THE HISTOCHEMICAL DEMONSTRATION OF HYDROXYSTEROID DEHYDROGENASES IN HUMAN SKIN

SUMMARY

of a Thesis presented for the

degree of

Doctor of Philosophy

by

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The first histochemical demonstration of hydroxysteroid dehydrogenases in human skin was in 1965 (Baillie, Calman, Milne, 1965) and since that time the scope of the investigation has been considerably expanded and extended. This thesis presents the results of a personal investigation into the distribution and significance of these enzymes in human skin.

Biochemical investigations have shown that many steroids, and notably androgens, are metabolised by human skin (Strauss & Pochi, 1969). Further, it is known both in experimental animals (Ebling, 1963) and humans (Strauss & Pochi, 1963) that sebaceous growth is controlled by oestrogens and androgens. It was in the light of these results that the present study was carried out.

The histochemical method for the demonstration of hydroxysteroid dehydrogenases depends on the oxidation of the hydroxyl group of the steroid to a ketone group, with transfer of the hydrogen, via the hydrogen acceptor NAD, to a tetrazolium salt which is then reduced to black diformazan granules. It will be noted firstly that a second enzyme, NADH oxidoreductase is involved, and secondly that the diformazan granules are deposited at the site of the enzyme reaction. These two features are of great importance and form the basis of the histochemical demonstration of the enzyme reaction.

This thesis set out specifically to answer a series of six questions in relation to these enzymes. The first of these was concerned with the histochemical reaction, and how it might be modified. The basic technique used was that of Muir et.al. (1968 a&b) and the following additional

features were studied.

1. The effect of pre-incubation fixation on enzyme activity. The results showed that most of the fixatives used had a detrimental effect on enzyme activity.

2. The effect on polyvinyl pyrrolidine on the histochemical reaction. This compound was introduced in an attempt to reduce the diffusion of enzymes, but was found to have no beneficial effect.

3. The effect of storage on enzyme activity. It was found that the enzymes could be stored either as a block of tissue, or as sections mounted on coverslips for periods of up to five weeks without loss of enzyme activity.

4. The localisation of NADH oxidoreductase activity in human skin. This enzyme, essential to the histochemical reaction was found in all tissues and cells of skin, an important fact to be remembered when the distribution in skin of hydroxysteroid dehydrogenases is discussed.

The second problem was to investigate which hydroxysteroid dehydrogenases which could be demonstrated in human skin. The major enzymes were found to be the 3β- hydroxysteroid dehydrogenase, the 17β-hydroxysteroid dehydrogenase and the 16β- hydroxysteroid dehydrogenase. Complimentary to this question was the establishment of the validity of the histochemical technique both in terms of specificity of substrates used, and of localisation. This was done using a series of experiments which confirmed that the observed localisation of the diformazan granules was indeed specific and not related simply to diffusion,

selective solubility of the diformazan or the distribution of NADH diaphorase.

The third problem was to review the distribution of these enzymes in human skin in relation to age, sex and site. A series of 214 human skin biopsies were used and it was concluded that these enzymes were present at all ages, and both sexes in the sebaceous glands of the skin surface. No activity was noted in any other skin structure, particularly high activity was noted in the sebaceous glands of the face and scalp where the greatest number of sebaceous glands were found. An interesting feature to come out of this survey was the different distribution of the enzymes within the sebaceous glands themselves. With the 3β- and 16β- hydroxysteroid dehydrogenases, the diformazan granules were deposited fairly uniformly throughout the sebaceous glands. With the 17β- hydroxysteroid dehydrogenase however the activity was located in small areas around the periphery of the glands, and in the secretory ducts.

The fourth question which was investigated was the distribution of these enzymes in the skin of the human fetus. Once again the activity was found only in the sebaceous glands, the activity first appearing at 16 weeks of gestation. In contrast to the adult situation, the enzyme with the highest activity was the 17β- hydroxysteroid dehydrogenase. No fetal skin over 32 weeks of gestation was used so that the changeover from fetal to pre-pubertal pattern could not be investigated in detail. The sebaceous glands in the fetus represent a major organ for steroid metabolism and the relation between the sebaceous glands, the feto-placental unit and the liquor amnii was discussed.

The fifth problem to be investigated was the use of the histochemical method for the screening of anti-androgens in human skin. This method, based on the inhibition of the enzymes by anti-androgens, has been used to assay the activity of these compounds. In all, seven compounds were studied, and it was found that inhibition of the 17β- hydroxysteroid dehydrogenase correlated well with the known biological activity of the anti-androgens in various other bioassay systems. The various advantages and disadvantages of the method as a screening technique were discussed.

The final question was to relate the histochemical results to the known biochemical reactions of steroids which occur in skin. In general, especially with androgen metabolism, the histochemical results correlate well with the biochemical ones. The important enzymes 3β- and 17β- hydroxysteroid dehydrogenases are known to occur biochemically and have an important role in sebaceous glands physiology (Strauss & Pochi, 1969).

Using the histochemical results which were found a hypothesis of the pathogenesis of acne vulgaris was put forward, based on the localised activity of the 17β- hydroxysteroid dehydrogenase. Testosterone acting at these sites increases the mitotic rate causing blockage of the ducts. The various arguments for and against this hypothesis were detailed and a number of deductions from this outlined.

Finally the role of the sebaceous gland in the metabolism, excretion, storage and secretion of steroids was discussed. The human sebaceous glands form a large mass of tissue which may be more important in the general turnover and metabolism of steroids then was formerly realised.

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A thesis submitted for the degree of

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CONTENTS

1. PROLOGUE

The work for this thesis began late in 1964 as an extension of an investigation into the distribution of hydroxysteroid dehydrogenases in various human organs, the results with human skin being published in 1965 (Baillie, Calman & Milne, 1965). Since that time, under the direction of Professor J.A. Milne, the study of these enzymes in human skin has been extended and diversified.

This thesis is firstly a description of the experimental work which has been personally carried out in this field. Secondly, it is an attempt at placing the results of the investigation in their proper perspective in terms of current knowledge of skin physiology, pathology and steroid metabolism.

In the introduction the current status of the structure and function of sebaceous glands and related topics will first be reviewed. This is followed by a description of the methods used in the study and then the results will be presented together with a discussion of their significance. Finally in the conclusions a more general view of the relationship between sebaceous gland function and steroid metabolism will be taken.

2. INTRODUCTION

1. Development and morphology of human sebaceous glands.
2. Human sebaceous gland function.
3. Chemical composition of sebum.
4. Hormonal control of sebaceous glands.
5. The histochemistry of human sebaceous glands.
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1. Development and morphology of human sebaceous glands.

Sebaceous glands are found in all areas of skin of the human body, except on the palms, soles or on the lower lip. They vary greatly in number and size, being largest and most numerous on the back and on the face. They are also present in large numbers on the scalp, external auditory meatus and anogenital surfaces. In these areas 400-900 large glands are present in each $cm.^2$ of skin surface. Over the remainder of the body there are fewer than 60 glands per $cm.^2$ (Montagna, 1963).

In general sebaceous glands are associated with hair follicles forming pilosebaceous units. Some glands however are not associated with hairs and these occur mainly on the face, forehead and around the nose. These are often very large and are known as gigantic sebaceous follicles (Kligman & Shelly, 1958).

The sebaceous glands are tubulo-acinar in type and their histological characteristics are similar in all situations. Each gland discharges into an excretory duct which is lined with stratified squamous epithelium (Fig. 1). This duct is continuous with the piliary canal in which the hair shaft lies. The piliary canal opens onto the skin surface.

The sebaceous glands comprise cells of varying maturity and size. In the centre of the gland the cells are large and full of lipid, and they may be anucleate. Around the outside of the gland the cells are smaller and have a well stained nucleus. The acini of any one gland may show different states of maturity; some acini being small

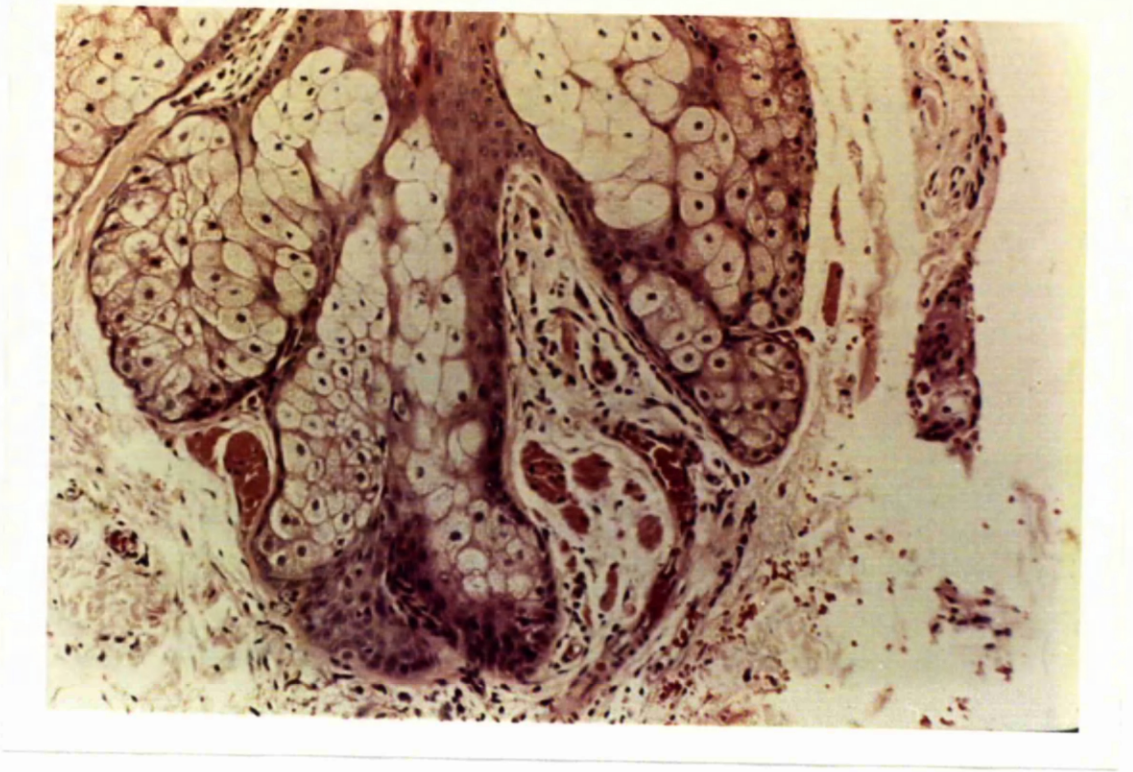

Figure 1. Human Adult Sebaceous Glands Haematoxylin and Eosin.

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and full of nucleated cells and others composed of large foamy fat-laden cells.

Sebaceous glands show a holocrine type of secretion which implies that the cell dies, disintegrates and is discharged onto the skin surface with its products. As "sebaceous differentiation" (as the process of disintegration has been called) proceeds the cells become progressively laden with lipid, lose their nuclei and eventually disintegrate, liberating the sebum. Sebum itself is a complex mixture of lipids, proteins, cell membranes, enzymes and other products of cellular disintegration which is discharged onto the skin surface. The cells on the periphery of the gland are rich in ribonucleoprotein which is progressively lost as they mature and become filled with lipid.

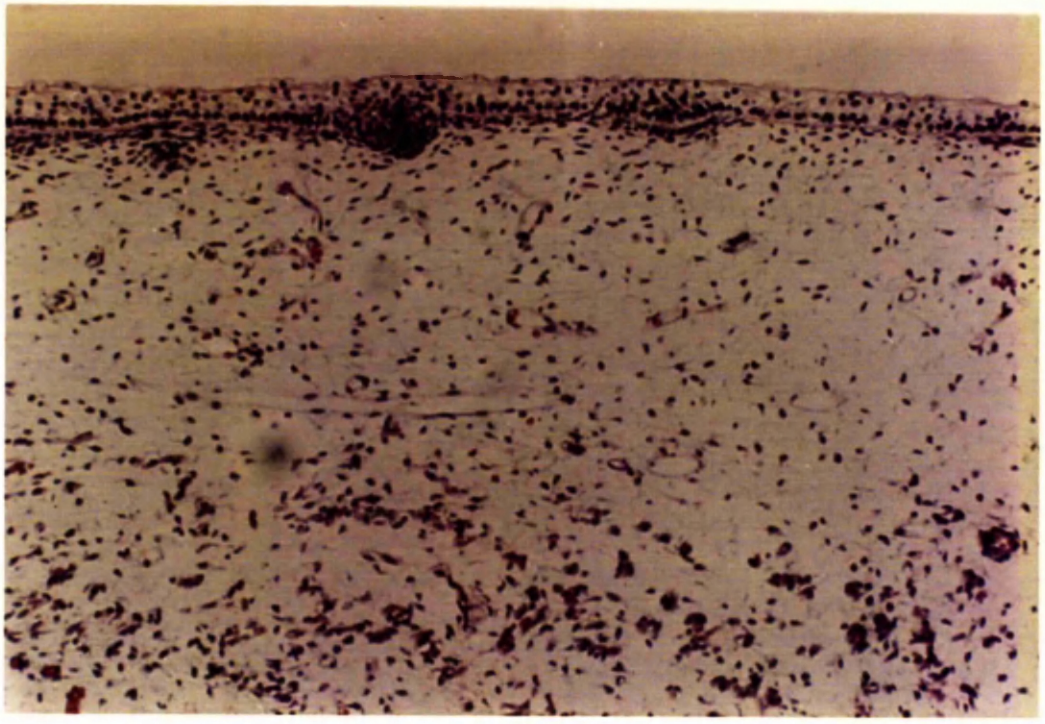
The ultrastructure of the sebaceous glands in man has been investigated by Ellis and Henrikson (1963). During the period of lipid synthesis, when the cells are differentiating, smooth membrane systems predominate. The cells are full of small vesicles and the golgi apparatus is well visualised. The golgi apparatus may offer sites for accumulation of lipid. Mitochondria are numerous and are presumably concerned with lipid synthesis.

The development of the sebaceous glands is intimately associated with the development of the hair follicle. This field has been reviewed by Serri & Huber (1963) and Montagna (1962). In the third week of fetal life, the epidermis is composed of a single layer of

undifferentiated cells rich in glycogen. By the fourth week, two layers of cells can be distinguished. The basal layer is known as the stratum germinativum and the outer layer, the periderm. It is from the basal layer that cutaneous appendages such as sebaceous glands, hair follicles and arrectores pilorum muscle arise. By the tenth week the stratum intermedium can be recognised and this differentiates further by the thirteenth week to form the spinous layer. The cells of the periderm also differentiate further becoming flattened and polygonal. Vernix caseosa, which is the greasy material which coats the fetus first appears towards the fourth or fifth month and comprises periderm cells, lanugo hairs, sebum and other debris.

The precursor of the hair follicle can be seen as early as nine weeks; palisades of cells occur at various points in the basal layer which are always accompanied by changes in the surrounding mesenchymal tissue. This mesenchymal tissue forms the lower layers of the skin including the dermis and blood vessels. The cells which will eventually form the hair root grow and extend into the mesenchyme which has become differentiated into the hair papilla. As the hair root develops further, its end becomes bulbous and the true papilla is formed.

At this stage two epithelial swellings appear on the wall of the hair follicle. The lower one is the area to which the arrector pilorum muscle will later become attached. The upper swelling is the beginning of the sebaceous gland. In certain areas a third bud,



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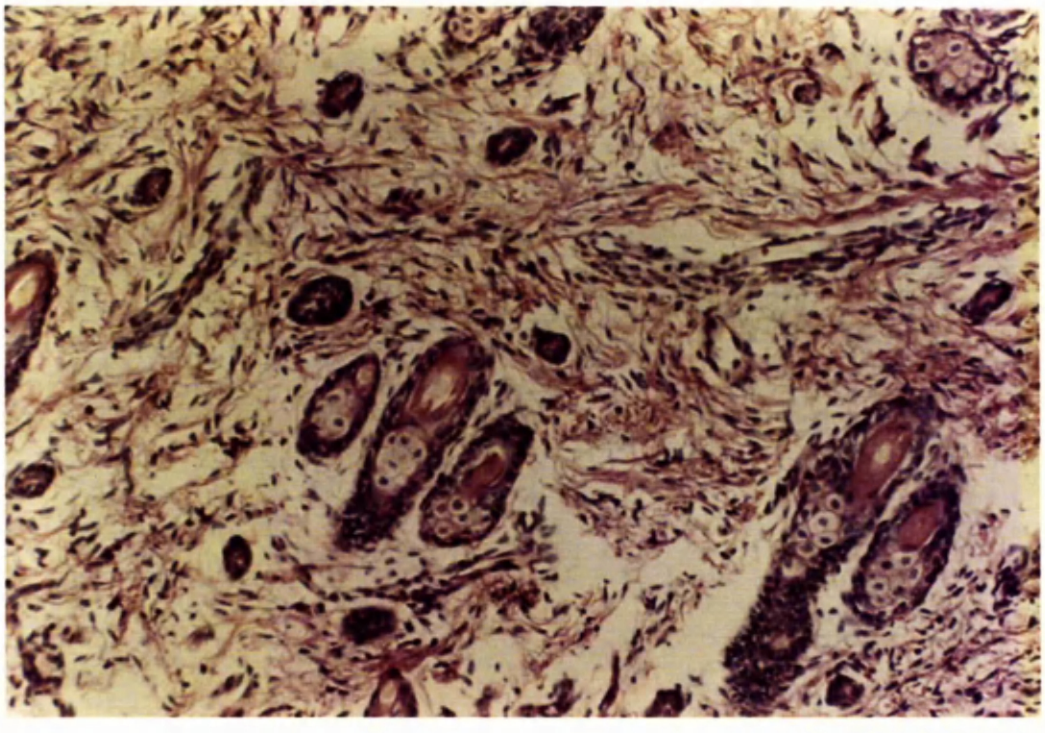

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Figure 2. Human Fetal Sebaceous Glands Haematoxylin and Eosin.

above the sebaceous gland, develops and is the forerunner of the apocrine glands.

The cells of the sebaceous bud are at first rounded and contain moderate amounts of glycogen. The cells however, soon lose their glycogen and acquire a foamy appearance (Fig. 2). As with adult glands, the cells differentiate and disintegrate and sebum is discharged into the piliary canal.

From their earliest differentiation at 13-15 weeks, the sebaceous glands show changes histologically which indicate that the glands are functional in that they contain enzymes and secretion products. In the fetus, the largest glands are on the face and the chest and all stages of functional activity can be seen in the acini of one pilo-sebaceous unit. After birth, the size of the glands is rapidly reduced, enlarging again at puberty.

2. Human sebaceous gland function.

One of the major functions of human sebaceous glands is the production of sebum. In animals the sebaceous glands are also associated with the hair follicles, and, from the embryological point of view the sebaceous gland is an appendage of the hair follicle and not of the epidermis. The sebum which is secreted protects the hair follicle and the hair shaft which in turn protects the skin. The lipid prevents overwetting and maceration of the skin and insulates the animal. Kligman (1963) suggests that in man, hair

has little functional significance and therefore the sebaceous gland itself has become redundant. Other workers, however, have suggested that in man sebum is of importance.

One of the suggested functions of human sebum, is its protection of the skin. The amount of lipid secreted however, is very small and the thickness of the lipid film is 3-10 μ (Miescher & Schonberg, 1944). The skin is however not uniformly covered with sebum. Certain areas of the body, notably the face and scalp, are rich in sebum while other areas contain only small amounts. In addition, the lipid collects in small skin creases and wrinkles and is not therefore spread evenly across the skin surface. In the prepubertal child, the sebaceous glands are relatively inactive yet the skin seems to suffer in no way from this loss of protection.

Sebum has also been thought to be both antifungal and antibacterial. Kligman (1963) however, has shown that the antifungal property of sebum is essentially an *in vitro* phenomenon. Bacteria, when placed on the skin, die rapidly. This fact however does not necessarily mean that it is the sebum itself which has antibacterial properties. The "acid mantle" of the skin, so called because of its high content of fatty acids, was at one time thought to be the factor responsible for skin sterilisation. Pilsbury & Rebell (1952) however showed that both resident and pathogenic bacteria grew well at pH values found on the skin surface. Richetts et. al. (1951) showed that extracts of sebum, and in particular oleic acid, were in fact streptococcidal *in vitro*. It is more likely however, that the fatty acids in skin do not protect

against bacteria but are produced by them (Scheimann et.al., 1960). In evidence to the contrary, it has been shown that areas with most sebaceous glands, and which therefore produce most sebum, also support the most organisms (Pilsbury & Kligman, 1954).

One function of sebaceous glands which is not often emphasised and is relevant to the present investigation, is that the sebaceous gland may be an end organ for steroid metabolism and function as an excretory organ for steroids. Evidence, which will be presented later, suggests that the sebaceous gland is readily modified by the action of steroid hormones. This however may be a secondary event; the real function being the excretion or further metabolic change of the steroid hormone. This represents a fairly new concept of sebaceous gland function.

3. The chemical composition of sebum.

The chemical composition of sebum is exceptionally complex. In the first place, the amount of sebum secreted is very small and thus only limited amounts can be collected. Developments in the characterisation of skin lipids therefore had to wait on the more recent advances in chemical techniques, thin layer chromatography, gas chromatography, counter-current distribution systems and mass-spectroscopy for elucidation (Powell, 1969). The second problem is that lipids are also secreted from the epidermal surface, sweat glands and apocrine glands and it is almost impossible to separate them once they have mixed. Direct cannulation of a sebaceous duct could be attempted

but the technical problems, both of collection and analysis would be considerable. Extraction of lipid from the skin surface and from hair fat however are the methods commonly employed.

The chemical composition of sebum has been reviewed by Wheatley (1963) and Nicolaides (1963). Glycerol, triglycerides and fatty acids, steroids and sterols and squalene can be extracted by relatively simple procedures. Various cholesterol esters have been found including 7-dehydrocholesterol, a precursor of vitamin D. This however, is more likely to arise from the epidermis and not from the sebaceous glands (Nicolaides & Rothman, 1955). Ketosteroids have also been found in human sebum and of course are relevant to the present study. Insufficient material however is available at present to analyse them in detail. It is likely that the fatty acids are hydrolysis products from the neutral triglycerides, formed either by bacterial action or by esterases in the sebaceous glands and sebaceous ducts. These esterases can be demonstrated histochemically (Ballantyne & Burch, 1967).

The amount of sebum secreted has been quantitated by Strauss and Pochi (1961), and the secretion rate determined in a variety of clinical states and endocrine abnormalities, including breast cancer (Krant et.al., 1968). After cleansing the forehead, a piece of thin tissue paper is applied. After a standard time the paper is removed, the sebum eluted and then weighed. This method has recently been extended by Shuster (Cuncliffe & Shuster, 1969), and the results with breast cancer repeated and confirmed (Burton et.al., 1970).

4. Hormonal control of sebaceous glands.

It is a matter of general clinical knowledge that sebaceous gland growth and function are related to changes in the hormonal status of the individual. A few examples will suffice; the change from the clean, non-greasy skin of the child to the seborrhea of the adolescent; the occurrence of acne at changes in the biological life cycles of the human associated with hormonal changes, premenstrually, at puberty and at the menopause. In various clinical conditions associated with changes in hormone production - Cushing's Syndrome, Stein-Leventhal Syndrome, adrenogenital syndrome, acromegaly - seborrhea or acne vulgaris can occur. With many of the steroid drugs given for a variety of conditions, rheumatoid arthritis, the collagen diseases, or as oral contraceptives, acneiform eruptions are found. These changes, which can be recognised clinically, allow us to state that sebaceous gland activity is modified by changes in the hormonal status of the individual.

In experimental animals, where the conditions can be more easily controlled, the effect of various steroids on the sebaceous glands has been more fully worked out, notably by Ebling (1963). Using the rat as his experimental model, Ebling investigated both the size of the sebaceous gland and the mitotic rate in response to various steroids. Testosterone for example, produced cell proliferation and the increase in the size of the gland was mainly due to this factor. There was however, some increase in cell volume. With oestradiol the situation was more complex. In general however, oestradiol reduced sebaceous

gland volume but not by an inhibition of mitosis. Although sebaceous gland size varied during the oestrous cycle, this was not associated with a change in the mitotic rate. When oestradiol and testosterone were given together, the sebaceous glands were small but the mitotic rates were still high. This suggested that oestradiol did antagonise the effect of testosterone but had a different site of action. With progesterone, Ebling found that only with very large doses could an increase in gland size and mitotic rate be demonstrated.

In humans the same effects have been looked for using the techniques of sebum collection already described. Strauss, Kligman & Pochi (1962) found that androgens, even in small doses, caused great enlargement of the sebaceous glands of the prepubertal male and female. However, with adult males, no increase in the size of the glands occurred, probably because they were maximally stimulated. With adult females some increase in sebaceous gland size was found. When given topically, testosterone exerted a local effect. Estrogens, on the other hand, suppressed androgenic action only at high doses, and in agreement with Ebling's results, did not antagonise androgens. Thus it is androgens in particular which appear to influence sebaceous gland growth. This had been previously reported by Hamilton (1941) and Jarrett (1959) and has been reviewed by Strauss & Pochi (1963) and Owens and Knox (1967).

The effects of progesterone and progesterone-like compounds has also been investigated in humans by histological and sebum production

studies (Strauss & Kligman, 1961). They have, again in agreement with Ebling, shown that natural progesterone has no effect on sebaceous gland size or function. However, with some of the synthetic compounds with androgenic activity, sebaceous gland enlargement did occur. They also noted that sebum production is not altered during the menstrual cycle. This however does not eliminate the possibility of a qualitative change in sebum production or of a change in other parameters of sebaceous gland function.

There is also some evidence, especially in rats, that the anterior pituitary produces a "sebotropic" factor which stimulates sebum production (Lorincz & Lancaster, 1957) and it may be that oestrogens act by blocking the action of this sebotropic factor. The evidence for the presence of such a sebotropic factor has been reviewed by Lorincz (1963). Various pituitary extracts were made and tested for their ability to increase the size of sebaceous glands. It was found that progesterone with the sebotropic fraction had a synergistic effect. Such a factor may be of importance in the seborrhea associated with mid brain lesions such as post encephalitic Parkinson's disease. There is still however, some debate about the existence of the sebotropic factor

5. <u>The histochemistry of human sebaceous glands.</u>

There have been many histochemical studies on sebaceous glands of human skin. Histochemical techniques, because they are able to localise the site of the cell constituent or enzyme reaction, are

especially useful with such a heterogenous tissue as skin. Generally, the standard histochemical approaches have been used but in certain studies, a combination of microdissection procedures and microchemistry have given more accurate quantitation.

As soon as the sebaceous glands can be recognised in human fetal skin, metabolic activity can be demonstrated within the glands. A wide variety of enzyme reactions can be demonstrated including enzymes of glycogen metabolism, acid phosphatase, β-glucuronidase, alkaline phosphatase, non-specific esterase, succinic and lactic dehydrogenases, cytochrome oxidase and aminopeptidase (Serri & Huber, 1963). Perhaps the most significant feature in these findings is that the human sebaceous gland is metabolically active by 13 weeks in fetal life. This makes it one of the earliest functioning human glands.

Histochemical lipid stains have been used in an attempt to characterise the type of fat found in the sebaceous gland. It was thought at one time that the different lipids were arranged concentrically within sebaceous glands (Melczer & Deme, 1942) but this has now been shown to be incorrect (Susskind, 1951). Cholesterol esters, triglycerides and phospholipids can all be demonstrated within sebaceous glands. Lipid synthesis begins in the centre of the gland, filling the central cells with fat and then extending to the periphery. In mature glands, then, most of the cells are laden with lipid. More recent work on lipid staining has been done by Nasr (1965).

Glycogen is found in most cells of the sebaceous acini and there appears to be an inverse relationship between the amount of glycogen

and the amount of lipid within the cell (Montagna, Chase & Hamilton, 1951). With older subjects there appears to be more glycogen than lipid, perhaps indicating a slower rate of synthesis of fat.

Many of the histochemically demonstrable enzymes can be observed within adult sebaceous glands (Montagna & Ellis, 1962; Montagna, 1963). All of the enzymes present in human fetal glands can be identified in adult glands. Of particular interest is the distribution of the enzymes within the acini themselves. Some enzymes, for example, cytochrome oxidase and NADH diaphorase, are found in almost all of the cells of the acinus. Other enzymes, and amylo-1, 4-1.6-transglucoside activity is a good example of this, are present particularly in the peripheral cells of the gland. Alkaline phosphatase activity is mainly found in the capillaries around the sebaceous gland and is an indicator of the vascular supply to the gland.

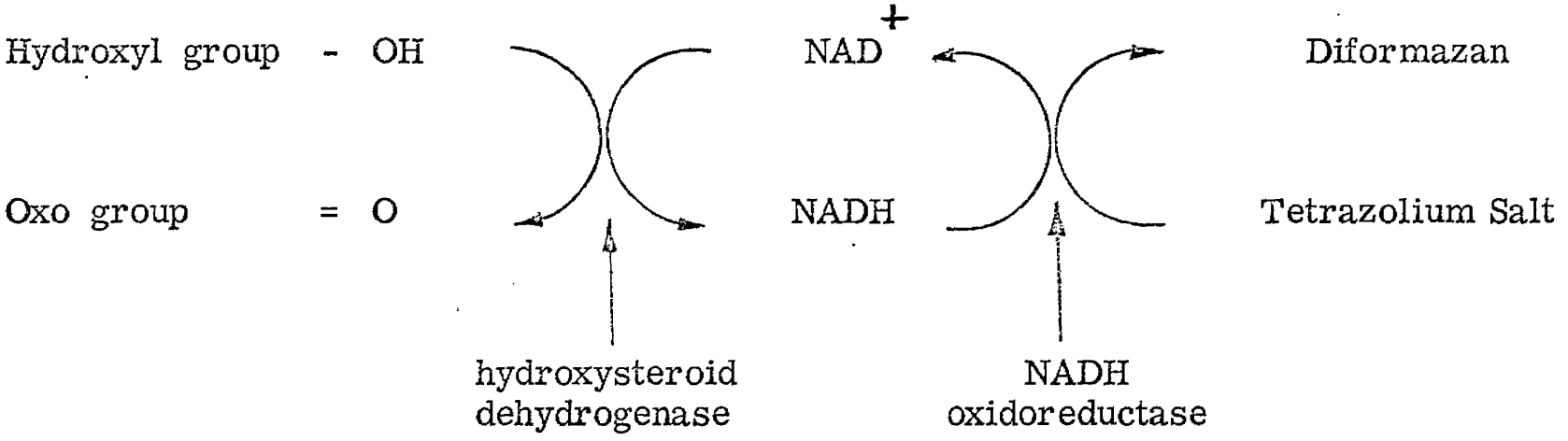
These last few facts highlight the important place of histochemical techniques in biological research. They enable the localisation of enzymes and other substances to be studied even in very small organs such as sebaceous glands. The differences in localisation which are found can then, in conjunction with other techniques, be used to elucidate the metabolic activity and functional significance of the organ. It is this theme which has been followed in the present study.

6. Development of Steroid Histochemistry.

Wattenberg in 1958 was the first to histochemically demonstrate

Fig. 3

HISTOCHEMICAL FLOW SHEET for the DEMONSTRATION of HYDROXYSTEROID DEHYDROGENASES



a steroid dehydrogenase and the enzyme he demonstrated was the 3β-hydroxysteroid dehydrogenase (HSD). Since that time numerous other hydroxysteroid dehydrogenases have been described and fully reviewed by Baillie, Ferguson and Hart (1966).

Basically, the reaction involves the removal of hydrogen from the hydroxyl group of the steroid. The hydroxyl group is then oxidised to a ketone group. The hydrogen is transferred via a physiological hydrogen acceptor to a soluble tetrazolium salt which is reduced to an insoluble diformazan (Fig. 3). The reaction is thus comparable with other dehydrogenase reactions. Dehydrogenase histochemistry has been fully reviewed by Pearse (1960) and Novikoff (1963). The hydrogen acceptors normally used are the pyridine nucleotides, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). It is the basic assumption of all histochemical techniques that the dye is deposited at the site of the enzyme reaction making localisation possible.

The histochemical method for the demonstration of hydroxysteroid dehydrogenases, however, has a number of drawbacks which must be appreciated if meaningful results are to be obtained.

The substrate for a steroid histochemical procedure requires particular attention. The reason for this is that any steroid with more than one source of available hydrogen, cannot be used in a histochemical system. Steroids such as dehydroepiandrosterone or testosterone, contain one hydroxyl group and therefore only one source

of available hydrogen. Steroids such as androstenediol, cortisol and oestriol, contain more than one hydrogen source in the form of hydroxyl groups, and cannot be used. Similarly, compounds with a 5 -hydrogen in addition to a hydroxyl group cannot be used unless special controls are employed, since theoretically this hydrogen could also be used histochemically. It becomes apparent then that only a limited number of substrates can be used and this is certainly a major disadvantage.

Another major disadvantage is that the number of enzymes which can be looked for with histochemical techniques is limited. At present, with relation to steroid metabolism, only dehydrogenase reactions can be studied. The very important steroid biosynthetic enzymes such as the side-chain splitting enzymes or the hydroxylases cannot be demonstrated. This then limits the scope of the histochemical technique even further. At present the major enzymes which can be visualised are the 3β-hydroxysteroid dehydrogenase, and the 17β- hydroxysteroid dehydrogenase. These are of importance in the biosynthetic and metabolic pathways of androgens and oestrogens. Various other enzymes, such as 20α-20β-HSD, 6β-HSD, 11β-HSD, 3α-HSD and 16β-HSD have been described, but their exact role is not fully understood.

The practical aspects of dehydrogenase histochemistry have been reviewed by Pearse (1960) and Novikoff (1963). Certain points however, peculiar to the steroid histochemical technique must be mentioned.

With most dehydrogenases such as lactic, succinic or glucose-6-phosphate dehydrogenase, the solvent for the substrate is an aqueous one. With steroid substrates on the other hand lipid solvents are required. Since many lipid solvents contain hydroxyl groups, it is obvious that the choice has to be restricted to those that are not utilised histochemically. Ethanol, methonal and propylene glycol for example cannot be used because of their available hydrogen (Ferguson et.al., 1966). Dimethyl formamide (D.M.F.) has therefore been used since it has no histochemically available hydrogen. Similarly, when inhibitors of the steroid dehydrogenases are to be studied it is important that these compounds do not donate hydrogen which can be used histochemically.

To demonstrate the dehydrogenases histochemically, requires the presence in the tissues of a second enzyme, the NADH diaphorase, or NADH oxidoreductase (Fig. 3). It is thus of importance that this enzyme is also present in the tissues. Not only must the enzyme be present in the tissue but it must be present throughout the organ, otherwise accurate localisation of the first enzyme will be impossible. In addition the enzyme must be present in such a quantity that it is not a rate limiting step. This is obviously of great importance if quantitation is to be attempted. In all routine investigations of the hydroxysteroid dehydrogenases then the presence of this enzyme should be checked from each biopsy specimen.

The validity of conclusions about localisation of enzymes which are NAD or NADP dependent has been questioned by Farber, Sternberg,

and Dunlap (1956) and further investigated by Kalina, Gahan and Jones (1965). Using two different tissues, liver and heart, they found that the activity of the glucose-6-phosphate neotetrazolium reductase system could be "donated" to a washed tissue section simply by incubating in the same incubation medium. It is implied then, that with a soluble enzyme system, localisation of the enzyme is simply related to the localisation of the NADH diaphorase. As will be seen from the succeeding paragraphs, the definition of "soluble" enzyme is a wide one, and in fact applies to almost all enzyme systems. Before any conclusions then about localisation of HSD's in human skin can be made experiments similar to this must be performed.

From the histochemical point of view, many of the tissue enzymes can be considered to be soluble in nature. This does not imply that the enzyme is soluble in aqueous media but simply that it can be leached out of the tissue section on incubation with buffer. Histochemically, this can be disastrous from the localisation of the enzyme. If the enzyme can be "solubilised" and extracted into the incubation medium, then the enzyme reaction can take place in the medium; the diformazan can then be deposited all over the tissue section. According to Jones (1965) over 70 per cent of the tissue protein and nucleic acids can be removed by short periods of washing.

To overcome the problem of "soluble" enzymes two methods have been used. The first is to "fix" the tissues either by the routine histological fixatives such as formaldehyde etc. or by vapour fixation

or freeze drying. With the first of these, there is denaturation of the proteins and inevitably, loss of activity of the enzyme (Wyllie, 1965). This however, may be justified if tissue localisation is improved.

The second method is to incorporate into the incubation medium, substances which will limit diffusion of the enzyme. Such substances are gelatin, polyvinyl alcohol and polyvinyl pyrollidine (Scarpelli & Pearse, 1958). The reaction tends to be slower but in general, the localisation is improved if the enzyme is "soluble".

The electron acceptor for dehydrogenase histochemistry is usually one of the tetrazolium salts. When reduced, these salts form insoluble diformazan granules. This reduction is thought to be a two-stage process in which a monoformazan is formed as an intermediate step. A number of tetrazolium salts are available and nitro-blue tetrazolium has been used extensively for this work, since one of its characteristics is that it is not readily removed from the tissues when it has been reduced to diformazan granules. Neotetrazolium on the other hand can be relatively easily removed by ethyl acetate extraction. Nitro B.T. thus gives a sharper histochemical picture than neotetrazolium.

The diformazans of many of the tetrazolium salts are selectively soluble in lipid. Their deposition, and therefore enzyme localisation in tissue which are rich in lipid such as adrenal, testis and sebaceous glands can be criticised because of this. Nitro B.T. on the other hand, does not appear to be selectively soluble in this way and it is thus

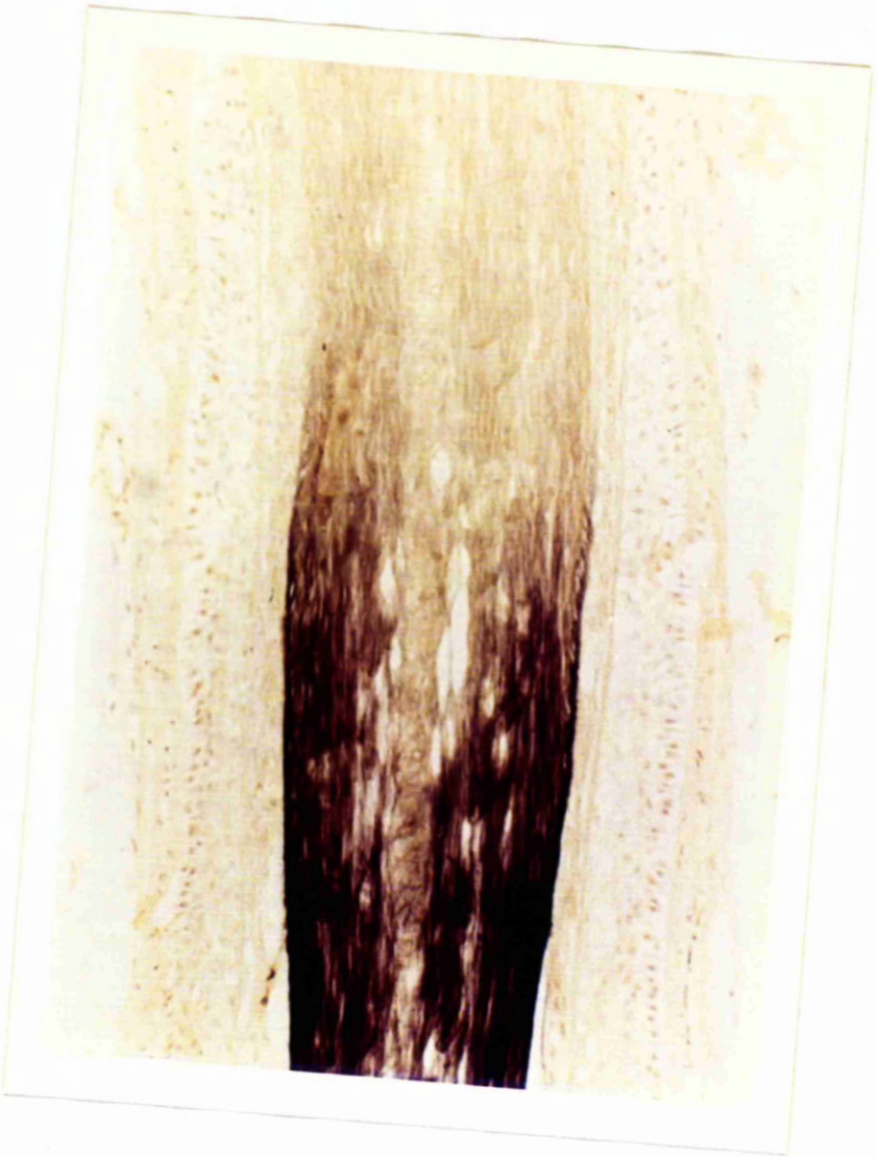


Figure 4. Hair Shafts. Control Incubation. Haematoxylin and Tartrazine. × 275

particularly useful with sebaceous glands, and other lipid rich organs.

The major disadvantage of dehydrogenase histochemistry at present is the inability to accurately quantitate the reaction. Various methods have been tried with varying degrees of success. Perhaps the most used method is of visual assessment of the reaction. In this method the activity of one section is arbitrarily compared to another. Results can either be expressed as positive or negative, or the activity graded as +, ++, or +++, again in an arbitrary way. This method has been used in most of the experiments in this study. Such a technique, of course, is very subjective but is simple and rapid and with experience it can give satisfactory results.

The second method is to extract the diformazan formed in the tissue sections. As has been mentioned before, with nitro B.T., extraction is not easy and one is not sure whether or not all the diformazan has been removed. If other tetrazolium salts are used, then extraction procedures may be worth trying but at the expense of poorer localisation. There is one feature in skin however, which makes extraction procedures difficult or impossible. Hair shafts stain deeply with tetrazolium salts, even in control sections, so that extraction procedures would not be able to eliminate this variable (Fig. 4). The non-specific reduction is most likely due to the high concentration of sulphydryl groups in the hair shaft.

A third method is to measure the amount of reduced nucleotide formed by biochemical procedures using spectrophotometric or fluorimetric methods. Such techniques however, unless microdissection

methods are also employed, will only give an overall picture of the activity in a section of skin. With other dehydrogenases this has been attempted in skin (Hershey et.al., 1960).

The fourth method, and one which has been used in this study, involves the counting of the diformazan granules. The technique has been described by Muir et.al. (1968) and is based on planimeter studies (Freere & Weibel, 1967). In essence, the number of diformazan granules per unit area of sebaceous glands is computed. The method has numerous drawbacks including, non-uniformity of diformazan deposition, variation in size of granules, and the fact that the activity of the whole gland but only two to three sections of it is measured. Another major drawback is that the amount of diformazan deposited may not accurately reflect the metabolism of the substrate within the tissues. It represents only a part of such metabolism and the percentage of conversion of a substrate is not known. On the other hand, it is a rapid technique, requires little expensive apparatus and can be used to give reproducible results if its limitations are known. The technique can be used to give reproducible results if serial sections of a single biopsy specimen are compared and the results read by a single observer (Muir et.al., 1968).

More recently an integrating microdensitometer has been introduced (Bitensky, 1970) which is capable of counting the diformazan granules, and is a considerable advance.

As with all enzyme histochemical procedures, special attention has to be paid to the collection and storage of skin biopsy material.

The skin, when removed, is rapidly frozen and stored in a block until required. It is usual to store the tissue at -20° since this is the temperature of the cryostat which will later be used to prepare the frozen sections. Freezing itself has marked effects on tissues and these have been reviewed by Meryman (1966). The initial freezing of the tissue causes ice crystal formation which is predominantly extra-cellular. However, even at -20° there is sufficient change in the crystal structure to make significant intra-cellular alterations. Enzymes may be destroyed and the phospholipid membranes broken. Localisation of the enzyme may therefore become difficult to interpret. It is of great importance then to know the storage characteristics of these ehzymes; whether they are stored better as a block or as tissue sections; what the rate of deterioration of the enzyme activity is, and what changes in localisation of the enzyme occur during storage.

7. Histochemical demonstration of hydroxysteroid dehydrogenases in Human Skin.

Hydroxysteroid dehydrogenases were first described in human sebaceous glands in 1965 by Baillie, Calman and Milne. In a survey of the steroids utilised histochemically, they found that the 3β HSD and the 17β-HSD could readily be demonstrated, but that many other steroids could be utilised to a limited extent. In particular, the 16β-HSD was found to be present in all the specimens assessed. In the original paper, no indication of the distribution of the enzymes within sebaceous glands was given but it was noted that no diformazan

was found in the epidermis or other cutaneous appendages.

In the logical extension of this, Baillie, Thomson and Milne (1966) surveyed 147 skin biopsies in an attempt to define the anatomical limits of the activity of these enzymes. In summary, they recorded activity of 3α-3β-11β-, 16β- and 17β- hydroxysteroid dehydrogenases in areas prone to acne vulgaris, the face, neck, anterior chest and scapular regions. Other areas showed only slight activity, in particular, only one out of four specimens from scalp skin showed any activity for these enzymes. In addition, no activity was found in abdominal skin with the exception of umbilical and epigastric regions and there was no activity in the perineal region or in the upper or lower limbs.

This study can be criticised from a number of standpoints. Firstly, the tissue was not counter-stained and traces of activity in certain areas of the gland may have been missed. Secondly, there is no record of the number of specimens in which no sebaceous glands were found. Personal experience had shown that with many skin biopsies, no sebaceous glands could be found even with repeated sectioning and this was particularly true of biopsies from the limbs and abdomen. Thirdly, the concentration of steroid utilised was higher than that which Muir et.al. (1968a) showed to be most effective.

Hydroxysteroid dehydrogenases in human skin were also investigated by Muir et.al. (1968a,b) and the characteristics of these enzymes, in particular the pH optimum, the substrate and co-factor concentrations worked out. A counter-staining technique and a method of semi-

quantitation were introduced.

The experiments of Muir et.al. (1968) considerably improved the histochemical localisation of these enzymes and during the study it was noted that certain skins outwith acne areas did show evidence of activity of these enzymes. Further experiments were therefore designed to re-investigate the distribution of these enzymes over the skin surface and to extend the knowledge of these enzymes. This will be reported later. The previous work on the hydroxysteroid dehydrogenases has been recently reviewed by Milne (1969).

8. Steroid Metabolism in Human Skin.

Steroid metabolism in human skin has only recently begun to receive the attention comparable with the other steroid producing organs such as adrenal, testis and ovary. Accordingly, the biosynthetic pathways and metabolic transformations have not been fully worked out in skin. In all the experimental work, whole skin has been used and in no instance have preparations of sebaceous glands alone been studied.

Dubovii (1954) is credited with the first isolation of ketosteroids from sebum. These he found present in small amounts and the steroids were detected by the Zimmerman reaction. These findings were confirmed by Carrie and Ruhrmann (1955) in 61 patients. Using enzymes prepared from pseudomas testosteroni, Cook and Lorincz (1963) found that 3β-, 3α and 17β steroids were found in human scalp soakings. Thus, though no enzymes were isolated by this method their presence was inferred. In

three patients with clinical acne vulgaris, there was no difference between the normals and the patients with acne.

More recent investigations into steroid metabolism have been along three main lines; hydrocortisone metabolism, androgen metabolism, and oestrogen metabolism.

Cortisol metabolism has been investigated by Hsia and his group. (Hsia, Witten & Hao, 1964, Hsia & Hao, 1966, Hsia & Hao, 1967). They have used both foreskin and abdominal skin and shown that cortisone and hydrocortisone are interconvertible and that other reduced derivatives could also be formed. Of particular interest was that saturation of the Δ^4 bond and reduction of the 3-oxo group occurred in foreskin only.

Little has been done in the area of oestrogen metabolism. In a preliminary report in 1966 (Frost, Weinstein & Hsia, 1966) it was shown that oestradiol could be converted to oestrone in foreskin, abdominal skin and vagina. This was later confirmed (Weinstein, Frost & Hsia, 1968) and extended in the same tissues. It was noted that in foreskin and abdominal skin that the direction of the interconversion was from oestradiol to oestrone. With vagina on the other hand the direction of the reaction is from oestrone to oestradiol. It was also found that the activity of the reaction was greatest in foreskin.

Because of the great importance of androgens in the regulation of sebaceous gland development, much more work has been done in this field and Strauss and Pochi (1969) have reviewed the most recent

advances.

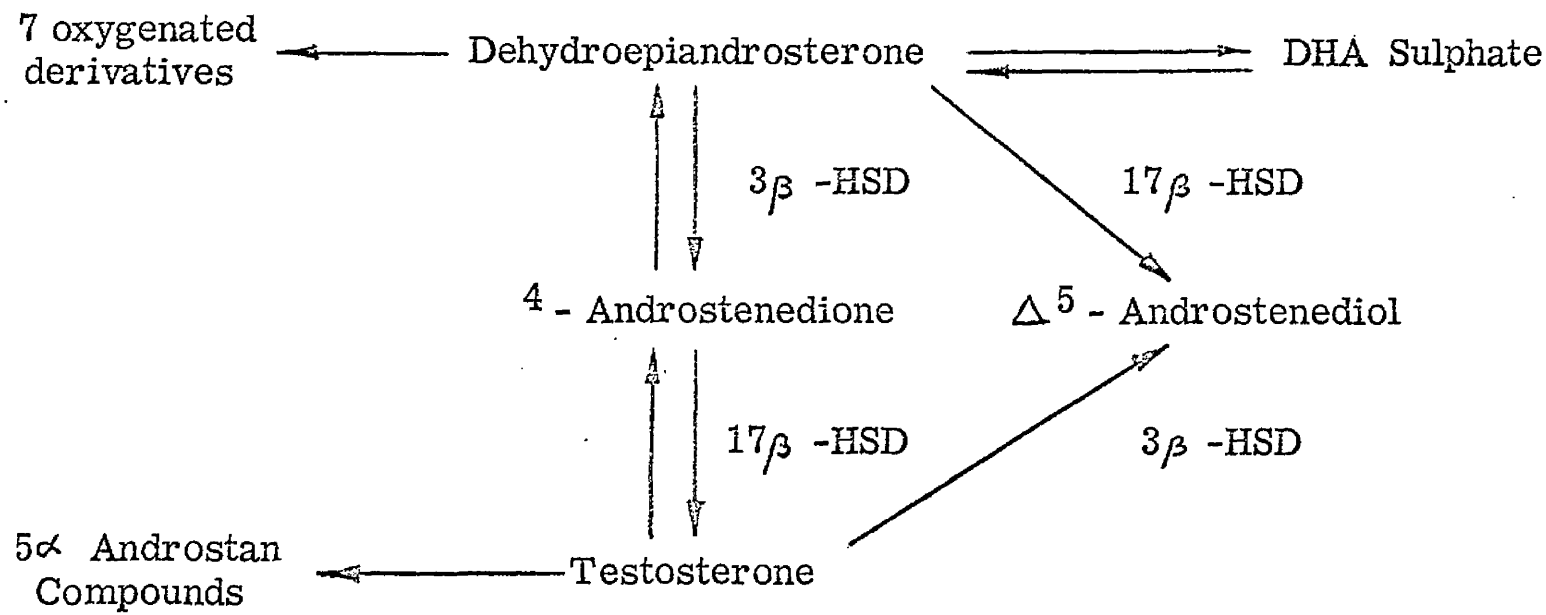
Rongone in 1966 showed that testosterone could be metabolised to androstendione and a number of 5α-androstan compounds. Cameron et.al. (1966) found that dehydroepiandrosterone could be transformed to testosterone implying that the 3β- and 17β- hydroxysteroid dehydrogenases were present in human skin. Gomez and Hsia (1968) showed that testosterone could, in agreement with Rongone (1966), be converted to androstenedione, but no 3α-ol compounds were formed. Berliner et.al. (1968) also found evidence for 3β- and 17β- hydroxysteroid dehydrogenases in anterior abdominal wall skin. Faredin et.al. (1969) found that 7-oxygenated derivatives of DHA could be identified following the incubation of normal human male and female skin with dehydroepiandrosterone.

Testosterone metabolism in testicular feminisation has been investigated in skin (Mauvais-Jarvis et.al., 1969, Northcutt et.al., 1969). They found that in the syndrome of testicular feminisation the transformation of testosterone to 5α-testosterone was deficient. It was suggested that the 5α-testosterone was the active form of the hormone, and that in this syndrome instead of testosterone being transformed to the 5α- form it was instead metabolised to oestradiol which was responsible for the clinical features of the syndrome.

Although steroid sulphatases and sulphokinases were not found in human skin by Warren and French (1967) their presence has been reported by other authors. Gallegos and Berliner (1967) found that DHA could be conjugated to DHA sulphate in abdominal skin and this was confirmed

Fig. 5

METABOLIC TRANSFORMATIONS of STEROIDS in HUMAN SKIN



by Faredin et.al. (1968) in slices of normal female skin. DHA sulphate itself was found to be metabolised by human skin and vaginal mucosa (Kim and Herrman, 1968) and this has recently been confirmed by Oertel and Treiber (1969) who used skin biopsies from patients who had been given intravenous DHA sulphate.

In summary then, it can be said that human skin is an active organ in the metabolism of many steroid hormones; androgens, oestrogens and corticoids. The inter-relationship of these compounds is shown in Figure 5. What is significant from this figure is the central place of the 3β- and 17β- hydroxysteroid dehydrogenases. These enzymes can readily be demonstrated histochemically, and this is the key to the relationship between the histochemical and biochemical findings. Many of the hypotheses to be detailed later in this thesis are based on this relationship.

9. Anti-androgens and the Skin.

It will be apparent from the preceding discussion on hormonal aspects of sebaceous glands, that androgens are of prime importance in the growth and differentiation of such glands. It is therefore not surprising that it has been suggested that anti-androgens, that is substances which antagonise the action of androgens, might be of use clinically in such diseases as female hirsuitism, acne vulgaris and perhaps male type baldness.

The difficulty arises in the assessment of anti-androgenic

substances. One of the major problems is that these substances may have dangerous side effects if given systemically. They could cause adrenal, testicular and ovarian hormonal depression, which could be serious and even life-threatening and prior screening in experimental animals is therefore mandatory. The use of experimental animals however introduces other variables, and the species differences may be of great importance. It is important therefore to assess the effect of antiandrogens in human skin and realise that other methods of antiandrogen assay may not be directly transferrable to the human situation. Further, it is well recognised that these animal assays may give different results with the same antiandrogen.

A number of methods are available for the assay of antiandrogens. One of the earliest of these was to use the cock's comb to test the effect of these substances. On administration of androgen, the cock's comb increased in size and this effect can be modified by introducing antiandrogens (Dorfman, 1962). Such a technique has been used by various workers and has given reproducible results.

A second method is to use the response of rat seminal vesicles, prostrate and levator ani muscles to androgens. On administration of androgen, the weight of these organs increases and this can be used as a biological assay system for antiandrogens (Dorfman, 1962, Saunders and Ebling, 1969, Saunders, Holden and Kerwin, 1964).

Another method, again using the rat is to estimate the effect of androgens on a specialised sebaceous structure, the rat preputial

gland. On administration of testosterone the rat preputial gland increases in weight and can be measured sufficiently accurately to give reproducible results. This technique introduced by Jones and Woodbury (1964), has been used as a screening method for anti-androgens. In their paper Jones and Woodbury suggest that the mechanism of anti-androgen action is either to reduce the ability of the target organ to respond to androgens, to reduce the release of androgens, or to reduce the release of pituitary hormones which influence sebaceous gland growth.

More recently, Ebling (1967), has introduced a technique of sebum collection in rats. The animals are first washed in a detergent to remove hair fat, then given an androgen or other preparation. The hair is then clipped after a few days and the ether extractable hair fat measured. He found that certain anti-androgens depressed the secretion of sebum when the animal had been given testosterone. He also suggested that the anti-androgen he used, 17α methyl -β- nor testosterone acted at the site of action of testosterone. This method has also been compared with the rat seminal vesicle assay (Saunders & Ebling, 1969). Shuster has also used a method of sebum collection in rats as a bioassay method for androgens and anti-androgens (Archbold & Shuster, 1967, Shuster, 1970).

The hamster has in its costovertebral region a sebaceous structure which can also be used for the purposes of anti-androgen assay (Burdick, 1970).

The methods described so far have all used experimental animals. However Strauss and Pochi (1961, 1963) introduced a method for the

collection of sebum in humans. After cleansing of the forehead, a piece of cigarette paper is applied and the sebum produced over a 3 hour period is measured by weighing after extraction. This technique gives good quantitative results which are reproducible. Using children from an institution for the mentally defective, the effects of androgens, oestrogens, and progestogens on sebum production was worked out and they suggest that this is a suitable method for the assay of androgens. As a screening method for anti-androgens, at least in the initial stages of experimental work, this would be ethically unacceptable with human subjects. As a technique for the estimation of sebum production in normal and pathological states however, the method is of great value. Cuncliffe and Shuster (1969) have investigated sebum production in a large series of normal subjects and in subjects with acne using this technique.

From the preceding sections it is known that androgens are metabolised within skin, and in particular 3β - hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase are present. It seemed reasonable then to extrapolate this fact in conjunction with the histochemical technique and use this as a screening method for anti-androgens. The anti-androgen, when incubated in the presence of testosterone or dehydroepiandrosterone with the full histochemical medium, would cause a reduction in the number of diformazan granules if inhibition of these enzymes occurred. It must be pointed out of course that this method would not by itself measure the anti-androgenicity of any compound, simply its ability to interfere with certain

limited enzymes of androgen metabolism. Thus only compounds with a direct action on androgens would be picked up in this way. An anti-androgen then, in this limited sense, is a substance which will inhibit the 3β- or 17β- hydroxysteroid dehydrogenases in a histochemical system in human skin, at the level of the sebaceous glands. Compounds such as oestrogens, which though anti-androgenic, have a different mode of action to other anti-androgens, might not be picked up in this way. The results of some of the histochemical investigations have already been published (Calman, Grant & Milne, 1969, Calman, 1970).

10. Scope of the present investigation.

This introductory review has given the background to the problems to be studied, and has discussed some of the related questions. It is evident that any study of sebaceous gland function or metabolism must be multidisciplinary in origin and the histochemical investigations to be described represent therefore, only one facet of the problem.

It is necessary perhaps at this point to justify the use of histochemical techniques in these investigations. In the first place, the techniques are simple and rapid and if necessary can be used without expensive apparatus. This is of particular relevance to the use of the technique as a screening method and for its use in non-specialised laboratories.

Secondly, only small amounts of tissue are required, about five

per cent of that required for biochemical investigations by current techniques. This again is of importance if large numbers of samples are to be processed.

Thirdly, human tissue can be used throughout. This is of considerable importance since inevitably differences in sebaceous gland function between different species will occur. From the point of view of the assessment of anti-androgens for clinical use, then this is of definite relevance.

Lastly, and perhaps the most important of all, the localisation of these enzymes within the tissues and within sebaceous glands, can be studied and data of anatomical, physiological and pathological importance, determined.

In the preceding review certain problems which could be specifically answered by histochemical methods have already been mentioned. These and other questions which constitute the aims of the present investigations will now be detailed.

1. Certain conditions affecting the histochemical demonstration of the enzymes would be investigated and the validity of the technique would be established using recognised criteria.

2. A review of the hydroxysteroid dehydrogenases which can be demonstrated in human sebaceous glands would be carried out and attention paid to the localisation of these enzymes within the sebaceous glands.

3. The regional variation in activity of these enzymes would be studied. In all biopsies, identification of sebaceous glands would be mandatory, as would be the demonstration of NADH diaphorase.

4. The activity of these enzymes in human fetal skin would be studied.

5. The effects of certain compounds thought to be anti-androgens, would be investigated, with a view to using the technique as a rapid screening method for such compound.

6. Relation of the histochemical findings to known biochemical work would be reviewed.

These investigations are original in that they have not been studied by the more recently introduced techniques of Muir et.al. (1968 a & b). New areas of research have been approached and the results of previous observations re-appraised. Throughout this thesis, only work which has been personally carried out will be reported. The results with fetal skin were done in collaboration with Dr. F. Sharp, Department of Obstetrics, the University of Glasgow.

3. MATERIALS AND METHODS

1) Skin biopsy specimens.

2) Fixation.

3) Steroid substrates.

4) Incubation methods.

5) Control incubations.

6) Counterstaining technique.

7) Semi-quantitative technique.

8) Inhibitors of hydroxysteroid dehydrogenases.

9) NADH diaphorase.

10) Gram staining.

11) Specificity of localisation experiments.

MATERIALS AND METHODS

(1) Skin biopsy specimens.

All specimens used throughout the investigation were from human skin. Adult human skin biopsies were obtained at operation, and were frozen on carbon dioxide snow within 2 minutes of removal. In general small ellipses of skin from the wound edge, 5-7 mm. x 2-3 mm. were used. Some wound biopsies, particularly those taken during a thoracotomy procedure were larger than this. Biopsies were taken from representative areas of fetal, juvenile and adult human skin, so that the anatomical pattern could be established.

Skin was also obtained, post mortem, from autopsy material. In all cases, scalp skin was used and the age, sex, and post mortem interval recorded. Following removal, the skin was frozen on carbon dioxide snow, and the skin processed in a similar manner to other specimens. Skin from episiotomy wounds, and caesarean sections was obtained from pregnant women.

Human fetal skin was obtained from a variety of sources. (a) Fetuses obtained at therapeutic termination of pregnancy, (b) fetuses obtained at spontaneous abortion, (c) fetuses obtained following stillbirth, (d) fetuses succumbing in the immediate neonatal period. With categories (a) and (d) fetal skin could be obtained in a fresh condition, approximating to that of adult skin biopsies. With categories (b) and (c) however, a variable period of time will

have elapsed between fetal death and collection of skin. If fetal death could be timed, then an attempt was made to use only fresh material and other fetuses were not used. Biopsies of human fetal skin were taken from four representative sites; scalp, anterior aspect of chest, posterior aspect of chest, and thigh. These were also frozen as soon as possible on carbon dioxide snow.

All portions of skin were then processed in an identical manner. While still frozen, excess subcutaneous fat was removed and the biopsy specimen orientated with a small amount of water. This was then frozen to a cryostat chuck and stored at -20° in this manner until sectioning. All sections were cut in a "Slee" cryostat at $-20^{\circ}C$; the section thickness was 8-10 u. Sections were attached to cover slips by momentary thawing, and stored in this form. All incubations were performed within one week of obtaining the skin biopsy. Specimens were stored during this time, either as the block of skin, or as sections mounted on coverslips.

Because the activity of the enzymes might vary with storage, a series of experiments was set up, in which sections were taken at intervals for estimation of the enzyme activity. Specimens stored in block form, and in section form, were used.

To investigate the distribution of these enzymes within sebaceous glands in some instances serial 10 μ thick sections were cut and incubated with testosterone and dehydroepiandrosterone.

(2) Fixation.

For the great majority of the enzyme incubations fresh frozen sections were used, without pre-incubation fixation. Post-incubation fixation by alcohol during the counterstaining procedure however, was used. In a short series of experiments, using adult skin, the effect of various fixatives was assessed. Sections, mounted on coverslips were incubated with cold fixative for 30 minutes, then incubated to demonstrate the presence of the enzymes. Fixatives used were neutral buffered calcium formalin, formal saline, Bouins fluid and cold acetone. A control incubated for the same time in cold buffer was also used.

(3) Steroid substrates.

Steroids used in these investigations were obtained from "Steraloids". Preliminary purification was not considered necessary with the histochemical system.

Steroid	Hydroxyl Group
dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one)	3 β
pregnenolone (3β-hydroxy-5-pregnene-20-one)	3 β
5-androsten-3, 17-dione	———
3β, 16β-dihydroxy-5-androsten-3-methyl ether	16 β
3β-hydroxy-5-androsten-3-methyl ether	———
testosterone (17β-hydroxy-4-androsten-3-one)	17 β
oestradiol-17β (1, 3, 5(10) estratriene-3, 17β-diol)	17 β
11β-hydroxy-4-androsten-3, 17-dione	11 β

17α-hydroxy-4-androsten-3-one	17 α
3α-hydroxy-5α-androstan-17-one	3 α
3α-hydroxy-5β-androstan-17-one	3 α
5α-androstan-3, 17-dione	———
5β-androstan-3, 17-dione	———
17β-hydroxy-5α-androstan-3-one	17 β
20β-hydroxy-4-pregnen-3-one	20 β
3β-sulphoxy-5-androsten-17-one	3β-SO_4

For routine use only four steroids, oestradiol, dehydroepiandrosterone, testosterone, and 3β, 16β-dihydroxy-5-androsten-3-methyl ether were utilised. Steroids were used at a concentration of 30 ug/ml. of incubation medium (Muir et.al., 1968). Throughout the experiments the steroids were dissolved in dimethyl formamide (DMF), and stored at 4° in that solvent.

(4) Incubation Methods.

The incubation medium was made up freshly for each batch of experiments and contained the following:

Steroid substrate (30 ug/ml. final conc.) 300 ug. in 0.3 ml.

Dimethylformamide (Analar) B.D.H.

Nitroblue Tetrazolium (Sigma) 1 mg. (1 ml. of 1 mg/ml. stock solution)

Nicotinamide adenine dinucleotide (NAD); 2 mg. (Sigma)

0.1 M phosphate buffer, pH 7.4; 7.7 mls. (Hopkins & Williams).

The section was then incubated with this medium, (pre-warmed to 37°)

for 2 hours at 37°. All incubations were performed in duplicate.

Various methods have been used to incubate the sections but in this investigation only two have been consistently used. The first, known as the meniscus method, involves placing the incubation medium on top of the section, mounted on a coverslip, and incubating in this form. Evaporation inevitably occurs, but with short incubation periods, this is of little importance. Evaporation can be considerably reduced, by incubation in a closed space with humidification. The second method is to enclose the incubation medium under a small glass dome. This reduces evaporation and small quantities of fluid only are required.

One additive, of potential importance is polyvinyl pyrrolidine (P.V.P.) which theoretically, will reduce diffusion of enzymes into the medium. A few experiments were performed using P.V.P. at a final concentration of 3% (W/V) to assess the effect of this substance.

(5) Control incubations.

During this investigation several controls were used to establish the validity of the technique.

(a) Incubation with the section without addition of steroid substrate. 0.3 ml. of D.M.F. was added.

(b) Incubation without addition of N.A.D. All other factors added.

(c) Incubation without steroid substrate or D.M.F.

(d) Sections containing no sebaceous glands.

With the first two controls, if no diformazan granules are deposited, then it can be said that the enzyme is an NAD dependent hydroxysteroid dehydrogenase, since only when both steroid substrate and NAD are present, does the reaction occur. The third control, that is eliminating both the steroid substrate and DMF, is not used routinely, but would be useful if positive controls were obtained in ruling out contamination of the DMF with a nitro-BT reducing substance.

The fourth control is used for histochemical localisation purposes. It is possible that although the enzymes are present in other areas of skin, that because of factors such as selective lipid solubility, or dye diffusion, they can only be demonstrated within sebaceous glands. Incubation of a section without sebaceous glands, containing the enzyme activity.

In those sections where diformazan granules were deposited in control incubations, a method to overcome this was devised. Sections were washed for varying periods of time in cold phosphate buffer, then directly transferred to the incubation medium. A series of experiments was performed with skin sections, pre-incubating in buffer for one to 90 seconds, then incubating with the steroid medium. In all incubations whose controls showed diformazan deposition, the incubation was repeated with fresh sections after a 15 second wash with cold phosphate buffer. With each skin, a control incubation was included.

Figure 6. Method of Semiquantitation used. The observer views simultaneously the microscope slide and the graph paper. The outline of the sebaceous gland and the density of diformazan granules are recorded as shown.

(6) Counterstaining technique.

Following the incubation, the sections were first rinsed in water to remove excess substrate, then stained with Haematoxylin for 2 minutes. The section is then dehydrated with alcohol, and stained with tartrazine in cellusolve (saturated solution) for 2 minutes. The sections are then washed in cellusolve, xylol, and mounted in H.S.R. (Eastman Kodak). The section can be stored in this form for many months, without loss of tissue staining or diffusion of granules.

In some experiments, the sections, after washing were mounted directly in an aqueous medium (Hydramount, G.T. Gurr) without counterstaining.

(7) Semiquantitative technique.

A method of semiquantitation, based on the counting of the deposited diformazan granules was used and has been described before (Muir, et.al., 1968a). The technique involves the principle of the camera obscura, in which the observer looks at the skin section through a binocular "Wild" microscope, and simultaneously observes, via a drawing tube attachment, a sheet of centimeter square graph paper (Fig. 6). The outline of the sebaceous gland is drawn and by suitable calibration, its area can be determined. The sebaceous gland is then visually scanned, and the number of granules in each 5 mm. square assessed and scored. If more than 5 granules are

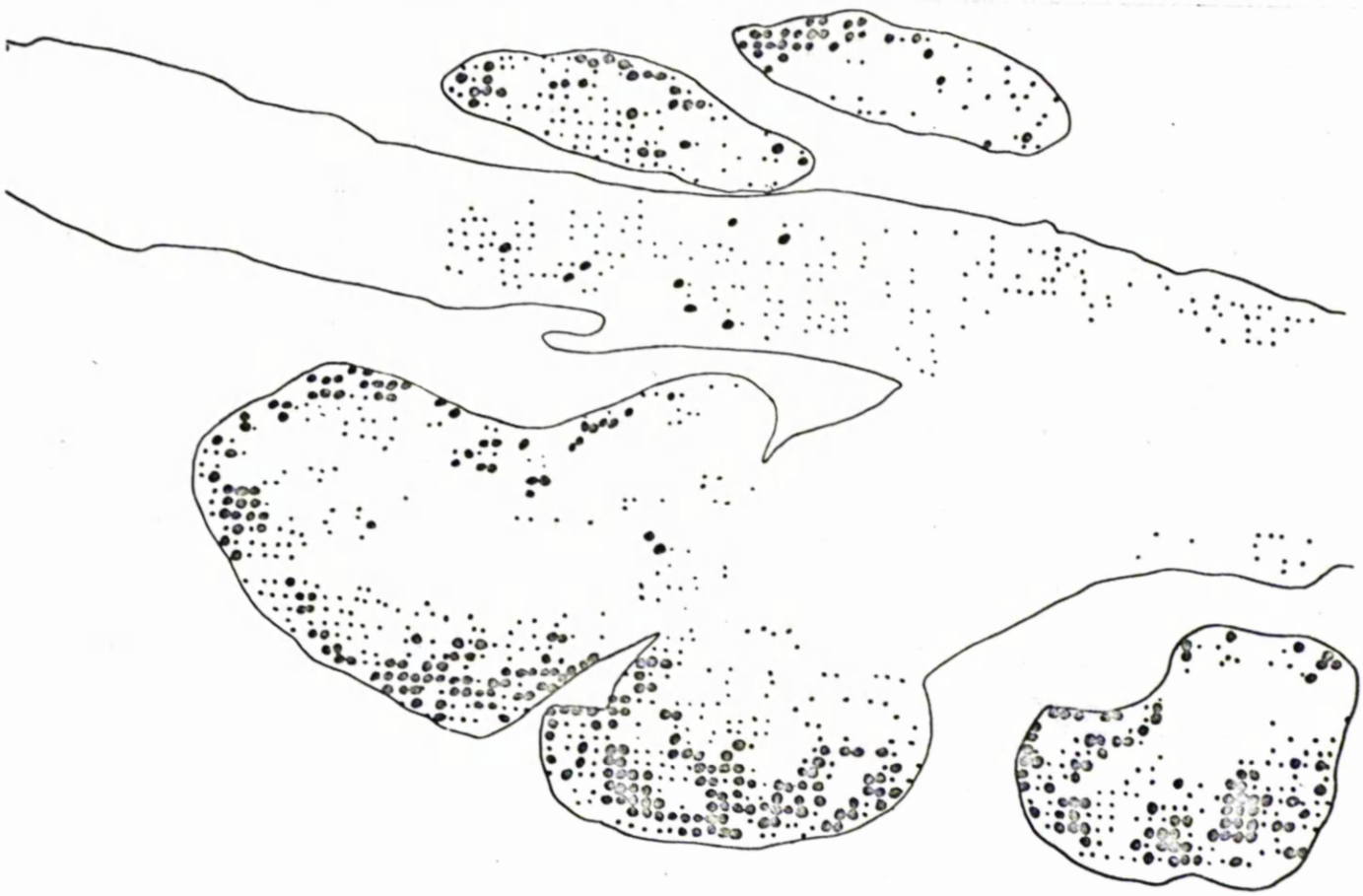


Figure 7. Typical granule count using the method of semiquantitation previously described.

found a score of 2 is given, if less than 5 granules, a score of 1 is given. If no granules are found then the score is zero. These are then totalled, and a granule count per unit area is estimated. In all semiquantitative experiments, results were set up in duplicate, and the mean figure used. The results can easily be stored on the graph paper as a permanent record, and the results assessed at leisure (Fig. 7).

(8) Inhibitors of hydroxysteroid dehydrogenases and anti-androgens.

In a series of experiments, the effects of a number of commercially available anti-androgens on these enzymes was investigated. The inhibitors were used at a range of concentrations, from 30-200 ug/ml. final concentration. These substances were made up in dimethyl formamide and added in a volume of 0.1 ml. per ml. incubation medium. All assays were set up in triplicate, and for the semi-quantitative experiments, concentrations of 100-200 ug/ml. were used. Results with the semi-quantitative experiments are expressed as a percentage of the control. With each substance controls were used to eliminate the possibility of the substance itself being used in the histochemical system. The substances used were:-

1. cyproterone (6-chloro-17-hydroxy, 1α, 2α, methylene pregna-4, 6-diene-3, 20, dione) A.G. Schering, Berlin.
2. cyproterone acetate (6-chloro-17-hydroxy, 1α,2α methylene pregna-4, 6-diene-3, 20 dione acetate) A.G. Schering, Berlin.

3. tetracycline (Pfizer, Ltd).

4. 6-chloro-17α hydroxy pregna-1, 4, 6-triene-3, 20-dione acetate (Compound A) Syntex Research.

5. 6-chloro-17α-hydroxy pregna-1, 4, 6-triene-3, 20-dione (Compound B) Syntex Research.

6. 6-chloro-16α, 17α-dihydroxy pregna-1, 4, 6-triene-3, 20-dione 16, 17-acetonide (Compound C) Syntex Research.

7. 6-chloro-17α hydroxy pregna-4, 6-diene-3, 20-dione-17 acetate (chloramidone acetate) Compound RS 4529 Syntex Research.

The steroids used in this investigation were Testosterone, Dehydroepiandrosterone, oestradiol and 5-androsten 3β-16β-diol-3 methyl ether. These were used at final concentrations of 30 ug/ml.

(9) NADH diaphorase (NADH oxido-reductase).

Throughout the experiments each skin biopsy was histochemically assessed for the presence and localisation of NADH diaphorase. The technique used was as follows:

NADH - 2 mg. (as solid)

Nitroblue Tetrazolium - 1 mg/ml. (1 ml. added)

Phosphate buffer 0.1 M pH 7.4 - 9 mls.

Sections were incubated for 20 minutes at 37° and counterstained as described previously. The technique is similar to that of Nachlas, Walker and Seligman (1958), and Scarpelli, Hess and Pearse (1958).

(10) Gram staining.

With a series of skins, the frozen sections obtained were stained by Gram Jensen or Gram Kirkpatrick methods. Following this the section was scanned for evidence of bacteria using an oil immersion lens.

(11) Specificity of localisation.

In a series of experiments, sections of skin which had been washed or incubated in fixative to remove hydroxysteroid dehydrogenase activity were incubated, side by side, with unwashed sections. Incubation media containing DHA, testosterone, and NADH were used. These experiments were designed to investigate the effects of the solubility of these enzymes in relation to localisation.

4. RESULTS AND DISCUSSION

A. Introduction.

B. Histochemical methods.

C. Hydroxysteroids metabolised by human sebaceous glands.

D. Distribution of hydroxysteroid dehydrogenases in prepubertal and adult human skin.

E. Hydroxysteroid dehydrogenases in human fetal skin.

F. Antiandrogens and hydroxysteroid dehydrogenases.

A. Introduction.

For the purposes of clarity the results and discussion of each section are dealt with together. Though the sections are inter-related, each is complete in itself, and has a separate summary.

Throughout the results section certain abbreviations will be used. The main steroid substrates used are :-

dehydroepiandrosterone - DHA

testosterone - TEST

oestradiol - OEST

5-androsten - 3β - 16β diol - 3 methyl ether - 16β

When these abbreviations are used it indicates that the tissue section has been incubated with these steroids at a concentration of 30 ug/ml. final concentration, unless otherwise stated.

The enzymes demonstrated, the hydroxysteroid dehydrogenases, are abbreviated to HSD's, and NADH oxidoreductase activity is shortened to NADH.

When the activity of the enzyme has been visually assessed the results are expressed as :

tr - weak activity, but diformazan granules present.

\+ - good activity present.

++ - very high activity.

B. Histochemical methods.

1. Fixation of tissue.
2. Effects of polyvinyl pyrrolidine.
3. NADH-oxidoreductase.
4. Control incubations.
5. Counterstaining, monoformazan formation & semiquantitation.
6. Bacteriological staining.
7. Effects of storage on enzyme activity.
8. The use of post-mortem skin.
9. Summary.

B. Histochemical Methods.

1. Fixation of tissue.

Preincubation fixation was carried out using a variety of fixatives, which have previously been described. The results are shown in Table 1.

Table 1.

	Control	Buffer	Formal Saline	Calcium Formalin	Acetone	Bouins
DHA	+	+	-	+	-	-
TEST	+	+	-	tr	-	-
OEST	+	+	-	tr	-	-
16	+	+	-	tr	-	-
NADH	++	++	++	++	++	-

The control indicates that the incubation was without either a wash for 30 minutes in cold buffer or with the fixative.

From these results it will be noted that (a) a wash in cold buffer does not remove all enzyme activity, so that any decrease following fixation is not due to simple washing out of the enzymes. It is in some ways surprising that the tissue can be washed for such long periods without much loss of activity, since, as has been previously mentioned, tissue protein rapidly diffuses into the incubation medium. The 3β-HSD is known to be a particle bound enzyme, and this could explain the fact that there is little loss of activity when the substrate is DHA.

The 17β-HSD is however a soluble enzyme and an explanation of activity being present even after washing may be due to its retention within intact membrane systems.

(b) In all instances, except with Bouin's medium, NADH diaphorase is present and its activity is high. Any loss of activity then when incubated with fixative is not due to a decrease in NADH diaphorase activity.

(c) It will be readily appreciated that only with calcium formalin is any activity of these enzymes retained, and even with this fixative, the activity is diminished.

These results are in accord with many others in the field of fixation in enzyme histochemistry. It is generally assumed that the use of fresh frozen sections means that the quality and localisation of the enzyme activity will be poor. Recently Wyllie (1965) has looked at the question of fixation in enzyme histochemistry, using a wide variety of enzymes. He found that the quality of enzyme activity was poorer after fixation, and that there was a marked decrease in activity in all cases, with each fixative. He also noted that the localisation was no different in fixed and unfixed sections. These results are in agreement with those presented in this study.

It is concluded therefore that routine pre-incubation fixation, at least with the hydroxysteroid dehydrogenases in human skin is undesirable, and has not been used in subsequent experiments.

2. Incubation with polyvinyl pyrollidine.

Polyvinyl pyrollidine (P.V.P.) has been used in the past to prevent diffusion of soluble products into the incubation medium (Scarpelli & Pearse, 1958). Using this substance it was hoped that localisation would be improved.

In a series of experiments polyvinyl pyrollidine (PVP) was added to the incubation medium at a final concentration of 3%. In no case with any of the steroids used was any beneficial effect noted. With control incubations, that is incubations where no steroid was added, diformazan granules were found in some cases, which were not present in the controls without PVP. This could be due to the retention within the sebaceous glands of small molecular weight substances, capable of donating hydrogen to the histochemical system and this will be more fully discussed later.

It is concluded that PVP is of little use in the histochemical demonstration of HSD's in human skin. With some animal skins however, PVP does aid localisation (Muir, 1969 unpublished observations).

3. NADH oxidoreductase.

Since the histochemical demonstration of HSD's depends on the presence of a second enzyme, it is of great importance that the NADH oxidoreductase is present in all skin biopsies used. In addition to this the localisation of this enzyme will inevitably reflect the localisation of the HSD's. In assessing the results of over 150 skin biopsies the following conclusions can be drawn.

(a) NADH oxidoreductase was present in all but 1 skin biopsy,

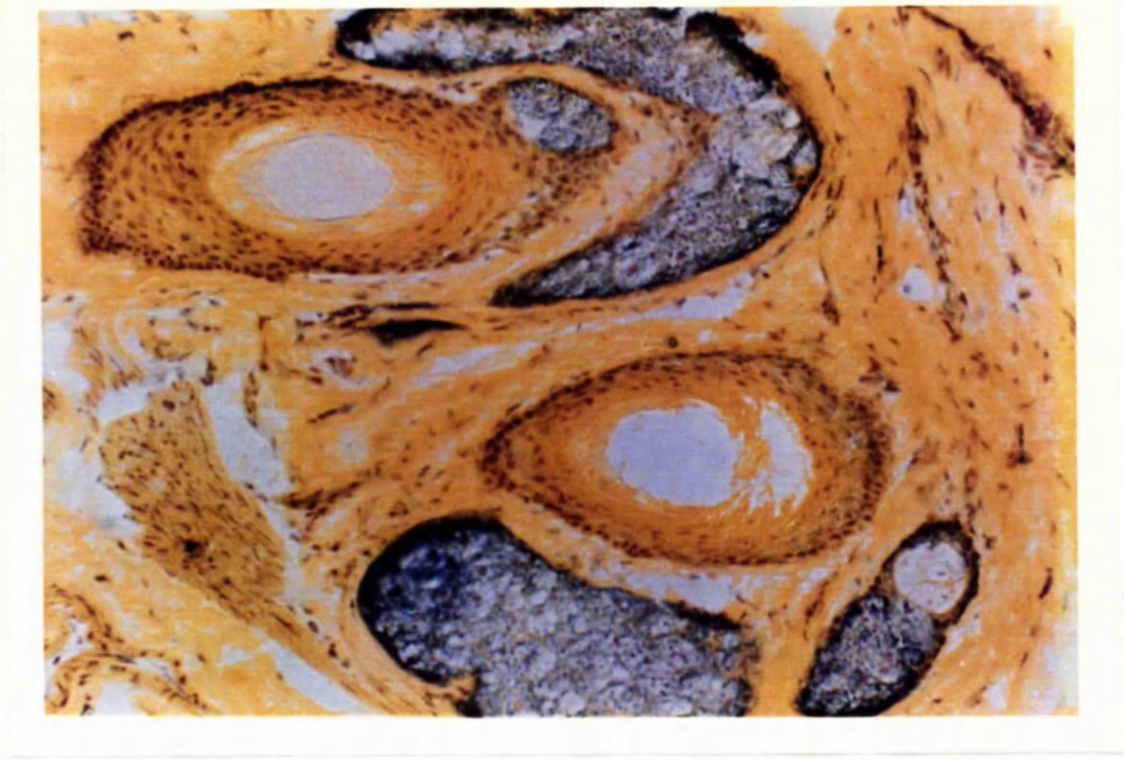


Figure 8. NADH Diaphorase Activity Sebaceous Glands. (x240)

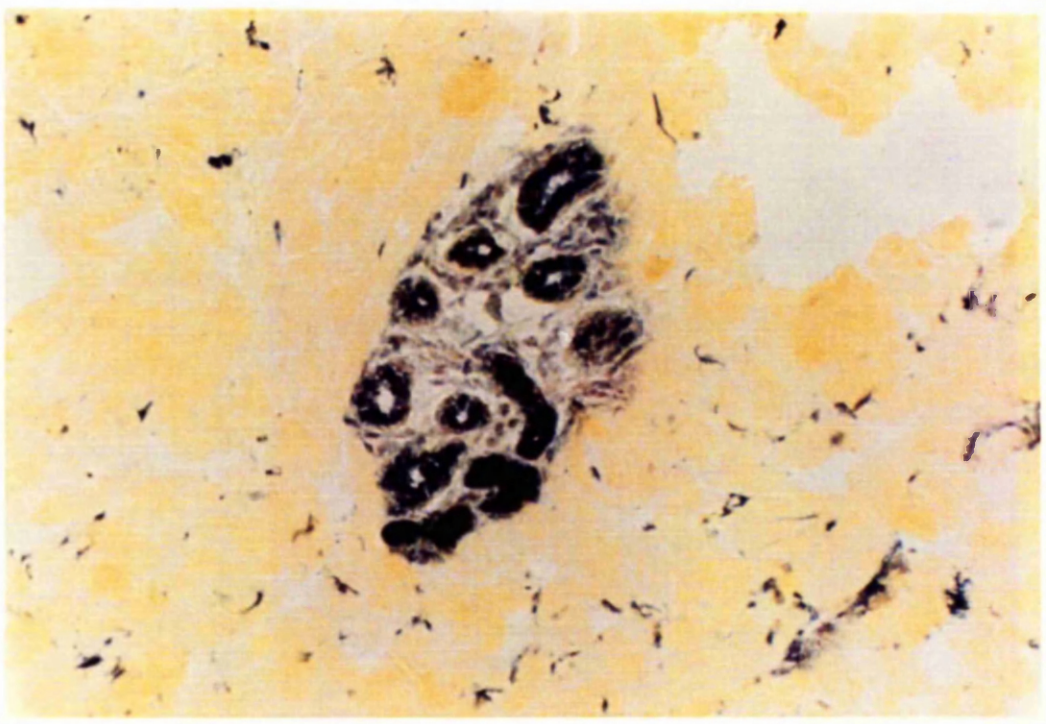


Figure 9. NADH Diaphorase Activity Sweat Glands. (x175)

and was present in high activity.

(b) The enzyme was found in all structures found within the skin; epidermis, sebaceous glands, sweat glands, fibroblasts, blood vessels and the piliary canal (Figs. 8 & 9). It can be assumed then, that any difference in localisation, or any specific localisation of the HSD's is not due to a selective distribution of NADH oxidoreductase, since that enzyme is present throughout the skin.

(c) Within sebaceous glands themselves, no regional variation in activity was apparent. Thus any regional distribution of HSD activity within sebaceous glands is not due to difference of NADH diaphorase localisation.

This is an important observation since it is basic to the whole concept of localisation of dehydrogenases that the activity, or localisation of the NADH diaphorase does not influence the tissue distribution of the dehydrogenase.

4. Control incubations.

Reference has been made in the Methods Section to the control incubations used throughout this study. The value of these controls cannot be too highly emphasised. With histochemical techniques in which no accurate quantitative method is available, the control section is the final reference standard. To make results comparable therefore it is imperative that no diformazan granules are deposited in control sections. In the series of skin biopsies used in this

study, only 6 specimens showed diformazan granules in control incubations. However, in the initial work done with this technique positive controls occurred more frequently than this, and accordingly the question was investigated more fully. In most instances the control used contain all reactants except the steroid substrate. If no granules are deposited in the control section, but are deposited in the section incubated with steroid substrate, then it can be said, that the reaction demonstrated is due to an NAD dependent HSD. If positive controls are obtained then one has to investigate further.

The first obvious check is to make sure that the various reactants contain no contaminants. The NAD for example, following prolonged storage or contamination, may be converted to the reduced form. The dimethylformamide, again with storage, may be converted to other amine derivatives which could potentially reduce the nitro-blue tetrazolium. These can be readily discovered and eliminated by using fresh NAD and redistilled dimethyl formamide. The steroid substrate itself may be contaminated by material capable of directly (that is non-NAD dependent) reducing the nitro-BT. This can also be readily checked by incubation without NAD. In no instance however have any of these factors been responsible for positive controls.

Since the tissue used throughout this study had not been subject to any extraction or purification procedure, simply treated by freezing and sectioning, it is likely that small molecular weight substances, such as lactic acid, malic acid, succinic acid or even endogenous NADH may still be present within the tissue. If this is the case then

simple washing, before incubation, would remove these materials, and give negative controls. It must be remembered however that washing will also remove enzymes, so that the shortest possible incubation time should be used.

Using a series of skin biopsies in which positive controls were obtained, sections were washed for various periods with cold phosphate buffer, then incubated with the control solution. The results are summarised in Table 2.

Table 2.

(a)	Control solution 1. (NAD. Nitro-BT. Buffer. DMF) ——— +							
	Control solution 2. (Nitro-BT. Buffer. DMF) ——— -							
(b)	Preincubation time	0	1	5	10	15	20	mins.
	Control solution 1.	+	+	tr	-	-	-	
	Full incubation (including steroid)	+	+	+	+	+	+	

Part (a) indicates that the cause for the positive controls is most likely to be NAD dependent, and part (b) shows that a positive control section can be made negative by a brief wash in buffer, without impairing the activity of the enzyme. From these results it is concluded that should a skin biopsy give a positive control, then a wash in cold buffer for 10 minutes, will render it negative. It is suggested that this is

due to the presence within the tissues of small molecular weight substances which cause NAD dependent reduction of nitro-BT.

One other observation of interest is that if a skin biopsy gives a positive control, when assayed within a few hours of removal, then if this is repeated a few days later the control will often be negative. The tissue in the intervening period being stored at -20°. This may be due to slow metabolism of these compounds so that their effective concentration is reduced. Post-mortem skin is another interesting example of this phenomenon, and will be discussed later.

Reference has already been made to the presence of diformazan granules in the hair shafts in control incubations. It is thought that this is due to the high concentration of sulphydril groups in the hair shafts.

One other control which is important in the establishment of the localisation of these enzymes, is to use skin specimens not containing sebaceous glands, In the survey to be described later, over 60 of these have been studied. It might be assumed that if the HSD's are present in other skin appendages, or the epidermis, that the deposition in the sebaceous glands of the diformazan could be due simply to the lipid solubility of the end product. In other words, the high lipid content of the sebaceous glands could mask the diformazan deposition in other sites in other sites in skin. If this was the case, then in sections containing no sebaceous glands, diformazan deposition could be more easily visualised. In these 60 specimens in no instances

have diformazan granules been visualised in other skin appendages. It is unlikely therefore that the diformazan granules have been prevented from depositing at other sites because of the presence of the lipid-rich sebaceous glands.

5. Counterstaining, monoformazan formation, and semiquantitation.

Counterstaining is an essential prerequisite in any investigation using histochemical techniques, when the tissue is as complex as skin. Frozen sections, without counterstaining are difficult to interpret. The collagen bundles in the dermis can often look like sebaceous glands, so that localisation of the granules is impossible. The exact outline of the gland is difficult to define and the area cannot be accurately measured. Nuclei cannot be seen so that the relation of granule density to the number of nuclei present cannot be assessed. The method introduced by Muir et.al. (1968), that of haematoxylin and tartrazine, has been used throughout and found to be very satisfactory. The black diformazan granules contrast well with the red nuclei and the yellow cytoplasm. As has been shown before there is no reduction in the number of granules found following counterstaining so that no loss of activity results.

As was mentioned in the Introduction, the histochemical reaction involving the reduction of nitro-BT to diformazan, is a two-step process, an intermediate monoformazan being formed. With nitro-BT this is a pink coloured material and it was assumed in earlier work

(Baillie, Fergusson & Hart, 1966) that the presence of monoformazan indicated a weak reaction. However, at least with neotetrazolium (Burtner, Bahn & Longley, 1957) the monoformazan and diformazan formed do not represent succeeding stages in the reduction. Since the counterstaining method has been introduced, and the optimum concentration of the incubation mixture worked out, no monoformazan has been observed in the present study. No monoformazan has been noted in unstained preparations so that the loss is not simply due to removal during counterstaining.

The method of semiquantitation introduced by Muir et.al. (1968) has been used only in the assessment of the antiandrogens. The drawbacks of this method are considerable and these have been summarised in the Introduction. On the whole visual assessment of the reaction has been used. Further attempts to quantitate the histochemical procedure should concentrate on rapid biochemical or histochemical measurement of the reduced nucleotide produced. Further attempts to quantitate the reaction either by counting the diformazan granules, or by extraction methods would seem unjustifiable, in view of the uncertainties regarding the method. Granule counting has been used in this study simply to put figures to the results obtained, and as will be seen later, these correlate well with the visual assessment. No value in terms of micromoles substrate transformed is to be taken from such data since only changes in activity can be measured.

Recently the introduction of a reliable scanning microdensitometer (Bitensky, 1970) will make the quantitation of diformazan granules

much easier.

6. Bacteriological staining.

Eight separate samples of skin, including one skin obtained at 18 hours post-mortem, were stained with Gram Jensen, or Gram Kirkpatrick. The sections were scanned and evidence of bacteria within the sebaceous glands, secretory ducts, epidermis or other skin appendages sought. The results showed that in no instance could definite evidence of bacteria be found in any of these areas.

This experiment was performed to exclude the possibility that the activity of the hydroxysteroid dehydrogenases within the sebaceous glands was due to bacterial contamination. It is fully realised that simply to say that no bacteria could be found does not constitute absolute proof that no bacteria were present. What it does do however, is to make it very unlikely that the deposition of diformazan granules within the sebaceous glands is due to gross bacterial contamination. The bacteria may be present, and may even metabolise the steroid substrates, but it is inconceivable that they could cause the kind of diformazan deposition which is found in sebaceous glands.

Other indirect evidence is available which makes bacterial contamination as a significant factor in the reaction, extremely unlikely. In the first place bacterial contamination would be expected to involve other skin appendages, and in particular the sweat glands and this does not occur. Secondly, if bacterial contamination were important, then

most of the activity would be found on the epidermis, the secretory duct and then the sebaceous gland. No such distribution of activity is found. In fact the reverse is the case, most of the activity being found in the glands and very little in the ducts. The third piece of evidence came from studies on fetal skin. The fetus is surrounded by a sterile medium, the amniotic fluid. Skin biopsies taken from the fetus however, show activity of these enzymes under circumstances in which bacteria are absent.

In conclusion then it is unlikely that the deposition of diformazan which results when skin specimens are incubated with steroids is due to bacterial contamination.

7. Effects of storage on enzyme activity.

Following removal of the skin biopsy and freezing, the block was divided into 2 portions. The first portion was stored as a block, at - 20°, sections being cut from it at intervals. The second portion was immediately cut and the sections stored, mounted on coverslips, until use. Three separate skin biopsies were used in this experiment. The results can be summarised as follows :-

1. Throughout the storage period of 6 weeks, there was no obvious difference in the morphology of the tissue sections over this period, the block remained easy to cut.

2. Over the 6 week period of storage, there was no decrease in activity of the NADH oxidoreductase.

3. There was good activity up to 4 weeks storage in all skins examined. Beyond this time, there was some fall off in activity in one skin sample.

4. There was no difference in enzyme activity between storage in block form or in tissue sections.

5. Once counterstained the tissue section can be stored for at least 1 year without losing activity when left at room temperature.

This observation has an important practical aspect. Human skin is not easy to obtain, in particular fetal skin and some areas of adult skin, notably face. Skin biopsies may become available at inconvenient times and it is important to know that simple freezing will be sufficient to preserve enzyme activity for at least a few weeks. This means that skin can be stored or sent from a distance, if suitably refrigerated, and the activity of these enzymes tested for at a later date.

It is concluded then that skin biopsy specimens can be stored at -20° for at least 4 weeks without loss of enzyme activity. It does not seem to matter whether the tissue is stored as a block or in tissue sections.

8. Enzyme activity in post-mortem skin.

Skin was obtained at post-mortem from the scalp. Sebaceous glands were noted in good numbers in all except one skin sample. Morphologically the biopsy specimen was fairly good, and there was no evidence of gross autolysis. The storage of the cadaver involved a warm time of 2-3 hours, with subsequent cooling to 4°C in a refrigerator for varying periods of

time. Following removal the biopsy was frozen with CO_2 and acetone, and then processed as with other skin biopsies. The results are shown in Table 3.

Table 3.

ENZYME ACTIVITY IN POST MORTEM SCALP SKIN

Age	Sex	P.M. Interval	3β-HSD	16β HSD	Test.	Oest.	NADH
68	F	18 hours	+	+	+	+	+
75	F	24 hours	+	+	+	+	+
63	M	18 hours	+	+	+	+	+
68	M	19 hours	+	+	+	+	+
73	F	12 hours	+	+	+	+	+
44	M	31 hours	+	+	+	+	+
58	M	12 hours	+	+	+	+	+

From these results, it is clear that activity for all four enzymes is present in post-mortem skin, up to 31 hours of post-mortem interval. Further, the activity is high and is very similar to that obtained in

fresh scalp skin. The 17β-HSD is particularly active. In all cases NADH oxidoreductase activity was present. One difference which was noted in the localisation of these enzymes was with the 17β-HSD. The picture was more diffuse than normal, suggesting diffusion of the enzyme during storage.

The most significant difference, however, was in the control incubations. Without exception, control incubations were positive, and clearly so. As with other positive controls, it was possible to make them negative with a brief wash in cold buffer. The reason for these controls being positive, is however, not fully worked out. A possible hypothesis to explain this would be that on death, with cessation of blood supply, anaerobic metabolism within the skin continues. This would result in the production of the products of glycolysis, lactate, pyruvate and other small molecular weight substances. These, in turn, might be present in the tissue at a sufficiently high level to reduce the nitro-BT.

It is concluded then, that although enzyme activity is present within the sebaceous glands of post-mortem skin, the problems of positive control solutions, and diffusion of enzymes, makes the use of such material for investigative purposes of little use.

9. Summary.

The results presented in this section were designed to expand the histochemical technique previously described by Muir et.al. (1968 a & b).

The emphasis here has been placed on the improvement of the histological and histochemical picture rather than a detailed modification of biochemical environment of these enzymes. Several useful pieces of information have been established. Fixation of the skin before incubation, has been shown to be of little use. Similarly the effect of including P.V.P. in the incubation medium has been shown to be of little value in human skin. The use of post-mortem skin, and its disadvantages have been described. By using post-mortem skin the hypothesis that anaerobic metabolism caused the production of small molecular weight substances which were responsible for the non-specific deposition of diformazan granules in control sections, has been put forward. The effects of storage in the activity of these enzymes has also been investigated and it has been found that enzyme activity will remain for at least 4 weeks if the tissue is stored at $20^{o}C$. One of the most important features of this section has been emphasis on control incubations and on the problems associated with "positive" controls.

One of the major difficulties in dehydrogenase histochemistry is the problem of quantitation. In this study only one method, that of granule counting has been used. This is unsatisfactory, though for the present limited purposes it is sufficient. Histochemical advances with dehydrogenases, would be greatly increased if a rapid and reliable quantitative method was available. It would be preferable if such a method was based on an end-product of the reaction, such as the steroid-formed or the NADH produced, rather than on the reduction at second

hand of a coloured dye.

C. Hydroxysteroids metabolised by human sebaceous glands.

1. Hydroxysteroids utilised histochemically.
2. Relation to known biochemical work.
3. Specificity of hydroxysteroid dehydrogenases.
4. Specificity of localisation of hydroxysteroid dehydrogenases.
5. Summary.

1. Hydroxysteroids used histochemically.

This part of investigation was designed to study those steroids which could be used in the histochemical system. In all, 16 steroids were assessed, both NAD and NADP being used as co-factors. The results are summarised in Table 5. Six separate skin samples were used in the experiment. These results are similar, but not identical to those reported previously (Baillie, Calman & Milne, 1965).

The clearest and best histochemical results were obtained with 3β, 16β-, and 17β-HSD's and this agrees with the previous work. All other enzymes showed either trace, or no activity. It was for this reason that these enzymes were studied in detail in succeeding experiments.

DHA sulphate was included in this investigation in the hope that the sulphatase activity could be demonstrated. Removal of the sulphate group would leave a 3β- hydroxyl group, which would in turn be oxidised by the 3β-HSD, and the activity recorded. Little activity was, however, found but it is likely that the reaction conditions were not ideal. Since the activity of sulphatases in human skin is likely to be low, it is probable that such activity could not be demonstrated by the histochemical method.

Another feature of interest is the lack of activity when NADP is used as co-factor. In previous work (Baillie, Calman & Milne, 1965) in skin and other organs (Baillie, Ferguson & Hart, 1966) NADP dependent hydroxysteroid dehydrogenases have been demonstrated. This may be due to the technique which is now used, that human skin does not

contain such NADP dependent enzymes. On the other hand, it could mean that the technique is not capable of detecting such enzymes since it is known that, with biochemical methods, the 3α, 17β-and 11β-enzymes all can use NADP.

The fact that some of these enzymes, for example the 11β-HSD and the 20β-HSD, can be demonstrated only in very small amounts could mean either, that enzymes are present in small amounts, or that the steroids are being oxidised by the 3β- or 17β- enzymes due to their lack of specificity. This question will be dealt with later.

Several control steroids were included in the series. Androstendione, containing no hydroxyl groups showed no activity histochemically. This is an important control since it indicates that the deposition of diformazan granules depends on the presence of hydroxyl groups, and not just the steroid nucleus. The 3-methyl ether of 5-androsten-3β ol, was included to make sure that the activity with the 16β- hydroxyl compound was not due to removal of the methyl group with oxidation of the 3β- hydroxyl group.

The reduced 5-androstan compounds showed no activity, so that the diformazan deposition which occurred when the 17β- hydroxyandrostan compound was incubated was due solely to the 17β- hydroxyl group.

The relationship of these results to known biochemical work in skin will now be discussed.

Table 5.

Hydroxysteroids utilised histochemically in human skin

	OH group	NAD	NADP
Dehydroepiandrosterone	3β	+	-
Pregnenolone	3β	+	-
5-androsten-3, 17-dione	—	—	—
3β, 16β-dihydroxy-5-androsten-3-methyl ether	16β	+	-
3β-hydroxy-5-androsten-3-methyl ether	—	—	—
Testosterone	17β	+	-
Oestradiol	17β	+	-
11β-hydroxy-4-androsten-3, 17-dione	11β	tr.	-
17α-hydroxy-4 androsten-3-one	17α	tr.	-
3α-hydroxy-5α-androstan-17-one	3α	tr.	-
3α-hydroxy-5β-androstan-17-one	3α	tr.	-
20β-hydroxy-4-pregnene-3-one	20β	tr.	-
Dehydroepiandrosterone Sulphate	$3\beta\text{-}SO_4$	-	-
5α-androstan-3, 17-dione	5αH	-	-
5β-androstan-3, 17-dione	5αH	-	-
17β-hydroxy-5α androstan-3-one	17β	+	-

2. Relation of the histochemical results to known biochemical work.

The mere demonstration of these enzymes is of little significance unless they can be related to known biochemical results, and correlated with the function of the sebaceous glands.

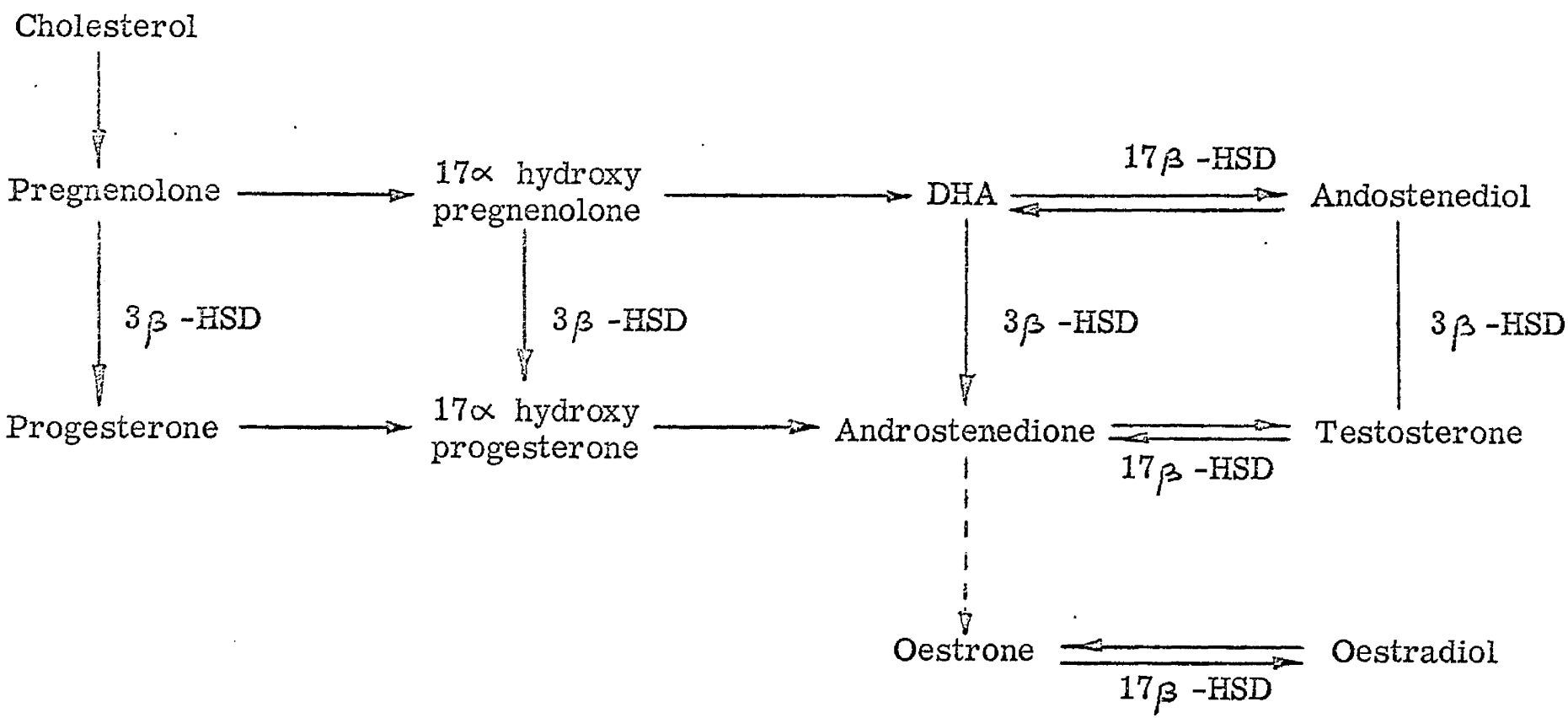
From the results detailed in Table 5, it will be noted that the major enzymes which can be demonstrated histochemically are 3β-, 17β- and 16β- hydroxysteroid dehydrogenases.

The 3β-HSD is of importance in both dehydroepiandrosterone (DHA) and pregnenolone metabolism, and the histochemical results indicate that both these compounds can be utilised. These results are confirmed by the biochemical results of Cameron et.al. (1966), Cook & Lorinz (1963) and Berliner et.al. (1968) with DHA, and other 3β-hydroxysteroids. This indicates that the human sebaceous gland can become involved in the further metabolism of androgens. The precise function of the sebaceous glands in this respect is, however, not known. It may be that the sebaceous glands are definite target organs for androgens, in which case the further metabolism of these compounds may either be related to their excretion, or to their metabolism into more active steroids.

On the other hand, sebaceous glands may simply be involved in the secretion and excretion of androgens, any effect on sebaceous gland growth and development being secondary to this. Histochemical methods do not enable us to examine these two functions in detail though it is likely that they are interrelated.

Fig. 10

PREGNENOLONE & HYDROXYSTEROID DEHYDROGENASES



The demonstration that pregnenolone can also be oxidised within sebaceous glands is of greater significance from the point of view of biosynthesis of steroids. Pregnenolone is at the beginning of the pathway for androgen, progesterone, and oestrogen biosynthesis (Fig. 10). Although this reaction occurs, however, it may simply be due to the presence in sebaceous glands of a 3β-HSD, which will react with any 3β- hydroxysteroid, rather than the demonstration of the start of a metabolic pathway.

The fact that the metabolism of DHA sulphate cannot be investigated by histochemical methods is unfortunate since there is good evidence (Gallegos & Berliner, 1967, Kim & Herrman, 1968, Faredin et.al., 1968) that this substance is utilised by human skin. From work in other tissues notably adrenal (Wallace & Lieberman, 1963, Bostram, Franksson & Wengle, 1964, Killinger & Solomon, 1965) and ovary (Wallace & Silberman, 1964), it is evident that steroid sulphates are of considerable significance in steroid metabolism. There is evidence that these sulphates act as biosynthetic intermediates (Calvin, Vaudeneile & Lieberman, 1963) and they may be the active form in plasma. It is of considerable interest in the pathogenesis of acne vulgaris that plasma DHA sulphate levels increase in plasma at puberty (Yamaki & Ibayashi, 1969). There has been no such investigation, however, in patients suffering from acne vulgaris. Steroid sulphates, and in particular DHA sulphate, deserve much more investigation in human skin.

The presence of 17β-HSD has also been confirmed biochemically in

human skin using testosterone as the substrate or end product of the reaction (Cameron et.al., 1966, Gallegos & Berliner, 1967, Kim & Herrman, 1968, Rongone, 1966). Once again this indicates that androgen metabolism can occur within human sebaceous glands. Testosterone is a much more potent androgen than DHA, and its presence therefore of greater significance. Oestradiol can also be used as a substrate in this histochemical system. In a recent investigation of oestrogen metabolism in human skin, this reaction was also demonstrated to occur biochemically (Weinstein, Frost & Hsia, 1968). The oestradiol 17β-HSD is known in placenta to be associated with a transhydrogenase function (Adams, Jarabac & Talalay, 1962) and this may also be its function in human skin.

Of considerable interest is the high activity of the 16β-HSD in human sebaceous glands. In almost all human organs examined (Baillie, Ferguson & Hart, 1966) this enzyme is particularly active, yet its function has not been adequately explained. 16β- hydroxysteroids are not common and those which have been isolated are oestrogens (Levitz, Rosen & Twombly, 1960, Ryan, 1960, Breuer & Knuppen, 1958, Breuer & Nocke, 1959, Trachewsky & Hobkirk, 1964). These have been isolated as end products of oestrogen metabolism and their exact function is not known. In the present investigation, the 16β- hydroxysteroid was an androstan compound thus its significance is even less apparent. The high activity, present in every section of skin with sebaceous glands present indicates that the enzyme is either of quantitative importance or is a gross histochemical artefact.

The enzyme pathways described are, however, not the only ones possible in human skin. Faredin et.al. (1969) have suggested that the 7 - oxo derivatives of DHA are of importance in the metabolism of DHA in skin. In addition it is likely that the active metabolite of testosterone is the 5α- steroid (Northcutt, Island & Liddele, 1969, Mauvais-Jarvis, Bercovici, Gauther, 1969). Although theoretically the 5α hydrogen could be used histochemically, in practice (Table 5), this was not found to be the case. The utilisation of 5α- testosterone in this system is, therefore, of considerable interest.

3. Specificity of hydroxysteroid dehydrogenases.

In any investigation of enzymic function, particularly when non-purified preparations are used, it is important that the specificity of the enzyme is known. In the histochemical demonstration of hydroxysteroid dehydrogenases, the specificity of the method is open to question from a number of points of view.

In the first place, the reaction is non-specific in the sense that any compound with an oxidisable hydroxyl group will react in this system. It is important therefore, that suitable controls to eliminate non-specific reactions are incorporated and this aspect has already been fully dealt with. The specificity in terms of substrate can therefore be established and this point has been made in the results described in Section 1. It was concluded that the reaction depended on the presence of a hydroxylated steroid.

The second question is the specificity of the reaction in terms of the actual steroid utilised. With histochemical techniques, apart from inference and indirect methods, it is impossible to state whether there are specific 17β-HSD's or 3β-HSD's or whether all the reactions are catalysed by a single enzyme which non-specifically oxidised all hydroxysteroids. Only by using biochemical separation techniques can such a statement be made. Talalay (1965) presents evidence that these HSD's are in fact distinct, and other biochemical evidence has shown that there are specific 11β- and 20β-HSD's. It is by direct transfer from these biochemical results that specific enzymes are assumed to be present.

From the histochemical results so far described, two features are important in relation to the specificity of the reaction. The first concerns the differences in localisation when different substrates are used. In human skin there is a difference in localisation between the 17β- and 3β-HSD's within sebaceous glands and this will be dealt with in detail later. This fact, therefore suggests that the two enzymes are distinct.

The second feature is related to the quantitative difference which occurs when different steroids are used as substrates. Thus, as has already been described in a previous section, poor activity was found with 20β- and 11β- steroids in contrast to the high activity with 3β- and 17β- steroids. This could of course be due to differences in specificity of a single enzyme, but again this cannot be investigated by histochemical methods.

The third question concerns the specificity of each individual enzyme. For example, does the 3β-HSD, in addition to oxidising 3β- hydroxysteroids, also oxidise 17β- hydroxysteroids?; does the specificity of the 17β-HSD extend to both testosterone and oestradiol or are separate enzymes involved? Again histochemical techniques cannot help in answering this question, but the problem is ripe for further biochemical investigations.

4. Specificity of localisation of hydroxysteroid dehydrogenases.

Although histochemical techniques have disadvantages, their main advantage is that the localisation of the enzyme or substrate being investigated can be established. Other problems such as quantitation of the reaction of limitation of enzymes which can be demonstrated become insignificant if the localisation of the enzyme can be assured. The following experimental results are presented in favour of specificity of localisation of these enzymes in the sebaceous glands in human skin.

(a) The ubiquitous presence of NADH diaphorase, the second enzyme in the histochemical reaction. Since this enzyme is present in all tissues and cells in human skin, any specificity of localisation cannot be explained simply on differences in NADH diaphorase localisation.

(b) The absence of any demonstrable histochemical reaction when no sebaceous glands are present in human skin. In no skin section in which sebaceous glands were not present did deposition of diformazan

granules occur. Thus the specific localisation of HSD's within sebaceous glands is not simply related to lipid solubility of the diformazan end product since without sebaceous glands the presence of these enzymes in other tissues and cells would be more easily visualised and this has not been the case.

(c) The specificity of localisation with different steroids. Although this question will be dealt with later, it is important to note that the 3β-HSD and the 17β-HSD have different distributions even within sebaceous glands. It is unlikely then that the localisation is non-specific, at least to a significant extent.

(d) Changes in localisation due to solubility of the enzymes. If the enzyme is soluble in the sense that it will diffuse into the incubation medium then the localisation may be non-specific because the diformazan formed will be deposited at random over the tissue section. From the histochemical point of view, two pieces of evidence can be put forward which negate this suggestion in human skin when the enzymes used are the HSD's.

(i) The use of P.V.P. in the prevention of diffusion of these enzymes, as has been reported (Section 1), is of little use in human skin to improve the demonstration of the hydroxysteroid dehydrogenases. There is no noticeable effect, suggesting that these enzymes are not particularly "soluble". Thus in the first place diffusion may not occur to a great extent with this system.

(ii) With glucose-6-phosphate dehydrogenase in liver, Kalina,

Table 4.

HYDROXYSTEROID DEHYDROGENASE ACTIVITY RELATED TO DIFFUSION

		CONTROL	TESTOST.	OEST.	16β	DHA	NADH
1.	untreated sections	-	+	+	+	+	++
2.	formalin washed sections	-	-	-	-	-	++
3.	(a) untreated	-	+	+	+	+	++
	(b) formalin washed	-	-	-	-	-	++

In experiment 3 the sections were incubated side by side.

Gahan & Jones (1965) detailed an experiment, by which the diffusion of enzymes could be investigated. This experiment has been repeated in human skin with hydroxysteroid dehydrogenases and is described below.

A number of sections of human skin were incubated with formalin until the activity of the hydroxysteroid dehydrogenases was destroyed, but the activity of NADH diaphorase retained. Two control series of incubations were set up. The first contained the normal steroid incubation medium (with DHA, testosterone or oestradiol) and sections which had not been washed in formalin were used (untreated sections). The second series was made up of sections washed in formalin and incubated with the same media (formalin washed series). The third group was a series of washed and unwashed sections, incubated side by side in the various incubation media. By this technique, any diffusion of the enzymes from the unwashed section into the incubation medium would cause the reaction to occur in the incubation medium with deposition of the diformazan on the washed sections. The results are shown in Table 4.

The results of this experiment are different from that of Kalina et.al. (1965) with liver. In their experiment, there was diffusion of the enzyme from the unwashed to the washed sections with consequent non-specific localisation of the diformazan granules. From this experiment they concluded that the localisation of dehydrogenases was suspect and therefore of little value. They emphasised that with all experiments with dehydrogenases similar studies should be performed before statements about localisation were made.

In the experiment described here, the opposite results were obtained with hydroxysteroid dehydrogenases. In the first control series, untreated sections, activity of all of the HSD's was demonstrated. When formalin washing was used no activity of these enzymes was noted. In the third experiment there was no transfer of enzyme activity from the unwashed to the washed section. It will be noted that the washed sections still retained high activity for NADH diaphorase so this was not the cause of the failure to demonstrate transfer of activity. At a much cruder level there was no evidence, throughout the work presented in this thesis, that diformazan granules were formed in the incubation medium.

From this experiment then, and from the inferences and results previously mentioned it seems very likely that the specific localisation of these enzymes within the sebaceous glands in human skin is a correct interpretation of the results. The validity of the technique is therefore, established, although the presence of trace quantities of activity in other tissues cannot be excluded by these results.

5. Summary.

In this section the hydroxysteroids which can be metabolised by human sebaceous glands have been studied. The most active enzymes are the 3β-, 17β-, and 16β-HSD's using dehydroepiandrosterone, pregnenolone, testosterone and oestradiol as substrates. It was found that these enzymes were NAD dependent and localised in the

sebaceous glands of human skin.

The specificity of these enzymes for steroid substrates was then discussed. Most of the evidence that specific enzymes are present is based on their biochemical isolation and characterisation. Differences in localisation is the only histochemical finding which is consistent with specificity of enzyme activity.

Specificity and accuracy of localisation is the most important feature in any histochemical reaction. Evidence based on

(a) the distribution of NADH diaphorase,

(b) absence of any demonstrable histochemical reaction when no sebaceous glands are present,

(c) specificity of localisation of different steroids and

(d) relationship between localisation and solubility of the enzyme

was presented to support the view that the localisation of these enzymes within sebaceous glands was correct. This last piece of evidence is of the greatest significance, and established the validity of the technique. Hydroxysteroid dehydrogenases are found only in the sebaceous glands in human skin and in no other skin appendages.

The relationship between the histochemically demonstrable enzymes and the known biochemical pathways was shown to correspond well. It was suggested that the sebaceous glands could act either as target organs for steroids, in particular androgens, or may be concerned in their further metabolism and excretion. The function of these enzymes in relation to the known biochemical findings was discussed, and the

enigmatic role of the 16β-HSD mentioned. Alternative pathways of steroid metabolism in skin were outlined.

D. Distribution of hydroxysteroid dehydrogenases in prepubertal and adult human skin.

1. General aspects of survey.
2. Age distribution of enzymes.
3. Sex distribution of enzymes.
4. Localisation of enzyme activity.
5. Summary.

D. Distribution of hydroxysteroid dehydrogenases in prepubertal and adult human skin.

1. General aspects of survey.

The following results are based on a consecutive series of 214 human skin biopsies. 19 of these biopsies were not used for technical reasons; these included inadequate data on the specimen, too small a biopsy, loss of material during processing and poor technical results. These technical failures were from no particular site or age group and were randomly distributed in the series.

The feature of note is the number of skin biopsies which did not contain sebaceous glands. In most instances, if a sebaceous gland could not readily be found, serial sections from different levels of the block were taken. In this series of 214 specimens 61, or 31%, contained no detectable sebaceous glands. This is one of the most significant findings of the survey and requires further comment. In Table 6 the percentage of skin biopsies shown to have sebaceous glands from each of the areas examined is recorded. With face and scalp only 2 skins out of 36 contained no sebaceous glands in contrast to the anterior chest and leg where 55% of skin biopsies contained no glands. There is also a difference between the anterior and posterior aspects of chest, though this difference is probably not statistically significant. One other feature of this table is the high proportion of sebaceous glands present in perianal skin. These samples were taken from the mucocutaneous margin of the anus, or from the surrounding

Table 6.

DISTRIBUTION OF SEBACEOUS GLANDS IN SKIN BIOPSIES

TOTAL NUMBER OF SAMPLES - 214

	Present	Not Present	% of Skins with Seb. Glands
Face & Neck	21	1	95
Scalp	15	1	94
Anterior Chest	16	10	62
Posterior Chest	14	6	70
Upper Abdomen	16	13	55
Lower Abdomen	18	6	76
Leg	16	14	53
Arm	4	2	66
Perianal Region	14	5	75
Other	3	3	0
TOTAL	134	61	69%

19 skins not used for technical reasons.

Table 7.

MALE AND FEMALE DISTRIBUTION OF BIOPSY SPECIMENS AND AGE GROUPING

Total of 134 specimens (72 males, 62 females)

		Male	Female
Age	0-10 years	6	1
	11-20 years	5	4
	21-30 years	13	10
	31-40 years	12	17
	41-50 years	13	10
	51-60 years	10	11
	60 + years	13	9

skin.

In Table 6 the skin surface has been divided into regions and in all areas, except for the arm, at least 14 separate skin samples containing sebaceous glands have been analysed. Under the term "other", skins from various regions not included in the table have been detailed. These were from axilla, scrotum and prepuce.

As well as there being a marked difference in the distribution of sebaceous glands throughout the body, there was also a noticeable difference in the numbers of glands and in their morphology. In the face, neck and scalp the glands are large and have many acini. Even within a single section of skin numerous glands can be seen. In contrast to this, the sebaceous glands of the arm, leg and abdomen are small, have fewer acini, and there is usually only one visible per section. In samples of skin from prepubertal patients the sebaceous glands are also small, the acini are not well developed and they are not easily visualised. These observations are of course not original. They simply confirm previous quantitative findings (Montagna, 1963). They are however, of considerable importance in the interpretation and significance of the histochemical findings. The importance of this observation to the histochemist, that there are more sebaceous glands in certain areas of the skin than others, becomes clear if a specific example is taken. If the enzyme to be studied is present only in small amounts and not in every sebaceous gland, then the chances of recording activity of the enzyme in any one skin section will depend on the number of glands present. Thus the activity

Table 8.

AGE DISTRIBUTION OF ENZYMES

	3β-HSD	16β-HSD	17β-HSD (TEST)	17β-HSD (OEST)	Total No. of skins
0-10 years	6	7	2	2	7
11-20 years	9	9	4	4	9
21-30 years	23	23	19	19	23
31-40 years	25	29	16	16	29
41-50 years	22	23	13	13	23
51-60 years	21	21	12	13	21
61 + years	18	21	16	15	22

recorded may well be artificially low in areas where few sebaceous glands are found, the activity recorded being simply a function of the total number of sebaceous glands in the area, rather than measuring any fundamental difference in the metabolic pattern of the sebaceous glands in the different areas.

The age and sex distribution of the biopsy specimens is shown in Table 7. In general males and females are fairly evenly represented except in the younger age group. The youngest specimen was from a male aged 1 year and the oldest from a 83 year old male.

2. Age distribution of enzymes.

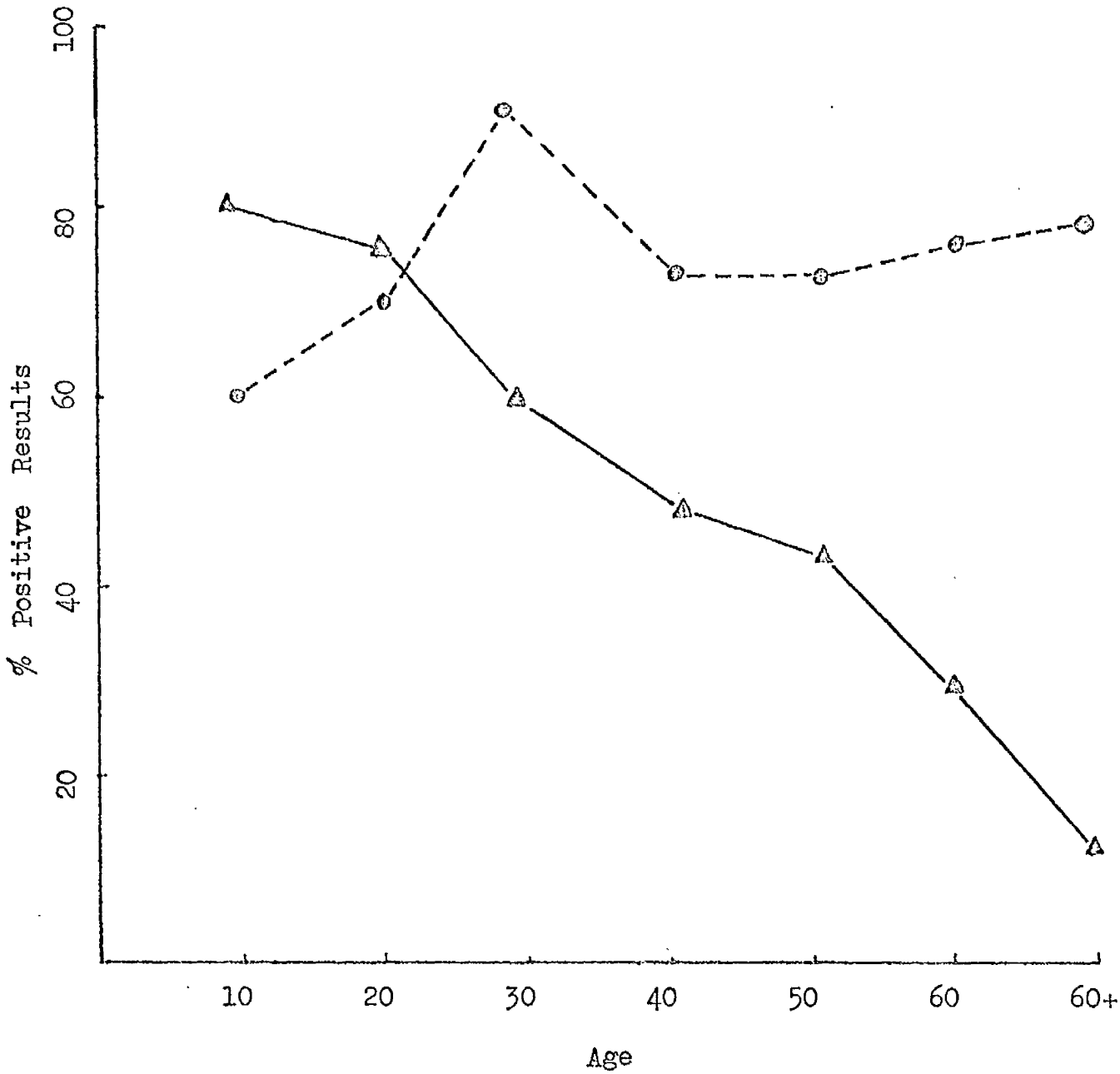
The age distribution in the 134 skins of the four enzymes is shown in Table 8. From this table several features are apparent. The first is that these enzymes are present in all age groups. The oldest skin used, that of an 83 year old man from the left iliac fossa, contained activity for all four enzymes. The differences in activity between age groups, is in general not significant for the 3β and 16β HSD's. These enzymes are present in almost all skins examined. With 17β HSD however there is a difference between the various age groups, though because of small numbers this difference has not been analysed statistically. In general before puberty the 17β HSD is not present in the majority of skins. However in the age group 20-30 the enzyme is present in almost all samples examined. After this age group the enzyme activity falls off again, though there is a small increase over the age of 60. Baillie, Thomson and Milne

Table 9.

AGE DISTRIBUTION OF ENZYME ACTIVITY

	Actual/Theoretical	%
0-10 years	17/28	61
11-20 years	26/36	72
21-30 years	84/92	91
31-40 years	86/116	74
41-50 years	71/92	77
51-60 years	67/84	79
60 + years	70/88	80

The actual number of positive results is expressed as a percentage of the theoretical maximum.



Present Survey.

Baillie, Thomson, Milne, 1966.

Figure 11. Relation Between Percentage Positive Results and Age.

(1966) found that there was a decrease with age of the number of enzymes present within the sebaceous glands. While the present study does not contradict this, it is by no means as clear cut as was expressed in that paper. In their paper, Baillie, Thomson and Milne expressed the activity of each age group as a percentage of the total number of enzymes which could theoretically be demonstrated. They used 5 enzymes in this way, the 3α, 3β, 16β, 11β and 17β HSD's. If a similar manoeuvre is performed with the results in Table 8; that is to express the total number of positive results in each age group as a percentage of the theoretical maximum then the graph shown in Figure 11 is obtained (Table 9). This is compared with that of Baillie, Thomson and Milne (1966). It is obvious that there is a difference in the number and type of enzymes involved in the two graphs, and the differences between the two are striking. It is recognised that this method is not a conventional or particularly good method of expressing results. It is used in this context simply by way of comparison. From the results obtained in this study then, it is concluded that the level of activity of the 17β-HSD increases at the age of 20, and in the decades following this the activity falls off slightly. It is interesting to note that the sebum secretion rate when related to age, mirrors very closely that of the 17β-HSD activity. Cuncliffe and Shuster (1969) have measured the sebum secretion rate in subjects, both with and without previous evidence of acne vulgaris. They found that with patients with acne the highest secretion rate was in the 20-30 age group, the rate falling off after this, but remaining steady at about 50% of the

Table 10.

SEX DISTRIBUTION OF SKIN BIOPSIES

	Male	Female
Face	14	7
Scalp	7	8
Ant. Chest	8	8
Post. Chest	7	7
Upper Abd.	10	6
Lower Abd.	9	9
Leg	6	10
Arm	3	1
Perianal	8	6

Table 11.

SEX DISTRIBUTION OF ENZYMES

	3β-HSD	16β-HSD	17β-HSD (Test)	17β-HSD (Oest)	Total
Male	65	72	57	49	72
Female	57	61	28	28	62

maximum. This pattern is clearly similar to that described here, though no attempt has been made to separate subjects with or without previous acne vulgaris.

3. Sex distribution of enzymes.

The sex distribution of the biopsy specimens is shown in Table 10. The biopsy specimens are comparable, except for the number of biopsy specimens from the face and neck, in which there were more male than female samples.

When the sex distribution of these enzymes is compared, a most interesting feature is noted (Table 11). With the 3β HSD and the 16β HSD, there is no statistical difference between males and females in the occurrence of these enzymes. Both are present to an equal extent and occur in almost all skin samples. With 17β HSD however, both with testosterone and oestradiol as substrates there is a statistically significant difference in the occurrence of these enzymes between males and females. They occur much more frequently in males.

The reason for this quite distinct difference is not exactly clear. Several hypothesis however might be put forward. Firstly, as will be apparent from later results, the occurrence of 17β HSD in sebaceous glands follows a definite pattern. The enzyme is located around the periphery of the gland in small patches. Very often whole glands contain no traces of enzyme activity. The difference between males and females could then be due simply to there being more sebaceous

glands in human male skin. This hypothesis however could not be tested, since no quantitative assessment of the number of sebaceous glands was made.

The second reason for the increase in male skin which could be put forward is that the higher endogenous androgen secretion known to be present in the male, may in some way prime the sebaceous glands. This higher androgen level may cause an increase in the enzyme levels. The fact that there is also an increase in 17β HSD which reacts with oestradiol can be explained simply by the lack of specificity of the 17β-HSD.

The third reason of course is that there is an absolute increase in the amount of the enzyme present in male skin. If this is so then it would be important to find out if this occurs at any particular age group, or if it occurs in all age groups. From the small numbers available no statistical data can be obtained, but it is not obviously apparent that any one age group either contains, or does not contain the 17β HSD in either sex.

If then, this is an absolute increase in the number of samples that the enzyme can be demonstrated in males it could be that this is an expression of the ability of the sebaceous gland to metabolise androgens, and act as an end organ of steroid transformation and excretion.

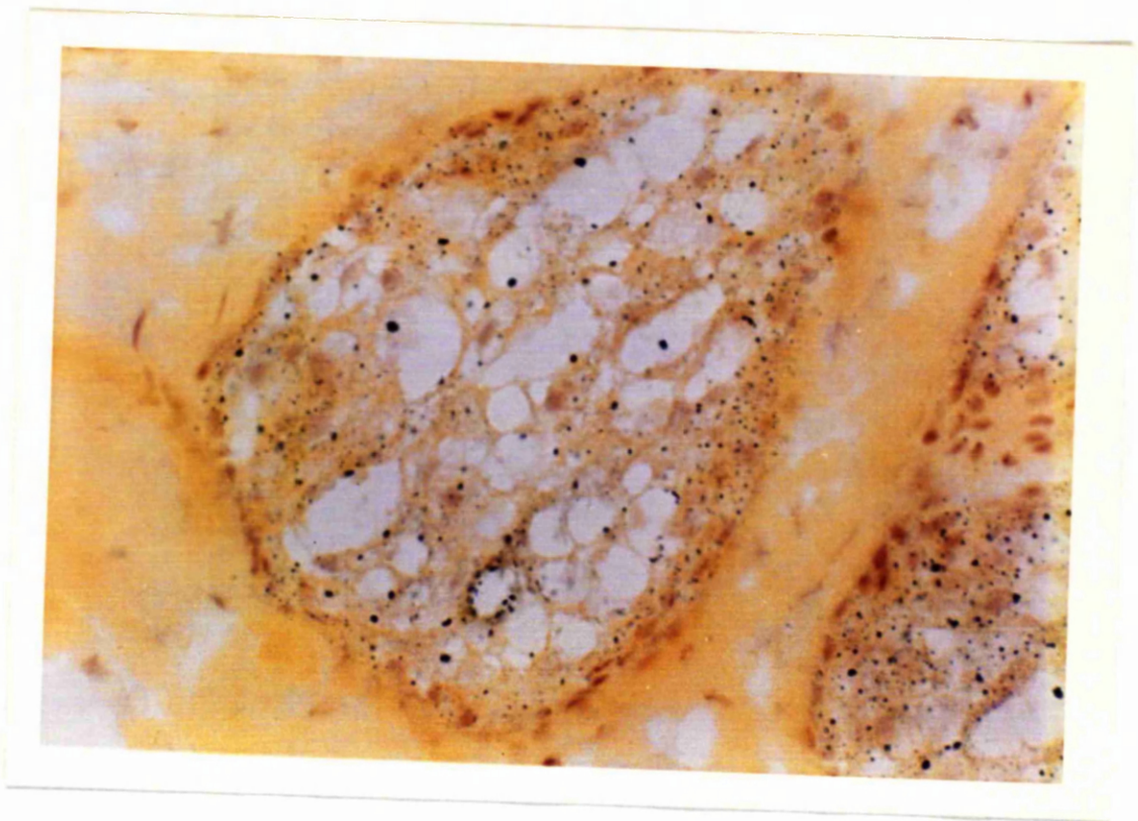


Figure 12. Distribution of Diformazan Granules in Sebaceous Glands. Dehydroepiandrosterone as Substrate. (×420)

4. Localisation of enzyme activity.

Before any statements about the localisation of these enzymes can be made, it is important that the specificity of localisation is established. This has been dealt with in a previous section. In this section the localisation will be reviewed under three headings; localisation within skin; localisation within sebaceous glands; localisation over the skin surface.

a. Localisation within skin.

From reviewing well over 200 skin specimens from all sites and ages, it becomes very clear, that the activity of these enzymes is only in the sebaceous glands when histochemical methods are used. In no instance were diformazan granules deposited in the epidermis, dermis, sweat glands or other skin appendages. The granules are clearly within the glands, (Fig. 12) and unless there has been "smearing" in cutting the section, the diformazan is entirely within the cells of the gland. With 17β-HSD, as will be described later, there is some activity in the cells of the secretory ducts.

It is perhaps surprising that these enzymes are located so precisely to the sebaceous glands. Cholesterol metabolism certainly occurs in the epidermis. With androgen metabolism most biochemical studies have been with whole skin; no definite localisation has been established. This observation then is of importance in establishing the sebaceous gland as an end organ of steroid metabolism.

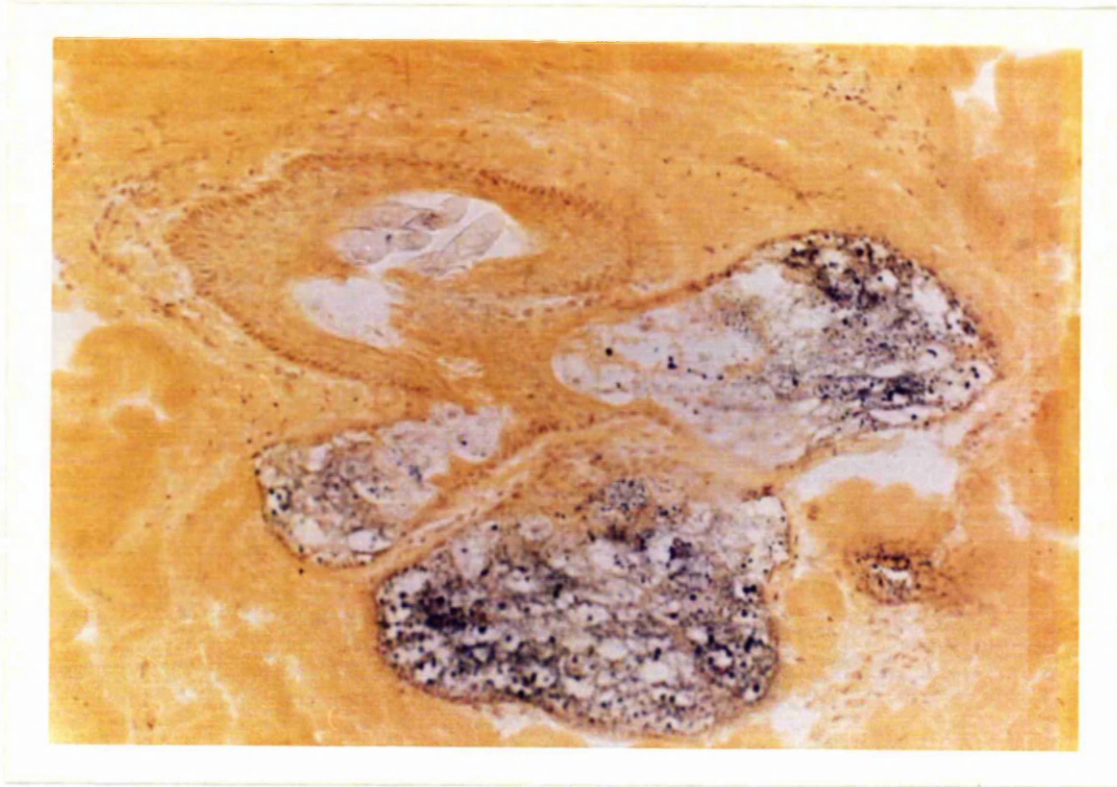


Figure 13. Distribution of Diformazan Granules in Sebaceous Glands. Dehydroepiandrosterone as Substrate. Granules Deposited Throughout the Glands. (×175)

Two factors, however, must be mentioned before definite conclusions can be drawn. The first concerns the validity of the localisation by this method. This has been previously dealt with and the technique itself is reliable. The second factor relates to a special property of the sebaceous gland itself, namely its high lipid content. With many of the tetrazolium salts, deposition of the diformazan occurs selectively in lipid. With nitro-BT, this is not nearly so much of a problem (Pearse, 1960). Further, in sections without sebaceous glands; no deposition of diformazan occurs in other areas of skin.

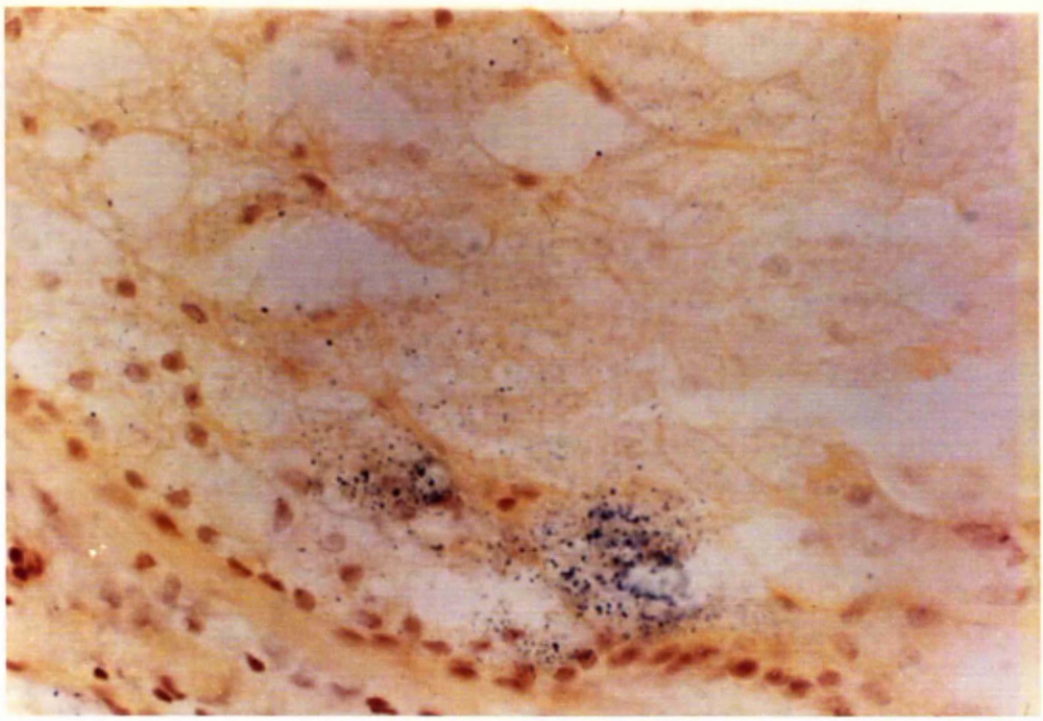
It is also possible that the histochemical technique is just not sensitive enough to pick up activity in other skin structures.

b. Localisation within sebaceous glands.

This aspect of localisation is in many ways the most interesting and informative. With the 4 steroid substrates which have been looked at most closely, two distinct patterns have emerged.

With DHA and the 16β-substrate, the diformazan granules are distributed fairly evenly throughout the acinus (Fig. 13). There are usually a few more granules round the areas containing most muclei; conversely there are fewer granules in the areas containing less nuclei and more lipid. With these enzymes there is no deposition in the secretory ducts.

With the 17β-substrates, testosterone and oestradiol, the picture is different. The diformazan is deposited in small areas around the



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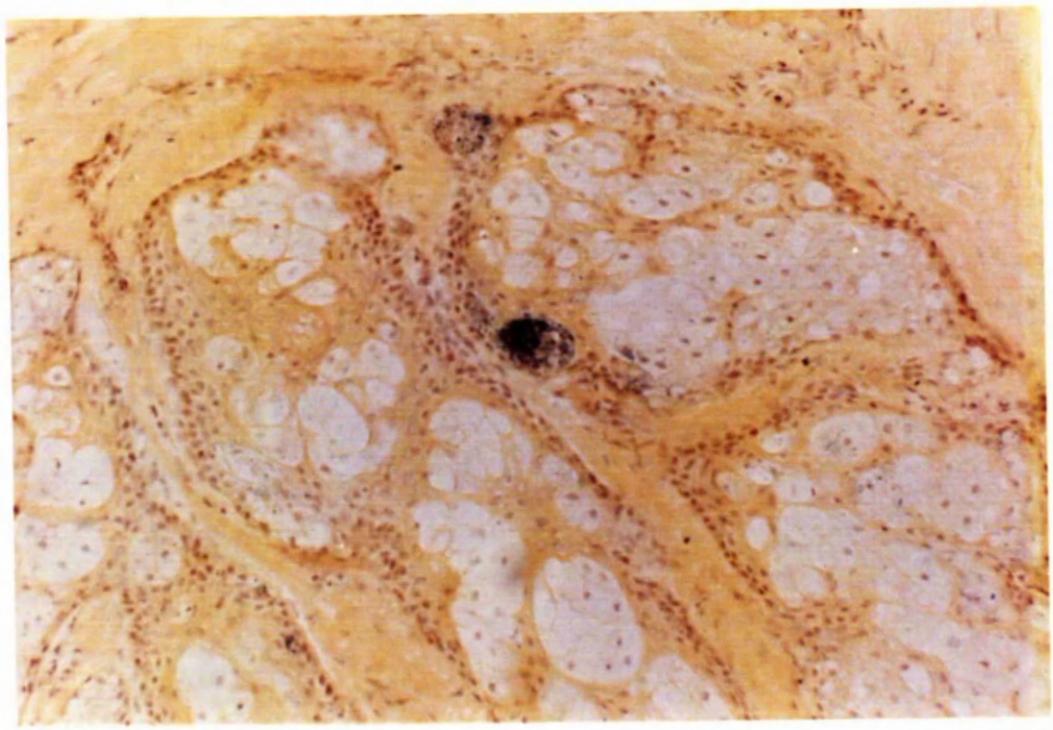

(X 420)

Figures 14, 15. Distribution of 17β-Hydroxysteroid Dehydrogenase Activity.

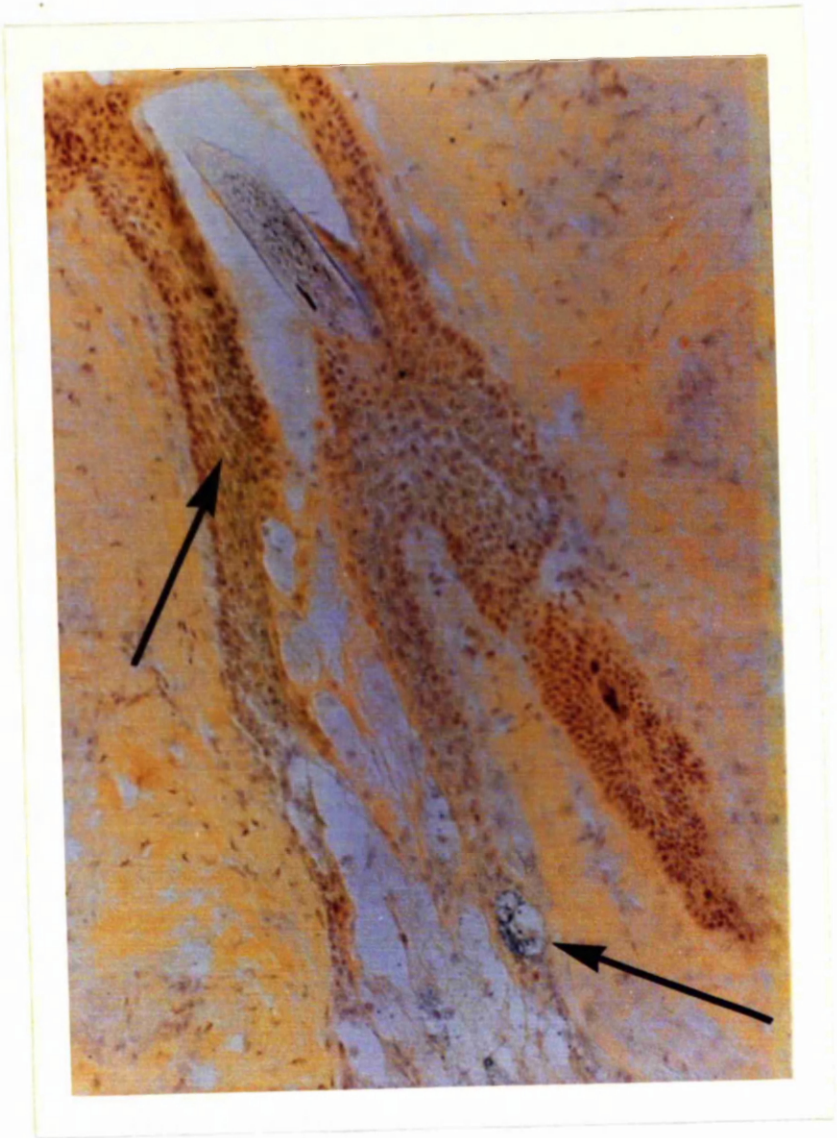

Figure 16. 17β-Hydroxysteroid Dehydrogenase Activity. Diformazan Granules in Secretory Ducts of Sebaceous Glands, with a Localised Area of Activity in the Gland Itself. (×175)

sebaceous glands (Figs. 14, 15). Very few, if any granules, are found throughout the glands. The other interesting feature is that the diformazan granules are also located in the lower parts of the secretory ducts (Fig. 16), and this occurs only with the 17β-HSD. The significance of this observation is not entirely clear; but indicates that the metabolism of 17β-HSD is different from the 3β-HSD. It is known from the work of Ebling (Ebling, 1963) that testosterone stimulates mitosis in the sebaceous glands of the rat, and it could be that this observation in humans indicates that testosterone acts on a few cells around the periphery of the gland. This observation must be followed up by further studies, which should include mitotic counts on the sebaceous cells to correlate enzyme activity with cell division.

c. Localisation over the skin surface.

In Table 12 the results of the survey in relation to enzyme activity over the skin surface are shown. The first feature of note is that these enzymes are present in all skin areas of the human body. This is in contrast to the original paper by Baillie, Calman & Milne (1965) in which enzyme activity was thought to be confined to areas known to be prone to acne vulgaris.

Of course there appear to be some regional variations, notably that the skin of the face and scalp regularly contain activity for all these enzymes. With the anterior chest region, on the other hand, there are fewer skins containing all four enzymes. Of particular note are the positive results in scalp and leg, which were previously reported to

Table 12.

ENZYMES PRESENT WITHIN SEBACEOUS GLANDS

	3β-HSD	16β-HSD	17β-HSD (TEST)	17β-HSD (OEST)	Total No. of skins
Face & Neck	21	20	17	16	21
Scalp	15	15	11	10	15
Anterior Chest	13	16	5	4	17
Posterior Chest	10	13	5	5	13
Upper Abdomen	15	16	9	7	16
Lower Abdomen	15	18	7	9	18
Leg	15	15	9	10	16
Arm	4	4	3	1	4
Perianal	13	14	13	13	14

The figures above record the number of skin biopsies in which positive results with each enzyme were obtained.

show no activity.

The interesting results with perianal skin must be mentioned more fully. The perineum is an area rich in sebaceous glands, and since skin can readily be obtained from this region, without mutilating results it is a useful source of human skin.

Once again it is likely that some of the negative results with the 17β-HSD are because of low enzyme activity occurring in areas of skin with few sebaceous glands, the enzyme activity simply being missed.

It is interesting to speculate on the reasons for the difference in the results of the present study with the previously reported work. The most obvious difference is in the use of counterstaining methods which have made the visual assessment of these results much easier to make. Further the histochemical technique itself is more refined than in the previous investigation. Finally the experience gained over the period of this investigation in the histology of human skin has greatly helped in the interpretation of the results.

5. Summary.

In this section a survey of 214 skin biopsies from all areas of human skin were studied for their content of hydroxysteroid dehydrogenases. Thirty-one per cent of the skin biopsies contained no sebaceous glands and this result is discussed in the light of the known distribution of sebaceous glands. The age and sex distribution of the biopsies show that the samples were representative of all ages

and were comparable in relation to sex.

The age distribution of the enzymes described show that these enzymes are present in all age groups, though the peak of activity is at 20-30 years.

The enzymes are present in both sexes though there appears to be a greater activity in the male biopsy specimens of the 17β-HSD.

Localisation of the enzymes was described in three ways. Firstly by the histochemical technique the enzymes are found only in the sebaceous glands of human skin, and in no other skin appendages. Secondly, within sebaceous glands there is a difference in distribution of the enzymes. The 3β-HSD and 16β-HSD occur throughout the gland whereas with 17β-HSD the activity is located in small areas around the periphery of the gland, and in the secretory ducts. Thirdly, the localisation of these enzymes over the skin surface has been described. These enzymes occurred in all areas of human skin, in contrast to previous investigations in which the activity of the enzymes was confined to specific areas.

E. Hydroxysteroid dehydrogenases in human fetal skin.

1. Clinical details.
2. Morphology of fetal skin.
3. Demonstration of HSD's in fetal skin.
4. Relation to known metabolic functions of fetal skin.
5. Summary.

E. Hydroxysteroid dehydrogenases in human fetal skin.

This part of the investigation was carried out in conjunction with Dr. Frank Sharp of the Department of Obstetrics, The University, and a report of this is to be published (Sharp et.al., 1970).

1. Clinical Details.

In all twenty-five fetuses were examined, with maturities ranging from 8 weeks to 32 weeks. The clinical details are summarised in Table 13. In twenty of these cases, there was minimal time lag between death and collection of skin specimens. Fresh abortion material was the main source of material. In case 24, neonatal death occurred $1\frac{1}{2}$ hours after delivery from extreme prematurity. In case 25, intrauterine death was known to have occurred 6 hours before delivery.

In any study of enzyme activity in relation to fetal maturity, it is of great importance that the gestational age is determined as accurately as possible. In most cases in this series the maturity was confirmed by ultrasonography. By this technique, the biparietal diameter of the fetal skull is measured and this can be accurately correlated to fetal maturity (Donald, 1969, Campbell, 1969). The maturity as measured by the date from the last menstrual period was also known.

The time lag between death and collection of fetal material is also important to establish accurately. In this series, apart from case 22, the fetus was known to be viable until just before delivery,

Table 13.

CLINICAL DETAILS OF FETUSES EXAMINED

FUS NO.	GESTATION in WEEKS	ULTRASONIC CONFIRMATION	VIABILITY	DIFFERENTIATED SEBACEOUS GLANDS
1	8	YES	YES	NONE PRESENT
2	9	YES	YES	"
3	10	YES	YES	"
4	12	YES	YES	"
5	15	NO	YES	"
6	16	YES	YES	"
7	16	YES	YES	PRESENT IN SCALP ONLY
8	18	YES	YES	"
9	18	NO	YES	"
10	18	NO	YES	NONE PRESENT
11	22	YES	YES	PRESENT ALL SITES
12	22	NO	YES	"
13	22	NO	YES	"
14	22	YES	YES	"
15	23	YES	YES	"
16	23	YES	YES	"
17	24	YES	YES	"
18	24	NO	YES	"
19	24	NO	YES	"
20	25	YES	YES	"
21	25	YES	YES	"
22	25	NO	YES	"
23	28	NO	YES	"
24	31	NO	NEONATAL DEATH	"
25	32	YES	DIED 6 HRS BEFORE DELIVERY	"

by Doptone recording of the fetal heart beat. Case 25 deserves special mention since death occurred 6 hours before delivery. Because of this the enzymes would be left at 37^{o} in an anaerobic environment for this time. It is known, however, (Section 1) that adult human skin will retain enzyme activity for up to 48 hours post mortem, and it was on the strength of this that this skin was included in the series.

It proved difficult to obtain material from fetuses of 32 weeks gestation and over. The reasons for this are many. In the first place most fetuses of this gestation are viable and do not succumb so readily in the neonatal period. From the administrative point of view, it is also more difficult to obtain skin from such sources since the baby is removed to a special neonatal unit. Secondly, the number of fetuses obtainable in this period is less than in the earlier months. Thirdly, some of the material obtainable in this third trimester is abnormal and cannot strictly be included in this series.

Because of this, the transition from fetal to prepubertal skin cannot be fully worked out. Since many changes occur close to the period of parturition, notably in the adrenal, it is not unreasonable to assume that major changes could also occur in this period in the steroid metabolism in skin. Unfortunately, this cannot be investigated until more material is available, and this is being actively studied.

2. Morphology of human fetal skin.

The morphological changes in the differentiation of sebaceous glands in human fetal skin, is in accord with that previously described (Serri & Huber, 1963). Small sebaceous buds were noted at 9 weeks, but fully differentiated sebaceous glands with acini were not clearly seen until 18 weeks of gestation. This is rather later than usual and could be due to poor histological orientation of the very small portions of skin. Since at least 50 sections were taken from each site however it is unlikely, that this was the only cause of the failure to recognise the glands. When fully differentiated, the cells of the sebaceous acini are fully of lipid-laden cells and as in the adult, the gland is connected to the piliary canal by a short excretory duct.

As in the adult, the number of sebaceous glands in scalp and face is greater than in other regions. It is noteworthy, however, that in no fetus over 18 weeks of gestation could sebaceous glands not be demonstrated in any of the sites examined. This is, of course, in contrast to the adult situation in which up to 30% of skin biopsies, randomly taken, will contain no demonstrable sebaceous glands. It raises the question as to whether the absolute number of sebaceous glands in any individual does not change over his life time, the distribution merely being modified by the change in surface are of the skin.

Table 14.

HSD ACTIVITY IN HUMAN FETAL SKIN

FETUS NO.	GESTATION in WEEKS	SEX	ENZYME ACTIVITY				NADH
			STEROID SUBSTRATES				
			TEST.	OEST.	16	DHA	
7	16	M	++	++	-	-	++
8	18	F	++	++	-	-	++
9	18	M	+	+	-	-	++
10	18	M	(0)	(0)	(0)	(0)	(0)
11	22	F	++	++	-	-	++
12	22	M	+(a)	++	-	-	++
13	22	F	++	++	tr(b)	-	++
14	22	M	++	++	-	-	++
15	23	M	++	++	+(c)	-	++
16	23	M	++	++	+	-	++
17	24	F	++	++	+	-	++
18	24	F	+	++	+(b)	+	++
19	24	M	++	++	+	+	++
20	25	F	++	++	+	-	++
21	25	M	++	++	+(b)	-	++
22	25	M	++	++	+	-	++
23	28	F	++	++	+	tr	++
24	31	F	+	+	+	+	++
25	32	F	+	+	+	tr	++

(a) Only in forearm.
(b) Scalp and anterior chest only.
(c) Scalp only.
(0) No differentiated sebaceous glands present any site.

Abbreviations: TEST = testosterone
OEST = oestradiol - 17
16 = 5-androsten-3-16-diol-3 methyl ether
DHA = dehydroepiandrosterone

tr = trace positive + = positive ++ = strongly positive - = negative

bracketed pairs are twin fetuses.

3. Demonstration of HSD's in human fetal skin.

In this series, activity of 17β-HSD, 3β-HSD and 16β-HSD was investigated. In all cases NADH diaphorase activity was assessed. The results are summarised in Table 14 and were assessed separately by two observers. The main features of note are -

(a) NADH diaphorase activity was found to be present in all skin biopsy specimens (Fig. 17). As with adult skin, activity was found in all cellular elements including epidermis, fibroblasts, and sweat glands. Activity was high in all cases.

(b) Hydroxysteroid dehydrogenase activity was found to be present only in sebaceous glands, and in the secretory ducts (Fig. 18). No activity was noted in any other cutaneous appendage or epidermis. This accords well with the observations previously recorded with adult skin. Before the development of differentiated sebaceous glands, no activity for any of these enzymes was noted, that is up to 18 weeks gestation. After this, if activity was present, then it could be recorded in all sites examined. It is for this reason that the results for each fetus are given in summary form. Exceptions to this generalisation are detailed in the table.

(c) The activity of 17β-HSD, both with testosterone and oestradiol, was high between eighteen and twenty weeks, and was in fact the first and only HSD to be demonstrated up to 22 weeks of gestation. After 28 weeks the activity tended to be less, but not sufficient skin specimens are available to make this certain. There was no difference

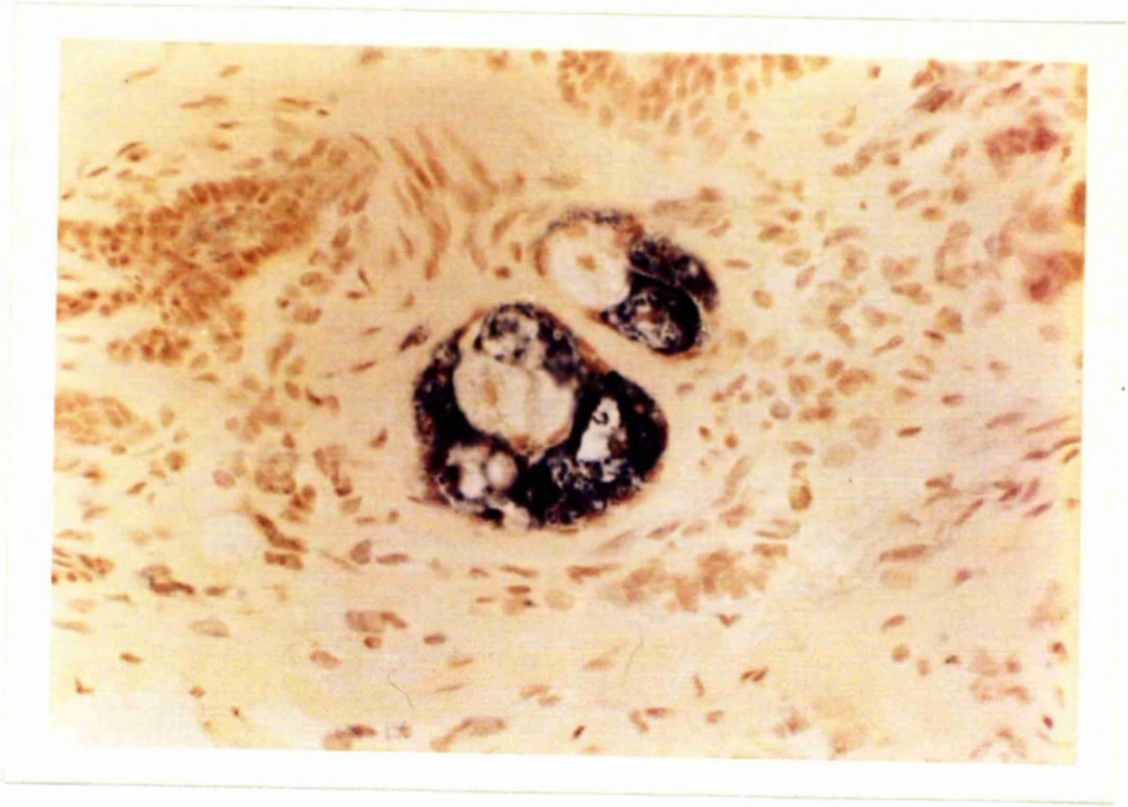

Figure 17. NADH Diaphorase Activity in Human Fetal Skin. (x 175)

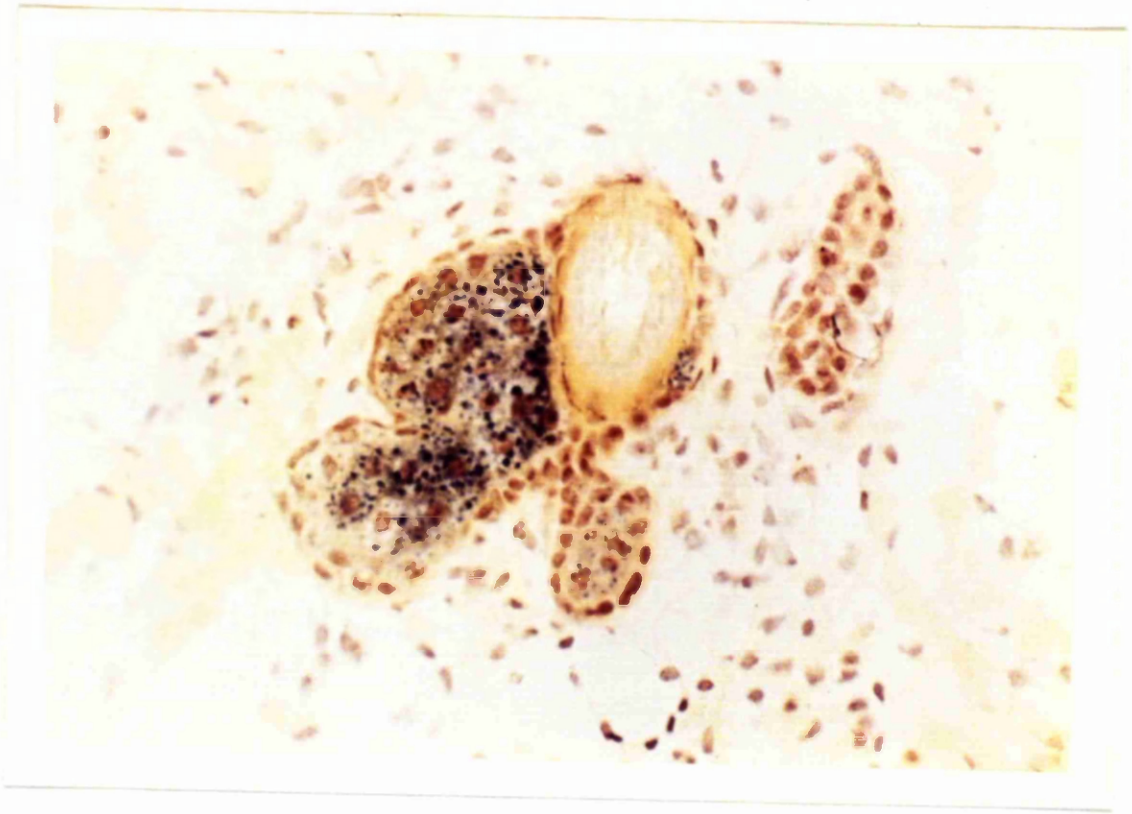

Figure 18. 17β-Hydroxysteroid Dehydrogenase Activity in Human Fetal Skin. (× 175)

in activity when testosterone or oestradiol were used.

(d) With DHA activity was weak, and was noted first at 24 weeks of gestation, and was consistently present though in trace amounts after 28 weeks.

(e) Activity of 16β-HSD was demonstrated first at 22 weeks, and remained present at all gestations after this.

(f) From 16 weeks gestation onwards numbers of male and female fetuses examined were approximately equal, and no significant difference in the pattern of HSD activity was observed in relation to sex. This observation was confirmed by comparing the activities in twin pairs of the opposite sex (Table 14) on the basis of examination of the external genitalia, no attempt was made to sex the fetuses before 16 weeks, as this is not generally possible (Hamilton, Boyd & Mossman, 1957).

Although the localisation of these enzymes is similar to adult skin, there was an obvious difference in the activity of the enzymes at various gestational ages. In particular there was a marked difference between the 3β-HSD, and the 17β-HSD activity. Whereas in the adult the 17β-HSD activity was poor, in the human fetus, it was high. The pattern is therefore different, and it is unfortunate that the changeover from fetal to prepubertal activity cannot be more clearly delineated.

As with adult skin, the 17β-HSD is found only in sebaceous glands. In the adult, however, the 17β-HSD activity is located in small patches

in contrast to the situation in the fetus in which activity is found throughout the gland, and in the secretory duct. The 3β-HSD is also localised throughout the sebaceous gland. It is of interest, that in almost every fetus examined, there was activity for these enzymes at all sites. This is also in contrast to the adult situation in which there occurs a substantial number of skin specimens in which enzyme activity does not appear to be present at all sites. This may simply be due to the fact that there are more sebaceous glands per unit area in the fetus, and thus more are visualised on each section. The arguments which were described in detail in Section 2 concerning the specificity of localisation of these enzymes also applies to fetal skin.

4. Relation to known metabolic functions of fetal skin.

Human fetal skin is a relatively large organ and any changes in its metabolic pattern could have significant consequences for the fetus as a whole. As a corollary to this, metabolic changes in the fetus may be reflected in similar or related changes in the skin. In addition, the fetal skin is intimately related to the liquor amnii and changes in the metabolic activity of the skin, could both modify and be reflected in the composition of the liquor. Any study of human fetal skin must relate its results both to the known internal metabolic changes of the fetus, and to its external environment, the liquor.

In recent years there has been growing interest in the steroid content and metabolism in the liquor amnii. It has been suggested that the products of steroid metabolism found in the liquor may be

used to predict the well-being of the fetus (Abramowich & Wade, 1969). Steroid components in the liquor amnii may, of course, come from many sources, urine, gastrointestinal secretions and the placenta and fetal membranes. The production of steroids in the feto-placental unit, as measured steroids found in the maternal blood or urine, have also been used to give information about fetal growth and development (McNaughton, 1969). In these investigations the role of fetal skin has received very little attention.

From the results of the present investigation, and previously established work, it can be said that -

(a) Human fetal skin is a large and metabolically active organ.

(b) Steroids are known to be metabolised histochemically in fetal sebaceous glands.

(c) Sebaceous differentiation and secretion involves holocrine secretion in which cell products, which presumably in the fetus as in the adult contain steroids, are discharged onto the skin surface.

Therefore :-

(d) Human fetal skin is a potential source of steroids for amniotic fluid.

It is known, for example, that the lipid content of liquor increases towards term, and this is associated with the developing functional activity of the sebaceous glands (Sharp, 1968). Insufficient work has been done to make an accurate comparison between steroid metabolism in skin and in liquor. As an offshoot of this, it would be of interest to harvest amniotic fluid cells, and also the vernix caseosa, and look at

them histochemically in the hope of picking up activity of HSD's outside the fetus. Work on this is in progress, but is hampered by the difficulties in preparing clean specimens of amniotic fluid cells.

Changes in the steroid metabolism in human fetal skin in relation to the total activity in the feto-placental unit, has also received little attention. It is reasonable to suppose, however, that these overall changes might be reflected in their metabolism within human skin. It is of interest therefore that the greatest activity recorded with these enzymes was of 17β-HSD and this may be related to the high circulating level of oestrogens in the fetus. The sebaceous glands may therefore be involved in the further metabolism of oestrogens.

From the reaoning above it may therefore be possible in the future to use skin as a "fetal biopsy" by which the overall metabolism of the fetus could be assessed. This concept is also fairly new, and applies equally well to other parameters of fetal well-being such as glycogen metabolism which has already been investigated (Shelley, 1969). Techniques for amnioscopy are available, and it is only a matter of time before an ethically acceptable method of obtaining human fetal skin in vivo will be developed. Using this, the overall metabolic pattern of the fetus may be derived.

5. Summary.

Skin specimens from 25 human fetuses from 8 to 32 weeks of gestation were examined to demonstrate the presence of hydroxysteroid dehydrogenases.

Skin was obtained absolutely fresh in 23 cases, and in one instance the skin used was obtained 6 hours after death. Fetal maturity was accurately determined by ultrasonography. The difficulties in obtaining fetal material over 32 weeks gestation were discussed.

The morphological changes in the differentiating human sebaceous gland were described. Sebaceous buds are noted early on, but fully differentiated cells were not seen until 18 weeks of gestation.

Of the hydroxysteroid dehydrogenases studied, 17β-HSD with both testosterone and oestradiol as substrates, was found at 18 weeks, and had good activity. The 3β-HSD and 16β-HSD activity was much less and appeared at a later date. NADH diaphorase activity was found in all skin samples, and was located in all cellular elements. This is in contrast to the HSD's which were found only in sebaceous glands. The comparison between the adult and fetal pattern of HSD activity was drawn. No sex difference in activity was noted.

These results were then related to the known metabolic functions of human fetal skin. Its role in the production of steroids in the amniotic fluid was commented on, and the idea of human skin being used as a "fetal biopsy" in relation to fetal maturity developed.

F. Antiandrogens and hydroxysteroid dehydrogenases.

1. Results.

2. Technical problems.

3. Discussion of results.

4. Significance of the method.

5. Summary.

1. Results.

The basis for the use of histochemical methods for the screening of antiandrogens in human skin has already been discussed. The results of these investigations will now be presented; and have been reported elsewhere (Calman, Grant & Milne, 1969, Calman, 1970).

Table 15 shows the results obtained, when visual assessment of the reaction is employed. None of the inhibitors used reacted alone with the nitro-blue tetrazolium in control incubations, nor did any inhibition of NADH diaphorase occur.

From the results with visual assessment, several conclusions can be drawn.

(1) Even with the relatively crude histochemical procedures, it is possible to pick out compounds which do inhibit the reaction. This is in most cases not difficult, and can be done rapidly and simply without any special instruments.

(2) The table illustrates the way in which differences in action between various compounds can be picked up. With testosterone for example, the metabolism is inhibited by compounds A, B and cyproterone, but not by compound C or cyproterone acetate.

(3) The table does not give any assessment of the degree of inhibition, but is useful in giving a preliminary screening. The results however, do correlate well with the semiquantitative results.

The next table (Table 16) shows the results of a semi-quantitative

Table 15.

VISUAL ASSESSMENT

	Control	A	B	C	CY	CYA	TETRA	RS. 4529
DHA	+	+	+	+	-	+	+	+
16 β	+	-	-	-	-	-		
Test.	+	-	-	+	-	+	+	-
Oest.	+	+	-	+	-	-	+	

\+ = No Inhibition

\- = Inhibition

Compounds A, B and C are 6-chloro substituted pregnenolones

CY = cyproterone

CYA = cyproterone acetate

Tetra = tetracycline

RS. 4529 = chloramidone acetate

Table 16.

SEMIQUANTITATIVE ASSESSMENT

	Control	A	B	C	CY	CYA	TETRA	RS. 4529
DHA	100	100	100	100	45	100	100	100
16	100	65	55	70	30	70	-	-
Test.	100	40	40	100	10	100	100	30
Oest.	100	30	10	25	10	75	100	-

Results are expressed as a percentage of the control.

assessment of these reactions. It should be pointed out that these results are based simply on counts of diformazan granules and may not be related to the end product of the reaction. Probably the smallest difference which can confidently be picked up is between 10 and 20%. These results are closely related to those by visual assessment.

Before such a method can be accepted as being of use, it requires to be compared with the standard biological assay techniques. Such a comparison has been constructed in Table 17, based on previously published work on these compounds.

2. Technical problems.

As with all histochemical techniques currently available for HSD measurement, quantitation is again a problem. The visual assessment is probably as accurate as the semi-quantitative method, the latter simply giving a figure for comparison purposes.

From the control point of view, it is important to note that in no case did the antiandrogen used inhibit the NADH-diaphorase, nor did it react in a control system. This means then that any difference in activity between the assay system which includes the suspected antiandrogen and a control assay is not simply due to these factors, and implies that the difference if any is a real one.

From the technical point of view, it will be noted that inhibition, if it occurred was present only at relatively high concentrations of the inhibitor (in excess of 100 ug/ml) when compared to that of the steroid

Table 17.

COMPARISON BETWEEN BIOASSAY AND HISTOCHEMICAL METHODS OF ANTIANDROGEN ASSAY

Compound	Bioassay	3 HSD	17 HSD
A	++	-	++
B	+	-	++
C	+	-	-
CY	+	++	++
CYA	++	+	+
RS. 4529	+	-	++

\+ = Inhibition

\- = No Inhibition

(30 ug/ml). This naturally raises the suspicion that the substance in question is not inhibiting the enzyme, but combining, either physically or chemically with the physiological substrate. Unfortunately, neither of these possibilities can be adequately investigated by histochemical methods. It could also mean that the histochemical assay procedure is just not sensitive enough to detect any differences in activity at a lower concentration.

With these high concentrations of antiandrogen, many of which are complex, lipid soluble molecules, their solubility in aqueous media becomes a problem. Thus with compound RS. 4529, at concentrations above 100 ug/ml, there was a definite cloudiness in the incubation media. The effective concentration is not therefore known with certainty. Lipid solvents, other than dimethylformamide would be worth investigating and to this end dimethylsulphoxide is at present undergoing assessment.

It is hoped that by using at least three different samples of human skin, and by assaying at different concentrations, that at least some of these problems will be minimised and only reliable and reproducible data obtained.

3. Discussion of results.

The compounds chosen for this investigation comprise only a limited number of the potentially enormous list of antiandrogenic compounds.

It is well recognised that tetracycline is of clinical importance in the treatment of acne vulgaris (Smith & Waterworth, 1961, Lane &

Williamson, 1962, Juhlin & Liden, 1965, Smith & Mortimer, 1967 and Verbov, 1968). The dosage which is administered, however, is less than the normal therapeutic dose, which would be given if its effect were solely due to its antibacterial action. The mechanism by which tetracycline is effective is not known though it has been found by fluorescent microscopies studies, that it is localised in the basal epidermis, the hair follicle, and the sebaceous glands (Marks & Davies, 1969). Tetracycline was, therefore, included in this series of experiments to evaluate its effect on the inhibition of hydroxysteroid dehydrogenases. No inhibition was noted, however, and its mechanism of action therefore, remains obscure.

Cyproterone, and cyproterone acetate are antiandrogenic compounds, which have been studied much more fully. They were introduced by Neumann and his colleagues in 1964 (Neumann & Kramer, 1964, Junkmann & Neumann, 1964). They assessed the androgenicity of the compound by its anti-masculinisation effect on male rat fetuses. It was found that cyproterone was five times less effective than cyproterone acetate, and accordingly most work was done on this latter compound. It was found (Neumann, Elger & Elger, 1966) that cyproterone acetate decreased the size of sebaceous glands in mice, and that the action of testosterone on sebaceous glands was inhibited. Gonadotrophin excretion (Neumann, 1966), vaginal conification and ovulation were also decreased (Neumann, Elger & Swordt-Wallrabe, 1966) suggesting that the compounds also had an effect on the hypothalamus.

Cyproterone acetate also inhibits benign prostatic hypertrophy in

the dog (Neri et.al., 1968) and when implanted locally in the seminal vesicles, their weight was decreased suggesting that it had a local action (Wollman & Hamilton, 1968).

In a clinical trial in man, cyproterone acetate was found to have no effect on sebum secretion or on clinical acne vulgaris, when the substance was applied topically (Cuncliffe, Shuster & Cassels-Smith, 1969).

The results presented in this investigation show that cyproterone acetate is less effective than cyproterone when tested histochemically. This is perhaps unexpected when it is known that cyproterone acetate inhibits the action of testosterone. It is likely, however, that the site of action is such that its inhibitory action cannot be picked up by this method.

The 6 - chloro-substituted pregnenolones were supplied by Syntex Ltd. Their biological activity is summarised in Table III. Little is known about their clinical effects as antiandrogens, but the histochemical results correlate closely with the biochemical results, as assayed in the chick, and rat ventral prostrate (Moore-Robinson, 1969)

Compound RS. 4529, also supplied by Syntex Ltd. is an active antiandrogen in a topical chick antiandrogen assay, and in the inhibition of androgen induced cock's comb growth. The histochemical results therefore once again are in accord with the bioassay experiments.

4. Significance of the method.

With any new method of antiandrogen assay must be assessed in the light of previously defined parameters of antiandrogen potency. Such a comparison drawn in Table 17 is of particular interest.

In the first place, it will be noted that the results of inhibition of the 17β-HSD, correlate well with the previously published results. With 3β-HSD, however, although an essential enzyme in androgen metabolism, no such correlation is found. This would indicate that the 17β-HSD, with testosterone as substrate may be a sensitive indicator for antiandrogen assay. It will be remembered from other results (Section 3) that the 17β-HSD activity is located in small patches around the sebaceous gland, and in the secretory duct. Further, androgens are known to stimulate mitosis in the cells of the sebaceous gland, and it may be that the antiandrogens which inhibit these enzymes are effective because of this relationship. Many other compounds will require to be assessed before this relationship can be known for certainty.

Secondly it should be noted that comparisons between different assay methods are extremely difficult, and it is not altogether surprising that some results will be at variance with the generally accepted results. Thus with cyproterone acetate the bioassay results, and the histochemical results do not correlate well at all.

This brings us therefore to mention the particular advantage of this method. It is simply that the results are obtained using human

skin, at the level of the sebaceous glands. No other method of antiandrogen assay can, at present, do this successfully with the exception of the method of Strauss & Pochi (1963) . With antiandrogens, of unknown character, their method is ethically unacceptable. It is thus the use of human skin, which makes the histochemical method unique.

The other major advantage of the method is its simplicity. No expensive equipment is required if it is to be used solely as a screening technique. The incubation time is 2 hours, and many sections (up to 100) can be conveniently incubated at one time. A considerable number of substances and many different skin specimens can thus be assayed in a very short time.

The disadvantages of the method are numerous and must be appreciated if the method is to be of any use. Perhaps one of the major disadvantages is in obtaining sufficient supplies of human skin. Several possibilities however, can be explored. In the first place, close liaison with surgical colleagues is the best way to obtain fresh human skin. The biopsies required are small and it is ethically acceptable to remove small ellipses of skin from the wound edges before closure. It is particularly important to have access to patients undergoing plastic surgery, since specimens of face, neck and scalp skin may be obtained in this way. It will be remembered (Section 3) that perineal skin contains sebaceous glands which are metabolically active and are present in good quantity. Prior to haemorrhoidectomy, therefore, the Surgeon should be requested to save the perineal skin removed. The

last useful source of skin is post-mortem scalp material. This has definite disadvantages, but if no other specimens are available, then it may be of use.

A more serious disadvantage is that only a limited number of substrates can be investigated. Only those having hydroxyl groups can be used in this system. This inevitably restricts the value of the method. Moreover, the number of inhibitors which can be used is also a theoretically limiting factor. Only those substances which do not react directly with the system on their own or do not inhibit the NADH diaphorase, being suitable. So far no such substance has been found, but the possibility should be checked for in each case. In addition, the number of enzymes which be investigated is by the same token restricted. Thus at present only the hydroxysteroid dehydrogenases can be demonstrated histochemically out of the whole range of steroid metabolic transformations. Other major enzymes such as the side chain splitting enzymes and the hydroxylases are excluded by this method.

In a more general way, one major disadvantage is that only a restricted mode of action of these compounds can be studied. The method restricts the mode of action to inhibition of hydroxysteroid dehydrogenases, in a histochemical system, at the level of the sebaceous gland. This indeed is the definition of an antiandrogen when this technique is used. It excludes such mechanisms as inhibition of penetration of the androgen into the sebaceous gland; binding of

steroids in the blood; inhibition of other enzyme systems.

From the clinical point of view, the method offers only limited information concerning its overall effects. The fact that the enzyme can be inhibited in vitro, is no guarantee that the same substance, applied topically will be effective. Further, no information about the dosage, route, or vehicle of administration can be obtained.

The advantages, disadvantages, and technical limitations have been described here in detail in the hope that the method will be placed in perspective in relation to other methods of antiandrogen assay. Because of its simplicity and the fact that human skin is used throughout, it is felt that the method certainly has potential and merits further investigation.

5. Summary.

A method for the assay of antiandrogens, based on the inhibition of hydroxysteroid dehydrogenases in a histochemical system has been described. The results were assessed both visually, and by a semi-quantitative method.

The results showed that inhibition could be readily detected, and that the technique was useful for the preliminary screening of compounds.

Various technical problems, such as control incubations and interference of the reaction by the compounds used were discussed, the solubility of the compound in the incubation medium being a particular problem.

The results of the study were then discussed in the light of the known antiandrogenicity of the compounds. In most cases, there was agreement between the biological assay system, and the histochemical technique, but the difference with cyproterone acetate was noted.

The significance of the method in relation to other systems for assaying antiandrogen was discussed, and the conclusion drawn was that the inhibition of the 17β-HSD correlated well with the known results, and that the method might be useful in the initial screening of anti-androgenic compounds.

5. CONCLUSIONS.

"The basic texture of research consists of dreams into which the threads of reasoning measurement and calculation are woven".

Albert Szent-Gyorgi, 1960.

This thesis set out specifically to answer a series of questions concerning the histochemical distribution, characteristics and clinical relevance of hydroxysteroid dehydrogenases in human skin. Perhaps the most significant feature of the study was that human skin was used throughout. In all, over 400 separate skin biopsies were used making the results more applicable to the clinical situation, and more easy to apply to problems of human sebaceous gland function in health and disease. In this section a critical review of the results of the questions posed in the introduction will be undertaken. Particular attention will be paid to future investigative work on steroid histochemistry in human skin and its relation to sebaceous gland function.

The first aim of this thesis was to investigate further the optimum conditions for the histochemical reaction. The basic reaction had already been established, and the additional material presented here has added greater depth to the method. In particular the storage characteristics of the enzymes which were investigated are important in any long term study of these enzymes. From the results presented here it can be safely concluded that human skin can be stored for periods of up to 6 weeks without appreciable loss of enzyme activity which can be detected histochemically.

The effect of fixation on the activity of these enzymes, and the usefulness of compounds which reduce tissue diffusion such as polyvinyl pyrrolidine were also essential facts to have before any major project could be started. In neither case was there any substantial benefit

from using these agents and with some there was a definite inhibition of activity.

In this first part of the thesis a major aspect covered was the problem of control incubations. No histochemical work can proceed unless the control solutions give no histochemical reaction. In this study the question of positive controls was investigated and it was suggested that a major factor causing positive control solutions was the presence of small molecular weight, oxidisable substances present in the tissue at the time of death. These substances could be removed by brief washing with buffer and the sections rendered negative when incubated with controls solutions while still retaining enzyme activity. The problem became even more acute when post-mortem skin was studied because sections with positive controls occurred regularly. Once again the controls became negative after washing and it was suggested that the high incidence of positive controls in post-mortem skins was due to anaerobic accumulation of small molecular weight reducing substances which could be easily removed.

As was explained in the earlier sections a major problem with the histochemical demonstration of dehydrogenases is quantitation. It was solved in this study partly by ignoring it, and partly by using a rather crude counting system. From the histochemical point of view, the technique for the demonstration of HSD's as it stands at present requires little in the way of revision apart from an adequate method of quantitation. This is most certainly a problem for the future.

Several lines of study are available. In the first place, computer assisted integrating microdensitometers have recently become available and though very expensive would be a major advance if applied to dehydrogenase histochemistry of steroids. It must be remembered however that the densitometer must be calibrated and with steroids, at the concentrations used histochemically, this might be a problem.

The second method would be to use tetrazolium salts other than the nitro-blue tetrazolium used in the present investigation. Such compounds, more soluble in the formazan form than nitro-BT would be readily eluted from the tissue section and estimated spectrophotometrically. A good deal of work has been done on this subject, notably by Jones (1970). Investigation on this line, however, evades the real problem, that of quantitation of the end product of the reaction, an oxidised steroid. Further investigation therefore to this end, as well as being time consuming, is of little relevance to the problem in hand. On the other hand extension of the investigation using the techniques of electron histochemistry would be of great interest, and would be of more value than extending the technique for light microscopy. Specific cell types and the intracellular distribution of the enzymes could be studied by these techniques.

Finally the amount of reduced NAD in the tissue could be readily measured using the elegant techniques of Glick (1949) and Lowry (1962). These quantitative methods could well be applied to the study of hydroxysteroid dehydrogenases, and would be a significant advance.

Each of these methods has drawbacks, and it is prudent to ask whether, with histochemical methods, quantitation is justified at all. It depends really on whether one feels that histochemical techniques should be used as ends in themselves, or simply as a first line investigation of a biological problem. The author has no doubts that it is the second view which is the correct one. Histochemical methods are only part of a larger and more extensive investigation of the problem. If the technique is not capable of resolving a particular problem, then it should be discarded, and different and more refined methods should be used. Histochemical methods form then a suitable starting point to an investigation into sebaceous gland structure and function. They provide some answers, but pose many more questions which can only be solved by other techniques.

Perhaps the main reason for using histochemical methods is that the localisation of the enzyme reaction can be established. The validity of the method depends therefore on the technique being accurate on this account. Various arguments in favour of the technique for the histochemical demonstration of hydroxysteroid dehydrogenases being valid were outlined, and it was concluded that the technique was reliable and did give an accurate picture of tissue localisation of enzyme activity.

The second question was to review the HSD's which could be demonstrated in human skin, and to establish their localisation within the skin itself. A range of HSD's was investigated using a variety

of substrates. Of these, three enzymes were found to be important; the 3β-HSD, the 17β-HSD, and the 16β-HSD, each enzyme using several substrates. In the major part of the thesis these enzymes alone were studied. Several other features however should be noted. No activity was observed with DHA sulphate, though this was not entirely unexpected for reasons which have already been mentioned. Further, the 5α testosterone is used as readily, and has the same histochemical distribution as testosterone itself. This is significant in that the 5α compound is thought to be the active form of testosterone.

When the distribution of these enzymes in human skin was looked at several interesting observations were made. In the first place it was confirmed that the enzymes were present only in sebaceous glands, and in other skin appendages or in the epidermis. This is an important finding which must be confirmed by further biochemical work using preparations of isolated sebaceous glands or on micro-dissected skin sections. At first glance it seems unlikely that all HSD activity in skin should be concentrated solely in the sebaceous glands. Several explanations have been put forward in the relevant section of the results to account for this including the fact that the histochemical technique may be too insensitive a method to detect small amounts of enzyme activity. It certainly poses a question for further and more refined biochemical work.

The localisation of these enzymes within sebaceous glands has also been studied. In general, there appears to be two patterns of enzyme

localisation; one with 3β- and 16β-HSD's, and one with 17β-HSD. In the former the diformazan granules are scattered over the sebaceous glands in a random manner. In the latter the granules are deposited in small areas around the periphery of the gland. The physiological relevance of this observation is not clear at present and requires confirmation by biochemical studies, or by using techniques of electron histochemistry which might be able to identify the specific cell types involved.

The third problem to be investigated was the distribution of these enzymes over the skin surface of the body. In general, it can be concluded that the hydroxysteroid dehydrogenases can be found in all areas of human skin bearing sebaceous glands, and in both sexes. There are, of course, differences between the various regions of the skin, but these can be accounted for by the differences in numbers of sebaceous glands in these regions. There is no real need to invoke an absolute difference in enzyme activity in these cases.

When the original observations on HSD's in human skin were made in 1965, it was thought that the distribution of these enzymes, in areas prone to acne vulgaris, might indeed be related to the pathogenesis of the condition. With the results presented in this thesis this hypothesis becomes less likely. It is still true that the highest activity, and hence number of sebaceous glands, occurs in facial regions, which are particularly prone to acne vulgaris. A major stumbling block however is that with scalp, in which the enzyme activity is as

high as in the face acne vulgaris does not occur. Other explanations for this difference can be offered, and will be presented later.

The pathogenesis of acne vulgaris is obscure. It is known that seborrhoea occurs in such patients, and Cuncliffe & Shuster (1969) have investigated the sebum secretion rates in patients with acne vulgaris. They postulate that acne cannot develop without seborrhoea, but since the seborrhoea persists after the acne regresses they postulate that a second factor, which can only operate in the active phase of the disease, must be involved.

Histological observations give some pointers to the pathogenesis of acne vulgaris. Strauss & Kligman (1960) found that it was the pilo-sebaceous unit with a small hair follicle which was particularly associated with acne. With blockage of the duct, sebum was discharged into the interstitial tissue and a foreign body giant cell reaction set up, giving the clinical lesion of acne vulgaris. Van Scott and McCardle (1956) went further and found that the earliest change in the pathogenesis of acne vulgaris was the hyperkeratinisation of the excretory duct which eventually blocked the sebaceous duct and the lesion of acne vulgaris occurred.

It seems then that there is a definite anatomical factor related to the pathogenesis of the disease, and it is known that the pilo-sebaceous units vary greatly over the body surface.

From these histological observations, and the histochemical work reported in this thesis a further hypothesis of the pathogenesis of

acne vulgaris can be tentatively put forward. The first essential feature of the hypothesis is that there is a definite anatomical arrangement of the pilosebaceous unit in acne vulgaris. In those units with wide excretory ducts and piliary canals acne lesions are less likely to occur than in units with narrow ducts and piliary canals. This would for example explain the difference between the scalp region and the facial regions if there was an anatomical difference between the types of sebaceous glands in these two areas. The number of sebaceous glands is similar, but the lesions are found only in the face.

In the second place, the regional distribution of the disease must be explained. This difference may be due simply to the number of sebaceous glands in the various regions. If this is so, then although some lesions should occur mainly on the face, back and chest, some should also be found in other regions unless there is a definite histological reason why this should not be so.

The third feature to mention is the known physiological response of the sebaceous glands to androgenic stimulation. It is known that the glands increase in size (Strauss & Pochi, 1961) and that the mitotic rate is increased (Ebling, 1963) when androgens are given. At puberty hormonal stimulation is maximal, and it is at this period that acne vulgaris occurs. It is conceivable that hormonal stimulation at this period causes the increase in size and activity of the sebaceous glands, and in those glands with narrow excretory ducts and piliary

HYPOTHESIS of PATHOGENESIS of ACNE VULGARIS

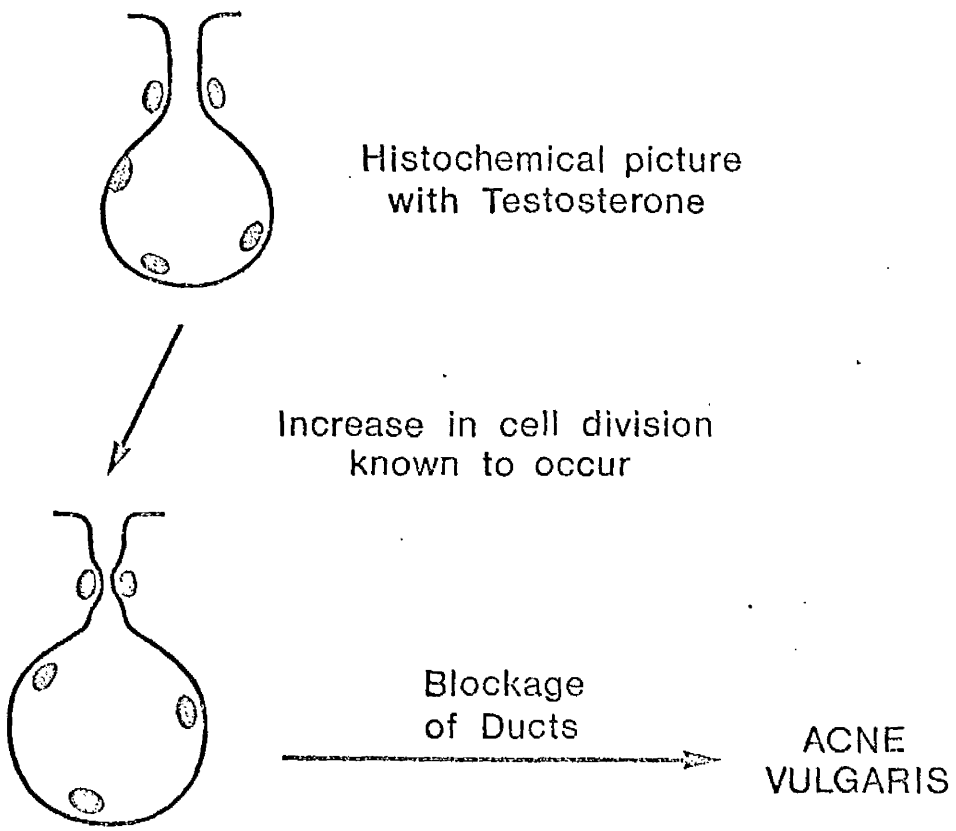

Figure 19. Hypothesis for the pathogenesis of acne vulgaris.

canals blockage occurs with development of the acne lesion. At the end of puberty then, either all the sebaceous glands now have large excretory ducts and cannot develop acne vulgaris, or the glands with the smaller ducts have been destroyed by the pathological process. This fact would then explain the decreasing incidence of the disease after puberty while the seborrhoea continues. In other words after puberty the sebaceous glands which were capable of becoming sites of acne lesions would be either destroyed or have become fully developed and could no longer have their excretory ducts and piliary canals blocked.

With these hypotheses as a base line what further can the histochemical results add. The main point is the unusual distribution of 17β-HSD activity around the periphery of glands and in the secretory ducts. Is it possible that testosterone or other androgen acts locally on small groups of cells causing mitosis to be stimulated, with hyperplasia of these cells? If so, then the secretory duct could become blocked when this occurred, and the pathological process leading to the clinical picture would be the result.

The hypothesis can then be summarised as follows (Fig. 19) -

1. In prepubertal skin two varieties of sebaceous gland occur. The first has a wide excretory duct and piliary canal and will not give rise to the acne lesion. The second type has narrow ducts which may block during the pubertal growth of the gland.

2. During puberty androgenic stimulation rises to a maximum. This causes localised areas of mitosis in the secretory ducts and sebaceous glands, with accompanying seborrhoea indicating functional maturity of the glands.

3. These localised areas of mitosis may in fact cause blockage of the secretory ducts leading to the clinical lesion of acne vulgaris.

4. After puberty the sebaceous glands are fully developed and the development of acne vulgaris is unlikely. This is either due to destruction of the susceptible glands, or to their conversion to fully dilated ducts and glands.

This hypothesis, of course, is a tentative one but it raised several lines of investigation which may stimulate further work. These are -

1. A further examination of the anatomy of prepubertal pilo-sebaceous units in acne and non-acne areas, particular attention being paid to the secretory ducts and the piliary canal.

2. Further clinical examination of non-acne areas for the occurrence of acne lesions and biopsy of these areas with full histological examination.

3. Examination of the sebaceous glands for areas of localised mitosis to confirm the hypothesis as detailed above.

4. Histological examination of post-pubertal sebaceous glands for the size of the secretory ducts. If the hypothesis is correct then there should be very few pilosebaceous units with narrow sebaceous

glands.

The fourth question to be investigated was how these enzymes were distributed in the skin of the human fetus. It was found that these enzymes were present early on in fetal life, and it was suggested that the enzymes may influence the hormonal changes which occur in the liquor amnii. As far as future investigation is concerned, it is likely that an extension of the study to fetuses nearer term and abnormal fetuses would yield interesting results. It is likely, for example in the post-mature fetus, that hormonal changes occurring both in the liquor, and feto-placental unit, may be reflected in changes in the skin. A skin biopsy, in utero, at this stage may therefore give an indication of the internal hormonal environment of the fetus. Further examination of the cells of the liquor amnii would also be interesting since one of the basic questions which remains unanswered about the liquor amnii is the origin of the cells in the amniotic fluid. Some of these cells certainly come from the sebaceous glands and the histochemical techniques used in this thesis may help to differentiate between different amniotic fluid cells.

The fifth point was to investigate the use of the histochemical method for the screening of substances thought to be antiandrogens. As with any new method the initial results are favourable and it takes some time for the technique to find its true level. It is important that the technique is used in other centres and the results either confirmed or refuted.

It is emphasised again that the method is in the experimental stage and requires much more work to be done. To this end an investigation of 12 compounds whose anti-androgenic properties are known in various systems is being undertaken. These compounds differ only minimally in their chemistry and this, it is hoped, will establish the sensitivity of the method.

If, using this technique, a compound is found with good anti-androgenic properties, then animal bioassay methods will be required before any clinical trials with the compound can be carried out.

It is perhaps useful to ask why the search for a suitable topical anti-androgen is being carried out so intensively in many laboratories throughout the world. Such a compound might be of use in the treatment of acne vulgaris, the assumption being that the cause of acne vulgaris is in some way related to androgenic stimulation of sebaceous glands. This common disease causes extensive morbidity because of its unsightly appearance both in the florid state and in its burnt out scarring form. A topical substance, which of necessity would require to have no side effects because of the benign nature of the disease, would be of definite clinical value. Further, conditions such as female hirsutism and male-pattern baldness, which are also assumed to be due to abnormalities of androgen metabolism, might well be helped by such a compound. Once again it should be pointed out that such compounds must have virtually no side-effects since the conditions themselves are so mild.

STEROID METABOLISM and SEBACEOUS GLANDS

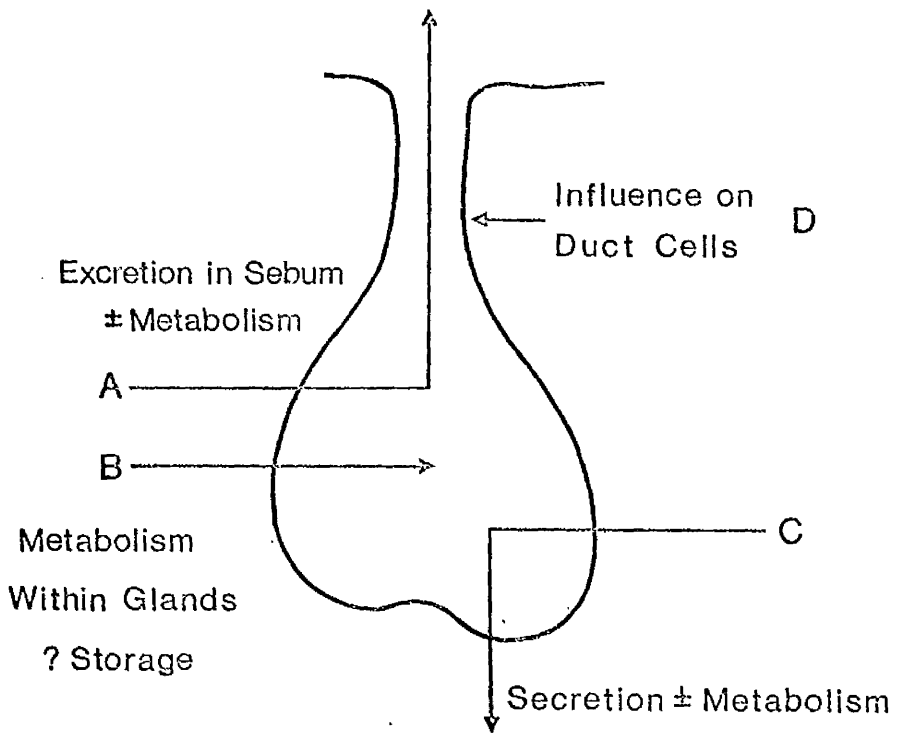

Figure 20. Sebaceous glands and steroid metabolism.

The final question was to relate the histochemical findings to the known biochemical evidence for steroid metabolism in human skin. Many of the biochemical results which have previously been reported in human skin were confirmed by the histochemical technique. The disquieting feature however was the localisation of these enzymes solely in the sebaceous glands. It seems likely that they are also present in other skin appendages and epidermis, and that the histochemical technique is not sufficiently refined to pick up small amounts of activity. It is hoped therefore that more elegant biochemical work will shed more light on this problem.

It remains therefore to speculate on the relationship between sebaceous gland function and the metabolism of steroid hormones. In the diagram (Fig. 20) the various ways by which the sebaceous glands could be related to steroid metabolism are shown.

In the first place the sebaceous glands may act simply as a means of steroid excretion from the body. Steroids reaching the sebaceous gland via the blood stream could be taken up by the gland, and, by the process of holocrine secretion, discharged onto the skin surface. It is probable that during this process, there would be further metabolism of the steroids within the sebaceous glands. If such a route of excretion does occur, then it may be a very important one because of the total number of sebaceous glands on the human skin surface.

Secondly, the sebaceous glands may act as a target organ for the

hormones. This is a fairly recently proposed view of sebaceous gland function, and if it is confirmed, then once again because of the large number of cells involved, the sebaceous gland becomes a major end organ in steroid metabolism. The histochemical results show clearly that the sebaceous glands themselves can definitely metabolise many of the steroid hormones. The histochemical methods however do not allow us to observe the physiological effect of such hormones, and this has to be approached by the use of other techniques. Just what function the sebaceous gland would have by virtue of it being an end organ or target organ for steroid hormones is not so clear. From the evidence discussed before it is unlikely that this would be related only to the production of sebum. Regulation of sebaceous cell differentiation would seem the most likely possibility.

The sebaceous glands could also act by storing hormones, and liberating them back into the circulation as required. In addition, the hormone on reaching the sebaceous gland may be modified, prior to release into the blood stream, and this is the third way in which steroid hormones and sebaceous glands may be related.

Finally, the steroids after modification by the gland, could act on the secretory duct of the gland and be responsible for hyper-keratosis of the ducts, the significance of which has already been discussed in relation to the pathogenesis of acne vulgaris.

In conclusion then, the six questions which were posed at the beginning of this investigation have at least been partly answered,

and our knowledge of the histochemical aspects of steroid metabolism in human sebaceous glands significantly extended. As usual in any such study, many more problems have been raised than answered, and it is hoped that these questions will be pursued in the future by the use of more refined histochemical, biochemical and electron microscopical techniques.

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