THE DISTRIBUTION AND CHARACTERISATION OF

ESTERASES IN SKIN

A thesis presented for the degree of

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by

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To

Lynne, Rex

and Alan
I wish to express my appreciation to my supervisors Dr D. Benford, Prof. J W Bridges, and especially Dr D G Upshall for their help and guidance during these studies. I would also like to thank my fellow students and colleagues Dr J Connelly, Dr S Maxwell, Dr P N Price, Dr G Wishart, Mr J Hopley, Mr T J Grace, Mr D B Coult, Mr C Owen, and many others for their help and encouragement. Thanks are also due to Mr M Yeadon for technical help and discussion during physical separation of esterases.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>BuChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>ASCh</td>
<td>Acetylthiocholine</td>
</tr>
<tr>
<td>BuSCh</td>
<td>Butyrylthiocholine</td>
</tr>
<tr>
<td>pNPac</td>
<td>p-Nitrophenylacetate</td>
</tr>
<tr>
<td>Ind.Ace.</td>
<td>Indoxylacetate</td>
</tr>
<tr>
<td>I50</td>
<td>Concentration of inhibitor producing 50% inhibition</td>
</tr>
<tr>
<td>BW284C51</td>
<td>1,5-Bis(4-allyldimethylamioniumphenyl)pentane-3-one diiodide</td>
</tr>
</tbody>
</table>
Two techniques of homogenising skin tissue have been examined for their efficiency to produce homogenates of rat skin containing subcellular organelles with the minimum amount of damage.

The subcellular distribution of three hydrolase enzymes, two esters of thiocholine and p-nitrophenylacetate, have been determined in the subcellular fractions produced from rat skin by homogenisation using grinding in liquid nitrogen. All three hydrolases were found to be solubilised by this technique and remained in the 100,000xg supernatant.

The 100,000xg supernatant from rat skin was subject to molecular exclusion chromatography and the three solubilised hydrolases were followed through this procedure. Each of the hydrolase activities was found to be associated with a number of proteins that were different by molecular size. The two thiocholine ester hydrolases were associated with the same proteins, but the p-nitrophenylacetate hydrolase was predominantly associated with other proteins.

The 100,000xg supernatant was also subjected to analysis of by polyacrylamide gel electrophoresis and staining with naphthylacetate. This procedure showed the presence of at least eight different hydrolases, and some of these electrophoretic bands could be associated with specific proteins of specific molecular size as determined by...
molecular exclusion chromatography.

5 - The hydrolases present in the 100,000xg supernatant from rat skin were characterised using four chromogenic substrates and four selective inhibitors. A cholinesterase with a substrate selectivity similar to that of acetylcholinesterase but an inhibitor selectivity similar to butyrylcholinesterase was found. Different esterases were responsible for the hydrolysis of p-nitrophenylacetate and indoxylacetate.

6 - The 100,000xg supernant from human skin was found to contain hydrolases that were different to those in rat skin. An enzyme hydrolysing p-nitrophenyl acetate that was insensitive to diisopropylfluorophosphate and did not hydrolyse this inhibitor was detected. The cholinesterase present behaved in the same way as butyrylcholinesterase. Only one electrophoretic band of naphthylacetate hydrolysing activity could be detected by polyacrylamide gel electrophoresis.

7 - Naphthylacetate and 5-bromoindoxylacetate hydrolases were histochemically located in skin from rat and man.
CHAPTER 1

INTRODUCTION
The skin is the organ with which we are all most familiar. It is the organ which gives visual form to the body and protection to the organs within. In its role as a protective barrier it is subject to many types of chemical and physical assault from washing with soap and water to the injection of ink below its surface to form a tattoo. The bulk of chemicals encountered domestically, such as soaps and some cosmetics, that come into contact with the skin do little or no damage, and are not absorbed through it into the body to any significant extent. Some chemicals however damage the skin and may be absorbed into the body percutaneously possibly causing damage to internal organs. A detailed knowledge of the way in which the skin can interact with chemicals as they pass into or through it, is essential to the treatment and prevention of their adverse effect, and also to developing methods of treatment of a variety of skin diseases.

The following review is in two parts. The first deals with the anatomy of the skin and its appendages, and the second with the biochemical capabilities and esterases associated with it.
1.1 THE ANATOMY OF THE SKIN

A knowledge of the anatomy and physiology of an organ is essential in assessing, and designing, biochemical and pharmacological studies of it. The following is a brief outline of the anatomy of the skin. It is not intended as a comprehensive review of the literature on this subject since several excellent reviews already exist. (Barr, 1962; Montagna and Parakkal, 1974; Jarratt, 1973, and 1974; and Odland, 1983).

The major structures of the skin are shown in fig. 1.1 and are described below. The skin is divided into two main layers, the upper epidermis and the lower dermis, or corium. The epidermis is further subdivided into several histologically distinct layers, described in turn below. Figure 1.2 shows the various layers of the epidermis.

1.1.1 THE EPIDERMIS

1.1.1.1 STRATUM BASALE (basal layer)

The stratum basale is the innermost layer of the epidermis, and is a single layer of cuboidal, or columnar, mitotically active cells, (Montagna and Parakkal, 1974). Basal layer cells divide to produce either further basal layer cells or cells that migrate upwards into the stratum spinosum. The melanocytes are contained within this layer. (Riley, 1974)
FIGURE 1.1

Transverse section of the skin of the rat (x4), stained with massons trichrome stain.
Fig. 1.2

1 - Stratum corneum
2 - Stratum granulosum
3 - Stratum spinosum
4 - Stratum basale
5 - Basement membrane
1.1.1.2 STRATUM SPINOSUM (prickle cell layer)

This is a layer of polyhedral cells derived from the stratum basale. As they migrate upwards, the cells of the stratum spinosum become progressively more flattened, their nuclear volume: cytoplasmic volume ratio increases and eventually they lose their nuclei completely, (Jarratt, 1973; Montagna and Parakkal, 1974)

1.1.1.3 STRATUM GRANULOSUM (granular layer)

Between the stratum spinosum and the stratum corneum is a layer of granulated cells, the stratum granulosum. This layer is particularly notable biochemically for its intense hydrolytic activity; it was thought to exclusively contain dendritic Langerhans cells. These cells are immunocompetent phagocytic cells that play a role in the defence of the body against potentially pathogenic organisms on the skin surface, and in sensitisation reactions. Langerhans cells have now been shown to be present in all the suprabasal layers of the epidermis, all epidermal appendages and the dermis (Stingl and Aberer, 1983). The keratohyalin granules first become visible in this layer and are thought to represent a late stage in keratinisation.

1.1.1.4 STRATUM CORNEUM (dead cell layer)

This is the layer of tightly packed keratin that remains after the process of keratinisation is complete. The stratum corneum varies in thickness from one area of the body to another
being thickest on the palms and soles. In areas where the stratum corneum is thick, it is possible to discern another epidermal layer, the stratum lucidum, between the stratum corneum and the stratum granulosum, (Montagna and Parakal, 1974). This is a poorly staining hyalin layer that is difficult to see in man, (Odland, 1983).

1.1.2 THE DERMIS

The dermis can be divided into two layers, the upper stratum papillaris and the lower stratum reticularis. The stratum papillaris accounts for the smaller proportion of the dermal mass, but has a higher water content.

The stratum papillaris is more pliable than the stratum reticularis. In addition to the role as a supporting structure, the dermis acts as a reservoir of water and fat. Skin and muscle between them contain 75% of available (extracellular) body water, but the skin contains four to five times as much per unit weight as does muscle tissue (Elden, 1971). In addition to collagen the dermis contains mucopolysaccharides, reticulum, reticulin, and elastin.

1.1.2.1 CELLS OF THE DERMIS

Some dermal cells are resident whilst others migrate into the dermis from the blood so as to protect the body from bacterial invasion. The resident dermal cells are neutrophilic and eosinophilic polymorphonucleocytes that enter the dermal tissue,
enlarge and take on a resident phagocytic role. The resident
cells are of four types 1) fibrocytes, between the bundles of
collagen, are inactive fibroblasts, the cells that secrete
collagen; 2) tissue macrophages, the resting "wandering cells";
3) dermal melanocytes and melanophores; and 4) tissue mast cells.

1.1.3 THE PANNICULUS ADIPOSUS AND THE PANNICULUS CARNOSUS

Below the dermis are two more layers of tissue. The
panniculus adiposus is a layer of fatty tissue of variable depth,
just below the dermis. It is one of the largest depots of fat in
the human body but is much smaller in other animals (eg. the
rat). This layer of tissue is a site of intense lipid metabolism.

The panniculus carnosus is a layer of skeletal muscle below
the panniculus adiposus. This layer is vestigial in man, the
platysma of the neck being all that remains of it (Montagna and
Parrakal, 1974). In animals however this layer of muscle can be
quite thick and is not easily separated from the remainder of the
skin tissue.

1.1.4 EPIDERMAL APPENDAGES

The many thousands of glands and hair follicles present in
the skin are derived embryologically from epidermal tissue and
are therefore termed "epidermal appendages".
1.1.4.1 THE HAIR FOLLICLE

Mammals are unique in being the only animals to possess body hair. The hair follicle is produced embryologically by the penetration of epidermal cells into the dermis, to form a bulb from which the hair develops, (Spearman, 1977; Holbrook, 1983).

Hair grows in cycles. Each hair passes through a growth phase (anagen) an intermediate phase (catagen) and a resting phase (telogen). During anagen the follicular cells proliferate and penetrate deep into the dermal tissue. In some rodents the hair growth runs from the throat region proceeding caudally to the tail. In man however there is no definite cycle, at any particular time a certain percentage of our hairs are in anagen, some in catagen, and the remainder in telogen, but there is no apparent co-ordinated pattern to their growth, (Johnson, 1958).

1.1.4.2 THE SEBACEOUS GLANDS

The sebaceous glands develop from the same epidermal penetration as the hair follicles. They remain attached to the follicle and secrete fats and oils onto the growing hair and hence onto the skin. Not all the functions of this secretion are known but it helps in the waterproofing of the skin and probably contributes to the maintenance of the structure of the stratum corneum.
1.1.4.3 THE APOCRINE GLANDS

Whilst apocrine and eccrine glands are collectively referred to as "sweat glands", apocrine secretion is not sweat. Apocrine glands respond slowly, if at all, to thermal stimulation, and their secretion is an oily substance varying from greyish-white to almost black in colour. Apocrine secretion has a variety of functions. The secretion of the glands in the external auditory meatus forms ear wax, whilst the secretion in the axilla areas is degraded by bacteria to produce odourous compounds and may be a vestigial pheromone.

1.1.4.4 THE ECCRINE SWEAT GLAND

Eccrine sweat glands are anatomically similar to apocrine glands, they differ in the nature of their secretion and in that they cover the whole body. The secretion of the eccrine glands is an aqueous fluid, secreted in response to high external temperatures, and increases in body heat due to exercise. The main biological role of eccrine secretion would appear to be that of thermoregulation, cooling by evaporation. In addition to this a layer of eccrine sweat aids the spread of apocrine and sebaceous secretions over the skin surface, (Montagna and Parakkal, 1974; Sato, 1983).
1.2 THE ORGANS OF FOREIGN COMPOUND METABOLISM

Until the 1960s the liver was thought to be the only organ capable of metabolising drugs to any significant extent. In other organs drug metabolising systems were either undetectable, or present at such low levels that they were thought to be inconsequential. Recent research however (reviewed by Connelly and Bridges, 1980; Pannatier et al., 1978; Gram, 1973), has shown that drug metabolism takes place in most organs of the body, all be it at low levels. In organs that are primary portals of entry such as skin, intestine and lung, this metabolic activity may assume an important role in metabolising foreign compounds before they enter the blood stream.

It has also been shown that different organs have different spectra of drug metabolising enzymes (Philpot and Smith, 1984; Bend and Hook, 1977; Orrenius et al., 1973). Clearly, the precise profile of the drug metabolising capacities of a tissue forming a portal of entry will have a profound influence upon the types and levels of foreign compounds to which other organs of the body are exposed.

Organs that metabolise drugs are also potential sites of action for those drugs and their metabolites. There are numerous examples of this including tumor production in skin by benzo(a)pyrene, and carbon tetrachloride induced liver damage as outlined in table 1.1.
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>METABOLITE</th>
<th>ORGAN</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycyclic</td>
<td>epoxides</td>
<td>skin</td>
<td>Weston et. al. (1982)</td>
</tr>
<tr>
<td>aromatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrocarbons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbon tetrachloride</td>
<td>trichloromethyl</td>
<td>liver</td>
<td>Butler (1961)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>radical</td>
</tr>
<tr>
<td>Betamethasone valerate</td>
<td>betamethasone</td>
<td>skin</td>
<td>Rawlins et. al. (1979)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>nitrosamines</td>
<td>liver</td>
<td>Bowman and Rand (1980)</td>
</tr>
<tr>
<td>Hexamethyl</td>
<td>formaldehyde</td>
<td>nasal</td>
<td>Dahl (1983)</td>
</tr>
<tr>
<td>phosphoramidemucosa</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3 FOREIGN COMPOUND HANDLING BY SKIN

1.3.1 PENETRATION OF COMPOUNDS THROUGH SKIN

A metabolic capacity is biologically irrelevant if the compound concerned never reaches the site of metabolism within the tissue. For instance if a chemical is metabolised by an enzyme in the lower layers of the epidermis but does not penetrate deeper than the stratum corneum, its metabolism at this site is of no importance. When exposed topically the ability of the skin to metabolise drugs must be considered in relation to its function as a barrier to the entry of foreign compounds into the body. In this role the skin can be considered as two layers, dermis and epidermis, with a net of capillaries sandwiched between. Foreign compounds that penetrate the epidermis will be absorbed into the blood via these capillaries. Any metabolic activity present in the dermis will therefore have a smaller effect on the amount of a percutaneously applied compound absorbed than would a similar capacity in the epidermis.

There are several possible routes by which chemicals may penetrate the skin. 1) Across the epidermis, 2) via the hair follicles, 3) down the eccrine sweat ducts. The contribution of each of these of these routes to the total penetration of a chemical through the skin has been the subject of much controversy.

The precise location and nature of the epidermal barrier to penetration of topically applied compounds is not known. Some
workers have demonstrated a barrier layer at the junction of the stratum corneum and the stratum granulosum (Rothman, 1954) that behaves as a negatively charged membrane, excluding anions and trapping cations. This barrier is not the stratum lucidum however. Large molecules stop at this barrier and Mali (1956) claims to have isolated a fibrillar continuous membrane. Kligman (1964) could not demonstrate such a membrane however and points out that the cells are histologically continuous through the layers of the epidermis. Tregear (1966) demonstrated that penetration increases gradually with the sequential removal of layers of the stratum corneum by stripping with adhesive tape, not suddenly as would be expected if a discrete membrane exists.

Penetration of compounds through the epidermal appendages probably plays only a small part in transepidermal passage. The staining of pilosebacious units, eccrine, and apocrine glands by dyes during penetration through skin (MacKee, 1945; Rothman, 1954), and the formation of wheals (Scheuplein, 1967), is not conclusive evidence for the appendages as an important route of penetration because the selective binding of dye could also be resolved as preferential passage through the appendages.

For a more detailed discussion of the evidence for these various routes of penetration the reader is referred to Katz and Poulson (1970).
The search for alternative methods of drug administration has led to the development of a "transdermal patch" designed to administer agents through the skin (Karim, 1983). Such devices may be useful for drugs that produce unwanted side effects when administered by other routes (e.g., nausea or vomiting when given by mouth, or irritation of the site of injection if given intravenously). Devices that administer nitroglycerin are now available and are an improvement on the ointments previously tried (Sved et al. 1971) though some preparations have failed to produce the required antiangina effects (Crean et al., 1983) possibly due to the dose in the patch being too low (Bennet and Davis, 1985). Scopolamine has also been administered in this way (Fara, 1983), and the method offers an alternative route for the administration of any compound that can penetrate the skin. Clearly the degree to which a drug given in this way is metabolised in the skin will critically effect the blood concentration achieved.

1.3.2 XENOBIOTIC EXCRETION BY SKIN

The skin has been shown to excrete foreign compounds in sweat and by incorporation into the keratin of hair. Aminopyrine, antipyrine, and some sulphonamide antibiotics have been demonstrated in the sweat of treated animals (Johnson and Mailbach, 1971). The partition of compounds into sweat was found to be dependent upon their pK, those with a pK value of about 5.0 were maximally ionised in sweat and best excreted by this route. Several compounds have been found to be incorporated into hair, for example albino rats treated with 9-phenyl-5,6-benzoiso-
alloxazime developed orange-yellow pigmentation of the hair (Haddow et. al., 1945), Polychlorinated biphenyls, DDT and DDE have also been found in hair (Matthews et. al., 1976).

1.3.3 SYSTEMIC EXPOSURE OF SKIN TO XENOBIOTICS

The skin can also be exposed to foreign compounds present in the blood, via the dermal circulation and the capillary net at the dermal-epidermal border. These compounds may be present in the form that they enter the body or they may be metabolites formed in other tissues such as the liver. The precise exposure of any particular area of skin by this route will be dependent upon blood flow. Skin that is highly perfused, for the purposes of thermoregulation for instance, will be subject to a higher exposure than poorly perfused skin.
1.4 The biochemical capabilities of skin.

As a collection of many small organs of different cell types the biochemical capabilities of the skin as a whole are understandably diverse. By necessity the organs of the skin possess the biochemical apparatus to perform the specialised functions attributed to them, and the basic chemical processes of life.

1.4.1 Glucose metabolism.

Freinkel (1960) showed that the skin was capable of utilising glucose as an energy source. Using labeled glucose, lactate was shown to be the major product of glucose metabolism in skin. The relative conversion of the C-1 and C-6 glucose carbon atoms suggested that the main pathway involved was the hexose monophosphate shunt, and that the Krebs cycle was of only minor importance (see also Yardley and Godfrey, 1963).

Halprin and Ohkawara (1966) showed hexokinase to be in large excess to the skin's needs (1/6 of total capacity was utilised), and glycogen metabolism to be detectable at low levels. Curiously glycogen is only visible histologically in epidermal basal cells after injury (Lobitz et al., 1962).

The metabolic state of the organ under scrutiny also has an influence on the rate of glucose metabolism. The activity of the krebs cycle has been shown to be higher in the growing human hair follicle than in the resting organ (Adachi and Uno, 1966). In addition, Kahlenberg and Kalant (1966) have shown that the
metabolism of glucose in human skin can be increased by insulin.

1.4.2 Lipid metabolism

The skin is a site of intensive lipid metabolism and inhibition of that metabolism with drugs such as those that inhibit sterol biosynthesis, results in scaly lesions like those of ichthyosis (Yardley, 1969). This finding, and other evidence such as the abnormal keratinization observed in young animals and infants deficient in essential fatty acids, suggest that skin lipids have an important role in maintaining the integrity of the stratum corneum.

The skin surface lipids are secreted by the epidermis and the sebaceous glands and have been shown to have the composition shown in Table 1.2. The skin does not synthesise its own arachidonic acid, as shown by incorporation studies using labelled acetate (Vroman, 1969). In this study however, labelled carbon was incorporated into all other lipid fractions.

1.4.3 Protein metabolism in skin

As is obvious by the production of collagen and keratin by the skin, the organ has the ability to synthesise not only these structural proteins but the enzymes that it uses to carry out such synthesis. The amino acids utilised by the skin are predominantly those used in the production of structural proteins, proline, glycine, and cysteine, though all amino acids are needed for construction of other cellular constituents.
**TABLE 1.2.**

Types and sites of synthesis of skin surface lipids.

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>% of total surface</th>
<th>main site of synthesis</th>
</tr>
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<tbody>
<tr>
<td>wax and sterol esters</td>
<td>23-25%</td>
<td>epidermis</td>
</tr>
<tr>
<td>triglycerides</td>
<td>20-44%</td>
<td></td>
</tr>
<tr>
<td>free fatty acids</td>
<td>2-30%</td>
<td></td>
</tr>
<tr>
<td>squalene</td>
<td>8-17%</td>
<td>sebaceous glands</td>
</tr>
<tr>
<td>diglycerides</td>
<td>5-14%</td>
<td></td>
</tr>
<tr>
<td>monoglycerides</td>
<td>2%</td>
<td></td>
</tr>
</tbody>
</table>

(distribution data from Nicoliades (1963); data on location of synthesis from Hsia et. al. (1970) and Kiistala and Mustakallio (1964))
1.5 Drug metabolism in skin

In recent years the skin has been much studied as a xenobiotic metabolising organ. Most of the metabolic systems that are present in the liver have also been found in skin, all be it at much lower levels. The following is a short summary of the types and functions of xenobiotic metabolising systems that have been demonstrated in skin. For more detailed accounts the reader is referred to the reviews quoted in each section and to the references in table 1.3 that refer particularly to skin enzymes.

1.5.1 Cytochrome P-450

The cytochrome P-450s (Paine, 1981) are the terminal systems haemoproteins in a microsomal monooxygenase that is widespread in different species, and in different tissues (Connelly and Bridges, 1980). The term covers a number of different isoenzymes that collectively have the ability to bind and subsequently oxidise a wide variety of substrates (Guengerich and Macdonald, 1984). These enzymes are induced by classical inducing agents such as phenobarbitone and 3-methylcholanthrene (Ryan et. al., 1979) in addition to many other chemicals (e.g. isosafrole, clofibrate, and arochlor 1254). The ability to respond in this way to changes in the chemical environment is an important consideration when applying data obtained experimentally to the free living organism.

The cytochrome P-450 monooxygenase system is responsible for the activation of many procarcinogens, such as the polycyclic aromatic hydrocarbons (Levin et. al., 1982), and as
such is a factor in the toxicology of industrial oils, and smokes, that gain access to the body topically.

In recent years much work has been done to define the reasons why cytochromes P-450 have such a broad substrate specificity. This is due in part to the multiplicity of the enzymes and isoenzymes (Nebert and Negishi, 1982) that have been demonstrated in animals (e.g. rabbit, Haugen et al. 1975; and rats, Ryan et al. 1979) and man (Davies and Boobis, 1984).

1.5.2 Epoxide hydrolase

In hepatic microsomes this 53,000–54,000 Mw enzyme has a wide substrate specificity but is particularly notable for its hydrolysis of epoxides produced from polycyclic aromatic hydrocarbons, (Oesch et al., 1979). It has been demonstrated in skin by several groups of workers (table 1.3), and one group have demonstrated both a microsomal and a cytosolic form. The possibility that this finding may result from the solubilisation of microsomal enzyme by the harsh homogenisation procedures that were employed cannot be fully discounted (see also Guengerich and Davidson, 1982; Oesch et al., 1980; Hammock and Ota, 1983).

1.5.3 Glutathione transferases

These are a family of enzymes that transfer glutathione from the cell's glutathione pool to a range of reactive xenobiotics. The reaction with some of the more reactive groups involved (e.g. epoxides, ethers, and peroxides) may occur rapidly without enzymic catalysis depending upon pH. Evidence for the multiplicity of the enzymes involved comes from experiments that separate the enzymes
1.5.4 Glucuronyl transferases

Glucuronyl transferases are a group of enzymes that catalyse the transfer of glucuronic acid residues from the activated form, 5-uridyldiphosphoglucuronic acid, to xenobiotics with hydroxyl, aglycone, carboxyl, sulphhydryl, amino, and imino groups. The heterogeneity of these enzymes has again been demonstrated by the separation of different forms with activities towards different substrates (work reviewed by Bock et al., 1983; Pollard and Dutton, 1982).

1.5.5 Sulphotransferases

Are a group of enzymes little studied in skin, the references given in table 1.3 are the only demonstrations of them to date. This is probably due to the fact that, in the liver, the cellular reserve of activated sulphate is small by comparison to that of glucuronic acid, though this may not be so in as yet uninvestigated extrahepatic tissues, such as skin. The enzymes transfer sulphate from activated PAP-sulphate to hydroxyl groups of xenobiotics. In liver several isoenzymes have been demonstrated, but the existence of multiple forms in skin has yet to be established.

Drug metabolism in skin has been the subject of several reviews in recent years. The reader is referred to:- Pannatier et al. (1978), Bickers and Kappas (1980), and Bickers (1980).
Table 1.3
Xenobiotic metabolising enzyme systems demonstrated in skin.

<table>
<thead>
<tr>
<th>ENZYME SYSTEM</th>
<th>REFERENCES</th>
</tr>
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<tbody>
<tr>
<td>Cytochrome P-450</td>
<td>Pannatier et. al. (1981)</td>
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<td></td>
<td>Weston et. al. (1982)</td>
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<td></td>
<td>Pohl et al. (1976)</td>
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<td>Fox et. al. (1975)</td>
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<td>DelTito et. al. (1984)</td>
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<td>Dehydrogenases</td>
<td>Davis et al. (1972)</td>
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<td>Deaminases</td>
<td>Hanhanson and Moller (1963)</td>
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<td>Epoxide hydrolase</td>
<td>Bentley et al. (1976)</td>
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<td></td>
<td>Oesch et al. (1976)</td>
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<td></td>
<td>Mukhtar and Bickers (1983)</td>
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<tr>
<td>Glucuronyl transferases</td>
<td>Harper and Callcut (1960)</td>
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<td>Anylan and Starr (1952)</td>
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<td></td>
<td>Dutton and Stevenson (1962)</td>
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<td></td>
<td>Moloney and Bridges (1962)</td>
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<tr>
<td>Sulphotransferases</td>
<td>Farein et al. (1968)</td>
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<tr>
<td></td>
<td>Berliner et al. (1968)</td>
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<td></td>
<td>Moloney et al. (1982)</td>
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<tr>
<td>Methylation</td>
<td>Hanhasson and Moller (1963)</td>
</tr>
<tr>
<td>Glutathione transferases</td>
<td>Mukhtar and Bresnich (1976)</td>
</tr>
</tbody>
</table>
|                        | Mukhtar et al. 1981)
1.6 HYDROLYSIS AND HYDROLASES

1.6.1 ESTER HYDROLYSIS BY ANIMAL TISSUES

An esterase is an enzyme that hydrolyses ester bonds. Such bonds are formed by the condensation of an organic acid and an alcohol, esterases reverse this condensation by adding water across the ester bond. The important structural requirement for this hydrolysis is the presence of a carbonyl carbon carrying a partial positive charge, a criterion also fulfilled by amides and peptides. Most esterases will therefore also hydrolyse amides, and enzymes denoted as amidases for their ability to hydrolyse amides are capable of hydrolysing esters. Thus the definition of an esterase/amidase is dependent upon the substrate under discussion (see Krisch, 1971; and Heymann, 1982).

Many drugs are esters or amides and the hydrolysis of their ester/amide bond plays a part in their metabolism, though often in competition with other routes of metabolism. In general there does not seem to be any rigorous structure activity relationship within the substrates for animal tissue esterases. This is in part because of the heterogeneous nature of the esterases in animal tissues, but is also due to the broad substrate specificity of individual enzymes (Heymann, 1975)

1.6.2 AMIDASES IN ANIMAL TISSUES

Earlier definitions of amidases reviewed by Dixon and Webb (1971) were based largely on work in liver using only amides as
substrates (Bray et. al., 1949; 1950). These definitions were used to assign the enzyme commission number, E.C. 3.5.1.4, to arylycamidase. Subsequent studies however have shown that the amidase activity of liver is associated with the same enzymes as the carboxylesterase activity and has led the establishment of the term carboxylesterase/amidase as reviews below (see section 1.6.7).

Simple amides are hydrolysed by almost all soft tissues. A list of amides known to be hydrolysed by animal tissues is given in table 1.4 but the precise identity of the enzymes catalysing this hydrolysis has not been established for all the compounds listed. As the techniques of protein separation are applied to amidases the nature of the responsible proteins and their relationship to esterases is being resolved and it is clear that the liver amidase is not the only enzyme which appears to be identical with an esterase. The amidase hydrolysing o-nitroacetanilide in rat and human serum has been shown to be the same enzyme as that which hydrolyses methylbutyrate and is classically regarded as serum "aliesterase" or carboxylesterase, though serum cholinesterase could be separated from it (Tsujita and Okuda, 1983). Another group of workers showed the serum arylacylamidase from human serum to be associated with cholinesterase (George and Balasubramania, 1981) though that in liver was not. Moreover the enzyme in human serum was sensitive to seratonin whilst that in liver was not.

Similar to esterases the amidases in some tissues have been shown to be heterogenous groups of enzymes (Lampelo, 1982; Hsu
<table>
<thead>
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<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>LOCAL ANAESTHETICS AND ANTI-INFLAMMATORY</strong>s</td>
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<td>butanilicain</td>
<td>Arndt et. al. (197 )</td>
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<td>penicillin G</td>
<td>Satoh and Moroi (1972)</td>
</tr>
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<td>Tolnaftate</td>
<td>Bowman and Rand (1980)</td>
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<td>chlodimethorm</td>
<td>Arndt et. al. (1973)</td>
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</tr>
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<td>fluorenylacetamide</td>
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</tr>
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<td>biphenamine</td>
<td>Bowman and Rand (1980)</td>
</tr>
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</table>

(after Heymann, 1982)
et. al., 1982; Jacobs et. al. 1982). The full hydrolytic capacities of the individual enzymes in these groups has yet to be defined.

Some of the amidases demonstrated in various tissues are shown in table 1.5.

1.6.3 ESTER HYDROLYSIS IN ANIMAL TISSUES

Table 1.6 shows some of the esters to which the skin may be exposed as drugs and environmental contaminants. The pharmacological and toxicological effects of esters can be due to the ester itself and/or to one or both of the hydrolysis products, the alcohol and the acid. Hydrolysis of an ester can therefore have a profound influence on its biological effects.

In the following sections the literature available on the major esterases present in animal tissues will be reviewed, and related to the known ester hydrolysing capacities of the skin.
<table>
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<td>Valinger et. al. (1984)</td>
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<td>rat</td>
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<td>hog</td>
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<td>rat</td>
<td>Gros et. al. (1985)</td>
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<td>eel</td>
<td>Majumbar and Balasubramania (1984)</td>
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<tr>
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<td>hog</td>
<td>Oellgen and Taylor (1985)</td>
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<td>Junge and Krisch (1975)</td>
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<td>● cocaine</td>
<td>Steward et. al. (1979)</td>
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<td>● betamethasone valerate</td>
<td>Goodman &amp; Gilman (1975)</td>
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<td>● hydrocortisone valerate</td>
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<td>ethylbutyrate</td>
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<td>• pincolinates</td>
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<td>• kuron</td>
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<td>• pyrethrin II</td>
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<td>• malathion</td>
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<td>Brooks (1979)</td>
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<td>• zectram</td>
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<td>• carbofuran esters</td>
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</tr>
</tbody>
</table>

**MISCELLANEOUS**

- Phthalates
  - benzylsalicylate
  - amyldimethylamino
  - benzoate

(alternate source: Heymann, 1982)
1.6.4 NOMENCLATURE OF ESTERASES

To date no attempt to apply a systematic nomenclature system to esterases has been universally accepted. Augustinsson (1959, 1961) distinguished between esterases hydrolysing aliphatic and aromatic esters, a system that is now considered unsatisfactory due to the activity of some enzymes towards both types of substrate. Aldridge (1953) proposed a functional nomenclature system based on the interaction of esterases with organophosphate esters, and is probably the most successful system currently in use. Esterases that hydrolyse organophosphates without being inhibited are designated 'A esterases', those that are irreversibly inhibited by organophosphates are 'B esterases' and 'C esterases' do not interact. The 'C esterases' have been demonstrated in only a single report on hog kidney, and their widespread existence therefore remains to be proven. Attempts to equate Augustinsson's and Aldridge's systems have led to the terms 'A esterase' and 'arylesterase', and the terms 'B esterase' and 'aliesterase' becoming synonymous however these terms are still often used to denote more specific enzymes. This is probably misleading since all the terms refer to heterogenous groups of enzymes. For example authorities that reject Augustinsson's system on the basis of cross reactivity often use the term 'carboxylesterase' to cover all esterases hydrolysing esters of carboxylic acids (Heymann, 1982). However this term is also applied to a more specific enzyme that is synonymous with the aliesterase of liver (Krisch, 1971; Junge and Krisch, 1975)

The use of the term 'nonspecific esterase' by some authors
may cause some confusion. The term refers to the use of substrates such as naphthylacetate and indoxylacetate that are hydrolysed by many enzymes, and demonstrations of the presence of nonspecific esterase must by interpreted in this light.

The separation of multiple forms of liver carboxylesterase by several groups of workers (Junge and Krisch, 1973; Arndt et. al. 1978; Lombardo et. al., 1978) further confuses the situation and brings the I.U.B. nomenclature system into question, as pointed out by Walker and Mackness. (1983). Clearly the terms:-

3.1.1.1  -  Carboxylesterase (aliesterase)
3.1.1.2  -  Arylesterase (A esterase)

refer to heterogenous groups of enzymes and are hence out of place in the enzyme commissions lists.

The cholinesterases form an important exception in the nomenclature of esterases, in that both forms of cholinesterase have been sufficiently well characterised to enable their optimal substrates to be stated with some degree of certainty. (see section 1.6.5).
1.6.5 THE MECHANISM OF ESTER HYDROLYSIS

The mechanism of ester hydrolysis is nucleophilic attack on the carbonyl carbon in the ester link, thus:-

\[ R-C-O-R \rightleftharpoons R-C-O-R \rightleftharpoons R-C-O^{-} + \text{HOR} \]

Where "N" is a nucleophile, the most common being the hydroxyl ion. The unstable intermediate so formed spontaneously rearranges to form, where hydroxyl is the nucleophile, a carboxylic acid and an alcohol.

An esterase catalyses this reaction by stabilising the intermediate and hence lowering the activation energy for the reaction. The enzyme catalysed reaction can therefore be written:-

\[ R-C-O-R \rightleftharpoons R-C-O-R \rightleftharpoons R-C-O^{-} + \text{HOR} \]

At the end of this process the enzyme is regenerated and can catalyse further reactions. If however the ester is of either phosphoric or carbamic acid and the enzyme is a 'B esterase' the rate of hydrolysis of the enzyme substrate complex can be severely reduced and the enzyme inhibited. For example:-

\[ iPr-P=O_{s+} \rightleftharpoons iPrC=O_{\Theta} \rightleftharpoons iPr-P=O + F_{\Theta} \]

Diisopropylfluorophosphate hydrolysis by a 'B esterase'
The enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine is among the most studied of all enzymes. Given the vital biological role of this enzyme its widespread occurrence in animal tissues is to be expected. Acetylcholinesterase has been found in nervous tissue and erythrocytes from many species (Nachmansohn and Rothenberg, 1945; Augustinsson, 1949), in snake venom (Mountner, 1950), snail blood (Augustinsson, 1946, 1948, 1949), and insect brains (Casida, 1955). In view of the vast amount of data available on the subject any review must seek to emphasis certain aspects of the field and give others only a brief coverage. In the present study aspects of the catalytic behavior and physicochemical properties of skin esterases have been measured, therefore this review is divided into two parts. The first deals with the molecular biology of acetylcholinesterase and the second with aspects of its catalytic mechanism. For more detailed reviews the reader is referred to Koelle (1963), Freode and Wilson (1971), and Massoullie and Bon (1982).

Molecular biology

Acetylcholinesterase consists of globular protein units (monomers) of 85,000 daltons each (Dudai and Silman, 1979; Bon and Massoullie, 1976; Rossenberry and Richardson, 1977). These units are glycoproteins containing about 15% carbohydrate by
weight (Lawler, 1963; Leuzinger and Baker, 1967). In the early 1960's isomers with different electric charges were separated by electrophoresis (Bernsohn et. al., 1962; Maynard, 1964 1966). Early work on enzymes purified by column chromatography as reviewed by Froede and Wilson (1971) indicated four active sites per molecule and a molecular weight of 250,000 (Leuzinger et. al., 1969). The demonstration of isomers of acetylcholinesterase led to work that showed molecular aggregates of various sizes, by ultracentrifugation (Massoulie and Rieger, 1969). Brimijion (1983) describes six different molecular forms in his review. The first three consist of one, two, and four globular subunits, linked together by disulphide bridges. The other three forms are linked to a "collagen tail" so as to produce forms that have four, eight, and twelve globular subunits. (see also Rosenberry and Richardson, 1977; and Anglister and Silman, 1978). Although the original studies on acetylcholinesterase isomers used enzyme from Electrophorus electrus, the same basic chemistry appears to hold for all the enzymes so far studied, including mammalian esterase (Vigny et. al., 1979). Brimijion (1983) suggests that this "collagen tail" is to anchor the enzyme to the membrane.

Acetylcholinesterase of similar quaternary structures have similar sedimentation coefficients as summarized by Brimijion (1983). The faster sedimentation of avian acetylcholinesterase has been attributed to a 40,000 dalton peptide covalently linked to the main protein chain (Allemond et. al. 1981).
Catalytic aspects

Within the family of esterases acetylcholinesterase is quite specific in its structural requirements for the structure of its substrates. The enzyme shows a characteristic order of hydrolysis rate in an homologous series of choline esters, of acetyl>propyl>butyl (Bergman et. al., 1950; Underhay 1957). The butyl ester has a very low hydrolysis rate but still binds to the enzyme producing good competitive inhibition (Cohen, 1949). The specificity of acetylcholinesterase for choline esters has led to the widespread acceptance of a simple model of the active site of the enzyme proposed by Adams and Whittaker (1950), and by Wilson and Bergmann (1950):–

![Proposed structure of the catalytic site of acetylcholinesterase](image)

Mountner and Whittaker (1950), Mountner (1951), and Whittaker (1953) showed acetylcholinesterase to be relatively intolerant of branching in the acyl and alkyl chains of a series of aliphatic substrates.
Acetylcholinesterase is inhibited by high concentrations of acetylcholine, a phenomenon thought to be caused by the binding of two substrate molecules to the active site (Augustinson, 1949). The cationic site is not essential for binding of the substrate as demonstrated by the high rate of hydrolysis of the uncharged acetylcholine analogue 2,2-dimethylbutylacetate (Adams, 1949). Curiously some uncharged esters such as dimethylaminoethylacetate, do not show this substrate inhibition (Wilson, 1953; Wilson and Bergman, 1950), possibly indicating that the binding to the cationic site is important in the mechanism of this inhibition. In contrast uncharged haloacetates (Adams and Whittaker, 1949; Bergman et. al., 1956) do show substrate inhibition.

Acetylcholinesterase will also split esters of substituted phenols (Whittaker, 1951; Underhay, 1957), indoxyl and naphthyl acetates (Underhay, 1957), and salycyl- and acetylsalycyl-choline (Bergman et. al., 1958; Koelle and Freidenwald, 1949). Early work showed that thioesters were also hydrolysed (Glick, 1941). Thioacetic acid is hydrolysed to acetic acid and hydrogen sulphide (Wilson, 1953).

Hosken and Trick (1955) have shown that the D-form of acetyl-β-methylcholine is preferentially hydrolysed by acetylcholinesterase. Ammon and Meyer (1959) however showed that L-form of mandelic acid choline esters was preferred by brain acetylcholinesterase.

When hydrolysing acetylcholine, acetylcholinesterase has a pH
optimum of 8.25 (Bergman, 1958). The hydrolysis of phenylacetate and acetylthiocholine was pH independent between 8 and 10. Bergman et. al. (1958) suggest that this is because the enzyme forms a hydrogen bond with the ethereal oxygen of acetylcholine that is not possible at high pH or when the nature of the ester link is changed, such as in a thioester.

Acetylcholinesterase from electric organ of the eel (Wilson and Cabib, 1956) and from erythrocytes (Shukuya, 1953) was found to vary little in activity between the temperatures of 15°C and 30°C.

Inhibition of acetylcholinesterase with labelled DFP has led to an accurate determination of the amino acid residues surrounding the serine residue in the active site. They are:-

Glu-SerP-Ala

(P = phosphorylated)

Based on experiments that showed the two basic groups in the active site were 9A (0.9nm) and 5A (0.5nm) from the anionic site, Krupka (1966) proposed the following mechanistic model of the active site:-
1.6.6.2 BUTYRYLCHOLINESTERASES (E.C.3.1.1.8)

Another form of cholinesterase found in plasma and most tissues is different from that classically associated with nervous tissue and erythrocytes. This type of cholinesterase is called "butyrylcholinesterase" because of its substrate specificity. This enzyme is often referred to as "pseudocholinesterase".

Molecular biology

The molecular size and quaternary structure of "butyrylcholinesterase" is very similar to that of acetylcholinesterase. The enzyme is made up of 85,000 dalton monomers, slightly larger than those of acetylcholinesterase, aggregated into dimers and tetramers. A form of butyrylcholinesterase isolated from skeletal muscle has a "collagen tail" similar to the acetylcholinesterases (Allemond et. al., 1981; Vigny et. al. 1978). In a similar way to acetylcholinesterase, several isomers of butyrylcholinesterase can be separated by electrophoresis (LaMotta et. al., 1968; Berry, 1960), and chromatography (Harris and Robson, 1963; Svensmark, 1965)

Catalytic aspects

The butyrylcholinesterase found in plasma is generally, perhaps too readily, considered to be representative of butyrylcholinesterases in general. Work with horse and human
plasma butyrylcholinesterases has produced a characterisation of the enzyme comparable with that outlined above for acetylcholinesterase.

It is in its substrate specificity that butyrylcholinesterase differs most from acetylcholinesterase. The order of hydrolysis rate for short chain fatty acid esters is reversed: -butyl > propyl > acetyl (Augustinsson, 1959). The optimum acyl chain length was found to be butyl for both choline and aliphatic esters (Glick, 1941; Adams and Whittaker, 1949; Sturge and Whittaker, 1950). Alteration of the choline moiety affects hydrolysis by butyrylcholinesterase in a similar way to acetylcholinesterase. Esters of 3,3-dimethylbutanol are hydrolysed with high rates compared to other aliphatic esters (Adams and Whittaker, 1949). Methylation at the $\beta$-carbon atom of the alkyl chain (e.g. acetyl- $\beta$-methylcholine) lowers the hydrolysis rate of both aliphatic and choline esters (Koelle, 1950; Augustinsson and Isachsen, 1957; Heilbrohn, 1959).

Butyrylcholinesterases have been found in serum from other animals, but at different levels and/or with different substrate specificities. Levels in ruminant plasma are low and substrate specificities vary with species, but a butyrylcholinesterase identical with that of horse or human serum has not been identified (Myers, 1953; Hardwick, 1956). Swine plasma contains a cholinesterase that hydrolyses phenylacetate at a rate only slightly lower than that of butyrylcholine (Levine and Suran, 1950; Augustinsson and Olsson, 1959). The same enzyme is present in sow's milk (Hines and McCance, 1953). In plasma from rat,
rabbit (Koelle, 1953) and chicken (Earl and Thompson, 1952) a "propionylcholinesterase", with maximum activity against propylcholine, has been detected. This finding illustrates the folly of classifying all "serum" cholinesterases as "butyrylcholinesterases" and also supports Augustinsson's view that no effective classification of serum cholinesterase can be made on the basis of substrate specificity alone (Augustinsson, 1963).

"Cholinesterases" in other tissues vary between species (Goldstien, 1951; Myers, 1953), and between tissues (Sawyer and Everett, 1947; Koelle, 1951; Ord and Thompson, 1950). Ord and Thompson (1951) showed that rat brain, skeletal muscle, and adrenal glands contain mainly acetylcholinesterase; heart, intestine and skin mainly propionylcholinesterase; and stomach, lung, liver and salivary glands a mixture of both. Experiments with esterases in tissues are however complicated by the preparations used. In unperfused tissue with high blood content, for instance, the tissue activity may be masked by the activity of blood enzymes. Crude homogenates and subcellular fractions contain many enzymes capable of hydrolysing simple esters, and proteins that may be capable of binding such substrates, leading to results that are difficult to interpret.

1.6.7 CARBOXYLESTERASE/AMIDASE

Molecular characteristics

In recent years the "aliesterases" of liver have been
extensively studied, and the term carboxylesterase/amidase has been used to denote these enzymes (Krisch, 1971; Junge and Krisch, 1975). The enzymes have a mixed activity against ester and amides and have been purified and extensively characterised.

The purification of liver carboxylesterase has enabled a large volume of data on the molecular properties of this enzyme to be accumulated. The procedure of Alder and Kistiakowsky (1961) first led to the purification of an enzyme with a molecular weight of between 150,000 and 200,000 from pig liver. The same enzyme was purified by Krisch (1963) from pig liver microsomes. Both these purified forms were found to be associated with amorphous material when examined microscopically (Krisch, 1971; and Barker and Jencks, 1969). Table 1.7 summarises some of the properties of liver carboxylesterases from various species (for a fuller account see Heymann, 1980). In general the enzymes are made up of subunits with molecular weights of between 45,000 and 80,000 and are associated into trimers, although dimers and tetramers have been reported.

Carboxylesterases have been purified from extrahepatic tissues such as mouse kidney (Goeppinger et. al., 1978), rat small intestine (de Jong et. al., 1978), and human brain (Hoejring and Svensmark, 1977). In terms of molecular weight these extrahepatic forms were similar to those in liver. Further support for a subunit weight of about 60,000 was obtained by Heymann et. al. (1971) using sodium dodecyl sulphate dissociation and gel filtration in the presence of 6M guanidine, to determine the subunit weights of cat liver, pig kidney, and ox liver.
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<tr>
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<td>60,000</td>
<td>AT,AU, CR,DE</td>
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58,000- pI=6.0 IF isoenzymes Mentlein et. al. (1980)

60,000 180,000 pI=5.2-6.4

PAGE 46 Cont'd...
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<th>COMMENTS</th>
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</thead>
<tbody>
<tr>
<td>RABBIT</td>
<td>60,000</td>
<td>GF</td>
<td>isoenzymes</td>
</tr>
<tr>
<td>LIVER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUINEA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIG</td>
<td>56,000</td>
<td>GF</td>
<td>isoenzymes</td>
</tr>
<tr>
<td>LIVER</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(after Heymann, 1980)

DE = Dodecylsulphate Electrophoresis
GF = Gel Filtration
AT = Active site Titration
AU = Analytical Ultracentrifugation
CR = Cross linking with dimethylsulphimidate
IF = Isoelectric Focusing
N-terminal analysis showed that glycine was the terminal amino acid in the pig enzymes and leucine in the ox.

Amino acid compositions have been determined for the pig liver (Barker and Jencks, 1969; Klapp et. al., 1970), and kidney (Klapp et. al., 1970), and ox liver (Klapp et. al., 1970). The composition of all three of these esterases was similar; isoleucine, aspartate, glutamate, glycine, alanine and valine were the most numerous residues (50% of total composition).

The isoelectric points of carboxylesterases are normally in the range 4.7 - 6.5, as reviewed by Heymann (1980). Mentlein et. al. (1980) separated five esterases from rat liver with isoelectric points of 5.2, 5.6, 6.0, 6.2, and 6.4, of which esterase 5.6 was heterogeneous. Of these esterases, esterase 5.6 is probably the esterase EA of Arndt et. al. (1978), and esterase 6.0 is esterase E1. The carboxylesterases of pig liver have been shown to be very heat stable, surviving 47°C for 1 hr, or 53°C for 10 mins.

Catalytic characteristics

Carboxylesterase has a wide substrate specificity, and its ability to hydrolyse both aliphatic and aromatic substrates casts further doubt on the enzyme commission nomenclature. Some of the more common esters that have been used as substrates in the determination of carboxylesterase activity are listed in table 1.8 (for a more detailed lists see Krisch, 1971 and Heymann, 1980). Other enzymes such as endopeptidases (Hartley and Kilby, 1952;
### TABLE 1.8

**SUBSTRATES USED TO DETERMINE CARBOXYLESTERASE ACTIVITY**

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenylformate (acetate, propionate, butyrate, valerate)</td>
<td>Downy and Andrews (1965)</td>
</tr>
<tr>
<td>ethylformate (acetate, propionate, valerate)</td>
<td>Levy and Ocken (1969)</td>
</tr>
<tr>
<td>p-nitrophenylacetate</td>
<td>Levy and Ocken (1969)</td>
</tr>
<tr>
<td></td>
<td>Franz and Krisch (1968a)</td>
</tr>
<tr>
<td></td>
<td>Barker and Jencks (1969)</td>
</tr>
<tr>
<td></td>
<td>Benohr and Krisch (1969)</td>
</tr>
<tr>
<td></td>
<td>Krisch (1967)</td>
</tr>
<tr>
<td></td>
<td>Mentlein et. al. (1980)</td>
</tr>
<tr>
<td>methylbutyrate</td>
<td>Benohr and Krisch (1969)</td>
</tr>
<tr>
<td></td>
<td>Franz and Krisch (1968a)</td>
</tr>
<tr>
<td></td>
<td>Franz and Krisch (1968b)</td>
</tr>
<tr>
<td></td>
<td>Ardnt et. al. (1973)</td>
</tr>
<tr>
<td></td>
<td>Mentlein et. al. (1980)</td>
</tr>
<tr>
<td>methylbutyrate (3-methylbutyrate, 3 &amp; 4-methylpentanoate, hexanoate, heptanoate)</td>
<td>Krenitsky and Fruton (1966)</td>
</tr>
</tbody>
</table>

Cont'd....
<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethylbutyrate</td>
<td>Stoops et. al. (1969)</td>
</tr>
<tr>
<td></td>
<td>Krisch (1963)</td>
</tr>
<tr>
<td></td>
<td>Benohr and Krisch (1967)</td>
</tr>
<tr>
<td>o-, m-, p-nitrophenylbutyrate</td>
<td>Franz and Krisch (1968a)</td>
</tr>
<tr>
<td>ethylbenzoate (benzenesulphonate, lactate, acetoacetate, fumarate, diethylsuccinate, aspartate; p-hydroxybenzoate, bromomalonate terephthalate)</td>
<td>Levy and Ocken (1969)</td>
</tr>
<tr>
<td>procaine</td>
<td>Krisch (1963)</td>
</tr>
<tr>
<td></td>
<td>Benohr and Krisch (1967)</td>
</tr>
<tr>
<td></td>
<td>Franz and Krisch (1968)</td>
</tr>
<tr>
<td></td>
<td>Kenitsky and Fruton (1966)</td>
</tr>
<tr>
<td>thiophenylacetate</td>
<td>Greenzaid and Jencks (1971)</td>
</tr>
<tr>
<td>p-nitrothiophenylacetate</td>
<td>Stoops et. al. (1969)</td>
</tr>
</tbody>
</table>

Cont'd....
### TABLE 1.8 cont'd

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetanilide</td>
<td>Franz and Krisch (1968a)</td>
</tr>
<tr>
<td></td>
<td>Benohr and Krisch (1967)</td>
</tr>
<tr>
<td></td>
<td>Franz and Krisch (1968b)</td>
</tr>
<tr>
<td></td>
<td>Arndt et. al. (1978)</td>
</tr>
<tr>
<td></td>
<td>Mentlein et. al. (1980)</td>
</tr>
<tr>
<td>phenacetin</td>
<td>Franz and Krisch (1968b)</td>
</tr>
<tr>
<td></td>
<td>Berhammer and Krisch</td>
</tr>
<tr>
<td></td>
<td>(1965)</td>
</tr>
<tr>
<td></td>
<td>Krisch (1966)</td>
</tr>
</tbody>
</table>

(after Krisch, 1971)
Kilby and Youatt, 1954) hydrolyse the more labile esters in the list such as o- and p-nitrophenylacetate, but their activity is generally low when compared to "true" esterases (Krisch et. al. 1966; and Stoops et. al., 1969). Though some proteinase substrates such as L-tyrosine ethyl ester are hydrolysed by pig liver carboxylesterase, other substrates such as proteins (casein and haemoglobin), olive oil, \( \text{OC-} \)naphthylpalmitate, and acetylcholine (Krisch, 1963), and butyrylcholine (Arndt and Krisch, 1973), are not.

Investigations into the effect of chain length on activity have shown that increasing the length of the fatty acid chain of esters of simple alcohols up to 12 carbon atoms increases the activity (Hofstee, 1954), whereas a chain length of 4 for the alcohol moiety is required for maximum activity (Dixon and Webb, 1971). Charged esters, such as those of choline, are either very poor substrates or are not hydrolysed at all (Dixon and Webb, 1971; Levy and Ocken, 1969; Bernhammer and Krisch, 1965), this indicates that hydrophobic bonding plays an important part in the binding of the substrate to the enzyme. Arndt et. al. (1978) isolated two isoenzymes from rat liver, one had a maximum activity at an alkyl chain length of 4-5 carbon atoms, and the other a maximum at an alkyl chain length of 2. These enzymes showed maximum activity at acyl chain lengths of 5 and 2 carbon atoms respectively. Junge and Krisch (1975) describe experiments with two forms of pig liver esterase. The form with high activity towards methylbutyrate (H form) shows a \( V_{\text{max}} \) that has a maximum at an acyl chain length of five carbon atoms, and a distinct minimum \( V_{\text{max}} \) at an alkyl chain length of four to five atoms. The
form with a low activity towards methylbutyrate (L form) shows highest Vmax values for longer chain fatty acids, and a small peak in Vmax values at a six to seven carbon alkyl chain length. Both forms behave similarly with respect to Km variation, and acyl chain length did not affect Km whereas increasing the alkyl chain length up to nine atoms produce a slight increase.

Carboxylesterases from pig (Levy and Ocken, 1969), and monkey (Bamann and Gelber, 1960), have been shown to hydrolyse both optical isomers of mandelic acid esters with equal rates. Similar results were obtained for the hydrolysis of hydrophobic amino acid esters by beef liver (Wynne et. al., 1973). The finding by Stoops et. al. (1969) however, that pig liver esterase hydrolyses p-nitrophenyl carbenzoxy-L-tyrosine 27 times faster than the D form of the same ester leaves the question of stereospecificity open.

As shown in table 1.8 carboxylesterases hydrolyse thioesters and aromatic amides in addition to esters of carboxylic acids. The esterase from chicken liver (Drummond and Stern, 1961) that hydrolysed S-acetoacetyl dihydrolipoic acid and some other similar esters is very similar to carboxylesterase from other sources. Purified esterases from goat intestine (Malhotra and Philip, 1966) and pig liver (Stoops et. al., 1969; Greenzaid and Jencks, 1971) also hydrolyse thioesters. The pig liver enzymes have a higher activity against phenylthioacetate, ethylthioacetate, and butyrylthioacetate than against their corresponding oxygen analogues. The hydrolysis of arylamides such as acetanilide and phenacetin is generally slower than that of
carboxylic acid esters. The reaction is inhibited by organophosphorus compounds in the same way as the hydrolysis of carboxylates (Krisch, 1963; Krisch, 1966). The hydrolysis of the local anaesthetic N-(n-butylamino)acetyl 2-chloro-6-methylanilide was competitively inhibited by ethylacetate and vice versa, indicating that the same catalytic site was responsible for both hydrolyses (Reimann, 1969; as reported by Krisch, 1971).

Carboxylesterase is also capable of transferring acyl groups to nucleophilic receptors other than water. Bergmann and Wurzel (1953) demonstrated transfer of the acyl moiety from glycine ethyl ester to hydroxylamine to form the hydroxamic acid. The ability of highly purified beef liver carboxylesterase to synthesise dipeptides from appropriate amino acid esters is now well established (Krenitsky and Fruton, 1966; Benohr and Krisch, 1967; Goldberg and Fruton, 1969).

The pH optimum for mammalian carboxylesterase has been consistently reported to be between 7.5 and 9.0 (Krisch, 1963; Benohr and Krisch, 1967; Connors et al., 1950), and maxima are broad. Rat liver microsomes hydrolysing methylbutyrate showed an activity maximum between 7.5 and 10, but a peak at 7.5 when inhibited by acetone (Arndt et al., 1973).

The active site of carboxylesterase has been elucidated using radiolabelled diisopropylfluorophosphate. Enzymes from chicken liver, ox liver, sheep liver, pig liver (Augusteyn et al., 1969), horse liver (Jansz et al. 1959), and pig kidney (Heyman et al. 1970) all have the same four residues surrounding the
serine in the active site:

Gly-Glu-SerP-Ala-Gly
(P = phosphorylated)

1.4.6 INHIBITION OF ESTERASES

The inhibition of acetylcholinesterase by various agents disturbs the function of cholinergic nerves to the point where they can become totally inoperative. Such inhibition is usually fatal and the individual dies of respiratory failure. Curiously the chronic inhibition of butyrylcholinesterase (Kutty, 1980), and carboxylesterase/amidase has no apparent deleterious effect. The most important inhibitors of esterases are the organophosphorus compounds that were developed initially as pesticides and later as "nerve gases" in the 1940s. The chemistry and biology of these compounds has been reviewed at length by Saunders (1957), Holmstedt (1963) and Aldridge and Heiner (1972). In addition to the organophosphorus compounds there are many inhibitors that reversibly inhibit esterases. Both reversible and irreversible inhibitors of varying selectivities exist, and a summary of the more commonly used is given in table 1.9..

Reversible inhibition

There are four types of reversible inhibitors. The first type, competitive inhibitors, act so as to decrease the affinity of the substrate for the enzyme (decrease \( K_m \)). They do this by either competing for the same binding site as the substrate, or
## TABLE 1.9

**ESTERASE SELECTIVITIES OF SOME INHIBITORS**

<table>
<thead>
<tr>
<th>ESTERASE</th>
<th>AChE</th>
<th>BuChE</th>
<th>carboxyl</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>INHIBITOR</td>
<td></td>
<td></td>
<td></td>
<td>esterase</td>
</tr>
<tr>
<td>284C51</td>
<td>x</td>
<td></td>
<td></td>
<td>Austin &amp; Berry (1953)</td>
</tr>
<tr>
<td>3116CT</td>
<td>x</td>
<td></td>
<td></td>
<td>Funke et. al. (1954)</td>
</tr>
<tr>
<td>WIN8077</td>
<td>x</td>
<td></td>
<td></td>
<td>Arnold et. al. (1953)</td>
</tr>
<tr>
<td>DFP</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>Hawkins &amp; Mendel (1947)</td>
</tr>
<tr>
<td>Mipafox</td>
<td></td>
<td></td>
<td></td>
<td>Aldridge (1953)</td>
</tr>
<tr>
<td>isoOMPA</td>
<td></td>
<td>x</td>
<td></td>
<td>Aldridge (1953)</td>
</tr>
<tr>
<td>Ethopropazine</td>
<td></td>
<td>x</td>
<td></td>
<td>Todrick (1954)</td>
</tr>
<tr>
<td>Astra 1397</td>
<td>x</td>
<td></td>
<td></td>
<td>Augustinsson (1955)</td>
</tr>
<tr>
<td>bisPNP</td>
<td></td>
<td></td>
<td>x</td>
<td>Mentlein et. al. (1979)</td>
</tr>
</tbody>
</table>

Structures given overleaf.
by combining with the enzyme so as to sterically hinder the binding of the substrate. The second type, noncompetitive inhibitors, combine with the enzyme at a site distant from the catalytic site so as to alter the tertiary structure of the enzyme and prevent the binding of substrate. In noncompetitive inhibition the Km remains unaffected but the overall activity of the enzyme is reduced (decreased Vmax). Whichever mechanism applies the end result is the same. There is a net decrease in the amount of substrate hydrolysed per unit time. The third type is a mixed competitive and noncompetitive inhibition where the inhibitor can bind either to the enzyme or the enzyme-substrate complex. This type of inhibitor produces an increase in Km but a decrease in Vmax. The fourth type is uncompetitive inhibition where the inhibitor combines with the enzyme-substrate complex causing a decrease in Km and Vmax.

Irreversible inhibition

Some irreversible inhibitors of esterases are used as insecticides, these inhibitors are predominantly carbamate and organophosphate esters. As detailed above (p35), these compounds are hydrolysed in an analogous way to substrate esters with the exception that the carbamylated or phosphorylated enzyme intermediate is stable.

Inhibitors are useful analytical tools when they are selective for one type of esterase. Selectivity of reversible inhibitors is a function of the affinity of the inhibitor for the enzymes involved. If an inhibitor has 100 times more affinity for
DFP

$$(\text{CH}_3)_2\text{CH} \quad \text{P} \quad \text{F}$$

Diisopropylfluorophosphate

3116CT

$$\text{HO} \quad \text{C} \quad \text{H}_2 \quad \text{O} \quad \text{N}(\text{CH}_3)_3 \quad \text{OH}$$

Bis (3-dimethylamino-5-hydroxyphenoxy)-1,3-propane diiodide

BW 284C51

$$\text{CH}_2=\text{C} \quad \text{H}_2 \quad \text{CH} \quad \text{N} \quad \text{+} \quad \text{CH}_2 \quad \text{CH} \quad \text{C} \quad \text{H}_2$$

1,5-bis (4-allyldimethylaminoniumphenyl)pentane-3-one diiodide

ethopropazine

$$\text{CH}_3 \quad \text{N} \quad \text{CH}_2 \quad \text{CH}_3 \quad \text{CH}_2 \quad \text{CH}_3$$

10H-(2-diethylamino-1-propyl)phenothiazine hydrochloride

WIN8077

$$\text{Cl} \quad \text{CH}_2 \quad \text{N} \quad \text{+} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{NH} \quad \text{C}_2 \quad \text{H}_5 \quad \text{C}_2 \quad \text{H}_5$$

$$\text{Cl} \quad \text{2Cl}^-$$

N,N-bis(diethyl-2-chlorobenzyl) oxamide dichloride

isoOMPA

$$\text{CH}_3(\text{CH}_3), \text{CH} \quad \text{NH} \quad \text{HN} \quad \text{CH}(\text{CH}_3), \text{CH}_3$$

$$\text{CH}_3(\text{CH}_3), \text{CH} \quad \text{NH} \quad \text{HN} \quad \text{CH}(\text{CH}_3), \text{CH}_3$$

tetramonoisopropyrophosphortetramide

Mipafox

$$\text{F} \quad \text{P} \quad \text{N} \quad \text{CH}_3 \quad \text{CH}_3$$

N,N'-Diisopropyl phosphorodiamidic chloride

bis-pNP

$$\text{O}_2 \quad \text{N} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{N}_2$$

bis-pNitrophenylphosphate

Formulae of some esterase inhibitors
enzyme A than enzyme B it will inhibit enzyme A by 50% at 1/100 the concentration necessary to inhibit enzyme B by 50%.

Aldridge and Reiner (1972) point out that for organophosphate inhibitors the relative rates of phosphorylation for different enzymes determines the selectivity. If enzyme A is phosphorylated 500 times faster than enzyme B at a given point in time enzyme A will be 99% inhibited whilst enzyme will be only 1% inhibited. At this time the inhibited portion of a mixture of A and B will be 99% A. Timing is clearly critical in such experiments.

For both reversible and irreversible inhibitors the degree of selectivity is concentration dependent. Increasing the concentration will eventually inhibit sensitive and insensitive enzymes alike.

1.7 ESTERASES IN SKIN

Since Porter (1916) first showed that skin was capable of hydrolysing tributyrin, several groups of workers have attempted to further define and utilise the hydrolytic capacity of this tissue. The use of esters of choline and of naphthyl and indoxyl acetates has led to the description of both "cholinesterases" and "nonspecific esterases" in skin.

Choline esterases

Magnus and Thompson (1954) showed that the activity of butyrylcholine hydrolysis falls postmortem, but that of
acetylcholine remains similar to that in premortem samples. These workers also claim that female skin has a higher activity than male skin, but the variation in their data between subjects is large and the results are therefore not conclusive. Butyrylcholine was shown to be hydrolysed by a different esterase to that which hydrolyses tributyrin by its greater sensitivity to eserine. The butyrylcholine esterase was located preferentially in the dermis while epidermal hydrolysis of acetyl-β-methylcholine could not be detected.

Studies on the percutaneous absorption of the organophosphorus compounds sarin (Fredriksson, 1958), parathion (Fredriksson, et. al., 1961), and paraoxon (Fredriksson, et. al., 1961; Fredriksson, 1964) indicated that the hydrolysis of these esters occurs during absorption. Further studies in skin homogenates from rats, mice, and guinea pigs, showed the hydrolysis of sarin catalysed by homogenates of skin was virtually abolished by heating the homogenates to 90 C for 25mins. Non-heated skin homogenates were capable of hydrolysing acetylcholine, butyrylcholine and methylcholine (Fredriksson, 1958).

Cotton et. al. (1973) confirmed the hydrolysis of choline esters by human skin. Cotton et.al. (1973) identified two hydrolases, on the basis of their kinetic parameters, in both soluble and particulate fractions from skin of both normal and atopic patients. Cotton and Van der Hurk (1972) developed a fluorometric assay for the hydrolysis of 2-naphthylacetate by guinea pig skin. Using this assay they showed that the skin
esterases were insensitive to neostigmine at concentrations that inhibited bovine erythrocyte acetylcholinesterase. The multiplicity of the enzymes hydrolysing 2-naphthylacetate was indicated by the kinetically complex inhibition with propanolol.

Nonspecific esterases

Findlay (1955) showed homogenates of human skin to be capable of hydrolysing short chain fatty acid esters of naphthol. The esterases had a maximal activity towards the butyl ester, but inclusion of polyethylene glycol in the incubates shifted the maximum to the pentyl ester.

Pannatier et al. (1981b) investigated the activity of mouse skin homogenates against a series of p-nitrobenzoate esters. The activity was found to increase up to an acyl chain length of four carbon atoms, and branching of the chain alpha to the ester link caused a fall in activity. These enzymes were found to be associated with the cytosol fraction and to be susceptible to heat denaturation (60°C for 10 mins reduced the activity by about 75%).

Findlay (1955) also showed, histochemically, that the naphthylacetate hydrolases were located in the basal layer of the epidermis and in the linings of the sweat ducts. These results should be compared to those of Angyris (1956) who showed naphthylacetate hydrolases in the epidermis, follicular tissue, and panniculus carnosus of the mouse. The activity of these esterases increased as the tissues containing them proliferated.
following the stimulation of the hair growth cycle by plucking.

Maruyama et al. (1980) have located the naphthylacetate esterases in the stratum granulosum of the mouse within the dendritic Langerhans cells.

1.8 OBJECTIVES AND SCOPE OF RESEARCH

The topical application of drugs in the form of esters that are hydrolysed by the skin has been exploited as a method of administering drugs. This combined with toxicological effects of esters used as solvents industrially (table 1.6), make a knowledge of the esterolytic capacity of the skin useful. Practically it is often sufficient to know that a particular ester is hydrolysed by the skin when applied to its surface, but a knowledge of the esterases present in the skin would have considerable predictive value. The differences between the esterases in human and in rat skin would also be useful, since most toxicological testing is done on rodents. A knowledge of the characteristics of skin esterases might also lead to a better understanding of their biological role(s).

A full characterisation of skin esterases would ideally entail the purification of the individual enzymes to a high degree of purity and the use of many substrates and inhibitors of different specificities. In this study a limited number of substrates and inhibitors have been used in conjunction with electrophoretic and physicochemical separation techniques to define the number and types of enzymes present in the skin, and
to compare them with esterases in other tissues.
2.1 MATERIALS

Acetylthiocholine, butyrylthiocholine, 1-naphthol and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), were obtained from BDH (Poole, Dorset). Electrophoresis reagents and standard proteins were from the British Drug Houses 'electran' range. Ethopropazine, UDP-glucuronic acid, N-ethylmaleimide, 1,5-bis(4-allyldimethylaminoniumphenyl)pentane-3-one (BW284C51), diisopropylfluorophosphate (DFP) 5-bromoindoxylacetate, indoxyl acetate, p-nitrophenyl acetate, p-nitrophenyl phosphate, NADPH, NADP, glucose-6-phosphate, and 2(4-indophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium were obtained from Sigma Chemicals ltd (Poole, Dorset).

All other reagents were analytical grade unless otherwise specified.
2.2 ANIMALS

The rats used in this study were male albino Porton or Surrey University strain rats aged 45 to 54 days.

The human tissue used was frozen in liquid nitrogen as soon as possible after excision. The tissue was obtained from mammary reduction operations and showed no overt pathology. Subcutaneous fat was scraped away with a scalpel before freezing in liquid nitrogen where possible, or before mincing when the tissue arrived in the laboratory already frozen.

2.3 STORAGE OF TISSUE AND TISSUE EXTRACTS.

Rat tissue was used within 15 mins of sacrifice. Human tissue, frozen in liquid nitrogen, was thawed overnight on ice in a cold room, prior to homogenisation. Subcellular fractions were stored frozen at -20°C after preparation, when they could not be assayed on the same day. Between successive stages in the separation procedures protein extracts were freeze dried in an Edwards model EF03 freeze drier. This served to both store (at -20°C), and to concentrate the protein. The high speed supernatant that was used for characterisation experiments was also stored freeze-dried at -20°C.
2.4 TISSUE PREPARATION

2.4.1 HOMOGENATE PREPARATION

Rat tissue used for biochemical studies was taken from a 9cm by 3 cm area of the mid dorsal surface. This area extended 1.5cm either side of the mid-dorsal line and from the point of articulation of the fore limbs to the point of articulation of the hind limbs. The full thickness of the skin, down to and including the panniculus carnosus was taken for study.

Initial disruption of the tissue was achieved using a Spong mechanical mincer. The minced tissue was then weighed and homogenised by one of two methods.

Method 1 - Silverson homogenisation

The minced tissue was added to four times its own volume of 0.15M KCl and homogenised for 40 seconds in ten second bursts, cooling in ice between periods of homogenisation sufficient to keep the homogenate temperature between 0°C and 4°C. The resulting homogenate was strained through two thicknesses of surgical gauze and further homogenised with three passes of a Potter-Elvehjem glass-teflon homogeniser. This homogenate was then centrifuged at 800xg for ten minutes and the supernatant from this centrifugation used as the starting material for enzyme studies and purification procedures.

Method 2 (Moloney, 1980) The minced material was spread as thinly as possible in a porcelain mortar. It was then frozen by pouring liquid nitrogen on to it and was maintained in a frozen
state by the addition of more liquid nitrogen as required. The tissue was then pulverized using a wooden handled pestle (until the pieces were small enough to be homogenised with a glass/teflon homogeniser). Using a plastic spatula the ground tissue was scraped from the mortar and resuspended in four times its initial wet weight of 0.15M KCl prior to homogenisation by three passes of the Potter-Elvejhem homogeniser. The frozen tissue thawed during resuspension and the temperature of the suspension was not allowed to rise above 4°C during this process.

2.4.2 PREPARATION OF SUBCELLULAR FRACTIONS

Subcellular fractions were prepared from homogenates by ultracentrifugation. Homogenates were first centrifuged at 800xg for 10 mins. in an M.S.E. HS 18 centrifuge, to remove any unhomogenised material and nuclei. The supernatant was then centrifuged at 14,000xg, in an M.S.E. HS 18. The 14,000xg supernatant was further centrifuged at 100,000xg for 60mins, in a Beckman L2-65B, or similar refrigerated ultracentrifuge.

Pellets were resuspended in 0.15M KCl (buffered with 0.05M tris-HCl to pH 7.4). Pellets were not washed to keep the procedure as short as possible.

2.5 SEPARATION PROCEDURES

2.5.1 AMMONIUM SULPHATE PRECIPITATION

Saturated ammonium sulphate (pH 7.4 with ammonia) solution at
4°C was added, slowly with stirring, to samples of the 100,000xg supernatant, prepared as described above, to give the required final percentage saturation. The volume added was calculated using equation 2.1.

\[ V' = \frac{V(S - S')}{100 - S} \]

Where \( V' \) = volume original solution
\( V \) = volume saturated ammonium sulphate added
\( S' \) = required % saturation
\( S \) = % saturation original solution

Solutions were allowed to equilibrate for 1 hour at each level of saturation before the precipitate was sedimented by centrifugation at 30,000xg in an M.S.E. HS18 centrifuge. The sediment was resuspended in phosphate buffer. The pH of both saturated ammonium sulphate and resuspension buffer was 7.4, and 8.0 in later experiments (see chapter 4). Each resuspended pellet was either dialysed twice against more than ten times its own volume of buffer for at least 8 hours, or desalted on Sephadex G25.

2.5.2 MOLECULAR EXCLUSION CHROMATOGRAPHY

Samples chromatographed on Sephadex G200 were applied to the top of a 1.6cm(diam) x 70cm column of gel in a volume of 2ml. The density of samples was increased by the inclusion of 4% sodium chloride and the samples were applied either directly under the eluent or with a sample applicator loop. The protein was then
eluted with 0.1M phosphate buffer (pH 7.4, or pH 8.0 in later experiments) at a flow rate of 4ml/hr; and 2ml samples were collected.

Samples desalted on Sephadex G25 were treated in the same way as those described above. They were made up in a volume of less than 10ml, applied to a 2.4cm(diam) x 30cm column, and eluted at 20ml/hr; 4ml fractions were collected. To concentrate the samples by freeze drying without simultaneous precipitation of buffer salt, the protein was eluted using 0.01M phosphate buffer.

Glass columns (L.K.B. and Amicon), the L.K.B. 2113 "Microperpex" peristaltic pump, and 2112 "Redrac" fraction collector were used. The absorbance of the eluent at 280nm was measured using an L.K.B. 2300A Uvicord II.

2.6 ENZYME ASSAYS

Acid phosphatase

Acid phosphatase was assayed by the method described by Moloney (1980), in a medium containing 75mM citrate-citric acid buffer pH4.9. Up to 0.1mg of tissue protein was added into a final volume of 3.5ml and the reaction was started by the addition of 4-nitrophenylphosphate (12mM final concentration). Incubation was continued for 20mins at 37°C. The reaction was stopped by the addition of 1.0M NaOH (2.5ml) and the reaction mixture was centrifuged in a bench centrifuge, for 10mins. The formation of p-nitrophenol was estimated by the increase in
β-Galactosidase was assayed by the hydrolysis of p-nitrophenylβ-D-galactoside. The final incubate volume of 1ml contained 0.1M acetate buffer (pH 5.0), 5mM p-nitrophenyl -D-galactoside, and 1 to 2 mg tissue protein. The reaction was started by the addition of enzyme source after a preincubation of 5mins. at 37°C. The reaction was stopped by the addition of 2.5ml of 1M NaOH after a 45min incubation at 37°C and the p-nitrophenol formed determined by its absorbance at 400nm. Tissue and substrate blanks were essential and p-nitrophenol standards were run concurrently with each assay.
absorbance at 405nm. The concentration was then calculated using an extinction coefficient of 18530M⁻¹cm⁻¹.

Succinic dehydrogenase

Succinate dehydrogenase was assayed by a modification of the method of Pennington (1961), and Porteus and Clark (1965). The incubation medium contained 75mM sodium succinate (or 75mM sodium malonate in the blanks), 50mM phosphate buffer pH 7.4, 2mM EDTA, approximately 0.3mg of tissue protein and 0.25mg 2(4-indophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium (INT) in a final volume of 0.5ml. The reaction was started by the addition of the tissue. Following a 15min incubation at 37°C the reaction was stopped by the addition of 10% w/v trichloroacetic acid (1ml) and measured spectrophotometrically at 490nm using an extinction coefficient of 20100M⁻¹cm⁻¹.

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was assayed by the method described by Molony (1980) in a medium containing 1.0mM NADP, 6mM magnesium chloride, up to 0.3mg tissue protein and 0.1M Tris-HCl buffer pH8.0 in a final volume of 3ml. Samples were incubated at 25°C for 5mins and the reaction started by the addition of glucose-6-phosphate (0.1mM final concentration). The production of NADPH was followed at 340nm. An extinction coefficient of 6220M⁻¹cm⁻¹ was used to calculate the initial rate.
Glucuronyl transferase

Glucuronyl transferase was assayed by a modification of the method of Bock (1974) for the fluorimetric determination of 1-naphthol glucuronide. The incubation mixture contained 5mM UDPGA, 5mM magnesium chloride, tissue equivalent to 0.6mg of microsomal protein, 0.4mM naphthol (added in 100µl of distilled water), 0.1M Tris-HCl buffer pH 7.6 and, when added, 0.1% w/v Brij 35 in a final volume of 0.5ml. The solutions were incubated for 4mins at 37°C after which the reaction was stopped by the addition of ice cold 0.5M glycine-TCA buffer pH 2.2 (1ml). UDPGA was without effect on the fluorescence assay and could be omitted from the control and standard incubations. A standard of 1-naphthyl glucuronide was added to the appropriate tubes. The incubates were extracted for ten minutes with chloroform (6ml) and centrifuged (2000 rpm for 10mins) to remove the unreacted naphthol and precipitate the protein. An aliquot of the aqueous layer (1ml) was added to 1.0M glycine-sodium hydroxide buffer pH 10.6 (1ml) and the fluorescence read at 300nm (excitation) and 334nm (emission), in a Perkin Elmer LS-5 luminescence spectrometer.

Nonspecific esterase (indoxylic acetate)

Indoxylacetate esterase activity was assayed by a modification of the method described by Shephard and Hubscher (1969). The incubation medium contained in a final volume of 3ml, 50mM phosphate buffer pH 6.8, 1mM EDTA, 0.1% Triton X-100 and up to the equivalent of 0.8mg of cytosolic protein. After a five
minute preincubation at 25°C the reaction was started by addition of indoxylacetate to give a final concentration of 2mM (added in 50μl of methanol) and the reaction was followed at 386nm for up to 10mins. An extinction coefficient of 3000M⁻¹cm⁻¹ was used to calculate initial rates.

Nonspecific esterase (p-nitrophenylacetate)

p-Nitrophenylacetate esterase was assayed by a modified method of Huggins and Lapides (1947). The incubate contained the equivalent of 0.8mg cytosolic protein in 0.1M phosphate buffer (pH7.0) After 5mins preincubation at 25°C the reaction was started by the addition of substrate (8mM) in 0.3ml ethanol. The production of p-nitrophenol was followed at 400nm for up to 10mins in a Pye-Unicam SP1800. An extinction coefficient of between 90,000 and 120,000M⁻¹cm⁻¹ was determined for each experiment.

Choline esterases

Thiocholine esterase was assayed by the method of Ellman et al. (1961). The final incubate volume of 3ml contained, 0.1M phosphate buffer pH 8.0, 0.01M DTNB, and up to the equivalent of 0.8mg of cytosolic protein. After a 5min preincubation at 37°C, the reaction was started by the addition of substrate, (either acetylthiocholine (2mM) or butyrylthiocholine (10mM), in 80μl of 0.1M phosphate buffer pH 7.0), and monitored for up to 30 mins at 412nm, in a Pye-Unicam SP1800. An extinction coefficient of 13600M⁻¹cm⁻¹ was used in calculations.
2.7 INHIBITORS

Inhibitors were added to the incubation mixtures (10μl/ml incubate) to give the desired concentration in the incubate. The solvents used differed with each individual inhibitor and are shown in table 2.1. Control incubations contained 10μl/ml of the appropriate solvent. DFP was added directly to the enzyme preparation and preincubated at 37°C. Aliquots were then diluted to a final volume of 3ml with incubation medium containing substrate and the reaction was monitored at the appropriate wavelength for at least 5mins.

2.8 PROTEIN ASSAYS

Lowry et. al. assay

Protein assayed by the method of Lowry et. al. (1951) was treated as follows. Tissue protein was diluted with water into the range 50μg/ml to 250μg/ml. 2.5ml of stock solution C was then added to 0.5ml of diluted protein, and incubated at room temperature for 10mins. To this solution 0.5ml of solution D was added and incubated at room temperature for 30mins. The resulting blue colour was read at 750nm. All solutions were added with vortex mixing, and bovine serum albumin (fraction V) standards were run concurrently with each set of test solutions (50-500μg/ml).

Solution A - 2% sodium carbonate in 0.1M sodium hydroxide
<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>SOLVENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethopropazine</td>
<td>ethanol</td>
</tr>
<tr>
<td>BW284C51</td>
<td>ethanol</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.1M phosphate buffer</td>
</tr>
<tr>
<td></td>
<td>pH 7.0</td>
</tr>
<tr>
<td>Di-isopropyl-fluorophosphate (DFP)</td>
<td>propan-2-ol</td>
</tr>
</tbody>
</table>
Solution B - 0.5% cupric sulphate pentahydrate and 1% sodium tartrate in water

Solution C - 1 part B in 50 parts A

Solution D - 1:3 dilution Folin-Ciocalteu's reagent in water

The Lowry assay was used in all routine protein determinations unless otherwise stated.

The Biorad assay was carried out according to the instructions published by Biorad laboratories (Watford, Hertfordshire, England). The reagent was diluted 1:4 with water and filtered prior to use. 5ml of reagent was then added to 0.1ml of standard/sample, mixed with a vortex mixer, and read at 595nm between 5mins and 1 hour later. Bovine serum albumin (fraction V) standards (0.2 to 1.4 mg/ml) were run concurrently with each set of samples.

2.9 POLYACRYLAMIDE GEL ELECTROPHORESIS (P.A.G.E.)

Polyacrylamide gel electrophoresis was used to separate and analyse the protein and esterases of subcellular fractions. The method of Laemmli (1970) was used to analyse the proteins, and this method was modified by the omission of SDS and 2-mercaptoethanol from the buffers when used to assay enzymes. The protein solutions were first diluted with the appropriate dilution buffer then separated on a discontinuous buffer system. Table 2.2 summarizes the solutions used and their proportions.
### TABLE 2.2

Table of mixtures for polyacrylamide gel electrophoresis

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>RUNNING GEL</th>
<th>STACKING GEL</th>
<th>ADDITION FOR SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>7.5%</td>
<td>3%</td>
<td></td>
</tr>
</tbody>
</table>

30%(w/v) acrylamide

0.8%(w/v)

N,N'-Methylenebis-acrylamide in dd-water

13.3ml 10ml 1ml

1.5M Tris-HCl (pH 8.0) 10ml 10ml - 0.4%(w/v) SDS

500mM Tris-HCl (pH 6.8) - - 2.5ml 0.4%(w/v) SDS

Tetramethyl-ethylenediamine 20μl 20μl 20μl -

10% ammonium persulphate 240μl 240μl 60μl - (made up fresh)

Cont'd......

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TABLE 2.2 cont'd

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>RUNNING GEL</th>
<th>STACKING GEL</th>
<th>ADDITION FOR SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>16.7ml</td>
<td>20ml</td>
<td>6.5ml</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>7.5%</td>
<td>3%</td>
</tr>
<tr>
<td>62.5mM Tris-HCl (pH6.8) plus</td>
<td>Buffer used to dilute samples prior to application to gel (glycerol diluted to 10% in final solution)</td>
<td>2.3%(w/v) SDS</td>
<td>5%(w/v) mercapto-ethanol</td>
</tr>
<tr>
<td>40% glycerol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001% bromophenol blue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25mM Tris-HCl (pH8.3) plus</td>
<td>Buffer used to fill tank reservoirs</td>
<td>0.1%(w/v) SDS</td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
in the different gels.

The gel was made in a glass and perspex cuvette, clipped together with "Bulldog" clips and sealed with molten agar. The internal dimensions of the assembled cuvette were approximately 120mm x 100mm x 1.5mm. Electrophoresis was carried out at room temperature. The glass plates and perspex spacers were kept in a solution of "Decon" when not in use. Immediately prior to use they were rinsed in distilled water, ethanol, and finally (glass plates only) acetone, then allowed to dry.

The cuvette was clamped in a vertical position and the seal produced by the molten agar checked by filling with water whilst the gel solutions were prepared. With the water removed from the cuvette the running gel was poured in up to a height of 8cm to 9cm above the inner edge of the bottom perspex spacer. A layer of water was carefully run onto the surface of the gel to prevent dehydration during setting.

When the running gel had set the water layer was removed and the remaining space in the cuvette filled with stacking gel. A perspex comb was then introduced into the top of the cuvette so as to produce wells in the stacking gel that would hold discrete samples. When the stacking gel had set the comb and bottom spacer were removed from the cuvette and it was clamped upright in the electrophoresis tank. Both lower and upper reservoirs were filled with buffer and a potential applied across the gel using a constant current power pack (Vokam SAE 2761). For the SDS system a current of 20mA was maintained until the bromophenol blue line
entered the running gel when the current was increased to 40mA for the rest of the separation. The running time was 3-4hrs. In the non-SDS system it was found that the current had to be increased to 30mA and 50mA respectively to maintain the same running time. This could be done without causing excessive heating of the gel. Alternatively the experiment was run at a constant voltage of 40vts/gel(3.3V/cm), then at a constant current of 40mA to completion. When the Bromophenol blue line was within 5mm of the bottom of the gel the cuvette was removed from the tank, the plates separated and the gel was stained with the appropriate stain.

The solubilising agent saponin (1%) was included in all the buffers for the electrophoresis of particulate fractions and homogenates.

2.10 PREPARATIVE P.A.G.E.

Preparative electrophoresis was carried out using the same solutions as the analytical, slab gel, electrophoresis described above. On a preparative scale however the gel was cast in an apparatus manufactured by Shandon Ltd. (Fig 2.1). With the central portion of the apparatus placed firmly on a polythene covered perspex board, the running gel was poured into the canal between the outer water jacket and the "cold finger", and allowed to set with a layer of water on its upper surface to prevent drying out. When the running gel had set the water layer was poured away and the stacking gel poured on top of the running gel. After the running gel had set the apparatus was assembled as
shown in fig.2.1 and running buffer added to the upper and lower buffer reservoirs. Air was aspirated from the bottom of the lower cone using a syringe and plastic capillary tube. The apparatus was run overnight and cooled by running water through the water jacket and "cold finger" (8-12°C).

The lower cone was filled with gel by inverting it on a polythene covered perspex board, a thin layer of silicon grease on the cone flange ensured a good seal. The inverted cone was then filled with liquid gel, and covered with a piece of polythene sheet to prevent the gel drying out as it set. Care was taken to expel all the air from between the gel and this polythene cover. The gel filled cone was good for two or three experiments provided that it was stored in a cold room and covered with polythene to ensure that it did not dry out.

Elution buffer (25mM Tris-HCl, pH8.3) was pumped through the collecting ring at 0.25ml/min, unless otherwise stated. Fractions of 2.5ml were collected in an Gilson TDC 80 fraction collector and protein elution was monitored by absorbance at 280nm using an LKB Uvicord.
Diagram of the Shandon preparative electrophoresis apparatus
2.11 STAINING GELS

2.11.1 STAINING FOR ESTERASE ACTIVITY

Naphthylacetate

Gels were removed from the cuvette, the stacking gel scraped away and incubated in 0.25M phosphate buffer (pH 6.25) for 15mins at 37 C. The buffer was then removed with a large syringe and replaced with stain mixture. The stain mixture was made up immediately prior to use: to 50ml of distilled water was added 0.8ml of naphthylacetate stock solution (1% naphthylacetate in 1:1 acetone:water), with stirring. Approximately 20mg of fast blue B salt was then added. If necessary the mixture was filtered prior to use (Paul and Fottrell, 1960). Gels were destained in 5:5:1::methanol:water:acetic acid.

When the gels were to be quantified by scanning densitometry, the staining time was 10mins and the loading of the gel was not more than 2 nmol/min p-nitrophenylacetate hydrolysing capacity.

Inhibitors were included at the required concentration in both the preincubation medium and the stain mixture.

2.11.2 STAINING FOR PROTEIN

Gels that were stained for protein were stained overnight in Coumassie Blue (0.5g Coumassie Blue in 2.5:1:6.5 propan-2-ol:acetic acid:water). The gels were then destained in
1:1:8 propan-2-ol:acetic acid:water.

2.12 HISTOLOGY

Skin was fixed in Bouins fixative for 48 hours and then stored in 70% ethanol until processed. Samples were embedded in wax after the following treatment in a Histokinette (Shandon).

80% ethanol.....................3 hours
90% ethanol.....................3 hours
90% ethanol.....................3 hours
Absolute ethanol..............2 hours
Absolute ethanol..............2 hours
Absolute ethanol..............2 hours
Xylene/ethanol................2 hours
Cedar wood oil................2 hours
Cedar wood oil................2 hours
Xylene..........................1 hour
Wax.............................2 hours
Wax.............................2 hours

Sections were cut on a Sledge Microtome set at 5mm, floated on water (60°C) and transferred to microscope slides and dried.

Sections were then stained with Massons trichrome stain as follows:-

1)Sections to water.
2)Stained in Weigarts Haematoxylin (25mins)
3)Rinsed in tap water, differentiated in acid alcohol if
necessary.

4) Stained in red solution - 5mins
5) 5% phosphotungstic acid for 15mins
6) Rinsed briefly in distilled water.
7) Stained in 2% light green in 0.8% acetic acid (2mins)
8) Rinsed in distilled water.
9) 1% acetic acid for 3-5mins
10) Dehydrated, cleared and mounted in dibutyl phthalate in xylene (DPX)

Red solution

1% Biebrich scarlet.............. 90ml
1% Acid Fuchsine................ 10ml
Glacial acetic acid............... 1ml

2.13 HISTOCHEMISTRY

Sections were fresh frozen by placing skin tissue on a metal block in a steam of carbon dioxide from a compressed gas cylinder. A drop of distilled water placed between the tissue and the block served to hold the tissue in place once frozen. The orientation of the tissue on the block was critical and in these experiments transverse sections of the skin were cut. The block was secured in a Slee crystat microtome and 10nm sections cut using disposable blades. The sections were transferred direct from the microtome blade to microscope slides and stained with either 5-bromoindoxyylacetate or naphthylacetate.
5-Bromoindoxylacetate (from Chayen et. al., 1973)

Reaction medium

0.2M Tris buffer (pH8.5) .................. 2ml
Oxidant .................................... 1ml
1M calcium chloride  ...................... 0.1ml
2M sodium chloride ........................ 5ml
Distilled water ............................ 2ml

Oxidant

Potassium ferricyanide .................. 210mg
Potassium ferrocyanide ............ 155mg
Distilled water .......................... 100ml

To the reaction medium was added 0.25ml of absolute ethanol containing 1.3mg of 5-bromoindoxyl acetate (with stirring). Sections were incubated for 20mins in this solution and counter stained for a few seconds with nuclear fast red, dehydrated, cleared, and mounted in DPX.

Naphthylacetate (modified from Chayen et. al., 1973)

To 50ml of 0.25M phosphate buffer (pH6.25) was added 0.8ml of a 1% solution of naphthylacetate in 50:50 acetone:water, with stirring. Approximately 20mg of fast blue B salt was then added and the final stain mixture filtered if necessary.

Sections were incubated in this mixture for 10mins at 37°C dehydrated, cleared, and mounted in DPX.
CHAPTER 3

TISSUE PREPARATION

AND THE

MULTIPLICITY OF ESTERASES

IN THE

SKIN OF THE RAT
3 TISSUE PREPARATION AND INVESTIGATION OF THE MULTIPLICITY OF ESTERASES IN THE SKIN OF THE RAT

3.1 INTRODUCTION

Many techniques for the study of foreign compound metabolism are available. Methodologies range from the simple administration of chemicals to whole animals, to the use of cell free extracts as a source of metabolic activity. Each procedure has its attendant advantages and disadvantages, for example it may be difficult to identify the relevance of findings in in vitro experiments to the likely effects in whole animals. While in vivo, such a large number of factors may contribute to the observed effect that interpretation of experimental results becomes difficult.

The aim of the first part of the investigation was to decide which method of preparation could be used to study the esterase content of skin. Two methods of homogenisation were selected for assessment of their efficiency in rupturing cells and for maintaining the integrity of subcellular organelles. The first used the Silverson rotating blade homogeniser with a 0.076mm clearance between the cutting surfaces which operates by repeatedly cutting the tissue. The second utilised the fact that tissue becomes brittle when frozen in liquid nitrogen and may therefore be readily ground and pulverised to produce a preparation suitable for homogenisation in a glass-teflon homogeniser. To assess cell rupture during Silverson homogenisation, the release of the cytoplasmic enzyme
glucose-6-phosphate dehydrogenase into the 100,000xg supernatant was measured.

To assess the organelle damage the distribution of "classical" marker enzymes within the subcellular fractions was determined (ie. succinate dehydrogenase for the mitochondria and glucuronyl transferase for the microsomes. The extent of organelle damage was also assessed by measuring the release of the lysosomal marker acid phosphatase into the medium during Silverson homogenisation.

The second part of this chapter describes the investigation of the multiplicity of the esterases of rat skin using three chromogenic substrates and two inhibitors.

3.2 RESULTS

3.2.1 TISSUE PREPARATION

3.2.1.1 ASSESSMENT OF SILVERSON HOMOGENISATION

Rat skin from three animals was pooled and minced as described in the methods (Chapter 2, p55). The minced tissue was homogenised in separate aliquots using the Silverson homogeniser for different periods of time. Each homogenisation was made up of 10 second periods separated by a period of cooling in ice, sufficient to maintain the homogenate temperature between 0 C and 4 C. These homogenates were then used to prepare 800xg and 100,000xg supernatants for the assay of acid phosphatase and
glucose-6-phosphate dehydrogenase.

As shown in fig 3.1(a), glucose-6-phosphate dehydrogenase was released rapidly into the homogenate so that 72%+/−16% of the experimental maximum was reached after 40 seconds of homogenisation, and 92%+/−4% after 60 seconds. The specific activity of glucose-6-phosphate dehydrogenase rose to a maximum after 20 seconds and remained at that level until 120 seconds when it decreased. This decrease in specific activity in the 100,000xg supernatant is associated with an increase in protein released. Figure 3.1(b) shows that acid phosphatase was also rapidly released into the 800xg supernatant so that 60% of the experimental maximum was attained after 20 seconds. The amount of enzyme released rose to 60% of the experimental maximum after 20 seconds of homogenisation, and then steadily to a maximum after 120 seconds. The specific activity and total protein behaved in a similar manner.

3.2.1.2 ASSESSMENT OF ORGANELLE INTEGRITY

Homogenates prepared by grinding minced tissue in liquid nitrogen and by Silverson homogenisation were used to prepare subcellular fractions. The distribution of protein and the "marker enzymes", succinate dehydrogenase (inner mitochondrial membrane), glucuronyl transferase (microsomal), and β-galactosidase (lysosomal), in the 800xg supernatant (starting material), 14,000xg sediment, 100,000xg sediment, and 100,000xg supernatant, is shown in figure 3.2.(a to d).
Fig. 3.1

AMOUNT OF ENZYME RELEASED INTO HOMOGENATE WITH DURATION OF HOMOGENISATION WITH SILVERSON HOMOGENISER. FOR:

□ - PROTEIN
○ - TOTAL ACTIVITY OF ENZYME
◊ - SPECIFIC ACTIVITY OF ENZYME
■ - ACTIVITY OF 100,000xg SUPERNATANT AS PERCENT OF ACTIVITY IN 800xg SUPERNATANT AFTER 120secs.
DISTRIBUTION OF PROTEIN IN SUBCELLULAR FRACTIONS FROM THE SKIN OF THE "SURREY UNIVERSITY" RAT (MEAN+/-S.E.M., n=3 experiments)

1-800xg SUPERNATANT (STARTING MATERIAL FOR FRACTIONATION)
2-14,000xg SEDIMENT
3-100,000xg SEDIMENT
4-100,000xg SUPERNATANT
DISTRIBUTION OF SUCCINATE DEHYDROGENASE IN SUBCELLULAR FRACTIONS FROM THE SKIN OF THE "SURREY UNIVERSITY" RAT (MEAN+/-S.E.M., n=3 experiments).

1-800xg SUPERNATANT (STARTING MATERIAL FOR FRACTIONATION)
2-14,000xg SEDIMENT
3-100,000xg SEDIMENT
4-100,000xg SUPERNATANT
Fig.3.2c

DISTRIBUTION OF GLUCURONYL TRANSFERASE IN SUBCELLULAR FRACTIONS FROM THE SKIN OF THE "SURREY UNIVERSITY" RAT (MEAN±S.E.M., n=3 experiments).

1-800xg SUPERNATANT (STARTING MATERIAL FOR FRACTIONATION)
2-14,000xg SEDIMENT
3-100,000xg SEDIMENT
4-100,000xg SUPERNATANT
HATCHED PORTION OF BARS REPRESENTS ACTIVATION BY BRIJ-35 (1%).
DISTRIBUTION OF β-GALACTOSIDASE IN SUBCELLULAR FRACTIONS FROM THE SKIN OF THE "SURREY UNIVERSITY" RAT (MEAN+/−RANGE, n=2 experiments)

1-800xg SUPERNATANT (STARTING MATERIAL FOR FRACTIONATION)
2-14,000xg SEDIMENT
3-100,000xg SEDIMENT
4-100,000xg SUPERNATANT

Fig.3.2d
The distribution of protein in the subcellular fractions from homogenates prepared by both techniques is shown in fig. 3.2(a). The largest amount of protein was found in the 100,000xg supernatant, the other fractions containing less than 10% of the total protein of the 800xg supernatant.

As shown in fig. 3.2(b), succinate dehydrogenase was distributed between the two particulate fractions (14,000xg sediments and 100,000xg sediment) using either homogenisation procedure. The 14,000xg sediment showed the highest specific activity of all the fractions whereas no enzyme was detected in the high speed supernatant. The fractions derived from the Silverson homogenisation technique had higher specific activities than those from the liquid nitrogen prepared tissue.

Figure 3.2(c) shows that glucuronyl transferase was also distributed between the two particulate fractions from both preparations. The 100,000xg sediment showed the highest specific activity of this enzyme in both cases, however, the percentage of total activity was the same in the two particulate fractions from the Silverson produced homogenate. The Silverson homogenates alone showed a small activity in the 100,000xg supernatant. Addition of Brij 35 to the incubations activated all the fractions; activation was greater in the 100,000xg sediment from the liquid nitrogen prepared tissue than from the Silverson homogenates (fig. 3.2(c))

Figure 3.2(d) shows that the lysosomal enzyme β-galactosidase was extensively solubilised by both methods of homogenisation.
The percentages of the total activity present in the 100,000xg supernatant from the Silverson produced homogenates and the nitrogen ground tissue were 81% and 77% respectively. Less than 3% of the total activity was present in the particulate fractions from homogenates prepared by either method. In both preparations the specific activity was highest in the 100,000xg supernatant and lowest in the 14,000xg sediment. The specific activity of the 800xg supernatant was only slightly lower than that of the 100,000xg supernatant.

3.2.2 ESTERASES IN RAT SKIN

The specific activity of the 800xg supernatant of a Silverson produced homogenate with each esterase substrate, and the saturating concentration for each substrate is shown in table 3.1. Substrate inhibition by acetylthiocholine above its saturating concentration was found to be small (fig. 3.3).

Figure 3.4 shows the manner in which ethopropazine inhibited the hydrolysis of acetylthiocholine and butyrylthiocholine by classical esterases used as references here, namely bovine erythrocyte acetylcholinesterase and horse serum butyrylcholinesterase respectively. Butyrylcholinesterase ([S]=10mM) was inhibited with an IC50 of 50µM, but acetylcholinesterase ([S]=1mM) was only inhibited by 15%+/-5% at 100µM ethopropazine.

Figure 3.5 shows the inhibition of the ester hydrolases contained in the 800xg supernatant of Silverson homogenised rat
### TABLE 3.1

SPECIFIC ACTIVITIES AND SATURATING SUBSTRATE CONCENTRATIONS DETERMINED EXPERIMENTALLY FOR SILVERSON HOMOGENATES OF RAT SKIN

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>SPECIFIC ACTIVITY (nmol/min/mg)</th>
<th>SATURATING SUBSTRATE CONCENTRATION (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetylthiocholine</td>
<td>4.29±0.29 (11)</td>
<td>4</td>
</tr>
<tr>
<td>butyrylthiocholine</td>
<td>2.22±0.16 (7)</td>
<td>10</td>
</tr>
<tr>
<td>p-nitrophenylacetate</td>
<td>7.5±2.95 (6)</td>
<td>8</td>
</tr>
</tbody>
</table>

Specific activities are means and standard errors of the number of determinations shown in parenthesis.
Fig. 3.3

RELATIONSHIP BETWEEN ACETYLTYCHOLINE CONCENTRATION AND INITIAL REACTION VELOCITY (V) FOR THE HYDROLYSIS OF ACETYLTYCHOLINE BY AN 800xg SUPERNATANT FROM RAT SKIN SILVERSON HOMOGENISED TISSUE

(RESULTS OF A SINGLE EXPERIMENT, PORTON RATS)
INHIBITION OF THE HYDROLYTIC ACTIVITY OF PURIFIED ESTERASES:

- HYDROLYSIS OF ACETYLTHIOCHOLINE BY BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE (E.C. 3.1.1.7), [S]=1mM.

- HYDROLYSIS OF BUTYRYLTHIOCHOLINE BY HORSE SERUM BUTYRYLCHOLINESTERASE (E.C. 3.1.1.8), [S]=10mM.

POINTS ARE MEANS AND S.E.M. (n=3 experiments)
Fig. 3.5

INHIBITION BY ETHOPROPAZINE OF THE HYDROLYTIC ACTIVITY OF THE 800xg SUPERNATANT FROM AN HOMOGENATE OF RAT SKIN (PORTON STRAIN, SILVERSON HOMOGENISED):

- HYDROLYSIS OF ACETYLTHIOCHOLINE ([S]=4mM)
- HYDROLYSIS OF BUTYRYLTHIOCHOLINE ([S]=10mM)
- HYDROLYSIS OF p-NITROPHENYLACETATE ([S]=8mM)

POINTS ARE MEANS +/- S.E.M. OF AT LEAST THREE EXPERIMENTS.
skin. The concentration of ethopropazine causing 50% inhibition (I50) of butyrylthiocholine hydrolysis by skin esterases and serum butyrylcholinesterase were similar (5 μM). In contrast ethopropazine inhibited the hydrolysis of acetylthiocholine ([S]=4mM) in skin to a greater extent than it inhibited either of the two purified enzymes (I50=500nM).

Comparison of the inhibition of the hydrolase enzymes in skin with the purified acetylcholinesterase and butyrylcholinesterase, using the specific acetylcholinesterase inhibitor BW284C51 (10uM) is shown in figure 3.6. Acetylcholinesterase was inhibited by 100% and butyrylcholinesterase by 6%+/-3%. However although the hydrolysis of butyrylthiocholine by the 800xg supernatant of skin was inhibited to the same extent as was purified serum butyrylcholinesterase, the hydrolysis of acetylthiocholine was only inhibited by 18%+/-5%.

The hydrolysis of p-nitrophenylacetate by skin was not inhibited by 10μM BW284C51 (fig 3.6) or by ethopropazine at concentrations up to 100μM (fig 3.5).

3.3 DISCUSSION

3.3.1 ASSESSMENT OF HOMOGENISATION

The homogenisation of a tissue is the first step in any biochemical analysis of its contents. With a soft tissue, such as liver, effective homogenisation can be achieved using a comparatively mild shearing force eg. by means of a glass/teflon...
INHIBITION BY BW284C51 (10μM) OF THE ESTERASE ACTIVITY OF THE 800xg SUPERNATANT FROM AN HOMOCENATE OF RAT SKIN (PORTON STRAIN, SILVERSON HOMOGENISED), AND PURIFIED ESTERASES:

OPEN BARS - HYDROLYSIS OF ACETYLTHIOCHOLINE
HATCHED BARS - HYDROLYSIS OF BUTYRYLTHIOCHOLINE
CROSS HATCHED BARS - HYDROLYSIS OF p-NITROPHENYLACETATE

BARS ARE MEANS +/- S.E.M. OF THREE EXPERIMENTS.
homogeniser (Potter, 1955). Tissues containing appreciable
amounts of collagen however require greater disruptive force to
homogenise the collagenous material and this may result in
considerable organelle damage. Homogenisation of a tissue such as
skin is thus a compromise between complete homogenisation of the
tissue and the maintenance of organelle integrity. In selecting
the correct procedure for a particular experiment the aims of
that experiment must be considered. If, for example, enzymes are
to be assayed in whole homogenates, damage to organelles may not
be critical, so long as the enzymes themselves are not damaged.
Such damage could occur as a direct result of homogenisation (eg.
through local heating effects) or indirectly by proteolytic
enzymes released during homogenisation, eg. from ruptured
lysosomes. Experiments aimed at determining enzyme location in
particular subcellular fractions however, would require more
discerning preparation to minimise cross contamination of
fractions.

The most "gentle" procedure used in this study, grinding
minced tissue in liquid nitrogen, appears to be sufficiently
harsh to cause considerable damage to the particulate fractions.
This was indicated by the contamination of the mitochondrial
fraction (14,000xg sediment) with the microsomal "marker"
glucuronyl transferase, and the contamination of the microsomal
fraction with the mitochondrial "marker" succinate dehydrogenase.

An attempt to reduce contamination of the microsomal fraction
was made by using a relatively high centrifugal force to sediment
the mitochondrial fragments (14,000xg for 20mins). Hepatic
mitochondria can be sedimented using a relative centrifugal force (r.c.f.) as low as 10,000xg for 10mins (Hogeboom, 1975), though r.c.f.s of 15,000xg for 30mins have been used. It is uncertain whether skin mitochondria are identical in this respect.

Previous subcellular fractionation studies in skin have shown cross contamination of particulate fractions (Moloney, 1980; Moloney et. al. 1982). There are several possible explanations of these findings. It is possible that the "marker" enzymes used here, and in previous studies, that are known to be located exclusively in certain hepatic organelles (Mahler and Cordes, 1970) are not restricted to the same organelles in skin. As discussed below there is some evidence to support this view for some lysosomal hydrolases. It is also possible that the skin organelles were damaged during homogenisation and/or tissue homogenisation is incomplete. The former would produce low density organelle fragments that sediment at a higher r.c.f. than the parent organelles, whereas incomplete homogenisation could result in high density organelle aggregates that sediment at a lower r.c.f. than their constituent organelles. The presence of large amounts of structural proteins that could co-associate with the organelles and form denser bodies might also promote low density organelle enzymes into higher density fractions.

It was apparent that for work on enzyme location in subcellular fractions Silverson homogenisation was too robust, because it produced contamination of the soluble fraction with glucuronyl transferase from the endoplasmic reticulum. In liver glucuronyl transferase is thought to be embedded deep within the
the endoplasmic reticular membrane on the basis of experiments where microsomes were treated with trypsin (Vanio, 1973). This accounts for its activation by detergents, storage, and autolysis. The finding that glucuronyl transferase in microsomes from skin is also activated by detergents (Moloney et. al., 1982), indicates that the enzyme in skin might be bound to the microsomal membrane in a similar way to that in liver. If this were so, a considerable amount of organelle damage would be indicated by the solubilisation of glucuronyl transferase during Silverson homogenisation. It is also possible that, since glucuronyl transferase is lipid soluble, there is some post homogenisation reassociation of the enzyme with membranous material. This might cause contamination of particulate fractions not containing microsomes.

In view of the above findings it must be assumed that enzymes that are not so tightly bound to the membranes as glucuronyl transferase would have been solubilised by the Silverson homogenisation, though they may have reassociated with either particulate fraction. Whether this happened to loosely bound enzymes during grinding in liquid nitrogen is not known. In general using either method of homogenisation, the specific activities of the enzymes assayed were highest in the fractions that would be expected on the basis of the study of marker enzymes in liver (Mahler and Cordes, 1971). An indication that the microsomal membranes were not severely damaged by either homogenisation method was given by the activation achieved by Brij 35. This activation was comparable to the activations previously reported in skin (Moloney et. al., 1982), but is only
a tenth of that reported for liver microsomes (Lucier et. al., 1971). This may indicate that the enzyme is nearer the surface of the membranes in skin or, perhaps more likely, partial activation occurs during homogenisation.

Some lysosomal damage is evident with both methods of homogenisation as shown by the distribution of the markers β-galactosidase and acid phosphatase. These enzymes were extensively solubilised, indicating that the lysosomes had been ruptured. During Silverson homogenisation the release of acid phosphatase into the soluble fraction was correlated closely with the release from the cells of glucose-6-phosphate dehydrogenase. This indicates that the release of the lysosomal enzymes was not simply due to "overhomogenisation" and therefore probably could not be avoided in the preparation of an homogenate from skin.

There is histochemical evidence that lysosomes have a special role in the skin. Single membrane bound vesicles staining for acid phosphatase (Eisen et. al., 1964; Olsen and Nordquist, 1966; Rowden, 1967; and Wolff and Schriener, 1970), and arylsulphatase (Olsen et. al., 1968), have been demonstrated in the basal layer of the epidermis. Attempts to isolate lysosomes from rat (Wynn and Iqbal, 1969), and human skin (Ockerman, 1966) have been largely unsuccessful. Typically in such studies the largest amount of enzyme remains in the high speed supernatant, although high specific activities have been obtained in lysosomal fractions prepared from rat and human epidermis (Dicken and Decker, 1966). A possible explanation for these findings is that the lysosomes are closely associated with the developing keratin
in the upper epidermal layers. This is also suggested by the observation that high levels of lysosomal hydrolases occur in the granular layer where the keratohyalin granules become visible. Just above the granular layer lysosome-like particles containing lamellar structures (Odland-Matoltsy bodies), and staining positively for acid phosphatase (Wolff and Schreiner, 1970) have been observed. These bodies subsequently appear to extrude their contents into the extracellular spaces as witnessed by the diffuse staining reported by Eisen et. al. (1964) and Olsen and Nordquist (1966). The extrusion of lysosomal enzymes into the extracellular space would also explain the soluble nature of these enzymes in subcellular fractionation studies of skin. If they were outside the cell before the tissue was disrupted they would obviously remain in the high speed supernatant. The exact role of the hydrolases in the granular layer is not known, though they may be responsible for the final conversion of the keratohyalin fibrils to keratin fibres. Odland-Matoltsy bodies may be concerned with the formation of a keratin glycocalyx that "cements" the cells together in the stratum corneum as speculated by Lazarus and Hatcher (1978).

In conclusion, for further work in which the concern was total tissue hydrolase activity the Silverson homogenisation would be adequate, since there is no apparent damage to the enzymes themselves. The specific activities of the enzymes measured after Silverson homogenisation were not significantly different from those measured after grinding in liquid nitrogen. If subcellular fractions are to be prepared however, grinding in liquid nitrogen is the preferred technique since this causes less
3.3.2 ESTERASES IN RAT SKIN

The first step in the analysis of the ester hydrolysing capacity of a tissue is to determine whether it contains one or several esterases. In this study of an homogenate of rat skin the use of three synthetic substrates and two inhibitors has indicated the presence of at least two esterases, namely:

1) An esterase(s) that hydrolyses butyrylthiocholine, that shows the same sensitivity to ethopropazine as does butyrylcholinesterase (E.C. 3.1.1.8) with an $I_{50}$ of 5 $\mu$M, but is relatively insensitive to BW284C51 (10 $\mu$M, 5.5% +/-3% inhibition).

2) An esterase that hydrolyses p-nitrophenylacetate and is not inhibited by ethopropazine (100 $\mu$M) or BW284C51 (10 $\mu$M).

The apparent higher sensitivity of the acetylthiocholinesterase to ethopropazine is probably an experimental artifact due to the higher substrate concentration used to assay butyrylthiocholinesterase. Increasing the substrate concentration would increase the percentage of enzymic sites occupied by substrate molecules at each concentration of inhibitor and give rise to a higher rate of hydrolysis. This phenomenon is called "substrate protection" of the enzyme.

Some conclusion may be drawn as to whether or not these enzymes are the same as the "classical" acetyl or
butyrylcholinesterases of blood. The butyrylcholinesterase of skin appears to be very similar to that of serum in that the inhibition by ethopropazine and BW284C51 was virtually identical. It is unlikely, however, that a red blood cell-like acetylcholinesterase is responsible for more than 18% of the hydrolysis of acetylthiocholine by skin, because the hydrolysis of acetylthiocholine was only inhibited by 18% by the very effective and specific acetylcholinesterase inhibitor BW284C51 (10μM), whereas acetylcholinesterase (from bovine erythrocytes) was totally inhibited at this concentration. Neither can butyrylcholinesterase be responsible for the remaining acetylthiocholine hydrolysis because the specific activity of the homogenate towards acetylthiocholine is higher than towards the butyryl derivative.

Lysosomal esterases are unlikely to be the source of these skin esterases because all the esterase assays described above were carried out at pH 7.0 and pH 8.0, whereas lysosomal esterases have a pH optimum of 5.0 (Barrett and Heath, 1977). Thus although the lysosomal esterase may retain some activity at higher pH in a similar manner to Cathepsin D (Lazarus and Poole, 1974), but this is likely to be small.

It is also improbable that the esterases cleaving the thiol esters are lipases since lipases require an oxygen rather than a sulphur atom in the ester link (Desnuelle, 1971).

In view of the unknown nature of the esterases identified in
rat skin they have been characterised further with a view to
differentiating between A, B and C type esterases (Aldridge and
Reiner, 1972), cholinesterases, and carboxylesterase. This work
is reported in the following chapters.
CHAPTER 4

THE SEPARATION

OF THE

ESTERASES IN THE SKIN

OF THE RAT
4. THE SEPARATION OF THE ESTERASES IN THE SKIN OF THE RAT

4.1 INTRODUCTION

The separation of biologically active macromolecules is based upon the differences in their physico-chemical properties, and in this respect is similar to the separation of any mixture of simple organic molecules. In contrast to the organic analyst however the enzymologist is limited by the need to retain the biological activity of the enzymes for further analysis. In practice it is often the exception rather than the rule that total recovery is achieved. Dixon and Webb (1971) list some of the many causes of loss of activity during the analysis and separation of enzymes, including temperature, pH, and ionic strength fluctuations, though particularly sensitive enzymes can even be denatured by such mild assault as excessive shaking.

In this study an attempt has been made to separate the esterases in rat skin on the basis of their subcellular location, solubility in water, molecular size, and their electric charge. The objective was to develop a procedure that would produce sufficient amounts of purified enzymes to permit partial characterisation of the individual esterases.
4.2 RESULTS

4.2.1 SEPARATION BY SUBCELLULAR FRACTIONATION

The distribution of p-nitrophenylacetate acetylthiocholine and butyrylthiocholine hydrolases among the subcellular fractions of full thickness rat skin (Surrey University strain) is shown in figure 4.1. All three hydrolases had rather similar distributions. Between 60% and 70% of the total activity remained in the soluble fraction (100,000xg supernatant) the other subcellular fractions contained between 9% and 17% of the total activity each. The specific activity however, was higher in the particulate fractions (14,000xg and 100,000xg sediments) than in the soluble fraction (100,000xg supernatant), and was highest in the 14000xg sediment.

Recoveries of these esterases during subcellular fractionation are shown in table 4.1. The recovery of acetylthiocholinesterases was highest, 109+/-8%, and that of p-nitrophenylacetate lowest at 84+/-4% (n=3).

Figure 4.2 shows the separation of the nonspecific esterases in the subcellular fractions by electrophoresis (stained with naphthylacetate). The three major bands present in the whole homogenate were also present in the soluble fraction (a, Rf=0.18; b, Rf=0.26; and e, Rf=0.57). Two other bands (c, Rf=0.30; and d, Rf=0.42) not visible in the whole homogenate were shown to be present in the particulate fractions upon concentration by fractionation. Cross contamination of each fraction with
DISTRIBUTION OF ESTERASES IN SUBCELLULAR FRACTIONS FROM RAT SKIN (means+/−S.E.M., n=3 experiments)

1 800xg SUPERNATANT (STARTING MATERIAL FOR FRACTIONATION)
2 14,000xg SEDIMENT
3 100,000xg SEDIMENT
4 100,000xg SUPERNATANT (SURREY UNIVERSITY STRAIN)
POLYACRYLAMIDE GEL ELECTROPHORESIS OF SUBCELLULAR FRACTIONS FROM AN HOMOGENATE OF RAT SKIN (SURREY STRAIN)

Fig. 4.2

1 2 3 4

origin

a

b

c
d
e

1800xg SUPERNATANT

2 14,000xg SEDIMENT

3 100,000xg SEDIMENT

4 100,000xg SUPERNATANT

STAINED FOR HYDROLYTIC ACTIVITY WITH NAPHTHYLACETATE AS SUBSTRATE

1 800xg SUPERNATANT

2 14,000xg SEDIMENT

3 100,000xg SEDIMENT

4 100,000xg SUPERNATANT

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esterases predominantly contained within other fractions was evident on the original gel (although this is not clear on the photograph due to lack of contrast).

4.2.2 SEPARATION BY DIFFERENCES IN AQUEOUS SOLUBILITY

The results of an experiment to investigate the effects of the progressive addition of ammonium sulphate to the 100,000xg supernatant, from Porton rats, are shown in figure 4.3. The separation by electrophoresis of samples taken from pellets sedimented after increasing the saturation with ammonium sulphate from 20% to 80% in 10% steps is shown in fig. 4.4. Five electrophoretically distinct bands of naphthylacetate esterase activity were visible in the fractions precipitated between 40% and 80% saturation (tracks 5-8, fig. 4.4).

The least electrophoretically mobile bands precipitated first and the most mobile last. The least mobile bands (a, Rm=0.18; and b, Rm=0.29) were stained most strongly in the 50% to 60% fraction (track 6), but were also stained in the 40% to 50% (track 5) and the 60% to 80% (track 7) fractions. The most mobile band stained most strongly in the 70% to 80% fraction (track 8) but was also stained in the 60% to 70% (track 6) and 70% (track 7) fractions. Bands of intermediate mobility (c, Rm=0.30; and d, Rm=0.35) stained in the 60% to 70% (track 7) and 70% to 80% (track 8) fractions.

Figure 4.3 shows that the acetylthiocholine, butyrylthiocholine, and p-nitrophenylacetate hydrolysing
Fig. 4.3

DISTRIBUTION OF ESTERASE IN AMMONIUM SULPHATE FRACTIONS FROM THE 100,000 x g SUPERNATANT FROM "PORTON" RATS (BARS ARE MEANS OF DUPLICATE DETERMINATIONS)

SPECIFIC ACTIVITY OF ORIGINAL 100,000 x g SUPERNATANT

PAGE 115
ELECTROPHORETIC SEPARATION OF AMMONIUM SULPHATE FRACTIONS FROM THE 100,000xg SUPERNATANT OF THE PORTON RAT

STAINED FOR HYDROLYTIC ACTIVITY WITH NAPHTHYLACETATE

1 100,000xg SUPERNATANT
2 0-20%
3 20-30% SATURATION
4 30-40% WITH
5 40-50% AMMONIUM
6 50-60% SULPHATE
7 60-70%
8 70-80%
9 80-100%

Fig. 4.4

origin
Rm
0.18
0.29
0.30
0.35
0.58

anode
activities are distributed throughout all the ammonium sulphate fractions, 0-40%, 40-70%, and 70-80%, from Porton rats. The p-nitrophenylacetate hydrolase was also present in the 80-100% fraction.

The largest amount of acetylthiocholinesterase was present in the 70% to 80% fraction and the smallest amount in the 0% to 40% fraction, whereas the largest amounts of p-nitrophenylacetate esterase and butyrylthiocholinesterase were present in the 40% to 70% fraction, and the smallest in the 80% to 100% and 0% to 40% fractions respectively. The specific activity of acetylthiocholinesterase was highest in the 70% to 80% fraction and lowest in the 40% to 70% fraction, but the specific activities of the p-nitrophenylacetate and butyrylthiocholinesterase were highest in the 0% to 40% fraction, and lowest in the 80% to 100% and 70% to 80% fractions respectively.

Comparison of fig. 4.3 with fig 4.5 shows an apparent difference between the Porton strain and the Surrey University strain of rat. Similarly prepared 100,000xg supernatant fractions from the two strains of rat had different specific activities towards all three substrates, shown by the broken horizontal line in fig. 4.3 and fig. 4.5. The activities of the esterases in the 100,000xg supernatants from the Surrey rats were twice those from the Porton strain. The lower levels in the Porton strain was found to be associated with the absence of at least one electrophoretic band which will be discussed more fully in chapter 5. Recoveries of esterases, for both strains of rat,
Fig. 4.5

DISTRIBUTION OF ESTERASES IN AMMONIUM SULPHATE FRACTIONS FROM THE 100,000xg SUPERNATANT OF "SURREY RATS" (BARS ARE MEANS OF DUPLICATE DETERMINATIONS)

SPECIFIC ACTIVITY OF ORIGINAL 100,000xg SUPERNATANT
during separation are shown in table 4.1. Recoveries from the subcellular fractionations are similar for the two strains, whereas the percentage recoveries from ammonium sulphate precipitation were higher in experiments with the Porton strain. The esterases from the Porton strain were not purified further because the activities of the esterases in the skin from this strain were lower than those of the Surrey University strain.

4.2.2.1 PREPARATIVE SEPARATION OF SKIN ESTERASES

Preparative scale separation experiments were carried out using skin from Surrey University rats. Figure 4.5 shows the distribution of p-nitrophenylacetate, acetyltiocholine and butyrylthiocholine hydrolases in preparative ammonium sulphate fractions, from the 100,000xg supernatant of Surrey rats. The three hydrolases co-associate and are distributed between the 20% to 50% and the 50% to 80% with the highest activity in the 50% to 80% fraction.

4.2.3 SEPARATION BY MOLECULAR SIZE

The 50% to 80% fraction was further separated by molecular exclusion chromatography on Sephadex G-200 and the eluent fractions analysed for the three hydrolase activities measured during ammonium sulphate precipitation (Fig. 4.6). The acetyltiocholine and butyrylthiocholine hydrolases co-associated and gave qualitatively similar elution profiles, but activities were higher towards the acetyl ester. Several peaks of thioester hydrolysing activity were found between molecular sizes of 300Kd.
ELUTION OF 50% TO 80% AMMONIUM SULPHATE FRACTION FROM A 100,000xg SUPERNATANT OF RAT SKIN. (PORTON STRAIN, NITROGEN GROUND)

○ - ABSORBANCE AT 622nm OF DEXTRAN BLUE(x10)
□ - ABSORBANCE AT 280nm (PROTEIN)
◆ - ACETYLYTHIOCHOLINE HYDROLYSING ACTIVITY
● - BUTYRYLTHIOCHOLINE HYDROLYSING ACTIVITY
■ - p-NITROPHENYLACETATE HYDROLYSING ACTIVITY

FRACTION VOLUME = 2ml
FLOW RATE = 4ml/hr
<table>
<thead>
<tr>
<th></th>
<th>ACETYLTHIO-CHOLINESTERASE</th>
<th>BUTYRYLTHIO-CHOLINESTERASE</th>
<th>p-NITRO PHENYLACETATE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SURREY UNIVERSITY STRAIN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUBCELLULAR FRACTIONATION</td>
<td>109+/-6</td>
<td>91+/-6</td>
<td>84+/-4</td>
</tr>
<tr>
<td>AMMONIUM SULPHATE FRACTIONATION</td>
<td>57</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>G-200 FILTRATION</td>
<td>55</td>
<td>62</td>
<td>64</td>
</tr>
<tr>
<td><strong>PORTON STRAIN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUBCELLULAR FRACTIONATION</td>
<td>103</td>
<td>ND</td>
<td>90</td>
</tr>
<tr>
<td>AMMONIUM SULPHATE PRECIPITATION</td>
<td>61</td>
<td>79</td>
<td>55</td>
</tr>
</tbody>
</table>

ND = not determined

Recoveries are expressed as percentages of amount at start of each separation procedure.
and 80Kd. Two major peaks of activity were found close together at 130Kd and 160Kd, and there was a "shoulder" at 300Kd. The p-nitrophenylacetate hydrolysing profile was quite different. The major peak was at 85Kd with two smaller peaks, one at 200Kd and the other at 300Kd. The major p-nitrophenylacetate hydrolysing activity was associated with the main peak of protein.

Electrophoretic analysis of the ammonium sulphate and G-200 fractions is shown in figure 4.7. All electrophoretic bands present in the 100,000xg supernatant (track 1) were also present in both the 20%-50% (track 3) and the 50%-80% (track 4) ammonium sulphate fractions. The relative distribution of the electrophoretic bands was different in the G200 eluent fractions. Fraction 39 (track 7) contained the least mobile electrophoretic bands (Rf=0.23 and 0.25) and was associated with the first major thiocholine esterase peak (fig. 4.6). Fraction 41 (track 8) contained the second major thiocholine esterase peak, and there was less staining in the region of the low mobility electrophoretic bands. In addition to these low mobility bands this fraction contained some of the highest mobility bands (Rf=0.58 to 0.64). Fraction 48 (track 9) contained the major p-nitrophenylacetate esterase peak and was relatively enriched with an electrophoretic band of intermediate mobility (Rf=0.41). All three fractions contained two bands with mobilities between those of the slowest moving bands and that associated with the major p-nitrophenylacetate esterase peak (Rf=0.34, and Rf=0.37).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100,000×g supernatant</td>
</tr>
<tr>
<td>2</td>
<td>0–20%</td>
</tr>
<tr>
<td>3</td>
<td>20–50%</td>
</tr>
<tr>
<td></td>
<td>Ammonium sulphate</td>
</tr>
<tr>
<td>4</td>
<td>50–80%</td>
</tr>
<tr>
<td>5</td>
<td>80–100%</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Gel filtration</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Fractions</td>
</tr>
</tbody>
</table>

Fig. 4.7

PAGE 123
fractions by dissociative electrophoresis (fig. 4.8) showed the apparent purification of three monomers of 44 Kd, 39.5 Kd, and 34 Kd, by molecular exclusion chromatography. The predominant band in subcellular fractions, and ammonium sulphate fractions, at 66 Kd, was removed by this last step in the purification.

4.2.4 STABILITY OF SKIN ACETYLTHIOCHOLINESTERASE AT DIFFERENT pH VALUES

Figure 4.9 shows the results of an experiment where samples of 100,000xg supernatant were diluted 1:6 with 0.05M phosphate buffer at pH 5.0 and pH 8.0, and the activity remaining in samples of this dilution assayed at various times after dilution. Activity is expressed relative to the activity of undiluted 100,000xg supernatant stored in parallel to the test dilutions. Samples were diluted to equivalent protein concentrations, and pH corrected to 8.0 by dilution with a 0.1M phosphate buffer, when assayed. The activities in both dilutions did not vary with time but the activity in the pH8.0 dilution was always higher than the activity in the pH5.0 dilution.

4.2.5. ASSESSMENT OF DESALTING AND DIALYSIS TECHNIQUES

In all the experiments described so far in this Chapter, dialysis was used to remove any residual ammonium sulphate from the samples. The poor recoveries of enzyme and protein however, led to the investigation of the dialysis procedure as a possible point of protein loss. Dialysis of samples of 100,000xg supernatant as described in the methods, for a period of 48 hours
Dissociative P.A.G.E. in 7.5% Gel of Subcellular Fractions and Partially Purified Proteins from Rat Skin

Fig. 4.8

1 - Marker Proteins
2 - 800xg Supernatant
3 - 14,000xg Sediment
4 - 100,000xg Sediment
5 - 100,000xg Supernatant
6 - Marker Proteins
7 - Poled Fractions from Molecular Exclusion Chromatography Equivalent to 30 - 55 in Fig 4.6
8 - 50% - 80% Ammonium Sulphate Fraction
9 - Marker Proteins

Page 125
THE EFFECT OF STORAGE AT 4°C ON THE ACETYLTHIOCHOLINE ESTERASE IN SAMPLES OF 100,000xg SUPERNATANT STORED AT pH 8.0 (○) AND pH 5.0 (□). POINTS ARE MEANS OF DUPLICATE DETERMINATIONS. ACTIVITIES ARE RELATIVE TO UNDILUTED 100,000xg SIMILARLY STORED.
with one dialysate change, resulted in the recovery of 69% +/- 3.5% (n=4) of the original protein when assayed by the Lowry method. The same samples assayed by the Biorad protein assay however, showed only a 49% +/- 0.89% (n=4). Furthermore the protein estimations in the 100,000xg supernatant and the dialysed samples were 31% and 51% lower, respectively, when assayed by the Biorad method than by the Lowry method. Protein material was found adhering to the inner surface of the dialysis bags by staining with the coumassie blue stain used to stain polyacrylamide gels. Presoaking the dialysis tubing for 48hrs prior to use did not improve recovery. Inclusion of bovine serum albumin in the presoak buffer, in an attempt to saturate nonspecific binding sites, or suspending the knots above the dialysate, also failed to increase recovery.

Gel filtration was investigated as an alternative method of desalting. Adequate separation of salt and protein was achieved as shown in fig. 4.10. However this general method of desalting shows that total recovery of the protein is not suitable in these conditions because "tailing" of the protein peak results in some protein eluting in the fractions containing sulphate. The method does however have the advantage of speed over dialysis, taking only 3 hours compared to 24 hours for dialysis.

4.2.6. PREPARATIVE AMMONIUM SULPHATE PRECIPITATION AND MOLECULAR EXCLUSION CHROMATOGRAPHY

Further experiments to partially purify the esterases in skin on a preparative scale are summarised in table 4.2. The overall
GEL FILTRATION OF 40% TO 80% AMMONIUM SULPHATE FRACTION FROM
THE 100,000xg SUPERNATANT (SURREY STRAIN OF RAT) ON SEPHADEX
G-25. HATCHED AREA INDICATES FRACTIONS SHOWING POSITIVE FOR
SULPHATE BY BARIUM CHLORIDE PRECIPITATION.

PAGE 128
<table>
<thead>
<tr>
<th></th>
<th>% Activity</th>
<th>Specific Activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p-NITROPHENYLACETATE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESTERASE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800xg supernatant</td>
<td>100</td>
<td>14.94</td>
</tr>
<tr>
<td>100,000xg supernatant</td>
<td>67</td>
<td>14.39</td>
</tr>
<tr>
<td>40%-80% ammonium sulphate fraction</td>
<td>39</td>
<td>24.55</td>
</tr>
<tr>
<td>G200 fractions</td>
<td>20</td>
<td>15.11</td>
</tr>
<tr>
<td><strong>ACETYLLHIOCHOLINESTERASE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800xg supernatant</td>
<td>100</td>
<td>20.90</td>
</tr>
<tr>
<td>100,000xg supernatant</td>
<td>45</td>
<td>13.09</td>
</tr>
<tr>
<td>40%-80% ammonium sulphate fraction</td>
<td>21</td>
<td>9.74</td>
</tr>
<tr>
<td>G200 fractions</td>
<td>17</td>
<td>18.034</td>
</tr>
</tbody>
</table>
recoveries of the p-nitrophenylacetate and acetylthiocholine esterases after subcellular separation by centrifugation and ammonium sulphate fractionation followed by molecular exclusion chromatography were 20% and 17% respectively. There was no appreciable increase in specific activity for either enzyme.

4.2.6. PREPARATIVE ELECTROPHORESIS

Attempts to separate the esterases by P.A.G.E. on a preparative scale were unsuccessful due to the presence of a substance in the eluent that hydrolysed acetylthiocholine, butyrylthiocholine, and p-nitrophenylacetate. To overcome the problems involved with assaying the fractions from the preparative P.A.G.E. apparatus, such fractions were analysed by slab P.A.G.E.. Figure 4.11 shows the results of a preparative P.A.G.E. experiment. Naphthylacetate hydrolysing bands were found in pooled fractions eluting from the apparatus up to 14 hours after the bromophenol blue marker dye. Whilst adequate separation of the naphthylacetate esterases was achieved by preparative P.A.G.E. as shown in fig. 4.11, insufficient esterase activity was present in the pooled fractions, after desalting with Sephadex G25, to enable detection by the thiocholine ester or nitrophenylacetate assays.

Although preparative electrophoresis failed to produce sufficient amounts of purified enzymes to enable proper characterisation, it did show that the most mobile electrophoretic band was heterogeneous. Increasing the eluent flow rate increased the dilution of slower moving bands but
Fig. 4.11

PREPARATIVE ELECTROPHORESIS OF 100,000xg SUPERNATANT FROM RAT SKIN. FRACTIONS WERE POOLED AND SUBJECT TO ANALYTICAL SLAB GEL P.A.G.E., STAINED WITH NAPHTHYLACETATE.

1-ORIGINAL 100,000xg SUPERNATANT
a-h - POOLED FRACTIONS FROM ELECTROPHORESIS (INDICATED BY ARROWS)
increased the resolution of faster moving bands, with the result that two bands are visible in fig. 4.12 (tracks 6 and 7 in particular). These two bands have very similar Rf values (0.55 and 0.53) and if present in the same sample they would normally be indistinguishable as separate bands.

Prior to preparative P.A.G.E. samples of 100,000xg supernatant were concentrated to a sample size of 2ml, by freeze drying. As a result of this concentration the samples were of high ionic strength and were therefore subjected to buffer exchange on Sephadex G-25. Figure 4.13 shows the absorbance at 280nm of the eluent from a typical buffer exchange experiment. No acetylthiocholine hydrolysing activity could be detected in the 280nm peaks eluting between 4 hours and 8 hours, and these peaks were not detected when the ammonium sulphate fractions were desalted.

4.3 DISCUSSION

4.3.1 SUBCELLULAR DISTRIBUTION

It was established in chapter 3 that few conclusions could be drawn upon which subcellular organelle a particular enzyme was associated from distribution studies alone. The distribution of thiocholinesterases and p-nitrophenylacetate esterase (fig 4.7) in skin was, however, different from that of any of the "marker" enzymes used (fig 3.2). The hydrolases were not predominantly lysosomal since they have higher specific activities in the particulate fractions than -galactosidase (fig 3.2d). They were
POLYACRYLAMIDE GEL ELECTROPHORESIS

OF FRACTIONS FROM
PREPARATIVE ELECTROPHORESIS

Fig. 4.12

1 100,000xg SUPERNATANT
2 8
3 10
4 12 PREPARATIVE P.A.G.E.
5 14 FRACTION NUMBERS
6 16
7 18
8 20 (SIMILAR EXPERIMENT TO
9 22 THAT ILLUSTRATED IN FIG 4.11)
10 24

BROMOPHENOL BLUE FRONT PEAKED AT FRACTION No 4
ARROWS MARK DISTINCT BANDS. ELUENT FLOW RATE = 1ml/min

PAGE 133
Fig. 4.13

SEPHADEX G-25 FILTRATION OF 35mg OF 100,000xg SUPERNATANT PROTEIN FROM "SURREY" RAT SKIN.

$V_0$ - VOID VOLUME

$V_t$ - TOTAL COLUMN VOLUME
also not bound as tightly to the mitochondrial or microsomal membranes as succinate dehydrogenase or glucuronyl transferase, since these enzymes sedimented in the particulate fractions from homogenates of skin (fig 3.2b & c).

Electrophoretic separation of the esterases in each fraction elucidated their nature more fully. Clearly at least three esterases were present in all fractions plus two others, one "microsomal" and one "mitochondrial". The impure nature of the fractions, as discussed below, however means that the unambiguous assignment of the esterases to specific particulate fractions is not possible.

In a previous study of skin biochemistry (Moloney, 1980) indoxylacetate esterase was shown to be predominately located in the 100,000xg supernatant from Surrey University rats, prepared in a similar way to that used in the present study. The specific activity of this enzyme was however 6-7 times higher in the microsomal fraction than the mitochondrial or cytosolic fractions, in contrast to the results obtained with the three substrates used here. Clearly a microsomal esterase(s) was predominantly responsible for the hydrolysis of indoxylacetate, possibly the carboxylesterase described in liver by Junge and Krisch (1975) and Heymann (1980), but the largest proportion of the esterases studied here were present in the soluble fraction. In contrast to the indoxylacetate hydrolase the hydrolases investigated in this study showed no increase in specific activity in the microsomal fraction. This comparison must be made in the light of the differences in the preparative procedures.
used in the previous study and in this study. Moloney (1981) showed that a smaller amount of the microsomal marker glucuronyl transferase, remained in the supernatant after a 10,000xg (20min) centrifugation than after an 8,000xg (20min) centrifugation. Some of the microsomal esterases that would have been present in the 100,000xg sediment from the previous study (Moloney, 1980) might therefore be present in the 14,000xg sediment in this study. Unless the material transferred into the 14,000xg sediment was enriched in esterase however, this should not alter the specific activity of the 100,000xg sediment. Whilst increasing the relative centrifugal force used to sediment mitochondrial material leads to a higher purity of the microsomal sediment, it also decreases the recovery of microsomal material and increases the contamination of the mitochondrial sediment with microsomes.

Studies of the distribution of indoxylacetate and 1-naphthylacetate esterases in subcellular fractions from liver (Underhay et al., 1956) showed that they were of microsomal origin in this tissue. Shibko and Tappel (1964) confirmed this finding and showed that 2-naphthylacetate was hydrolysed by microsomal enzymes with a pH optimum of 8.0. This is in the range of optima for liver carboxylesterase quoted by Heymann (1980). These microsomal esterases can be solubilised by relatively "soft" techniques such as extraction with 20% glycerol in buffer (Junge and Krisch, 1975) and it is not inconceivable that the harsh methods necessary to homogenise skin give rise to the solubilisation of some microsomal carboxylesterase. Shibko and Tappel (1964) also demonstrated an "A type" esterase (Aldridge, 1953) that was bound to the outer surface of the lysosomal
membrane and had a pH optimum of 5.0. This enzyme was identified as lysosomal "acid esterase" and remained bound to the lysosomal membrane after repeated freeze/thaw treatment.

In the light of the above findings the subcellular location of the esterases in the 100,000xg supernatant used in the remainder of this study cannot be established with any certainty. It is unlikely that any of the enzymes was the lysosomal A esterase since this enzyme would have remained bound to the membrane, but the possibility that the lysosomal esterase in skin are released in vivo or are more easily solubilised than those of liver cannot be ruled out.

Similarly the possibility of association of esterases with membranous fractions after homogenisation, in a similar manner to one of the forms of lactate dehydrogenase (Keck and Choules, 1962), exacerbates the problem of specifying the true subcellular origin of the esterases in this study.

4.3.2 AMMONIUM SULPHATE PRECIPITATION

In general the more electrophoretically mobile naphthylacetate esterases were more soluble in water than those which were less mobile ie. they precipitated at higher percentage saturations of ammonium sulphate. This is not unexpected since higher electrophoretic mobility indicates a larger number of charged groups, which would tend to make the protein more soluble in a polar solvent like water. Exceptions to this, proteins that tend to precipitate at higher, or lower saturation
than would be expected by their electrophoretic mobility, may be due to differences in pH between the slab P.A.G.E. experiments and the precipitation experiments. The electrophoresis was performed at pH 8.9 but the precipitation experiments at pH 7.4. The two experiments cannot be directly compared therefore in assessing solubility, thus any protein with a significant proportion of its ionisable groups having a pK between 7.4 and 8.9 would have a different solubility at 7.4 from that at 8.9.

It is also apparent from these experiments, perhaps for the same reasons, the esterases measured spectrophotometrically cannot be correlated directly with the naphthyl acetate hydrolysing enzymes shown by electrophoresis. Some thiocholinesterase and p-nitrophenylacetate esterase activity was found in fractions not containing detectable naphthylacetate esterases (compare fig 4.4 with fig 4.3). This indicates that either the spectrophotometric substrates are not hydrolysed exclusively by the naphthylacetate hydrolysing esterases, or that the naphthylacetate staining method is not as sensitive as the spectrophotometric assays. However even given the above restrictions some conclusions can still be drawn. In particular the relative enrichment of the 70% to 80% fraction with an acetylthiocholinesterase indicates that one of the naphthylacetate esterase bands present in this fraction (track 8, bands c,d,e; fig 4.4) is responsible for some of the hydrolysis of this chromogenic substrate.

The difference in the protein concentrations assayed by the "Lowry" and the "Biorad" techniques show that the choice of
assay method is important in obtaining an accurate estimate of the total protein in a solution of biological origin. The discrepancy probably arises from the differences in composition of the sample solution and the bovine serum albumin solution used as a standard. The ideal standard protein to use for the assay of an biological sample is the protein contained in that sample. In solutions that contain mixtures of unidentified proteins this is not possible. Therefore chromogenic methods that develop different amounts of colour when used to assay the same protein will give different estimates of the total concentration of protein in a mixture, if a single protein is used as a standard in both assays. It has been shown that different estimates of the concentration of various purified proteins can be obtained using the "Lowry" and the "Biorad" assays. Sedmark and Grossberg (1977)
4.3.3 MOLECULAR EXCLUSION CHROMATOGRAPHY

The results from molecular exclusion chromatography have shown that the thiocholinesterases and the p-nitrophenylacetate esterases are different populations of proteins. None the less all substrates were hydrolysed by proteins of more than one molecular size.

A further correlation of the naphthylacetate staining electrophoretic bands with both the enzymes hydrolysing the spectrophotometric substrates was made by separating the molecular exclusion fractions containing the peak activities by slab P.A.G.E.(fig 4.7). This experiment revealed that the 130Kd peak was associated with the lowest mobility bands, the 160Kd peak with the low mobility and the higher mobility bands and the p-nitrophenylacetate hydrolysing 85Kd peak with an intermediate mobility band. Unfortunately separation was not complete and no peak fraction contained only one electrophoretic band. It is clear from fig. 4.7 and table 4.2 that no significant purification of any of the esterases assayed here, acetylthiocholine, butyrylthiocholine, p-nitrophenylacetate, or naphthylacetate, was achieved by ammonium sulphate precipitation and molecular exclusion chromatography. Pooling fractions equivalent to 30-55 in fig. 4.6 produced no higher specific activity towards acetylthiochoine and p-nitrophenylacetate. It was therefore apparent that if purification was to be achieved, different techniques would have to be used. The most promising appeared to be preparative P.A.G.E., because analytical P.A.G.E. produced good separation.

PAGE 140
The predominance of monomers of lower molecular size (between 34Kd and 44Kd) than the proteins discussed above, in the pooled molecular exclusion chromatography fractions and demonstrated by dissociative electrophoresis, indicates that the esterases may be polymeric molecules. Though the proteins separated on G-200 are not exact multiples of the monomers shown by SDS PAGE, they could still give rise to those monomers by the loss of associated polysaccharides or small protein subunits, during preparation for SDS PAGE.

4.3.4 PREPARATIVE ELECTROPHORESIS

Unfortunately the preparative P.A.G.E. did not produce sufficient purified esterases for the intended purpose because of the presence of a substance in the eluent, that eluted with the dye front, which was able to catalyse the hydrolysis of acetyltiochochine, butyryltiochochine and p-nitrophenylacetate. This hydrolysis was not related to pH nor was it due to the presence of the Tris buffer. However it was found that the glycine which was used in the electrode buffer, but not in the elution buffer, was able to hydrolyse the above substrate esters. Glycine would have been present in significant concentrations in the elution buffer as the ion front eluted, since at pH8.9 it would have a higher mobility than the most mobile protein (Ornstein and Davis, 1964; Williams and Reisfeld, 1964). It is however difficult to conceive of a mechanism whereby this catalysis could take place. It is unlikely that under normal
circumstances glycine would be a sufficiently strong nucleophile to attack an ester bond (see chapter 1 p35).

The results illustrated in fig. 4.10. show that the most mobile band is heterogenous. This casts doubt on the homogeneity of the other electrophoretic bands as although the most mobile band is the broadest band other bands could represent several co-migrating esterases. The possibility that different electrophoretic bands are a result of a single polymeric protein breaking down during the preparation of the enzymes for electrophoresis is eliminated by two findings. Firstly it is possible to precipitate the electrophoretically different proteins separately using ammonium sulphate and secondly these proteins can be distinguished in terms of molecular size by exclusion chromatography. These findings indicate that the electrophoretically different proteins were present as functionally separate molecules in the 100,000xg supernatant from which they were separated. This does not however completely exclude the possibility that the electrophoretically different proteins could be derived from a common polymeric protein at an earlier stage of the preparation, for instance during homogenisation or storage.

4.3.5 LOSS OF ENZYME AND PROTEIN DURING SEPARATION AND PURIFICATION

Both protein and enzyme activity were lost during the purification procedures in equal quantities so that the specific activity remained unchanged. This loss could be the hydrolysis of
protein nonselectively, by lysosomal proteases which occur in soluble extracts of skin (Lazarus and Hatcher, 1975). Alternatively the proteins could be "sticking" to the surfaces of dialysis bags, and molecular exclusion gels, as indicated by the staining of dialysis bags by coumassie blue (section 4.2.5. pl27), and the interaction with Sephadex G-25 (fig 4.11). This loss of protein alone however could not account for the loss of enzyme activity unless the enzyme was associated with the protein that was lost.

Loss of enzyme activity was not observed during the pH stability experiment described in section 4.2.4 (pl24), an observation that is hard to reconcile with the findings during other separation procedures. The pH stability experiment would need to be repeated to confirm this result, but if it proved to be reproducible the possibility that dilution eliminated the protein and enzyme loss might be considered.

4.3.6 THE IDENTITY OF RAT SKIN ESTERASES?

A tentative identification of the esterases in rat skin is possible on the basis of their molecular size. The molecular sizes of some purified esterases are shown in table 4.3. Purified butyrylcholinesterase and acetylcholinesterase have comparable monomeric sizes (85Kd and 80Kd respectively) but these enzymes are unlikely to be the 85Kd enzyme in rat skin because this enzyme does not hydrolyse acetylthiocholine or butyrylthiocholine. It is possible that the 160Kd thiocholine esterase is the dimeric form of either acetylcholinesterase or
butyrylcholinesterase. To discover if this 160Kd protein is a dimer, molecular exclusion chromatography could be repeated in the presence of dissociating agents such as urea, or at high ionic strength, a condition that promotes the dissociation of cholinesterases into monomers (Brimijion, 1983). The 130Kd protein is not divisible by any simple number to produce the size of any known purified esterase monomer. It is possible of course that this protein is identical with the 160Kd protein except for an associated protein or carbohydrate that has been lost during the preparation of the enzymes.

The ease with which esterases may be electrophoretically separated and stained with chromogenic substrates such as naphthylacetate has led to such "zymograms" being performed on many different tissues from various species. LaDu and Snady (1971) has reviewed the data available on esterases from human tissues. Human serum contains five electrophoretically different naphthylacetate hydrolysing proteins. Erythrocytes contain 7 zymogram bands, liver has 11 or 12 bands in three zones (Ecobichon and Kalow, 1962, 1963), kidney has 3 zones similar to those in liver (Ecobichon and Kalow, 1964) skeletal muscle has 10 bands, smooth muscle has 9 bands (Ecobichon and Kalow, 1965), and brain apparently has no less than 15 bands (Ecobichon, 1966).

These zymogram experiments were performed in starch gels at pH7.4, and so are not directly comparable to those in this study. The zymograms described by LaDu and Snady (1971) are however quite similar to those from rat skin demonstrated in this study, in as much as they exhibit three "zones" of esterase activity, one
more mobile than the other two as occurs in rat skin. Ecobichon and Kalow (1962, 1963, 1964, 1965) identified one of the zones that was common to liver, muscle, and kidney as containing "aliesterase", and the other two zones as containing a mixture of esterases including "acetylesterase" and "serum type" butyrylcholinesterase. There is a marked similarity between the esterase content of liver, kidney and that found in skin in this study. There is an acetylesterase present in all three tissues (160Kd species in rat skin) and an aliesterase (carboxylesterase/amidase) that does not hydrolyse charged substrates (85Kd species in rat skin). It should be noted that the presence of proteinases in an enzyme mixture could complicate the interpretation of zymograms by promoting the dissociation of polymeric enzymes into monomers, or smaller aggregations of monomers. Saeed et. al. (1971) showed that various proteases could dissociate serum butyrylcholinesterase into its constituent monomeric and polymeric forms, each of which retained catalytic activity but showed different electrophoretic mobility. There was also some suggestion that the parent oligomeric form was partially resistant to eserine whilst the smaller forms were not.

In conclusion, rat skin has been found to contain esterases capable of hydrolysing simple esters of choline and p-nitrophenol. These enzymes are heterogenous groups of proteins that consist of more than one subunit of 34Kd, 39.5Kd, and 44Kd, to give molecular sizes of between 85Kd and 160Kd by molecular exclusion chromatography. The esterases can also be separated electrophoretically to show the presence of at least five distinct bands and one of these has been shown to consist of more
than one electrophoretically distinct esterase. It should be emphasised however that physical parameters are not sufficient in themselves to positively identify an esterase. Some knowledge of the catalytic properties in the form of the range of substrates hydrolysed and the inhibitor specificity of the enzymes must be determined.
CHAPTER 5

THE PARTIAL CHARACTERISATION

OF RAT AND HUMAN

SKIN ESTERASES
5 THE PARTIAL CHARACTERISATION OF RAT AND HUMAN SKIN ESTERASES

5.1 INTRODUCTION

The catalytic behavior of an esterase is a major consideration in the determination of its biological significance. The relationship between the chemical structures of esters and their rate of hydrolysis, together with the structure of inhibitors that prevent or modify ester hydrolysis is of value in two ways. Firstly, in distinguishing a previously unknown esterase from other esterases in other tissues and species, and secondly, in enabling predictions to be made as to the likelihood that a novel ester will be hydrolysed by a given esterase. In the context of this study such information is of value in understanding the fate of esters absorbed through the skin.

Having shown that the purification of the individual esterases in rat skin was not possible using the techniques employed in this study, a partial characterisation of the mixture of esterases in the soluble fractions from rat and human skin was undertaken. Four selective inhibitors have been used to characterise the type of esterase(s) responsible for the hydrolysis of each of four substrate esters.
5.2 RESULTS

The following experiments were performed in duplicate on single preparations of rat and human 100,000xg supernatant. The rat skin was taken from 46 day old animals (Surrey University) and was homogenised by grinding in liquid nitrogen. Human skin showed no overt pathology, and was scraped free of fat prior to homogenisation by a similar method. Inhibition experiments were performed as described on p73.

5.2.1 CHARACTERISATION OF RAT ESTERASES

Table 5.1 shows the results of the partial characterisation of rat 100,000xg supernatant. The % inhibition of the esterases by ethopropazine is the similar to that described in chapter 3 for homogenates of "Porton" rat skin. The hydrolysis of butyrylthiocholine was inhibited with an \( I_{50} \) of 5\( \mu \)M similar to that of horse serum butyrylcholinesterase. Acetylthiocholine hydrolysis was inhibited with an \( I_{50} \) of 500nM by ethopropazine, but the hydrolysis of p-nitrophenylacetate and that of indoxylacetate were not inhibited at a concentration of 100\( \mu \)M. These results are further illustrated by in figure 5.1, as is the inability to totally inhibit the hydrolysis of acetylthiocholine with ethopropazine. In homogenates only 80\% of the activity was inhibited (fig. 3.5) and in 100,000xg supernatant only 60%-70\% could be inhibited. The fall in inhibition at 100\( \mu \)M in fig. 5.1 was confirmed by a second duplicate determination.

The specific acetylcholinesterase inhibitor BW284C51 did not
TABLE 5.1

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>ASCh $I_{50}=500nM$</th>
<th>BuSCh $I_{50}=5\mu M$</th>
<th>NPA 100μM</th>
<th>Ind. ace. 100μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethopropazine</td>
<td></td>
<td></td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>BW284C51 10μM</td>
<td>25%</td>
<td>NI</td>
<td>NI</td>
<td>3%</td>
</tr>
<tr>
<td>N-Ethylmaleimide 1mM</td>
<td>ND</td>
<td>ND</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>D.FP 1 μM</td>
<td>90% 30mins</td>
<td>100% 30mins</td>
<td>75% 30mins</td>
<td>90% 30mins</td>
</tr>
</tbody>
</table>

**TABLE 5.1**

**SUMMARY OF THE INTERACTION OF RAT SKIN ESTERASES WITH INHIBITORS.**

NI = not inhibited

ND = not determined

Times are preincubation times with inhibitors where applicable.

Values are expressed as % inhibition.
INHIBITION OF THE ESTERASE ACTIVITIES IN THE 100,000xg SUPERNATANT FROM THE SKIN OF THE RAT. Activities represented are:

- □ - acetylthiocholine esterase (4mM)
- ○ - butyrylthiocholine esterase (10mM)
- ◇ - p-nitrophenylacetate esterase (8mM)
- ● - indoxylacetate esterase (2mM)

points are means of duplicate determinations, not different by more than 10%. Numbers in parenthesis are concentrations of substrates.
inhibit the hydrolysis of butyrylthiocholine, p-nitrophenylacetate, or indoxylacetate, but did inhibit the hydrolysis of acetylthiocholine by 25% (at 10uM). There was no inhibition of hydrolysis of acetylthiocholine at concentrations of BW284C51 between 10nM and 1uM.

Investigation of the inhibition of the hydrolysis of the thiocholine esters with N-ethylmaleimide was not possible because the thiocholine product combined with the N-ethylmaleimide in preference to the Ellman reagent (DTNB). No inhibition of the hydrolysis of either p-nitrophenyl acetate or indoxylacetate was detectable after a 15min preincubation with 1mM N-ethylmaleimide. The N-ethylmaleimide was shown to be combining with sulphydryl groups using a known concentration of cysteine followed by the determination of the remaining cysteine using Ellman reagent.

Preincubation with Di-isopropylfluorophosphate (DFP) at a concentration of 1uM, for 30mins, produced 90%, 100%, 75%, and 90% inhibition of the hydrolysis of acetylthiocholine, butyrylthiocholine, p-nitrophenylacetate, and indoxylacetate, respectively. These results are expanded upon in fig. 5.2 which shows the time course of inhibition for the hydrolysis of each substrate by DFP. In each experiment the activity of the enzyme mixture decreased with time between 10 and 30 mins to a "plateau" level. For acetylthiocholine this "plateau" activity was between 0% and 10% at 1uM DFP, and 90% at 10nM DFP. For butyrylthiocholine however, both 1uM and 10nM reduced the activity to 35%. For p-nitrophenylacetate the plateau activities
ONSET OF INHIBITION BY DI-ISOPROPYLFLUOROPHOSPHATE (DFP) FOR THE ESTERASE ACTIVITIES IN THE 100,000xg SUPERNATANT FROM RAT SKIN. Activities represented are:-

- acetylthiocholine esterase (4mM)
- butyrylthiocholine esterase (10mM)
- p-nitrophenylacetate esterase (5mM)
- indoxylacetate esterase (2mM)

Points are means of duplicate determinations not different by more than 10%. Numbers in parenthesis are substrate concentrations.
were 25%, 45%, and 80% for 1μM, 100nM, and 10nM respectively. For indoxylacetate the "plateau" activities were 5% at 1μM and 15% at 100nM.

5.2.2 CHARACTERISATION OF HUMAN ESTERASES

Table 5.2 summarises the results of a similar set of experiments performed on a 100,000xg supernatant from human skin. The results are rather similar to those obtained for the rat preparation.

Ethopropazine inhibited the hydrolysis of acetylthiocholine with an IC50 of 900nM, and the hydrolysis of butyrylthiocholine with an IC50 of 5μM; it did not however inhibit the hydrolysis of p-nitrophenylacetate or indoxylacetate (at 100μM). These results are presented more fully in fig. 5.3. The hydrolysis of the acetylthiocholine and butyrylthiocholine were inhibited between 100nM and 100μM. The hydrolysis of indoxylacetate and p-nitrophenylacetate were not inhibited up to 100μM ethopropazine.

BW284C51 (10μM) inhibited the hydrolysis of acetylthiocholine by 29% and butyrylthiocholine by 7%, but did not inhibit the hydrolysis of p-nitrophenylacetate or indoxylacetate.

N-Ethylmaleimide (10nM) did not inhibit the hydrolysis of p-nitrophenylacetate or indoxylacetate after a 30min incubation.

The inhibition of human skin esterases by DFP is summarized in table 5.2 and illustrated more fully in figure 5.4. The
<table>
<thead>
<tr>
<th>Substrate/Inhibitor</th>
<th>ASCh</th>
<th>BuSCh</th>
<th>NPA</th>
<th>Ind.Ace</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethopropazine</td>
<td></td>
<td>$I_{50} = 900\text{nM}$</td>
<td>$I_{50} = 5\mu\text{M}$</td>
<td>NI</td>
</tr>
<tr>
<td>BW 284C51</td>
<td></td>
<td>29%</td>
<td>7%</td>
<td>NI</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>ND</td>
<td>ND</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>1mM</td>
<td></td>
<td></td>
<td>30 mins</td>
<td>30 mins</td>
</tr>
<tr>
<td>DFP</td>
<td></td>
<td>95%</td>
<td>95%</td>
<td>25%</td>
</tr>
<tr>
<td>1 \mu M</td>
<td>30 mins</td>
<td>30 mins</td>
<td>30 mins</td>
<td>30 mins</td>
</tr>
</tbody>
</table>

**TABLE 5.2**

**SUMMARY OF THE INTERACTION OF HUMAN SKIN ESTERASES WITH INHIBITORS.**

NI = not inhibited  
ND = not determined  

Times are preincubation times with inhibitors where applicable.  
Values are expressed as % inhibition.  
Tissue was mammary skin from a young female.
Fig. 5.3

INHIBITION OF ESTERASE ACTIVITIES IN THE 100,000xg SUPERNATANT OF HUMAN SKIN. Activities represented are:

- □ - acetylthiocholine esterase (4mM)
- ○ - butyrylthiocholine esterase (10mM)
- ◇ - p-nitrophenylacetate (8mM)
- ● - indoxylacetate (2mM)

Points are means of duplicate determinations not different by more than 10%. Numbers in parenthesis are concentrations of substrates. Source tissue was mammary skin from a young female.
ONSET OF INHIBITION BY DI-ISOPROXYLFLUOROPHOSPHATE (DFP) FOR ESTERASE ACTIVITIES IN THE 100,000Xg SUPERNATANT FROM HUMAN SKIN.

Activities represented are:

- acetylthiocholine esterase (4mM)
- butyrylthiocholine esterase (10mM)
- p-nitrophenylacetate esterase (0.1mM)
- indoxylacetate esterase (2mM)

Points are means of duplicate determinations not different by more than 10%. Numbers in parenthesis are concentrations of substrate. Source tissue was mammary skin from a young female.
hydrolysis of acetylthiocholine, butyrylthiocholine, and indoxylacetate were all inhibited by between 90% and 100% at both concentrations of DFP (1uM and 10nM) after 30mins preincubation. The hydrolysis of p-nitrophenylacetate however was inhibited by 25% by 1uM DFP after 30mins, and could not be further inhibited by the addition of more DFP.

Table 5.3 shows the specific activities of the rat and human 100,000xg supernatants towards each of the substrates used in the study. The specific activities with respect to the thiocholine esters in human 100,000xg supernatant were the reverse of those in the rat. Human esterases showed a preference for the butyryl rather than the acetyl ester. The specific activity of the human esterases towards acetylthiocholine was almost twice that of the rat, but the specific activity towards the butyrylthiocholine was some eight times greater than that of rat enzymes. In contrast the rat esterases possessed higher specific activity towards the p-nitrophenylacetate and the indoxylacetate.

During the spectrophotometric investigations it was found that 10% ethanol totally inhibited the hydrolysis of the thiocholine esters, whilst 2% ethanol produced no inhibition. However 10% ethanol did not interfere with the reaction of cysteine standards with DTNB reagent indicating that this inhibition originated with the production of the thiocholine. The assay of the p-nitrophenylacetate was performed in the presence 10% ethanol, to keep this substrate in solution, thus any activity recorded under these conditions was probably not due to
### TABLE 5.3

**SPECIFIC ACTIVITIES OF ESTERASES IN RAT AND HUMAN SKIN**

*(100,000xg SUPERNATANTS)*

<table>
<thead>
<tr>
<th>substrate</th>
<th>rat</th>
<th>human</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetylthiocholine</td>
<td>8.74</td>
<td>15.52</td>
</tr>
<tr>
<td>butyrylthiocholine</td>
<td>4.77</td>
<td>31.66</td>
</tr>
<tr>
<td>p-nitrophenylacetate</td>
<td>3.33</td>
<td>1.84</td>
</tr>
<tr>
<td>indoxylacetate</td>
<td>33.65</td>
<td>4.71</td>
</tr>
</tbody>
</table>

Activities are expressed as nmol/min/mg protein.

Figures are means of duplicate determinations not different by more than 10%.
the thiocholine ester hydrolases.

Additional information upon the nature of the ethopropazine sensitive enzymes present in the 100,000xg supernatant from rat skin was obtained by including this inhibitor in the naphthylacetate stain used in a nondissociative electrophoresis experiment. (fig. 5.5). This experiment showed that the least electrophoretically mobile esterases (Rf 0.18 and 0.2) were sensitive to ethopropazine (100μM) but the more mobile bands were not.

5.2.3 ELECTROPHORETIC ANALYSIS OF THE ESTERASE CONTENT OF HUMAN SUBCELLULAR FRACTIONS.

The esterase content of human skin was further investigated by electrophoresis. Aliquots of subcellular fractions (800xg supernatant, 14,000xg sediment, 100,000xg sediment, and 100,000xg supernatant) from both rat and human tissue were subject to nondissociative electrophoresis on the same slab gel as described in the Materials and Methods. The results of this experiment are shown in fig. 5.6. The electrophoretic pattern of the extracts of rat tissue were the same as those described in chapter 4 (p112). The human extracts however showed a quite different pattern. No band visible in the human fractions had a mobility higher than the lowest mobility band in the rat fractions (Rf 0.18). In addition the human fractions contained a densely staining band with a lower mobility than any of the bands present in the rat fractions. This band was present in all the fractions but stained most prominently in the 800xg and 100,000xg supernatants, indicating that the responsible protein was probably soluble.
THE EFFECT OF ETHOPROPAZINE ON THE ELECTROPHORETIC GRAMS OF RAT SKIN ESTERASES

Fig. 5.5

Tracks are identical loading (0.29 mg) with 100,000 xg supernatant protein from Surrey University rats. The gel was divided after running and both parts were stained for identical periods with the naphthylacetate stain.

PAGE 160
Fig. 5.6
POLYACRYLAMIDE GEL ELECTROPHORESIS OF SUBCELLULAR FRACTIONS FROM RAT AND HUMAN SKIN HOMOGENATES

This photograph has been retouched due to the poor quality of the original.

Shading key:— heavy / light

SURREY UNIVERSITY RATS
(0.1mg protein/track)

HUMAN (0.04mg protein/track)
An homogenate of cultured keratinocytes prepared as described by Hopley (in preparation) was also subjected to nondissociative electrophoresis in the same experiment. This preparation showed two bands of naphthylacetate esterase activity neither of which corresponded to any of the bands in rat or human tissue.

At the end of a 15min incubation at 37°C the gel was divided between tracks 4 and 5 and the right hand section, containing the bands of low staining intensity (human and keratinocyte fractions) was incubated with fresh stain for a further 15mins. No additional bands were visualised.

As described earlier there were differences between Porton and Surrey University strains of rat, in order to determine the precise nature of these differences electrophoresis of the 100,000xg supernatant samples from the skin homogenates were performed. The results of this experiment are shown in fig. 5.7. Though the protein was not equivalent in these two tracks, the obvious absence of one electrophoretic band (Rf 0.26) from the Porton rat offers a possible explanation for the reduced esterase activities described in chapter 4 (p117).
1 - PORTON RAT
2 - SURREY RAT
arrows indicate position of band missing from Porton animals
protein :- PORTON 0.26 mg/track
          SURREY  0.29 mg/track
5.3 DISCUSSION
5.3.1 THEORETICAL CONSIDERATIONS

Identification and characterisation of nonspecific enzymes, such as esterases on the basis of substrate or substrate type is not reliable. Inhibitors should be more specific for particular esterases but may still demonstrate some inhibitory activity towards other enzymes.

For reversible competitive inhibitors selectivity is a function of substrate and inhibitor concentration. The most specific inhibitor is clearly one for which a concentration can be found at which one esterase is totally inhibited whilst another is not at all inhibited. Such a case is illustrated in fig. 5.8. Unfortunately such ideal inhibitors do not exist for most enzymes. However as described in chapter 1 (p55) some inhibitors have been shown to be selective for certain esterase types. This study has attempted to use this information to partially characterise some of the esterases in skin.

Irreversible inhibitors may not be as dependent upon inhibitor concentration. Provided the inhibitor concentration exceeds that of the enzymic sites, the enzyme should eventually be totally inhibited. The only phase of such inhibition that is dependent upon the concentration of the inhibitor is the period of onset of inhibition.

As illustrated in fig 5.9(A) the maximum inhibition is constant at all inhibitor concentrations and represents the
Fig. 5.8

Schematic representation of "ideal" inhibition vs lg concentration of inhibitor profiles of two enzymes. The sensitive enzyme (A) can be distinguished from enzyme (B) using concentration "X" of inhibitor.
Schematic representations of onset of inhibition profiles for two mixtures of sensitive and insensitive enzymes. Mixture "A" does not contain any enzyme or binding protein, other than the enzyme being inhibited, that reduces the concentration of inhibitor during onset of inhibition. Mixture "B" does contain such an enzyme or binding protein.
The single exception to this is when inhibitor is rapidly removed from the incubation mixture during the period of onset of inhibition. Such phenomena may result from a mixture which contains an enzyme that inactivates the inhibitor, a protein that binds it, or when the inhibitor itself is unstable. The results of a time to onset of inhibition experiment in such circumstances are represented in fig 5.9(B). When an irreversible inhibitor is progressively removed from the system during onset, lower initial concentrations of inhibitor will produce lower final inhibitions; since a larger proportion of a lower initial concentration will be removed. This argument assumes the inactivating enzyme, or binding protein, to be saturated at both concentrations.

The inhibitors used in this study are selective for the following enzymes:

- N-ethylmaleimide - sulphhydryl containing enzymes (Miyader and Kosower, 1972)
- DFP - serine based hydrolases (Aldridge and Reiner, 1971)
- BW284C51 - acetylcholinesterase (Austin and Berry, 1953)
- ethopropazine - butyrylcholinesterase (Todrick et al., 1954; Edwards and Brimijion, 1981)
5.3.2. CLASSIFICATION OF ESTERASES IN RAT AND HUMAN SKIN

In the light of the above discussion the information contained in tables 5.1 and 5.2 and their associated figures (5.1, 5.2, 5.3, and 5.4) show that there are two distinct enzyme populations in the skin of rat and of man.

The first group hydrolyse p-nitrophenylacetate and indoxylacetate and are insensitive to the butyrylcholinesterase inhibitor ethopropazine, and to the acetylcholinesterase inhibitor BW284C51. The second group hydrolyse acetyl and butyryl esters of thiocholine and are inhibited by ethopropazine but not BW284C51, indicating that they are more similar to butyrylcholinesterase than acetylcholinesterase.

The first group is not inhibited by the sulphydryl combining agent N-ethylmaleimide indicating that sulphydryl groups are not important in the catalytic mechanism of these enzymes. Both groups are inhibited by the serine alkylating agent DFP. The time course of this inhibition in the rat however strongly suggests that there is either a binding protein for DFP, or a DFP hydrolysing enzyme present in the mixture. This finding is consistent with those of Fredriksson (1958) who showed the apparent "hydrolysis" of various organophosphate esters during passage though the skin of the rat, mouse, guinea pig, rabbit, cat, and dog.

It is unlikely that the concentration of enzymic sites was greater than that of DFP since in the case of the indoxylacetate
hydrolysis (where an increase on inhibition was achieved by increasing the concentration above $10^{-7}$M) the concentration would have to exceed $10^{-7}$M (assuming one site/molecule). Taking the molecular weight of the esterase to be 130,000Kd as indicated by molecular size (chapter 4, p119) the enzyme represented about 5% of the total protein in the 100,000xg supernatant.

This was not seen in the human extracts, as both $10^{-6}$M and $10^{-8}$M DFP produced almost total inhibition of the indoxylacetate and p-nitrophenylacetate hydrolyses. The observation that 75% of the hydrolysis of p-nitrophenylacetate was resistant to DFP, when taken in conjunction with the lack of DFP hydrolysis indicated by the equivalent inhibition at the two concentrations mentioned above, and the insensitivity to N-ethylmaleimide, indicates the presence of a nonsulphydryl dependent enzyme that does not interact with DFP. This apparent anomaly is not easy to explain. Under normal circumstances the only two amino acid residues that are sufficiently nucleophilic to attack the carbon or phosphorus centre of an ester link are serine and cysteine; via their hydroxyl and a sulphydryl group respectively. Yet this p-nitrophenylacetate esterase appears to be dependent upon neither of these groups. It is possible that the enzyme still utilises one of these groups but that it protects its active site in some way, (eg by sterically hindering the binding of the inhibitor molecule).

The interaction of skin esterases with DFP enables them to be categorised under the nomenclature system proposed by Aldridge (1953). As detailed in Chapter 1 (p33) this system names...
esterases that hydrolyse organophosphate esters, such as DFP, as "A type" esterases, those that are inhibited by DFP as "B type" esterases, and those that do not interact as "C type" esterases.

Clearly the thioesters and indoxylacetate used in this study were hydrolysed by "B" esterases in both rat and human skin. The hydrolysis of p-nitrophenylacetate in rat skin was also catalysed by a "B" esterase, but in human skin only 25% of the hydrolysis of this ester was catalysed by a "B" esterase. Since the absence of any differential inhibition at different concentrations of DFP indicates that there was apparently no "DFPase" operating in the human 100,000xg supernatant, it might be assumed that this DFP resistant enzyme is a "C" esterase. To date "C" esterases have only been demonstrated in hog kidney (Bergmann et. al. 1957). Another possibility is that it is active against DFP at such low levels as not to affect the preincubation experiments, but is still capable of hydrolysing DFP, this would lead to its classification as an "A" esterase.

It is possible that the rat skin extract contained an "A" esterase that was responsible for hydrolysing the DFP but that this enzyme was not hydrolysing any of the substrate esters used and did not therefore show up as an uninhibited enzyme in the onset experiments. Conclusive evidence for the hydrolysis of DFP could be gained by using a fluoride electrode to measure the release of fluoride during such hydrolysis. This was not however attempted in the present study, since such an electrode was not available to the author.

Though DFP is somewhat unstable in aqueous solutions, at pH 7.0
and pH8.0 as used in these experiments it would not be appreciably hydrolysed during the 60min preincubation. (Saunders, 1957).

The identity of the "cholinesterases" in skin have not been fully elucidated. In human skin it is likely that the enzyme or enzymes responsible for thiocholine ester hydrolysis is a "butyrylcholinesterase" as indicated by its sensitivity to ethopropazine, its insensitivity to BW284C51, and its preference for the butyryl ester of thiocholine. In the rat however the evidence is contradictory. Whilst the enzyme, or enzymes, have an insensitivity to ethopropazine and BW284C51 which is characteristic of a "butyrylcholinesterase" their preference for the acetyl ester contradicts this conclusion. These characteristics of rat skin "cholinesterase" are similar to those of the "cholinesterase" found in rat skeletal muscle by Skau (1981,82), who reported an ethopropazine sensitive acetylcholinesterase. The possibility that the "cholinesterase" in rat skin homogenates originates from the subdermal muscle layer (panniculus carnosus) cannot be discounted.

These findings suggest the existence of a "cholinesterase" in rat skin that is different from that found in the serum of the horse and in man (both red cell, and serum cholinesterases), and is comparable with early studies of rat tissues. Levy (1951) and Myers (1953) found that rat tissues contained a "cholinesterase" with an optimal activity for the propyl acyl chain. This suggests the wisdom of using propionylcholine or propionylthiocholine in order to further elucidate their
"cholinesterase" properties.

It is not possible to more fully characterise the esterases in the skin of rat and man from the data presented to date. The identification of specific types of esterases such as neurotoxic esterase (Johnson, 1975), or proteases capable of hydrolysing the esters used, would require the use of additional substrates and inhibitors (eg. phenylvalerate and mipafox for neurotoxic esterase).

5.3.3. COMPARISON OF ELECTROPHORETICALLY SEPARABLE ESTERASES FROM RAT AND HUMAN SKIN

The presence of a different set of esterases in human skin from those in rat skin is shown in fig. 5.6. It is unlikely that the differences between esterases in rat and human skin are the result of lower protein loading in the human tracks, because a) the less mobile bands in the human extracts stained as quickly as the rat esterases, and b) increasing the staining time of the human extracts showed no further bands.

The completely different electrophoretic pattern produced from the homogenate of cultured keratinocytes leads to several possible conclusions: namely keratinocytes contain different esterase in culture than they do in vivo, or the esterases in an homogenate of full thickness rat skin originate from nonepidermal tissue, or the enzyme composition of rat tongue, the source tissue of the keratinocytes, differs from that of skin from the same species. The absence of the esterases present in
keratinocytes from the electrophoretograms of tissue extracts does not of course preclude their presence in epidermis in vivo. The epidermis represents such a small proportion of the total tissue mass of full thickness skin that epidermal esterases could escape detection.

In conclusion rat and human skin contain a biochemically heterogeneous population of esterase capable of hydrolysing short chain fatty acid esters of thiocholine, p-nitrophenol and indoxyl. These esterases are identifiable as "B" type esterases with the exception of an esterase in human skin that is capable of hydrolysing p-nitrophenylacetate but none of the other substrates used, and is tentatively identified as a "C" esterase. Two types of esterase could also be distinguished by using selective "cholinesterase" inhibitors, the thiocholine substrates were mainly hydrolysed by acetylcholinestase and butyrylcholinesterase "type" enzymes, whereas the acetates of p-nitrophenol and indoxyl were not. These mixtures of esterases, contained in the soluble fraction from skin of both species, can be separated on polyacrylamide gel to show several electrophoretically distinct naphthylacetate hydrolysing esterases. The naphthylacetate esterases present in the rat are not detectable in the human extracts; and there was no similarity between the esterases of either species and those present in an homogenate of cultured rat keratinocytes.
CHAPTER 6

THE LOCALISATION OF ESTERASES

IN SKIN
6. THE LOCALISATION OF ESTERASES IN SKIN

6.1 INTRODUCTION

In a heterogenous tissue like skin the localisation of enzyme activities is critical to the determination of their effects on foreign compounds. If a xenobiotic does not reach the site of enzyme activity (eg. if it is absorbed into the blood at the epidermal/dermal border and the enzyme is located in the sebaceous glands), then the enzyme is of academic interest only, in so far as metabolism during percutaneous absorption of that compound is concerned.

Of the methods used to determine the location of an enzyme in skin (that are discussed later in this chapter) histochemistry was chosen for this purpose in the present investigation.
6.2 RESULTS

Routine histology

Samples of the skin used in the experiments described in chapters 3, 4, and 5 were routinely fixed and sectioned to provide a "histological" check on the tissue used in biochemical studies. No lesions or other histological abnormalities were found and the rat skin was always in the growth stage of the hair cycle.

Histochemistry

Skin from 46 day old rats was fresh frozen with carbon dioxide and cut on a cryostat microtome as described in materials and methods. Sections were stained for indoxylacetate esterase activity and the results from one animal are presented in figures 6.1 to 6.5.

Figure 6.1 shows a section of a rat skin stained with the massons trichrome stain to show the hair follicles, sebaceous glands, panniculus carnosus and other organs labelled in fig. 1.1 (p6).

Figure 6.2 shows the staining of a section of rat skin from the same animal for indoxylacetate esterase activity. The activity shows, in blue, in the sebaceous glands with a slight staining of the piloerector muscles. Some diffuse stain is visible in the lower layers of the epidermis.

Figure 6.3 shows the results of "overstaining" with indoxylacetate, blue colour appears in the keratin sheaf of the hair. This is probably due to the diffusion of product away from
the site of formation and subsequent binding to other areas of the section for which it has an affinity.

Figure 6.4 shows the results of a section that has been cut so that the hair follicles are in transverse section. The sebaceous glands stain well in this section as do small areas of piloerector muscles around the glands. This pattern of staining was seen in all sections made from the skin of four separate animals in four separate experiments.

Figure 6.5 shows the staining of a section of rat skin with naphthylacetate. The distribution of esterase is different from that of the indoxylacetate esterase. The sebaceous glands did not stain but the epidermis, piloerector muscles, follicular linings, and panniculus carnosos did. This pattern of staining was seen in several sections from two separate animals in two separate experiments.

Figure 6.6 shows a section of human mammary skin used for the biochemical experiments detailed in chapter 5 stained with masson's trichrome stain. This skin differed from the rat in the major respect of having almost no hair follicles. In all two hair follicles were found in 15 sections from the human skin, one of these had a sebaceous gland associated with it and this gland stained faintly with indoxylacetate (photograph not shown).
FIGURES 6.1 TO 6.6

Massons trichrome stain:–

  dark red to black – nuclei
  red – cytoplasm, muscle, keratin
  blue/green – collagen

Indoxylacetate esterase:–

  esterase – blue
  counter stain – red

Naphthylacetate esterase:–

  esterase – red/brown
  counter stain – none
Transverse section of skin from a 46 day old "Porton Strain" rat.

See Fig. 1.1 (p6) for identification of organs.

Massons trichrome stain, 10µm section.
Fig. 6.2

TRANSVERSE SECTION OF 46 DAY OLD RAT SKIN. 5-BROMINDOXYLACETATE STAIN SHOWING STAINING FOR ESTERASE IN SEBACEOUS GLANDS AND PILOERECTOR MUSCLE. SOME DIFFUSE STAIN VISIBLE IN LOWER LAYERS OF THE EPIDERMIS.
Fig. 6.3

TRANSVERSE SECTION OF 40 DAY OLD RAT SKIN. 5-BROMOINDOXYLACETATE STAIN, 10μm SECTION, SHOWING STAINING FOR ESTERASE IN SEBACEOUS GLANDS AND PILOCRETOR MUSCLES, ALSO SHOWING THE EFFECTS OF "OVERSTAINING" BY STAINING OF THE KERATIN SHEATH OF THE HAIRS.

PAGE 180
Fig. 6.4

TRANVERSE SECTION OF 40 DAY OLD RAT SKIN. 5-BROMINDOXYLACETATE, 10μm SECTION SHOWING STAINING OF ESTERASE IN THE SEBACEOUS GLANDS AND IN THE PILOERECTOR MUSCLES. IN THIS SECTION THE HAIR FOLLICLES HAVE BEEN SECTIONED TRANSVERSALLY.

PAGE 161
Fig. 6.5

Transverse section of 46 day old rat skin. Naphthylacetate stain, 10μm section, showing staining of the epidermis and lining of the hair follicles. The panniculus carnosus also stained in this section.

Page 182
Fig. 6.6

Transverse section of human mammary skin from a young female.

Massons trichrome stain, 10μm section

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6.3 DISCUSSION

Methods of determining the site of enzyme activity within the skin.

Several different methods have been employed to define the location of enzymes within the skin. In the first the skin was split using a dermatome or similar device to remove the epidermis and leave the dermis and deeply embedded organs behind. Treatment with sodium iodide will remove the epidermis with sebaceous glands attached and Adachi and Halprin (1967) have developed a method of performing "micro" biochemistry on single hair follicles from skin. The epidermis may also be removed by heating the skin to 52°C for 30secs. (Thompson and Slaga, 1976).

Each of these techniques provides sufficient material for biochemical analysis but could cause damage to the enzymes of interest. The sodium iodide might inhibit enzymes, and in the experiments where heat was used to separate epidermis prior to arylhydrocarbon hydroxylase assay control of temperature and duration of heating was critical to prevent loss of activity (Thompson and Slaga, 1976).

Mechanical separation reduces the risk of damaging the enzymes but produces more heterogeneous preparations, leaving some epidermis attached to the dermis, or removing some dermis with the epidermis depending upon the precise depth to which the cutting blade is set. In addition the need to use potentially
destructive depilatory creams when using this method on hairy skin again raises the question of enzyme damage.

Histochemical techniques permit the detailed location of enzymes without the need to use such potentially denaturing treatments but have many artifacts associated with them. The techniques are limited by the need for insoluble coloured products, thus not all enzymes can be histochemically determined, and rarely using their preferred substrates. The techniques were however particularly suited to the aims of this investigation since "nonspecific" esterase is routinely localised by histochemical methods using 5-bromoindoxylacetate, and naphthylacetate, with subsequent derivatisation with Fast Blue. In addition histochemical methods are not easily quantifiable.

Many other substrate have been used in the histochemical determination of esterases, Since the result of ester hydrolysis is the release of two molecules that have a higher solubility in water than the original ester, the need to combine one of these products, normally the alcohol, with some insoluble molecule after hydrolysis has arisen. This can be done in one of two ways, the derivatised; agent can be added to the incubation mixture so as to combine with the product immediately upon its formation, or the hydrolysis can be allowed to take place and the derivating agent added afterwards to develop the colour. The former of these methods is open to criticism in that the derivatising agent may be inhibiting the enzyme, whereas the later allows the product to diffuse away from the site of formation leading to erroneous
localisation. Whichever method is chosen these limitations must be considered in assessing results. In addition the possibility that some soluble enzymes could diffuse away into the incubation mixture is particularly true in the case of esterases, since some esterases are notably soluble (i.e. butyrylcholinesterase). Since the esterase studied in chapters 3, 4, and 5 were soluble proteins the possibility that they have diffused out of the tissue during the early stages of the incubations cannot be ruled out.

Given the above limitations the experiments described here have confirmed the results of earlier histochemical studies in this area. Angyris (1956) and Findlay (1955) found naphthylacetate esterase in the epidermis, follicular linings and subdermal muscle layer. Similarly Healey et. al. (1971) found indoxylacetate esterase in the sebaceous glands of mouse skin during their studies on the irritancy of tobacco smoke.

Implications of the localisation of esterases in skin

The naphthylacetate esterases will have a greater effect upon xenobiotics applied percutaneously than "indoxylacetate esterase" due to the former's location in the epidermis. However xenobiotics that are preferentially concentrated in the sebaceous glands, are available for hydrolysis by the indoxylacetate esterases. Moreover the degree to which foreign compounds that reach the skin by way of the systemic circulation may be metabolised by these two populations of esterases will be determined by the relative perfusions of the various areas of the organ with blood, and thus upon the temperature, hormonal, and pathological status of the skin.
Most significant is that the enzymes responsible for the hydrolysis of naphthylacetate are different to those responsible for that of 5-bromoindoxylacetate. Thus the term "non-specific esterase" when applied to skin must be supplemented with the identity of the substrate used.
CHAPTER 7

FINAL DISCUSSION
The first part of this investigation showed that the esterase population in rat skin homogenate could be demonstrated as consisting of at least two types by the use of selective substrates and inhibitors. One of these "types" of esterases was a cholinesterase and the other an esterase capable of hydrolysing p-nitrophenylacetate but resistant to cholinesterase inhibitors.

Attempts to purify these esterases, though unsuccessful in producing purified esterases in quantity, showed them to be heterogeneous populations of proteins. The different esterases tended to be found together in the "subcellular fractions" prepared by ultracentrifugation, the largest quantity of cholinesterase and p-nitrophenylacetate esterase remaining in the 100,000xg supernatant. Electrophoresis however showed that there were different naphthylacetate esterases present in the different fractions, in particular a "microsomal" and a "mitochondrial" enzyme. At least sixty percent of the total activity present in the 100,000xg supernatant is likely to have been composed of soluble proteins that originated in the cytosol of the cell or were only loosely associated with cellular organelles.

These "soluble" esterases coassociated through ammonium sulphate precipitation, but when subject to molecular exclusion chromatography they were shown to be heterogenous populations of proteins with molecular sizes of between 80Kd and 200Kd. Dissociative P.A.G.E. revealed a purification of monomers of between 32Kd and 45Kd during chromatographic separation.

The biochemical characterisation of these enzymes was
carried out using the 100,000xg supernatant as the source of proteins. This again showed the presence of two biochemically distinct populations of esterases, in both rat and human 100,000xg supernatants. Most of the esterases from both species were "B" esterases that were inhibited by the organophosphorus ester DFP. One portion of the esterase activity against p-nitrophenylacetate in human skin however appeared to be the rare "C" esterase that did not interact with DFP.

The cholinesterases in rat and human skin behaved in a similar way to butyrylcholinesterase from serum in their interaction with specific inhibitors. The rat cholinesterase however displayed a preference for the acetyl ester rather than the butyryl ester of thiocholine, demonstrating a major difference between the two species. Another difference between man and rat in this respect was shown by the electrophoretic separation of the esterase in the subcellular fractions from the two species, the rat showed three major bands of naphthylacetate esterase, with several minor bands in the rat, whereas the there was only one major naphthylacetate hydolysing band with one other minor band in human skin. Evidence was also obtained that showed one of the bands in the rat 100,000xg supernatant was composed of more than one protein.

This investigation has raised some interesting questions that merit further study:-

1) Which of the electrophoretically separable esterases correspond to the esterase responsible for the hydrolysis of indoxylacetate, p-nitrophenylacetate, and the thiocholine esters?
In this study the addition of the butyrylcholinesterase inhibitor ethopropazine in the stain mixture used on the electrophoresis gels has not resolved the identity of this enzyme (chapter 5).

2) What other esters are hydrolysed by skin esterases?

3) Are there esterases present in skin that may be responsible for the hydrolysis of xenobiotics that have not been measured in this study?

4) Are the esterases in the particulate fractions similar to those in the soluble fraction? Electrophoresis showed the presence of different enzymes in these fractions.

The complete identification of the skin esterases is not possible from the data available at present. Subsequent studies must involve the use of a wider range of substrates than has been used here. This would enable more complete characterisation of the enzymes and identification by comparison to existing data on enzymes from other tissues and species.

A fruitful line of further research would therefore combine the techniques of electrophoresis, with subsequent staining with many different substrates in the presence and absence of inhibitors, and protein separation and purification techniques to provide adequate quantities of purified enzymes for biochemical characterisation of individual esterases. The histochemical localisation of each of the enzymes, preferably using the high specificity of antibodies raised to the purified proteins would also be useful in assessing their significance in the hydrolysis of xenobiotics reaching the skin by topical or systemic routes.
The precise biological roles of the esterases in the skin is as yet undetermined, but some idea has been gained during this study. The 5-bromoindoxylacetate, and perhaps the indoxylacetate esterases might be associated with the production of sebum because of the localisation of the former in the sebaceous glands. Staining of the piloerector muscle with 5-bromoindoxylacetate however may indicate a heterogeneity in the enzymes responsible for its hydrolysis in skin, or the localisation of the same enzymes in two areas of the skin. The naphthylacetate hydrolysis has been shown here, and in other studies, to be the function of a heterogeneous population of enzymes and as such probably has a wide range of different biological roles.

The cholinesterases in skin have a similarly undefined biological role, as do their counterparts in other tissues. To date no attempt to assign a biological role to these enzymes is regarded as "proven" and it is possible that they are vistigial. (Kutty, 1980). The main reason for this is the absence of a physiological substrate. Butyrylcholine remains the best substrate for butyrylcholinesterase, but neither this compound, nor any resembling it occur physiologically. The best substrate for the cholinesterases in skin appears to be acetylthiocholine which may indicate a role for these enzymes in the hydrolysis of acetylcholine from the cholinergic nerves in the skin. In the absence of further evidence to support this suggestion however it remains speculative.

A possible role for the esterases in the epidermis is the
hydrolysis of bacterial endotoxins produced on the surface of the skin. In this regard it is noteworthy that splenic "nonspecific" esterases are capable of hydrolysing such endotoxins. In addition the bacteria on the surface of the skin might in themselves be a source of esterases that find their way into skin homogenates and further studies should explore this source of hydrolytic enzymes.

In conclusion, the ability of the skin to metabolise foreign compounds has been the subject of much research in recent years. Many enzymes have been positively identified and partially characterised in the skin, but the full identity of the esterases in skin remains elusive. Oral administration and topical application of therapeutic compounds have long been the most popular routes of administration, and interest in this area is growing with the development of various transdermal delivery systems. This interest combined with the power of hydrolytic cleavage to terminate and activate pharmacological activity will undoubtedly stimulate many more studies of the biochemistry of skin esterases.
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