STUDIES OF ANDROGEN METABOLISM IN SKIN

by

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CHEMICAL NOMENCLATURE AND ABBREVIATIONS

Most abbreviations used in this thesis are those set out in the Biochemical Journal Instructions to Authors (Biochem.J. (1972) 126, 1).

The abbreviation sp.act. denotes the Specific Activity (d.p.m./unit mass) of a radioactive compound.

The names of certain enzymes involved in steroid metabolism are abbreviated as follows:

3β-Hydroxysteroid dehydrogenase Δ^{4-5} isomerase :- Δ^{5}-3β-HSD.

3β-Hydroxysteroid dehydrogenase :- 3β-HSD.

3α-Hydroxysteroid dehydrogenase :- 3α-HSD.

17β-Hydroxysteroid dehydrogenase :- 17β-HSD.

Steroid nomenclature is according to the IUPAC-IUB (1967) Revised Tentative Rules for Steroid Nomenclature, as reproduced in Steroids (1969) 13, 277. Many steroids dealt with have been given trivial names. Common names such as testosterone, androsterone, pregnenolone do not need to be defined further. Other trivial names are defined as follows:

IUPAC-IUB	TRIVIAL
5α-androstane-3α, 17β-diol	3α-androstanediol
5α-androstane-3β, 17β-diol	3β-androstanediol
5-androstene-3β, 17β-diol	androstenediol
17β-hydroxy-5α-androstan-3-one	dihydrotestosterone
3β-hydroxy-5α-androstan-17-one	epiandrosterone
3β-hydroxy-5-androsten-17-one	dehydroepiandrosterone DHA
3α-hydroxy-5β-androstan-17-one	etiocholanolone
4-androstene-3, 17-dione	androstenedione
5α-androstane-3, 17-dione	5α-androstanedione

Derivatives of the above trivial names are coined by adding the

name of the substituent as a prefix or suffix :

3β-sulphoxy-5-androsten-17-one	DHAsulphate
5-androstene-3β,17β-diol-3, 17-diacetate	androstenediol diacetate
Sulphate ester of androstenediol where number (1 or 2) and position (3 or 17) are not defined	androstenediol sulphate

Steroid β-D-glucosiduronides are termed glucuronides.

When a group of compounds is referred to, a centre of unsaturation in the steroid nucleus is denoted by the symbol $\triangle$
eg: $\triangle^4$-3-oxosteroids = C4-5 unsaturated 3-oxosteroids.

Similarly the A-ring saturated steroids with 5α-configuration are referred to as 5α-steroids, those with 5β-configuration as 5β-steroids.

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Part. I.

INTRODUCTION

1. EFFECTS OF STEROID HORMONES ON SKIN.

The skin is a complex organ derived from the embryonic ectoderm (epidermis and its appendages) and mesoderm (fibrous dermis). The epidermis and its appendages (hairs, sebaceous glands, sweat glands) require the presence of dermis for normal development (see Montagna, 1962). Androgens and oestrogens have effects on skin of man and animals. In man, androgen appears to cause an increased dermal collagen content (Black et al., 1970) and in both human and rat, skin collagen and skin thickness are greater in males than females (Shuster and Bottoms, 1963; Dickerson and John, 1964). However the most striking and well studied effects of steroid hormones on skin are those on hair growth and sebaceous gland function.

The relationship between sexual development, hair growth and sebaceous gland growth (causing oily skin and acne) is common knowledge. The fact that individuals castrated as children do not develop body hair, baldness, greasy skin or acne, emphasises this relationship.

i. Endocrine Aspects of Sebaceous Glands.

Sebaceous glands arise as outgrowths of cells from the developing hair follicles, early in foetal life. The growth of sebaceous glands in man and various other animals has been shown to be androgen stimulated. This includes such specialised sebaceous glands as rat preputial glands, mongolian gerbil ventral sebaceous patch, and hamster costovertebral glands among others (for a recent review see Ebling, 1970). Avian preen glands which are homologues of mammalian sebaceous glands are androgen controlled (Kar, 1947). Androgens of gonadal and adrenal origins stimulate sebum secretion in rats, and the growth response of the rat sebaceous glands has been used to bioassay androgens (Archibald and Shuster, 1969; Nikkari and Valavaara, 1970).

The growth of human sebaceous glands is stimulated by androgens, particularly testosterone. This is a direct effect

on the glands (Strauss and Pochi, 1963). However, the sebaceous glands of different body areas develop to different degrees under androgenic stimulation. The glands of the scalp, face, chest, upper back and perianal region become large multilobed structures. Those found on the abdomen and limbs are much smaller, usually unilobed structures. With the sebaceous glands of the forehead and nose, the usual size relationship of hair follicle and sebaceous gland is reversed, the hair follicle remaining an almost vestigial structure beside the huge sebaceous gland (Strauss and Pochi, 1963; Montagna, 1963). Most studies of human sebaceous glands have used those of the forehead area. Many experiments have established that testes, ovaries and adrenals are all involved in maintaining sebaceous gland growth in man (Strauss and Pochi, 1963; Pochi et al., 1963; Pochi and Strauss, 1967). Although sebaceous glands are androgen target organs they are actively growing in both sexes. The demonstration in recent years that testes, ovaries and adrenal cortex all synthesize and secrete androgens (Slaunwhite and Samuels, 1956; Gandy and Peterson, 1968; Kase et al., 1961; Horton, Romanoff and Walker, 1966; Ichii et al., 1962; Wieland et al., 1965) supports the theory of their common role in controlling sebaceous gland growth.

From soon after the time they first differentiate, the sebaceous glands are active throughout foetal life and their lipid secretion goes to form the vernix caseosa (Serri and Huber, 1963). After birth, the sebaceous glands regress to small buds of undifferentiated cells on the side of the hair follicles. They start to grow once again around the time of puberty (from 9-10 years of age) in response to the increased circulating androgens. In adult men, the sebaceous glands are maximally stimulated by testicular androgens and sebaceous secretion cannot be increased by testosterone administration (Strauss and Pochi, 1963). According to Strauss and Pochi (1963), sebaceous secretion does not fall appreciably with age in men and castration of aged men caused a great fall in sebum secretion, suggesting that testicular secretion of androgen is sufficient to maintain sebaceous secretion even in the aged male. In male castrates, the low rate of sebum secretion, which is

still significantly greater than in children, is maintained by adrenal androgens (Pochi and Strauss, 1967). In women, both ovarian and adrenal cortical secretions are important for maintaining sebaceous gland function. After about 30 years of age the sebum secretion in women decreases and drops abruptly around the time of the menopause (Strauss and Pochi, 1963; Krant et al., 1968). Sebum secretion in healthy premenopausal women can be reduced by adrenal suppression but not to the same extent as in castrated men (Pochi and Strauss, 1967). Progesterone and glucocorticoids in physiological doses do not appear to stimulate human sebaceous secretion; oestrogens suppress sebum secretion (Strauss and Pochi, 1963) so it has been concluded that adrenal and ovarian androgens are the main sebaceous gland stimulators in women.

ii. Effects of Sex Hormones on Hair Growth in Man

This subject has been critically reviewed (Hamilton, 1958; Rook, 1965; Hamilton et al., 1969). In man, the growth of hair in certain regions of the body is obviously associated with sexual development. In both sexes at puberty, changes occur in the hair follicles of pubic and axillary regions which result in formation of coarse terminal hairs. Similar changes in the beard, trunk and limbs are normally, much greater in the male than in the female. As with sebaceous gland growth androgens of gonadal and adrenal origins are involved in development and maintenance of adult body hair pattern. However, unlike sebaceous glands, whose development in different body areas is similar in both sexes, the hair follicles of different body regions differ in their sensitivity to the levels of androgens in male and female, eg: while axillary and pubic hair develop in both sexes, the growth of coarse sternal and beard hair is limited normally to the male.

iii. Endocrine Diseases and Skin. Abnormal Growth response to Androgens.

As sebaceous gland and hair growth are controlled by steroid hormones, it is not surprising that abnormalities of sebaceous glands and body hair pattern are associated with diseases in which there are disturbances of steroid hormone production and

metabolism., e.g.: association of seborrhoea, acne and hirsutism with Stein Leventhal syndrome, Cushing's syndrome, congenital adrenal hyperplasia.

There are however, certain conditions in which abnormal hair growth patterns or abnormalities of sebaceous glands are present without any obvious underlying disturbances of plasma steroid concentrations, steroid secretion or urinary excretion, e.g.: idiopathic hirsutism in women and acne vulgaris in both sexes (see Strauss and Pochi, 1969). More detailed studies of variations in androgen and oestrogen secretion, plasma protein binding or day to day fluctuations in plasma concentrations may yet demonstrate an underlying abnormality of the endocrine glands in some cases of acne and hirsutism; e.g.: detailed analysis of testosterone concentrations in the plasma of some women with idiopathic hirsutism has shown that even though they may have normal plasma concentrations, the blood production rate and metabolic clearance rate of testosterone from blood are increased (Bardin and Lipsett, 1968). Thus more testosterone enters the blood while its concentration is not raised. It is thought that the increased testosterone production represents direct secretion. It was recently shown (Harkness et al., 1968) that males with severe acne had greater day to day fluctuations in urinary testosterone glucuronide excretion than a non-acne group. Previously, no abnormalities of urinary 17-oxosteroids or plasma testosterone could be detected in acne sufferers.(Pochi et al., 1965).

However, it has also been considered possible that some cases of abnormal hair and sebaceous gland growth are due to an abnormal target organ response to hormones. The best example is the testicular feminization syndrome in which individuals are genetically male but appear superficially as normal females, except for absence of axillary and pubic hair. They have undescended testes which can synthesize androgens and oestrogens and they may have plasma testosterone concentrations within the normal male range (Griffiths et al., 1963; Southren et al., 1965). However there appears to be a complete unresponsiveness of target tissues to androgenic stimulation. This includes

sebaceous glands and body hair (Gwinup et al., 1966; Strauss and Pochi, 1969). To explain idiopathic hirsutism on such a basis an "over response" to androgens by some hair follicles must be proposed. The different sensitivity of hair follicles in different body areas to male and female levels of androgen is well recognised; the influence of genetic/racial factors on this has been observed (Hamilton, 1958; Hamilton et al., 1969). Thus it may be imagined that facial hair follicles of some women could develop a similar sensitivity to androgen as their axillary hairs. As will be seen later, it may be possible to explain such a change on the basis of the "prehormone concept" and changes in target organ steroid metabolism.

It is not so easy to explain acne on the above basis. This is because there is no clear cut evidence that an increase or decrease of sebaceous gland activity is a direct causative factor in acne (Pochi and Strauss, 1964; Pochi et al., 1965; Cunliffe and Shuster, 1969; Powell and Beveridge, 1970). To quote Strauss and Pochi (1964), "While there may be a statistically significant difference in mean sebum secretion between groups with and without severe acne, it is not possible to characterize any individual as having or not having acne by their sebum secretion". Undoubtedly androgenic stimulation of sebaceous glands is necessary for acne to occur (eunuchs do not develop acne, but if treated with androgen they may do so). Possibly some other factor is required to cause the lesion in the androgen stimulated gland. It may be that this other factor is androgen itself, causing a change in sebum composition (Powell and Beveridge, 1970) or a blockage of the sebaceous gland duct by inducing excessive keratinization (Van Scott and MacCardle, 1956). In this case, the development of acne might be termed as an abnormal target organ response to androgen. In those individuals with acne who do have abnormally high sebum secretion rates while at the same time having normal secretion and plasma concentrations of androgens, there may be an over-response of the sebaceous glands to normal androgenic stimulation.

2. THE ROLE OF THE SKIN IN METABOLISM AND EXCRETION OF ANDROGENS.

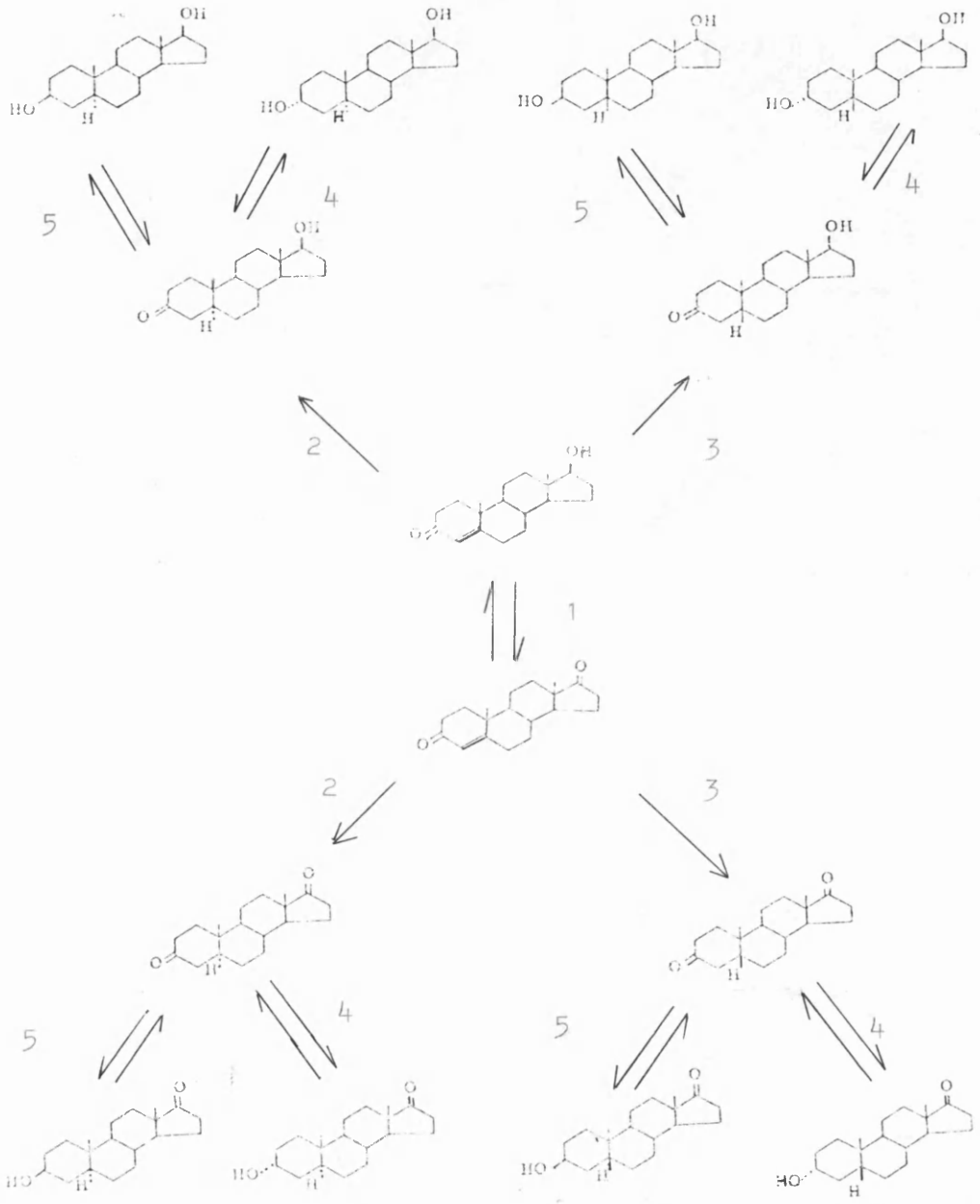
Traditionally, the liver was considered as the main organ in which the secreted steroids are metabolized. The classical idea being that a steroid hormone was secreted by an endocrine gland, acted on its target organ was then catabolized in the liver and excreted in urine (see Figs. 1 and 2 pp. 7 and 8 for metabolic transformations of androgens). However, evidence has accumulated to show that the liver is not the only site of androgen metabolism. It was shown that kidney tissue would convert testosterone to androstenedione (West and Samuels, 1951). Then it was shown that a number of human tissues metabolized testosterone (Wotiz et al., 1954). These included prostate, endometrium, myometrium, intestine, smooth muscle, mammary gland tissue and skin. The skin was found to be one of the most active sites of testosterone metabolism (Wotiz et al., 1954, 1956). Prostatic tissue from human and rat was shown to metabolize testosterone (Farnsworth and Brown, 1963; Shimazaki et al., 1965; Chamberlain et al., 1966). A-ring reduction to 5α-steroids and oxidation of the 17β-hydroxyl group were the major reactions.

If this extrahepatic metabolism is quantitatively important then the pattern of excreted steroid metabolites will reflect this complex situation and it becomes necessary to investigate the steroid metabolism of different organs in order to discover the origin of different excreted metabolites. Indeed, recent studies by Mauvais-Jarvis et al., (1969a, 1970a) have shown that different urinary metabolites may represent the androgen metabolism of different organs, e.g.: urinary 5α-androstanediols appear to mainly reflect testosterone metabolism in tissues other than the liver or intestines while 5β-androstanediols and etiocholanolone are more representative of hepatic metabolism.

Another complication arises over the actual excretory routes for androgen metabolites. In man, C_{19} steroids are excreted mainly in urine as sulphates or glucuronides, in

Fig. 1.

IMPORTANT METABOLIC TRANSFORMATIONS OF ANDROGENS.



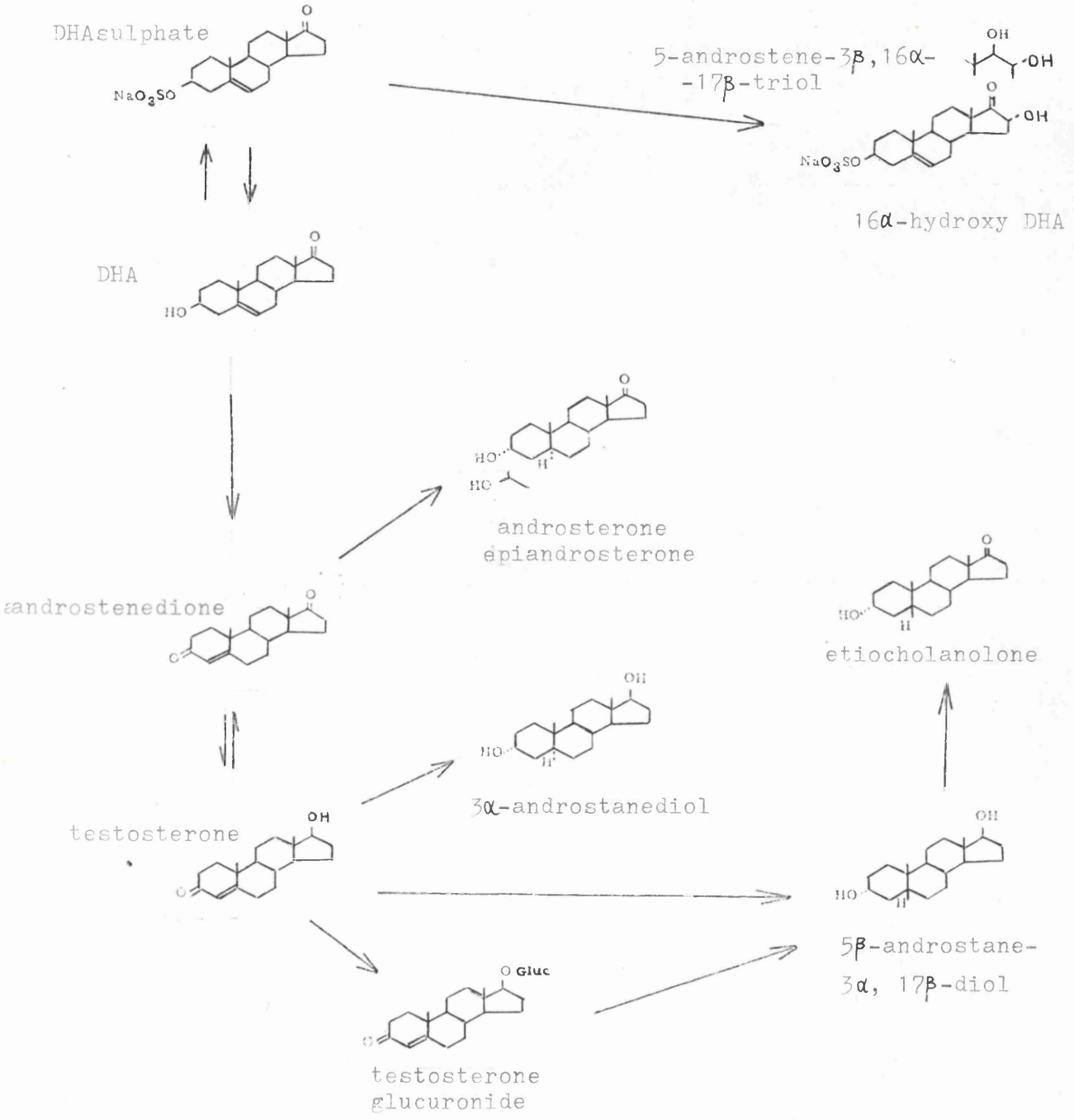
Reactions catalysed by:

1. 17β-HSD 2. 5α-reductase 3. 5β-reductase 4. 3α-HSD 5. 3β-HSD.

The major metabolic transformations which testosterone androstenedione and their metabolites may undergo are shown. The reactions have been detected in various tissues of a number of species but particularly in the liver (see Dorfman, P.I and Ungar, F. (1965) Metabolism of Steroid Hormones. Academic Press, London and New York). Hydroxylation of the steroid nucleus at positions 1, 2, 6, 7, 16 also occurs. Hydroxyl groups at 3 and 17 form conjugates with sulphuric and glucuronic acids. Conversion of testosterone and androstenedione into oestrogens is not shown.

Fig. 2

FORMATION OF SOME OF THE MAJOR ANDROGEN METABOLITES IN MAN



In the left hand column are the main androgens secreted into the blood of man. Transformation into some of their major metabolites which are excreted in the urine as sulphate or glucuronide conjugates is shown.

other species elimination in faeces after excretion in bile is important (Gallagher et al., 1951). This route also has some importance in human infants (Gustafsson et al., 1969). Another possible route for excretion is through the skin (sweat, sebum, epidermal keratin, hair). The skin may be an excretory route for some steroids and also for certain drugs, e.g.: tetracyclines (Rashleigh et al., 1967; Marples and Kligman, 1971). C_{19} steroids have been tentatively identified in extracts of human sebum, sweat and hair. Earlier experiments (Carrié and Ruhrmann, 1955; Dubovyi, 1960) in which the Zimmerman reaction was carried out on skin washings or hair extracts were unreliable as this is not a steroid specific reaction. However further studies showed that compounds could be extracted from human axillary and pubic hairs which had similar chromatographic properties to DHAsulphate and which gave a positive Zimmerman reaction. After acid hydrolysis of these extracts, compounds having similar chromatographic properties to DHA and androsterone were isolated (Julesz et al., 1966; Julesz, 1968). Dubovyi (1967) claimed to have isolated androsterone from extracts of human hair fat. Cook and Lorincz (1963) had used another approach; they showed that human skin surface fat contained compounds which could be oxidized by bacterial hydroxysteroid dehydrogenases, NAD^+ or $NADP^+$ being required as cofactors. They claimed that the lipids excreted on to the human scalp in 24 hours could contain up to 400μg of steroids which were oxidizable by the bacterial enzymes. Oertel and Treiber (1969), injected [7α-^{3}H] DHA-[^{35}S] sulphate into a man and were able to isolate a ^{3}H and ^{35}S labelled fraction from skin surface washings. About 1.4% of the injected radioactivity was recovered in this fraction obtained by washing arms and legs with an ether: ethanol: water solution 6, 12 and 24 hours after injection of the labelled DHAsulphate. This could be a significant proportion of the radioactivity when it is considered that only 30% of the body surface was extracted and this an area with only low density of active sebaceous glands and eccrine sweat glands. One would consider these glands

to be the most likely organs in skin to be involved in steroid excretion. From the isolated radioactive fraction, Oertel and Treiber extracted and identified a number of C_{19} steroids. Most of these were present as sulphate conjugates as judged by chloroform/water partition, chromatographic behaviour and solvolysis. DHA, androsterone, etiocholanolone, 5α-androstanedione, androstenediol and 5-androstene-3β, 16α, 17β-triol were identified by reversed isotope dilution with authentic carriers. Constant specific activity of free steroids and 2,4-dinitrophenylhydrazone derivatives was demonstrated after t.l.c. in several solvent systems. Oertel suggested that because the $^{3}H/^{35}S$ ratio of the isolated metabolites was similar to that of the injected DHAsulphate, the latter compound had been metabolized without losing its sulphate group. However, it is difficult to explain a direct conversion of DHAsulphate to androsterone sulphate without loss of the sulphate, through the action of known steroid metabolizing enzymes.

In addition to these isotope experiments, Oertel and Treiber (1969) extracted human thermal sweat and also direct skin surface washings in an attempt to isolate any endogenous steroids. Using techniques similar to those used in their tracer studies, they claimed to have isolated about 60μg of C_{19} steroids from an extract of 100 cm^2 of skin surface from the thigh. Most of the steroids were present as sulphate conjugates. From the sweat extracts were isolated about 170μg of C_{19} steroids. This time most of the steroids were unconjugated. Oertel suggested that the metabolites of DHAsulphate isolated from the skin surface might reflect steroid metabolism in the skin. Only direct incubation or perfusion experiments could test this theory.

So far two problems concerning androgen metabolism and skin have arisen:

1) The role of skin in whole body metabolism of C_{19} steroids.
2) The specific problem of the formation of steroids found on the skin surface, notably the metabolites of DHAsulphate.

The other major problem of skin steroid metabolism is related to 1) above. If a hormone sensitive organ such as skin, possesses the enzymes necessary to degrade active androgens it might also possess the enzymes necessary to synthesize them from inactive secreted precursors. This idea has come to be known as the "Prehormone Concept" and will be discussed in the next section.

3. PREHORMONES: BIOSYNTHESIS OF HORMONES BY PERIPHERAL TISSUES.

It has long been considered that testosterone is the most important androgen. The androgenicity of DHA and androstenedione, which may both act as intermediates in testosterone biosynthesis, could be due to conversion in the body to testosterone (Mahesh and Greenblatt, 1962). Similarly DHAsulphate the major C_{19}-steroid secreted by human adrenals might act as a precursor of testosterone (Baulieu et al., 1965). It has been shown that endocrine and non-endocrine tissues from various species can interconvert the above steroids (Ungar and Dorfman, 1954; Klempien et al., 1961; Pearlman and Pearlman, 1961; Smith and Ryan, 1961; Aakvaag et al., 1964; Cameron et al., 1966; Kim and Herrmann, 1968; Fahmy et al., 1968; Jones et al., 1970; Collins et al., 1970). However 'in vivo' studies indicated that in men the major fraction of testosterone in the blood was secreted directly by the testes; secreted DHAsulphate, DHA and androstenedione appeared as unimportant contributors to the plasma testosterone pool (Tait and Horton, 1966; Bardin and Lipsett, 1968; Migeon et al., 1968). In women, because direct secretion of testosterone is small compared to that of androstenedione, more than half of the blood production of testosterone may arise through peripheral metabolism of secreted androstenedione (Tait and Horton, 1966; Bardin and Lipsett, 1968). Also in women peripheral DHA metabolism may account for up to 15% of blood testosterone production. The fraction of blood testosterone arising from DHAsulphate is very small in both men and women

even though the plasma concentration of the latter compound is more than 100 x that of testosterone (Tait and Horton, 1966). It appears therefore that if blood production rate of testosterone is taken as an index of biologically active androgen formation in man, then secretion and peripheral metabolism of "prehormones" is important only when testosterone secretion is small compared with that of secreted precursors such as androstenedione. Even then DHAsulphate would appear to have only a minor role as an androgen precursor although the finding that ovarian and mammary gland tumours will convert it to DHA and androstenedione (Fahmy et al., 1968; Jones et al., 1970) leaves the possibility that DHAsulphate secreted by the adrenals might be a source of testosterone in some disease states.

The above arguments were based on the premise that the blood production rate of testosterone is a good indicator of the formation of biologically active androgen and that the plasma concentration of testosterone is a reliable measure of the amount of biologically active androgen available to the target organs. As already noted, this appears to be true for the adult man. However, it has to be considered whether the conversion of DHAsulphate, DHA and androstenedione to blood testosterone truly reflects the importance of their secretion for supplying active androgen. There are three reasons why this may not be so:-

1) These steroids may possess biological activity independent of any metabolism.

2) They could be converted to testosterone in peripheral tissues. This testosterone might not mix with the blood testosterone pool. For instance, it could be further metabolized.

3) Testosterone itself may be converted in the target organs to a metabolite which exerts the biological effect. Other androgens might also be converted to this metabolite either, via testosterone, or via a pathway not involving testosterone.

It has been demonstrated that various androgen target organs metabolize testosterone, one of the earliest studies was that of Wotiz et al. (1954). Further studies of prostate gland, chicken comb, and preen gland showed that testosterone was

rapidly metabolized, particularly by reduction of the 4-5 double bond to give 5α-steroids (Farnsworth and Brown, 1963; Shimazaki et al., 1965; Baggett,1962; Rongone et al., 1967). Dihydrotestosterone(17β-hydroxy-5α-androstan-3-one), one of the principal testosterone metabolites formed in prostatic tissue (Farnsworth and Brown, 1963; Shimazaki et al., 1965) was known to have high androgenic activity. In fact, Dorfman and Dorfman (1962, 1963a,b) had shown that it was as active or more active than testosterone for stimulating growth of chick comb and rat prostate. Evidence has since been accumulating that dihydrotestosterone may be one of the biologically active forms of androgen, and that conversion of testosterone to this compound in the target tissue may be important in mediating its androgenic action (Bruchovsky and Wilson,1968a,b; Anderson and Liao, 1968; Mainwaring, 1969a,b; Gloyna et al., 1970). If this is the case the importance of the other secreted androgens should be assessed by their conversion to dihydrotestosterone rather than by their conversion to blood testosterone. In addition to the possibility of DHAsulphate, DHA and androstenedione being converted to dihydrotestosterone in target tissues it is quite likely that other naturally occurring androgens exert their effects through conversion to this compound. For instance, androsterone, epiandrosterone, 5α-androstanedione, 3α-androstanediol may all be converted to dihydrotestosterone by the appropriate 3α, 3β and 17β-hydroxysteroid dehydrogenases (fig. 1 p. 7). There was, however, very little information about the metabolism of androgens, other than testosterone, in target organs. Most studies had been concerned with conversion of DHAsulphate and DHA to testosterone little attention being paid to other steroids.

The work described in this thesis has been aimed at elucidating some of the aspects of androgen metabolism in skin. The skin is one of the most interesting androgen target organs (or collection of androgen target organs) and many disturbances of the endocrine system are reflected by changes in skin,

particularly by disturbances of sebaceous gland and hair growth.

The differing sensitivities of hair follicles to androgens, secreted by testes, ovaries and adrenals in the human, might result from differing abilities to utilize secreted DHAsulphate, DHA, androstenedione and testosterone as hormones. Possibly such differences could be due to varying abilities of the target organs to convert the secreted compounds to "active forms" (testosterone, dihydrotestosterone...?). The postulated abnormal target organ response in hirsutes might then arise from disturbances of this androgen metabolism. Similarly, the postulated abnormal response of sebaceous glands in acne might result from disturbances of androgen metabolism in skin.

4. REASONS FOR STUDYING STEROID METABOLISM IN HUMAN SKIN.

These can now be stated as:

1. The role of the skin as a site of androgen catabolism (part 1, section 2).

2. Conversion in the skin of secreted steroids to biologically active species and its relation to sebaceous gland activity and hair growth (part 1, sections 1 and 3).

5. EXISTING KNOWLEDGE OF STEROID METABOLISM IN SKIN.

Wotiz et al. (1956) reported studies of testosterone metabolism in skin from back, axilla and scalp of men aged 22 - 30 years. Unfortunately, in this study the possible testosterone metabolites were not identified, but an autoradiogram of a paper chromatogram of the skin steroid extracts showed that possibly 8 metabolites of testosterone were formed. They all gave a positive Zimmerman reaction and one was identified as androstenedione by further chromatography and infra-red spectra. Thus the presence of a testosterone 17β-HSD in the human skin was indicated.

Baillie et al. (1965) reported that the human sebaceous glands were sites of NAD^+ and $NADP^+$ dependent hydroxysteroid dehydrogenase activity, which was demonstrated using histochemical

techniques. A wide variety of hydroxysteroids reacted as substrates in this system. Particular importance was attached to the presence of 3β-HSD (utilizing DHA as substrate) and 17β-HSD (utilizing testosterone). These enzymes could catalyse the conversion of DHA and androstenedione to testosterone in the sebaceous cells. A further study of 3β-HSD led to the conclusion that the enzyme was restricted to the sebaceous glands of acne prone areas (face, upper back, chest), that the activity was highest in the 15 - 20 age group, then declined with increasing age (Baillie et al., 1966). It has however, been recently shown, using more refined techniques, that both 3β-HSD and 17β-HSD are present in sebaceous glands from all over the body (Calman et al., 1970). In order to directly test the possibility that DHA could be converted to testosterone in sebaceous glands, Cameron et al. (1966) incubated slices of shoulder skin from men with [7α-^{3}H] DHA. They were able to demonstrate an extremely small conversion of DHA to testosterone (0.05% / 4 hours). At about the same time, Rongone (1966) reported the metabolism of testosterone by mammary skin from a man with Klinefelter's syndrome. Metabolites were identified by g.l.c. Testosterone was converted to androstenedione, 5α-androstanedione, androsterone and etiocholanolone. These studies indicated that human skin contained 17β-HSD, 3α-HSD, 5α-reductase and possibly 5β-reductase enzymes. The 5α-reduction of cortisol had previously been shown to take place in human foreskin (Hsia et al., 1964). Faredin et al. (1967) reported some experiments on the metabolism of [4-^{14}C] DHA in pubic skin from an agonadal male. Evidence for formation of androstenedione testosterone and androsterone was presented along with tentative evidence for identity of various highly polar metabolites. These were later identified as 7-hydroxylated DHA metabolites (Faredin et al., 1969). In another study, Gallegos and Berliner (1967) incubated human abdominal skin with DHA for 5 days in Eagle's medium; radioactive androstenedione, testosterone and androstenediol were isolated and identified. In addition, the skin was shown to form the sulphate esters of DHA and androstenediol (Berliner et al., 1968) indicating the presence of a steroid

sulphotransferase in male human skin. Faredin et al.(1968) confirmed that this reaction could also take place in female abdominal skin. Chakraborty, in this laboratory, had been investigating the metabolism of DHA in skin biopsies taken from individuals of different ages with and without acne vulgaris (Chakraborty et al., 1970). By incubating thin (10μ) frozen sections of skin in buffer with NAD^+ and [7α-^{3}H]DHA, he was able to demonstrate the formation of androstenedione and androstenediol. Skin from the shoulder region was metabolically much more active than skin from the lateral thoracic wall or thigh (non-acne regions). The rate of conversion of DHA to androstenedione appeared to decrease with increasing age after the third decade. This agreed with the earlier histochemical studies. However, in no experiment was Chakraborty able to demonstrate the conversion of DHA to testosterone by skin slices. He criticised the results of other workers as they did not appear to have purified the radioactive DHA before use and he had found that commercial [7α-^{3}H]DHA contained amounts of testosterone comparable to the quantities isolated from metabolic experiments with skin and DHA. Using [4-^{14}C]androstenedione as substrate and an NADPH generating system to supply coenzyme, he was able to demonstrate the conversion of androstenedione to testosterone in skin. It was quite likely that his earlier failure to find a conversion of DHA to testosterone was due to a lack of NADPH in his incubating system. However, when experiments were carried out with NAD^+ and an NADPH generating system, the result was an increased conversion of DHA to androstenediol, but still no testosterone was formed (Chakraborty, 1968 unpublished work). There remained a conflict regarding the quantitative importance of conversion to testosterone as a metabolic pathway for DHA in human skin. Kim and Herrmann (1968) studied the metabolism of DHAsulphate in human neonatal foreskin homogenates and were able to isolate DHA, androstenedione and testosterone as metabolites. The [7α-^{3}H]DHAsulphate used was extensively purified before use and the testosterone was finally identified by repeated crystallization with authentic carrier steroid.

These results contrasted with those of an earlier study by Warren and French (1965), who had been unable to detect steroid sulphatase activity in adult skin. Chakraborty attempted to repeat the experiments of Kim and Herrmann using adult skin. He was able to show a low conversion of DHAsulphate to DHA but no other metabolites could be detected (Milne, 1969). Gomez and Hsia (1968), had reported further studies on metabolism of testosterone and androstenedione in human skin. Interconversion of the two steroids was demonstrated and both compounds were converted to A-ring saturated metabolites. Androsterone, epiandrosterone, and 5α-androstanedione were major metabolites of both incubated steroids. In addition, testosterone was converted to dihydrotestosterone. Neonatal foreskin produced a higher yield of this compound than adult abdominal skin. In contrast to the earlier experiments of Rongone (1966) no etiocholanolone or 5β-androstane-3,17-dione were detected in the experiments of Gomez and Hsia.

The relatively active conversion of both androstenedione and testosterone to 5α-metabolites in human skin suggested that the failure to detect a convincing conversion of DHA to testosterone in skin incubation experiments might be due to rapid further metabolism of testosterone, once formed. There was however, no information about metabolism of DHA to dihydrotestosterone or androstanediols in human skin. The experiments of the Hungarian workers had shown that in human pubic skin DHA could be converted to androsterone (Julesz, 1968) but no other information was available. We therefore set out to investigate the problems of in-vitro skin steroid metabolism outlined in Section 6. While these experiments were being carried out other groups were also making further studies of skin steroid metabolism. This recent work will be discussed in relation to the results presented here.

6. AIMS AND PURPOSE OF RESEARCH.

To study:

1. The conversion of DHA and DHAsulphate to testosterone and dihydrotestosterone in human skin. Any formation of these compounds would have to be related to the total metabolism, therefore identification and quantitation of all major metabolites would have to be achieved.

2. The possible conversion of DHAsulphate to other steroid sulphates in skin.

3. The metabolism of DHAsulphate and DHA compared with that of androstenedione and testosterone in the same type of skin from the same individual.

4. The metabolism of DHAsulphate, DHA, androstenedione and testosterone in skin from body regions characterized by abundance or lack of sebaceous glands and/or terminal hairs. This would be a first approach to characterizing the tissues involved in skin steroid metabolism.

Part. II.

MATERIALS AND METHODS

1. GENERAL OUTLINE OF INCUBATION AND ANALYTICAL METHODS.

i. Tissue and Incubation.

Tissue obtained at surgery was kept wrapped in 0.9% NaCl soaked gauze, on ice for up to one hour before use. The skin was dissected free of subcutaneous fat and cut into 0.5 - 0.7 mm thick slices using a specially made, multibladed cutter (photo. 1 p. 20). The slices were incubated with shaking either in Erlenmeyer flasks or glass scintillation counting vials with the appropriate radioactive steroid, dissolved with the aid of propane-1, 2-diol in Krebs Improved Ringer I medium (Krebs, 1950) at 37°C under an atmosphere of 95%O_2: 5%CO_2 for three hours as described for each experiment (Part III, Experiments and Results). The [7α-^{3}H] labelled DHA, androstenedione and testosterone were purified the day before use by t.l.c. on silica gel in the system chloroform: acetone, 185:15 (v/v), then stored dissolved in methanol: benzene, 4:1 (v/v) at -20°C. [7α-^{3}H]DHAsulphate was purified the day before use by t.l.c. on silica gel in the system ethyl acetate: 2-methyl-propan-2-ol: 5M-NH_4OH, 100: 82: 40 (v/v), then stored dissolved in methanol at -20°C. Control incubations containing steroid but no tissue were run with each batch of incubations.

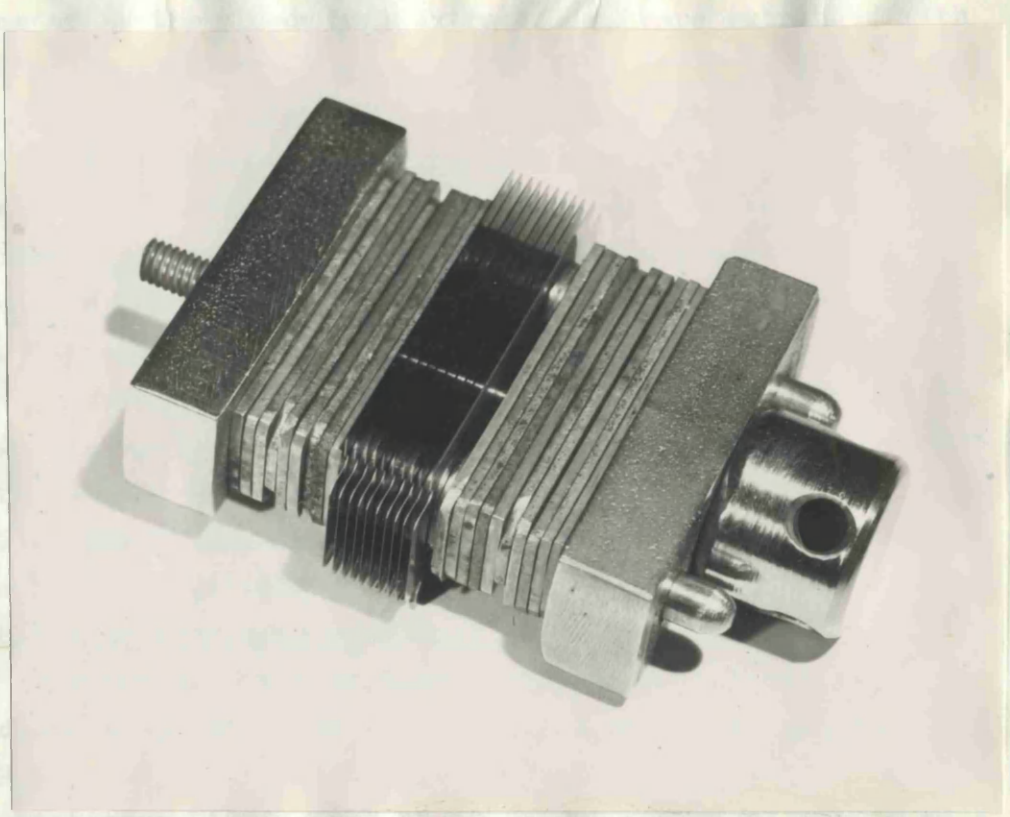
ii. Extraction of Steroids from Tissue and Medium.

Incubations were stopped with 5 volumes of acetone, and 200-300 μg of each of the appropriate carrier steroids dissolved in ethanol were added. The carriers are listed with each experiment. The mixtures were kept at -20°C.

Total lipids were extracted by homogenizing the mixtures with 100 ml of diethyl ether: ethanol, 3:1 (v/v). The extracts were filtered through sintered glass then evaporated to dryness at 40°C in a rotary evaporator. The residues were suspended in 15 ml of water and adjusted to about pH 8. Neutral steroids were then extracted with 3 x 2 vol. of chloroform. The chloroform extracts were evaporated to dryness and partitioned between equal volumes of 70% aqueous methanol and light petroleum (b.p. 40-

Photo 1.

A TISSUE CUTTER FOR SLICING SKIN



The cutter consists of double edged stainless steel razor blades mounted on the frame as shown. Steel washers slipped between the blades on to the support rods provide the correct spacing between blades. The assembled cutter is screwed up tight and pressed down on to the tissue. Skin is placed, epidermis down, on a sheet of thick polythene (1 mm) for cutting.

60°C) to separate steroids from less polar lipids. The methanolic extracts were evaporated to dryness and the residues were dissolved in methanol: benzene, 4:1 (v/v).

The steroid conjugates, which remained in the aqueous phase after the chloroform extractions, were extracted with 3 x 1 vol. of ether: ethanol, 3:1 (v/v) after adjustment of the aqueous solution to 50% saturation in ammonium sulphate and 0.5 M in ammonium chloride (Edwards et al., 1953). The ether: ethanol extracts were evaporated to dryness and the residue, containing steroid sulphates and glucuronides, dissolved in methanol.

iii. Isolation and Identification of Radioactive Steroids.

The steroid sulphate-glucuronide fractions were run on t.l.c. in system IX (table 1, p.27) to remove traces of neutral steroids and to separate sulphates from glucuronides (Pierrepoint, 1967). Bands corresponding to steroid sulphate and glucuronide standards were eluted (p.26) and counted for radioactivity; glucuronide fractions contained only traces of radioactivity and were not analysed further. Sulphate fractions were solvolysed by dissolving the dried extracts in a saturated solution of 1M-H_2SO_4 in ethyl acetate and leaving overnight at 37°C (Burstein and Lieberman, 1958; Wang et al., 1968). The solutions were back washed to neutrality with 20% sodium carbonate solution, then water; carrier neutral steroids were then added to the solvolysates, as at the end of the tissue incubation and the extracts were analysed in the same way as the neutral metabolite fractions.

The neutral steroid extracts (neutral metabolites and neutral steroids obtained by solvolysing the sulphate fractions) were separated by thin-layer and paper chromatography using the solvents listed in table 1 (p.27). Fig. 5 (p.30) shows the basic sequence of solvents for separating the main groups of steroids investigated. In some experiments the androstenediol-androstanediol fractions were further separated as shown in Fig. 6 (p.31).

Aliquots of each isolated steroid fraction were taken for

radioactivity determination (p. 32) and measurement of carrier recovered (p.33). Identity and radiochemical purity of the isolated radiometabolites was then established by one of two methods:

a. The radioactive steroid was further purified by chromatography and preparation of derivatives (p39) until constant specific activity was obtained (p.49and p.55). When the specific activity of the steroid and one or two derivatives agreed to within 10%, the steroid was considered radiochemically pure (Berliner and Salhanick, 1956).

b. A further aliquot (size depending on the amount of radioactivity in the fraction) of the isolated steroid was re-crystallized to constant specific activity with 10-20 mg of authentic carrier (p.38). When the specific activites of four successive crops of crystals agreed within 10% the steroid was considered radiochemically pure (Axelrod et al., 1965). This method was used in most experiments.

iv. Calculation of the Percent Conversion to Each Radiometabolite.

di d.p.m. of tracer are incubated with the tissue. A carrier quantity of the metabolite X (Mx) under investigation is then added and this unlabelled material mixes homogeneously with the radioactively labelled X produced in the incubation. The now radioactively labelled carrier is isolated in pure form and its specific activity (Sx) measured. Assuming the mass of radioactive X produced on incubation to be negligible compared with the mass (Mx) of carrier added, the percent conversion of tracer to X can be calculated:

$$\% \text{ conversion to } X = \frac{Sx.Mx}{di} \cdot 100 \quad (1)$$

The above calculation is used with method iii.a for identifying and estimating radiometabolites. Sx is the mean of the agreeing specific activities obtained after chromatography and derivative formation.

When the re-crystallization method for identification is used (iii.b) formula (1) must be modified to correct for the fact that the isolated compound X may not be 100% radiochemically

pure before crystallization.

Equation (1) becomes :

$$\% \text{ conversion to } X = \frac{Sx.Mx.100}{di} \cdot \frac{Sf}{St} \quad - \quad (2)$$

Sf = Final constant specific activity of crystals.

St = Specific activity of crystals if X was 100% pure before crystallization, i.e.: if all radioactivity of the aliquot used for crystallization was in X.

Now Sx = $\frac{\text{d.p.m. in aliquot of final t.l.c. eluate taken for crystallization.}}{\text{mg of carrier X in same aliquot (y)}}$

St = $\frac{\text{d.p.m. in aliquot of final t.l.c. eluate taken for crystallization.}}{\text{mg of carrier X in same aliquot (y) + mg excess carrier added for crystallization (Y).}}$

Mx is now measured in mg.

$$\% \text{ conversion to } X = \frac{Sf.Mx.100}{di} \cdot \frac{Y + y}{y} \quad - \quad (3)$$

In practice, $Y \gg 10$mg while $y \ll 0.2$ mg

By accepting an extra error of $\ll 2\%$ equation (3) simplifies to:-

$$\% \text{ conversion to } X = \frac{Sf.Mx.100}{di} \cdot \frac{Y}{y} \quad (4)$$

The radioactivity of the final chromatographic eluate is always measured even though it is not needed for the final calculation. This is so that appropriate aliquots can be taken for crystallization or further purification.

N.B. The final constant specific activity of the crystals (Sf) cannot be used to calculate the % conversion unless the amount of initially added carrier in the aliquot taken for crystallization (y) is known. This contrasts with the derivative method of purification (formula 1) where Sx (specific activity of the purified, labelled carrier) can be used directly to calculate the % conversion.

See appendix 1.

Fig. 3.

SUMMARY OF THE CHROMATOGRAPHIC METHOD FOR IDENTIFYING AND ESTIMATING RADIOMETABOLITES.

Skin + [^{3}H] steroid (di) incubated in Krebs Ringer medium.

↓

5 vol. Acetone + Carrier Steroids (0.2-0.3 mg) (Mx) added.

↓

Fractionation of free and conjugated steroids by solvent partition.

↓

Each [^{3}H] steroid purified by paper chromatography and t.l.c.

↓

Specific activity of each steroid measured - carrier recovery by g.l.c., E240. Radioactivity by liquid scintillation counting.

↓

Form a derivative of steroid. Purify by paper chromatography or t.l.c. Measure specific activity.

↓

Repeat, until 3 constant specific activities are obtained. (Sx)

$$\% \text{ conversion to metabolite X} = \frac{Sx.Mx}{di} \cdot 100$$

Fig. 4.

SUMMARY OF THE CHROMATOGRAPHY - RECRYSTALLIZATION METHOD FOR IDENTIFYING AND ESTIMATING RADIOMETABOLITES.

Skin + [^{3}H] steroid (di) incubated in Krebs Ringer Medium.

↓

5 vol. Acetone + Carrier Steroids(0.2-0.3 mg each)(Mx).

↓

Fractionation of free and conjugated steroids by solvent partition.

↓

each [^{3}H] steroid purified by paper chromatography and t.l.c.

a. aliquots for carrier recovery (g.l.c., E240) (y)

b. aliquots crystallized to constant specific activity (Sf) with 10 mg authentic carrier (Y).

c. aliquots for radioactivity measurement.

$$\%\text{conversion to metabolite X} = \frac{Sf.Mx}{di} \cdot 100 \cdot \frac{Y}{y}$$

2. CHROMATOGRAPHY

Thin layer Chromatography (t.l.c.) was done on 250 μ thick layers of silica gel Hf254+366. The plates were activated for one hour at 110°C before use. The solvent systems used for t.l.c. are shown in table 1 (p. 27). The Rf values for various steroids are shown in tables 2 and 3 (pp. 28,29).

After the chromatograms had been developed the steroids were vizualised on the plates under an ultra-violet lamp. Steroids which absorbed light at 240 nm were visualized under u.v. light of this wavelength while other steroids were visualized under light of 360 nm.

The steroids were eluted from the silica gel as follows:-

The steroid band was scraped from the plate into a small glass column with a filter paper disc in the bottom. The steroid was then eluted by pouring through 2 - 4 ml of a suitable solvent:- Ethyl Acetate:Methanol, 3/1 (v/v) for neutral steroids and their acetates. Methanol:Ammonium Hydroxide, 20/1 (v/v) for steroid sulphates. Recoveries of steroids from the plates were 85 - 98%.

Descending Paper Chromatography was done on sheets of Whatman No.2 Chromatography paper, 57cm long. The solvent systems used were of the volatile type described by Bush (1961), (table 1). Glass tanks were lined with Whatman 3MM filter paper. Solvent (stationary and mobile phases) was poured into the bottom of the tank, which was then sealed and left 24 hours before use. Prepared chromatograms were then hung in the tanks and left to equilibrate for 2 - 3 hours. They were then developed with mobile phase at 22°C. After development the papers were dried in air. Δ^4-3-oxosteroids were visualized by their absorption at 240 nm. Other steroids were then located by staining strips cut from the chromatogram in ethanolic phosphomolybdic acid solution (10%, w/v). The colour was then developed by heating the strips at 90°C for 2 minutes when steroids showed up as green spots on a yellow background, (Bush, 1961). Bands corresponding to the steroid

table : 1.

SOLVENT SYSTEMS FOR CHROMATOGRAPHY

A. Thin Layer Chromatography on Silica Gel.

I.	chloroform:acetone	185:15 (v/v)
II.	toluene:ethyl acetate	160:40 (v/v)
III.	chloroform:ethanol	190:10 (v/v)
IV.	toluene:ethyl acetate:cyclohexane	80:50:20 (v/v)
V.	cyclohexene:cyclohexanone	180:20 (v/v)
VI.	benzene:ethyl acetate	180:20 (v/v)
VII.	hexane:ethyl acetate	150:50 (v/v)
VIII.	diethylether:ethyl acetate	190:10 (v/v)
IX.	ethyl acetate: 2-methyl-propan-2-ol: 5M-NH_4OH.	100:82:40 (v/v)
X.	chloroform:methanol:water	200:9:0.75 (v/v)

B. Paper Chromatography.

XI.	light petroleum (b.p.100:120°C) - methanol - H_2O.	100:85:15 (v/v)
XII.	light petroleum (b.p. 100:120°C) - toluene : methanol - H_2O	60:40:85:15 (v/v)

System V was taken from Wright (1968)
System X was taken from Vandenheuvel (1968)
System IX was taken from Pierrepoint (1967)

The solvent sequences used in steroid separations are shown in Figs. 5 and 6.(pp. 30 and 31).

table 2. CHROMATOGRAPHIC PROPERTIES OF STEROID CARRIERS. 1.

	Rf in Solvent System				
	I	II	III	IV	V
1. androstenediol	0.15	0.07	0.35		
2. 3β-androstanediol	0.15	0.07	0.35		
3. 3α-androstanediol	0.15	0.07	0.35		
4. 1-3 diacetates			0.71		
5. testosterone	0.23	0.09			
6. " acetate	0.63				
7. DHA	0.29	0.17			
8. " acetate	0.71				
9. etiocholanolone	0.27	0.11		0.39	
10. epiandrosterone	0.29	0.17		0.45	
11. dihydrotestosterone	0.32	0.17		0.52	
12. androsterone	0.32	0.17			
13. 9-12 acetates					
14. androstenedione	0.43	0.19			
15. 5α-androstanedione	0.47	0.32			0.45
16. 5β-androstane-3,17-dione	0.45	0.30			0.40

	Rf in Solvent System				
	VI	'VII	VIII	+XI	°XII
1. androstenediol	0	0.12	0.34		1.0
2. 3β-androstanediol	0	0.11	0.30		1.25
3. 3α-androstanediol	0	0.13	0.38		1.70
4. 1-3 diacetates	0.52				
5. testosterone	0.02			0.5	
6. " acetate	0.19				
7. DHA	0.05			1.0	
8. " acetate	0.32				
9. etiocholanolone				1.28	
10. epiandrosterone				1.28	
11. dihydrotestosterone				1.28	
12. androsterone				1.75	
13. 9-12 acetates	0.3				
14. androstenedione					
15. 5α-androstanedione					
16. 5β-androstane-3,17-dione					

' 3 x run 15 cm. + 16 hour run, Rx relative to DHA = 1.0

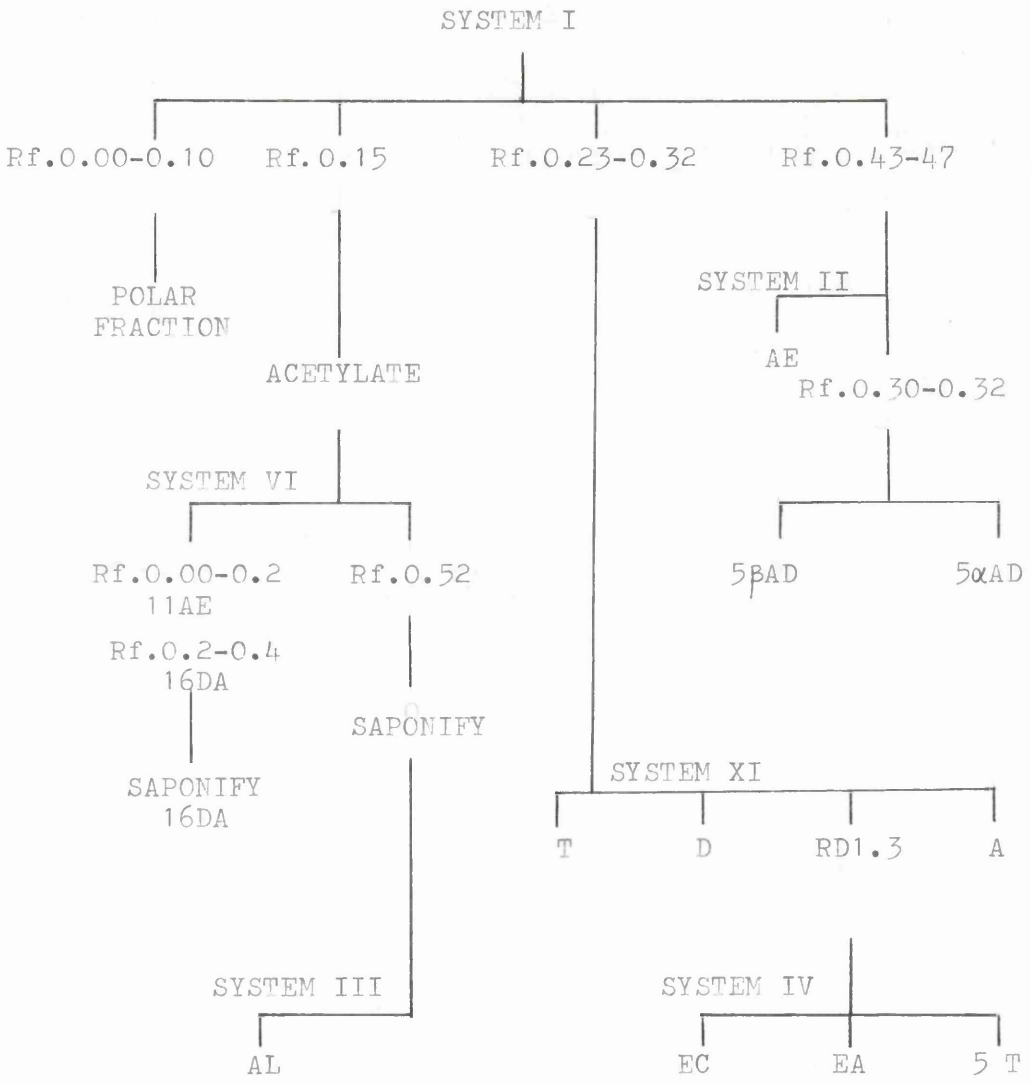
° 14 hour run, Rx relative to androstenediol = 1.0

table 3. CHROMATOGRAPHIC PROPERTIES OF STEROID CARRIERS. 2.

	Rf in Solvent System IX
Testosterone Sulphate	0.30
DHAsulphate	0.30
DHAglucuronide	0.01
Neutral steroids	0.75 – 0.9

Fig. 5.

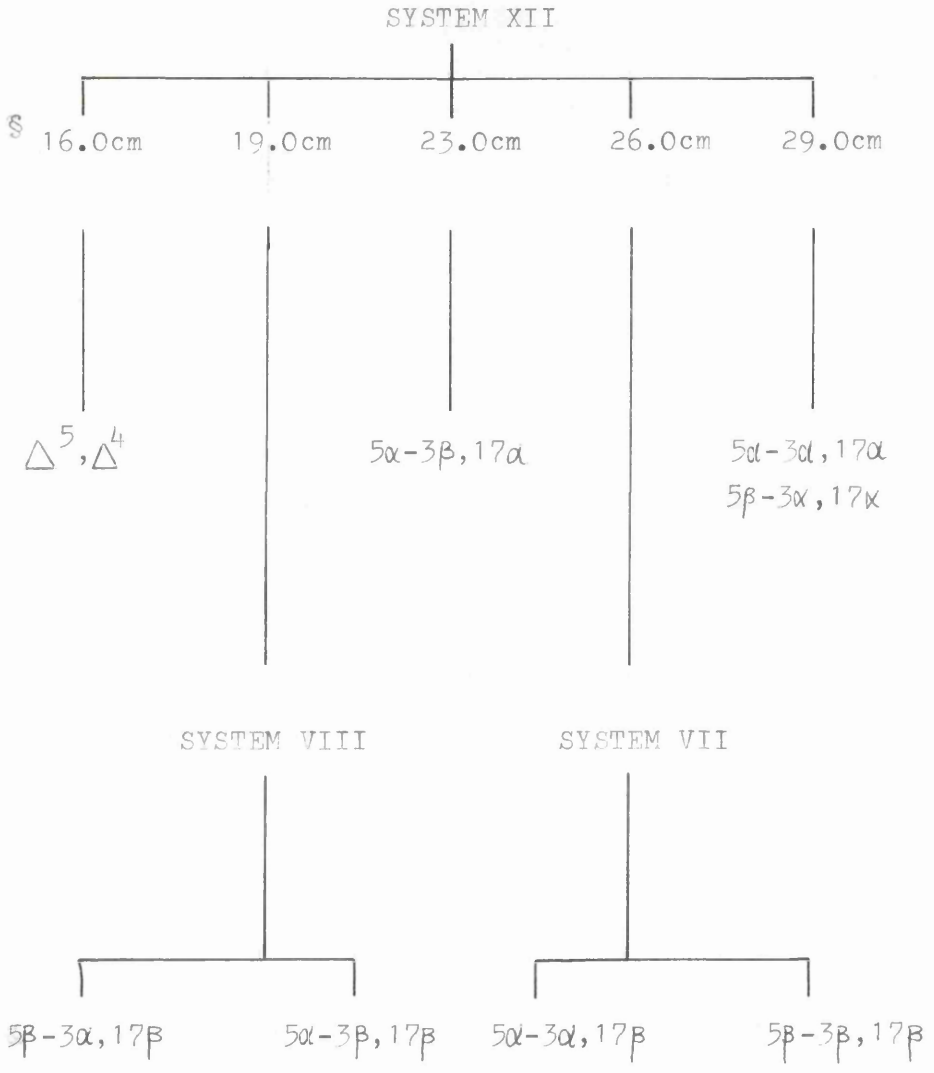
BASIC CHROMATOGRAPHIC SCHEME FOR SEPARATING C_{19}-STEROIDS



AE = androstenedione
11AE = 11β-hydroxyandrostenedione
5αAD = 5α-androstanedione
5βAD = 5β-androstane-3,17-dione
AL = androstenediol + androstanediols
A = androsterone
D = DHA
16DA = 16α-hydroxy DHA-3,16-diacetate
5 T = dihydrotestosterone
EA = epiandrosterone
EC = etiocholanolone
T = testosterone
RD 1.3 = Rf relative to DHA = 1.3

Fig. 6.

SEPARATION OF ANDROSTENEDIOLS AND ANDROSTANEDIOLS



§ No. in cm refers to the distance of the centre of the carrier band from the origin after a typical 14 hour run in system XII.

Δ^5 = androstenediol
Δ^4 = 4-androstene-3β,17β-diol
5α-3β,17α = 5α-androstane-3β,17α-diol
5α-3α,17α = 5α-androstane-3α,17α-diol
5β-3α,17α = 5β-androstane-3α,17α-diol
5β-3α,17β = 5β-androstane-3α,17β-diol
5α-3β,17β = 3β-androstanediol
5α-3α,17β = 3α-androstanediol
5β-3β,17β = 5β-androstane-3β,17β-diol

spots visualized by staining were cut from the rest of the chromatogram and the steroids eluted by soaking in 10.0 ml ethyl acetate:methanol, 3:1 (v/v) for 2 hours. This gave recoveries of 80-90%.

3. MEASUREMENT OF RADIOACTIVITY.

Radioactivity was measured in a Liquid Scintillation Spectrometer (NUCLEAR CHICAGO, Mark I).

Tritium was counted in one channel. At least 5000 counts and, in most cases, 10,000 counts were collected for each sample. Samples were dissolved in scintillator solution in glass counting vials:-

1. Crystals (0.5 - 2.0 mg.) were placed in the vial with the weighing pan.
2. Neutral steroids (and derivatives) dissolved in organic solvents were evaporated to dryness in the vial at 50°C, under a jet of filtered air.
3. Steroid conjugates or any compounds in aqueous solution were not evaporated.

Basic scintillator solution was toluene containing dissolved,
2, 5 Diphenyloxazole (3.0 g/l) and
1, 4-bis- 2-(4-Methyl-5-Phenyloxazolyl) -Benzene
(0.1g/l)

Samples 2 and 1 excepting androstenediol and androstanediols were dissolved in 6.0 ml of this toluene scintillator.
For milligram quantities of androstenediol and androstanediols and for group 3 a toluene:ethanol scintillator was used:-

toluene scintillator + ethanol 175:75 (v/v)
6.0 ml. for androstenediol and androstanediols
10.0 ml. for steroid conjugates or steroids in aqueous solution
(this dissolves up to 0.2 ml of water)

Counting Efficiency:

For samples in toluene scintillator efficiency was 45-50%.

For samples in toluene:ethanol scintillator efficiency was 20-25%.

To correct for counting efficiency a standard solution of [^{3}H] hexadecane (containing about 40,000 d.p.m.) in appropriate scintillator was counted with each batch of samples.

Quenching.

No quenching was caused by up to 3 mg of neutral steroids dissolved in toluene or toluene:ethanol scintillator. There was no quenching of toluene:ethanol scintillator caused by steroid sulphates added in 0.2 ml of ethanol or methanol. Counting efficiency of aqueous samples was estimated by reference to a standard of [^{3}H]hexadecane in toluene:ethanol scintillator containing 0.2 ml of saturated NaCl solution. This was adequate as it was seldom necessary to count aqueous samples with a high degree of accuracy. When accurate counting of quenched samples was needed internal standardisation with [^{3}H] hexadecane was used to calculate counting efficiency.

4. ESTIMATION OF STEROID CARRIER RECOVERIES.

This was done by u.v. absorption of by g.l.c.

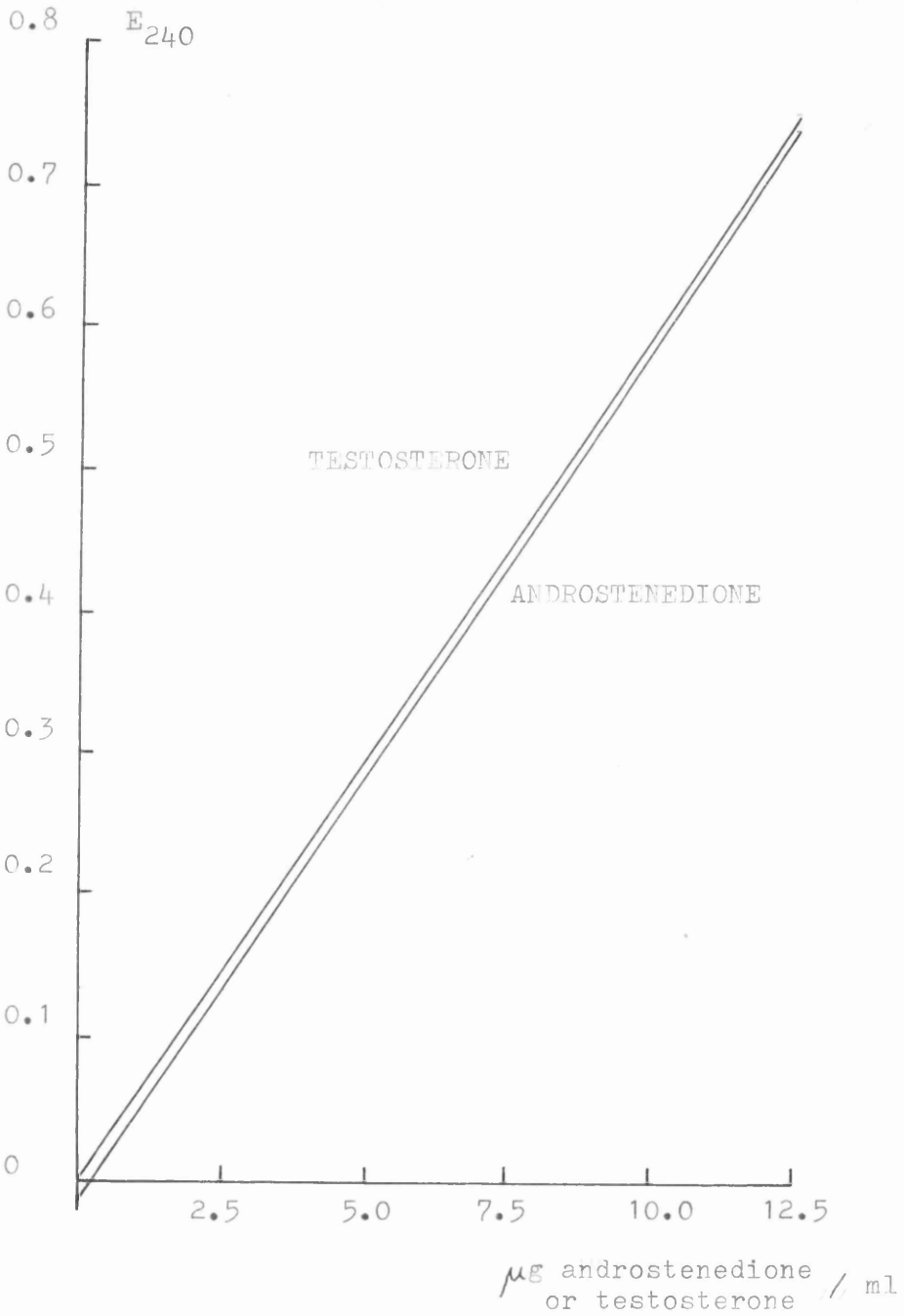
i. u.v. Absorption:

Δ^4-3oxosteroids and their acetates were measured by their absorption of light at 240 nm; a Unicam S.P.500 spectrophotometer with matched silica cells was used.

An aliquot of the chromatographic eluate was evaporated to dryness and then dissolved in 4.0 ml of ethanol. Absorption of light at 240 nm was then measured against an ethanol blank. The concentration of steroid in the sample was then estimated from a calibration curve prepared from standards of varying concentration and plotted as E240 .v. steroid concentration. This always gave good straight lines for testosterone, testosterone acetate and androstenedione (Fig. 7 p.34). The absorption from blank areas of silica gel or paper eluted in the same way as the

Fig. 7

CALIBRATION CURVES FOR ANDROSTENEDIONE AND TESTOSTERONE



sample was measured with each batch of samples. These blanks generally gave E240 $<$0.02 and were subtracted from sample values. High blank values of E240 were found if there was contamination of the t.l.c. eluate by silica gel particles and care was taken to exclude them from eluates.

ii. Gas Liquid Chromatography (g.l.c.)

The following steroids were measured by flame ionization detection after g.l.c. :

1. androstenediol
2. 3β-androstanediol
3. 3α-androstanediol
4. 1-3-diacetates
5. epiandrosterone
6. androsterone
7. etiocholanolone
8. DHA
9. DHA acetate
10. dihydrotestosterone
11. 5α-androstanedione
12. 5β-androstane-3,17-dione.

A Pye series 104 gas chromatograph with hydrogen flame ionization detector linked to a Speedomax W. chart recorder was used.

Glass columns 4 ft long and 4 mm internal diameter were silanized and packed with 3% S.E.30 coated on acid washed silanized Gas-Chrom Q (100-120 mesh).

Column temperature was 230°C.

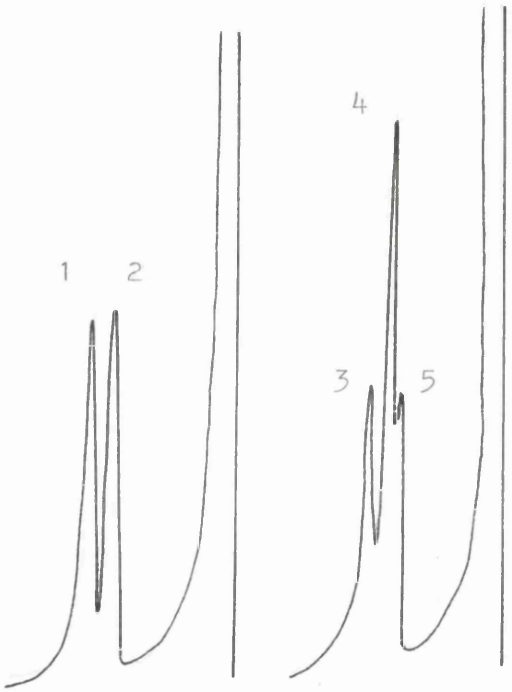
Carrier gas was nitrogen. Flow rate = 50 ml/min.

Under the above conditions, the free steroids all had retention times (relative to 5α-cholestane = 1) of 0.38 - 0.46. However, as shown in Fig. 8 (p. 36) there was sufficient separation between compounds 5 - 8, 10, and 11 - 12 to make g.l.c. a useful check on their resolution by paper chromatography and t.l.c.

An aliquot (usually 1/20th) was taken from the eluate, containing the carrier to be measured. To this was added a

Fig. 8.

CARRIER STEROIDS WHICH SEPARATE ON G.L.C.



Conditions. 3% SE.30 on Gas Chrom Q (100-120mesh)
Column temperature 230°C
Carrier gas N_2. Flow rate 50ml/min

1. = 5α-androstanedione
2. = 5β-androstane-3,17-dione
3. = dihydrotestosterone
4. = DHA, epiandrosterone, androsterone
5. = etiocholanolone

Table 4.

ACCURACY OF G.L.C. MEASUREMENTS.

DHA. Internal standard : progesterone.

known µg/sample	Estimated µg/sample. mean ± S.E. (4)
2.0	2.03 ± 0.07
4.0	3.90 ± 0.11
6.0	6.11 ± 0.14
8.0	8.05 ± 0.25
10.0	10.60 ± 0.23

androstenediol diacetate. Internal standard: DHA acetate.

known µg/sample	Estimated µg/sample. mean ± S.E. (5).
2.5	2.53 ± 0.10
5.0	4.94 ± 0.10
7.5	7.52 ± 0.18
10.0	9.66 ± 0.23
15.0	14.8 ± 0.30

androstenediol. Internal standard: pregnenolone acetate.

known µg/sample	Estimated µg/sample. mean ± S.E. (4)
2.5	2.56 ± 0.10
5.0	5.02 ± 0.14
10.0	10.5 ± 0.28
12.5	12.7 ± 0.50

known amount of another steroid to serve as an internal standard. The solution was evaporated and taken up in ethanol. 1-2µl were then injected into the gas chromatograph. Aliquots generally contained 2-10µg of carrier. 10µg of internal standard were added and the final volume of the ethanolic solution was 0.05 ml. Thus 0.04 - 0.2µg were injected into the gas chromatograph. The peak heights of sample and internal standard shown on the chart recorder were measured and the ratio of peak height of sample to peak height of internal standard calculated. The mass of sample steroid was then determined from a calibration curve, constructed using varied quantities of sample steroid with a constant amount of internal standard and plotted as peak heights ratio v. mass of steroid.

The following internal standards were used:-

Carriers (p.35)	Internal Standard
1 - 3	pregnenolone acetate
1 - 3 diacetates	DHA acetate
5 - 8, 10 - 13	progesterone
9	androstenediol diacetate.

Accuracy of g.l.c. measurement:

Linear calibration curves were obtained. The accuracy of the g.l.c. measurements was assessed by constructing standard curves and using them to estimate the concentrations of known standard solutions. Data are shown for DHA, androstenediol, and androstenediol diacetate (table 4 p.37).

5. RECRYSTALLIZATION OF STEROIDS.

A known quantity of radioactive sample was taken in a test tube. To it was added 10 - 20 mg (accurately measured) of carrier for crystallization. If a large number of samples were to be crystallized the carrier was made up as a volumetric solution and added by pipette. Otherwise the carrier was weighed on the microbalance and transfered directly to the tube.

The sample and carrier were thoroughly mixed and then the solution was evaporated to dryness at 50°C under a jet of filtered air. The steroid was then dissolved in an appropriate solvent mixture and crystallized. Solvents used were acetone: hexane, acetone:water, benzene:hexane, chloroform:methanol: hexane, methanol:water. The tubes were left in a fridge at -20°C and then the mother liquor was removed. The crystals were washed and washings were combined with the mother liquor, which was then evaporated to dryness. Crystals and mother liquors were then left overnight in a vacuum dessicator to dry.

Weighing of Crystals:

Crystals and mother liquor residues were weighed on a Cahn Microbalance, 0.5 - 2.0 mg of sample were weighed and then transfered directly to a scintillation counting vial with the weighing pan (a 1 cm square piece of aluminium foil) for radioactivity measurement.

Accuracy:

The 5 mg range of the balance was used. This allows 1 mg to be weighed to four significant figures. When a 1 mg weight was weighed at different times on different pans the maximum variation between recorded weights was 0.5%, and with a 0.5 mg weight the variation was 1% of the mean value.

6. PREPARATION OF DERIVATIVES.

Methods of Derivative formation were taken from Bush (1961) and Griffiths et al. (1963).

i. Acetylation of Secondary Hydroxyl Groups.

The solution containing the steroid was evaporated to dryness then equal volumes of anhydrous pyridine and acetic anhydride were added and the solutions left overnight in stoppered tubes at room temperature. For up to 300µg steroid, 0.1 ml pyridine and 0.1 ml acetic anhydride were used. After

reaction methanol was added and reagents were evaporated under a stream of filtered air.

For larger quantities of steroids (up to 5 mg) 0.5 ml of each reagent was used and after reaction the steroids were precipitated by adding water.

ii. Chromic Acid Oxidation of Secondary Hydroxyl Groups.

For up to 300 µg of steroid the Kiliani Reagent, prepared according to Fieser and Fieser (1967), was used (5.3g CrO_3 + 8.0g H_2SO_4 + 40 ml H_2O). 0.2 ml of Kiliani Reagent was mixed with 10 ml of acetone. The precipitate which formed was removed by centrifuging then 0.5 ml of the supernatant was added to the dry steroid extract. The solution was left for 30 min. at room temperature then 2 ml of water were added and the steroids extracted with ethyl acetate. 1 - 5 mg of steroid were oxidized by dissolving in a small volume of glacial acetic acid then adding an equal volume of a 2% aqueous chromic oxide solution and leaving overnight at room temperature. Water was then added to precipitate the steroids. If precipitation was not satisfactory the steroids were extracted with ethyl acetate.

iii. Reduction of Ketonic to Hydroxyl Groups with Sodium Borohydride.

To the dried steroid extract kept at 0 - 4°C were added 0.3 ml of an ice-cold methanolic sodium borohydride solution (5 mg $NaBH_4$ in 10 ml methanol). The solution was left at 0 - 4°C for 20 min., 1 drop of glacial acetic acid and 1.0 ml H_2O were then added and the steroids were extracted with ethyl acetate.

iv. Saponification of Acetates.

For up to 300µg steroid, 0.25 ml of 2% aqueous potassium carbonate were added to the extract which had been dissolved in 1.0 ml of methanol. The solution was left overnight at room temperature, then 3.0 ml H_2O were added and the steroids extracted with ethyl acetate. For larger amounts of steroid proportionately larger quantities of reagents were used.

7. CONTROL INCUBATIONS.

A control incubation with each substrate was carried out along with every batch of tissue incubations. The controls were taken right through the analytical procedure or until the radioactivity associated with any carrier, except the incubated substrate, was less than 0.02% of the total radioactivity. In the case of an incubation with 4×10^6 d.p.m. of starting substrate, this amounts to there being a total of less than 200 c.p.m. in any carrier after purification. In practice, this was routinely achieved and reported % conversions to any metabolite from tissue incubations were always equal to or more than 10x the maximum possible % conversions in the control.

androstenediol : After acetylation and t.l.c. in system VI
androstanediols: there was $<$0.02% conversion to this group of compounds in controls.

testosterone : In general, after purification by either derivative formation or recrystallization there was $<$0.02% conversion to testosterone in controls (except testosterone controls of course). However, in experiments 5D and 5AE (pp.93-109) there was about 0.1% conversion to testosterone in the controls.

epiandrosterone, etiocholanolone) After recrystallization there
androsterone, dihydrotestosterone) was $<$0.02% conversion to these in all controls.

androstenedione : Controls from DHA and testosterone incubations contained $<$0.1% conversion to androstenedione and DHAsulphate controls contained $<$0.01% androstenedione. This was usually 10x less than the % conversion in corresponding tissue incubations.

5α-androstanedione : The amounts of radioactivity associated with the carrier could be reduced to $<$0.02% in DHAsulphate and DHA controls. In androstenedione and testosterone controls, there was sometimes about 0.1% conversion to 5α-androstanedione. However, this was negligible compared to the % conversions in tissue incubations.

DHA : $<$0.5% in all controls (except DHA incubations). In DHAsulphate experiments the % conversions to DHA in tissue incubations were always 10x greater than those for controls.

DHAsulphate : $<$0.02% in androstenedione and testosterone controls. $<$0.1% in DHA controls - always 10x less than in tissue incubations.

epiandrosterone sulphate : About 0.1% conversion after recrystallization of epiandrosterone to constant specific activity from DHAsulphate controls and tissue incubations. $<$0.02% in all other controls.

Polar Neutral Fraction : $<$1% in all controls compared with 10% in most tissue incubations.

Sulphates of testosterone, androstenedione, 5α-androstanedione, androstenediol, androstanediols, androsterone, etiocholanolone, dihydrotestosterone: $<$0.02% conversion after solvolysis, chromatography and recrystallization of the appropriate fractions.

In practice the average recovery of the four substrates used in control incubations was 90% (75 - 100%). However in only one DHA control was the low recovery of 75% obtained. The recoveries of substrate from the other 11 controls ranged from 89 - 100%.

8. HISTOCHEMISTRY.

Histochemical localization of hydroxysteroid dehydrogenase activity was done by the method of Muir et al. (1968). This consists of the following steps :

1. Tissue was frozen on solid CO_2 then 10μ thick sections were cut in a cryostat at -20^{o}C and attached to glass coverslips by momentary thawing.
2. The section on the coverslip was covered with 1.0 ml of incubating medium and incubated for 2 hours at 37^{o}C.
3. The sections, attached to coverslips were washed in water, stained with Mayer's Haematoxlin, dehydrated in

alcohol and counterstained with a saturated solution of tartrazine in methyl cellosolve.

4. The coverslips were mounted on glass slides and examined to localize the blue-black deposits of nitrobluetetrazolium diformazan which indicate sites of hydroxysteroid dehydrogenase activity.

Incubating medium contained in 9.7 ml of 0.1M-sodium phosphate buffer pH7.4 :- 0.3 mg steroid dissolved in 0.3 ml dimethyl formamide, 2.0 mg NAD^+ or $NADP^+$ and 1.0 mg nitrobluetetrazolium. Blank incubations which contained no steroid were carried out. Control incubations were carried out to demonstrate the presence of NAD(P)H-diaphorase activity in the sections; the incubating medium contained in 10.0 ml of 0.1M sodium phosphate buffer pH7.4 : 2.0 mg NADH or NADPH and 1.0mg nitrobluetetrazolium.

9. AUTORADIOGRAPHY.

Autoradiographic demonstration of the uptake of [^{3}H]thymidine into cell nuclei was done using a modification of the method of Lachapelle and Gillman (1969). This consisted of the following steps :

1. Slices of skin 0.5 - 0.7 mm. thick were cut with the multibladed slicer (p.20). The slices were incubated in 20.0 ml of tissue culture medium 199 (Morgan et al., 1950) containing 2.0μCi/ml [6-^{3}H]thymidine (sp. act. 25Ci/mmol.) at 37^{o}C for two hours under an atmosphere of 95%O_2 : 5%CO_2.
2. The slices were removed from the medium, washed three times with 10.0 ml of tissue culture medium to remove unincorporated [^{3}H]thymidine and frozen on to chucks for sectioning in a cryostat at -20^{o}C.
3. Thin (7 - 10μ) frozen sections were cut, mounted on glass slides by momentary thawing and fixed in calcium-formol solution (8% aqueous formaldehyde containing 10%, w/v, calcium chloride).
4. Autoradiography was done with Kodak AR - 10 stripping

film (see Ruthmann, 1970). The exposure was for four weeks, during which time the film covered slides were kept in a light-tight box at $4^{o}C$. After developing and fixing (D.19 Eastman-Kodak developer) the autoradiographs, the sections were stained through the film with haematoxlin and eosin.

Part III

EXPERIMENTS AND RESULTS.

1. AUTORADIOGRAPHIC AND HISTOCHEMICAL DEMONSTRATION OF VIABILITY OF SKIN INCUBATED IN VITRO.

Slices of skin composed mainly of whole cells were incubated in a Krebs Ringer-Bicarbonate-buffered medium (Krebs Improved Ringer I medium (Krebs, 1950). It has been shown that human and animal skin slices take up oxygen and evolve carbon dioxide when incubated in such media (Wohlgemuth and Klopstock, 1926; Gilbert 1962). This avoided the addition of cofactors such as NAD^+, $NADP^+$, ATP.

Histological examination of skin slices which had been incubated for up to 4 hours in Krebs Improved Ringer I medium showed that they retained a relatively normal appearance (photo. 2, p. 46).

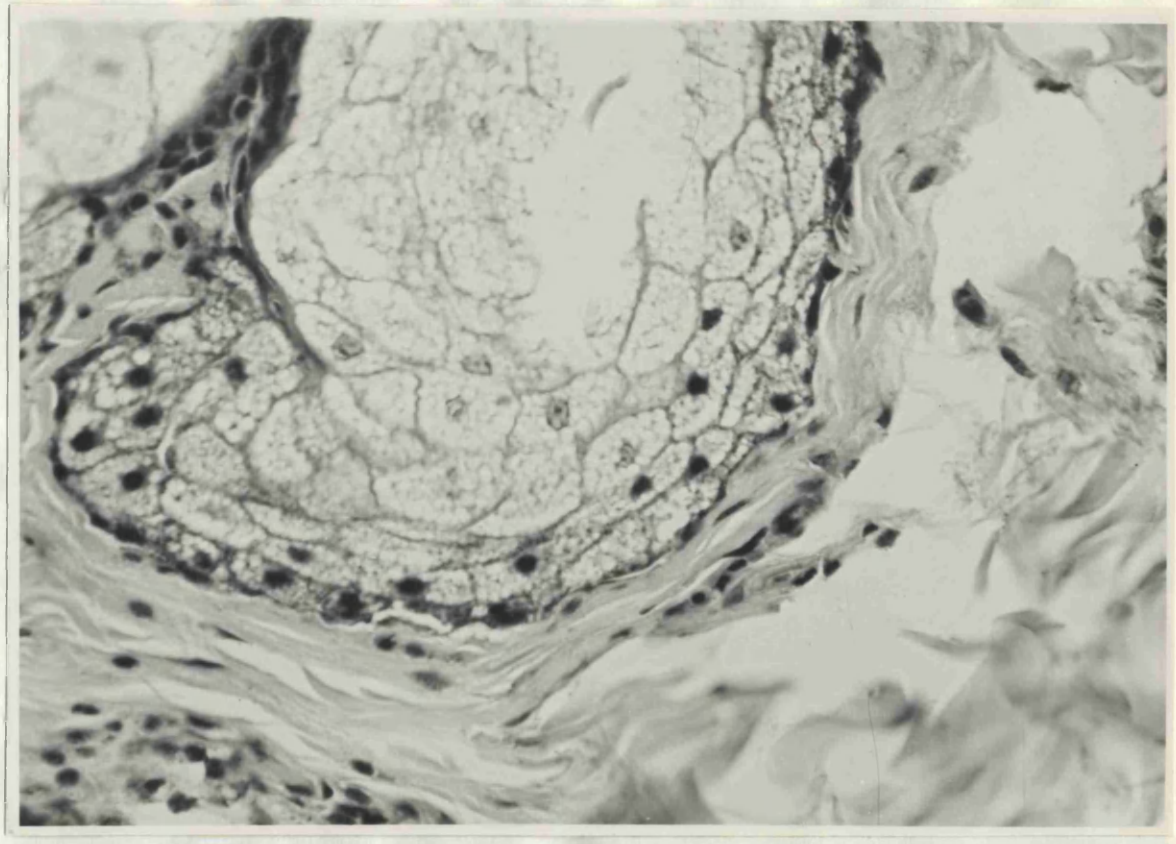
An autoradiographic experiment with male forehead skin showed that [^{3}H]thymidine was incorporated into cell nuclei of sections cut from all levels of 0.5 - 0.7 mm thick skin slices incubated in vitro.

The 0.5 - 0.7 mm thick slices, cut with the multibladed cutter, were incubated with [^{3}H]thymidine in medium 199 (Morgan et al., 1950) under 95%O_2 : 5%CO_2 at 37°C. for 3 hours. The slices were then washed to remove excess [^{3}H]thymidine and frozen on solid CO_2. 10μ thick transverse sections were cut parallel to the largest face of each slice in a cryostat at -20°C. Every other section from a slice was taken on to a glass slide, fixed in calcium formol, dehydrated and then treated to demonstrate incorporation of radioactivity into DNA by the autoradiographic technique (p. 43). The other sections from each slice were treated to demonstrate NADH-diaphorase, NADPH-diaphorase and NAD^+ dependent testosterone 17β-HSD (p. 42).

Results: Uptake of [^{3}H]thymidine into cell nuclei could be demonstrated in sections cut from all levels of the skin slices (photo. 3, p. 47). Cells in the middle of sebaceous acini did not usually show any radioactive nuclei. This is not surprising as the nuclei of these cells are gradually being destroyed (Montagna, 1963) and mitotic figures are rare in the central acinar cells. Most

Photo 2.

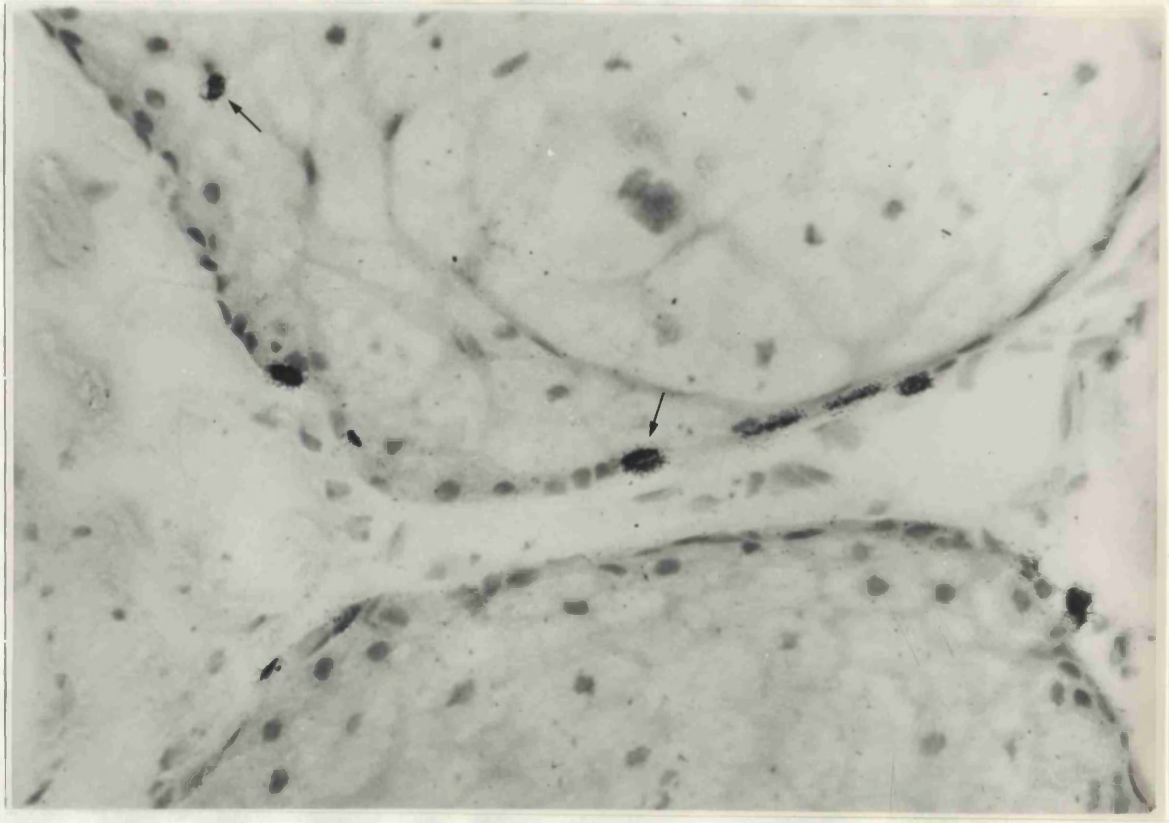
SKIN HISTOLOGY AFTER IN VITRO INCUBATION



Breast skin was cut into 0.5 mm thick slices and incubated for 4 hours in Krebs Improved Ringer I medium at 37°C under an atmosphere of 95% O_2 : 5% CO_2. The skin was fixed in calcium-formol solution, dehydrated with alcohol and embedded in wax. Sections 5μ thick were cut and stained with Haematoxlin and Eosin. The relatively normal appearance of sebaceous gland and surrounding tissue is seen.
Magnification X 650.

Photo 3.

UPTAKE OF [^{3}H]THYMIDINE INTO SEBACEOUS GLAND CELLS.



0.5 mm thick slices of forehead skin were incubated with [^{3}H] thymidine in medium TC.199 as described (p. 45). Sections were cut and the incorporation of ^{3}H into cell nuclei demonstrated by autoradiography.
Incorporation of [^{3}H]thymidine (arrows) into the nuclei of sebaceous gland cells is shown.
Magnification X6650.

cell division in sebaceous glands occurs around the edge of the acini. An occasional cell with radioactive nucleus was seen in the centre of a sebaceous acinus.

After 3 hours of incubation, NADH and NADPH diaphorase still showed a histochemical reaction in all cells of sections cut from all levels of the skin slices. This indicated that the part of the electron transport chain involved in transfer of electrons from NADH to the acceptor nitrobluetetrazolium was still functional. Sebaceous gland cells showed 17β-HSD activity when testosterone was used as substrate and NAD^+ as cofactor.

Conclusion : The 0.5 - 0.7 mm thick whole skin slices are satisfactory for incubation experiments as far as viability and diffusion of small molecules is concerned.

2. PRELIMINARY INCUBATION EXPERIMENT WITH [7α - ^{3}H] DHA.

i. Experimental.

Full thickness skin was obtained from the midline side of the breast of a 40 yr. old woman who underwent mastectomy for breast cancer.

The skin was trimmed of subcutaneous fat, sliced and 2 g of slices were incubated with 43 nmol of [7α- ^{3}H] DHA (sp. act. = 500 mCi/mmol) in 25.0 ml of Krebs Original Ringer Bicarbonate medium (Krebs, 1950) containing glucose (0.23%, w/v). Incubation with shaking was for 3 hours at 37°C. under 95%O_2: 5%CO_2. The steroid was dried down in the incubation flask from methanol: benzene solution containing 4 drops of propylene glycol. The Krebs medium was then added to dissolve the steroid. A control incubation with steroid but no skin was carried out.

The incubations were terminated by adding 50 ml. of acetone. Then the following unlabelled carrier steroids dissolved in ethanol were added to each flask: DHA (500μg), testosterone 500μg), androstenedione (500μg), androstenediol (500μg) and DHA-sulphate (500μg). About 1 g of skin slices were added to the

sulphate (500μg). About 1 g of skin slices were added to the

control and then all flasks were kept at -20°C overnight.

The radioactive steroids were then extracted, as described in Part II Sections 1.i and 1.ii. The neutral steroid extracts were separated in system I. The isolated steroids were then purified to constant specific activity by t.l.c. and derivative formation (Part II section 1.iii - iv) as shown in table 5 below.

table 5.

CHARACTERIZATION OF RADIOACTIVE STEROIDS BY CHROMATOGRAPHY AND DERIVATIVE FORMATION WITH AUTHENTIC CARRIERS.

Compound isolated and chemical reaction	Solvent system	Rf.same as	Specific activity (d.p.m./nmol)
DHA	I	DHA	27,600
acetylated	VI	DHA acetate	26,300
DHAsulphate	IX		
solvolysed	I	DHA	742
acetylated	VI	DHA acetate	743
saponified	I	DHA	790
androstenediol	I		
acetylated	VI	androstenediol diacetate	93.1
saponified	I	androstenediol	92.5
androstenedione	I	androstenedione	34.5
reduced	I	testosterone	30.3
acetylated	VI	testosterone acetate	29.8
testosterone	I		
acetylated	VI	testosterone acetate	12.6
saponified	I	testosterone	7.0
oxidized	I	androstenedione	1.2

(ii) Results :-

There was very little metabolism of DHA in this experiment. Evidence was obtained for formation of [^{3}H]androstenediol, [^{3}H] androstenedione and [^{3}H]DHAsulphate (table 5). Percent conversions are shown in table 6 (p. 50). Radioactivity associated with testosterone carrier fell to 0.005% of the total radioactivity after purification by t.l.c., acetylation and chromic acid

oxidation. The DHA fraction was chromatographed on paper in system XI. Scanning of this chromatogram showed only one peak which had the mobility of DHA. The recovered [^{3}H]DHA accounted for 92% of the total starting radioactivity. Some radioactive material remained at the origin after t.l.c. in system I. This was about 5% of the total radioactivity and in the control about 1%.

---

table 6.

METABOLISM OF [7α-^{3}H] DHA IN BREAST SKIN FROM A WOMAN AGED 40 YEARS.

Radioactive Steroid	% conversion/3 hours/2.0 g tissue
§DHA	§92
DHAsulphate	1.9
androstenediol	0.3
androstenedione	0.1
testosterone	⩽0.005
polar neutral fraction	4

2.0 g of skin slices were incubated for 3 hours at 37°C. in 25.0 ml of Krebs Ringer Bicarbonate Glucose medium with 43.0 nmol [7α-^{3}H] DHA under an atmosphere of 95% O_2 : 5% CO_2.

§ This figure represents initial substrate remaining after 3 hours, ie:% not converted.

(iii) Discussion :-

The conversion of DHA to DHAsulphate confirmed the presence of a steroid sulphotransferase enzyme in human female skin. In the same skin specimen, the activity of Δ^5-3β-HSD and 17β-HSD must have been low. This was shown by the meagre formation of androstenediol (0.3%) and androstenedione (0.1%) and the absence of testosterone formation.

3. METABOLISM OF DHA IN HUMAN FEMALE BREAST SKIN.

EXPERIMENTS 1D AND 2D.

After the preliminary experiment it seemed that the rate of DHA metabolism in breast skin was very slow. As Chakraborty et al. (1970) had found previously that they could increase the percent conversion of DHA by lowering the DHA concentration from 340μM to 17.0μM, it was decided to try some further experiments with breast skin using lower DHA concentrations. In addition, if the 17β-HSD of skin could act on both DHA and androstenedione then the presence of a large excess of DHA over a much smaller concentration of its metabolite androstenedione could effectively inhibit the conversion of the latter steroid to testosterone, if the concentration of the 17β-HSD was small enough to be saturated by the DHA. In such a case, it might be possible to increase formation of testosterone by using a lower initial DHA concentration.

i) Experimental :

Skin samples from the midline side of the breast were obtained from two women who underwent mastectomy as treatment of breast cancer. They were aged 52 years (specimen 1, experiments 1a and 1b) and 48 years (specimen 2, experiments 2a and 2b). The skin appeared grossly normal. The piece from the woman aged 48 showed two coarse hairs but the piece from the woman aged 52, showed no sign of any hairs. Histological examination (Dr. F.M. Vint) showed each specimen to have a normal appearance.

From each skin specimen were cut two pieces weighing about 1 g. The rest was frozen at -20°C and kept for histology. Each piece was trimmed free of subcutaneous fat, weighed and cut into slices 0.5 - 0.7 mm thick. The slices were then incubated in Krebs Improved Ringer I medium (10.0 ml/g tissue) containing [7α-^{3}H] DHA (sp.act. 500 mCi/mmol). For one lot of slices from each specimen (experiments 1a and 2a) the DHA concentration was 3.5μM (17μCi) and for the other lot (experiment 1b and 2b) it

was 0.35μM (1.7μCi). The steroid was dried down from methanol solution containing 50μl of propylene glycol and then dissolved in Krebs medium. Incubation with shaking was for 3 hours at 37°C under 95%O_2 : 5%CO_2.

The incubations were stopped by adding 40 ml acetone. The following carrier steroids dissolved in ethanol were then added to each flask:

DHA	(300μg) (600 μg DHA in control 1c)
DHAsulphate	"
androstenediol	"
testosterone	"
dihydrotestosterone	"
androsterone	"
epiandrosterone	"
etiocholanolone	"
androstenedione	"
5α-androstanedione	"

All flasks were kept at -20°C overnight. The radioactive metabolites were extracted, purified and estimated and their identity and radiochemical purity was established after repeated t.l.c., derivative formation and recrystallization to constant specific activity with authentic carriers (Part II sections 1. ii - 1.iv, 2. Fig.5).

ii) Results :

A radiochromatogram scan after t.l.c. of the neutral fractions in system I showed that there had been much more metabolism of [7α-^{3}H] DHA than in the first experiment. The evidence for identification of those [^{3}H] metabolites purified by derivative formation is given in table 7 (p. 55) and of those purified by recrystallization in tables 8a - h (pp. 56-60). Percent conversions to the different metabolites are shown in table 9 (p. 65). Identification methods for certain DHA metabolites deserve further mention :

(a) The androstenediol fraction isolated after t.l.c. of the diacetate and free steroid could have contained various isomers of androstanediols. The fact that the radioactivity remaining

with androstenediol after recrystallization accounted for 85% or more of the radioactivity in the androstenediol carrier fraction (table 8d) eliminated the possibility of a significant formation of most androstanediols (5α and 5β). However, in view of the evidence for some dihydrotestosterone formation the androstenediol fraction was also checked for the presence of 3α-androstanediol and 3β-androstanediol. Aliquots of the androstenediol fractions from experiments 1a and 2a were crystallized with 3α-androstanediol. After two crystallizations less than 2% of the radioactivity remained with the crystals. Therefore it was concluded that neither androstenediol fraction contained 3α-androstanediol in measurable quantity (<0.01% conversion). Further aliquots from the same experiments were crystallized with 3β-androstanediol. After five crystallizations, the specific activities had fallen to 51% and 62% of the theoretical values (for 100% purity) without having become constant. It is probable that androstenediol and 3β-androstanediol cocrystallize like DHA and epiandrosterone (see below). There was insufficient material for any further investigations. However, as crystallization with androstenediol gave constant specific activity of 85% or more of the theoretical value, it was concluded that this compound was the major component of this fraction.

(b) epiandrosterone :

Evidence for conversion of [7α-^{3}H] DHA to [^{3}H] epiandrosterone appears in table 8f. It was noticed that the epiandrosterone fraction from the control incubation, which contained radioactivity after chromatography, could not be cleared of this radioactivity after crystallizations with authentic epiandrosterone. Similarly, the specific activity of the epiandrosterone fraction from experiment 1a, fell gradually over 4 crystallizations without becoming constant. Chromatographic separation of epiandrosterone and DHA is difficult and possibly the epiandrosterone fractions from the control and experiment 1a were contaminated by [^{3}H] DHA which might cocrystallize with epiandrosterone carrier. Possible cocrystallization of DHA and epiandrosterone

was therefore checked in the following way :
26,000 c.p.m. of DHA (purified chromatographically in systems I and XI) were added to 12.0 mg of unlabelled epiandrosterone, which was then recrystallized from acetone-hexane:-

Specific Activity of the Crystals (c.p.m./mg.)	No. of Crystallizations	% Theoretical sp.act.
1690	1	79.2
1445	2	67.7
1380	3	64.8
1165	6	54.6
1000	+ 8	46.8

+ carrier acetylated before 7th crystallization.

Obviously, recrystallization of neither free steroids nor acetates was satisfactory for distinguishing epiandrosterone from DHA.

When a Δ^5-3β-hydroxysteroid is oxidised with chromic acid, it forms a Δ^4-3,6 diketone, however an A-ring saturated 3β-hydroxysteroid simply forms the corresponding 3-monoketone. If [7α-^{3}H] DHA is oxidized to 4-androstene-3,6,17-trione tritium is lost from the steroid molecule, possibly due to enolization of the 6-ketone (Pierrepoint et al., 1967). Such a reaction should be useful for distinguishing [7α-^{3}H] DHA from [7α-^{3}H] epiandrosterone. 43,000 c.p.m. of [7α-^{3}H] DHA were mixed with 5.16 mg of unlabelled epiandrosterone. The mixture was oxidized with chromic acid (p. 40), and the recovered 5α-androstanedione precipitated from water then recrystallized once from aqueous acetone. The specific activity of the crystals was 67 c.p.m/mg (0.8 % theoretical value), showing that this was a good method of distinguishing [7α-^{3}H] epiandrosterone and [7α-^{3}H] DHA. The epiandrosterone fractions from experiments 1, 2 and control were therefore oxidized with chromic acid after 4 crystallizations. In experiments 1b, 2a, 2b, the specific activity of the crystals (which was constant before oxidation) remained unchanged. This

Table 7.

METABOLISM OF DHA IN HUMAN FEMALE BREAST SKIN

INCUBATIONS 1a 1b 2a 2b CONTROL 1c.

CHARACTERIZATION OF RADIOACTIVE STEROIDS BY CHROMATOGRAPHY AND DERIVATIVE FORMATION WITH AUTHENTIC CARRIER STEROIDS.

Steroid investigated and chemical reaction	Rf. same as that of	Solvent system	S 1a
like DHA	DHA	XI	25800
acetylation	DHA acetate	VI	22600
like androstenedione	androstenedione	II	653
reduction	testosterone	I	678
acetylation	testosterone acetate	III	606
like testosterone	testosterone	XI	-
acetylation	testosterone acetate	VI	32.6
saponification	testosterone	I	21.8
oxidation	androstenedione	I	21.3
reduction	testosterone	I	20.2
like androstenediol	androstenediol	I	-
acetylation	" diacetate	VI	448
saponification	androstenediol	III	398
like 5α-androstanedione	5α-androstanedione	II	-
"	"	III	1740
"	"	V	1700
like DHAsulphate	DHAsulphate	IX	-
solvolysis	DHA	I	1210
	DHA	XI	983

Table 8a-8h.

METABOLISM OF DHA IN HUMAN BREAST SKIN.

EXPERIMENTS 1a, 1b, 2a, 2b AND CONTROL 1c.

IDENTIFICATION OF RADIOACTIVE STEROIDS BY RECRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY.

Breast skin was incubated with [7α-^{3}H]DHA at a concentration of 3.5 μM (17.0 μCi) in incubations 1a, 2a and 1c or 0.35 μM (1.7 μCi) in incubations 1b and 2b.
Radioactive steroids isolated by chromatography were mixed with authentic carrier and recrystallized to constant specific activity.
Specific activities (1, 2, 3) were measured after successive crystallizations.
+c = sp.act. of crystals, m = sp.act. of mother liquors.

Table 8a

steroid : [^{3}H]DHA derivative crystallized: acetate.
solvents : acetone-water.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3		
	+ c	m	c	m	c	m	
1a	8220	8020	8020	8150	7500	7750	98
1b	2165	2180	2170	2170	2185	2060	103
2a	12400	12700	12500	12800	12500	-	100
2b	1900	2360	1920	1990	1930	-	97
1c	17900	17300	16800	17400	17100	-	102

Table 8b.

steroid : [^{3}H]DHAsulphate. derivative crystallized: DHA.

solvent : acetone-hexane.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3		
	c	m	c	m	c	m	
1a	6130	5920	6020	-	6030	5350	99
1b	695	718	685	-	705	625	92
2a	3510	3390	3510	-	3480	3430	97
2b	495	506	487	-	482	484	85

Table 8c.

steroid : [^{3}H] dihydrotestosterone carrier : 20.0mg

derivative crystallized : parent steroid and acetate.

solvents : acetone-hexane, acetone-water, methanol-water.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2	3	4	5 +	
	c	m	c	c	c	c	
1a	247	1480	46	52	41	33	22
2a	502	1300	399	377	393	353	42
2b	184	219	162	172	161	-	54

+ Because of poor recoveries of crystals from the first and second recrystallizations, there was not enough material left to recrystallize for a fifth time. Therefore the mother liquor residues obtained after crystallizations 1 - 4 were combined, crystallized 4 times, acetylated and the acetates crystallized a further 3 times before specific activity measurement.

Table 8d.

steroid : [^{3}H] androstenediol

derivative : -

solvent : acetone-hexane.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3	4	
	c	m	c	m	c	m	
1a	1470	1465	1605	1590	1380	1505	90
1b	161	210	133	-	188	-	92
2a	1060	1090	1080	1315	932	1083	87
2b	221	251	205	161	173	211	85

Table 8e.

steroid : [^{3}H] androsterone carrier : 20.0 mg

derivative : -

solvent : acetone-hexane.

	Specific Activity (d.p.m./mg)								% calculated sp.act.
	1		2		3		4		
	c	m	c	m	c	m	c	m	
1a	1590	1680	1420	-	1480	-	1550		92
1b	585	615	605	-	642	588	600	-	86
2a	14800	15200	14900	-	14900	14300	15100	-	91
2b	4070	3910	4420	-	4270	4380	4500	-	103

Table 8f.

steroid : [^{3}H] epiandrosterone carrier : 20.0 mg

derivative : parent steroid; 5α-androstanedione

solvents : acetone-hexane, acetone-water.

	Specific Activity (d.p.m./mg)										% calculated sp.act.
	1		2		3		4	5	6+	7+	
	c	m	c	m	c	m	c	c	c	c	
1a	3110	6080	3100	4475	2740	-	2540	2490	343	-	8
1b	161	380	124	202	161	166	121	161	159	-	89
2a	2820	4620	2920	3000	3020	3000	3050	3050	2900	2950	73
2b	309	648	305	245	259	-	284	326	290	285	70

+ crystals oxidized to 5α-androstanedione before the 6th recrystallization.

Table 8g.

steroid : [^{3}H] androstenedione. derivative crystallized: testosterone acetate.

solvents : acetone-water, acetone-hexane.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3		
	c	m	c	m	c	m	
1a	868	1051	881	880	888	-	90
1b	64.8	87.3	62.9	78.4	59.7	-	65
2a	607	656	610	648	598	-	92
2b	122	157	115	134	119	-	76

Table 8h.

steroid : [^{3}H] 5α-androstanedione.

solvents : acetone-water

Specific Activity (d.p.m./mg)

	1		2		3	4	% calculated sp.act.
	c	m	c	m	c	c	
1a	3260	2800	3240	-	2730	3040	98
1b	not crystallized						
2a	5350	4850	5070	-	5020	4780	100
2b	1930	1390	1910	1900	1925	1770	102

was taken as good evidence that the radioactivity in these fractions was [^{3}H]epiandrosterone. Radioactivity was lost from control crystals after oxidation. The specific activity of theepiandrosterone crystals from 1a fell to 8% of the value immediately before crystallization. This residual radioactivity possibly represented [^{3}H]epiandrosterone.

(c) etiocholanolone :

Radioactivity was present in this fraction isolated from tissue and control incubations. However, after recrystallization twice with authentic etiocholanolone only 5% of this remained with the crystals ($\ll$0.001 % conversion).

(d) 5β-androstane-3,17-dione :

100μg of this carrier was added to the 5α-androstanedione fractions before t.l.c. in system V, which separates these two isomers. After t.l.c., some radioactivity remained with 5β-androstane-3,17-dione carrier. This carrier was seen on g.l.c. to be contaminated with about 10% of 5α-androstanedione. After another t.l.c. in system V all measurable radioactivity was lost from 5β-androstane-3,17-dione carrier.

(e) Sulphate Fraction :

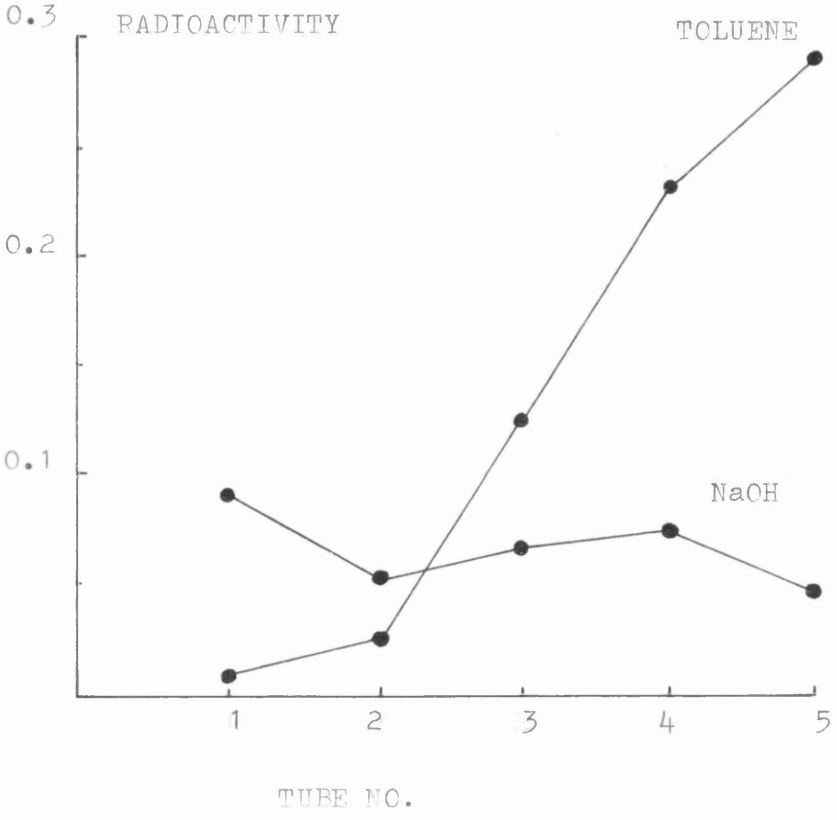
The neutral steroid fraction obtained after solvolysis of the sulphate fractions was separated according to Fig. 5 (p.30). Only the DHA carrier was associated with a significant amount of radioactivity. This provided evidence for conversion of DHA to DHAsulphate (tables 7, p. 55 and 8b p.57).

(f) Polar Fraction :

This fraction remained at the origin after t.l.c. of the neutral steroid fraction in system I. It accounted for 10 -23% of the total radioactivity in tissue incubations, no correction being made for extraction losses. In the control incubation, it accounted for 0.8% of the total radioactivity. The polar fractions from 1a and 2a were combined. An aliquot was partitioned 4 times countercurrently between toluene and 0.1 M NaOH (Fig. 9 p.62).

90% of the radioactive material was extracted into the toluene fractions. As seen in Fig. 9, there was some residual

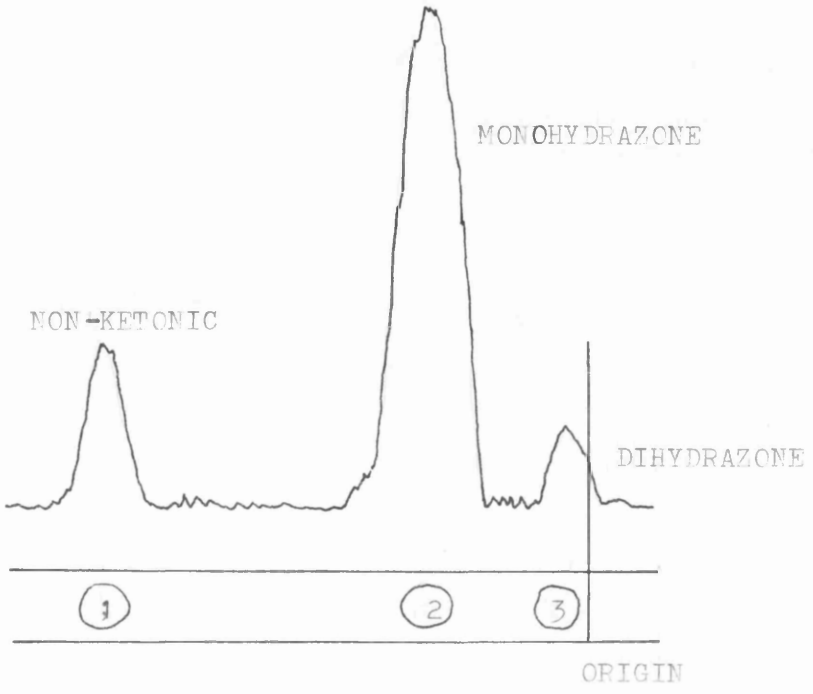
FOUR TRANSFER COUNTERCURRENT DISTRIBUTION OF POLAR FRACTIONS POOLED FROM INCUBATIONS 1a AND 2a BETWEEN TOLUENE AND 0.1M-NaOH.



27,000 c.p.m.of polar fraction (non-conjugated material remaining on the origin after t.l.c. in system I) were partitioned four times countercurrently between 2.0 ml volumes of toluene and 0.1M-NaOH. Aliquots of the toluene and NaOH phases from each tube were removed for counting. Radioactivity in each phase is expressed as a fraction of the total c.p.m. partitioned .

Fig. 10

REACTION OF COMBINED POLAR FRACTION FROM EXPERIMENTS 1a AND 2a WITH GIRARD'S REAGENT-T



20,000 c.p.m. of polar fraction were applied to a silica gel t.l.c. plate and reacted in situ with Girard's reagent-t overnight, by the method of Lisboa (1966). Marker steroids were treated similarly on the same plate. The plate was then run in a solvent system consisting of the upper phase obtained after shaking together equal volumes of butan-1-ol, 2-methyl-propan-2-ol and water. Scanning of the chromatogram for radio-activity revealed two peaks, a major one having the mobility of a steroid monohydrazone and a minor one having the mobility of a free steroid. Marker steroids were visualized by spraying the plate with a solution of conc.H_2SO_4 in ethanol (50%, v/v) and heating at 110°C.

1. = 3β-androstanediol standard + unreacted DHA and androstenedione.

2. = DHA and androstenedione monohydrazones.

3. = androstenedione dihydrazone.

radioactivity which partitioned in favour of NaOH solution. This alkali soluble material could not be any more readily extracted into toluene or diethyl ether after acidification of the aqueous solution, showing that it was not necessarily phenolic.

Another aliquot of the polar fraction (20,000 c.p.m.) was run on t.l.c. in system X (chloroform : methanol : water, 200 : 9 : 0.75, v/v). The plate was scanned and one radioactive peak was seen, which appeared to run just behind a 5-androstene -3β,16α,17β-triol standard (Rf.0.16). Another aliquot of the polar material was acetylated. This changed the mobility in System X to Rf. 0.78, suggesting the presence of esterifiable hydroxyl groups. t.l.c. in system VI (benzene : ethyl acetate, 180 : 20 v/v) showed an Rf. 0.12; this is less than that of 5-androstene-3β,16α,17β-triol triacetate (Rf. 0.26), 16α-hydroxy-DHA-diacetate (Rf. 0.29) or testosterone acetate (Rf. 0.13), suggesting that the material had ketone or unesterifiable hydroxyl groups. An aliquot of the original polar fraction was reacted with Girard's reagent-t (trimethylacetohydrazide) by the method of Lisboa (1966) and the resulting products run on t.l.c. as described in Fig. 10 (p.63). Two similarly sized peaks were seen on scanning, one of Rf. 0.21 indicating a monohydrazone and another of R.f. 0.70 indicating an unreacted steroid. When another aliquot of polar fraction was reacted with a greater excess of Girard's reagent the monohydrazone peak accounted for about 80% of the radioactivity on the t.l.c. plate and the unreacted material about 15% (Fig. 10). It was concluded that the polar material had one ketone and at least two hydroxyl groups.

(g) 16α-hydroxy DHA (3β,16α-dihydroxy-5-androstene-17-one).

200μg. of this steroid were added to the androstenediol fraction from experiment 2a after t.l.c. in system I. Acetylation and t.l.c. in system VI separated the two carriers. There was radioactivity in the 16α-hydroxy DHA diacetate fraction. The fraction was saponified and chromatographed on silica gel in cyclohexane:ethyl acetate (1:1, v/v). The carrier was still

Table 9.

METABOLISM OF DHA IN HUMAN FEMALE BREAST SKIN.

PERCENT CONVERSION/3 HOURS.

	Female 52 years		Female 48 years	
	1a	1b	2a	2b
DHA [§]	58.5	48.3	72.3	40.0
DHAsulphate	2.4	3.1	1.6	2.9
androstenediol	1.0	0.7	0.8	1.0
androstenediol sulphate	-	-	-	-
androsterone	0.5	3.1	5.5	14.6
epiandrosterone	0.1	0.6	0.8	1.1
testosterone	0.05	0.08	0.03	0.20
dihydrotesterone	0.05	-	0.2	0.7
androstenedione	1.5	1.8	1.2	4.1
5α-androstanedione	4.2	6.4	4.5	9.8
polar neutral steroids	20	18	10	23

[+] Initial DHA concentration: a = 35μM b = 0.35μM

[§] = % steroid substrate recovered.

Breast skin was incubated in Krebs Improved Ringer I medium with [7α-^{3}H]DHA for 3 hours at 37°C under an atmosphere of 95%O_2 : 5%CO_2.

The radioactive products were isolated and estimated as described in the text.

radioactive and remained so after further t.l.c. in cyclohexane: ethyl acetate:ethanol (8:8:2, v/v). The radioactivity in the 16α-hydroxy DHA fraction was now about 0.1% of the total starting d.p.m. (uncorrected for losses). However, when this fraction was recrystallized with 6.0 mg of authentic 16α-hydroxy DHA constant specific activity could not be obtained (specific activities of crystals (d.p.m./mg) = 312, 261, 245, theoretical value = 426).

iii) Discussion.

Experiments 1a/b and 2a/b showed that there are four reactions of DHA in human female breast skin:

1. Oxidation of the 3β-hydroxyl and isomerization of the C 5-6 double bond to form androstenedione.
2. Reduction of the 17-ketone to form androstenediol.
3. Hydroxylation probably on at least two different carbon atoms.
4. Conjugation with sulphate to form DHAsulphate.

The percent conversions to androstenedione were 1.2 - 4.1%. On their own, these results would give a considerable underestimate of Δ^5-3β-HSD activity. The percent conversions to 5α-androstanedione, androsterone and epiandrosterone add up to 5.7 - 28%. These three steroids are most likely formed from androstenedione by the action of 5α-reductase, 3α-HSD and 3β-HSD, (Fig.1, p. 7). Therefore, the rate of conversion of DHA to androstenedione measured from the total of all 4 metabolites ranges from 7% - 32% conversion/3 hours. It is worth noting that the percent conversion to androsterone always exceeded that to epiandrosterone (ratio 5/1 - 14/1). This shows that in breast skin, the 3α-reduction of 5α-androstanedione predominates over 3β-reduction. In theory, epiandrosterone could be formed by direct reduction of the C5-6 double bond of DHA. This reaction has never been demonstrated in other tissues while the 3β-reduction of 5α-androstanedione is well known (Ungar, 1960; Rubin and Strecker, 1961). However the occurrence of direct conversion of DHA to epiandrosterone would represent a potential route to dihydrotestosterone involving neither androstenedione nor testosterone. It might be noted that evidence has been

obtained for direct conversion of cholesterol (3β-OH -Δ^5) to 5β-cholestan-3β-ol (Rosenfeld et al., 1954).

[^{3}H]testosterone and [^{3}H]dihydrotestosterone were isolated from experiments 1 and 2 (tables 7 & 8c.pp.55&57). This is proof that these steroids can be formed from DHA in skin. The higher % conversion to dihydrotestosterone than to testosterone could indicate a rapid further conversion of testosterone by 5α-reductase or a conversion of 5α-androstanedione to dihydrotestosterone. There is no direct evidence that 5α-androstanedione can be a substrate for 17β-HSD in skin, however the reverse reaction (i.e. oxidation of dihydrotestosterone) has been demonstrated histochemically in sebaceous glands (Calman, 1970a). The other possible route to testosterone and dihydrotestosterone is via androstenediol. The existence of this pathway depends on whether the Δ^5-3β-HSD can use androstenediol as a substrate. Whatever the pathway of testosterone and dihydrotestosterone formation, it is obvious that in experiments 1 and 2, these compounds were only minor metabolites compared to the formation of 17-oxosteroids.

It is difficult to assess the importance of androstenediol formation relative to that of androstenedione without knowing whether androstenediol is further metabolized. If the percent conversions to testosterone and dihydrotestosterone are added on to the conversions to androstenediol this only brings the total to a maximum of 1.9% in experiment 2b. However, the possibility that androstenediol is a precursor of polar fraction cannot be dismissed on the evidence available.

On the basis of the chromatographic experiments, it is suggested that the polar fraction contains one or more steroids with one ketone and 2 or 3 hydroxyl groups. There are three important possible metabolites which can be excluded as components of the polar fraction:

7α-hydroxy DHA. Formation of this compound from [7α-^{3}H] DHA would have resulted in a loss of [^{3}H].

7-keto DHA. Formation of this compound would also result

in loss of [^{3}H].

Faredin et al. (1969) reported the conversion of [4-^{14}C] DHA to the above steroids in human skin. Experiments 1 and 2 provide evidence for formation in skin of other hydroxylated DHA metabolites.

5-androstene-3β,16α,17β-triol. The fact that the polar material is ketonic rules out this possibility. Oertel & Treiber (1969) isolated the sulphate of this compound from skin after injection of [7α-^{3}H]DHA-[^{35}S]sulphate into a man. Our results show that it is not a major metabolite of DHA in-vitro.

4. [^{3}H]DHAsulphate was the only [^{3}H] compound which could be isolated from the sulphate fraction. This indicates that it was not further metabolized. In addition, the fact that there was no evidence for [^{3}H] androsterone or [^{3}H] polar fraction in the sulphate fraction, indicated that these steroids are not acted on by sulphotransferase in human skin.

The results from experiments using 3.5μM DHA (experiments 1a and 2a) and 0.35μM DHA (1b and 2b) show some interesting differences.

If the activity of Δ^{5}-3β-HSD is measured from the total conversion to androstenedione and 5α-steroids then a 10-fold reduction of DHA concentration (i.e. from 3.5μM to 0.35μM) resulted in a 5-fold reduction in the rate of oxidation of DHA (i.e. a 2-fold increase in % conversion/3 hours) as shown by the % conversion ratios for experiments 1a and 1b and 2a and 2b (1a/1b = 0.53, 2a/2b = 0.41). If it is assumed that the Δ^{5}-3β-HSD reaction followed Michaelis-Menton type kinetics, then if both DHA concentrations were sufficient to saturate the enzyme (i.e. reaction zero order with respect to substrate concentration) a 10-fold change in substrate concentration should not alter the true reaction rate, i.e. for a 10-fold reduction the % conversion should increase 10 times. If neither DHA concentration was sufficient to saturate the enzyme (i.e. reaction first order with respect to substrate concentration for 3.5μM and 0.35μM) then a 10-fold decrease in substrate concentration should result in a 10-fold decrease in reaction rate, i.e. the

% conversion should not be altered. The observed results fall between these two extremes. This suggests that the 3.5μM DHA concentration was sufficient to saturate the enzyme while the 0.35μM DHA concentration was not.

The % conversions to testosterone and dihydrotestosterone were higher in experiment 2b than 2a, but the increase was not strikingly greater than that for the other steroids.

The % conversions to androstenediol were similar (0.7 - 1.0%) in all experiments. Without a knowledge of whether androstenediol is further metabolized, it is not possible to interpret this finding.

All the above arguments are based on highly simplified assumptions. However, one practical point emerged from the experiments with the two DHA concentrations. This was that there was no great advantage to be gained by using DHA concentrations less than 3.5μM as the increased % conversions obtained did not compensate for the smaller amount of radioactivity which could be used (the specific activity of the [7α-^{3}H]DHA available being 500 mCi/mmol). This point was particularly relevant later when experiments had to be carried out with much smaller skin samples.

Experiments 1 and 2 confirmed the conversion of DHA to testosterone in human skin and further showed that DHA was converted to dihydrotestosterone. However, these two compounds were only minor metabolites compared to formation of polar fraction, androstenedione and other 17-oxosteroids. It is therefore fair to say that under the experimental conditions used, DHA was converted mainly to compounds of low androgenic activity by human female breast skin. In addition, it has been shown that in an incubation system such as was used in experiments 1 and 2, the measurement of conversion of DHA to androstenedione and testosterone alone gives a considerable underestimate of the rate of DHA metabolism because of the high activity of 5α-reductase in skin.

4. METABOLISM OF DHASULPHATE IN HUMAN FEMALE BREAST SKIN.

EXPERIMENT 1 DS

i. Experimental.

A piece of breast skin was obtained from a woman aged 56 years who underwent mastectomy for breast cancer. Histological examination (Dr. F.M. Vint) showed a normal appearance with rather small sebaceous glands. There were no visible coarse terminal hairs. The specimen was used for a preliminary investigation of DHAsulphate metabolism. The specimen was trimmed of fat, weighed and sliced as before. 1.0 g of slices were incubated in 10.0 ml of Krebs Improved Ringer I medium containing 3.5μM -[7α-^{3}H]DHAsulphate (8.96 x 10^6 d.p.m.). Incubation with shaking was for 3 hours at 37°C under 95% O_2 : 5% CO_2. A control containing steroid but no tissue was carried out. The DHAsulphate was dried down from methanol solution containing 100μl of propylene glycol and then dissolved in 22.0 ml of Krebs Improved Ringer I medium. 10.0 ml of this solution were used for the tissue incubation and 10.0 ml for the control.

The reactions were stopped by adding 40 ml of acetone. Carrier steroids were then added as in experiments 1 and 2. The radioactive steroids were then extracted, purified by t.l.c. and paper chromatography (Part II sections 1.ii, 2, Fig.5) and their identity and radiochemical purity established by recrystallization to constant specific activity (Part II sections 1.iv, 5, Fig.4).

ii. Results.

Evidence for identity of metabolites purified by recrystallization to constant specific activity is shown in table 10 (p.71). Table 11 (p.72) shows % conversions of DHAsulphate to the various metabolites.

The identification of [^{3}H]androstenediol sulphate was based on the recovery of [^{3}H]androstenediol from the sulphate fraction after solvolysis. This gave no indication of whether the sulphate group was at C3 or C17.

[^{3}H]epiandrosterone sulphate was identified in control and tissue incubations. Radioactive material which co-crystallized

Table 10.

METABOLISM OF DHASULPHATE IN HUMAN FEMALE BREAST SKIN.

IDENTIFICATION OF RADIOACTIVE STEROIDS AFTER RECRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY WITH AUTHENTIC CARRIERS.

Experiment 1DS. Breast skin from a 56 yr. old woman was incubated with [7α-^{3}H]DHAsulphate at a concentration of 3.5μM (8.96×10^6 d.p.m.) for 3 hours as described in the text.
Specific activities were measured after successive recrystallizations and were all determined on the crystals.

Steroid	Specific Activity (d.p.m./mg)			
	1	2	3	4
DHA	7170	8050	7380	-
DHAsulphate ^{o}	79600	76700	77800	-
androstenediol	79	95	78	73^{+}
androstenediol sulphate ^{o}	103	103	98^{+}	-
androsterone	281	276	259	278^{+}
androstenedione	186	178	180	184
5α-androstanedione	451	475	470	-

o crystallized as the free steroid after solvolysis with ethyl acetate/H_2SO_4 (p.21).

+ crystals acetylated before 3rd or 4th recrystallization and sp.act. measurement (p.39).

carrier for crystallization : 10.0mg

Table 11.

METABOLISM OF DHASULPHATE IN HUMAN FEMALE BREAST SKIN.

PERCENT CONVERSION/3 HOURS.

DHA	2.3
§DHAsulphate	91.0
androstenediol	0.05
androstenediol sulphate	0.1
androsterone	0.2
epiandrosterone	-
testosterone	-
dihydrotestosterone	-
androstenedione	0.06
5α-androstanedione	0.2
polar neutral steroids.	-

§ = % steroid substrate recovered.

Breast skin was incubated in Krebs Improved Ringer I medium with [7α-^{3}H]DHAsulphate for 3 hours at 37°C under an atmosphere of 95% O_2 : 5% CO_2. The radioactive products were isolated and estimated as described in the text.

with epiandrosterone was isolated after solvolysis of the sulphate fraction. The radioactivity remained with the epiandrosterone carrier after oxidation to 5α-androstanedione. It was concluded that [^{3}H]epiandrosterone sulphate (about 0.02%) was either present as an impurity in the [7α-^{3}H] DHAsulphate or was formed by decomposition during incubation. DHAsulphate, androstenediol sulphate and epiandrosterone sulphate were the only radioactive steroids detected after solvolysis of the sulphate fraction, even though the solvolysates were subjected to a complete chromatographic and recrystallization analysis.

iii. Discussion.

A conversion of DHAsulphate to neutral steroids in human female skin was demonstrated (2.8%/3hours). This indicates the presence of steroid sulphatase in skin.

It appeared to be the rate of hydrolysis of DHAsulphate which limited its metabolism. The isolation of [^{3}H]androsterone, [^{3}H]5α-androstanedione, [^{3}H]androstenedione and [^{3}H]androstenediol as well as [^{3}H]DHA shows that DHA liberated from DHAsulphate underwent a similar metabolism to that of exogenously administered DHA. Even though the % conversions to the different metabolites were small, the overall pattern of metabolites which accumulated after 3 hours was similar to that in experiments 1 and 2 (using [7α-^{3}H] DHA). The most obvious feature of the results (table 11) is the greater % conversion to androsterone and 5α-androstanedione than to androstenedione, once again indicating that there was an active 5α-reductase in the skin which metabolized the androstenedione as it was formed.

No formation of [^{3}H]testosterone or [^{3}H]dihydrotestosterone could be detected in experiment 1 DS. However, when the low % conversion of DHA (experiments 1 and 2) to these compounds is considered along with the low % conversion of DHAsulphate to neutral steroids in 1 DS it is not surprising that any formation of testosterone or dihydrotestosterone was below the sensitivity limit of experiment 1DS (<0.01%); e.g.: in experiment 2a, (table 9, p.65) the ratio of % conversions of DHA to:

$$\frac{\text{testosterone + dihydrotestosterone}}{\text{androstenedione + 5α-androstanedione + androsterone}} = 0.02$$

= 0.02. An equivalent ratio in experiment 1 DS would mean a total % conversion to testosterone + dihydrotestosterone of only 0.008%.

Another interesting result was the absence of any polar neutral steroid formation (tissue incubation = 0.1%, control = 0.2%). This contrasts with the DHA experiments where polar neutral steroids were major metabolites.

Analysis of the [^{3}H]steroids obtained after solvolysis of the sulphate fraction showed that [^{3}H]androstenediol sulphate had been formed from [^{3}H]DHAsulphate but there was no evidence for formation of sulphoconjugated androstenedione, testosterone, A-ring saturated steroids or polyhydroxylated steroids. This experiment therefore, provided no support for the hypothesis that DHAsulphate is extensively metabolized in human skin without loss of the 3β-sulphate. Oertel and Treiber (1969), put forward this idea when they isolated ^{3}H/^{35}S labelled C_{19}-steroid sulphates from human skin after injecting [7α-^{3}H]DHA[^{35}S]sulphate into a man.

5. METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN HUMAN MALE FOREHEAD SKIN.

EXPERIMENTS 3 AND 4 (DS, D, AE, T).

Pieces of forehead skin were obtained from two men aged 58 (specimen 3, expts. 3DS, 3D, 3AE, 3T) and 83 years (specimen 4, expts. 4DS, 4D, 4AE, 4T). This was normal skin obtained during plastic surgery. The forehead skin is characterized by its numerous, large sebaceous glands with only tiny vellus hairs (Montagna, 1963). Strauss and Pochi (1963) have shown that in men, there is no great decrease in sebaceous secretion up to around 80 years of age and that this secretion is still androgen stimulated as shown by the considerable drop in sebum secretion observed when old men were castrated. The two

skin specimens therefore provided an opportunity to study steroid metabolism in skin dominated by its sebaceous glands. This was the nearest possible approach to studying human sebaceous gland metabolism without actually dissecting the pure glands.

i) Experimental :

It was decided to study the metabolism of DHAsulphate, DHA, androstenedione and testosterone in each skin specimen. The incubation system was scaled down because of the small amounts of tissue available. The steroid concentration, tissue/steroid ratio and tissue/medium ratio were kept the same as in previous experiments.

A small piece from each specimen was taken for histology and histochemistry. The rest of each specimen was trimmed of fat and cut into 4 pieces which were weighed and cut into 0.5 - 0.7 mm thick slices as before. Slices were incubated in 2.0 ml, 1.0 ml, or 0.5 ml of Krebs Improved Ringer I medium with [7α-^{3}H] DHA (sp.act., 0.5 Ci/mmol) [7α-3H]DHAsulphate (sp.act., 20Ci/mmol) or [7α-^{3}H]androstenedione (sp.act., 5.0 Ci/mmol) or [7α-^{3}H] testosterone (sp.act., 1.5 Ci/mmol). Table 12 (p.76) shows the tissue weights, volume of medium, quantity of steroid, etc., for each incubation. The [^{3}H] steroids were dissolved as before in Krebs Improved Ringer I medium with propylene glycol (5μl/ml medium). Controls containing

Table 12

EXPERIMENT 3. INCUBATION CONDITIONS.

Wt.Skin(mg)	Steroid Incubated	nmol steroid	10^{-6} x d.p.m. ^3H	ml medium
63	DHAsulphate	3.5	12.5	1.0
180	DHA	6.2	6.9	2.0
88	androstenedione	3.5	16.9	1.0
190	testosterone	6.8	16.7	2.0

EXPERIMENT 4. INCUBATION CONDITIONS.

Wt.Skin(mg)	Steroid Incubated	nmol steroid	10^{-6} x d.p.m. ^3H	ml medium
89	DHAsulphate	3.5	12.3	1.0
96	DHA	3.5	2.45	1.0
48	androstenedione	1.75	4.76	0.5
44	testosterone	1.75	4.76	0.5

[^{3}H] steroids but no skin were incubated at the same time as the experiments. (CDS, CD, CAE, CT.)

Reactions were stopped with 5 ml. of acetone then the following carriers dissolved in ethanol (total volume 6 - 8 ml) were added:-

DHAsulphate	250µg.	)
DHA	"	) added to DHA and DHAsulphate incubations only
3α-androstanediol	"	
3β-androstanediol	"	
androstenediol	"	
testosterone	"	
dihydrotestosterone	"	
androsterone	"	
epiandrosterone	"	
etiocholanolone	"	
androstenedione	"	
5α-androstanedione	"	
5β-androstane-3, 17-dione	"	

The mixtures were kept at -20°C then the radioactive steroids were isolated, purified by t.l.c. and paper chromatography (Part II sections 1.ii and 2, Fig.5) and their identity and radiochemical purity established by recrystallization to constant specific activity (Part II, sections 1.iv, 5, Fig.4).

ii) Results.

The evidence for identity and radiochemical purity of those metabolites which were crystallized to constant specific activity with authentic carriers is given in tables 13a-m (pp. 80 - 86). % conversions to the different metabolites are shown in tables 15, 16 (pp. 89 & 90). As the tissue/steroid ratios, tissue/medium ratios and steroid concentrations were kept constant in the different incubations the results expressed as % conversions can be compared directly between incubations. An exception to this is experiment 3DS in which the mass of tissue used was less than that used in other incubations with 1.0 ml of medium.

The details of the identification of certain metabolites

need to be further explained.

a) The androstanediol and androstenediol carriers were isolated as a group and estimated together by g.l.c. using an SE.30 column. The three compounds all had the same retention time so only one peak was observed when the mixture was run on g.l.c. As all three compounds showed similar molar responses on the flame ionization detector measurement was made with reference to an androstenediol standard and the recovery of each carrier was taken to be one third of the total. This assumed that the recovery of all three carriers was similar. Those fractions which contained sufficient radioactivity were divided up and aliquots were crystallized with androstenediol, 3β-androstanediol and 3α-androstanediol (tables 13e, f, g. pp. 82 and 83). Evidence was obtained for the conversion of DHAsulphate and DHA to androstenediol and for conversion of DHAsulphate to androstenediol sulphate. Testosterone and androstenedione were both converted to 3α-androstanediol and 3β-androstanediol. The radiochemical purity of [^{3}H] 3β-androstanediol from experiments 3AE and 3T was further checked by oxidizing the 3β-androstanediol carrier with chromic acid after it had been crystallized to constant specific activity. The resulting 5α-androstanedione was purified by t.l.c. in system I, recrystallized and its specific activity measured. This was not significantly different from the specific activity of the 3β-androstanediol crystals before oxidation and therefore eliminated the presence of [^{3}H]4-androstene-3β,17β-diol or [^{3}H]androstenediol which might cocrystallize with 3β-androstanediol carrier.

b) In experiments with testosterone (3T, 4T) and androstenedione (3AE, 4AE) there was evidence for formation of [^{3}H]etiocholanolone. Radioactivity was associated with etiocholanolone carrier after chromatography and constant specific activity was obtained after recrystallization (table 13l, p. 86). However, the final constant specific activity of the crystals accounted for only about 30% of the radioactivity in the etiocholanolone fraction. Another possible metabolite which would be found in the etiocholanolone fraction is 17β-hydroxy-5β-androstan-3-one.

There was no evidence for formation of 5β-androstane-3,

17-dione in any of the experiments. Any radioactivity in this fraction was always lost after recrystallization.

c) Polar Neutral Fraction.

In experiment 3 with the skin specimen from a 58 yr. old man a major fraction of the radioactivity was associated with material which was extracted from water into chloroform but which remained on the origin when chromatographed in system I. These were called the polar neutral steroid fractions as before. Aliquots of these fractions from experiments 3D, 3AE, and 3T were run on t.l.c. in system X. The t.l.c. plates were scanned; 3D showed a major peak (Rf. 0.15) and a minor peak (Rf. 0.36). 3AE showed two equal sized peaks (Rf. 0.15 and Rf. 0.41). 3T showed a major peak (Rf. 0.16) and a smaller peak (Rf. 0.42). These peaks were eluted and subjected to acetylation, borohydride reduction and chromic acid oxidation reactions together with further t.l.c. The results are shown in table 14 (p. 87).

d) Sulphate Fractions.

After solvolysis all sulphate fractions were analysed by chromatography and recrystallization as were the neutral metabolite fractions. Evidence was obtained for conversion of DHA to DHAsulphate and androstenediol sulphate and of DHAsulphate to androstenediol sulphate. No evidence for formation of testosterone sulphate, polyhydroxy steroid sulphates, or A-ring saturated sulphates was obtained. Even though sulphate fractions from incubations with testosterone and androstenedione contained radioactivity, the only radioactive compounds isolated after solvolysis and chromatography were epiandrosterone and traces of material remaining on the origin after t.l.c. in system I.

Histochemical Investigation of Forehead Skin.

Samples of the forehead skin were investigated histochemically for the presence of hydroxysteroid dehydrogenase activity. 3β-HSD and 17β-HSD activities were demonstrated in the sebaceous glands. The results are considered later in more detail together with similar studies of axillary skin (p.109).

Table 13a-13m.

METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN HUMAN MALE FOREHEAD SKIN.

EXPERIMENTS 3DS, 4DS, 3D, 4D, 3AE, 4AE, 3T, 4T. CONTROLS CDS, CD, CAE, CT.

IDENTIFICATION OF RADIOACTIVE STEROIDS BY RECRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY.

Forehead skin from a man of 58 yrs. (expt. 3) and a man of 83 yrs. (expt. 4) was incubated with [7α-^{3}H] DHAsulphate (3DS, 4DS) [7α-^{3}H] DHA (3D, 4D) [7α-^{3}H] androstenedione (3AE, 4AE) and [7α-^{3}H] testosterone (3T, 4T) as described in the text.
Radioactive steroids isolated by chromatography were mixed with authentic carriers and recrystallized to constant specific activity.
Specific activities (1.2.3.) were measured after successive crystallizations.

c = sp.act. of crystals, m = sp.act. of mother liquors.

Table 13a.

steroid : [^{3}H] DHA

solvent : acetone-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
3D	79000	77000	81500	81800	107
4D	73300	74000	73800	78500	96
CD	362000	358000	-	369000	92
3DS	19800	19100	19200	20900	90
4DS	6210	6350	6450	-	85
CDS	371	378	388	-	89

Table 13b.

steroid : [^{3}H]androsterone

solvents : acetone-hexane, $CHCl_3$-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
3D	21800	20800	20800	19600	93
4D	4520	4470	4320	4420	104
3DS	1310	1310	1312	1250	75
4DS	540	512	500	515	92
3AE	61300	59300	56000	60200	90
4AE	12500	11700	11800	11600	91
3T	64000	64000	62300	61700	105
4T	20300	18700	19600	18500	96

Table 13c.

steroid : [^{3}H]epiandrosterone

derivatives crystallized : epiandrosterone, 5α-androstanedione.

solvents : acetone-hexane, acetone-water.

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)					% calculated sp.act.
	1	2	3	4	5	
	c	c	c	c	c	
3D	5070	4950	+3970	+3770	+3790	30
3DS	510	467	460	+343	+347	57
3AE	11300	11000	11700	+11200	+11500	68
4T	1850	1835	1705	+1495	+1490	53

+ crystals were oxidized to 5α-androstanedione which was precipitated from water, purified by t.l.c. in system I then recrystallized before further sp.act. measurement.

Table 13d.

steroid : [^{3}H]epiandrosterone sulphate

derivatives)
solvents) : as for epiandrosterone
carrier)

	Specific Activity (d.p.m./mg)					
	1 c	2 c	3 c	4 c	5 c	% calculated sp.act.
3D	484	465	492	355$^+$	325$^+$	67
3AE	795	870	800$^+$			73
3T	1120	1140	1165	952$^+$	960$^+$	80

$^+$ crystals oxidized to 5α-androstanedione which was precipitated from water and recrystallized before further sp.act. measurement.

Table 13e.

steroid : [^{3}H]androstenediol

derivative : "

solvent : acetone-hexane; chloroform-hexane: methanol-water

carrier : 10.97 mg 3DS, 10.12 mg 3D, 12.62 mg 4D.

	Specific Activity (d.p.m./mg)						
	1	2		3		4	% calculated sp.act.
	c	c	m	c	m	c	
3DS	119	105	100	113	123		not measured
3D	271	257	306	276	304	272	
4D	220	202	214				

Table 13f.

steroid : [^{3}H]3β-androstanediol

derivative : " + 5α-androstanedione

solvent : $CHCl_3$-Methanol (2/1)-Hexane;Methanol-Water

carrier : 11.02 mg 3D, 10.34 mg 3AE, 10.02 mg 3T, 11.02 mg 4T.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1	2	3	4		5	
	c	c	c	c	m	c	
3D	286	251	258	95[+]			not measured
3AE	106	79.5	73.2	73.5		70.5[+]	
3T	398	283	297	273	300	268[+]	
4T	65.6	46.0	54.5	54.4			

[+] carrier was oxidized to 5α-androstanedione which was then purified by t.l.c. (solvent I.) and recrystallized before further sp.act. measurement.

Table 13g.

steroid : [^{3}H]3α-androstanediol

derivative : "

solvent : $CHCl_3$-Methanol (2/1)-hexane

carrier : 10.04 mg 3T, 10.93 mg 4T 4AE, 10.08 mg 3AE.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1	2		3		4	
	c	c	m	c	m	c	
3T	442	423	390	416	465	398	not measured
4T	134	132	-	122	-	-	
3AE	247	204	-	188	-	-	
4AE	68.3	65.7	-	72.3	-	-	

Table 13h.

steroid : [^{3}H]testosterone

solvents : acetone-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)					% calculated sp.act.
	1	2	3	4	5	
	c	c	c	c	c	
3D	148	126	150	136	136	23
4D	324	322	303	333	305	87
3DS	88.1	100	100	-		78
4DS	407	293	307	307		48
3AE	559	590	533	557	581	51
4AE	713	647	662	679		68
3T	81500	79000	80700	84700		81
4T	56200	57300	54700	58500		89
CT	918000	937000	893000	-		84

Table 13i.

steroid : [^{3}H]5α-androstanedione

solvent : acetone-water

carrier : 10.0mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
3D	95700	100000	97800	98600	95
4D	28800	29000	29000	-	92
3DS	9880	10800	10500	11000	99
4DS	2140	2210	2195	2175	85
3AE	443000	462000	459000	457000	90
4AE	77500	80900	78100	79300	91
3T	443000	459000	462000	467000	98
4T	71300	73800	71100	73800	87

Table 13j.

steroid : [^{3}H]androstenedione

solvents : acetone-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)					% calculated sp.act.
	1	2	3	4	5	
	c	c	c	c	c	
3D	5750	5620	5380	5450		81
4D	7980	7950	8180	7950		91
3DS	2810	2420	2465	2435		76
4DS	1322	1260	1245	1220	1320	63
3AE	102000	94300	94000	93000		90
4AE	126000	122000	128000	122000		96
CAE	1010000	1040000	1010000	985000		94
3T	83500	77200	79700	77600		92
4T	63100	58600	56400	56500		89

Table 13k.

steroid : [^{3}H]dihydrotestosterone

solvents : acetone-hexane, $CHCl_3$-hexane.

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)					% calculated sp.act.
	1	2	3	4	5	
	c	c	c	c	c	
3D	990	913	808	802	832	18
4D	383	308	300	276	272	9
4DS	80.9	73.7	71.3	90.3	-	19
3AE	1620	1400	1395	1400	1415	68
4AE	369	307	331	333	319	55
3T	657	607	595	585	597	63
4T	7450	8070	7280	7450	7800	81

Table 131.

steroid : [^{3}H]etiocholanolone

solvents : acetone-hexane, $CHCl_3$-hexane.

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)					% calculated sp.act.
	1	2	3	4		
	c	c	c	c	m	
3AE	278	293	290	288	-	28
4AE	262	231	283	250	255	16
3T	252	257	278	248	-	30
4T	616	569	566	619	614	32

Table 13m

steroid : [^{3}H]DHAsulphate

derivative crystallized : DHA

solvent : acetone-hexane.

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
3D	5950	6020	5570	5880	100
4D	780	840	771	730	105
CD	119	125	-	-	-
3DS	236,000	235,000	232,000	-	96
4DS	292,000	302,000	307,000	288,000	90
CDS	141,000	154,000	156,000	147,000	99

Table 14.

INVESTIGATION OF POLAR NEUTRAL FRACTIONS FROM EXPERIMENT 3.

Treatment	Rf. of Radioactive Peak.					
	T_1	T_2	AE_1	AE_2	D_1	D_2
1.	0.16	0.42	0.145	0.41	0.15	0.36
2.	0.77	0.76	0.76	0.74	0.74	0.73
3.	0.10	0.04	0.12	0.05	0.13	0.04
4.	0.60	-	0.62	-	0.60	-
5.	0.21	-	0.19	-	0.23	-
6.	0.09	0.04	0.11	0.03	-	0.02
7.	0.74	0.72	0.72	-	-	0.70

1. Polar neutral steroid fractions were run on t.l.c. in system X. Scanning of the plate showed each fraction to be resolved into two radioactive peaks ($3D_1$, $3D_2$, $3AE_1$, $3AE_2$, $3T_1$, $3T_2$) with the recorded Rf. values. The Rf. of androstenediol run on the same plate was 0.50. Each peak was eluted from the plate.

2. An aliquot of each radioactive peak isolated from step 1. was acetylated (p. 39). A portion of the acetylated material was run in system X.

3. The rest of the acetylated material was run in system VI.

4. Aliquots of peaks $3D_1$, $3AE_1$ and $3T_1$ were acetylated, reduced with sodium borohydride (p.40) and then run in system X.

5. Aliquots of peaks $3D_1$, $3AE_1$ and $3T_1$ were acetylated, reduced with sodium borohydride, re-acetylated and run in system VI.

6. Aliquots of fractions $3D_2$, $3AE_1$, $3AE_2$, $3T_1$ and $3T_2$ were acetylated then oxidized with Kiliani's reagent (p. 40). A portion of the material was then run in system VI.

7. The rest of the acetylated-oxidized material was run in system X.

In each case the fractions from incubations with DHA (3D) androstenedione (3AE) and testosterone (3T) were run on the same plate. In the case of acetylated, reduced and oxidized derivatives, samples of the parent compounds or a previous derivative with which direct comparison of Rf. was to be made, were also run on the same plate.

Radioactive material was located on the plates with a Panax radiochromatogram scanner.

system X : chloroform:methanol:water ,200:9:0.75 (v/v)

system VI : benzene:ethyl acetate,180:20 (v/v)

Table 14.

INTERPRETATION OF THE RESULTS OF TABLE 14

Incubation of forehead skin with DHA, androstenedione and testosterone results in formation of at least two metabolites more polar than androstenediol, 16α-hydroxy DHA or 11β-hydroxy androstenedione (Rf.'s 0.50, 0.50, 0.60 in system X). Fractions $3T_1$, $3AE_1$ and $3D_1$ are probably compounds with at least two acetylable hydroxyl groups and one ketone group. The presence of a ketone group in fractions $3D_1$, $3T_1$ and $3AE_1$ was inferred from the change in Rf. after borohydride reduction of the starting material and borohydride reduction of the acetylated material followed by further acetylation. Absence of any reaction with chromic acid after acetylation indicated that there were no hydroxyl groups which resisted acetylation (e.g.: 11β-OH (Bush, 1961).

Fractions T_2, AE_2 and D_2 are compounds with at least one acetylable hydroxyl group and possibly have a ketone group where compounds T_1, AE_1 and D_1 have a hydroxyl. This conclusion is based on the reversal of the relative Rf.s of compounds 1 and 2 after acetylation.

It is pointless to speculate on the possible identities of the different fractions. It cannot even be deduced for certain whether D_1 and D_2 retain the Δ^5-3β-OH structure or whether T_1, T_2 and AE_1, AE_2 retain the Δ^4-3-oxo structure.

The possibility that compounds AE_1 and T_1 are 17β-hydroxysteroids while AE_2 and T_2 are 17-oxosteroids must be considered. If this were the case, it would mean considerably more conversion of androstenedione to 17β-hydroxysteroids than is apparent from analysis of the identified C_{19} O_2 metabolites. If $3AE_1$ had a 17β-hydroxyl group this would mean about 4% 17β-reduction of androstenedione in experiment 3AE. This is still, however, small compared with 17-oxidation of testosterone (which incidentally would be increased if formation of $3T_2$ was taken into account).

Table 15.

METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN HUMAN MALE FOREHEAD SKIN.

	% conversion/3 hours			
	3DS	3D	3AE	3T
DHA	2.70	+19.6	-	-
DHAsulphate	+83.3	3.65	-	-
androstenediol	0.03	0.32	-	-
androstenediol sulphate	0.36	0.09	-	-
3β-androstanediol	-	-	0.03	0.12
3α-androstanediol	-	-	0.07	0.11
androsterone	0.60	14.5	24.8	19.3
epiandrosterone	0.07	1.33	2.01	0.20
epiandrosterone sulphate	-	0.2	0.18	0.20
etiocholanolone	-	-	0.08	0.08
testosterone	0.02	0.04	0.07	+8.97
dihydrotestosterone	0.02	0.25	0.28	0.09
androstenedione	0.25	1.03	+7.24	7.53
5α-androstanedione	1.66	30.8	54.8	52.9
polar neutral steroids	-	21.0	8.2	8.5

+ = % substrate recovered

Slices of forehead skin from a 58 yr. old man were incubated for 3 hours in Krebs Improved Ringer I medium with one of the following: [7α-^{3}H]DHAsulphate (3DS), [7α-^{3}H]DHA (3D), [7α-^{3}H] androstenedione (3AE), [7α-^{3}H]testosterone (3T). Amounts of tissue and incubation conditions are shown in table 12 (p.76).

Table 16.

METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN HUMAN MALE FOREHEAD SKIN.

	% conversion/3 hours			
	4DS	4D	4AE	4T
DHA	1.07	+50.7	-	-
DHAsulphate	+93.2	1.30	-	-
androstenediol	-	0.32	-	-
androstenediol sulphate	0.18	-	-	-
3β-androstanediol	-	-	-	0.10
3α-androstanediol	-	-	0.06	0.28
androsterone	0.28	9.81	17.9	19.5
epiandrosterone	-	1.07	-	0.67
etiocholanolone	-	-	0.36	0.62
testosterone	0.05	0.19	0.26	+23.0
dihydrotestosterone	0.02	0.27	0.22	3.56
androstenedione	0.15	4.20	+37.3	16.8
5α-androstanedione	0.36	20.5	35.4	31.5
polar neutral steroids	-	3.0	3.5	3.9

+ = % substrate recovered

Slices of forehead skin from an 83 yr. old man were incubated for 3 hours in Krebs Improved Ringer I medium with one of the following: [7α-^{3}H]DHAsulphate (4DS), [7α-^{3}H]DHA, (4D), [7α-^{3}H] androstenedione (4AE), [7α-^{3}H]testosterone (4T). Amounts of tissue and incubation conditions are shown in table 12 (p.76).

iv) Discussion :

The first obvious feature of tables 15 and 16 is the extensive metabolism of DHA, androstenedione and testosterone in human male forehead skin. The total rates of metabolism in specimen 3 (male 58 yrs.) were greater than those in specimen 4 (male 83 yrs.). This difference was in part due to the much greater % conversions to polar metabolites by specimen 3. The conversion of DHA to androstenedione and of testosterone to androstenedione showed the presence of a Δ^5-3β-HSD and a 17β-HSD in forehead skin. This confirmed the histochemical studies which showed a reaction in sebaceous glands with DHA and with testosterone.

The rate of DHAsulphate metabolism was much less than that of DHA, androstenedione or testosterone. It appeared to be limited by the hydrolysis of DHAsulphate to DHA. (Total % conversion / 3 hours of DHAsulphate to neutral steroids = 5.33% 3DS; 1.92% 4DS). The metabolism of neutral steroids formed from DHAsulphate was essentially similar to that of [7α-^{3}H]DHA (3D and 4D).

Excluding the polar neutral fraction, 5α-androstanedione and androsterone were the major metabolites which accumulated in experiments with DHA, androstenedione and testosterone. This indicated the presence of 5α-reductase and 3α-HSD in the skin. The conversion of DHAsulphate, DHA and testosterone to androstenedione and the conversion of all four steroids to 5α-androstanedione + androsterone makes it likely that androstenedione is a common intermediate in the formation of these A-ring saturated steroids. As testosterone itself is a substrate for 5α-reductase the assumption that androstenedione was the major intermediate between testosterone and 5α-androstanedione + androsterone may not be valid. A proportion of these two steroids could have been formed via dihydrotestosterone (Fig.12, p.112) and it is not possible to deduce from experiments 3 and 4 whether the very low % conversions of testosterone to dihydrotestosterone were due to low rates of formation or rapid further metabolism of 5α-dihydrotestosterone.

In all experiments, the % conversions to 17β-hydroxysteroids were low. It appears that 17β-HSD present in the skin worked almost

exclusively to catalyse the oxidation of 17β-hydroxysteroids rather than reduction of 17-oxosteroids. This accounts for the very low % conversions to dihydrotestosterone and androstanediols even in experiments with testosterone.

[^{3}H]epiandrosterone was isolated from experiments with all four substrates. The conversion of testosterone and androstenedione to this steroid suggests the presence of a 3β-HSD in the skin specimens. This enzyme probably reduces the 3-oxo group of 5α-androstanedione to form epiandrosterone and also the 3-oxo group of dihydrotestosterone to form 3β-androstanediol. As in the previous experiments the 3α-reduction of 5α-androstanedione (to form androsterone) predominated over 3β-reduction.

The isolation of [^{3}H]etiocholanolone from incubations with androstenedione and testosterone raises the problem of how it was formed. [^{3}H]5β-androstane-3, 17-dione is the logical intermediate. However, no formation of this steroid could be detected in any experiment, although it could be an intermediate which, like androstenedione, did not accumulate. An alternative pathway could go via 17β-hydroxy-5β-androstan-3-one and 5β-androstane-3α, 17β-diol. The former compound would be found in the etiocholanolone fraction after chromatography. As the final specific activity after recrystallization never accounted for more than 30% of the radioactivity in the etiocholanolone fraction, it is possible that there was also some 17β-hydroxy-5β-androstan-17-one.

The conversion of DHA to DHAsulphate indicated the presence of a sulphotransferase in the skin specimens. The failure to detect formation of either testosterone sulphate or androsterone sulphate suggests that the sulphotransferase in skin acts preferentially on 3β-hydroxysteroids rather than on 17β-hydroxy or 3α-hydroxysteroids such as testosterone and androsterone.

6. METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN HUMAN FEMALE AXILLARY SKIN.

EXPERIMENT 5 (DS, D, AE, T).

A piece of skin was obtained from the armpit of a woman aged 20 years, who underwent a skin grafting operation. She was suffering from hyperactivity of the eccrine sweat glands. However, no histological abnormalities of the excised armpit skin could be detected (Prof. J.A. Milne, Dr. F.M. Vint). The skin specimen had numerous large terminal hairs and also apocrine sweat glands. The sebaceous glands (associated with each hair) were smaller than those of the face skin.

The growth of the axillary hair and apocrine glands is stimulated by androgens, therefore this piece of skin provided the opportunity to study:

a) steroid metabolism of an androgen responsive organ of the human female;

b) to compare steroid metabolism of hairy skin with that of skin dominated by sebaceous glands (forehead, section 5 expts. 3 and 4).

i) Experimental :

Four pieces, each weighing about 400 mg were cut from the specimen. Each piece was cut into 0.5 - 0.7 mm thick slices and incubated in 4.0 ml of Krebs Improved Ringer I medium with one of the four steroids; [7α-^{3}H] DHAsulphate, [7α-^{3}H] DHA, [7α-^{3}H] androstenedione or [7α-^{3}H] testosterone (table 17 p.94). The steroid concentration (3.5μM) and tissue/steroid ratio (10^5/1), were kept the same as in previous experiments. Incubation with shaking was for 3 hours at 37°C under 95%O_2 : 5%CO_2. Reactions were stopped with acetone, and carrier steroids were added as in experiments 3 and 4 (p.77). The radioactive steroids were then isolated, purified by t.l.c. and paper chromatography (Part II sections 1.ii and 2, Figs. 5, 6) and their identity and radiochemical purity established by recrystallization to constant specific activity (Part II sections 1. iv and 5, Fig. 4).

Table 17.

EXPERIMENT 5. Incubation Conditions.

Wt.Skin (mg)	Steroid Incubated	nmol steroid	10^{-6} x d.p.m. ^{3}H	ml medium
430	DHAsulphate	14	4.83	4.0
390	DHA	14	13.82	4.0
386	androstenedione	14	15.75	4.0
383	testosterone	14	14.85	4.0

ii) Results :

Evidence for identity and radiochemical purity of those [^{3}H]steroids which were recrystallized to constant specific activity is given in tables 18a - n (pp. 97-104). The percent conversions are shown in table 20 (p. 106) and are not corrected for tissue weights as these were similar in all experiments.

The methods for establishing radiochemical purity of certain steroids were modified in experiment 5 and will now be described more fully.

a) androstenediol and androstanediols (tables 18i, j, k, l, pp. 101-103).

Scanning of the first t.l.c. plate (solvent I) for radioactivity showed large peaks in the androstanediol region. This radioactivity remained with the carrier after acetylation and t.l.c. in system VI. The acetylated material was saponified then run on paper in solvent XII, which separated various isomers of the androstenediols and androstanediols (p. 31). Further purification by t.l.c. showed that radioactivity was associated with androstenediol (5DS, 5D), 3β-androstanediol (5D, 5AE, 5T) and 3α-androstanediol (5DS, 5D, 5AE, 5T). Aliquots of the radioactive fractions were therefore crystallized to constant specific activity with the appropriate carriers.

a.1.) [^{3}H]androstenediol was identified in the neutral steroid fractions and in the steroids obtained by solvolysis of the sulphate fractions from experiments 5D and 5DS. The chromatographic purification eliminated any possibility of 3β-androstanediol contaminating this fraction.

a.2.) [^{3}H]3α-androstanediol was identified in experiments 5D, 5DS, 5AE and 5T. The fact that the final constant specific activities of the crystals were around 100% of the theoretical value (table 18k) indicates that the improved chromatographic method was sufficient to purify 3α-androstanediol from any significant contaminants.

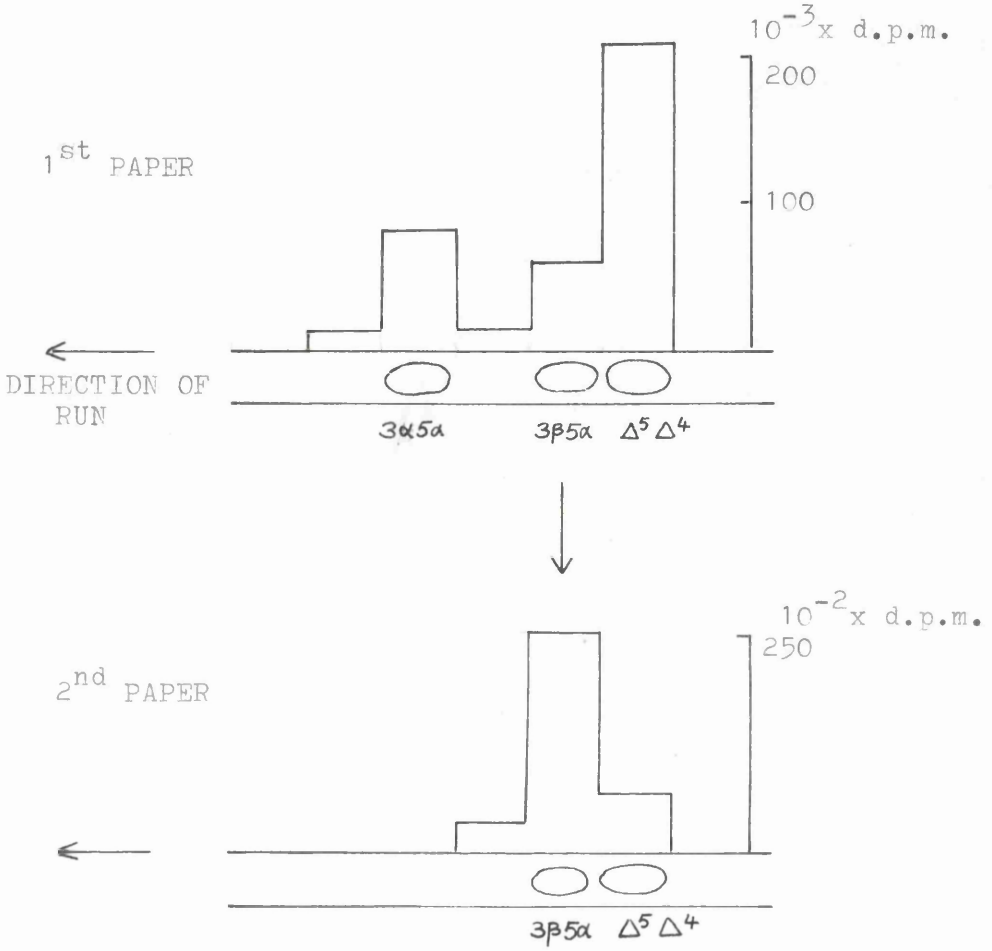
a.3.) 3β-androstanediol.

Paper chromatography and t.l.c. separated this compound from 4-androstene-3β, 17β-diol and androstenediol which might cocrystallize with it. Recrystallization then established the radiochemical purity of [^{3}H]3β-androstanediol from incubations 5AE and 5T. In experiment 5D a highly significant amount of radioactivity (3% conversion) remained with 3β-androstanediol carrier after chromatography. However, the sample was rechromatographed in system XII to remove any [^{3}H]androstenediol contaminant. Fig. 11 (p. 96) shows the distribution of radioactivity on the first and second paper chromatograms run in solvent XII, showing that there was radioactive material associated with 3β-androstanediol carrier which was not androstenediol. The 3β-androstanediol was recrystallized to constant specific activity and then oxidized to 5α-androstanedione. There was only a slight drop in specific activity of the crystals after oxidation (table 18l). This provided good identification of [^{3}H]3β-androstanediol in experiment 5D.

b) [^{3}H]testosterone from incubations 5DS, 5D, 5AE, was acetylated and purified by t.l.c., using solvent VI. The acetate was recrystallized to constant specific activity then saponified and the free steroid crystallized further (table 18m). However, in experiments 5DS and 5D, [^{3}H]testosterone was isolated from control incubations (0.1 %). This was probably due to contamination of a test tube by [^{3}H]testosterone. Because of this, no exact quantitation of testosterone formation in experiments 5D and 5DS could be made.

Fig. 11.

ISOLATION OF [^{3}H]3β-ANDROSTANEDIOL AFTER INCUBATION OF [7α-^{3}H] DHA WITH FEMALE AXILLARY SKIN



1st Paper: The androstenediol-androstanediol fraction isolated by t.l.c. in systems I and VI (p. 31) was chromatographed on paper in system XII. Bands corresponding to the carrier steroids and intermediate bands were cut out, eluted and counted to determine the distribution of radioactivity on the chromatogram.

2nd Paper: The 3β-androstanediol fraction isolated from the first paper was re-run in system XII. Three bands were eluted and counted, giving the distribution of radioactivity shown.

It can be seen that the 3β-androstanediol fraction contained radioactivity associated with neither androstenediol nor 4-androstene-3β,17β-diol.

3α5α = 3α-androstanediol
3β5α = 3β-androstanediol
Δ^5 = androstenediol
Δ^4 = 4-androstene-3β,17β-diol.

Table 18a-18n.

METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN HUMAN FEMALE AXILLARY SKIN.

IDENTIFICATION OF RADIOACTIVE STEROIDS BY RECRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY.

EXPERIMENTS 5DS, 5D, 5AE AND 5T. CONTROLS CDS, CD, CAE, CT.

Axillary skin from a woman aged 20 yrs. was incubated with [7α-^{3}H]DHAsulphate (5DS), [7α-^{3}H]DHA (5D), [7α-^{3}H]androstenedione (5AE) and [7α-^{3}H]testosterone (5T) as described in the text. Radioactive steroids isolated by chromatography were mixed with authentic carriers and recrystallized to constant specific activity. Specific activites were measured after successive crystallizations (1.2.3..)

c = sp.act. of crystals m = sp.act. of mother liquor.

Table 18a.

steroid : [^{3}H]DHA

solvent : acetone-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	c 1	c 2	c 3	c 4	
5D	123000	124200	125500	123500	92.5
CD	386000	378000	380000	372000	98
5DS	2880	2990	3110	3110	82
CDS	300	293	300	288	70

Table 18b.

steroid : [^{3}H]DHAsulphate

derivative crystallized : DHA.

solvent : acetone-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
5D	10600	10360	10400	10550	91
CD	77	65	74	-	-
5DS	201000	205000	205000	188000	85
CDS	215000	217000	225000	198000	87

Table 18c.

steroid : [^{3}H]epiandrosterone.

solvents : acetone-hexane, acetone-water

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1	2	3	4	5	6	
	c	c	c	c	c	c	
5D	5350	5050	4980	4930	+5130	-	87
5DS	214	226	193	182	200	+130	52
5AE	1270	1340	1255	1280	+1350	-	92
5T	1680	1600	1535	1510	1670	+1560	85

+ carrier oxidized to 5α-androstanedione which was precipitated from water, purified by t.l.c. in system I, then recrystallized before sp.act. measurement.

Table 18d.

steroid : [^{3}H]epiandrosterone sulphate

derivative crystallized : epiandrosterone.

solvents : acetone-hexane, acetone-water

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1	2	3	4	5	6	
	c	c	c	c	c	c	
5D	641	623	667	667	+482	+521	70.5
5AE	230	246	230	244	+293		91.5
5T	330	374	377	379	+261	+278	70.0

+ carrier oxidized to 5α-androstanedione which was precipitated from water then recrystallized before sp.act. measurement.

Table 18e.

steroid : [^{3}H]androstenedione

solvent : acetone-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
5D	2740	2640	2740	2700	80
5DS	no result				
5AE	65700	68000	65600	68600	94
5T	11600	12100	11900	12100	92

Table 18f.

steroid : [^{3}H] 5α-androstanedione

solvent : acetone-water

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
5D	24000	24400	24400	24100	80.5
5DS	588	596	596	-	83.7
5AE	53300	50200	52100	51000	80.5
5T	32000	32000	31200	31400	86.0

Table 18g.

steroid : [^{3}H]androsterone

solvents : acetone-hexane, $CHCl_3$-hexane.

carrier : 10.0 mg

	Specific Activity (d.p.m/mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
5D	49700	50800	51000	50200	107
5DS	1890	1930	1835	1870	93.0
5AE	72100	73200	73600	74200	102
5T	94200	96500	95000	96200	95.0

Table 18h.

steroid : [^{3}H]etiocholanolone

solvents : acetone-hexane, $CHCl_3$-hexane, benzene-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)				% calculated sp.act.
	1	2	3	4	
	c	c	c	c	
5AE	630	612	570	582	23.0
5T	477	414	465	-	30.0

Table 18i.

solvents : acetone-hexane, $CHCl_3$-methanol-hexane, acetone-water.

steroid : [^{3}H]androstenediol

carrier : 10.2 mg

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3		
	c	m	c	m	c	m	
5D	7050	6600	6530	6500	6800	6930	98.5
5DS	216	245	190	215	186	200	94.0

Table 18j.

steroid : [^{3}H]androstenediol sulphate

derivative crystallized : androstenediol

carrier : 10.2 mg solvents : as for 18i.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3		
	c	m	c	m	c	m	
5D	4500	4300	4680	4060	4620	4450	94.0
5DS	6650	6900	6300	6400	6630	6250	94.8

Table 18k.

steroid : [^{3}H] 3α-androstanediol

carrier : 10.0 mg solvents : as for 18i.

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1		2		3		
	c	m	c	m	c	m	
5D	5400	5210	5130	5210	5220	4750	103
5DS	485	585	520	483	500	503	78.4
5AE	8650	8150	8230	8100	8030	7200	104
5T	6520	-	6890	6800	6850	7000	100

Table 18l.

steroid : [^{3}H]3β-androstanediol

carrier : 10.7 mg solvents : as for 18i.

	Specific Activity (d.p.m./mg)								% calculated sp.act.
	1		2		3		4		
	c	m	c	m	c	m	c	m	
+5D	910	1680	969	915	855	1320	°785	-	62.2
5AE	520	665	503	565	550	502	532	523	80.6
5T	800	1020	792	830	762	825	802	757	77.5

+ crystallized twice between each measurement of sp.act.

o crystals oxidized to 5α-androstanedione which was precipitated from water, purified by t.l.c. in system I then recrystallized before 4th sp.act. measurement.

Table 18m.

steroid : [^{3}H]testosterone

derivative crystallized : acetate (except 5T, CT)

solvents : acetone-water, acetone-hexane, $CHCl_3$-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg§)					% calculated sp.act.
	1	2	3	4	5	
5D	2790	2970	2890	+2860	3010	102
5DS	265	302	311	+ 300	344	74.0
5AE	3500	3490	3770	3670	3790	
5T	64200	65100	64600	63900	-	80.0
CT	563000	555000	550000	555000	-	83.5

+ saponified to give testosterone which was then recrystallized before 4th sp.act. measurement.

§ all corrected to mol wt. of testosterone acetate. (except, 5T, CT)

Table 18n.

steroid : [^{3}H]dihydrotestosterone

solvents : acetone-hexane, $CHCl_3$-hexane

carrier : 10.0 mg

	Specific Activity (d.p.m./mg)						% calculated sp.act.
	1	2	3	4	5	6	
5D	20900	20700	18950	20900	+21050	-	93.5
5DS	990	947	942	925	987	+893	72.5
5AE	5200	3780	5180	4830	5100	+5050	96.0
5T	10430	10350	10020	10300	+11250	-	98.0

+ crystals oxidized to 5α-androstanedione which was precipitated from water and purified by t.l.c. in system I then recrystallized before sp.act. measurement.

c) A polar neutral steroid fraction was isolated from each of the incubations 5D, 5AE, 5T. It accounted for 10-25% of the radioactivity in each incubation. The fractions were analysed by t.l.c. in system X (table 19 below).

Table 19.

INVESTIGATION OF POLAR FRACTIONS FROM EXPERIMENT 5.

Rf. of Radioactive Peak in System X.

$5T_1$	$5T_2$	$5AE_1$	$5AE_2$	$5D_1$	$5D_2$
0.15	0.25	0.14	0.22	0.15	0.27

T = testosterone

AE = androstenedione

D = DHA

System X = t.l.c. on Silica Gel in $CHCl_3$/Methanol/H_2O, 200:9:0.75 (v/v).

The polar neutral steroid fractions isolated from incubations 5D, 5AE and 5T were run on t.l.c. in system X. Scanning of the plate showed each fraction to be resolved into two peaks ($5T_1$, $5T_2$ etc.) with the recorded Rf. values. The Rf. of androstenediol run on the same plate was 0.55.
Bands $5T_1$, $5AE_1$, and $5D_1$, had Rf. values similar to those of compounds 3T, 3AE, and 3D isolated from incubations of male forehead skin (table 14 p. 87). However, bands $5T_2$, $5AE_2$ and $5D_2$ had Rf. values only half those of the fractions $3T_2$, $3AE_2$ and $3D_2$ from forehead skin.

Table 20.

METABOLISM OF DHASULPHATE, DHA, ANDROSTENEDIONE AND TESTOSTERONE IN FEMALE AXILLARY SKIN.

	% conversion/3 hours			
	5DS	5D	5AE	5T
DHA	1.20	+14.65	-	-
DHAsulphate	+74.7	1.67	-	-
androstenediol	0.11	12.45	-	-
androstenediol sulphate	11.55	2.19	-	-
3β-androstanediol	-	1.21	0.27	0.48
3α-androstanediol	1.27	6.92	14.95	15.70
androsterone	1.72	12.81	34.0	24.90
epiandrosterone	0.07	1.32	0.84	0.56
epiandrosterone sulphate	-	§0.13	§0.05	§0.06
etiocholanolone	-	-	0.23	0.09
testosterone	0.18	0.44	0.70	+7.76
dihydrotestosterone	0.56	6.08	7.75	15.85
androstenedione	0.9	0.29	+8.23	1.20
5α-androstanedione	0.05	4.65	10.23	6.44
polar neutral steroids	-	25	11	18

+ = % substrate recovered

§ = tentative identification

Slices of axillary skin from a 20 yr. old woman were incubated for 3 hours in Krebs Improved Ringer I medium with one of the following: [7α-^{3}H] DHAsulphate (5DS), [7α-^{3}H] DHA (5D), [7α-^{3}H] androstenedione (5AE), [7α-^{3}H] testosterone (5T). Amounts of tissue and incubation conditions are shown in table 17 p.94.

iii) Discussion :

DHA, androstenedione and testosterone were extensively metabolized by the axilla skin, (86% - 93% conversion/3 hours). The formation of neutral steroid metabolites from DHAsulphate was slow (5% conversion/3 hours) as in face skin incubations. However, there was 11.6% conversion of DHAsulphate to androstenediol sulphate; this was not observed in previous experiments.

Conversion of DHAsulphate and DHA to androstenedione and testosterone occurred, as did interconversion of androstenedione and testosterone. This indicates the presence of Δ^5-3β-HSD and 17β-HSD enzyme systems in the female axilla skin. However, as in previous experiments, the A-ring saturated C_{19}-steroids were the major neutral metabolites which accumulated in all incubations. In contrast to the face skin experiments, the % conversions to dihydrotestosterone (5T) and 5α-androstanediols (AL) were similar to % conversions to 5α-androstanedione (AD) and androsterone (A); (5T+AL)/(AD+A) = 1.04, 5DS; 0.75, 5D; 0.52, 5AE; 1.01, 5T. This indicated that in the axilla the 17β-HSD reaction proceeded equally in oxidative and reductive directions, in contrast to the male face skin where the reaction was almost exclusively oxidative. The active reduction of 17-oxosteroids was also seen in the conversion of DHA to androstenediol (12.5%) and of DHAsulphate to androstenediol sulphate (11.6%).

This high % conversion of DHA to androstenediol raises the possibility that the latter compound was a precursor of testosterone, dihydrotestosterone and androstanediols. In the case of DHAsulphate, there is another possible metabolic sequence. That is DHAsulphate → androstenediol sulphate → androstenediol → testosterone. This would depend on the ability of the steroid sulphatase to hydrolyse androstenediol sulphate. Like the conversion of androstenediol to testosterone, the occurrence of this reaction in skin has yet to be proved. As found in previous experiments, the formation of the 3α-hydroxysteroids predominated

over the formation of 3β-hydroxysteroids (ratio 3α-androstanediol/ 3β-androstanediol = 56, 5AE; 32, 5T; 6, 5D. ratio androsterone/ epiandrosterone = 41, 5AE; 43, 5T; 10, 5D). However, it can be seen that in experiment 5D, the % conversions to the 3β-hydroxysteroids were considerably greater relative to formation of 3α-hydroxysteroids than were those in experiments 5AE and 5T (table 20 p. 106).

The formation of trace quantities of etiocholanolone in experiments 5AE and 5T confirmed the results of the forehead skin experiments. Once again no evidence of 5β-androstane-3, 17-dione formation could be obtained. However, the final constant specific activities of etiocholanolone after recrystallization accounted for only 23% and 30% of the radioactivity associated with etiocholanolone carrier after chromatographic purification (table 18h p. 101). 17β-hydroxy-5β-androstan-3-one could have been present in this fraction.

The female axilla skin showed steroid sulphotransferase activity. DHA was converted to DHAsulphate and androstenediol sulphate. It is not possible to deduce whether the androstenediol sulphate was formed by reduction of DHAsulphate or sulphation of androstenediol. Both reactions are possible.

There was very little radioactivity in the sulphate or glucuronide fractions isolated from experiments 5AE and 5T. Tentative evidence for formation of [^{3}H]epiandrosterone sulphate was obtained (table 18d p. 99) but no other steroid sulphates could be identified. This supports the earlier tentative conclusion that the skin enzymes will not sulphate 17β-hydroxy- or 3α-hydroxysteroids.

DHAsulphate was shown to undergo two reactions:

(a) Hydrolysis to DHA.

(b) 17β-reduction to androstenediol sulphate.

No other steroid sulphate metabolites could be detected in experiment 5DS, even though the solvolysates from all the fractions expected to contain steroid sulphates were subjected to a full chromatographic and recrystallization analysis. As with face

skin incubations, there was a notable lack of conversion of DHAsulphate to polar neutral steroids or polar steroid sulphates (i.e. compounds yielding polar neutral steroids after solvolysis).

7. HISTOCHEMICAL STUDIES OF FOREHEAD AND AXILLA SKIN.

Samples cut from the forehead and axilla skin specimens were examined histochemically for hydroxysteroid dehydrogenase activity. The method of Muir et al. (1968)(p.42) was used. The following steroid substrates were examined :

DHA	(3β-OH)
epiandrosterone	(3β-OH)
androsterone	(3α-OH)
testosterone	(17β-OH)
dihydrotestosterone	(17β-OH)

NADH-diaphorase and NADPH-diaphorase activities in the skin specimens were checked. Each steroid was incubated with NAD^+ and $NADP^+$ as cofactors in different experiments.

The histochemical staining method depends on the reduction of the dye, nitrobluetetrazolium by NADH or NADPH formed during oxidation of the steroid substrate. The reduced dye is deposited at the reaction site as insoluble blue-black granules. The transfer of hydrogen (electrons) from NAD(P)H to the dye requires the mediation of part of the electron transport chains of the cell. This is termed the "NAD(P)H diaphorase activity". Thus, for the histochemical method to give reliable localization of hydroxysteroid dehydrogenase, all the cells examined must show NAD(P)H diaphorase activity.

All types of cell examined in the skin sections gave a staining reaction for NADH-diaphorase. Staining was particularly intense in sebaceous glands and apocrine and eccrine sweat gland epithelia. Staining was much less intense in the cells of the terminal hair follicles below the point where sebaceous glands were connected. The staining reaction for NADPH-

diaphorase was generally less intense than that for NADH. Hair follicles showed only very weak staining when skin was incubated with NADPH.

Table 21.

HISTOCHEMICAL DEMONSTRATION OF HYDROXYSTEROID DEHYDROGENASE IN SEBACEOUS GLANDS.

	Forehead		Axilla	
	NAD^+	$NADP^+$	NAD^+	$NADP^+$
DHA	+	-	+	-
epiandrosterone	+	-	++	-
androsterone	-	-	-	-
testosterone	+		-	-
dihydrotestosterone	+		-	-

+ = reaction observed. - = no reaction.
Reactions were seen only in sebaceous glands.

The histochemical results provide some evidence that sebaceous glands are sites of steroid metabolism in face and axilla skin. However, they also show inconsistencies with the findings of the radioisotope studies.

1. Incubations of [^{3}H]-steroids with forehead and axilla skin showed the presence of 17β-HSD. However, only in the sebaceous glands of the forehead could a histochemical demonstration of 17β-hydroxysteroid dehydrogenase activity be obtained.
2. Incubation of [^{3}H]-steroids showed that both forehead and axilla skin contained 3α-HSD. The presence of this enzyme was inferred from the conversion of androstenedione and testosterone to androsterone and 3α-androstanediol. All attempts to localize the enzyme histochemically in the skin failed.
3. Incubation of [^{3}H]-steroids with breast, forehead and axillary

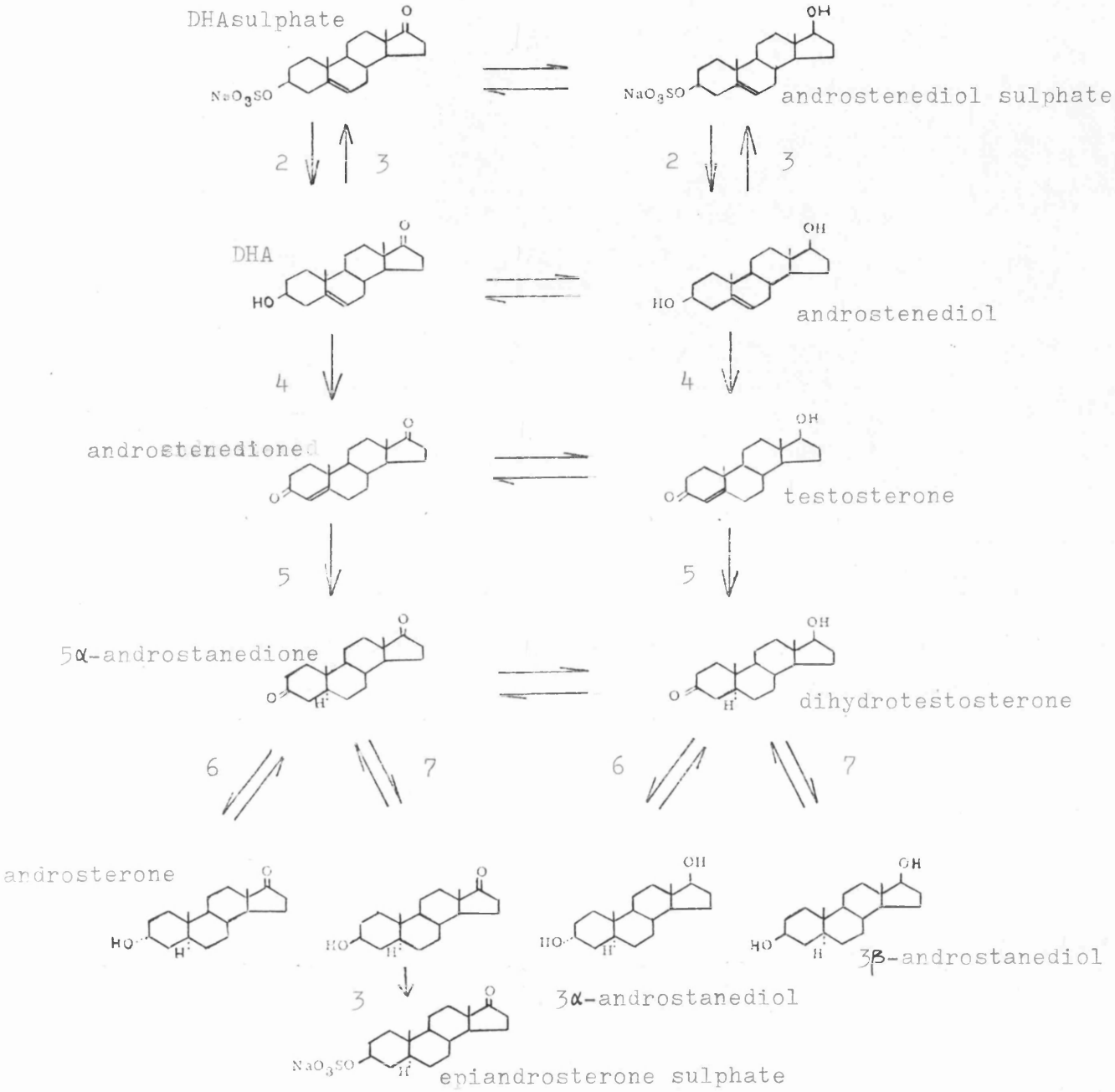
skin showed that % conversions to epiandrosterone (3β-OH) and 3β-androstanediol were usually at least 10 X less than total % conversions to 3α-hydroxysteroids. However histochemistry indicated that in sebaceous glands there was an intense 3β-HSD reaction with epiandrosterone as substrate in contrast to the apparent absence of any reaction with androsterone (3α-OH).

PART IV.

GENERAL DISCUSSION

Fig. 12

PROBABLE PATHWAYS OF C_{19}-STEROID METABOLISM IN SKIN IN VITRO.



Reactions catalysed by:

1. 17β-HSD 2. Sulphatase 3. Sulphotransferase 4. Δ^5-3β-HSD
5. 5α-reductase 6. 3α-HSD 7. 3β-HSD

In addition DHA, androstenedione and testosterone are converted to hydroxylated metabolites.

Androstenedione is converted to etiocholanolone, possibly via the intermediate 5β-androstane-3, 17-dione.

GENERAL DISCUSSION

I. PATHWAYS AND ENZYMES OF STEROID METABOLISM IN SKIN.

i) The pathways of androgen metabolism in skin, which have been deduced from the results of incubation studies, are shown in Fig. 12 (p.112). The experiments have shown that both DHA-sulphate and DHA are converted in skin to A-ring saturated neutral steroids. Androstenedione and testosterone appear to be intermediates in these transformations and it can be seen that common metabolic pathways for all four steroids investigated appear to exist in human skin.

The pathways of skin steroid metabolism found from 'in-vitro' studies are similar to the overall 'in-vivo' metabolic pathways for C_{19}-steroids, deduced from analysis of the fate of injected tracers and analysis of urinary metabolites (compare Figs. 2 and 12 pp. 8 and 112). There are some differences however; notably the skin did not form glucuronides of DHA, androstenedione or testosterone or of their major metabolites. Sulphates of testosterone, dihydrotestosterone, 3α-androstanediol and androsterone appear not to be formed in skin.

ii) Enzymes :

a. Steroid sulphatase (EC. 3.1.6.2.)

Hydrolysis of DHAsulphate by homogenates of human neonatal foreskin was reported by Kim and Herrmann (1968). The work described here confirms the presence of a steroid sulphatase in adult skin. The extent of DHAsulphate hydrolysis was least in old male (83 yrs.) forehead and female (56 yrs.) breast skin (1.9% and 2.9% conversion to neutral steroids) and greatest in the younger male (58 yrs.) forehead and female (20 yrs.) axillary skin (5.3% and 5.1% conversion). The rate of DHA-sulphate hydrolysis was much less than that of other metabolic transformations of androgens.

The anatomical localization of steroid sulphatase in skin is completely unknown; attempts to localize steroid sulphatase

in skin, histochemically, by coupling the reaction with further oxidation of DHA, have failed (Muir, 1971). Histochemically detectable aryl sulphatase (substrate: 6-Bromo-2-napthyl sulphate) gives an intense reaction in sebaceous glands, eccrine sweat glands and epidermis (Vezekenyi, 1968). However, the aryl sulphatases appear to be distinct from the steroid specific sulphatase, which has been isolated from liver (Roy, 1957).

b. 3β-Hydroxysteroid sulphotransferase (EC. 2.8.2.2.)

The experiments have extended the findings of Berliner et al. (1968), and Faredin et al. (1968), who showed that DHA was converted to DHAsulphate in human abdominal skin. The reaction has been shown to occur in male and female breast, forehead and axillary skin. The sulphotransferase, which presumably catalyses the reaction appears to act on DHA, androstenediol and epiandrosterone, but not on testosterone or androsterone. In forehead and axillary skin experiments the formation of sulphoconjugates from testosterone and androstenedione was sought. While about 1% of the incubated radioactivity was found in the sulphate fractions isolated from incubations with these two steroids, after solvolysis followed by chromatography and recrystallization only epiandrosterone and material remaining on the origin after t.l.c. in system I were radioactive.

It is interesting that the percent conversions of DHA to DHAsulphate were similar in all experiments, despite considerable variations in formation of other metabolites. Recently, it has been shown in this laboratory that conversion of DHA to DHA-sulphate could be demonstrated in rat skin but not in the preputial gland (Hodgins and Hay, 1971 unpublished work) and it is possible that, while total hydroxysteroid dehydrogenase and 5α-reductase activity in a piece of skin may be related to the presence of pilosebaceous units, the activity of steroid sulphotransferase is not.

c. UDP-Glucuronyl Transferase (EC. 2.4.1.17).

In no experiment was more than 0.05% (uncorrected for recovery) of the total radioactivity recovered in the glucuronide fraction. This suggests that "in vitro", neither DHA nor

testosterone nor any of their major metabolites are converted in significant amounts to glucuronides in skin.

d. 3β-Hydroxysteroid dehydrogenase- Δ^{4-5} isomerase.

(Δ^5-3β-HSD : abbreviation).

The conversion of a C5-6 unsaturated 3β-hydroxysteroid to the corresponding C4-5 unsaturated 3-oxosteroid is catalysed by this enzyme complex. The presence of Δ^5-3β-HSD in human skin was first inferred from the histochemical studies of sebaceous glands (Baillie et al., 1965). Cameron et al. (1966), then Chakraborty et al. (1970) used biochemical techniques to demonstrate the enzyme system in human skin. Chakraborty showed that DHA was converted to androstenedione by human skin and that NAD^+ was required as co-factor. Enzyme activity was higher in shoulder skin than in thigh or lateral thoracic wall skin. As the histochemical studies suggested that Δ^5-3β-HSD was localized in sebaceous glands, Chakraborty's finding could be interpreted in terms of the more numerous and larger sebaceous glands in shoulder skin than in the other two areas, (Montagna, 1963; Calman et al., 1970). Chakraborty's experiments also showed that enzyme activity in shoulder skin decreased with advancing age as had previously been suggested by Baillie et al. (1966). However, the experiments described here have shown the presence of active Δ^5-3β-HSD in forehead skin from an 83 year old man. This could be related to the presence of numerous large sebaceous glands in this skin specimen. Further support for the theory that the enzyme is localized in sebaceous glands has come from studies of the rat preputial gland, which is composed almost entirely of sebaceous cells. A histochemical 3β-HSD-reaction with DHA as substrate and NAD^+ as co-factor has been demonstrated in the lipid laden cells of this gland (Muir et al, 1970). The presence of Δ^5-3β-HSD has been confirmed by demonstrating conversion of DHA to androstenedione by preputial gland preparations (Hodgins and Hay, 1971 unpublished work).

Δ^5-3β-HSD is a key enzyme in biosynthesis of steroid hormones. In skin, it catalyses a first step in conversion of

DHA to the highly active androgens, testosterone and dihydrotestosterone. Thus the stimulation of sebaceous secretion by DHA (Pochi and Strauss, 1969) may be mediated through its metabolism by Δ^5-3β-HSD.

e. 17β-Hydroxysteroid Dehydrogenase

(abbreviation : 17β-HSD)

Wotiz et al. (1956), first demonstrated the conversion of testosterone to androstenedione by human shoulder and scalp skin in the presence of NAD^+. Histochemical studies have localized 17β-HSD (testosterone or oestradiol as substrate, NAD^+ as cofactor) in sebaceous glands from all over the body (Calman et al., 1970). There appears to be a localization of enzyme activity in groups of cells around the edges of sebaceous gland acini (Calman, 1970b).

It has been shown that DHA is converted to androstenediol in skin and that NADPH will act as a cofactor (Chakraborty, 1968 unpublished work). The experiments described here have established the conversion of androstenedione to testosterone in human male and female skin. They have also shown that DHAsulphate is converted to androstenediol sulphate in skin thus opening the possibility of conversion of DHAsulphate to testosterone by a pathway involving neither DHA nor androstenedione as intermediates (see Fig. 12 p.112).

However, the main point of interest arising from the breast, forehead and axillary skin experiments is the difference in the direction of the 17β-HSD reaction which predominated in the various skins. In male forehead skin there was extensive oxidation of the testosterone 17β-hydroxyl group but only minute conversions of DHAsulphate, DHA or androstenedione to 17β-hydroxysteroids. A similar situation may exist in female breast skin. This was not incubated with testosterone, but incubation with DHAsulphate and DHA showed very little conversion to 17β-hydroxysteroids. In contrast, the female axillary skin actively reduced DHAsulphate, DHA and androstenedione to 17β-hydroxysteroids which were major metabolites. Because of the high activities of Δ^5-3β-HSD and

5α-reductase in all skin specimens, it appeared to be the 17β-HSD reaction which was responsible for the differences in accumulation of androgenically highly active compounds (dihydrotestosterone, androstanediols) or relatively inactive compounds (androsterone, epiandrosterone) in the various experiments. It cannot yet be decided which factors control the interconversion of 17β-hydroxy and 17-oxosteroids in skin but a possibility is the availability of oxidized and reduced forms of NAD and NADP which are coenzymes in the reaction. Gurpide and Welch (1969) showed that interconversion of oestradiol and oestrone and of testosterone and androstenedione in human endometrial tissue was influenced by tissue slice thickness and oxygenation of the incubating medium. This was interpreted as an effect of tissue oxygenation on the generation of oxidized and reduced NAD and NADP. The experiments described here were carried out with uniformly thick tissue slices and conditions of incubation were similar in all cases. It is therefore likely that the different results obtained with the various skins reflect basic metabolic differences between the skins rather than artefacts due to varying incubation conditions.

Studies of rat liver have shown that the ratios of the concentrations of unbound NAD^+/NADH and $NADP^+$/NADPH differ widely between the mitochondrial and cytoplasmic compartments of the cell (Williamson et al., 1967; Veech et al., 1969). It has been shown that there exist in guinea pig liver two forms of 17β-HSD which interconvert testosterone and androstenedione (Endahl, et al., 1960). One form, localized in the soluble cytoplasm requires NADP(H) as coenzyme, the other, localized in the mitochondrial-microsomal fraction requires NAD(H). In view of the above it would be of interest to know the subcellular localization and coenzyme specificity of 17β-HSD of human skin from different body sites.

f. 5α-Reductase (E.C. 1.3.1.)

There was extensive 5α-reduction of testosterone and

androstenedione when they were incubated with skin. In addition, when DHAsulphate or DHA were incubated, the low % conversions to testosterone and androstenedione compared with those to 5α-steroids showed that active 5α-reduction had occurred. 5α-Reductase, a key enzyme in metabolism of testosterone and androstenedione, is present in many tissues - liver, gonads, accessory sex organs ... skin (Stylianou et al., 1961; Forchielli et al., 1963; Gloyna and Wilson, 1969). Most androgen target tissues so far investigated contain the testosterone 5α-reductase (Gloyna and Wilson, 1969), an exception is the rat levator ani muscle. In the rat prostate the enzyme has been detected in the cell nuclei and it has been proposed that conversion of testosterone to dihydrotestosterone in the prostate cell nucleus is followed by binding of the latter steroid to the chromatin (Bruchovsky and Wilson, 1968a,b). The further effects of androgen on the cell would then follow from this nuclear binding of dihydrotestosterone. However Gloyna and Wilson, (1969) found only low 5α-reductase activity in the prostates of mature rabbit and bull. In addition Jeffcoate and Short (1970) concluded that testosterone not dihydrotestosterone may be the active androgen in the bull testis, after their failure to isolate dihydrotestosterone from this organ. Voigt et al. (1970), could not find any 5α-reductase in the nuclear fraction prepared from human foreskin and all conversion of testosterone to dihydrotestosterone took place in the microsomal fraction. As pointed out by Voigt this does not exclude dihydrotestosterone from being the active form of androgen in skin, because it does not need to be formed in the cell nuclei to exert its effects, demonstrated by the androgenicity of injected or topically applied dihydrotestosterone (Dorfman and Dorfman, 1962, 1963a,b). Voigt et al. (1970) showed that conversion of testosterone to dihydrotestosterone by foreskin microsomal fractions had an absolute requirement for NADPH as coenzyme. It was also shown that the same enzyme catalyses 5α-reduction of progesterone and probably androstenedione. This finding supports the claim that the differences in percent conversions of testosterone

to dihydrotestosterone between axilla and forehead skin were the result of the action of 17β-HSD and not due to differences in activity of a 5α-reductase specific for testosterone.

As dihydrotestosterone may be the active molecular species of androgen it is important to consider the possible anatomical localization of 5α-reductase in skin. Northcutt et al. (1969) have shown that plucked pubic hair follicles contain the enzyme. The rat preputial gland and chicken uropygial glands, which are composed mainly of sebaceous cells, have high 5α-reductase activity (Gloyna and Wilson, 1969; Rongone et al. 1967). Thus we have evidence for the presence of 5α-reductase in hair follicles and sebaceous glands, on which basis the presence of 5α-reductase in breast, forehead and axillary skin could be explained. Wilson and Walker (1969) found that human neonatal foreskin had high concentrations of 5α-reductase. They used the stretching technique of Van Scott (1952) to separate foreskin into "epidermis", "dermis" and "sub dermal fibrous tissue" (Wilson and Walkers' terminology). The"sub dermal tissue" had highest 5α-reductase activity with dermis and epidermis (probably contaminated by dermis) having lower enzyme activity. It is difficult to know what the exact composition of Wilson and Walkers' "dermal" and "sub dermal"fractions were, but they claim the result shows that fibrous tissue cells may have high 5α-reductase contents. As foreskin contains many sebaceous glands some of the 5α-reductase could have been localized in them. However, Wilson and Walker also detected 5α-reductase activity in skin from palmar and plantar skin which have no sebaceous glands.

The presence of 5α-reductase in fibroblasts could help to explain the observation that 5α-reductase activity in foreskin decreased greatly with increasing age from birth (Wilson and Walker, 1969), when enzyme activity was expressed on a "per wet tissue weight" basis. In the foetus and neonate the fibrous tissues of the dermis are much more cellular than in adults (Montagna, 1962). (i.e. more fibroblasts, less fibrous material).

As the fibrous tissue constitutes most of the skin's dry weight, the number of enzyme containing cells per wet weight of tissue will decrease with increasing age and therefore lead to the observed decrease of 5α-reductase activity. This theory could be simply checked by expressing enzyme activity on a "per μg. of DNA" basis.

It might be considered that, if fibroblasts of skin do contain 5α-reductase, they may differ in enzyme content over different parts of the body. Their enzyme content could perhaps be related to the presence of pilosebaceous units, as areas of skin with many of these have high enzyme activity (facial skin, axilla, pubic skin, perineal skin).

It can be seen from the foregoing arguments that, while there is circumstantial evidence for the presence of 5α-reductase in sebaceous glands, hair follicles and fibroblasts, it will be necessary to isolate the different tissue types of skin and study them individually before direct information about their enzyme activities can be obtained.

g. 5β-Reductase (E.C. 1.3.1.)

5β-reduction of testosterone and androstenedione has been demonstrated in human and rat liver (Stylianou et al., 1961; Forchielli et al., 1963). The conversion of androstenedione and testosterone to etiocholanolone by forehead and axillary skin suggests that there is a 5β-reductase in human skin. However, no conversion ($<0.01\%$) to 5β-androstane-3, 17-dione could be detected after chromatography and recrystallization.

Rongone (1966) reported the isolation of etiocholanolone after incubating testosterone with mammary skin of a man with Klinefelter's syndrome. Gomez and Hsia (1968) were however unable to detect any conversion of testosterone or androstenedione to 5β-steroids by normal human abdominal and preputial skin. Likewise Frost et al. (1969) could demonstrate no 5β-reduction of progesterone in human skin. The results here lend support to Rongone's earlier studies, but it is obvious that in normal human skin

5β-reduction of testosterone and androstenedione is of minor importance, compared to 5α-reduction. From the results of 'in-vivo' studies of testosterone metabolism in man Mauvais-Jarvis et al. (1969a,1970a) have concluded that 5β-reduction takes place mainly in the liver and possibly intestines. They found that the 5α/5β ratio of urinary testosterone metabolites is greatest when testosterone is rubbed directly into the skin; the ratio is lower for intravenously injected testosterone and lowest for orally administered testosterone. In this last case, a large part of the testosterone perfuses the liver directly before entering the peripheral circulation; so that it would be catabolised by the 5β-reductase of liver.

h. 3α-Hydroxysteroid Dehydrogenase (E.C. 1.1.1.50.)

(abbreviation: 3α-HSD)

The conversion of testosterone and androstenedione to 3α-androstanediol and androsterone shows that there is, in the skin, a 3α-HSD. In liver the 3α-HSD reaction has been shown to be freely reversible, and the enzyme will use NAD or NADP as cofactor (Tomkins, 1956; Hurlock and Talalay, 1958). It has been suggested that because of this dual coenzyme specificity 3α-HSD can act as a transhydrogenase effecting the interconversion of oxidized and reduced NAD and NADP (Hurlock and Talalay, 1958). Recently, it has been shown that NADPH will serve as cofactor for the reaction in human skin (Voigt et al. 1970). Histochemical methods have been used to demonstrate 3α-HSD in rat skin; both NAD^+ and $NADP^+$ have been shown to act as coenzymes for the reaction (Muir, 1971). However, when the histochemical technique is applied to human skin, even to samples known from biochemical studies to actively form androsterone, no 3α-HSD can be detected with either NAD^+ or $NADP^+$ as cofactors. So far no satisfactory explanation for this phenomenon has been found.

i. 3β-Hydroxysteroid Dehydrogenase (E.C. 1.1.1.)

(abbreviation: 3β-HSD)

The conversion of androstenedione and testosterone to epiandrosterone and 3β-androstanediol by human skin shows the

presence of 3β-HSD.

In human prostatic tissues Chamberlain et al. (1966) detected 3β-HSD when testosterone was incubated with NADPH as cofactor. In human sebaceous glands, 3β-HSD activity can be detected histochemically with epiandrosterone as substrate; only NAD^+ will act as coenzyme (p. 110). It is unknown whether the histochemically observed oxidations of epiandrosterone and DHA in sebaceous glands are due to two different enzymes or to dual substrate specificities of one enzyme, i.e:

a) Does the histochemical reaction with DHA demonstrate activity of Δ^5-3β-HSD while the reaction with epiandrosterone demonstrates 3β-HSD?

b) Are both DHA and epiandrosterone being oxidized by Δ^5-3β-HSD?

c) Are both DHA and epiandrosterone being oxidized by a 3β-HSD not associated with any isomerase?

This last point is of some importance for interpretation of the histochemically observed oxidation of DHA in sebaceous glands, for while there is undoubtedly a Δ^5-3β-HSD in skin (conversion of DHA to androstenedione) the histochemical reaction in sebaceous glands may not be demonstrating its activity so much as that of a simple 3β-HSD.

In all the experiments with human skin, the % conversions to 3β-hydroxysteroids were much less than to 3α-hydroxysteroids ($3\alpha/3\beta > 10$). This was irrespective of body site, sex or age of the skin.

Mauvais-Jarvis et al. (1970b) have investigated metabolism of 3β-androstanediol "in-vivo" by injecting [^{3}H] 3β-androstanediol into men. They found that more than 80% of the radioactivity excreted in the urine was recovered in androsterone and 3α-androstanediol; so there had been extensive conversion of the 3β-hydroxysteroid to 3α-hydroxysteroids. When [^{3}H] 3α-androstanediol was injected, very little of the urinary radioactivity was present as 3β-hydroxysteroids, most of it being in 3α-androstanediol and androsterone showing that there was

little conversion of 3α-hydroxy to 3β-hydroxysteroids.

Interest in 3β-androstanediol has been centered around its specific stimulation of rat prostatic secretion (Baulieu et al., 1971) and more recently its effects on sebum secretion and composition in rats (Nikkari and Valavaara, 1970). It has been suggested that the biological effects of 3β-androstanediol are mediated independently of any conversion to dihydrotestosterone (Baulieu et al., 1971; Robel 1970). Therefore, the formation of this compound in human skin is of great interest, particularly as it is a more active stimulator of sebum secretion than is dihydrotesterone when tested on the hypophysectomized female rat (Nikkari and Valavaara, 1970).

j. Hydroxylases.

Human skin appears to contain an enzyme system which hydroxylates C_{19}-steroids. As yet, nothing is known about its properties and even the exact identities of the polar metabolites are unknown. Faredin et al. (1967, 1969) have reported the hydroxylation at positions 7α, 7β and 16α of DHA in human skin. However, as was argued earlier it is unlikely that either 7α-hydroxy or 7-oxosteroids were among the polar metabolites isolated here. Likewise, a search for formation of 16α-hydroxy DHA was unsuccessful (p. 64). In all the experiments, the androstenediol fraction isolated from the first t.l.c. plate was examined for the presence of 16α-hydroxy DHA, 11β-hydroxy androstenedione or any other compounds of similar polarity (see p. 30 Fig. 5). There was never more than about 0.1% of the total radioactivity associated with such compounds, compared to usually more than 10% associated with the more highly polar fractions.

The hydroxylation reaction, which decreases the biological activity of androgens can be thought of as antagonizing the actions of the hydroxysteroid dehydrogenases and 5α-reductase which may form potent androgens from less active precursors in skin.

k. 17-20 Desmolase and 17α-hydroxylase.

Recently in an extensive study of steroid metabolism by rat skin (Flamigni et al., 1970) it was shown that both pregnenolone

and progesterone were converted to their 17α-hydroxy derivatives in rat skin. In addition, 17α-hydroxy progesterone was converted to androstenedione and pregnenolone was converted (via 17α-hydroxy pregnenolone) to DHA.

This finding leads to the possibility that pregnenolone and progesterone may be converted to potent androgens in the skin. However, it should be remembered that Frost et al. (1969) showed that more than 85% of progesterone metabolites formed in human skin retained either the 4-pregnene or 5α-pregnane structures, and even in Flamigni's study C_{19}-steroids were only minor metabolites of progesterone. It is now generally accepted that in physiological doses progesterone does not stimulate the sebaceous glands of human or rat skin (Strauss and Pochi, 1963). However it has been shown that topical application of large amounts of pregnenolone acetate or progesterone to the aged human axilla elicit similar, although much less marked, changes to those seen after testosterone administration. The effects appeared to be restricted to the axilla under treatment and it was suggested that pregnenolone and progesterone were transformed locally into "androgenic substance" (Papa and Kligman, 1966).

2. VARIATIONS OF SKIN STEROID METABOLISM IN RELATION TO AGE, SEX AND BODY SITE.

The conversion of DHAsulphate and DHA to testosterone, dihydrotestosterone and androstanediols by skin from breast, face and axilla of male and female has been demonstrated. However, there are quantitative differences between skins in the % conversions to 17-oxo or 17β-hydroxy metabolites. The metabolic differences between axilla skin and forehead or breast skin might be interpreted in terms of:-

a) Body site differences related to the presence of pilosebaceous units;

b) Age differences between individuals (female axilla - 20 years, male forehead-83 years.)

c) Sex differences (male forehead, female axilla and breast);

d) A combination of a, b. c.

Female breast skin and male forehead skin are anatomically similar (large sebaceous glands, no terminal hairs) and have a similar metabolic pattern dominated by formation of 5α-androstanedione and androsterone (17-oxo pathway). Recent experiments with Dr. J.B. Hay have shown that forehead skin from a young man (20 yrs.) and cheek skin from an old woman (76 yrs.) also have a similar metabolic pattern. The total enzyme activity was, however, very much reduced in the cheek skin from the old woman (sebaceous gland atrophy? (Strauss and Pochi, 1963)). On the other hand, skin from the axilla of a 43 yr. old man had a similar metabolic pattern to that of the young female axilla (active formation of dihydrotestosterone and 3α-androstanediol). Jenkins and Ash (1971) have recently reported in-vitro studies of testosterone metabolism in suprapubic skin of men and women. They found a similar metabolic pattern to that which has been found in axilla skin and concluded that there were no differences between the sexes. While, at first sight, the results of Jenkins and Ash show no differences between male and female, if they are re-calculated as 17β-hydroxy/17-oxo ratios (dihydrotestosterone/androstenedione + 5α-androstanedione + androsterone) there are differences. The mean value for males is 0.71 $\pm$ 0.08 (7) S.E.; that for females is 1.04 $\pm$ 0.10 (7) S.E. This difference is just statistically significant ($0.02 < P < 0.05$. Student's-t. test) and shows a greater accumulation of dihydrotestosterone relative to 17-oxosteroids in female skin experiments. This was mainly due to a greater conversion of testosterone to 17-oxosteroids in male skin experiments than in female skin experiments. In view of the fact that old and young male forehead skin formed mainly 17-oxosteroid metabolites, it will be interesting to study steroid metabolism in facial skin of young women with active sebum secretion and compare it with facial skin from men of comparable age.

A recent paper by Sansone and Reisner (1971) has reported the conversion of testosterone to dihydrotestosterone by facial skin from men and women with acne and from "normal" individuals. They used the incubation system of Wilson and Walker (1969) with minced skin incubated in Krebs original Ringer Phosphate - Glucose medium for 3 hours.

One finding was that percent conversions to dihydrotestosterone were equal to or greater than those to 17-oxosteroids. There was very little androsterone formed in those incubations where its presence was sought. This is in direct conflict with the results of the experiments described here and those recently carried out on young male forehead skin. The metabolic pattern was in fact similar to that which was observed in axilla skin. It is possible that these differences are due to the incubation conditions used by both groups of workers. Sansone and Reisner used a testosterone concentration of 0.3μM, i.e.: 10 times less than that used here. In addition, they used an incubation medium with high phosphate concentration (22 mM)while a low phosphate medium (1.2 mM) was used here. The concentration of phosphate in a tissue has been implicated in control of glycolysis and reduction of cytoplasmic NAD^+ (Williamson et al., 1967). Any such effects on cytoplasmic NAD^+/NADH ratios might alter interconversion of 17β-hydroxy and 17-oxosteroids. Differences in tissue oxygenation caused by variation in thickness of slices or minced tissue or in the method of gassing the medium might have similar effects (Gurpide and Welch, 1969). These problems emphasize the care which must be exercised when results of "in-vitro" incubations under different conditions are compared and extrapolated to the "in-Vivo" situation.

3. STEROID METABOLISM AND ANDROGEN ACTION ON SKIN.

The differences detected between forehead or breast and

axilla skins in accumulation of 17β-hydroxy and 17 oxo-metabolites emphasise the control which 17β-HSD activity had over metabolism. Metabolism via the 17-oxo route could be termed "deactivating" as coupled with the high 5α-reductase activity of skin, it leads to accumulation of compounds with low biological activity - androsterone, epiandrosterone, 5α-androstanedione. Metabolism via the 17β-hydroxy route could be termed "activating", as it leads to accumulation of highly active androgens - dihydrotestosterone and androstanediols (Dorfman and Dorfman, 1962, 1963a, b).

It might be suggested as a hypothesis that target tissues have the necessary activating metabolism for converting steroids to dihydrotestosterone and androstanediols while non-target tissues have a mainly deactivating metabolism. Some support for this idea comes from studies of male accessory sex organs, which actively convert testosterone to dihydrotestosterone (Gloyna and Wilson, 1969) and of non-target tissues which convert testosterone to weakly androgenic substances, eg: human endometrium (Collins et al., 1969), canine urinary bladder (Morfin et al., 1970) and the submaxillary gland of the dog (Weiner et al., 1970). In a recent paper, Kelch et al., (1971) compared the metabolism of testosterone in various human adult and foetal tissues. Results were expressed in terms of conversion to dihydrotestosterone relative to conversion to 17-oxosteroids (17β-hydroxy/17-oxo ratio). It was found that target tissues - perineal skin, and vas deferens-had a ratio about 1 while presumed non-target tissues - fetal thigh skin, skeletal muscle, Müllerian duct, fallopian tubes, all gave ratios less than 0.1.

It would be interesting to know from further studies if there are any metabolic differences between androgen target organs which develop in both sexes (eg; axillary hair) and those which develop only in the male (eg. prostate). While prostatic tissue incubated in-vitro converted testosterone mainly to dihydrotestosterone there was little conversion of DHA or androstenedione to testosterone and dihydrotestosterone (Collins et al., 1970).

However the results of the experiments described here do not entirely conform to the above hypothesis. Both male and female chest, facial and axilla skin are sensitive androgen

target areas. Their numerous sebaceous glands are stimulated by adrenal and gonadal androgens (DHA, androstenedione, testosterone). The terminal hairs of the axillary region are similarly sensitive (Part I sections 1i -1iii). In spite of this, the in-vitro metabolic patterns of forehead and axillary skin are quite different. The conversion of DHAsulphate, DHA, androstenedione and testosterone to dihydrotestosterone and androstanediols in axillary skin fits well with the fact that the hair follicles of this region respond to testicular,ovarian and adrenal stimuli. However, in breast and forehead skin, the "deactivating" pattern of metabolism is predominant with little accumulation of testosterone or dihydrotestosterone. Yet the sebaceous glands of forehead are among the most sensitive of androgen target tissues. How can this paradox be explained?

i) The 'in-vitro' studies do not give the same metabolic patterns as 'in-vivo'. It has already been argued that the different results obtained with axillary or forehead skin 'in-vitro' represent real metabolic differences between the skins, which resulted in the different percent conversions to 17β-hydroxy and 17-oxo-steroids. It is however possible that such metabolic controls 'in-vivo' would give rise to other patterns of steroid metabolism. This is not to say that 'in-vivo' the control of 17β-HSD activity is unimportant in determining patterns of steroid metabolism but that control of 17β-HSD 'in-vivo' could give rise to different metabolic patterns to those observed 'in-vitro'. Possibly further studies of the effects of incubation conditions on 'in-vitro' steroid metabolism or 'in-vivo' perfusion experiments would help resolve this problem.

ii) Assuming that the patterns of steroid metabolism 'in-vitro' are similar to those 'in-vivo', it may be that, in spite of a predominantly deactivating metabolism in forehead and breast skin, enough biologically active androgen reaches and is retained in the sebaceous glands to maintain sebum secretion in male and female.

iii) Androgen metabolism in skin is related to some specific catabolic function of sebaceous glands. This idea comes from the suggestion that sebaceous glands are involved in catabolism and excretion of foreign compounds and steroids (Rashleigh et al., 1967; Cook and Lorincz 1963). In this case, an activating metabolism in a relatively few androgen target cells might be masked by deactivating metabolism in the differentiated sebaceous cells.

4. STEROID METABOLISM IN RELATION TO ENDOCRINE DISEASES INVOLVING SKIN.

i) Testicular Feminization:

Attempts have been made to explain the general target organ insensitivity to androgen in this condition by impaired conversion of testosterone to dihydrotestosterone in target tissues. Various in-vitro studies of skin have not provided any convincing evidence for this concept. Northcutt et al. (1969), claimed that suprapubic skin from individuals with testicular feminization had a deficiency of 5α-reductase. Jenkins and Ash (1971) could not confirm this and in two patients the suprapubic skin had normal testosterone metabolism. Wilson and Walkers' (1969) experiments on mons pubis skin aggree with those of Jenkins and Ash but they did claim a possible deficiency of 5α-reductase in perineal skin from subjects with testicular feminization. By applying [^{3}H] testosterone to the skin and simultaneously injecting [^{14}C] testosterone into subjects with testicular feminization, Mauvais-Jarvis et al. (1969a) have provided evidence that there may be a deficiency of direct A-ring reduction of testosterone in the extrahepatic tissues. There did not appear to be a similar deficiency in progesterone 5α-reduction (Mauvais-Jarvis et al., 1969b). However Voigt et al. (1970) showed that in skin it is probably the same enzyme which catalyses 5α-reduction of testosterone and progesterone. This is difficult to reconcile with

Mauvais-Jarvis's findings unless the properties of the 5α-reductase are altered in testicular feminization. Further evidence against a simple 5α-reductase deficiency being responsible for testicular feminization was provided by Strickland and French (1969) who showed that patients with this syndrome were unresponsive to dihydrotestosterone.

ii) Idiopathic Hirsutism:

It was suggested in the introduction (p. 5) that this condition might be explained in terms of the prehormone concept. It appears from the 'in-vitro' experiments that skin, characterized by hair follicles which are sensitive to stimulation by female androgen levels (axilla), has an "activating" C_{19}-steroid metabolism. This appears to result from active conversion of 17-oxo to 17β-hydroxysteroids coupled with high 5α-reductase activity and leads to formation of dihydrotestosterone and androstanediols. Skin characterized by hair follicles which do not normally respond to female androgen levels (breast skin) may have a "deactivating" metabolism, resulting in 5α-androstanedione and androsterone formation with little dihydrotestosterone. If the metabolism observed is characteristic of the hair follicles and if it is a property of the follicles before they have been androgen stimulated (i.e. a cause rather than an after-effect of the hormone response), it might be suggested that in idiopathic hirsutes the follicles of areas which are not normally sensitive to female androgen levels, become so through developing an"activating" type of metabolism. This hypothesis could be tested by studying the steroid metabolism of skin from the appropriate body sites of men, normal women and women with hirsutism. It would also be important to investigate steroid metabolism in areas of skin which differ in androgen sensitivity before they have been exposed to androgenic stimulus, so that it can be determined whether or not metabolic patterns characteristic of hairy areas or those with well developed sebaceous glands exist before these have developed under androgen stimulation. This would require studies of steroid metabolism in skin from prepubertal children and foetuses. In addition, the enzyme activites of the hair follicles must be established by direct studies on these organs.

iii) Acne Vulgaris:

Increased sebum secretion and hyperplasia of the sebaceous

gland duct in acne might be caused by an abnormal target organ response to androgens. Workers in Hungary have recently claimed that skin of acne patients contains more DHA (DHA + DHAsulphate) than skin of normals (Vadasz and Debreczeni, 1969). Drawing on the original hypotheses of Baillie, Milne and co-workers (Baillie et al., 1965, 1966) they suggest that increased conversion of DHA to testosterone in skin is a cause of acne. In a limited study, Chakraborty found no differences of $\Delta^5 3\beta$-HSD activity in shoulder skin of acne patients compared to normals (Chakraborty et al., 1970). Sansone and Reisner (1971) have studied the conversion of testosterone to dihydrotestosterone in acne patients. No significant differences between acne and normal were noted for face or back skin of men (although 2 normal forehead specimens had lower conversions to dihydrotestosterone than did acne specimens). There was, however, a highly significant increase of dihydrotestosterone formation in face and back skin of females with acne compared to normals. However, these workers give no indication of the ages of their normal and acne groups. An age difference of steroid metabolism could be important if total enzyme activities are at all related to the presence of active sebaceous glands. This will be especially so in the female, as sebaceous gland activity declines quite sharply with increasing age in women (Strauss and Pochi, 1963). Sansone and Reisner do not state whether increased conversions to dihydrotestosterone were associated with increases or decreases in conversion to other steroid metabolites (17-oxo, 3α-hydroxy, 3β-hydroxy). As the experiments described here showed that the % conversion of four different steroids to dihydrotestosterone were not dependent so much on the level of 5α-reductase in the skin as on the activity of 17β-HSD, it may well be that changes in dihydrotestosterone formation, by skin, in acne, are associated with alterations in the 17β-HSD reaction rather than with increases of 5α-reductase activity. In view of this, any attempts to study changes of steroid metabolism in skin of "normal" and diseased subjects must take into account the overall steroid metabolic patterns. Even so, the

observations of Sansone and Reisner (1971) are of great interest and a stimulus to further studies.

One particular problem relating to acne is that of the effects of testosterone metabolites on sebaceous glands. The work of Nikkari and Valavaara (1970) has shown that dihydrotestosterone stimulates sebum secretion in rats but its activity is only 50% of that of 3β-androstanediol or testosterone. Dihydrotestosterone is, however, a very active stimulator of preputial gland and prostate gland weight increases in the rat (Ebling et al., 1971; Dorfman and Dorfman, 1963a). It will be interesting to know whether there is any dissociation of effects between testosterone, dihydrotestosterone and 3β-androstanediol on sebaceous glands as has been demonstrated in organ cultures of rat prostate, where dihydrotestosterone caused a marked increase in the number and size of epithelial cells while 3β-androstanediol specifically stimulated the secretory activity (Baulieu et al., 1971). It could be that in sebaceous glands 3β-androstanediol stimulates sebum production while dihydrotestosterone causes hyperplasia of the gland epithelium and ducts (Sansone and Reisner, 1971). An effect of dihydrotestosterone independent of any increase in sebum secretion would be important in acne and could help explain the poor correlation between sebum secretion rates and the tendency to develop acne (p. 5).

However, even if some differences in effects or metabolism of androgens can be found between skin of acne and non-acne groups, the fact that one sebaceous gland may develop a lesion while adjacent ones remain unaffected will have to be explained. This is a major problem in acne as lesions may develop on all normal individuals around puberty; acne sufferers simply have more of them than others. Biopsies taken from acne sufferers, for 'in-vitro' studies, will invariably be of largely uninvolved skin from areas afflicted by acne, and 'in-vivo' measurements (such as sebum secretion) will usually measure activity of both unaffected and affected sebaceous glands. Therefore if any gross differences are detected in skin of normal individuals

and acne sufferers (sebum secretion, steroid metabolism) it will have to be determined whether:-

i) They are characteristics of the whole skin on the body area from which the specimen was taken (for 'in-vitro' study) or on which the measurement was made. If this is the case, a second factor must exist which determines whether or not a sebaceous gland will develop an acne lesion.

ii) They are due to abnormal characteristics of a few pilosebaceous units which will develop or have developed acne lesions.

Before a full understanding is possible of the pathogenesis of acne and of the role of steroid hormones in its development, it will be necessary to discover what causes sebaceous glands to differ in their tendencies to develop acne lesions. For the study of skin steroid metabolism in acne, this means that the precise anatomical sites of steroid metabolism in skin will have to be defined, so that enzymic changes in sebaceous glands themselves can be measured, before further progress can be expected.

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APPENDIX 1.

TABLES FOR CALCULATING THE % CONVERSIONS OF STEROID SUBSTRATES TO VARIOUS METABOLITES.

The following tables list the percentage recoveries of initially added carrier steroids in the samples taken for crystallization from incubations of human skin with different steroid substrates. This enables the % conversions to metabolites to be calculated from the specific activities obtained after crystallization.

Equation 4 (p.23) gives:

$$\% \text{ conversion to metabolite X} = \frac{Sf.Mx.100}{di} \cdot \frac{Y}{y}$$

Now $\frac{y}{Mx} \cdot 100$ = % recovery (%R) of initially added carrier in the sample for crystallization.

$$\% \text{ conversion to X} = \frac{Sf.Y.10^4}{di.\%R} \quad (5)$$

Sf = specific activity after crystallization (d.p.m./mg)
Y = mg. carrier added for crystallization
di = d.p.m. steroid substrate incubated

Thus % conversions can be calculated from the specific activities of the crystals and the % recoveries.

In experiments where characterization by derivative formation was followed by crystallization (expts. 1D, 2D: table 7, p.55 and tables 8a-h, pp.56-60) equation 2 (p.24) can be used to calculate the % conversion:

$$\% \text{ conversion} = \frac{Sx.Mx.100}{di} \cdot \frac{Sf}{S_t} \quad (2)$$

$\frac{Sf}{S_t} \cdot 100$ = percent calculated specific activity (%T)

$$\% \text{ conversion} = \frac{Sx.Mx.\%T}{di}$$

%T is given in the tables of crystal specific activities
Sx = specific activity of isolated steroid before carrier was added for crystallization.
Mx = mass of initially added carrier
di = d.p.m. substrate incubated.

%RECOVERY OF CARRIER STEROIDS IN SAMPLES TAKEN FOR CRYSTALLIZATION FROM INCUBATIONS OF HUMAN FEMALE BREAST SKIN WITH DHA

Carrier Steroid	Experiment No.			
	1a	1b	2a	2b
dihydrotestosterone	4.7	[illegible]	10.4	12.5
androsterone	16.0	10.4	14.4	15.7
epiandrosterone	18.2	13.1	19.6	14.2

The above data are used with the specific activities after crystallization (tables 8a-8h,pp.56-60) to calculate % conversions (table 9,p.65).

%RECOVERY OF CARRIER STEROIDS IN SAMPLES TAKEN FOR CRYSTALLIZATION FROM AN INCUBATION OF HUMAN FEMALE BREAST SKIN WITH DHASULPHATE

Carrier Steroid	Experiment No.
	1 DS
DHA	36.5
DHAsulphate	9.6
androstenediol	18.1
androstenediol sulphate	11.3
androsterone	15.3
androstenedione	33.8
5α-androstanedione	26.0

The above data are used with the specific activities after crystallization (table 10,p.71) to calculate % conversions (table 11,p.72).

% RECOVERY OF CARRIER STEROIDS IN SAMPLES TAKEN FOR CRYSTALLIZATION FROM INCUBATIONS OF HUMAN MALE FOREHEAD SKIN WITH FOUR STEROID SUBSTRATES

Carrier Steroid	Experiment No.			
	3D	3DS	3AE	3T
DHA	59.0	58.5		
androsterone	20.7	17.3	14.1	19.5
epiandrosterone	41.9	39.4	33.4	
epiandrosterone sulphate	24.6		27.0	
androstenediol	12.3	32.9		
3β-androstanediol			14.8	14.0
3α-androstanediol			16.7	22.4
testosterone	50.4	38.4	47.7	54.5
5α-androstanedione	46.1	50.8	49.2	51.8
androstenedione	78.1	81.0	78.3	63.2
dihydrotestosterone	47.2		29.9	39.7
etiocholanolone			21.2	19.4
DHAsulphate	23.2	22.5		

The above data are used with the specifc activities after crystallization (tables 13a-m , pp. 80-86) to calculate % conversions (table 15, p.89).

% RECOVERY OF CARRIER STEROIDS IN SAMPLES TAKEN FOR CRYSTALLIZATION FROM INCUBATIONS OF HUMAN MALE FOREHEAD SKIN WITH FOUR STEROID SUBSTRATES

Carrier Steroid	Experiment No.			
	4D	4DS	4AE	4T
DHA	60.3	48.1		
androsterone	18.4	15.0	13.7	20.8
epiandrosterone				44.7
epiandrosterone sulphate				27.0
androstenediol	34.0			
3β-androstanediol				12.6
3α-androstanediol			26.3	10.6
testosterone	68.2	49.2	54.6	51.8
5α-androstanedione	57.6	49.2	46.9	48.4
androstenedione	77.9	69.0	70.1	71.5
dihydrotestosterone	43.7	32.2	30.8	44.9
etiocholanolone			15.0	20.1
DHAsulphate	24.5	25.9		

The above data are used with the specific activities after crystallization (tables 13a-m, pp.80-86) to calculate % conversions (table 16, p.90).

% RECOVERY OF CARRIER STEROIDS IN SAMPLES TAKEN FOR CRYSTALLIZATION FROM INCUBATIONS OF HUMAN FEMALE AXILLARY SKIN WITH FOUR STEROID SUBSTRATES.

Carrier Steroid	Experiment No.			
	5D	5DS	5AE	5T
DHA	61.4	52.1		
DHAsulphate	45.5	55.4		
epiandrosterone	27.9	38.5	9.8	19.2
epiandrosterone sulphate	28.0		31.6	28.4
androstenedione	67.6	32.0	51.7	66.9
5α-androstanedione	37.8	24.6	32.1	33.1
androsterone	28.5	22.6	13.7	25.8
etiocholanolone			16.5	33.8
androstenediol	4.0	37.9		
androstenediol sulphate	15.0	11.9		
3α-androstanediol	5.4	8.1	3.5	2.9
3β-androstanediol	5.0		13.3	11.8
testosterone	47.8	36.2	33.1	55.9
dihydrotestosterone	24.4	33.0	4.2	4.5

The above data are used with the specific activities after crystallization (tables 18a-n, pp.97-104) to calculate % conversions (table 20, p.106).

APPENDIX 2.

SOURCES OF REAGENTS

Solvents and general reagents were of Analar grade. Silica Gel Hf 254+366 was bought from E.Merck Darmstadt, W. Germany. Steroids were bought from Steraloids Ltd., Croydon, Surrey. Standard reference steroids were obtained from the M.R.C. Steroid reference collection.

Radioactive steroids were bought from the Radiochemical Centre, Amersham or from New England Nuclear Corp., Frankfurt A.M., W. Germany.