steroid level, blood samples can be taken at regular intervals through the 24 hour period, e.g. every 30 minutes. This is an inconvenient procedure to both patient and experimenter. We have investigated the possibility of only taking 4 samples as a method of estimating the 24 hour mean plasma steroid level. The evidence for a diurnal rhythm for DHAS is not as strong as for the unconjugated steroids (Rosenfeld et al, 1975), however, few workers have studied plasma DHAS levels over the entire 24 hour period (Madden et al, 1978).

A seasonal variation for plasma DHAS has been reported in post-menopausal women with higher values found in autumn/winter than in spring (Deslypere et al, 1983). It is not known whether the unconjugated delta five steroids show the same pattern.

During the menstrual cycle the evidence suggests no alteration in the plasma DHA levels, however opinions vary on the plasma DHAS levels during the cycle. Abraham and Chakmakjian (1973) have shown an increase in plasma DHAS levels around the time of the LH peak in a bilaterally adrenalectomised patient and Jänne (1971) has shown a significant increase in urinary DHAS during the luteal phase. Few authors have measured plasma DHAS in the same normal subject through a complete menstrual cycle, a procedure that is required if small changes are to be detected.

The effect of old age on the plasma levels of the delta five steroids is well documented. Vermeulen (1980) has shown that the plasma levels of DHA, and DHAS, are negatively correlated with age in oophorectomised women. Crilly et al, (1980) have suggested that there is a discrete fall in plasma DHA levels after the menopause rather than a steady decrease. Crilly attributed this to a specific decrease in the adrenal secretion of androgen which was independent of an alteration in ACTH secretion. As the majority of DHAS is produced by the adrenal the measurement of this steroid over the menopausal period would be a good indicator of a specific decrease in adrenal androgen secretion. If a specific alteration does occur then a fault in this mechanism could lead to an over-production of androgen after the menopause.

A role for the adrenal androgens, DHA and DHAS, in the aetiology of breast cancer was suggested by the work of Bulbrook et al, (1967), with the demonstration of a lower urinary ratio of androsterone:etiocholanolone in British women over 40 years old compared to Japanese women. Japanese women are known to have a significantly lower incidence of breast cancer than British women.

As DHA and DHAS are the main plasma precursors to the urinary oxosteroids the plasma level of these steroids in breast cancer subjects has been investigated by many workers. Using mean values from plasma samples taken over 24 hours, Zumoff et al, (1981) demonstrated subnormal DHA and DHAS levels in premenopausal women and elevated levels in postmenopausal women. Thijssen et al, (1975) have shown a decreased urinary production rate of DHA and DHAS in postmenopausal women with breast cancer compared to normal subjects.

A number of theories have been proposed for the role of DHA and DHAS in breast cancer. In postmenopausal women the majority of oestrogen derives from peripheral conversion of androstenedione (Siiteri and MacDonald, 1973). The action of oestradiol unopposed by progesterone has been proposed to maintain tumour growth in postmenopausal women.

Kirschner et al, (1978) suggested there was another source of oestrone in postmenopausal women when they demonstrated that only 72% of oestrone was derived from androstenedione in these subjects. Knapstein et al, (1967) have suggested that oestrone sulphate could be formed from DHAS via the sulphate pathway and oestrone sulphate is a precursor to oestrone. The oestrone so formed being converted to oestradiol by the enzyme 17β hydroxy steroid dehydrogenase. There is therefore the possibility that the sulphate pathway may be responsible for some oestrone production in normal postmenopausal women and possibly excess oestrone in breast cancer patients. The sulphate pathway from DHAS to E_1S requires the formation of androstenedione 3-enol sulphate, the presence of which in plasma is controversial. To provide evidence for the postulated sulphate pathway an attempt was made to isolate, identify and quantify androstenedione 3-enol sulphate in plasma.

Reed et al, (1982) have shown a negative correlation between the conversion of oestrone to oestradiol and plasma DHAS levels in patients with either endometrial hyperplasia, endometrial cancer or cirrhosis. This raises the question of whether DHAS or a metabolite has an effect on the conversion of oestrone to oestradiol within the tissue. In these studies the assumption has been made that the levels of steroid in the tissue are related to the plasma steroid levels, however, the concentration of androgen and its cellular metabolism in diseased tissue may be totally different to normal tissue with no noticeable effect on the plasma levels of steroid.

The aims were:

i)   To obtain data on the normal variation of plasma steroid levels to enable the selection of appropriate controls for further studies.

ii) To investigate the plasma levels of DHA and DHAS in postmenopausal subjects with breast cancer and to investigate DHAS as a possible precursor to E_1S via the sulphate pathway by demonstrating the existence in plasma of the intermediate androstenedione 3-enol sulphate.

iii) We have attempted to measure the tissue concentration of the adrenal androgens in normal and diseased tissue and to relate the levels to those found in plasma.

3.2 Plasma steroid levels in normal subjects

Lithium heparin plasma samples were obtained from volunteers with no known endocrine disorders or taking any drug treatment including the oral contraceptive. The follicular phase was defined as the period of the menstrual cycle with low, <3.2 nmol/l progesterone values, up to fourteen

days after menses. Luteal phase samples had progesterone values in excess of 10 nmol/l. Blood samples from subjects with a normal menopause were taken more than 1 year after the last menstrual period. The normal ranges for the steroids measured using the methods described above are shown in table 3.1.

3.2a Diurnal variation of plasma steroid levels

Plasma levels of DHA, DHAS and cortisol were measured in blood samples taken at 30 minute intervals over 24 hours from 10 subjects (3 pre and 4 postmenopausal women and 3 males). The results were calculated as the percentage change of each sample from the mean 24 hour plasma steroid level for each steroid and subject. The percentage changes at each time point were averaged and plotted ±1 SD as shown in figure 3.1. A distinct diurnal rhythm can be seen (fig. 3.1) for cortisol, DHA and DHAS, with the secretory episodes of DHA and cortisol occurring in synchrony. Plasma DHAS did not demonstrate episodic secretion. Plasma DHAS levels are relatively constant from 1000 hrs. to about 2200 hrs. then fall to a nadir between 0100-0400 hrs. before rising again around 0600-0800 hrs.

To test the validity of using the results obtained from four plasma samples taken at 1030, 1530, 2200 and 0400 hrs. as an estimate of the mean 24 hrs. plasma steroid level, three subjects (one male, one premenopausal and one postmenopausal female) had blood samples taken at 30 minute intervals for 24 hours. Plasma DHA and DHAS levels were measured in all subjects and pregnenolone in one. The comparison of the mean 24 hrs. plasma steroid value with the 4 sample result is shown in table 3.2 and demonstrates a good agreement between the two methods.

Table 3.1   Plasma DHA, DHAS and pregnenolone levels in normal subjects

	DHA (nmol/1)			DHAS (µmol/1)			Pregnenolone (nmol/1)		
	mean	range	n	mean	range	n	mean	range	n
Male	18.0	7.4-53.0	19	6.7	3.7-15.0	19	1.2	0.7-3.0	12
Premenopause									
follicular	19.2	3.0-35.0	22	3.4	1.5-6.1	22	2.6	0.5-6.1	10
luteal	21.2	3.4-43.9	33	3.5	1.3-6.7	33	3.4	0.6-9.2	35
Postmenopause	8.5	1.3-25.7	68	1.9	0.1-7.9	68			
Early pregnancy									
< 12 weeks	19.6	5.0-82.5	60	4.8	0.7-9.7	60	5.4	2.0-14.2	45
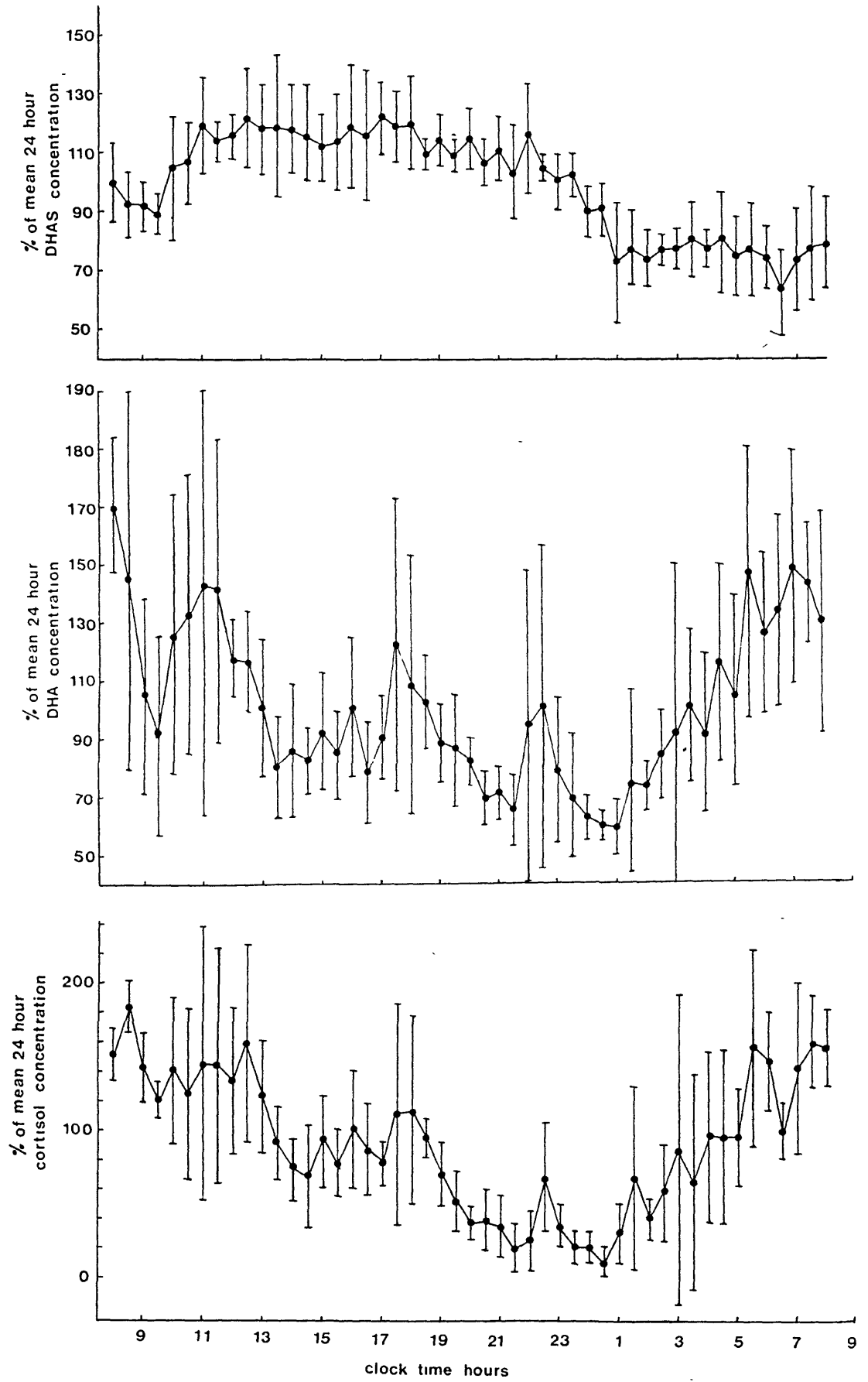
Fig 3.1 Diurnal rhythms of DHA, DHAS and CORTISOL in 10 normal subjects.

Table 3.2   Comparison of mean plasma steroid levels obtained from

40 and 4 blood samples

Subject	Mean diurnal value			Mean 4 sample value		
	DHA nmol/l	DHAS µmol/l	Pregnenolone nmol/l	DHA nmol/l	DHAS µmol/l	Pregnenolone nmol/l
Male	8.1	8.8	3.5	8.2	8.7	3.5
Premenopause	14.5	4.9	-	16.6	4.8	-
Postmenopause	24.3	0.9	-	24.6	0.8	-

3.2b Menstrual cycle variation of plasma steroid levels

Plasma steroid levels were measured in two groups of women, both with normal regular cycles. Group 1 had single blood samples taken at various stages of the cycle. No significant difference was seen between the follicular and luteal phase for any of the steroids measured (table 3.1).

Group 2 were studied with blood samples taken from each subject over the majority of the cycle and the plasma progesterone levels were measured using a fully validated method after hexane extraction of the progesterone and using a specific antisera.

As can be seen (fig. 3.2) there is a large variation in the DHAS levels with possibly a trend to increase during the luteal phase. DHA and pregnenolone levels fluctuate wildly with no distinct pattern. The increase in plasma DHA around day 14-19 is matched by the large peak in DHAS levels (subject 1). In general increasing plasma progesterone levels are associated with decreasing or unchanging DHAS levels and vice versa. Plasma cortisol levels remain fairly constant over the cycle suggesting that the large increase in DHA and DHAS in subject 1. is possibly of ovarian origin.

3.2c Effect of age on plasma steroid levels

To obtain more information on the relationship between advancing age and the adrenal contribution to plasma DHA and DHAS levels we have measured DHA and DHAS in pre and postmenopausal women.
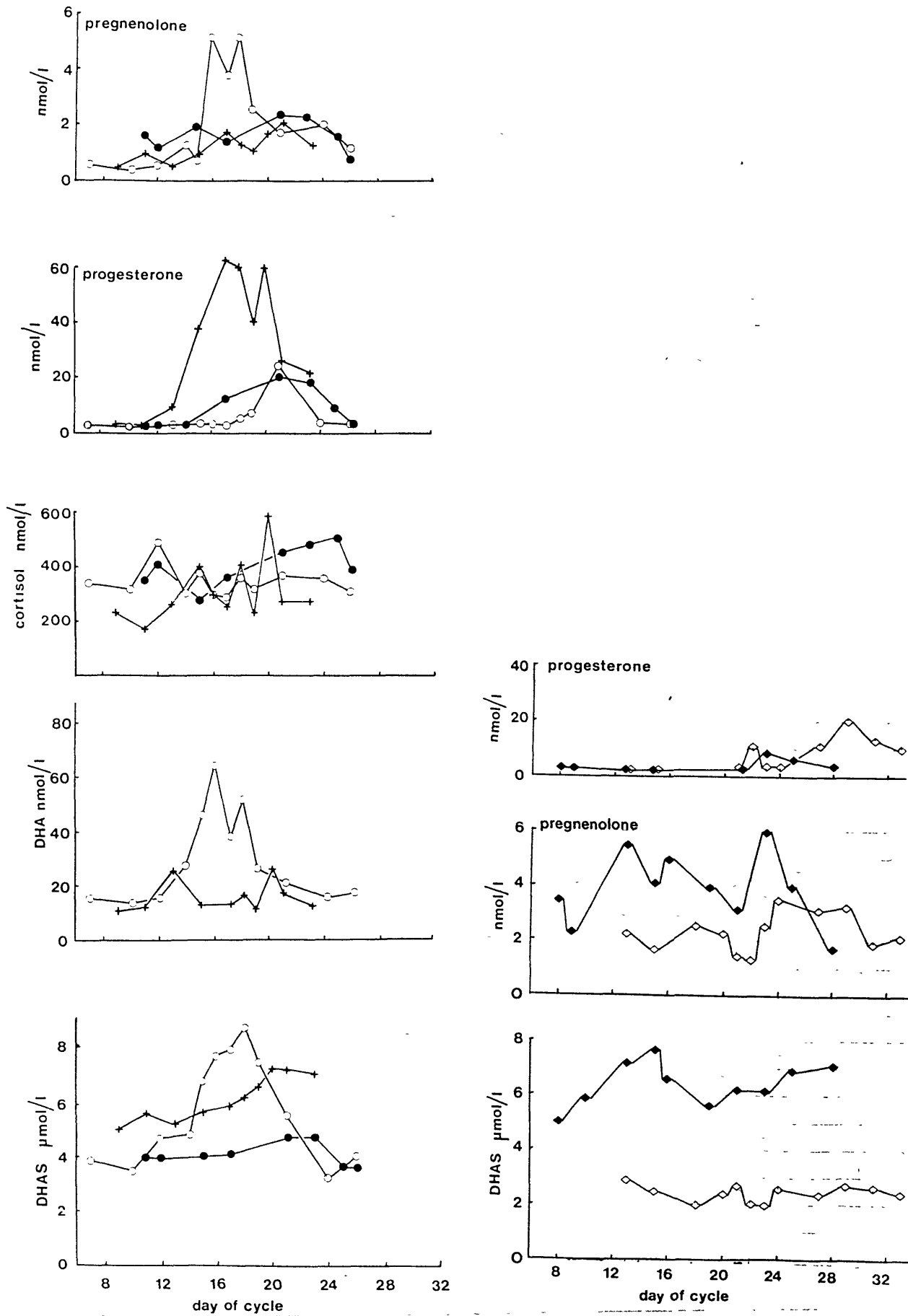
Fig. 3.2. Plasma levels of steroid measured over the menstrual cycle of 4 normal women. Subject 1 (27 yrs.) was studied on 2 consecutive cycles (o——o and ●——●). Subject 2 (32 yr.+——+) subject 3 (30 yr. ◇——◇) subject 4 (25 yrs. ◆——◆).

Single blood samples were taken either between 1000-1200 or 1400-1600 hrs. The results show a slowly decreasing plasma androgen level up to the age of 45-50 years for DHA and 50-55 years for DHAS, fig. 3.3. The plasma level of DHA and DHAS fall rapidly after these ages with the DHA level decreasing first followed by DHAS, table 3.3.

Table 3.3 The effect of age on plasma DHA and DHAS levels in women

Age Years		DHA nmol/l			DHAS μmol/l		
		mean	range	n	mean	range	n
1.	< 50	16.9	3.0-43.9	55	3.4	1.3-6.7	55
2.	50-60	8.8	3.3-25.7	31	2.4	0.6-7.9	31
3.	> 60	8.3	1.3-25.0	37	1.48	0.1-3.7	37
		1 vs 2 $p<0.005$			1 vs 2 $0.025<p<0.05$		
		2 vs 3 $p>0.1$			2 vs 3 $p<0.005$		
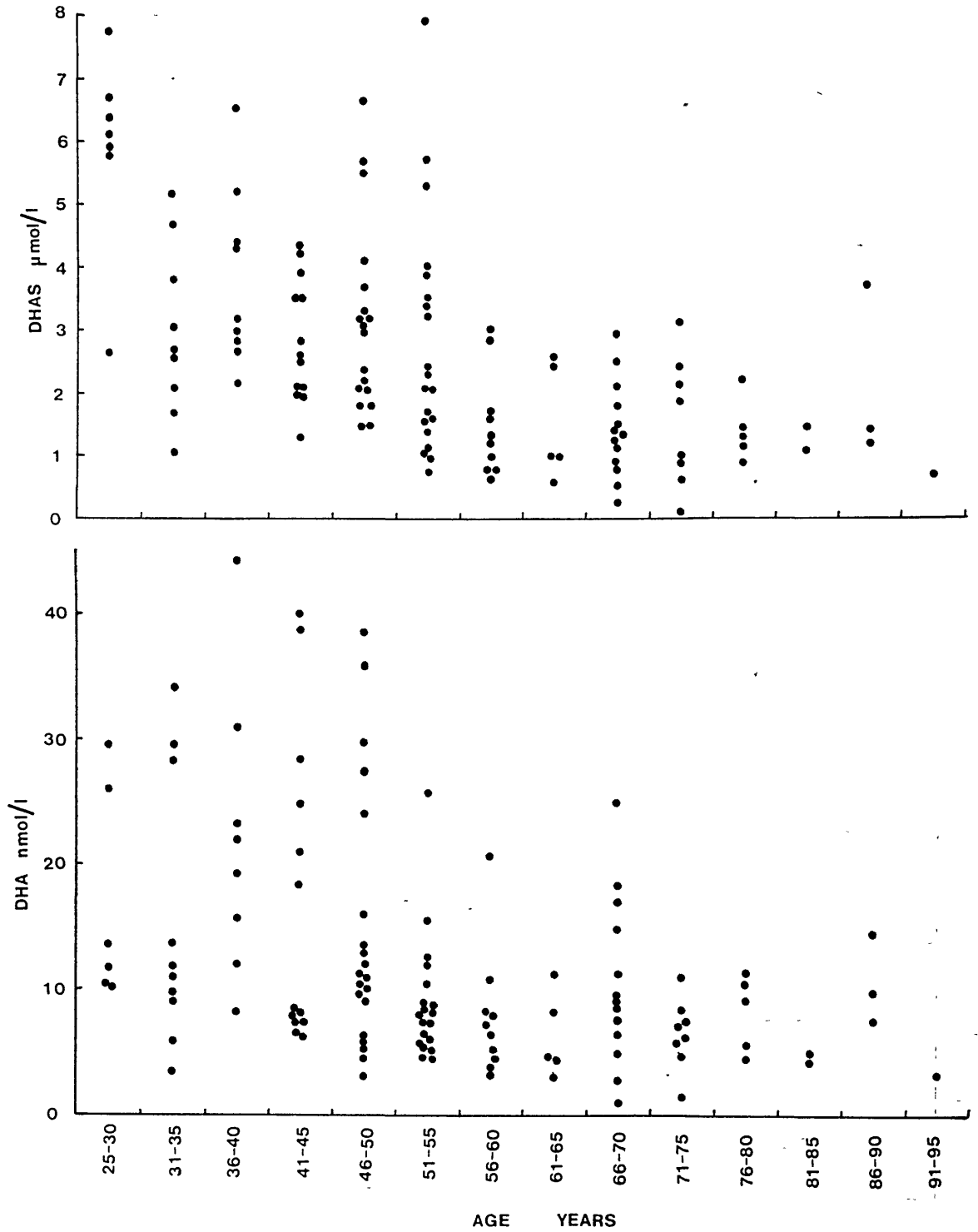
Fig. 3.3. Plasma levels of DHA and DHAS measured in normal women between the age of 25 and 91 yrs.

3.2d Effect of weight on plasma steroid levels

To ensure that obesity had no bearing on our studies 25 postmenopausal
women were studied. No relationship was found between the plasma level of
the adrenal androgens in postmenopausal women to the percentage ideal body
weight (%IBW) for either the single sample or the mean 24 hour values.
The % IBW was calculated from the Documenta Geigy Scientific tables (1970
edition) using the three parameters of height, weight and age. We have
no data on the relationship between either DHA or DHAS with weight in
premenopausal women or men.

3.2e Effect of the oral contraceptive on plasma steroid levels

In 38 premenopausal women taking the combined oral contraceptive pill the
mean plasma DHAS level was 5.4 μmol/l (range 1.7-10.4). There did not
appear to be any relationship between the constituents of the pill to the
plasma level of DHAS.

In 33 subjects taking the progesterone only pill the mean plasma DHA and
DHAS levels were 16.3 nmol/l (range 5.8-45.1) and 5.5 μmol/l (range 1.5-10.0)
respectively. Again there was no relationship between the type of pill
and the plasma DHA and DHAS levels. In both cases the number of subjects
on each type of pill were low, and only single blood samples were taken
without a control sample being taken.

In two subjects with severe acne treated over a period of six months with
a combined pill containing 50 μg ethinyl oestradiol and 1 mg norethisterone,
there was a decrease in the plasma DHA and DHAS level over the first four
months followed by an increase up to six months. In response to the
treatment the plasma cortisol levels increase, fig. 3.4. This data would
indicate that the combined oral contraceptive can affect the plasma level
of DHA and DHAS.
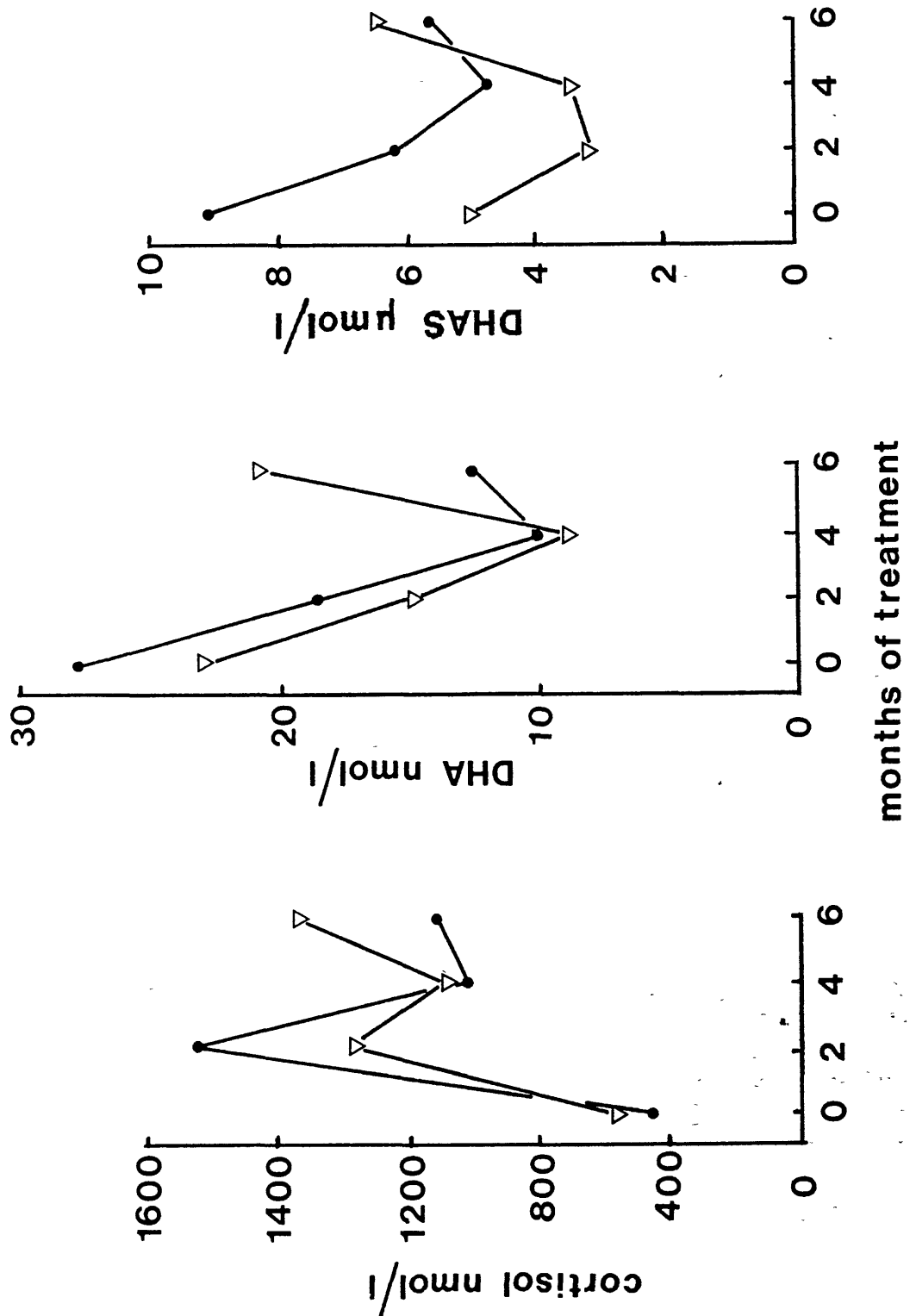
Fig. 3.4. Effect of the combined oral contraceptive (50 μg ethynyl oestradiol and 1 mg Norethisterone) on plasma cortisol, DHA and DHAS levels in two female patients with severe acne.

3.3 Relationship between plasma levels of DHA and DHAS

Plasma levels of DHA and DHAS were measured in 55 pre and 37 post-menopausal women and the results plotted, fig. 3.5. Calculation of the linear regression for each group of women demonstrated a significant correlation between plasma levels of DHA and DHAS in premenopausal (p<0.05) and postmenopausal (p<0.005) women. The regression lines for the two groups are very similar suggesting the relationship between DHA and DHAS is maintained in pre and postmenopausal women. The wide spread of points around the regression lines probably reflect the secretory nature of DHA against the relatively stable plasma DHAS level. The lower plasma levels of DHA and DHAS in postmenopausal women suggests an alteration in the secretion of either DHA and/or DHAS.

3.4 Plasma DHA and DHAS levels in patients with breast cancer

In 37 postmenopausal women with breast cancer no significant difference was found between the plasma levels of either DHA or DHAS in single blood samples compared to age matched normal postmenopausal women (table 3.4). The same was true for the mean 24 hour plasma levels of DHA and DHAS as determined from four samples taken at times of the day that we have shown to give a good estimate of the 24 hour value (table 3.5).

In postmenopausal breast cancer subjects a correlation approaching significance (0.05<p<0.1) was found between the mean 24 hour plasma levels of both DHA and DHAS with weight expressed as the % IBW. This is in contrast to the findings with normal subjects.
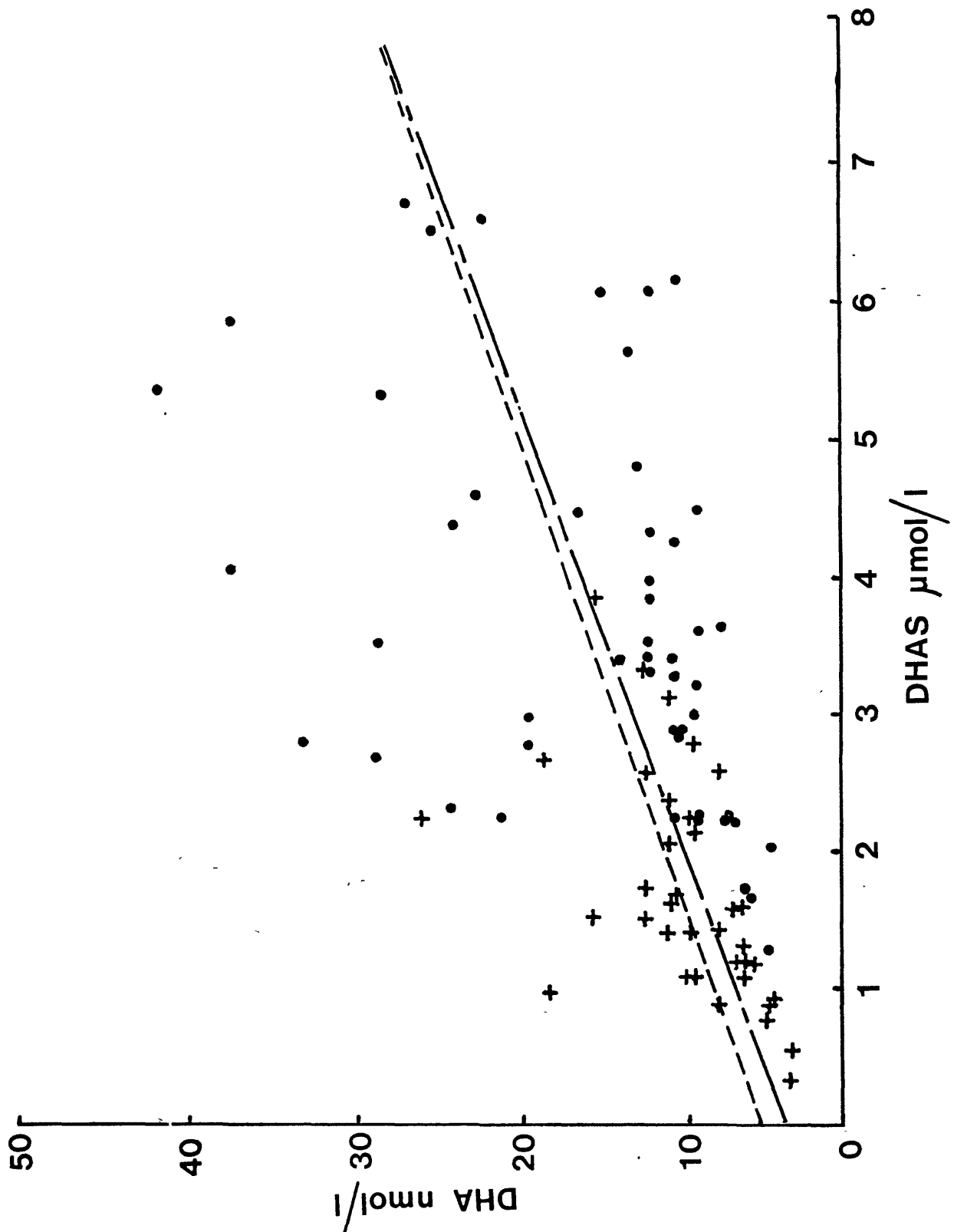
Fig. 3.5. Comparison of the relationship between plasma DHA and DHAS

levels in pre and >5 yrs postmenopausal women. Premenopausal

(; y = 3.1 x + 4.1, r = 0.46), postmenopausal (+; y = 3.2 x

+ 3.6, r = 0.52).

Table 3.4

Plasma steroid levels obtained with single blood samples from normal and cancer patients

	DHA (nmol/l)			DHAS (µmol/l)		
	mean	range	n	mean	range	n
Postmenopause						
1. <50 yrs. old	18.2	7.3-38.7	22	3.4	1.8-6.5	22
2. 50-60 yrs.	10.7	3.3-25.7	17	2.7	0.8-5.3	17
3. >60 yrs. old	7.4	1.5-25.0	32	1.4	0.1-3.5	32
Breast cancer (48-83 yrs. old)	10.5	1.9-19.1	32	2.6	0.6-5.0	32
Endometrial cancer (49-74 yrs. old)	12.6	4.1-32.9	7	2.3	0.7-4.5	8
	1 vs 2 $p<0.005$			1 vs 2 $0.025<p<0.05$		
	2 vs 3 $p>0.1$			2 vs 3 $p<0.005$		

Table 3.5

Steroid concentrations using mean 24 hour plasma values for postmenopausal control and breast cancer subjects

	DHA nmol/l			DHAS µmol/l			Cortisol nmol/l		
	mean	range	n	mean	range	n	mean	range	n
Normal	5.14	1.37-9.4	7	1.48	0.2-3.05	7	170	96-264	7
Cancer	6.6	1.86-12.8	10	1.78	0.29-4.34	10	262	180-400	10

3.5 Plasma DHA and DHAS levels in patients with endometrial cancer

The mean plasma levels of DHA and DHAS in 10 postmenopausal women with endometrial cancer were not significantly different to age matched controls (table 3.4).

3.6 Correlation between plasma DHA and DHAS in postmenopausal women with breast and endometrial cancer

The linear regression between the plasma levels of DHA and DHAS is not significantly different from that for normal postmenopausal women for either the breast cancer or the endometrial cancer group of subjects. This would suggest there is no alteration in the production or metabolism of the DHA and DHAS in these subjects compared to normals.

3.7 Measurement of androstenedione 3-enol sulphate in plasma

The results described above do not suggest a direct relationship between plasma DHA or DHAS levels with either breast or endometrial cancer. A role for DHAS in the aetiology of breast cancer has been proposed as a precursor to oestrone sulphate via the sulphate pathway. A probable intermediate between these two conjugated steroids would be androstenedione 3-enol sulphate. The evidence for the existence of this compound in the plasma is controversial, so an attempt was made to isolate and quantify androstenedione 3-enol sulphate from human plasma.

3.7a Investigation of available hydrolysis methods

As it has been shown that some hydrolysis procedures produce artifacts, Dorfman (1956), eight different hydrolysis procedures were tried in an attempt to obtain a clean hydrolysis of the sulphate moiety. Four methods

used acid hydrolysis after extraction of the steroid sulphate into ethyl

acetate using either hydrochloric acid (Azo et al, 1977), or sulphuric

acid (Burstein and Lieberman, 1958) or sulphuric acid in the aqueous

phase (Goodall et al, 1981). The remaining methods used sulphatase enzymes

obtained from either Helix pomatia, Pattela vulgata or Abalone. The

methods used for the sulphatase hydrolysis were as described in section 2.

^{3}H-DHAS was used as the substrate for the hydrolysis experiments as DHAS

is present in plasma in large amounts which could result in a small

percentage of artifact being produced which would be significant if low

plasma levels of that artifact were expected.

The effects of each hydrolytic procedure on the formation of artifacts was

monitored by running the ether extracted products of the hydrolysis on TLC

in DCM:ethyl acetate (93:7 v/v) or chloroform:acetone (95:5 v/v). The

chromatography strips were scanned using a Panax thin layer scanner to

detect the radioactive areas. Results are shown in table 3.6.

Most of the hydrolysis methods form artifacts, however the enzyme methods

all form a compound with the same mobility as androstenedione. Incubation

of increasing amounts of unlabelled DHAS with the sulphatase enzymes and

measuring the product that ran with androstenedione by radioimmunoassay,

using the method of Goodall et al, (1981), demonstrated a linear increase

in the amount of measurable androstenedione correlating with the increasing

mass of incubated DHAS. The acid hydrolysis methods did not appear to

produce as much androstenedione, but with the mass of DHAS present in

Table 3.6   Effect of hydrolysis procedure on the formation of artifacts

Hydrolysis Procedure	rF						
	0.03	0.10	0.18	0.34	0.37	0.60	0.82
1. Conc. H_2SO_4 % total	1.3	11.8	4.0	59.8	18.2	4.8	-
2. 4M H_2SO_4 % total	2.6	1.4	1.2	96.2	-	0.4	-
3. 1M H_2SO_4 % total	-	-	-	5.0	-	-	-
4. Conc. HCL 16 hr. inc. % total	-	-	-	68.6	-	-	31.4
5. Conc. HCL 1 hr. inc. % total	-	-	-	99.6	-	-	0.4

Sulphatase

Hydrolysis	rF	0.04	0.13	0.34	0.46
6. H.pomatia % total		3.8	3.8	89.2	3.2
7. P.vulgata % total		6.0	-	91.0	3.0
8. Abalone % total		-	-	99.5	0.5

Standards	rF	0.23	0.34	0.41	0.46	0.54
		Testosterone	DHA	Pregnenolone	Androstenedione	Progesterone

normal plasma (2-5 μmol/1) an 0.1% conversion would result in 2.5 nmol/1 of androstenedione.

The acid hydrolysis method of Goodall et al, (1981) was chosen for further work as Matsui et al, (1974) have claimed that androstenedione 3-enol glucuronide is unstable in acid conditions (pH 1.0) and there is little hydrolysis of DHAS to enable the formation of breakdown products.

3.7b Plasma levels of androstenedione 3-enol sulphate

To remove the possibility of the artifactual production of androstenedione during the hydrolysis stage it would be advantageous to isolate the androstenedione 3-enol sulphate prior to hydrolysis. Previous methods to isolate the conjugated steroids involve a solvent extraction followed by paper or column chromatography, both of which are tedious and time consuming. High pressure liquid chromatography is a very quick technique and initial reports have demonstrated its use for the separation of steroid conjugates, Laffosse et al, (1976). Initial sample purification and steroid concentration has been rapidly performed using Sep Pak cartridges (Waters) for the conjugated oestrogens, Helkkinen et al, (1981). It was therefore decided to couple these two procedures for the rapid purification of androstenedione 3-enol sulphate. The methods used were as described in section 2.13.

The plasma samples used for the assay of androstenedione 3-enol sulphate were obtained from normal male subjects, patients pre and post ACTH stimulation and from adrenal and renal cannulations of patients with Conns disease.

All fractions collected from the HPLC column up to 15 minutes after the DHAS peak were subjected to 1M H_2SO_4 hydrolysis and radioimmunoassay for androstenedione. From the structure of androstenedione 3-enol sulphate, and from preliminary data on urine the androstenedione 3-enol sulphate peak was expected to elute close to oestrone sulphate.

No significant amounts of androstenedione 3-enol sulphate were measurable, some androstenedione positive areas were found by radioimmunoassay with a retention time of 12 minutes and at the DHAS peak but both results were close to the sensitivity of the androstenedione assay. No further release of androstenedione was obtained after a short HCl hydrolysis (procedure 5) of the eluates remaining after the 1M H_2SO_4 hydrolysis.

The possibility that the androstenedione 3-enol sulphate had not eluted from the column was investigated by changing the elution solvent to water:methanol (65:35 v/v). This elutes DHAS in 7 minutes, and collecting aliquots for 30 minutes we were still unable to detect any androstenedione 3-enol sulphate.

3.8 Plasma and tissue levels of DHA and DHAS in subjects with breast and endometrial cancer

Primary breast adenocarcinomas and normal tissue from the same breast were obtained from eleven postmenopausal patients aged 50-79 years. Endometrial tissue and peripheral blood samples were obtained at the time of operation from women aged 20 to 53 years admitted for curretage or hysterectomy for non-malignant conditions and from postmenopausal women aged 49-79 years with endometrial carcinoma. None of the patients were receiving any form of hormonal treatment up to the time of surgery.

The mean level of DHA in tumourous tissue was significantly higher than normal tissue taken from the same breast ($p < 0.01$ n = 11, paired t-test). In 10 similarly matched pairs the mean DHAS levels were not significantly different (table 3.7).

The endometrial tissue level of DHA and DHAS are shown in table 3.7. The normal endometrial tissue levels of DHA, but not DHAS, are significantly lower in the 50-60 year old compared to the < 50 year old group ($0.001 < p < 0.01$). The number of subjects is too low in the other groups to allow statistical analysis. No significant difference was found between the levels of either steroid in normal compared to cancerous tissue taken from postmenopausal women (table 3.7).

3.9 Correlation of plasma and tissue steroid levels

Concentrations of DHAS and DHA in the plasma and endometrial tissue of 55 normal premenopausal women were significantly correlated ($r = 0.7$, $p < 0.001$, and $r = 0.323$, $p < 0.01$ respectively) (figs. 3.6 and 3.7). For normal postmenopausal women plasma DHAS but not DHA levels were correlated to the tissue levels, however the numbers are small (fig. 3.8). No correlation was found between plasma and tissue levels of either DHA or DHAS in patients with endometrial cancer, but the numbers are small (n = 6).

No data is available on the relationship between plasma and tissue steroid levels for the breast cancer subjects.

Table 3.7

Tissue steroid levels

	DHA (ng/g)			DHAS (ng/g)		
	mean	range	n	mean	range	n
Normal endometrial tissue						
1. <50 yrs. old	77.6	4.7-343	66	173	3.7-620	57
2. 50-60 yrs.	33.6	5.1-94.4	17	154	9.6-501	14
3. >60 yrs. old	32.5	11.5-53.1	3	80	29.0-131	2
4. Endometrial cancer (48-74 yrs. old)	23.4	2.4-53.1	8	184	29.0-530	7
Normal breast tissue						
5. <50 yrs. old	25.1	4.3-49.5	3	171	73.9-296	3
6. 50-60 yrs.	8.2	6.1-10.2	2	204	41.7-366	2
7. >60 yrs. old	6.5	1.6-20.0	9	92.4	6.0-554	8
8. Breast cancer (50-79 yrs. old)	15.8	3.3-41.4	11	155	9.5-857	10

1 vs 2 $0.001 < p < 0.01$	1 vs 2 $p > 0.1$
2 and 3 vs 4 $p > 0.1$	2 and 3 vs 4 $p > 0.1$
6 and 7 vs 8 $p < 0.01$	6 and 7 vs 8 $p > 0.01$
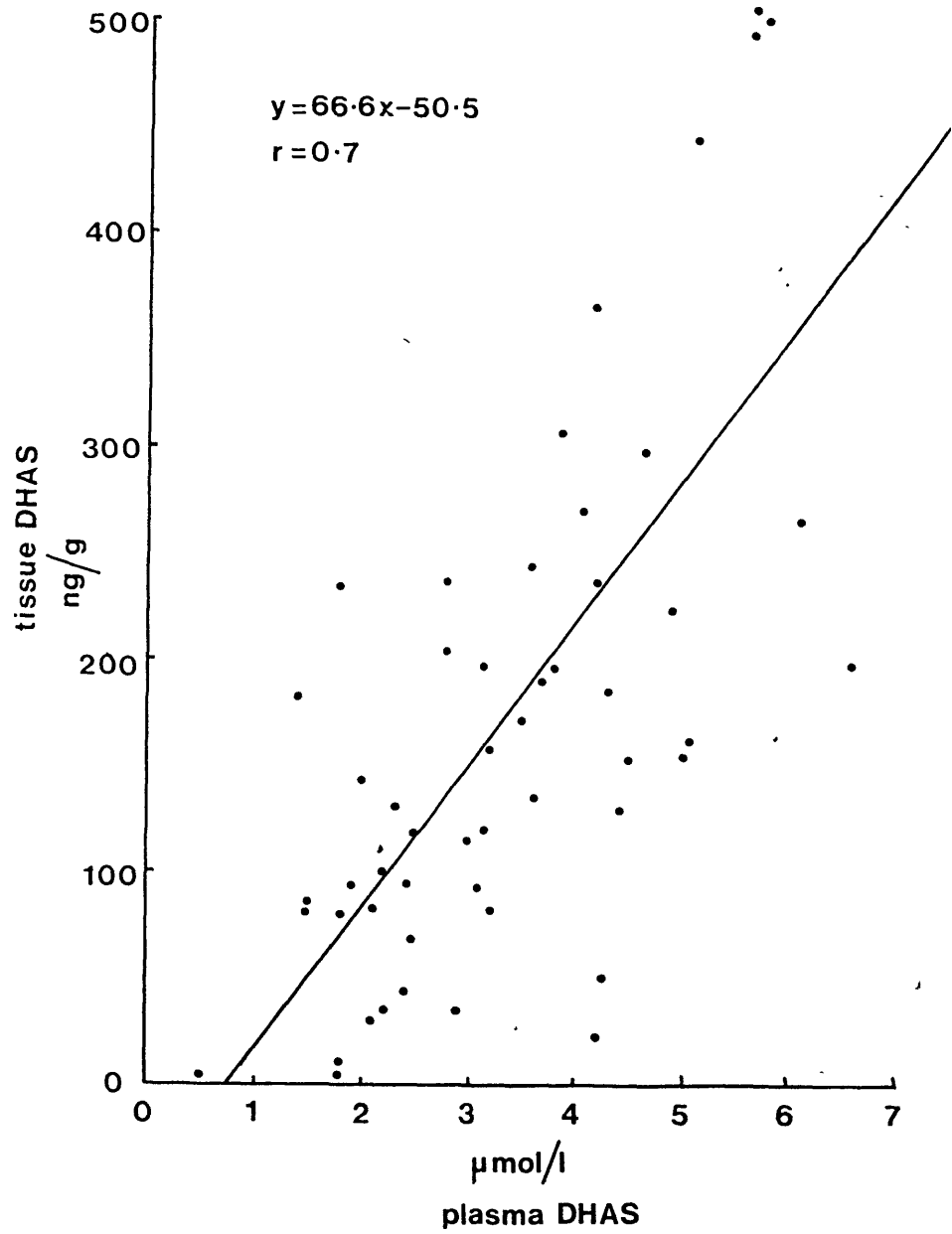
Fig.3.6   Correlation between plasma and endometrial tissue levels of
DHAS in normal premenopausal women.
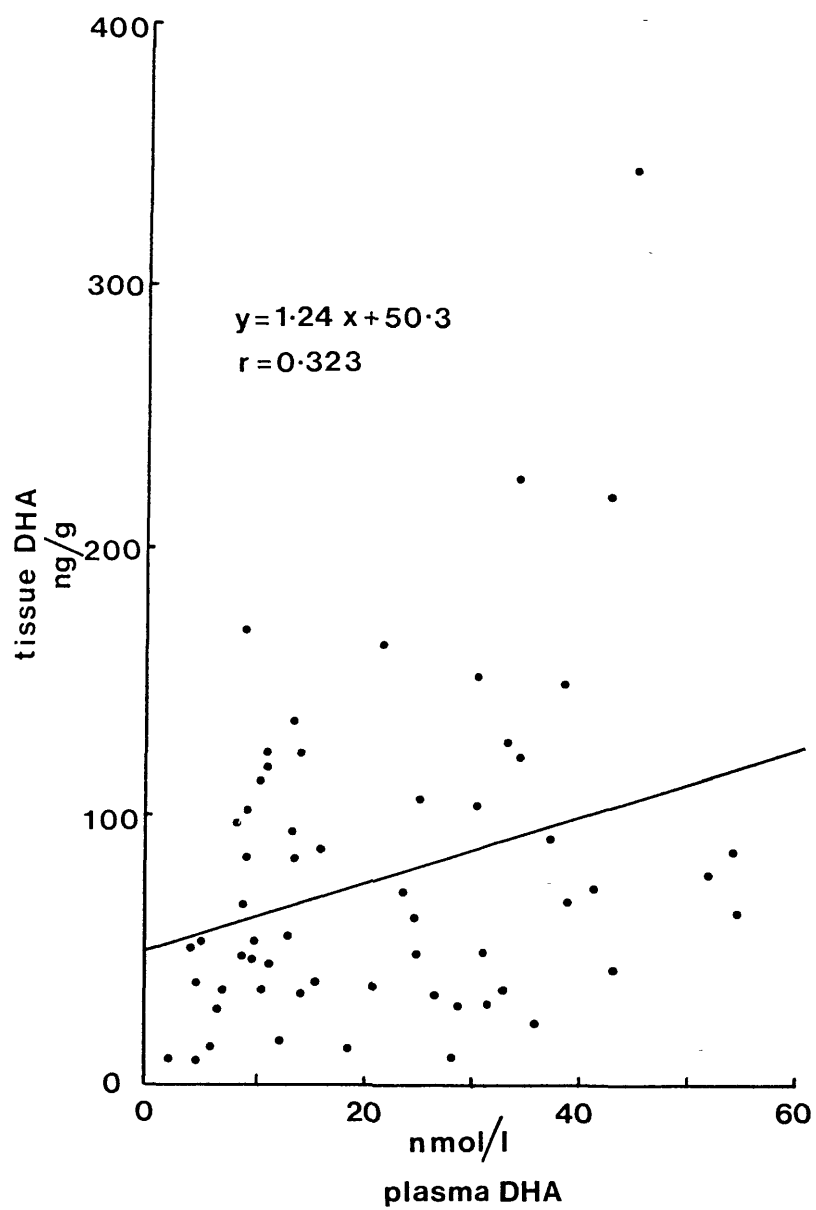
Fig.3.7   Correlation between plasma and endometrial tissue levels of
DHA in normal premenopausal women.

$y = 0.2x + 25$
$r = 0.05$
NS

tissue DHA ng/g

plasma DHA nmol/l

$y = 101x - 111.3$
$r = 0.889$
$p < 0.01$

tissue DHAS ng/g

plasma DHAS μmol/l
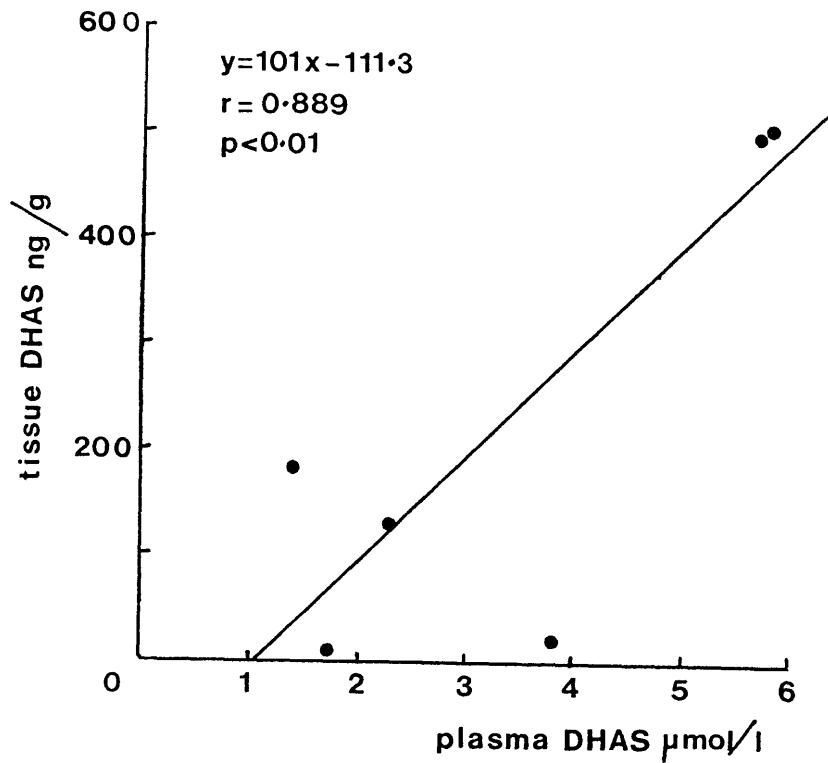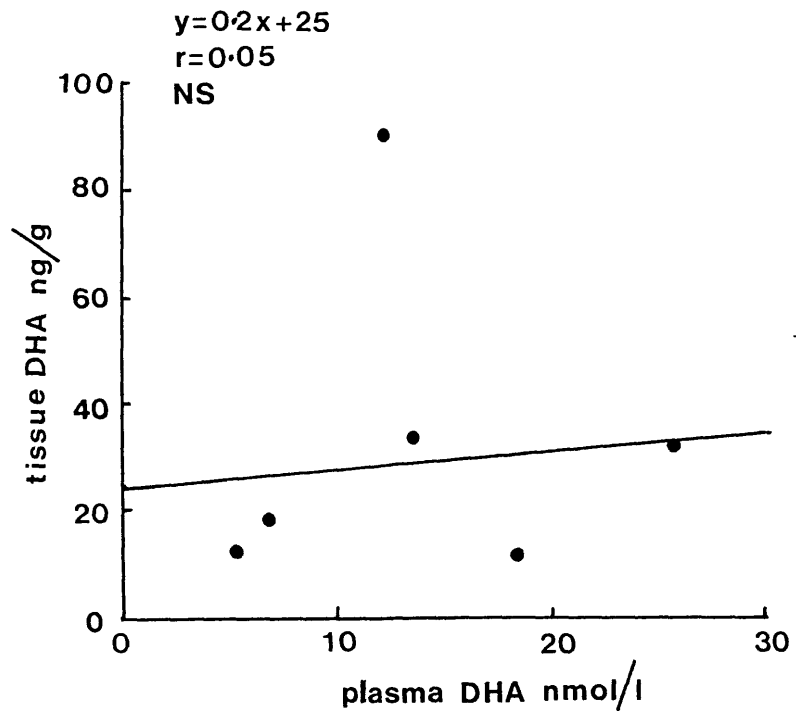
Fig. 3.8   Correlation between plasma and endometrial tissue levels of
a) DHAS and b) DHA in postmenopausal women.

3.10 Discussion

In order to develop a simple, rapid and reliable radioimmunoassay the main requirement is for a good specific antiserum. This is demonstrated in this thesis by the assay for DHA which is quicker and easier to perform than the methods available and gives results that compare favourably with the reported literature values (Rosenfield, 1971; Abraham et al, 1973). The pregnenolone antiserum on the other hand is a low titre and poor specificity and requires an elaborate, time consuming assay methodology to obtain reliable results that agree with reported plasma levels (Bermudez et al, 1970; Abraham et al, 1973; Di Pietro et al, 1972; Anderson et al, 1976).

Due to the lack of a specific antiserum to DHAS an initial hydrolysis procedure was required prior to immunoassay. The use of the sulphatase enzyme enabled the hydrolysis to be performed relatively easily and results obtained for the plasma DHAS levels are in good agreement with reported values (Buster and Abraham, 1972 a/b; Cattaneo et al, 1975).

The lack of antisera to conjugated steroids results in the need to hydrolyse the conjugate prior to assay, and as demonstrated here, can lead to erroneous results due to the formation of hydrolytic artifacts. As long ago as 1956, Dorfman et al, demonstrated that hydrochloric acid forms artifacts during hydrolysis of steroid conjugates and other workers have alluded to the problem (Azo et al, 1977). Our results support these observations and show that a range of artifacts are formed during acid hydrolysis. The production of artifacts being related to the concentration of acid present and the duration of hydrolysis.

Very little information is available on the production of artifacts using
an enzymic hydrolysis. Recently a paper by Messeri et al, (1984) reported
the conversion of DHA to androstenedione using Helix pomatia sulphatase.
Our results show that the commercially available sulphatase enzymes contain
a high degree of enzymic contamination that allows steroid metabolism to
occur at the acid pH of the hydrolysis buffer. It is not known how variable
the contamination is from batch to batch but in this case the Patella enzyme
appears to be the least contaminated. In all probability the contaminating
enzymes act on the free steroid liberated from the hydrolysis of the
conjugates rather than acting directly on the conjugates themselves.

If a hydrolysis stage is required then ideally the steroid conjugate should
be purified first. The work reported here demonstrates the combination of
Sep Pak cartridges and HPLC to extract and separate a number of conjugated
steroids rapidly and reproducibly from human plasma. Once collected from
the column the eluates can be measured colourimetrically or by immunoassay
either directly or after hydrolysis.

Using HPLC purification and acid hydrolysis prior to RIA we were unable to
detect a significant amount of androstenedione 3-enol sulphate in plasma.
It could be argued that we did not get complete extraction of this compound
from the plasma, however we have tried a methanol extraction, ethyl acetate
extraction of plasma saturated with sodium chloride and extraction using
Sep Pak cartridges and found no androstenedione 3-enol sulphate with either
method. Assuming this sulphate acts in a similar fashion to E_1S with regard
to binding to plasma proteins, then we obtained a 50% extraction efficiency

with the latter method. Using this figure and assuming 100% hydrolysis we found a maximum plasma androstenedione 3-enol sulphate concentration of 0.18 nmol/l. However, this figure was obtained from a mass of steroid extrapolated from a point on the standard curve which was near the sensitivity of the androstenedione assay.

It is possible that androstenedione 3-enol sulphate exists in plasma as an unstable intermediate bound to the enzyme during a metabolic conversion, similar to the role of testosterone 3-enol sulphate proposed by Toft et al, (1974), during the enzymatic conversion of testosterone to its saturated metabolite. However, it seems that androstenedione 3-enol sulphate is not present in plasma in the quantities previously reported.

The variation in the plasma levels of DHA and DHAS in normal subjects at different times of day, month and age, emphasise the need to obtain appropriate control subjects for any study. The episodic secretion and diurnal rhythm shown by DHA, DHAS and pregnenolone indicate the need to take more than one sample to obtain a good estimate of the mean 24 hour plasma steroid level. Four samples taken at 10.30, 15.30, 22.00 and 04.00 have been shown to give a good estimate of the mean 24 hour plasma steroid level. This study demonstrates that DHAS does have a distinct diurnal rhythm which agrees with the findings of Madden et al, (1978) of a nadir in the plasma DHAS level at 02.00-04.00 hours.

The demonstration of increased plasma DHAS levels during the latter half of the menstrual cycle in women with no or a low progesterone level has not been reported before. In previous studies on the level of plasma DHAS through the menstrual cycle only cycles with good progesterone peaks were reported or no progesterone results were given. This may be a reason for the differences of opinion on the existence of a change in DHAS levels through the menstrual cycle found in the literature. This study and the study on the effect of the contraceptive pill on plasma steroid levels demonstrate the need to investigate the same subject throughout the cycle and as their own control to detect an effect of a treatment regime. The large inter-person variability in plasma steroid level can result in the masking of small changes in plasma steroid level when results from a single blood sampling of two separate populations are compared.

The increase in plasma cortisol levels in response to the combined oral contraceptive treatment is probably due to the well documented increase in cortisol binding globulin caused by the oestrogen therapy (Schwartz and Hammerstein, 1974). However, the effect of oestrogen therapy on plasma levels of DHA and DHAS is controversial. Abraham and Maroulis (1975) have shown increased plasma levels of DHAS in postmenopausal women on oestrogen therapy while Madden et al, (1978) and Wild et al, (1982) have shown that the combined oral contraceptive decreases plasma levels of DHAS. Anderson and Yen (1976) with oestradiol infusions and ethinyl oestradiol treatment to premenopausal women, showed no effect on DHAS, as did Mahajan et al, (1978) with castrate women given ethinyl oestradiol.

Bulbrook et al, (1973) have demonstrated that the combined oral contraceptive has no effect on the plasma binding of DHAS. Our results agree with the findings of Madden et al, (1978) and suggest that a component of the combined oral contraceptive affects the biosynthesis of DHAS. Whether this effect occurs at the site of adrenal DHAS synthesis is not known.

When compared to age matched control patients there was no significant alteration in the mean plasma DHA or DHAS levels in patients with breast cancer. This contrasts with the results of Zumoff et al,(1981) who demonstrated increased plasma levels of DHA and DHAS in postmenopausal women with breast cancer. The majority of other workers report normal or subnormal levels of plasma DHA and DHAS in breast cancer subjects. The increased plasma levels of adrenal androgen reported by Zumoff et al, (1981) may reflect a specific alteration of adrenal androgen secretion or increased adrenal activity. Unfortunately Zumoff et al, do not give any values for plasma cortisol levels in the patients studied. For our subjects although the breast cancer group have a slightly elevated mean plasma androgen level, the mean cortisol level is also raised compared to control subjects suggesting an increased adrenal activity.

The results obtained for tissue DHA levels agree well with reported values (Maynard et al, 1978; Poortman et al, 1983; Guerrero et al, 1975). No reports have been made on the measurement of DHAS in breast or endometrial tissue.

Guerrero et al.,(1975) demonstrated a good correlation between tissue and plasma pregnenolone levels but not for DHA. Our results demonstrate a strong relationship between the concentration of DHAS in normal endometrial tissue and plasma. The less significant correlation between plasma and tissue DHA may reflect the measurement of steroid in single plasma samples which has the problem of episodic secretion which the tissue levels may not reflect. Plasma DHAS levels are relatively stable during the day and may therefore reflect tissue levels more accurately.

A relationship between tissue and mean 24 hour plasma levels of unconjugated steroid may be expected from the observations of Giorgi (1981), that unconjugated steroids cross the membrane by passive diffusion. Giorgi (1981) suggests that the intracellular concentration of steroid is related to the amount of unbound steroid in plasma and the solubility of the steroid in the lipid membrane. For DHA and DHAS approximately 5% and 1% of steroid is free in plasma. Using plasma levels found in normal postmenopausal women approximately 144 pg of DHA and 10.6 ng of DHAS is unbound in 1 ml of plasma. Expressing the mean tissue concentrations of these steroids as a ratio of the plasma concentration, ratios of 230 and 10 are obtained for DHA and DHAS respectively.

The partition coefficients of DHA and DHAS between n-octanol and water were obtained using the method of Giorgi (1981), and were 126 and 6.3 respectively. The partition coefficient between n-octanol and water has been reported to give a good indication of the solubility of the steroid

in the cell membrane (Giorgi, 1980). The 20 fold higher tissue to plasma ratio of DHA to DHAS corresponds to the relative partition coefficients found for these steroids.

This data suggests that for normal endometrial tissue the unbound plasma level and tissue level of DHA and DHAS are related and that the tissue levels agree with the hypothesis of free diffusion of steroid across the membrane, the degree of which is dependent on the polarity of the steroid.

Our data for breast tissue concentrations of DHAS and DHA demonstrate a significantly increased DHA level with an increased mean DHAS level for breast cancer versus normals. For DHAS however there were four samples in which the tumour tissue DHAS level was lower than the control tissue value while for DHA all tumour tissue values were raised compared to the controls. Whether a direct correlation exists between the concentration of DHAS in normal breast tissue and plasma as found with endometrial tissue is not known.

The possibility that the elevated tissue DHA concentration found in breast cancer tumour tissue has an inhibitory action on the 17β-OH steroid dehydrogenase has been investigated by Bonney et al, (1984). These workers found that for normal breast tissue there was a significant positive correlation between tissue DHA and DHAS levels and 17β-OH steroid dehydrogenase activity, while for tumour tissue only DHAS was significantly correlated. These findings would therefore disagree with the hypothesis of Adams (1977), that the increased adrenal androgen in tissue inhibits the conversion of oestradiol to oestrone.

Elevated tissue DHA levels may be due to increased passage of plasma DHA to the tissue, increased passage and intracellular metabolism of DHAS or other precursors to DHA, or inhibited metabolism of DHA to products such as androstenediol and androstenedione.

There is no alteration in the calculated regression line obtained from a plot of plasma DHA and DHAS levels for normal compared to breast cancer subjects. This suggests that the specific increase in tissue DHA seen with breast cancer subjects is not a reflection of an altered plasma DHA level. However, we have no data on the plasma unbound DHA level so it is feasible that this may be altered in postmenopausal breast cancer subjects. This also assumes that the breast tissue uptake of steroid is similar to endometrial tissue.

As reported by Bonney et al, (1984), there is a significant correlation between the concentration of DHA and DHAS in normal breast tissue ($r = 0.87$, $p < 0.001$, $y = 0.035$ x $+4.42$), but none for breast tumour tissue ($r = 0.23$, $p < 0.1$, $y = 0.01$ x $+15.38$). This would suggest there is an alteration in the metabolism of DHA and DHAS in the tumour tissue, possibly by an alteration in the tissue sulphatase/sulphokinase activity.

It would be expected that the increased tissue DHA level would result in an increased tissue androstenediol level provided the tissue 17β-OH steroid dehydrogenase enzyme was working normally. This has indeed been found to be the case (Bonney et al, 1984), with tissue androstenediol levels significantly elevated in subjects with breast cancer. This suggests there

is no inhibition of the intracellular delta five steroid metabolism which would result in increased DHA levels. There is no evidence of an increased plasma or tissue level of the possible precursors to DHA such as pregnenolone (O'Higgins et al, 1976) in breast cancer patients.

In conclusion there appears to be a difference in the DHA, DHAS ratio between normal breast and tumour tissue. Whether the increase in breast tumour tissue DHA is due to an increased tissue uptake or altered intracellular metabolism is not known. However, whichever is the case there is an increased tissue androstenediol level which may have some bearing on the aetiology of breast cancer.

4.1 INTRODUCTION

The human adrenal cortex produces glucocorticoid, mineralocorticoid and androgenic hormones. Production of the glucocorticoid and mineralocorticoid hormones is under the control of ACTH and renin-angiotensin secretion respectively. The mechanism for control of adrenal androgen secretion, particularly for DHAS, has not been fully elucidated.

A role for ACTH in the control of adrenal DHA secretion was suggested by Rosenfeld et al, (1975) with the demonstration of a synchrony between the episodic increases in the plasma levels of DHA and cortisol. Studies in humans have demonstrated the simultaneous increase in plasma DHA and cortisol levels in response to the injection of synthetic ACTH (Cutler et al, 1979). Further evidence for the involvement of ACTH has been obtained from acute and chronic studies with dexamethasone, which results in a complete suppression of plasma cortisol and a rapid fall in plasma DHA levels to 20-30% of normal levels. With chronic dexamethasone treatment of two oophorectomised postmenopausal women with metastatic breast cancer the plasma levels of DHA did not fall to below 26% of initial levels (Barkowski et al, 1977).

A possible inference from these findings is that ACTH is required for adrenal androgen production, but whether the ACTH acts alone or has a permissive effect to enable another hormone to stimulate adrenal androgen production is not known.

Similar investigations into the control mechanism for DHAS secretion have shown that ACTH and dexamethasone have stimulatory and inhibitory actions respectively on the plasma DHAS level, but the DHAS response is much slower than is seen for DHA. This has been attributed to the much larger pool size and longer half life of plasma DHAS compared to DHA.

Possible effects of ACTH on the adrenal secretion of DHAS may not be seen due to the large endogenous concentrations of the steroid present in plasma, which would result in small secretory episodes being masked. It was thought that measurement of the free DHAS in plasma may enable an acute effect of ACTH on DHAS secretion to be seen. As it has been shown by some workers that levels of steroid in saliva accurately reflect the free plasma concentrations (Umeda et al, 1981; Baxendale et al, 1982), experiments were performed to measure DHA and DHAS in human saliva. If synchronous increases in the saliva levels of DHA and DHAS were seen then this would add evidence to the direct secretion of DHAS by the adrenal in response to ACTH.

Most workers have concluded that DHAS is solely secreted by the adrenal and the measurement of plasma DHAS has been suggested to be an indicator of adrenal function (Korth-Schutz, 1976).

In a number of clinical situations secretion of ACTH, as measured directly by changes in the concentration in plasma or as reflected by alterations in the plasma cortisol level in response to ACTH, is divergent from changes in the plasma adrenal androgen level. This has been reviewed by Parker and

Odell (1980). These dissociations have led to the suggestion that the pituitary releases a factor other than ACTH which specifically stimulates the secretion of androgen by the adrenal cortex. This factor has been christened, cortical androgen stimulating hormone (CASH) by Parker and Odell (1977), and adrenal androgen stimulating hormone (AASH) by Grumbach et al, (1978).

Other suggestions to explain the dissociation between adrenal androgen and cortisol production during adrenarch have been made. High local levels of cortisol induce morphological and functional changes of the zona reticularis (Anderson, 1980), or that there is a primary shift in the adrenal biosynthetic pathways with advancing age (Rich et al, 1981).

Of the many compounds proposed as the postulated AASH (Parker and Odell, 1980), prolactin has received a great deal of attention. This was started by the report by Vermeulen et al, (1977) that subjects with hyperprolactin-aemia have elevated plasma DHAS levels. Since this time a number of papers have appeared supporting (Bassi et al, 1977; Carter et al, 1977; Kandeel et al, 1978) and contesting the hypothesis (Metcalf, 1979; Parker et al, 1978).

Most authors have concentrated on the adrenal as being the site of prolactin action, however prolactin receptors have been demonstrated in ovarian tissue (Costlow and McGuire, 1977). McNatty et al, (1974) have demonstrated that in the human Graafian follicle the prolactin concentration is inversely related to the progesterone level, and that this effect is

dose dependant. El-Ayat (1978) showed that ovarian tissue taken during the follicular phase converts pregnenolone to DHA rather than progesterone, and that the reverse occurs during the secretory phase. Hence high levels of prolactin could be associated with conversion of pregnenolone to DHA by ovarian tissue during the follicular phase. The conversion of the ovarian produced DHA to DHAS would therefore increase the plasma DHAS levels. To investigate this possibility plasma DHA, DHAS and progesterone were measured in patients with Addisons disease.

In the following experiments it was hoped to obtain further information on the relationship between ACTH secretion and plasma DHA and DHAS levels and to investigate some examples of a divergence between the plasma cortisol and adrenal androgen levels, with regard to the existence of the postulated AASH. The possibility that prolactin is the postulated AASH and its possible site of action was also investigated.

4.2 <u>Correlation between the secretion of DHA and DHAS with cortisol</u>

Regular blood samples were taken from a normal premenopausal female (age 34 yrs.) at 30 minute intervals throughout 24 hours, and plasma DHA, DHAS and cortisol levels were assayed. A good correlation between the secretory episodes of DHA and cortisol is seen (fig. 4.1). This correlation is maintained in males and both pre and postmenopausal females. No correlation is seen between the plasma levels of DHAS and cortisol.
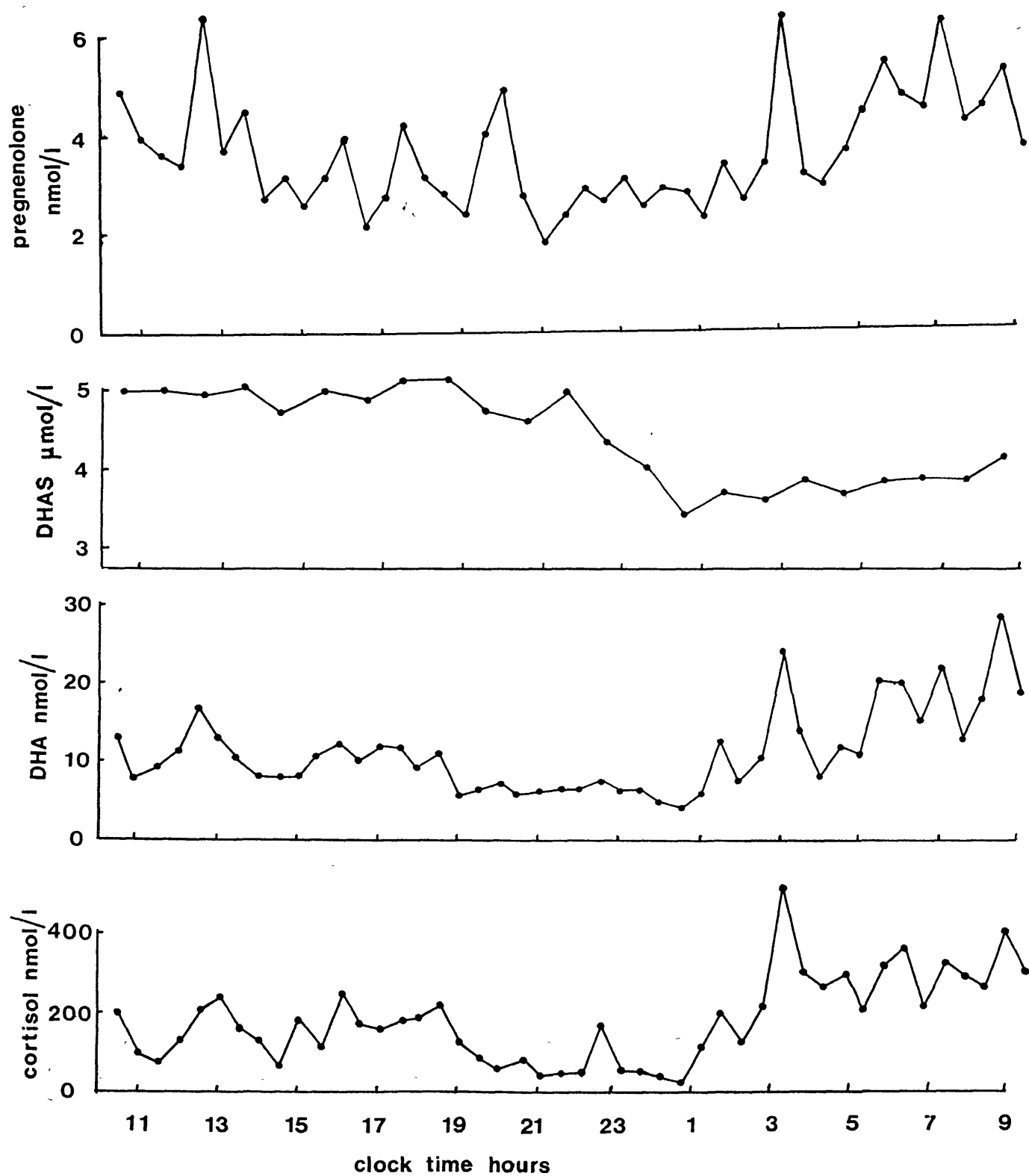
Fig. 4.1. An example of the diurnal profile in plasma levels of DHA, cortisol,
DHAS and pregnenolone measured in a normal 35 yr. old female
over 23 hrs.

4.3 Correlation between plasma ACTH, DHA and cortisol levels

In three normal subjects, two female and one male, studied over either 8
or 24 hours with regular blood sampling, the plasma ACTH concentration was
measured using ACTH kits obtained from Amersham International. The kits
are derived from the method of Ratcliff and Edwards (1971), for measuring
plasma ACTH by radioimmunoassay after extracting and eluting the ACTH from
porous glass beads. As can be seen (fig. 4.2) there is a good agreement
between the secretory episodes of ACTH with those of cortisol and DHA.
The plasma level of ACTH was within the expected normal range (10-100 pg/ml,
Berson and Yalow, 1968; Landon and Greenwood, 1968), although there were
fewer secretory episodes than expected for the female subject (Krieger
and Allan, 1975). With the timing of the samples it is possible that some
secretory episodes of ACTH were missed due to the short half life of ACTH
(8 minutes, Gallagher et al, 1973).

4.4 Normal ranges for salivary DHA and DHAS

Measurement of the salivary steroids was performed as described in section 2.
The normal levels found for DHA and DHAS in males and females in samples
taken at various times of the day and at different stages of the menstrual
cycle, are shown in table 4.1. None of the subjects were taking any
medication or the contraceptive pill.

There was no significant difference between the concentration of DHAS in
males and females $(0.5 < p < 0.1)$, although the number of subjects is small.
This contrasts with the higher level of DHAS found in the plasma of men
compared to women. Whether this reflects the relative amounts of bound
DHAS in plasma is not known. Results for salivary DHA agree with the reported
values but the DHAS values are higher than reported values (Gaskell et al,
1980; Finlay et al, 1982).
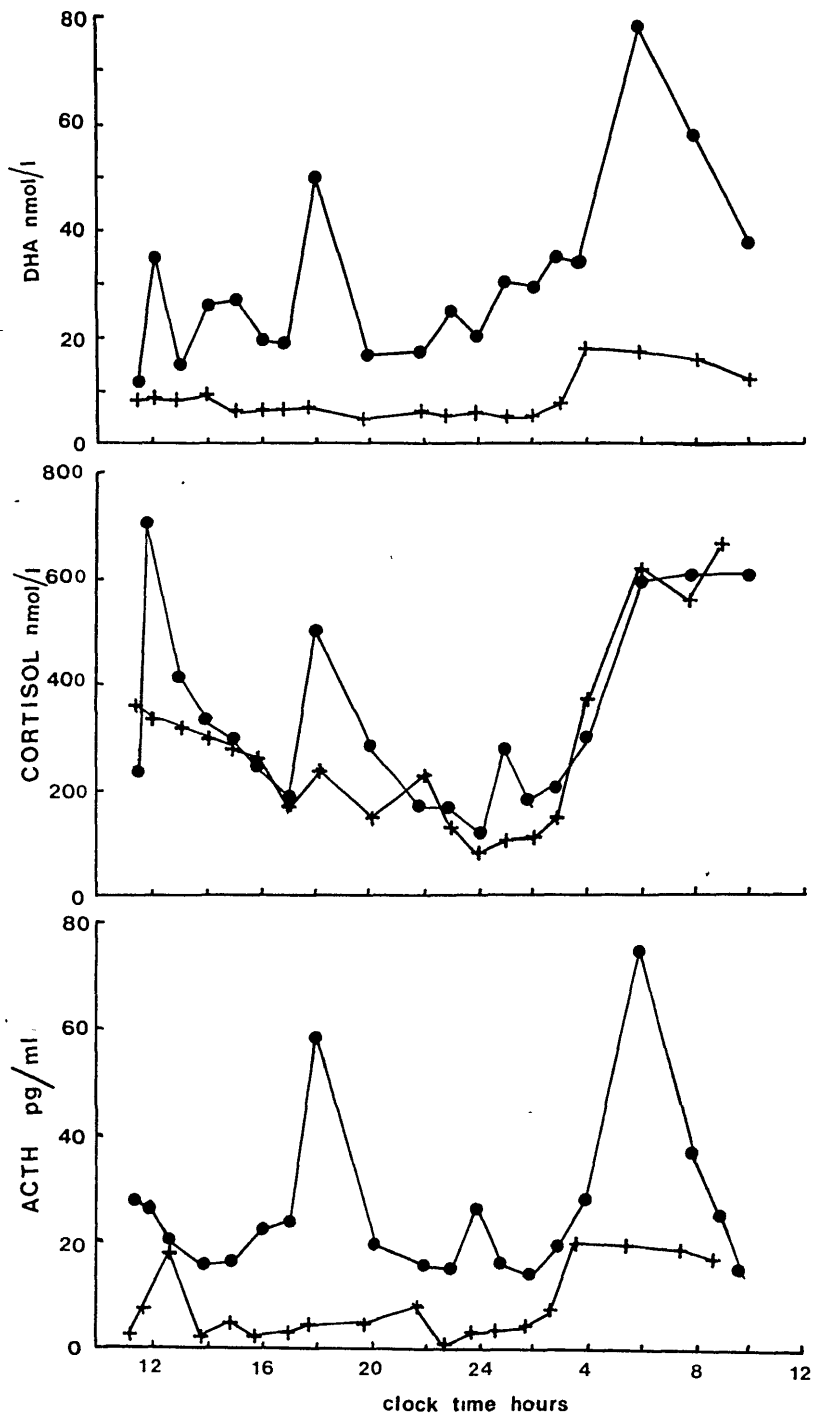
Fig. 4.2. Relationship between the secretory episodes of DHA and cortisol
to those of ACTH in a normal male (●——●) and premenopausal
female (+———+) studied over 23 hrs.

Table 4.1

Normal salivary DHA and DHAS concentrations

Males

Subject	DHA nmol/1	DHAS nmol/1
PB *	1.8	26.2
DJ *	0.5	16.1
JH	0.88	–
B1		9.9
B2		59.4
B3		16.3
B4		15.4
B5		18.1
B6		20.8
B7		8.8
B8		10.5
B9		31.1
B10		10.8
B11		12.2
Mean	1.06	19.7

Females

Subject	Age	Cycle stage	DHAS nmol/1
AA	–	–	10.6
AB	–	–	9.9
AC	–	–	10.9
AD	27	–	11.0
AE	25	Early	29.2
LH	27	Early	11.2
AF	15	Mid	9.6
AG	24	Mid	12.1
AH	26	Mid	11.7
PB	27	Mid	9.0
AI	43	Mid	10.3
AJ	37	Late	11.2
Mean			12.4

* = mean of diurnal data

4.5 Diurnal profiles of salivary and plasma DHA, DHAS and cortisol

Two normal male volunteers were studied through a twenty four hour period with samples collected every hour. Blood samples were taken via an indwelling cannula in the antecubital vein and saliva samples collected over 10 minutes, 5 minutes pre and post the blood sampling. A good agreement exists between the secretory episodes of plasma DHA and cortisol and for plasma cortisol with salivary cortisol (fig. 4.3). The agreement between plasma and salivary DHA secretory episodes is not so good, notably the increase in salivary DHA prior to an increase in plasma DHA at 01.00 and the peak at 20.00 hours.

The diurnal rhythm shown by plasma DHAS is similar to that seen in other subjects except the fall in the plasma steroid level occurs later at 01.00 hours to a nadir at 04.00 hours. The plasma and salivary levels of DHAS appear to be inversely related. The salivary DHAS diurnal profile shows no relationship to that of salivary cortisol and hence by inference with ACTH, but does agree with the salivary DHA profile.

4.6 Divergence of adrenal androgen secretion from cortisol

a) Effect of age

Single blood samples were taken from females between 10.00-12.00 or 14.00-16.00 hours and plasma DHA, DHAS and cortisol levels measured. As shown in fig. 4.4 there is a decrease with age in the plasma concentration of DHA and DHAS in females which is not shown by cortisol. This has been documented by other workers (Vermeulen et al, 1980) and has been interpreted to indicate a specific alteration in the adrenal secretion of the adrenal androgens, either by a decrease in an adrenal androgen stimulating hormone or through a decrease in the biosynthetic capability of the adrenal zona reticularis.
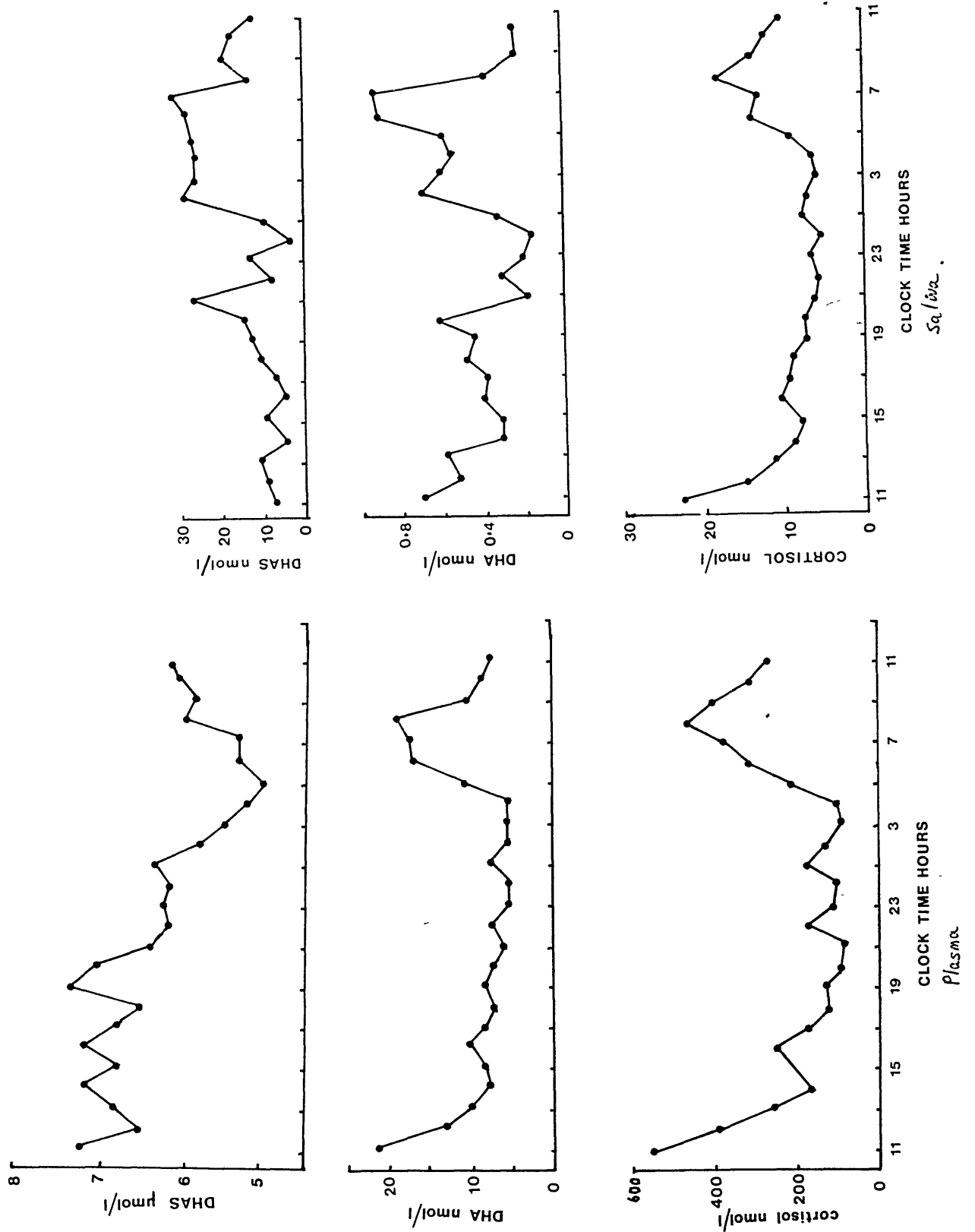
Fig. 4.3. Comparison of the diurnal profiles of DHA, DHAS and cortisol measured in the plasma and saliva of a normal male (28 yrs.) over 24 hrs.
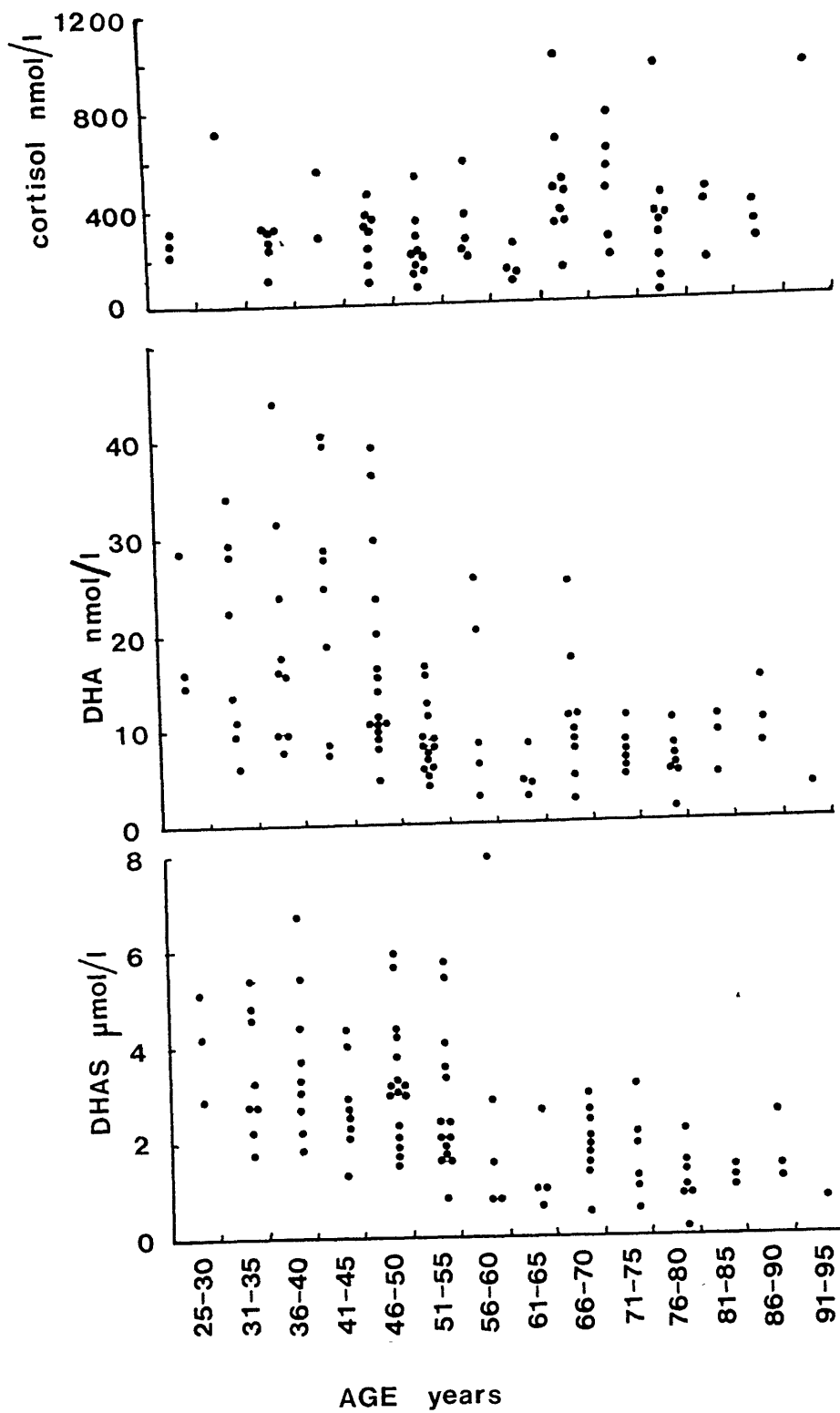
Fig.4.4 Effect of age on plasma DHA, DHAS and cortisol levels in women.

b) <u>Patient Car</u>

This patient was a 19 year old male who presented with hypogonadotrophic hypogonadism. He was an asthmatic and used Ventolin. Midnight and 8·a.m. cortisol levels were normal (52 and 408 nmol/l), with normal thyroid function and growth hormone levels. His testosterone level was <5 nmol/l with a plasma FSH level of 1.0U/L (normal range 2-8) and plasma LH level of 3.2 U/L (normal range 6-12 U/L). He has no body hair and was diagnosed as also having a 5 alpha reductase deficiency.

When studied over a period of 12 hours by regular blood sampling (every 30 minutes) his plasma cortisol and pregnenolone levels were found to be normal. DHA and DHAS levels were very low (approximately 10% of expected values), although there was still a good agreement between the episodic secretion of DHA and cortisol (fig. 4.5).

c) <u>Patient M.T.</u>

This patient was a 43 year old lady who presented with amenorrhoea. Her pituitary scan was normal but she had a raised plasma prolactin level (35 ng/l, normal range 0-12 ng/l). Upon further investigation it was discovered that she had minimal plasma cortisol and elevated DHA and DHAS levels. An initial study was performed with blood samples taken every 30 minutes for 24 hours. Plasma DHA and DHAS levels were measured and were high normal with a normal diurnal rhythm. The cortisol profile was abnormally flat with most values around the detection limit for the cortisol assay (27 nmol/l).
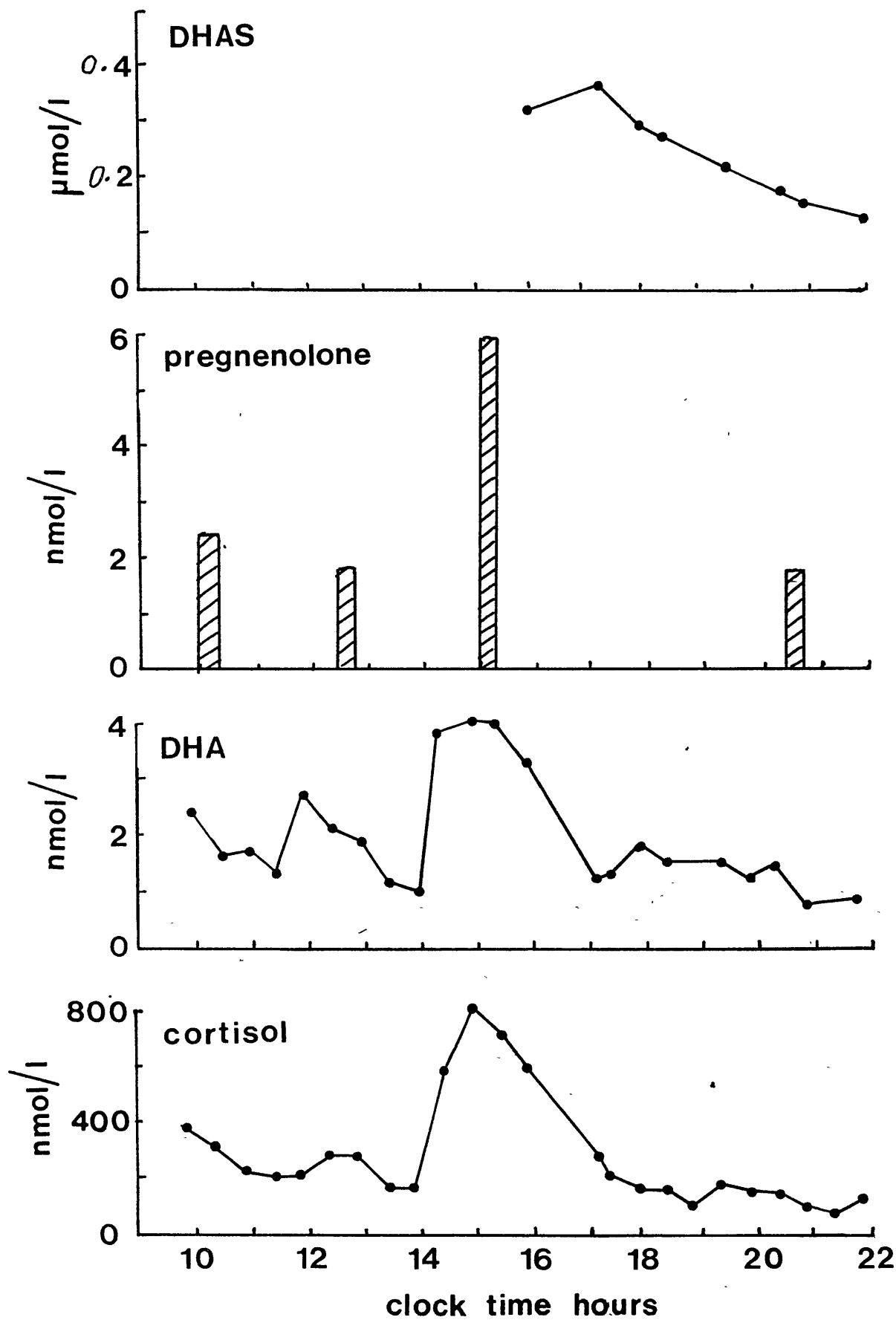
Fig. 4.5. The plasma levels of DHA, DHAS, cortisol and pregnenolone measured in a 19 yr. old male patient with hypogonadotrophic hypogonadism.

Measurement of plasma prolactin levels over the 24 hour study period

suggested a correlation between the secretory episodes of prolactin and

those of DHA (fig. 4.6). There was no agreement between the secretory

episodes of plasma DHA or DHAS with those of LH or FSH.

The plasma cortisol level in response to 250 µg of ACTH was attenuated

while DHA responded normally. In response to a single oral dose of

dexamethasone (2 mg at midnight), followed by an intravenous dose of

hydrocortisone at 08.00 hours, the plasma levels of DHA and androstenedione

fell, 47,5 and 75% respectively, the decrease in DHA being less than

expected (70% Nishida et al, 1979). The decrease in the plasma DHAS

level in response to the dexamethasone was very slow.

The half life for the injected cortisol was about 65 minutes which is in

agreement with the results of Hellman et al, (1970). This suggests that

the metabolic clearance rate of cortisol was normal, indicating a problem

with the production of cortisol. In response to an insulin stress test the

ACTH level responded poorly, pre test 17 ng/l peaking at 60 minutes to

46 ng/l (normal peak ACTH level at 60 minutes is about 200 ng/l).

This subject appears to have either a partial pituitary ACTH deficiency or

an increased sensitivity in response to cortisol by the target organs and

the pituitary. Two possible explanations for the secretion of DHA and

androstenedione with little cortisol are: i) the zona reticularis is more

sensitive to ACTH than the zona fasciculata, or ii) that the adrenal

androgens are stimulated by another hormone.
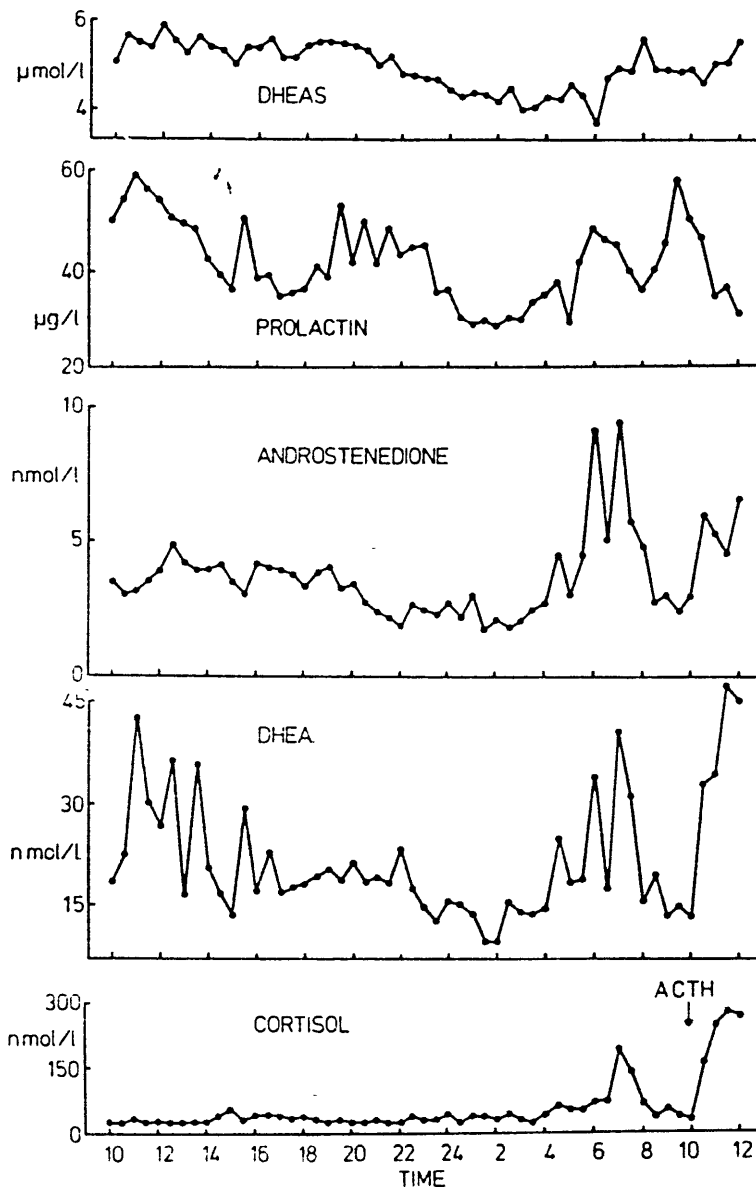
Fig. 4.6.   Plasma levels of hormones measured through 24 hrs. in a
43 yr. old women with hyperprolactinaemia.

Interest was raised in the second explanation with the observations of Vermeulen et al, (1977) that increased DHAS levels were seen in subjects with raised plasma prolactin levels. Our patient had a raised plasma prolactin level and some correlation between the secretory episodes of prolactin and DHA. To test the hypothesis that prolactin is the putative adrenal androgen stimulating hormone the following study was performed.

4.7 Investigation of patients with hyperprolactinaemia

Sixteen women with untreated hyperprolactinaemic amenorrhoea were studied, twelve over a 24 hour period with blood samples taken either ½ or hourly, and four with a single morning sample. Four women were given a single 2.5 mg tablet of bromocriptine midway through the 24 hour study. Hormone measurements were made on all samples from six patients and on four samples taken at 10.30, 15.30, 22.00 and 04.30 hours from the other women. In patients given bromocriptine the mean steroid level was calculated on the period prior to medication. Prolactin was measured by S. Franks from the Dept. of Obstetrics and Gynaecology, St. Mary's Hospital, according to the method of Franks et al, (1975), and the results expressed in terms of the VLS 1 standard prolactin preparation.

The mean plasma prolactin level for patients with hyperprolactinaemia was 66.8 µg/l with a range of 17-296 µg/l. The normal range is 7.2 $\pm$ 3.2 µg/l. The mean 24 hour plasma concentrations of DHA and DHAS were mean 20.6 nmol/l (range 6.1-34 nmol/l) and mean 7.4 µmol/l (range 1.2-15 µmol/l) respectively. The mean 24 hour concentration of DHAS was raised above the normal range in four patients, while all DHA levels were within the normal range.

The mean levels of cortisol, androstenedione, testosterone and oestradiol were measured over 24 hours in some of the patients and were in the normal ranges (table 4.2). Gonadotrophins were measured in six patients. In five the LH levels were below normal while FSH was elevated in two, depressed in one and normal in the remainder. No temporal relationship was found between the secretory episodes of either FSH or LH with DHA or DHAS.

A good linear correlation was found between the log of the mean plasma prolactin concentration, as determined by regular sampling over 24 hours, to the mean plasma levels of DHA ($r = 0.58$, $0.01 < p < 0.025$) and DHAS ($r = 0.57$, $0.025 < p < 0.05$).

A very good temporal relationship was demonstrated between the secretory episodes of cortisol and DHA. No such relationship was demonstrated between cortisol and DHAS nor between prolactin and cortisol, DHA or DHAS.

The diurnal profiles of cortisol, DHA and DHAS show the expected pattern while prolactin has lost the night time peak. One patient with a proven pituitary tumour and a prolactin level of 124 µg/l, the only complete profile with a prolactin level greater than 100 µg/l, had an abnormal diurnal rhythm of DHAS with no nadir at 02.00-04.00 hrs. DHA secretory episodes remained in good agreement with secretory episodes of cortisol through the profile but with a greatly elevated baseline level.

Table 4.2 Hormone levels in patients with hyperprolactinaemia

Hormone

Subject	Prolactin µg/l	DHA nmol/l	DHAS umol/l	Cortisol nmol/l	LH µ/l	FSH u/l	Testo nmol/l	E2 pmol/l	A'dione nmol/l
Cr	17.8	16.8	4.2	137	—	—	—	235	—
Ca	25.0	6.1	1.8	221	—	—	1.74	128	—
O'Sh	26.0	20.5	11.8	160	—	—	—	250	—
D'An	124.0	34.6	15.0	342	5.4	1.7	1.8	280	—
Do	23.7	14.2	5.3	282	—	—	—	—	—
La	50.0	18.7	5.5	200	5.5	2.5	—	—	6.3
Hi	20.0	8.3	1.2	209	—	—	—	224	—
MT.1976	32.0	16.7	6.1	71	—	—	1.5	—	—
MT.1979	40.0	19.7	5.4	40	2.3	3.9	—	—	—
Wa	116.0	13.7	4.0	—	9.7	4.2	—	—	—
Fu	132.0	13.3	5.1	—	1.2	0.3	—	—	—
Ch	296.0	28.2	11.9	—	3.7	2.5	—	—	—
†Fo (pre)	47.0	13.9	5.9	169	—	—	1.6	—	4.2
†Fo (post)	22.0	18.1	5.8	—	—	—	3.6	—	—
Ax *	25.0	40.3	14.3	—	—	—	—	—	—
Sm *	42.0	22.6	8.5	—	—	—	—	—	—
Smi *	20.0	40.3	9.3	—	—	—	—	—	—
Cr *	63.0	20.8	8.7	—	—	—	—	—	—
Normal range	7.2 ± 3.5	3.6-43.9	1.3-6.7	40-690	6.6-13.4	1.3-3.7	0.5-3.0	146-440	1.4-7.0

* Single sample results

† Results pre and post 1 month bromocriptine treatment

Acute treatment with a single 2.5 mg dose of bromocriptine resulted in a rapid decrease in the plasma prolactin concentration in all subjects, but had no effect on the plasma levels of either DHA or DHAS or on the secretory episodes of DHA and DHAS when compared to non-treated subjects. Chronic treatment with bromocriptine for one month decreased the mean plasma concentrations of prolactin (47 μg/l to 22 μg/l) and DHAS (7.5 μmol/l to 5.8 μmol/l) while testosterone and DHA both rose (1.6 nmol/l to 3.6 nmol/l, and 13.0 nmol/l to 18.0 nmol/l respectively). The post-treatment level was based on a single sample and may not be a good estimate of the 24 hour mean value. The comparison above is made with pre-treatment samples taken at a similar time of day.

Fig. 4.7 shows the relationship between the plasma levels of DHA and DHAS in female subjects with hyperprolactinaemia and normal premenopausal women. The different gradients for the lines of best fit suggest that there is an alteration in the blood production rate of either DHA or DHAS rather than an alteration in the metabolic clearance rate, as Vermeulen and Ando, (1978) have shown this is unchanged in subjects with hyperprolactinaemia. Both correlations were significant at the $p < 0.005$ level. The superior r value found for the group of prolactin subjects may reflect the use of mean 24 hour plasma steroid levels, which removes the effects of DHA episodic secretion.
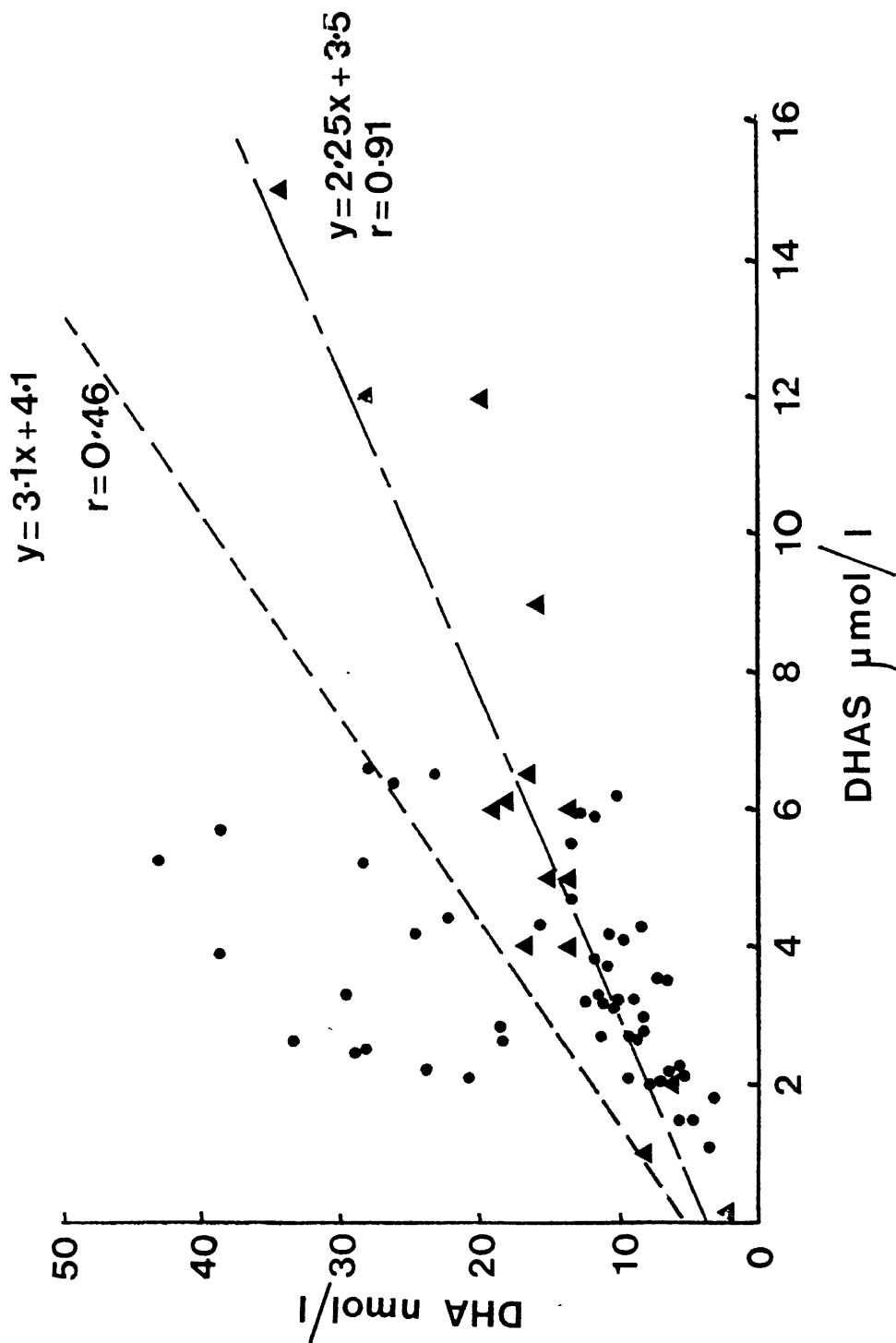
Fig. 4.7. Correlation between the plasma levels of DHA and DHAS in normal female subjects (●) and patients with hyperprolactinaemia (▲).

4.8 Investigations on subjects with Addisons disease

To investigate the production of DHA and DHAS by the ovary, four patients with Addisons disease were studied over 4-48 hours with regular blood sampling. One subject (MP) was studied on three occasions over a period of three years. Plasma DHA and DHAS levels were measured in the samples and because low levels of the hormones were expected, larger volumes of plasma were assayed. This resulted in the assayed mass of steroid falling on a reliable portion of the standard curve. All the assays have been tested for linearity over the range of volumes used in this section.

The mean plasma levels of the hormones over the periods measured are shown in table 4.3. As expected the DHA and DHAS levels are low, 2-10% of normal, however the plasma pregnenolone level was normal in the one subject tested. The plasma DHA and pregnenolone levels appear to alter synchronously (fig. 4.8). The secretory episodes of DHA and pregnenolone do not correlate with the secretory episodes of FSH or LH. DHAS exhibits no secretory episodes, however the fall in the plasma level between 22.00 hours and 04.00 hours demonstrated by normal individuals is still seen in these subjects.

4.9 Comparison of plasma progesterone and DHA levels

In patient Pau, who was studied on three separate occasions, plasma progesterone and DHA levels were measured over each complete 24 hour study. The mean 24 hour plasma progesterone levels were 6.2, 29.0 and 16 nmol/l with mean DHA levels of 2.2, 1.5 and 2.2 nmol/l respectively. There would seem to be an inverse relationship between the plasma levels of DHA and progesterone in this subject.

Table 4.3

Subject	Age	DHA nmol/l	DHAS umol/l	Pregnenolone nmol/l
Pay.	56	0.38	0.16	-
Fid.	Pre dex	1.0	0.06	-
Fid.	Post dex	0.9	0.07	-
Sav.	52	1.0	0.08	-
Pau. yr 0	43	2.2	-	-
Pau. yr 1	44	2.2	0.06	-
Pau. yr 2	45	1.5	0.06	1.9

Mean plasma steroid levels in patients with Addisons disease.
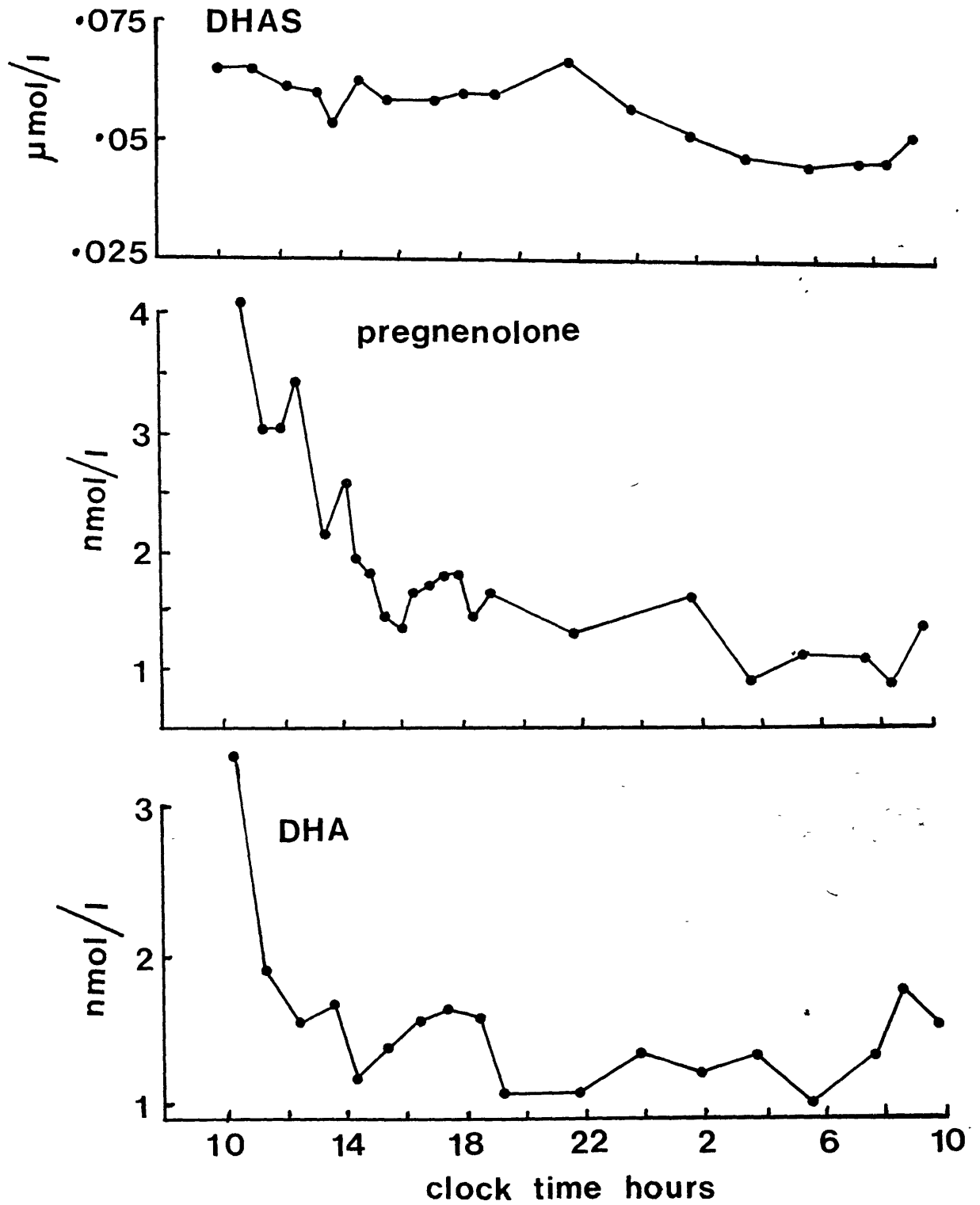
Fig. 4.8. Plasma levels of DHA, DHAS and pregnenolone through 24 hrs. in a female patient (45 yr.) with Addisons disease.

4.10 Discussion

The results described here demonstrate the excellent temporal correlation between the changing plasma levels of DHA and pregnenolone with cortisol and of DHA and cortisol with ACTH, supporting the hypothesis of an adrenal origin for these steroids which is stimulated by ACTH. We have been unable to demonstrate an acute effect of ACTH, as reflected by correlation with secretory episodes of cortisol, on the plasma levels of DHAS.

The evidence obtained from the measurement of DHAS in saliva supports the plasma evidence that there is no acute effect of ACTH on DHAS secretion. The reason(s) for the disagreement between the plasma and saliva levels of both DHA and DHAS while those of cortisol appear to agree are not known. The disagreement may be caused by secretion or metabolism of DHA and DHAS by the cells of the mouth, by bacterial growth or by a concentration effect caused by decreased nocturnal saliva flow. The latter reason would appear to be correct as the salivary levels of DHA and testosterone (Baxendale et al, 1982) also increase nocturnally, although cortisol does not show the same effect. It would appear that salivary measurements of DHA and DHAS can not be used as a reliable indicator of the plasma steroid levels as has been demonstrated with other hormones (Baxendale et al, 1982).

The three examples quoted of a divergence between plasma, cortisol and DHA levels suggest another mechanism for the control of DHA and DHAS secretion other than ACTH. The possibility that prolactin in the postulated A.A.S.H. was investigated and the results reported here clearly confirm the association of raised plasma DHA and DHAS concentrations with severe

hyperprolactinaemia. Our results and those of Facchinetti (1980), show a
dose-response relationship between the log prolactin concentration and the
plasma DHA and DHAS levels. We found no increase in the plasma levels of
testosterone, androstenedione, oestradiol or cortisol in patients with
hyperprolactinaemia, while the levels of FSH or LH ranged from below to
above normal. These findings are in agreement with those of Parker
et al, (1978), Bassi et al, (1977) and Aono et al, (1976).

As it has been shown that the increased plasma DHAS levels in subjects
with hyperprolactinaemia are due to an increased blood production rate
rather than a decreased metabolic clearance rate (Vermeulen et al, 1978;
Belisle et al, 1980), and that more than 90% of DHAS and 80% DHA is
produced by the adrenal (Nieschlag et al, 1973; Abraham and Chakmakjiam,
1973; Abraham, 1974), most authors have concluded that the adrenal is
the site of prolactin action by partial inhibition of the 3β-ol-dehydrogenase-
isomerase enzyme with ACTH required to maintain steroid biosynthesis. This
concept has been supported by the finding of prolactin receptors in rat
adrenal tissue (Costlow and McGuire, 1977).

That the elevated androgen levels are not caused by ACTH was suggested by
Caufriez et al, (1977) and Thorner et al, (1974), who demonstrated that
plasma levels of ACTH are low to normal in patients with hyperprolactinaemia.
The data from the ACTH stimulation tests of Vermeulen and Ando (1978),
Carter et al, (1977), and Facchinetti et al, (1980), and the data on
patient MT, who despite minimal ACTH and cortisol levels maintained
elevated and episodic androgen secretion, also support this hypothesis.

The data shown in fig. 4.7, illustrate that the ratio of DHAS to DHA concentration in the patients with hyperprolactinaemia is different from that of the control subjects, and this could be due to either a relative increase in production of DHAS, or an altered conversion of DHA to DHAS. To our knowledge the latter has not been investigated in relation to hyperprolactinaemia.

The results from the study of patients with hyperprolactinaemia and from the study of patient A.T, raise the alternative possibility that the source of the increased production of DHA and DHAS may be ovarian. The work of McNatty et al, (1974) and El Ayat (1978) support this with the suggestion that prolactin directs ovarian biosynthesis either through the delta four or delta five pathway depending on the stage of the menstrual cycle.

The observation of an inverse relationship between the plasma levels of DHA and progesterone in the patient with Addisons disease may lend in vivo support to the in vitro findings of McNatty et al, (1974). The mechanism for interchanging biosynthesis from the delta four to the delta five pathway may be mediated via regulation of the 3β-ol-dehydrogenase isomerase enzyme as Sano et al, (1981) have demonstrated low activity of this enzyme during the follicular phase and increased activity during the luteal phase of the menstrual cycle.

This work supports the hypothesis that the elevated DHAS level found in patients with hyperprolactinaemia may be due to the increased production of

DHA by the ovary under the influence of prolactin followed by conversion of the DHA to DHAS. The finding that in most of the patients with hyperprolactinaemia the DHA levels were within normal limits while DHAS levels were elevated, suggests that the sulphokinase enzyme acts to maintain plasma DHA levels within normal limits by conversion to DHAS.

The adrenal secretion of DHAS meets the following criteria of:
  i) there is a concentration gradient between the adrenal venous effluent and peripheral levels (Nieschlag et al, 1973),
  ii) dexamethasone suppression and ACTH stimulation tests (Vaitukaitis et al, 1969; Nieschlag et al, 1973).

With ACTH stimulation and dexamethasone suppression tests there is an initial lag period after administration of the test material and the response by plasma DHAS. This contrasts with the rapid response seen with plasma cortisol and DHA levels. This difference has usually been ascribed to the large endogenous plasma DHAS levels swamping any initial rapid response.

Two pieces of information suggest an alternative hypothesis to the direct secretion of DHAS by the adrenal in response to ACTH:
  i) the report by Rosenfeld et al, 1972, that within 30 minutes of an injection of ^{3}H-DHA 90% of the radioactivity in plasma was recovered as the conjugated steroids DHAS (55%) androsterone sulphate and etiocholanolone sulphate,

ii) with studies involving the continuous infusion of unlabelled DHA

into hirsute women with polycystic ovarian syndrome with and

without a prior seven day treatment with dexamethasone there was

a rapid conversion of the infused DHA to DHAS, fig. 4.9.

The mass of DHA infused was greater than the usual daily production rate

(38 mg/24 hours vs. 6-8 mg/24 hours). As can be seen in the patients taking

dexamethasone there is a rapid increase in plasma DHA levels to reach a

plateau, while the DHAS levels rise slowly but continuously. The pattern

in the non-suppressed patient is different showing a much slower increase

in plasma DHA levels to reach a plateau and a significant lag period

before plasma DHAS levels increase (1.5-2 hours). This pattern of a lag

period followed by a steady increase in plasma DHAS levels is the same as

seen in response to ACTH stimulus, Nieschlag et al, 1973; Carter et al,

1977; Cutler et al, 1979. It is also noticeable that during the 24 hour

profile studies the plasma levels of DHAS roughly follow those of DHA

but with a delay of 1-3 hours, and with episodic peaks of DHA merged

into one DHAS peak.

It is suggested that alterations in plasma DHAS levels are a reflection of

an altered plasma DHA level and the action of the sulphokinase enzyme.

Steroid sulphokinase enzyme activity has been demonstrated in a number of

tissues, liver, jejunal mucosa and adrenal, Bostrom and Wengle, 1967;

Cameron et al, 1969. The activity of the adrenal sulphokinase results

in the gradient seen between peripheral and adrenal venous DHAS levels,

by conversion of adrenal DHA secreted in response to ACTH. The conversion

of DHA to DHAS is ACTH independent as shown by the DHA infusions to

dexamethasone suppressed subjects.
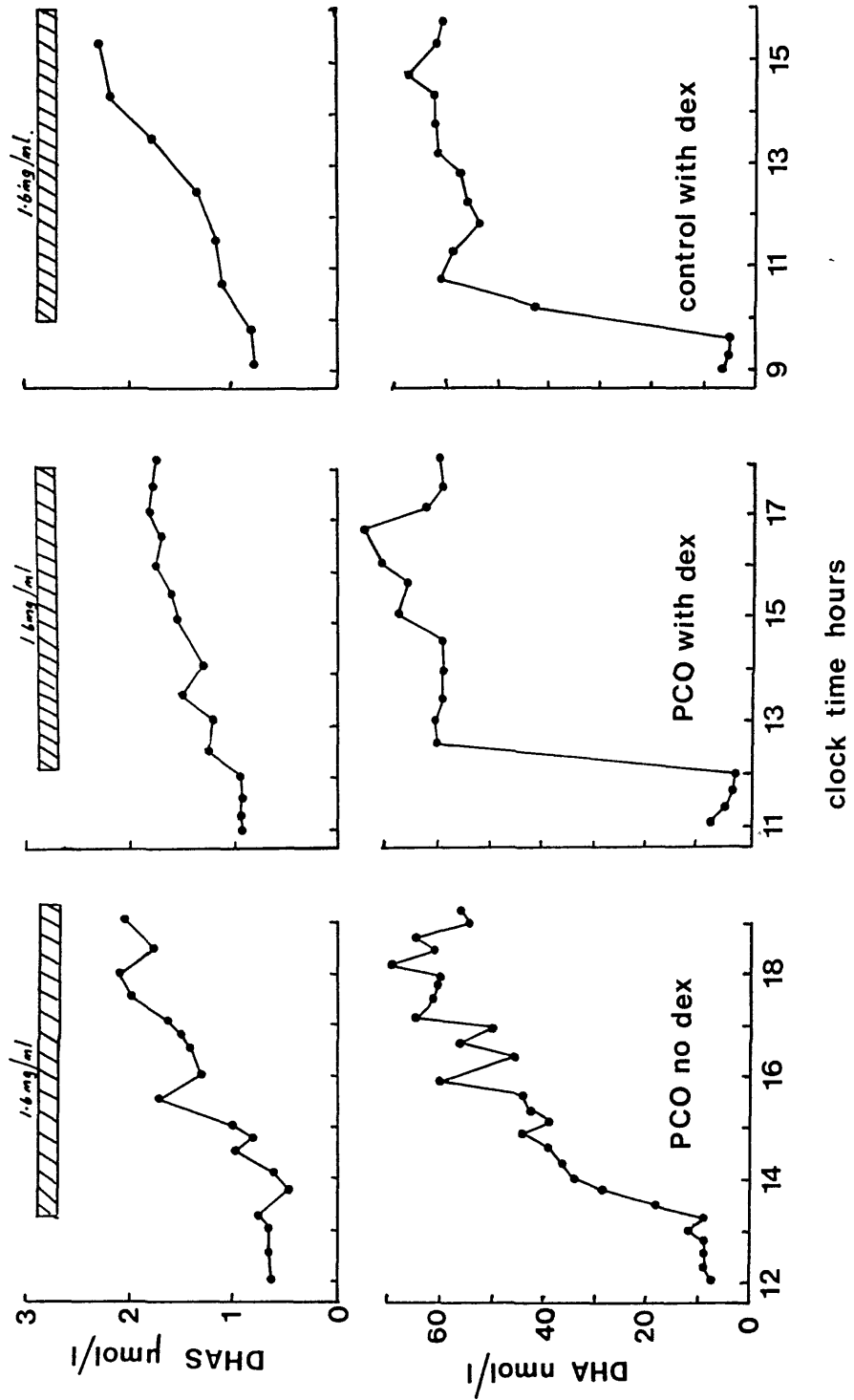
Fig. 4.9. Effect of the infusion of DHA on the plasma levels of
DHA and DHAS, in women with polycystic ovaries.

The measurement of plasma DHAS may therefore not be a good indication of adrenal androgen secretion. The decrease seen in plasma DHA and DHAS in hirsute and acne subjects on treatment with the combined oral contraceptive may be a result of the inhibition of LH mediated ovarian secretion of DHA due to the ethinyl oestradial rather than an effect of the treatment on the adrenal.

The inverse relationship seen between plasma DHAS and progesterone levels during the menstrual cycle may result from the inability of ovarian steroidogenesis during an inadequate luteal phase to divert from the delta five to the delta four pathway. The increased DHA production resulting in an elevated DHAS level by the action of adrenal and peripheral sulphokinase activity.

In conclusion, we have confirmed the relationship between the secretory episodes of DHA with those of ACTH and cortisol but have been unable to detect any relationship of ACTH to the secretory episodes of DHAS. Our work has suggested the existence of an adrenal androgen stimulating hormone, but that prolactin is not this hormone. Prolactin may have an effect on the human ovary but this has not been proven. We have also suggested that DHAS may not be directly secreted by the adrenal but that its plasma level reflects the blood production rate of DHA.

5. INTRODUCTION

Many non-endocrine tissues are capable of metabolising steroids in vitro, skin, adipose tissue, pulmonary endothelial cells and muscle tissue (Thomas and Oake, 1975, Siiteri and MacDonald, 1973; Milewich et al, 1983; Longcope et al, 1978). Within the tissue inactive precursor steroid can be converted locally to an active product (Thomas and Oake, 1975, DHA to DHT in skin). Although the tissues have been shown to be able to metabolise steroid, the quantitative importance of this metabolism is not known as no data is available on the endogenous concentration of precursor steroid within the tissue.

One cell which may be of importance for steroid metabolism is the red blood cell. These cells possess 17βhydroxy-steroid dehydrogenase activity (Jacobsohn and Hochberg, 1968; Mulder et al, 1972; Jacobsohn et al, 1975), and are capable of converting androstenedione to testosterone and DHA to androstenediol (Van der Molen and Groen, 1968; Mulder et al, 1972). Red blood cells of dogs and humans have been shown to contain appreciable amounts of pregnenolone and other steroids (Holzbauer, 1972).

If the red blood cell concentration of DHA and androstenedione is significant the large volume of red blood cells within the body could contribute to the peripheral production of androstenediol and testosterone. Indeed Fylling (1965) has reported a significant difference in the conversion of androstenedione to testosterone, in the red blood cells of hirsute women compared to normal women.

The aim of this work was to investigate the concentration of steroid within the human red blood cell and evaluate the possible role of the red blood cell in the peripheral conversion of steroid. If red blood cells contain a significant amount of steroid then heamolysis of blood samples could cause severe problems for the measurement of the plasma levels of each steroid.

5.2 Measurement of steroid in red blood cells

Fresh lithium heparin blood samples (10 ml) were taken and the haematocrit measured. The blood was centrifuged (1000g) for 5 minutes and the plasma decanted and kept. The cells were resuspended in normal saline to a volume of 10 ml. After mixing the cells on a rotary mixer for 10 minutes the cells were brought down by centrifugation (1000g) and the saline decanted and collected. This procedure was repeated three times and the washes kept. After the final wash the cells were lysed by resuspending in distilled water to a final volume of 10 ml.

Aliquots of plasma (500 μl), each wash (500 μl) and lysed cells (250 μl) were taken and 50 μl of ^{3}H-DHA and ^{3}H-pregnenolone added (10,000 dpm of each) as internal recovery. After equilibration for 30 minutes at 24°C the steroid was extracted with 10 ml of diethyl ether, the organic phase decanted after freezing the aqueous layer and evaporated to dryness under N_2. The residue was spotted onto silica gel TLC plates with 2 x 100 μl of diethyl ether and the plate developed in dichloromethane:ethyl acetate (93:7 ^{V}/v) twice.

Areas of radioactivity were located under a Panax beta·scanner and excised.
The steroid was eluted from the plate with 5 ml of diethyl ether for 2 hours
prior to decanting the ether and blowing to dryness under nitrogen. To each
sample residue add 1 ml of ethanol. Aliquots (3 x 200 µl) were taken one
for recovery estimation and two for assay. The assay methods were then as
described in section 2.

Fig. 5.1 illustrates the disposition of steroid between the plasma and cell
fractions in 10 ml of whole blood. In each case the final wash contained
no steroid therefore there was no significant amount of plasma left around
the cells. It is not known whether any steroid is leached from the cells during
the wash procedure, from the above observation this would seem unlikely though.

As can be seen, the mass of DHA in the cell fraction is fairly constant between
subjects but is more variable for pregnenolone. The cell concentration of
steroid does not follow the diurnal variation seen with the plasma steroid
level.

The majority of pregnenolone in blood (approximately 90%) and less than 40%
of DHA is associated with the RBC. To determine whether the steroid was
attached to the membrane or free within the cell, five blood samples were
taken and processed as above. The haemolysed blood was then divided into
2 aliquots and one aliquot centrifuged at 1000g for 30 minutes. Aliquots
of the supernatant and unspun haemolysate were taken and assayed. There was
no consistent difference found between the two samples for either DHA or
pregnenolone.
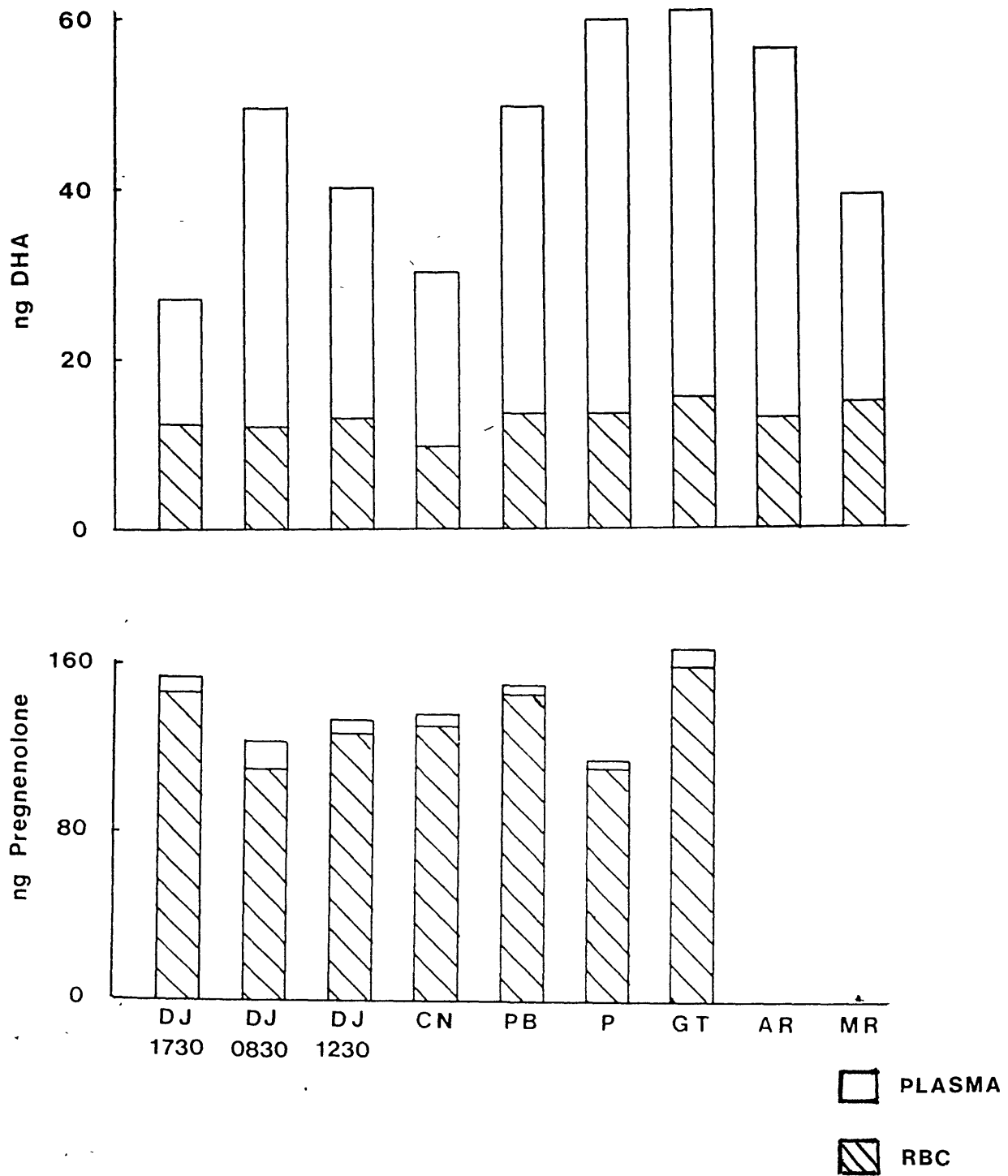
Fig.5.1 Disposition of steroid between plasma and red blood cell fractions of whole blood.

5.3 Metabolism of steroids by red blood cells

Due to the high intracellular concentration of steroid it was of interest to investigate the degree of intracellular metabolism. Tritiated steroid (800,000 dpm) in 50 μl of ethanol was added in quadruplicate to screw top 10 ml glass vials and blown to dryness under nitrogen. Two total count tubes were also taken. To one pair of vials 2 ml of freshly taken whole blood (Lithium Heparin) was added and 2 ml of sterile normal saline to the second pair. The vials were incubated with constant shaking in a water bath at $37^{\circ}C$ for 2 hours. After incubation 2 ml of distilled water was added to each tube and each incubate transferred to a separate 20 ml stoppered glass tube. The steroid was extracted with 3 x 10 ml of diethyl ether and each sample aliquots pooled and evaporated to dryness under nitrogen.

The residue was applied to TLC plates with 2 x 100 μl of diethyl ether and the plates developed in chloroform:acetone (85:15 $^{v}/v$). After development each lane was divided into 0.5 cm sections and each section eluted with 10 ml of toluene based scintillant prior to counting the eluted radioactivity.

The result for each section was expressed as a percentage of total counts incubated. Comparison of the test chromatograms with the saline controls indicated the presence of a metabolic product. The possible identity of the product steroids was obtained from reference steroids run in the same chromatography system, table 5.1.

Table 5.1

Steroid incubated	Possible product	% conversion	mean % conversion
Pregnenolone	Dihydropregnenolone	2.5 2.1	2.3
Androstenedione	Testosterone	5.9 6.2 5.9	6.0
DHA	Androstenediol	2.5 3.0 3.2	2.9
Testosterone	–		
Progesterone	–		
Androstenediol	–		

% conversion of steroids by incubation with RBC at $37^{o}C$ for 2 hours.

The results indicate that only the 17-hydroxy steroid dehydrogenase and possibly the 20-hyroxy steroid dehydrogenase enzymes exist in RBC. There was no evidence for the existence of aromatase, 3βol-dehydrogenase isomerase or 5 α reductase enzymes in RBC.

Incubation of 2 ml of red blood cells (resuspended in saline after 2 saline washes) with ^{3}H-DHA resulted in an increased conversion to androstenediol (10.4%). This presumably reflects the removal of the plasma binding proteins which would prevent entry of steroid into the RBC for metabolism.

During the course of the incubation experiments an area of radioactivity was detected that ran with the solvent front on TLC from the extractions of the DHA and pregnenolone incubations. No such radioactive area was seen with the control incubation.

This observation was of interest as a result of the report by Hochberg et al, (1977) of a lipoidal derivative of pregnenolone formed by incubation of pregnenolone with bovine adrenal corticol tissue that releases free pregnenolone after saponification. If human blood could form these lipoidal derivatives then this could be of major significance. It was therefore decided to further investigate these compounds.

5.4 Comparison of whole blood, washed cells and plasma for formation of non-polar steroid conjugates

To determine the site of the non-polar conjugate formation a fresh lithium heparin blood sample was taken and divided into four aliquots. Two aliquots were centrifuged and the plasma decanted. The cells were washed with 3 x 2 ml of sterile saline, the saline being decanted and discarded after each wash and centrifugation. The volumes of washed cells and decanted plasma were made to 3 ml with sterile normal saline. The whole blood, washed cells and plasma were incubated with either ^{3}H-pregnenolone or ^{3}H-DHA (8 x 10^5 dpm) at 37°C for 3 hours. 2 ml of distilled water was added to each sample and the steroids extracted with 3 x 10 ml of diethyl ether. The procedure was then as described in section 5.3. The production of non-polar conjugate by each fraction is shown in table 5.2. Results are expressed as a percentage of the total cpm added to the incubation.

The majority of the non-polar conjugate is formed by the plasma. The higher percentage formed by the whole blood compared to the sum of the plasma and washed cells may reflect uptake of the conjugate by the cell thereby removing a possible product inhibition effect, assuming an enzyme is responsible for the conjugate formation.

5.5 Comparison of steroids for formation of non-polar conjugates

To test the specificity of non-polar conjugate formation by plasma, 1 x 10^6 dpm of pure ^{3}H-steroid was incubated at 37°C with 3 ml of plasma. The results are shown in table 5.3. These figures represent mean values obtained from

Table 5.2 Conversion of DHA and pregnenolone to non-polar conjugates by whole blood, plasma and washed cells

	% conversion to non-polar conjugates		
	whole blood	plasma	washed cells
Pregnenolone	29.4	15.6	2.9
DHA	5.4	4.4	1.1

Table 5.3 Percentage conversion of steroids to non-polar conjugates

Steroid	% conversion to non-polar conjugate
Pregnenolone	20.5
DHA	12.4
Androstene 3β 17β diol	4.7
Oestradiol	0.2
Androstenedione	)
Testosterone	)
Oestrone	) Nil
Dihydrotestosterone	)
DHAS	)

five incubations using different extraction and incubation protocols. The pregnenolone conversion figure is underestimated due to a low recovery of labelled material (30%) from the incubation mixture. The extraction of pregnenolone from plasma using these solvents would be 80-90%, the 50% difference between the two figures may therefore represent unextracted non-polar conjugate. No polar conjugates have been detected for any of the incubations. The quantity of non-polar conjugate formed was dependent on the incubation time and was the same for both male and female plasma.

5.6 Measurement of non-polar conjugate in plasma

Plasma (1 or 2 ml) was added to stoppered glass tubes containing 10,000 cpm in 50 μl of chloroform of the tritiated non-polar conjugates of DHA and pregnenolone prepared by incubation of the steroid with plasma followed by extraction, TLC and elution as described above. The residue after evaporation was reconstituted in chloroform and in this form was stable for at least 8 weeks stored at 4°C.

The tube contents were vortex mixed and allowed to equilibrate for 30 minutes prior to extraction with 2 x 10 ml of ethyl acetate. Each extraction was for 10 minutes on a mechanical shaker, followed by centrifugation at 200 g for 10 minutes. The supernatants were decanted and pooled for each sample. A third extraction with 10 ml of diethyl ether was performed and the organic phase was decanted after freezing the aqueous phase and combined with the pooled aliquots.

The extracts were blown to dryness in a water bath at $45^\circ C$ under nitrogen.
The residue was applied to silica gel TLC plates with 100 µl of chloroform
and 100 µl diethyl ether. The plates were developed twice to improve
separation of the non-polar conjugates from free steroid in hexane: diethyl
ether:glacial acetic acid (80:20:1 v/v/v). This system has been reported to
separate the classes of lipids extracted from plasma, Smith and Stevens
(1969). The relative mobilities of the non-polar conjugates and the free
steroid are shown in table 5.4.

The tritiated non-polar conjugate added for recovery estimation enabled the
exact location of the conjugates on the TLC plate and these areas were
excised and eluted with 10 ml of diethyl ether for 16 hours.

After decanting, the ether was blown to dryness under nitrogen and the non-
polar conjugate saponified using the method of Smith and Stevens (1969).
To the residue left after evaporation of the ether extract 200 µl of distilled
water and 1 ml of 2M KOH in ethanol:water 90:10 $^V/v$ was added, the tube
contents vortex mixed and then incubated at $60^\circ C$ for 40 minutes. After
cooling the solutions 1 ml of distilled water was added to each sample, the
tube contents mixed and the liberated steroid extracted with 10 ml of
diethyl ether. The aqueous phase was frozen in an ethanol/dry ice bath
and the ether decanted, blown to dryness under nitrogen and the residue
applied to silica gel TLC plates with 2 x 100 µl diethyl ether. The
plates were developed with chloroform:acetone (85:15 $^V/v$). The area of
tritiated steroid was located and eluted with 5 ml of diethyl ether for
2-4 hours.

Table 5.4 Mobilities of steroid and non-polar conjugates on TLC run in
hexane:ether:gl.acetic acid (80:20:1)

Compound	rF
Pregnenolone	0.03
Non-polar pregnenolone	0.35
DHA	0.03
Non-polar DHA	0.25
Androstenediol	0.01
Non-polar androstenediol	0.14
Oestradiol	0.03
Non-polar oestradiol	0.18

After elution the ether was decanted and evaporated to dryness under
nitrogen. The residue containing the liberated steroid was then
reconstituted in ethanol, 1 ml for pregnenolone, 600 µl for DHA and
500 µl for E_2 and androstenediol. Aliquots of 100 µl were taken for
recovery estimation, evaporated to dryness and 1.7 ml scintillator added
prior to counting. Recoveries of labelled DHA, pregnenolone and
androstenediol non-polar conjugates taken through the above procedures
were 60.5%, 61.5% and 46.1% respectively. The ^{3}H-DHA and
^{3}H-pregnenolone extracted after this procedure had an identical Rf
value to authentic ^{3}H-steroid run on the same plate.

Duplicate aliquots of 200 µl were taken for each steroid estimation and
assayed by RIA. DHA and pregnenolone by the methods described in
section 2, entering the assay protocol at the evaporation of sample
and addition of antibody mixture stage. The androstenediol and
oestradiol assays were as described by Bonney et al, (1983) and
Braunsberg et al, (1981). With samples containing recovery counts
the assay standard curve was corrected by the addition of an
equivalent amount of labelled steroid as that found for the mean
recovery count.

For each steroid, specificity is conferred by the use of two TLC
purification stages prior to assay and the use of specific antisera
for each RIA.

5.7 Concentration of non-polar conjugates in plasma

The plasma concentrations of the non-polar conjugates of pregnenolone, DHA and androstenediol for normal subjects and patients with a range of endocrine disorders are shown in table 5.5. The comparison and significance levels between the groups of patients are also shown. The values for the conjugates of androstenediol in normal premenopausal subjects were less than the lowest standard as only 1 ml of plasma was assayed.

Plasma levels of the non-polar conjugates of oestradiol were not significantly different to the water blank values (10 pg/ml) for all samples. In one male subject blood samples were taken at 12.00 and 16.00 hours. Non-polar conjugates of pregnenolone and DHA measured in these samples demonstrated a possible diurnal rhythm. Plasma levels of pregnenolone and DHA conjugate were 168 ng% and 86 ng% at 12.00 hours, and 214 ng% and 40 ng% at 16.00 hours respectively.

The plasma levels of the non-polar conjugates reported for the postmenopausal and breast cancer subjects were obtained on plasma pools prepared from samples taken at 08.30, 11.00, 16.00 and 22.00 hours, thus minimising the effects of a diurnal rhythm. The remainder of the samples were taken singly at random times.

Plasma levels of the pregnenolone and DHA non-polar conjugates are significantly lower in postmenopausal compared to premenopausal women ($0.01 < p < 0.02$), while plasma pregnenolone and probably androstenediol conjugate levels are lower in premenopausal women than in men ($0.02 < p < 0.05$).

Table 5.5

Subjects	n NP-pregnenolone ng%	NP-DHA ng%	NP-Adiol ng%
Male (23-45 yrs)	9 210 ± 50.8	47.1 ± 29.2	10.8 ± 2.9
Females			
Premenopausal (20-30 yrs)	5 139.4 ± 47.3	47.2 ± 15.5	<6.4
Postmenopausal (53-79 yrs)	5 69.8 ± 25.5	29.5 ± 13.8	4.5 ± 0.8
Pregnant (12 weeks)	1 >1220.0	57.0	-
Premenopausal (Acne)	5 243.8 ± 92.0 **	81.8 ± 38.0 **	6.2 ± 1.2
Hirsutism (Idiopathic)	3 150.3 ± 16.5	157.3 ± 17.0 ***	7.7 ± 2.3
(PCO)	2 120/132	60/75	3.2/4.5
Eugynon 30	1 74.0	10.0	-
Postmenopausal			
Breast cancer	3 118.3 ± 33.0 **	39.7 ± 15.9	5.0 ± 0.6

** = $0.05 < p < 0.1$, *** = $p < 0.001$

Plasma levels for the non-polar conjugates of pregnenolone, DHA and androstenediol in normal and abnormal subjects.

Patients with acne or idiopathic hirsuitsm were compared to normal age matched premenopausal women. The breast cancer subjects were compared to the normal postmenopausal subjects.

5.8 Comparative uptake of free steroid and non-polar conjugate by washed RBC

To investigate the ability of the non-polar conjugates to cross the lipid cell membrane compared to free steroid, washed red blood cells were used as an _in vitro_ model. Fresh lithium heparin blood was taken and the RBC spun down and collected. The cells were washed twice with an equal volume of saline. The washed cells were resuspended in saline to give the original volume of whole blood and 1 ml aliquots dispensed in duplicate into stoppered glass tubes containing ^{3}H-DHA, ^{3}H-pregnenolone and ^{3}H-non-polar conjugate of pregnenolone (30,000 dpm of each). No incubation was performed with the non-polar conjugate of DHA due to the lack of ^{3}H-compound.

The samples were incubated with continuous mixing at 37°C for 15 minutes in a water bath. After incubation the samples were centrifuged at 1000g for 3 minutes and the supernatant decanted. The cells were then washed twice with 1 ml of sterile saline and then lysed by the addition of 500 μl of distilled water. Each sample and wash was extracted with 5 ml of diethyl ether and the organic phase decanted into plastic 5 ml counting vials prior to evaporating the ether to dryness under nitrogen and the addition of 3 ml of scintillant to the residue for radioactive estimation.

The results are shown in table 5.6.

Table 5.6

	% of total radioactivity in fraction		
	DHA	Pregnenolone	non-polar pregnenolone conjugate
Incubation supernatant	21.2	3.2	20.2
1st wash	2.1	2.5	11.9
2nd wash	1.3	3.4	7.6
Lysed cells	64.3	73.9	31.2

Uptake of ^{3}H-steroid by washed red blood cells after incubation at $37^{\circ}C$

for 15 minutes.

As can be seen less conjugated pregnenolone than free pregnenolone is taken up by the RBC. There is however a higher recovery of non-polar conjugate of pregnenolone in the two washes. Whether this represents the passage of non-polar conjugate through the RBC membrane or the detaching of loosely associated conjugate from the cell membrane is unknown. It is also noticeable that there is less uptake of ^{3}H-DHA than ^{3}H-pregnenolone by the RBC during the course of the incubation.

5.9 Discussion

The ability of the red blood cell to metabolise steroid has been known since 1948 when Werthessen et al, demonstrated the metabolism of oestrone by human red blood cells to compounds with different biological activity. Since this report the red blood cell has been shown to contain 16 α and β, 17α and β and 20α and β-hydroxy steroid dehydrogenase activity (Van der Molen and Groen, 1968). The enzyme activity and the percentage conversion of the steroids tested is in agreement with other reports. There have been no reports on the presence of aromatase or 3-β-hydroxy steroid isomerase dehydrogenase activity in the red blood cell, and this has been confirmed by our results.

The physiological importance of the enzyme systems within the red blood cells has received very little attention. Patt et al, (1970) and Fylling (1965) have both reported an increased reduction of androstenedione to testosterone by the red blood cells of hirsute women. This attention would only be of importance if significant quantities of androstenedione were to be found in the red blood cell or there was a rapid transfer of steroid across the red cell membrane.

Despite the first observation by Kemp and Bjorgaard in 1932 that the oestrogenic activity in the blood of pregnant women was equally distributed between plasma and the red blood cell, very few reports have appeared on the mass of steroid within the red cell. This point is also of importance for the interpretation of blood levels of hormone measured in plasma.

Our work shows there is an appreciable amount of steroid, especially pregnenolone, present within the red blood cell. Whether the red blood cell store of hormone acts as a further easily accessible store of steroid precursor for periods of high usage, or whether the hormone has a specific role within the red blood cell, is unknown.

Assuming a blood volume of 5.25 litres and a haematocrit of 45%, approximately 2.36 litres of red blood cells are available to metabolise steroid. With a mean mass of 12 ng/4.5 ml of red blood cells the total DHA mass in the cells is 6.3 μg. We have shown that 2.9% of DHA can be converted to androstenediol in 2 hours. Using these figures, 2.2 μg of androstenediol can be formed by this route in 24 hours. This is however only a small percentage of the quoted production rate of androstenediol, 680 μg/24 hours (Bird et al, 1982). Unfortunately no data are available on the red blood cell concentration of androstenedione, so no comment can be made as to whether the red blood cell may be an important site of testosterone production.

The suggestion has been made that the red blood cell steroid level may be of use in estimating the unbound plasma steroid level (Hiramatsu, 1983) with the demonstration of a good correlation between the plasma free and the red blood cell level of cortisol. No measurement of unbound plasma steroid was performed in this study, so no comment can be made on the possibility that the measurement of red cell steroid levels would be a valid technique for the estimation of unbound plasma androgens.

There have been no previous reports on the presence of the non-polar conjugates of pregnenolone, DHA or androstenediol in human plasma. The ratio of the plasma level of the non-polar conjugates in normal subjects are 2, 0.1 and 0.05-0.1 of the plasma pregnenolone, DHA and androstenediol levels respectively. The ratio for non-polar DHA conjugate to DHA is increased to 0.33 in idiopathic hirsute patients but is only 0.11 for patients with polycystic ovary syndrome. The subject taking the oral contraceptive Eugynon 30 has a decreased ratio (0.02). The plasma DHA conjugate level for the pure hirsute subjects was significantly raised compared to the control group ($0.01 < p < 0.02$). The greatest alteration in ratio of the conjugate to non-conjugate, > 6, is shown by the pregnant subject for pregnenolone conjugate to pregnenolone, but this is only based on one value, further work is needed to verify this finding.

In contrast to the report by Janoko and Hochberg, 1983, we were unable to detect any non-polar conjugates of oestradiol in human plasma. As the plasma levels of the conjugate reported by Janoko should have been measurable using our method, it is surprising that we did not detect any.

Possible reasons for this may be that due to the lack of a tritiated conjugate tracer, the area of the TLC plate containing the conjugate may have been missed, alternatively the saponification procedure used may have affected the immunoreactivity of the oestradiol.

The degree of specificity demonstrated by the plasma for the production of the non-polar conjugates suggests the presence of a specific, or group of specific enzymes in plasma. The enzyme(s) appear to be specific for the 3-β-hydroxy 5-ene ring structure. It is not known if the 21-hydroxy group of the corticosteroids is reactive to this enzyme. Whether the alterations in the ratio of plasma conjugate to steroid level for specific steroids represents the actions of different enzymes or one enzyme with a different Km value for each steroid is not known.

One possible candidate for the enzyme is lecithin cholesterol acyl transferase. This enzyme is responsible for the esterification of the 3-β-hydroxy position of cholesterol with fatty acids transferred from lecithin. The enzyme has been shown to be both present and active in human plasma.

The possibility that plasma is the only production site for these conjugates in the human is unlikely. From the work of Hochberg et al, (1977) and Albert et al, (1980) on bovine adrenal and corpera lutea, it is probable that these organs could also produce non-polar conjugates in the human. Whether these organs would demonstrate a similar degree of specificity for steroid conjugation to that seen in plasma is not known.

From the work of Albert et al, (1980) and Mellon-Nussbaum et al, (1980) it is anticipated that the non-polar conjugate is formed between the steroid and a fatty acid, although the work of Oertal and Benes (1974) suggests the possibility of conjugation to a diglyceride. The observation that each non-polar conjugate migrates as a single spot in a system designed to separate the lipid subgroups on TLC suggests conjugation to a single type of lipid. During the investigation of solvents for conjugate extraction, a gradient of extractions from iso-octane through diethyl ether to ethyl acetate and ethyl acetate:n-propanol (3:1 ^{v}/v) was used on the same plasma. Using iso-octane only a small proportion of the conjugate was extracted, the majority coming out with the latter two solvents. The efficiency of the different solvent extractions may indicate a range of different conjugated fatty acids or very tight binding of the conjugate by plasma proteins.

Giorgi (1980) has demonstrated that the permeability of the cell membrane to steroid is inversely proportional to the polarity of the steroid, and that for the majority of steroids passage through the cell membrane occurs by passive diffusion. Due to the very non-polar nature of these conjugates the possibility is raised that they will have a greater solubility in the cell membrane than free steroid. However, the initial results from the incubation of the red blood cells with the non-polar conjugate of pregnenolone would suggest that, certainly for the red blood cells, the non-polar conjugate is less well taken up by the red cell than is the free steroid. Whether this is true of other tissues and for the non-polar conjugate of DHA and androstenediol is not known.

In view of the elevated plasma levels of the pregnenolone and DHA
conjugates in hirsute and acne subjects, and the possibly greater
solubility in membranes, an increased uptake of these steroids by the skin
could. be postulated. Mellon-Nussbaum et al, (1980) has demonstrated that
the lipoidal conjugates of pregnenolone can be metabolised by incubation
with a mitochondrial-microsomal fraction from adrenal cortisol tissue to
lipoidal derivatives of 17-hydroxy pregnenolone and DHA. Whether the
further metabolism of the lipoidal DHA to testosterone or oestrogen
conjugates is possible is not known.

Thomas and Oake (1975) have shown that human skin is capable of
metabolising DHA to testosterone and DHT and that the degree of DHA
utilisation and conversion to the active androgens is greater in the
skin of hirsute women than normal controls. This suggests the
possibility that an increased uptake of the non-polar conjugates of
DHA or pregnenolone, followed by direct metabolism or cleavage to the
parent steroid followed by metabolism to the active androgens may have
a role in the aetiology of hirsutism.

It is interesting to speculate that the formation of the non-polar
conjugates and their increased tissue uptake and metabolism may be a
link between the increased risk of cancer and other illnesses associated
to obesity and dietary lipid.

In conclusion we have demonstrated the existence and plasma levels of a new group of non-polar conjugates formed from pregnenolone, DHA and androstenediol by human plasma. An alteration in the plasma levels of the non-polar conjugates when compared to age matched controls has been demonstrated in patients with hirsutism, acne, breast cancer and a subject taking the combined oral contraceptive pill. The physiological and pathological significance of these findings remain to be explored.

6. CONCLUSIONS

To develop a sensitive and specific immunoassay a good antiserum is of prime importance. Antibody specificity is essential for the measurement of a steroid in a direct assay system due to the number of structurally similar steroids that exist in plasma. In recent years the advent of the monoclonal antibody has enabled the development of highly specific antisera to free steroids and the glucuronide conjugates of oestrone and pregnanediol. No monoclonal antibody to sulphate conjugated steroid has yet been described to my knowledge. The monoclonal antisera allow the development of highly specific direct immunoassays with the attendant advantages of speed and simplicity. The cost of developing a good monoclonal antibody clone is high therefore only commercially viable compounds have had clones developed.

Good assays can be developed using a polyclonal antibody as described in this thesis by the development of a simple, quick and reliable RIA for DHA. The use of the DHA-7-CMO derivative in preference to the DHA-3-CMO or DHA-17-CMO derivatives in the preparation of the immunogen conjugate results is an antiserum with a high specificity for DHA. Unfortunately the antisera used did not possess sufficient specificity to allow the development of a direct or simple extraction assay for either DHAS or pregnenolone.

Using the assays described in this thesis the plasma levels of DHA, DHAS and pregnenolone were measured in normal subjects and patients with endocrine abnormalities. Wide variations in plasma steroid levels were seen between normal subjects and within the same subject depending on the chronological timing of blood sampling. The existence of a diurnal rhythm with episodic secretion of the unconjugated steroids has been known for many years (James et al., 1978), but few authors have noted that plasma levels of the conjugated steroid DHAS also exhibit a distinct diurnal rhythm.

An appreciation of the existence of a diurnal rhythm is needed for the correct interpretation of a comparison of plasma steroid levels between two populations of subjects. To minimise the error caused by the episodic secretion on the estimation of a mean 24 hour plasma level regular blood sampling would be ideal but is inconvenient to both subject and experimenter. Estimation of steroid levels in blood samples taken at 0400, 1030, 1530 and 2200 (all $\pm$ 30 mins.) have been shown in this thesis to give a good estimation of the mean 24 hour plasma levels of DHA, DHAS and pregnenolone.

The DHAS diurnal rhythm does not follow the secretory episodes of DHA or pregnenolone both of which are synchronous with cortisol secretory episodes. A possible inference of this is that the secretion of DHA and pregnenolone is regulated by ACTH while DHAS is not. The question of the existence of a specific adrenal androgen stimulating hormone acting either together with ACTH or separately is unanswered.

The evidence presented suggests that the secretion of the adrenal androgens is divergent from cortisol in some subjects but whether this is caused by a single agent or a number of interacting mechanisms is unclear. The conclusion can be made that prolactin is not the putative AASH but that prolactin does increase the plasma DHAS levels possibly by ovarian stimulation to produce DHA and then conversion of the DHA to DHAS.

Many workers have concluded that DHAS is solely produced by the adrenal and as such is a good indicator of adrenal function (Korth-Schutz, 1976). Some of the data presented in this thesis suggests that the plasma DHAS levels reflect the production of DHA, the majority of which is produced from the adrenal but which is also produced by the gonad and possibly by peripheral conversion. Overproduction of DHA at sites other than the adrenal may thus affect the plasma DHAS levels due to the long half life exhibited by this steroid without affecting the plasma DHA levels. This would agree with the suggestion that the sulphokinase enzyme acts to maintain the plasma DHA level within certain limits (Adams and McDonald, 1983).

Overproduction of the adrenal androgens has been implicated in some hormonally dependent conditions, e.g. hirsutism, acne and breast cancer. In this study no significant difference was found between the plasma levels of DHA and DHAS in patients with breast cancer compared to matched controls. There is however very little evidence available as to the relationship between the plasma levels of a steroid and the

level found within the tissue. The cellular concentration of a steroid in diseased tissue may be altered in relationship to the plasma level compared to normal tissue due either to increased uptake or altered intracellular steroid metabolism.

The tumour tissue levels of DHA were higher when compared to normal breast tissue, however it is not proven whether this reflects an increased passage of steroid across the cell membrane or altered intracellular metabolism. Recently Van Landeghem et al, (1981) have demonstrated in vivo the ability of breast tumours to metabolise or synthesise de novo a number of androgens and oestrogens by measuring the steroid content of the afferent and efferent blood supply to the breast. In a subsequent paper Poortman et al, (1983) demonstrated that the tissue/plasma steroid concentration ratio differed with the tissue type and a different subcellular distribution between the nuclear and cytosol fractions depending on the type of oestrogen measured.

This evidence suggests that the tissue may be able to alter its steroidal microenvironment independently of plasma levels of steroid. Whether this is by de novo synthesis or more likely by conversion of precursor steroids taken up from the blood is not known. The uptake of steroid by the tissue depends on a number of factors, the degree of tissue vascularisation, the speed of bloodflow, capillary volume and length and the ability of the steroid to cross the membrane.

In 1964 as a result of their studies on steroid dynamics Tait and Burstein proposed that only the "free" steroid in the circulation was available for tissue uptake. The "free" steroid included steroid loosely bound to albumin as Tait and Burstein noted that a greater proportion of cortisol was extracted from the circulation by the liver than could be donated by the unbound cortisol alone. The model proposes that during passage through the target tissue the free steroid is rapidly cleared by the tissue to be replaced by steroid dissociating slowly from specific binding proteins during passage in the general circulation.

A slightly different model was proposed by Robbins and Rall (1979) in their work with the thyroid hormones. This model proposed the rapid dissociation of bound hormone from the specific binding protein to maintain a constant "free" plasma hormone level during transit within the target tissue. This model implies that the rate of hormone delivery to the tissue is independent of blood flow rate and not on the delivery of fresh "free" hormone rich blood to the tissue as assumed by Tait and Burstein.

The protein bound fraction would therefore contribute to the uptake of steroid if the dissociation times are less than the tissue transport time. Dissociation times (t/2) vary greatly, albumin bound steroid dissociates in less than 1 sec. while E2, testosterone and DHT take 5 sec., 22 sec., and 100 secs. to dissociate from high affinity SHBG (Heynes and Demoor, 1971; Pardrige, 1981).

As transit time through skeletal muscle and brain is 1 sec. and through the liver takes 10 sec. (Gorsky and Rose, 1977; Pardrige, 1981) it can be seen that the SHBG bound fraction will contribute less than the albumin bound fraction. As DHA, DHAS and pregnenolone are mainly albumin bound it may be expected that the protein bound fraction will contribute to tissue uptake.

Steroid uptake by the cell membrane is generally believed to occur by passive diffusion at a rate inversely proportional to the polarity of the steroid (Giorgi, 1980). There is however the suggestion that the protein-steroid complex itself can cross the membrane. This has been suggested by the demonstration of SHBG in MCF 7 cells and CBG in human liver nuclei (Bordin and Petra, 1980; Werthamer et al, 1973; Amaral, 1974).

Most work on steroid transport across the cell membrane has been performed using unconjugated steroids. When tested, sulphate conjugated steroids were found, as expected, to cross membranes slower than the unconjugated steroid due to the higher polarity exhibited by these compounds. It is interesting to speculate that the plasma non-polar conjugates reported in this thesis may be more membrane permeable than the unconjugated steroids due to the non-polar characteristics shown by these compounds. The non-polar conjugates are capable of being metabolised without loss of the acyl side chain to other steroid derivatives and may thus form another source of intracellular steroid.

The question of the importance of intracellular metabolism has recently received more attention with the demonstration that a number of tissues are capable of metabolising steroids _in vitro_ and _in vivo_. The production of altered steroid micro-environments within the tissue would explain why often no relationship is found between plasma steroid levels and disease.

Recently Lieberman _et al_, (1984) postulated the existence of discrete steroid producing units within the cell which have been named "Hormonads", each of which produces a certain steroid product without the release of stable intermediates during the biosynthetic chain. If one accepts this hypothesis then the sulphate pathway from DHAS via androstenedione 3-enol sulphate through to the oestrogens may still be a possible biosynthetic pathway, despite the inability to demonstrate the presence of androstenedione 3-enol sulphate in plasma. The androstenedione 3-enol sulphate existing as a transient enzyme bound reactive species (Hochberg _et al_, 1975).

No specific role for any of the delta five steroids has been demonstrated other than the ability to be transformed to the more biologically active delta four steroids and the polar and non-polar conjugates. Recently a paper by Baulieu and Robel (1984) reported the concentration of total (i.e. free and conjugated) DHA and pregnenolone in rat brain to differ according to the part of brain measured. Baulieu and Robel also reported the presence of non-polar

conjugates in brain tissue but no quantitative data was given. Whether the presence of the delta five steroids in brain tissue has any bearing on the observation by Erb et al, (1981) that schizophrenic patients can be separated from normal controls by an altered DHA diurnal pattern with 100% success is open to speculation.

The conclusion to this thesis is that the tissue levels and intracellular metabolism of steroids is probably of more pathological significance than the total plasma concentration of steroid. More work is needed to decide whether the plasma free and/or bound steroid levels significantly affect tissue levels of biologically active steroid and to elucidate the controlling mechanisms and biosynthetic capability that exists within the cellular micro-environment. The role of the non-polar steroid conjugates within this framework also deserves more study.

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