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AL-SAIDAN, S.M.H.

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# AN INVESTIGATION OF SOME FACTORS AFFECTING TRANSDERMAL PERMEATION

SALEH MOHAMMED HAMAD AL-SAIDAN

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# AN INVESTIGATION OF SOME FACTORS AFFECTING TRANSDERMAL PERMEATION

S.M.H. AL-SAIDAN

## ABSTRACT

Factors affecting transdermal permeation were investigated using Differential Thermal Anlaysis (DTA) and in vitro penetration studies of neonatal rat stratum corneum (s.c.) with radiolabelled propanol and hexanol as penetrants. Thermogravimetric Analysis, Thin-Layer Chromatography (TLC) and Infrared Spectroscopy were used to provide additional information.

DTA of s.c. revealed four endothermic transitions. Those at 42°, 71° and 80°C were attributed to melting of crystalline lipid and at 110°C to water loss. TLC indicated that T71 and T80 were related to melting of free fatty acids. The T80 endotherm displaying an irreversibility dependent on both temperature and time of exposure to elevated temperature. This is explained as being due to an interaction between free fatty acids and sterols within s.c. Variations in peak temperature with species and human body site may explain corresponding variations in skin permeability.

Both hydration and type of vehicle were found to affect penetration rate. The effect of time and temperature of heat pre-treatment (40°-90°C) on s.c. permeability was studied. Large increases in the permeability were obtained above 70°C. This correlates with the irreversible changes in T80 seen with DTA suggesting distortion of lipid structures. S.C. exposed to DMSO vapour showed lower endothermic temperatures and higher permeability. Heat above 75°C plus DMSO vapour pre-treatments produced similar permeability as heat alone indicating a common site of action. Studies of the concentration dependance of aqueous DMSO action showed increasing s.c. permeability above 70% DMSO. The corresponding changes in T80 suggested a possible screen test for penetration enhancers which was confirmed with several materials.

It is concluded that penetration enhancers appear to disrupt the lipid barrier structures probably by hydrogen bonding. DTA linked to penetration studies has been shown to be a useful technique for studying the skin barrier and mode of action of penetration enhancers.

CONTENTS

# CHAPTER ONE

## GENERAL INTRODUCTION

Skin is a multilayered organ, whose main functions are to protect the body from environmental stress, regulate heat loss, resist mechanical shock, act as an electrical and radiation barrier, retard the loss of endogenous substances and the invasion of external chemicals and micro-organisms. The major role of the skin is, therefore, defensive, but, whilst it is passive, it also acts as a metabolic site for drugs penetrating the skin (1,2). The anatomy and physiology of skin has been reviewed by many workers (2-9).

In the past skin was considered to be totally impermeable to all substances except gases, and even the latter was doubted. However, at the turn of the century, scientists concluded and generalised from their systematic work, that skin is permeable to gases and to substances which are soluble in lipids, but that it is practically impermeable to water and electrolytes. Within the past few decades, following development of highly sensitive detection methods such as the use of radioactive isotopes, it has been shown that skin is permeable to practically every chemical substance, even high molecular weight materials such as human serum albumin. The rates of penetration vary by about 10,000-fold for the range of substances estimated (1,9-12).

When a substance is applied topically, three processes may be initiated.

(a) Penetration : when the substance enters a single layer of the skin.

(b) Permeation : when the substance migrates from one layer to another anatomically different layer.

(c) Resorption : when the substance is taken up by the vascular

system (lymph and/or blood).

Percutaneous absorption is the sum of these three processes and may be defined as the penetration of substances from the outside into the skin, through the whole skin and into the blood stream (9,10).

Data obtained from permeability studies are analysed mathematically to calculate the rate of penetration or flux and the permeability coefficient ($K_p$). The latter is commonly used as an index of permeability so that the higher the permeability coefficient the higher the permeability (1,2,4,6,11,13-17).

Percutaneous absorption is important in topical therapy, toxicity and as a possible alternative route for systemic use. The main advantage of using topical administration rather than the oral route is that, potentially, fluctuation in blood levels can be avoided. This transdermal delivery devices give almost constant, optimal blood levels. The route also avoids first-pass metabolism (2,12).

Observed variations in penetration rates are due to the ability of these different substances to cross the skin permeability barrier. The outermost layer of the epidermis is called the stratum corneum or horny layer. It is composed of several layers of dead keratinised cells. Sequential stripping or organic solvent extraction has shown that the skin barrier resides almost entirely in the stratum corneum (1,3,11). It has been recognised that materials may enter the skin by any of three routes, namely transcellularly, intercellularly or through the pilosebaceous system and sweat glands (2,6,11-14,18,19).

Stratum corneum contains both aqueous polar and lipid non-polar regions. Data from permeability studies suggests that, depending on the physicochemical properties of the penetrant, a different

pathway through the stratum corneum may be used. Thus a polar substance is likely to penetrate through the polar regions of stratum corneum, whilst a non-polar substance would pass through the non-polar region (11,13).

Stratum corneum has been found to be composed mainly of proteins (keratin), lipids and water (11). Extensive work on percutaneous absorption has led some workers to conclude that the main skin permeability barrier is a lipid-protein complex (11), although more recent work concluded that the intercellular lipids are the main barrier (20-22).

Skin permeability has been investigated using both human beings and animals for in vivo studies and excised skins from these species for the in vitro methods. Many experimental techniques have been devised for both in vitro and in vivo studies and data obtained from them have been found to correlate to a certain extent (1,2,4,9,18, 19,23,24).

This experimental work has shown that drug permeation through the skin is controlled by many factors which may be divided into two major groups. Biological factors are concerned with the skin whilst physicochemical factors are concerned with the penetrant and the formulation.

Studies on skin permeability have shown that the skin barrier becomes inferior when diseased, stripped or damaged by chemical and physical treatment. For intact skin, other factors such as species, age, body site, blood flow have been recognised. Thus, when different animals are used in permeability studies (such as mice, rats, rabbits, pigs, guinea pigs, monkeys, etc.) different penetration rates are found. Human skin shows the lowest permeability, whilst rabbit skin has the highest. Care is, therefore, needed in any comparative

work.

Physicochemical factors such as skin hydration, temperature, concentration of drug in a vehicle and lipid-water solubility of applied material have been investigated. Solvents have an effect on skin permeability and in particular aprotic solvents such as dimethylsulphoxide (DMSO), dimethylacetamide (DMAC) and dimethylformamide (DMF) have the effect of increasing skin permeability. They have been called Sorption Promoters (25). A considerable literature has been published on these factors affecting percutaneous absorption and have been reviewed by several workers (1,2,4,18,19,24, 26,27).

Apart from using permeability studies, skin has also been investigated using several analytical techniques such as wide-angle and small-angle X-ray diffraction, infrared spectroscopy (IR), electron microscopy and thermal analysis. The latter includes Differential Thermal Analysis (DTA), Differential Scanning Calorimetry (DSC), Thermomechanical Analysis (TMA) and Thermogravimetric Analysis (TGA) (reviewed by 28,29). Thermal analystical methods have enabled the identification and detection of desorption of water from skin, melting of crystalline lipids and ice and denaturation of protein (30-42), as well as detecting differences between normal and abnormal skin and between species. The effect of added materials on the thermal behaviour of skin has also been studied (33,37).

The aim of this work was to study, in vitro, the penetration of propanol and hexanol using neonatal rat stratum corneum in a diffusion cell, and to relate the data to DTA studies. With the latter, together with other physical techniques such as TGA, Thin-Layer Chromatography (TLC), IR, the aim was to identify the skin components responsible for the endotherms obtained. Once identified,

factors which are known to affect skin permeability can be studied. From this work should come a better understanding of the nature of skin permeability barrier and the factors which affect it. Because it has been established that the barrier lies within the stratum corneum, separated stratum corneum sheets were used in this work to avoid interfering variables. Neonatal rat was used because it does not have developed pilosebaceous units.

# CHAPTER TWO

# THERMAL ANALYSIS OF STRATUM CORNEUM

## Introduction

Dry stratum corneum has been shown to contain keratin (65%), soluble proteins and other low-molecular-weight dialyzable substances (20%), resistant plasma membrane (5%), and lipids (7-9%) (Matoltsy and Balsamo, 43).

During the past two decades, groups of workers have carried out general thermal analytical studies on the stratum corneum of both human beings and animals. In all these studies a number of endothermic transitions have been found. Each group of workers has attempted to relate the endotherms to changes taking place in the constituents of stratum corneum.

Bulgin and Vinson (30) were the first to investigate stratum corneum using Differential Thermal Analysis (DTA). They found that with hydrated samples of both human and neonatal rat stratum corneum, five endothermic transitions were observed, at 77-88°, 103°, 114°, 135° and 185°C. They interpreted the broad transition at 77-88°C as being due to lipid melting, and confirmed this by extracting the stratum corneum with the lipid solvent, chloroform-methanol (2:1 v/v). The dried extract showed the broad lipid transition at 70-88°C whilst the extracted stratum corneum no longer showed this endotherm. The transition at 103° was found to be due to the elimination of unassociated water from the skin, whilst the peaks at 114° and 135° were due to bound water held at different binding sites. The transition at 185°C was attributed to protein breakdown. In addition to this work on normal skin, those workers also investigated abnormal skin.

Wilkes et al (31) also used DTA for adult human and neonatal

rat stratum corneum. They reported a very small endothermic transition in some samples at 40°C, and a fairly broad endotherm at ca. 80°C. Also there was another broad endothermic peak at 110°C in some of neonatal rat stratum corneum samples. They found that on reheating samples, the 110°C endotherm was missing, but it could be regenerated by exposing the same sample to 81% relative humidity for a few hours. This was in line with Bulgin and Vinson and it was accepted that this transition was caused by water desorption. X-Ray diffraction of neonatal rat stratum corneum showed two lipid melting bands at 40° and 70°C in agreement with DTA. Human stratum corneum however showed an additional lipid reflection, indicating lipids melting at 40° and two between 80-90°C. The dynamic mechanical studies of Wilkes and Wildnauer (32) also indicate lipid melting around 40°C for both neonatal rat and human stratum corneum, although the latter is less distinct. Wilkes et al. (31) suggested that the 40°C transition was due to either a partial melting or some sort of morphological change of the type which occurs in liquid crystal or mesophase systems. Such mesophase transitions are known to occur in lipid systems and have smaller peaks of transition than melting. DTA of solid cholesterol has also shown a similar small endotherm at around 40°C (44,45). The broad peak at ca. 80°C was described as lipid melting. On reheating the sample, the peak was reduced and occurred at 70°C. This change was explained as being due to a reduction in the crystalline lipid content or by the lack of interference by water on this second heating.

In his work Van Duzee (33) used Differential Scanning Calorimetry (DSC), and reported four endothermic transitions in human stratum corneum at 40°, 75°, 85° and 107°C. Those at 40° and 75°C were attributed to lipid melting, this conclusion being supported by extraction with chloroform-methanol (2:1 v/v) following which two

transitions were observed in the dried extract although at lower temperatures. The two endotherms at 85° and 107° were attributed to denaturation of α-keratin and non-fibrous protein, respectively. In further work (33) he investigated the absorption and desorption of water and found that the quantity of freezable water (i.e. non-bound water) increased when stratum corneum was heated to the denaturation temperature of non-fibrous protein (107°C). The location of fibrous and non-fibrous proteins was studied by the use of urea solutions. He found that the endothermic transition of non-fibrous protein moved to a lower temperature following overnight soaking of stratum corneum in an aqueaous solution of urea, but no change occurred in the α-keratin endotherm temperature. From these observations, Van Duzee concluded that the non-fibrous protein is located either intercellularly or as part of the cell wall, whilst α-keratin is intracellular and cannot, therefore, be reached by urea. Apart from the endothermic transition around 70°C, the three reports (30,31,33) show disagreement in both the general pattern of endothermic transitions and the interpretation of results. For example Bulgin and Vinson (30) report three transitions above 100°C, at 103°, 114° and 135°, in contrast to the single peaks of the other two groups (31,33). Also, of the three groups, only Van Duzee reported protein denatuations around 100°C. Also the lipid transition at 40°C was found to be reproducible by Van Duzee, less reproducible by Wilkes et al and was not reported by Bulgin and Vinson.

Such variation in results are not surprising because of the differences in instrumentation, operating condition, and sample origin, preparation and size (46). The variables for the three groups (30,31,33) are presented in Table (2.1).

Table 2.1

Comparison of conditions used for thermal analysis by previous workers.

| Reference | Instrument | Source of stratum corneum | Sample weight (mg) | Heating rate °C $min^{-1}$ | Note |
|---|---|---|---|---|---|
| Bulgin and Vinson (30) | DTA | Human and neonatal rat | 50 | 2 | Open pan, static air |
| Wilkes et al (31) | DTA | Human and neonatal rat | Not stated | 20 | Open pan, sweeping gas |
| Van Duzee (33) | DSC | Human | Approx.10* | 20 | Hermetically sealed samples |

* Estimated from the size of stratum corneum sheet used.

There is evidence that a miscellaneous group of water-soluble substances, referred to as Natural Moisterizing Factor (NMF) (Jacobi, 47) have a role in retaining water in the stratum corneum (47-54). These NMF have been shown to consist of a mixture of amino acids, urea, sodium pyrrolidone carboxylate, lactates, polysaccharides, inorganic salts and water-soluble polypeptides (47,50-52,54). These hygroscopic materials can be removed by sequential extraction of stratum corneum with organic solvent (e.g. Ether) and water or by extraction with surfactants (35,47-53). They may also be extracted with water following pulverization of stratum corneum in presence of liquid nitrogen (52). Water loss can be quantitified by using TGA technique (31,35) as well as quantitative DSC (33,34). Walkley (34) was able to differentiate between bound and unbound water in both human and guinea pig stratum corneum. Skin of known total water content (bound and unbound) was frozen. The latent heat of melting of the ice gave the amount of unbound water, after which the bound water (non-freezable) can be calculated. The maximum amount of bound water (before the appearance of unbound water) was found to be 0.29 g/g for guinea pig and 0.34 g/g for human stratum corneum.

Using Thermomechanical analysis (TMA), Humphries and Wildnauer (36,37) carried out a study on both human and neonatal rat stratum corneum. Lipid as well as protein transitions were observed when stratum corneum was analysed from 25° to 325°C. Neonatal rat stratum corneum showed softening transtions at 45°, 155° and above 200°C. Similar transitions were obtained with human stratum corneum but at higher temperatures, 53°, 177° and above 200°C. They suggested that the softening transition at 45°C is partly due to lipid, since extraction with choroform-methanol (2:1 v/v) caused a reduction of this transition and DTA of the dried extract showed lipid endotherms

at 40° and 60°C. The softenings at 155° and higher were attributed to protein structural change, that at about 215°C being caused by a disordering of the α-keratin. The more recent work of Miller and Wildnauer (38) on the TMA of normal human stratum corneum, also showed softening transitions at about 50°, 150° and 260°C. Their DSC and TMA studies suggest that the softening at 50°C is related to lipid melting as well as protein side chain motion. The other two softenings were attributed to changes in the state of the protein organization and decomposition, respectively. The denaturation of α-keratin to the β-structure has been of some interest. Van Duzee (33) found that the irreversible endothermic peak at 85°C was lost when human stratum corneum was extracted with chloroform-methanol (2:1 v/v). He suggested that in the extraction the α-keratin was denatured. However both IR and X-ray studies by other workers indicate that lipid extraction does not appear to significantly affect the protein structure of stratum corneum (28,32,35). In the isometric contraction study of Baden and Gifford (39) the hydrated stratum corneum showed an inflection temperature between 82-85°C. X-Ray of samples heated above this temperature indicated conversion of α-keratin to β-structure. However, extraction with chloroform-methanol (3:1 v/v) did not affect the inflection temperature, but resulted in a flattening of the curve at about 90°C due to the loss of lipid. Other X-ray and IR studies have also indicated that α-keratin denatured when heated in water at about 85°C (40,55). In another IR study, conversion of α-keratin to the β-structure in dry stratum corneum was found to require temperatures in excess of 120°C (29). Wilkes et al (31) showed that dry stratum corneum heated at 157°C gave a similar X-ray pattern to control samples, implying no major change in the protein structure. They also did not detect the α-keratin reflection at 5.14Å with rat stratum

corneum and concluded that the α-structure can only be obtained with stretched, hydrated skin or drawn keratin fibres as reported by other workers (40,55,56).

The literature shows that X-ray diffraction and IR spectroscopy are not only useful for studying protein structure but also for detecting lipid within stratum corneum. IR spectra of stratum corneum showed characteristic absorption bands at $2920^{-1}$ and $2851cm^{-1}$ which disappeared after lipid extraction (35). The X-ray reflections at 4.2Å and 3.7Å were commonly found with lipids within stratum corneum (31,55-57).

Elias et al. (58) studied the location of lipids in neonatal mouse stratum corneum using both electron microscopy and X-ray diffraction analysis. They found that the membrane couplets provided X-ray reflections almost identical to those found with intact stratum corneum. Lipid lamellae were observed, by electron microscopy, to reside within these couplets. They concluded that lipids of stratum corneum are located intercellularly and not associated with the intracellular filamentous protein. This conclusion is in good agreement with the recent morphologic and histochemical studies of the stratum corneum which also suggest that lipids are located between cells rather than intracellularly (20-22) and contradicts the theory of a lipid-keratin complex as reported by other workers (11,31,56,57).

In the recent work of Rehfeld et al. (41) using DSC for both human and neonatal mouse stratum corneum sheets, three lipid endothermic transitions were reported at 33°C (23-41°C), 55° and 68°C. The more recent work of Rehfeld and Elias (42) using neonatal mouse stratum corneum and DSC found similar endothermic transitions at 38.2°, 61.6° and 67.6°C. Apart from solvent extraction techniques, they also confirmed lipids by the use of a cooling cycle which showed a

downward move of transition temperatures to 34.4°, 56.6° and 60.7°C. They also found that the extracted lipids showed five endothermic transitions on repeated heating and cooling of the same sample. This is similar to the number of bands found on thin layer chromatography (TLC) of the lipid extracts. A more detailed review of the fractionation and identification of lipids is presented in chapter 4.

There are obvious disagreements in some of the work reviewed above. It can also be seen that there are some differences between species. For example lipid and protein transitions of rat and human stratum corneum sheets were not the same (31,32,37). The health of the skin must also be considered. A number of workers have used abnormal skin (28,30,37) and observed differences between it and normal skin. For example the size of the lipid melting endotherm at 77-88° was significantly reduced in psoriatic scales and callus (30), and the dielectric properties and X-ray diffraction patterns of psoriatic scales were also found to be different from normal stratum corneum (28).

Apart from the use of solvents there is relatively little literature on the effect of added material on the thermal analysis of stratum corneum. The stabilizing and destabilizing effects of formaldehyde and formic acid on keratin has been mentioned (37) and the isometric contraction study of Baden and Gifford (39) showed that skin which had been treated with 6M urea produced inflection temperatures lower than control samples, suggesting an effect on protein integrity.

Van Duzee (33) showed that soaking stratum corneum in urea solution overnight reduced the temperature of the 107°C endotherm. Urea has also been reported to affect the elastic modulus of stratum corneum (59).

The effect of DMSO on the mechanical properties (60-62) and thermal shrinkage (63) of skin has been investigated. The data suggest that it may have an effect on the protein. No work has been published which has used Thermal Analysis to study the effect of DMSO on skin. From the literature Thermal Analysis offers a useful investigative tool. It has been established that there are lipid melting transitions in stratum corneum, but there is disagreement about the interpretation of the transitions above 80°C.

It has also been indicated that changes in thermograms may be a useful way of understanding the action of added materials. This section of the work had, therefore, two objectives. Firstly to clarify the interpretation of the endotherms found in stratum corneum and secondly to investigate the effect of DMSO on the constituents of stratum corneum.

**Experimental Methods**

Sources of skin, apparatus and reagents are given in the appendix.

**A. Preparation of Stratum Corneum**

Neonatal rats were killed within 12 hours of birth. A longitudinal cut was made along the abdomen and the skin pealed off from the rest of the body. The epidermis was separated from the dermis, using the heat method (64), by immersing the skin in water at 60°C for 10 secs. The epidermis was then digested for 45 mins in a solution containing 0.1% trypsin and 0.9% sodium chloride in phosphate buffer of pH 7.4 at 35°C. The digested soft tissue was gently removed using moistened cotton wool tips. The stratum corneum sheet was then rinsed with distilled water for 1 hour and floated onto a stainless steel mesh (120 mesh) and left to dry (at about 22°C, 50-60% R.H.).

Dry sheets were examined microscopically and were only used for Thermal Analysis if they showed cracks or holes. Stratum corneum sheets of neonatal mice and rabbits and adult humans were prepared in the same way except that human corneum was rinsed in hexane at 0°C for 5 mins to remove surface lipid contamination (33) and initial heating in water was for 1 minute. The human plantar stratum corneum was obtained using a sharp razor blade.

**B. Differential Thermal Analysis (DTA)**

Stratum corneum, weighing approximately 8mg dry weight, was folded (using forceps) neatly into an aluminium crucible (volume about 50µl) and held in place by folding the sides over an aluminium lid. This procedure was found necessary to ensure the good thermal contact between the sample and crucible necessary to give reproducible results and avoid hermetical sealing. 10mg alumina, in an identical crucible, was used as reference. Differential Thermal Analysis was carried out using a Stanton Redcroft Model 671B instrument with BD9 recorder. The heating rate was 10°C $min^{-1}$ in static air. Throughout the thermal studies, neonatal rat stratum corneum was used with these conditions detailed below.

Reduced pressure (100mm Hg) was obtained by connecting the instrument vacuum port to mains vacuum supply with the gas inlet valve closed. For subambient work, the gas inlet valve was closed and a desiccant tube attached to the vacuum port to prevent water condensation. Cooling was achieved using liquid nitrogen in the integral cooling system.

**Other Thermoanalytical Techniques**

Differential Scanning Calorimetry (DSC) was carried out using a Du Pont Model 900 fitted with a DTA/DSC cell. Samples of neonatal rat stratum corneum were prepared in a similar manner to that used for DTA. Samples weighing about 8mg were heated at 10°C $min^{-1}$.

TGA technique was carried out on home-made apparatus within the School of Chemistry at Robert Gordon's Institute of Technology on similarly prepared samples of neonatal rat stratum corneum. Samples weighing about 8mg were heated at 2°C $min^{-1}$.

**Investigations using DTA**

**1 - Effect of sample size**

Dry stratum corneum samples weighing 2,4,6,8,10 and 13mg were subjected to Thermal Analysis.

**2 - Effect of heating rate**

Dry samples weighing about 8mg were heated on DTA at 1,5, 10°C $min^{-1}$.

**3 - Effect of Hydration on Thermal Transitions**

The pretreatments listed in Table 2.2 were used prior to DTA.

Table 2.2 Pretreatment of Stratum Corneum

| |
|---|
| 1. Desiccated for at least 3-4 days. |
| 2. Stored at room humidity 50-60% for 4-5 days. |
| 3. Exposed to 100% R.H. for 3 hours. |
| 4. Hydrated with distilled water for 10 days followed by storage over silica gel for 3-4 days. |
| 5. Samples which had been heated up to 140°C were removed from the crucibles, exposed to either room atmosphere for 24 hours or hydrated in water for 2 hours then desiccated. |

In all subsequent experiments, stratum corneum was stored in a desiccator over silica gel (RH, 6-10%) for a minimum of 3 days unless specified to the contrary.

4 - Effect of extraction procedures

(i) Organic solvents (Lipid extraction)

Stratum corneum sheets were extracted for 24 hours with ether, carbon tetrachloride and chloroform-methanol (C/M 2:1 v/v). The extracted sheets were left open to the atmosphere for a few hours to evaporate the solvent, then stored over silica gel prior to DTA. The extracts were evaporated under reduced pressure (100mm Hg) in a nitrogen atmosphere at 30°C. 3-4mg of dry extract was used for DTA.

(ii) Extraction of water-soluble substances

Stratum corneum sheets, hydrated for 24 hours with distilled water, were wrapped in aluminium foil and slowly frozen to cause cell rupture. The frozen sheets were pulverized to a fine powder in a metal pestle and mortar in the presence of liquid nitrogen (65). About 8mg of the powder, after desiccation was thermally analysed. The powdered stratum corneum was homogenized for 10 min in distilled water at 20-22°C to extract the water soluble substances (52). The suspension was stirred for 24 hours with 4 changes of distilled water then centrifuged at 10,000g for 10 min. The extract was evaporated to dryness under reduced pressure (100mm Hg) at 30°C. 5mg was used for DTA.

(iii) Extraction of keratin

Extraction of the fibrous protein of stratum corneum was carried out using both 6M urea and Tris-urea-mercaptoethanol solutions in a similar manner as that of Baden and Co-worke.. 66,67).

a. 6M Urea

Following extraction with water (ii above), the pellet was stirred for 24 hours in a neutral solution of 6M urea at room temperature under nitrogen (with three changes of fresh solution of

6M urea). After centrifugation at 10,000g for 30 min the supernatant was dialyzed against several changes of distilled water. The clotted keratin was centrifuged (10,000g for 30 min), collected and dried in a stream of nitrogen and desiccated. The extracted pellet was also desiccated after rinsing with water to eliminate urea. 5mg of keratin and 8mg of dry pellet was used for DTA.

**b. Tris-Urea-Mercaptoethanol**

Pellet obtained after water extraction (ii above) was extracted with 6M urea containing 0.1M Tris, pH 9.0 and 0.1M mercaptoethanol (Procedure, as for 6M urea, iiia). The recovered keratin as well as the dry pellet was subject to DTA.

**(iv) Digestion with subtilisin, (Protease type viii, sigma)**

To ensure complete removal of keratin, the pellet remaining after treatment by method (iiib) above, was digested with 0.005% subtilisin in 0.03M tris-HCl buffer (pH 8.8) for 1 hour, at 25°C under gentle agitation (65,68). After centrifugation (10,000g for 30 min) the pellet was washed with water, and prepared for DTA (8mg) as before.

**5 - Effect of acidic and alkaline solutions and other substances**

Stratum corneum sheets were exposed to test solutions at room temperature, washed thoroughly with water for 1 hr and desiccated before thermal analysis (Table 2.3). Samples also exposed to formic acid for 1 hr, either rinsed with water and desiccated or excess acid was removed by filter paper, before thermal analysis.

Table 2.3 Pre-treatment of Stratum Corneum

| Test Solution | Contact Time (hr) |
|---|---|
| 1M Sod. hydroxide | 24 |
| 1M Hydrochloric acid | 24 |
| 9M Sulphuric acid | 1 |
| 2% Sod. dodecyl sulphate (SDS) | 4 |

**6 - Effect of Heat Treatment**

Stratum corneum was heated to a specific temperature in the DTA furnace at 10°C $min^{-1}$, removed immediately, and after cooling the furnace, reheated. The upper temperatures for the first run were 80°, 85°, 90°, 100°, 110°, 120° and 140°C. Other samples were pre-heated in the DTA furnace for fixed times at a particular temperature as detailed in Table (2.4). They were then subjected to DTA.

Table (2.4) Pre-heating of Stratum Corneum

| Temperature (°C) | Time (min) |
|---|---|
| 70 | 30,60 |
| 75 | 60,120 |
| 80 | 1,5,10,15,30,45,60 |
| 90 | 15 |
| 100 | 5 |

A Humidity Oven (Vinden Scientific Ltd.) was used to heat stratum corneum sheets at 50% RH for fixed times at a range of temperatures, as detailed in Table (2.5), then subject to DTA.

Table (2.5) Pre-treatment at 50% R.H.

| Temperature (°C) | Time (hr) |
|---|---|
| 70 | 12 |
| 80 | 1, 1½, 3, 4, 5, 6 |
| 90 | ½, 1, 2 |

In another approach, samples were heated to 86-87°C in the DTA and immediately cooled using the integral liquid nitrogen cooling system to below room temperature, followed by re-heating at 10°C $min^{-1}$. The process was repeated several times. Samples pre-treated with 9M sulphuric acid for 60 min, were heated similarly.

**7 - Effect of Regional/Species Variation**

Stratum corneum from neonatal mice and rabbits and adult human (Buttock, forearm, and plantar) were studied using DTA as follows:

Stratum corneum obtained from mice, rabbits and human buttock were heated at 10°C $min^{-1}$ up to 91°, 88° and 99°C, respectively followed by an immediate cooling (6°C $min^{-1}$ for that of mouse and rabbit, 10°C for human buttock stratum corneum) using the integral liquid nitrogen cooling system to below room temperature, followed by reheating at 10°C $min^{-1}$ up to 130-140°C, and finally a third heat run was carried out.

Lipid was also extracted using C/M (2:1 v/v)(see page 17) from stratum corneum of human buttock and plantar and neonatal mice and subjected to thermal analysis using 3-4mg samples.

**8 - Treatment of Stratum Corneum with aprotic solvents (DMSO and DMAC)**

**(i) Liquid treatment**

Stratum corneum sheets were soaked for 6 hours in 80% and 90% DMSO and for 24 hours in 100% DMSO followed by thorough rinsing in distilled water for 16 hours and desiccation

In another approach stratum corneum sheets were treated for 3-4 hours with 50%, 70% and 90% DMSO, pressed dry between filter papers and prepared for DTA.

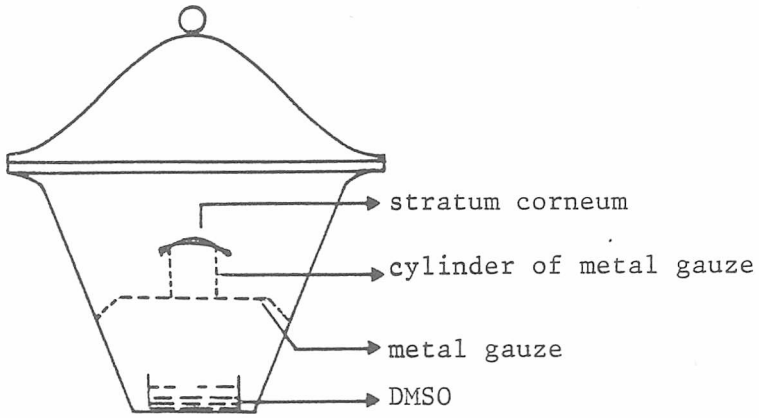



Figure 2.1. Apparatus used for exposing stratum corneum to a vapour.

**(ii) Vapour treatment**

Stratum corneum sheets were exposed to DMSO vapour at 25° and 30°C for 24 hours (Fig. 2.1) then put between two filter papers to remove any condensed DMSO on the surface of stratum corneum and immediately prepared for thermal analysis. Some of the samples treated in this way were also desiccated for two weeks, or thoroughly rinsed with water and desiccated for 3-4 days, before thermal analysis. Human stratum corneum sheets were also exposed to DMSO vapour for 24 hours at 25°C as in case of neonatal rat stratum corneum.

Lipid extracted with C/M (2:1 v/v) (Method 4i) for both human and neonatal rat stratum corneum were mixed with a small drop of DMSO before thermal analysis.

Similarly neonatal rat stratum corneum sheets were exposed for 24 hours to DMAC vapour at 30°C.

## Results and Discussion

Unless specified to the contrary, all results were obtained using neonatal rat stratum corneum .

### 1. Effect of Sample Size

Samples weighing 2,4,6,8,10,13 mg produced identical thermograms, except that the sensitivity of the DTA was increased for smaller samples. With the 2mg samples, the recorder was not very stable due to the high sensitivity required. For subsequent work, around 8mg samples were used.

### 2. Effect of Heating Rate

Heating rates of 1, 5 and 10°C $min^{-1}$ were investigated. It was found that a higher sensitivity and lower chart speed were required for samples heated at 1°C $min^{-1}$. When samples were heated at 1, 5 and 10°C $min^{-1}$, the endotherm temperatures were found to vary as shown in Table (2.6). The recording was unstable at 1°C $min^{-1}$ because of the high sensitivity. For all subsequent work a heating rate of 10°C $min^{-1}$ was chosen.

Table 2.6 Effect of Heating Rate on Endothermic Transitions of neonatal Rat Stratum Corneum up to 100°C (see Fig.2.2).

| Heating Rate, °C $min^{-1}$ | Endotherm Temperature, °C | | |
|---|---|---|---|
| 1 | 39 | 67 | 76.5 |
| 5 | 41 | 70 | 78 |
| 10 | 42 | 71.5 | 79.5 |

### 3. Effect of Hydration

The effect of hydration on the resolution of transitions is shown in Figure 2.2. Stratum corneum sheets which had been desiccated over silica gel for at least 3 days revealed distinct endothermic transitions at 42°, 71-72°, 79-80°, 105-115°C. Reproducibility was within the limits shown. For ease of reference,

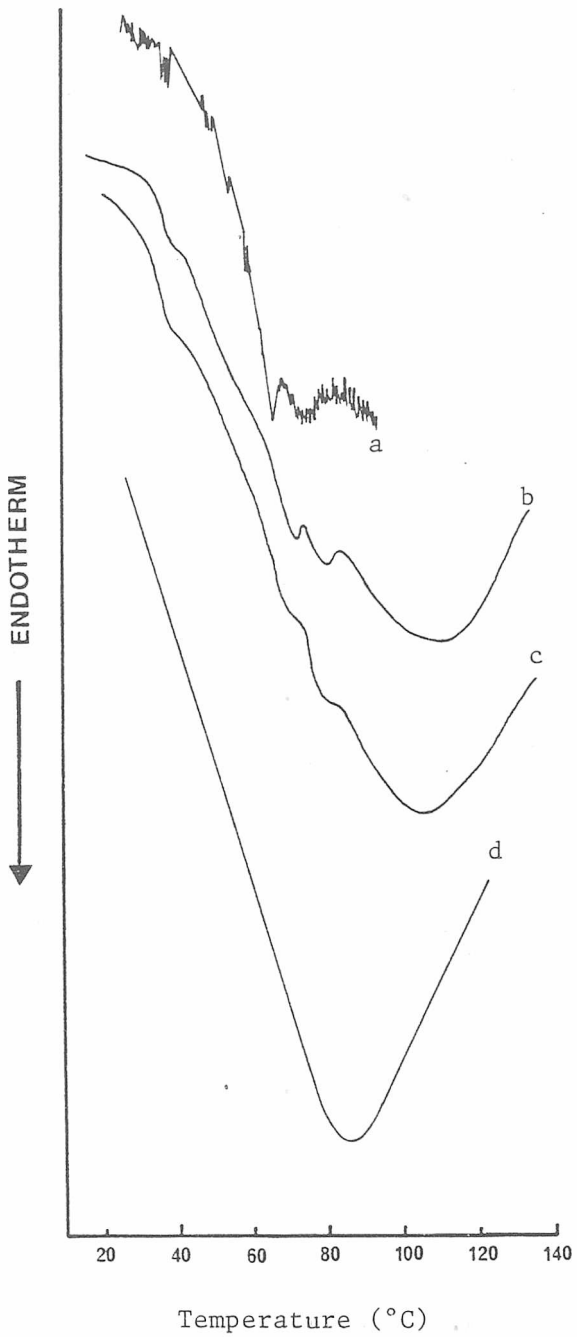



Figure 2.2 DTA thermograms of neonatal rat stratum corneum showing effect of heating rate and hydration on the endothermic transitions. (a) 1°C $min^{-1}$, desiccated sample, (b) 10°C $min^{-1}$, desiccated sample, (c) 10°C $min^{-1}$, sample stored at 50-60% RH for 5 days, (d) 10°C $min^{-1}$, sample stored at 100% RH for 3 hours.

they will be referred to subsequently as being at 42°, 71°, 80° and 110°C.

Similar, but less distinct transitions were obtained with samples stored at room humidity (50-60%). However only one large endotherm, at about 85.5°C, was obtained with samples stored at 100% R.H. for 3 hours. It is obvious that the presence of moisture reduced the resolution of the transitions. The heat of vapourisation of water is large in comparison with the heat requirements for the other possible transformations. Thermal gravimetry has shown that, as stratum corneum is heated, water evaporates over a wide temperature range. It is the energy requirement of this evaporation which progressively masks the smaller energy requirements of the 42°, 71°and 80°C endotherms as hydration is increased.

Stratum corneum soaked in distilled water for 10 days, followed by desiccation prior to thermal analysis gave transitions identical to control samples, with those at 42°, 71°and 80°C more clearly defined. The broad transition at 110°C was less broad, smaller and at 98°C (Fig. 2.3). This may be due to extraction of some water soluble materials (27).

It was, therefore, found that drying samples over silica gel (6-10% RH) was necessary in order to obtain good resolution of the transitions. This finding contradicts van Duzee (33) who found that distinct transitions were associated with the presence of moisture (he was using samples in sealed capsules). Wilkes et al. (31) suggested that the presence of moisture was not necessary for lipid crystallisation. This was confirmed in this work also. Van Duzee agreed that water is not necessary, but suggested that its presence adds to the order of lipid crystal.

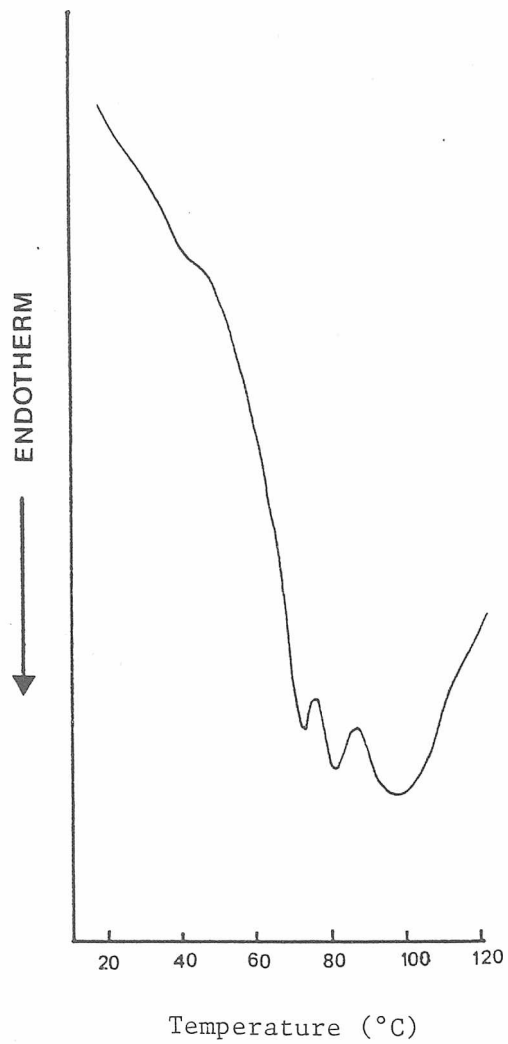



Figure 2.3 DTA thermogram of neonatal rat stratum corneum after soaking in water for 10 days followed by desiccation.

4. **Reversibility of Transitions**

When the temperature of a heating cycle had reached ca., 140°C the DTA was switched to a cooling cycle. Two exotherms were obtained at 35° and 62°C. The second heating cycle of the same sample gave two endotherms at 40° and 69°C (Fig. 2.4). When the resulting sample was removed and immersed in distilled water and desiccated, subsequent thermal analysis revealed three endotherms at 40°, 69° and a broad endotherm around 110°C (Fig. 2.4). A similar effect can be obtained by exposure to 50-60% RH for 24 hours.

DSC produced identical endotherms plus another endotherm at 59-61°C which was occasionally found on DTA and was found to be irreversible (Fig. 2.5). The transition at 80°C was never found to be reversible.

5. **Identification of Transitions**

**(a) Transition at 42° and 71°C**

These endotherms are reversible when heated up to 140°C. However, on a second heating cycle, their temperatures were lower at 40° and 69°C (Fig. 2.4). The cooling cycle gave two exotherms at 35° and 62°C. These observations agree with those of other workers (41,42) that the transitions are due to lipids which supercool during the cooling cycle. Stratum corneum extracted with the lipid solvents such as C/M (2:1 v/v) gave no endotherms around 40° or 70°C, but the dried extract had endotherms at 39°C and 68.5°C (Fig. 2.6).

Using DTA and X-ray diffraction techniques, Wilkes et al. (31) found lipid endotherms at 40° and 70°C in neonatal rat stratum corneum. Humphries and Wildnauer (37) also found that dry C/M (2:1 v/v) extract of neonatal rat stratum corneum gave endotherms at 40° and 60°C on DTA and attributed them to lipid melting. According to van Duzee (33) DSC of human skin gave reversible endotherms at 40°

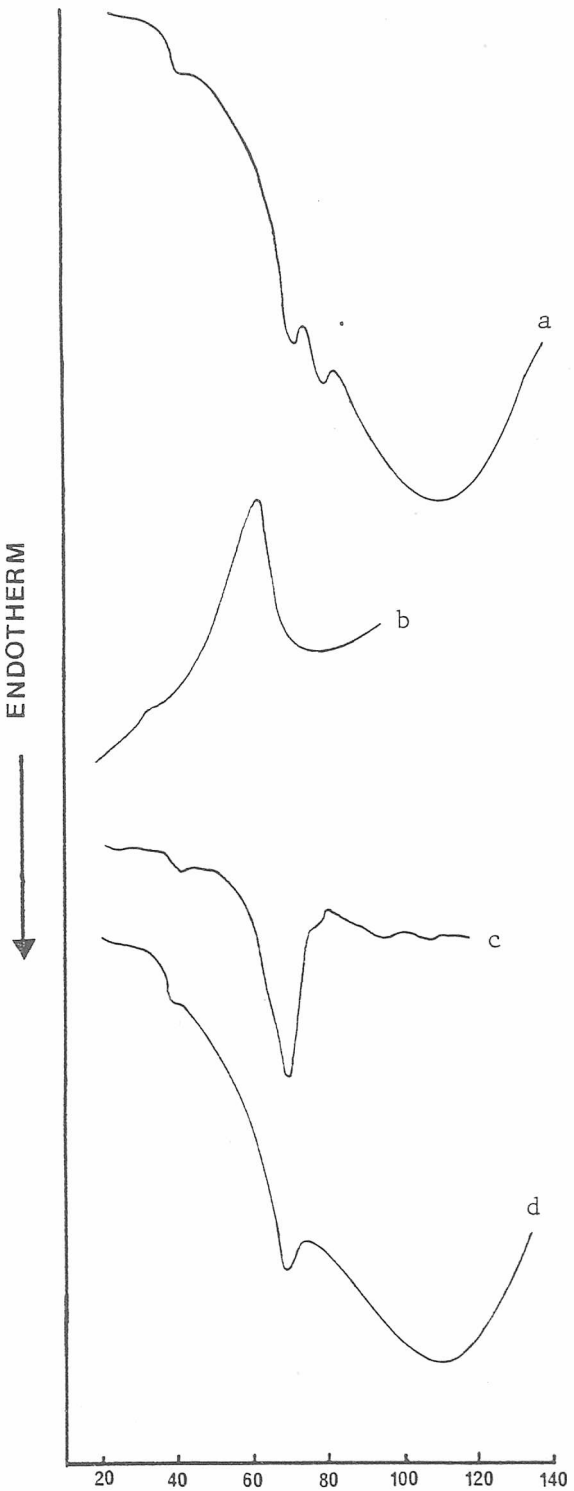



Figure 2.4 DTA thermograms of neonatal rat stratum corneum. (a) first heating run, (b) cooling cycle, (c) second heating run, (d) third heating run following storage for 24 hours at room atmosphere (50-60% RH).

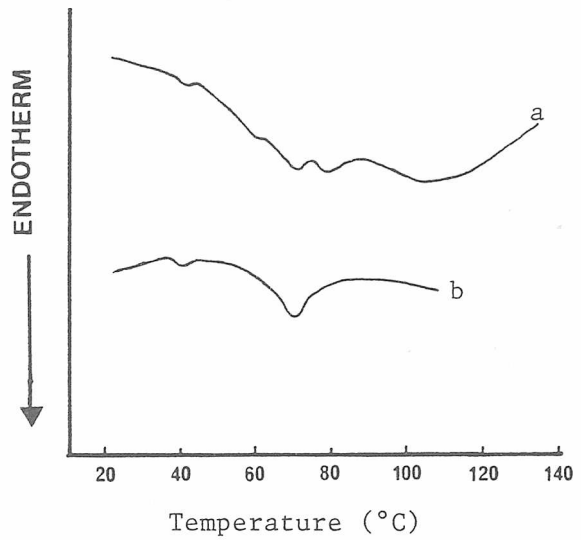



Figure 2.5 DSC thermograms of neonatal rat stratum corneum. (a) first run, (b) second run.

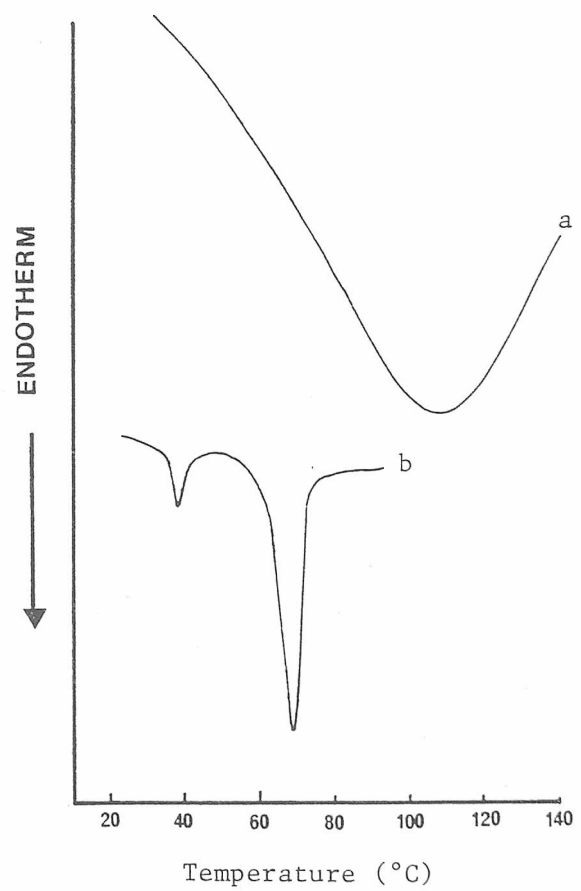



Figure 2.6 DTA thermograms of: (a) neonatal rat stratum corneum extracted with C/M 2:1. (b) the dried extract.

and 75°C with lower transition temperatures when extracted with C/M (2:1 v/v). These were also attributed to melting of lipids. Other workers (29,30,41,42) have also observed that lipid transitions are eliminated from stratum corneum by organic extraction and reappear, at lower temperatures, in the dried extract.

It is, therefore, concluded that the endotherms at 42° and 71° are due to lipid melting.

**(b) Transitions at 80° and 110°C**

These transitions were irreversible when the stratum corneum was recycled immediately after being heated to 140°C (Fig. 2.4). Unlike the sharp transition at 80°C, the broad transition at 110°C was reversible when the stratum corneum was rehydrated (Fig. 2.4). Chloroform-methanol extraction showed no noticeable effect on T110 (Fig. 2.6), but the T80 was eliminated with no sign of its presence in the dried extract. When DTA was carried out under reduced pressure (100 mm Hg) (Fig. 2.7), the transition at 80°C was unaffected, but the transition at 110°C was absent. From the above data it is clear that, whilst both endotherms are irreversible, they are quite different in origin. Their detailed identification will be dealt with separately.

**(i) 110° Transition**

This transition is thought to arise from water loss. A similar broad transition at 110°C has been reported in neonatal rat stratum corneum (31) and found to be regenerated easily by exposing the skin to moisture. It was attributed by Wilkes et al. to moisture release.

Most protein and polypeptides, unless specially dried, show an endotherm in the region of 100-150°C, associated with a weight loss and attributed to moisture desorption (69). The interpretation of the

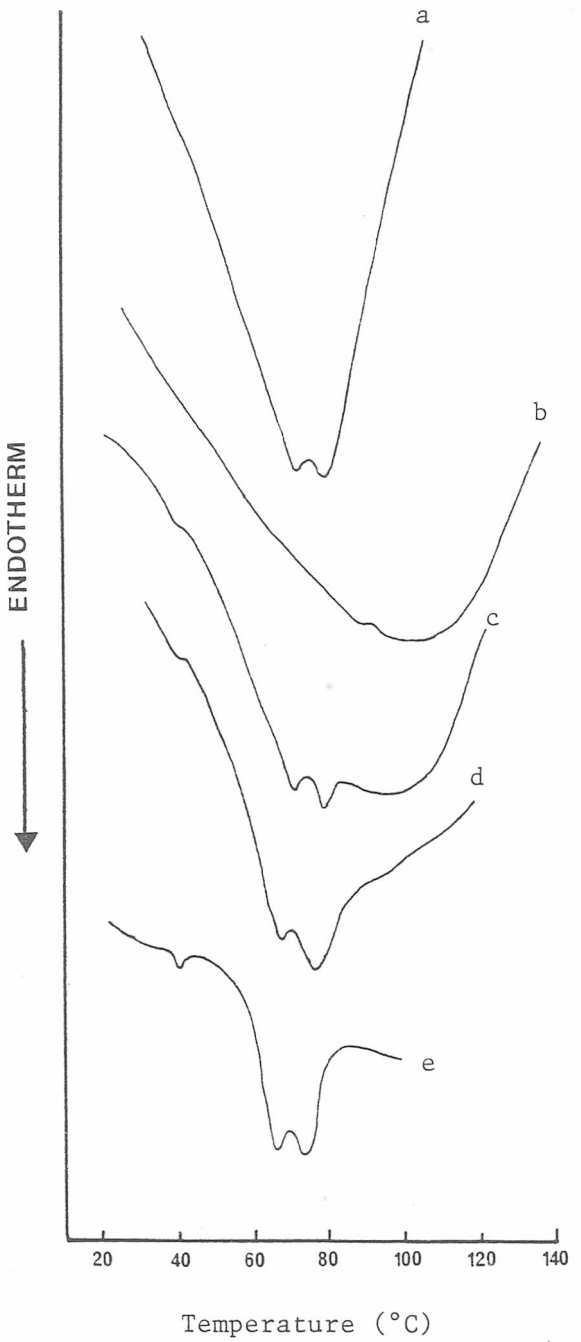



Figure 2.7 DTA thermograms of: (a) stratum corneum, under reduced pressure (100 mm Hg), (b) dried water extract of pulverized stratum corneum, (c,d,e) pulverized stratum corneum extracted with, water, 6M Urea, Tris-Urea-mercaptoethanol, respectively.

endotherm can be supported by TGA in which only one inflection point was observed, at around 110°C, when rat stratum corneum was analysed.

This broad endotherm is probably associated with the water extractable substances, because dry aqueous extract showed a broad peak at around 108°C on DTA (Fig. 2.7). A considerable reduction in the size of the endotherm at 110°C was found in stratum corneum powder extracted for 24 hours with distilled water (Fig. 2.7). The water soluble substances have been considered to be responsible for holding the water content of the stratum corneum (47-54). Since stratum corneum, having had water-soluble substances and keratin removed, showed no transition around 110°C (Fig. 2.7). Thus, these possibly together with keratin (30,35) will hold the water whose loss gives rise to the endotherm at 110°C.

**(ii) 80°C Transition**

From the experimental work already reported, it is very unlikely that the 80°C transition could be attributed to water loss. In the work of Wilkes *et al.* (31), neonatal rat stratum corneum gave an endotherm between 70-80°C on the first run, but the second run showed it only at 70°C. The higher endotherm temperature was attributed to moisture on the first run, although they failed to regenerate it by exposure to moisture (Fig. 2.8). In the work of van Duzee (33) a similar, irreversible endotherm, at 85°C, was observed with human stratum corneum (Fig. 2.9). Following C/M (2:1 v/v) extraction, neither the dry extract nor the extracted stratum corneum showed an endotherm at 85°C. Based on the work of Baden *et al.* (40), van Duzee concluded that the transition at 85°C was due to the denaturation of α-keratin.

In this work, an attempt was made to extract keratin from the

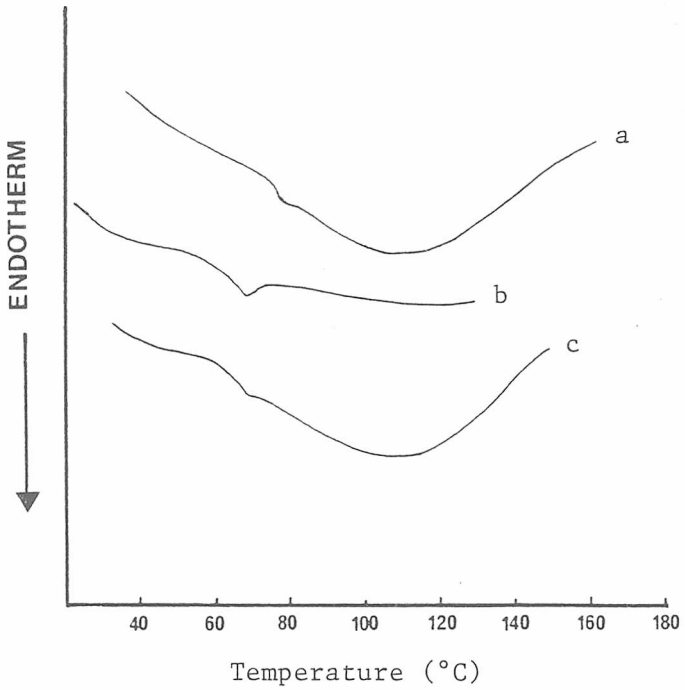



Figure 2.8 DTA thermograms of neonatal rat stratum corneum obtained by Wilkes et al. (31). (a) First run, (b & c) second run after exposure to 81% RH for 30 mins and 2 hrs, respectively.

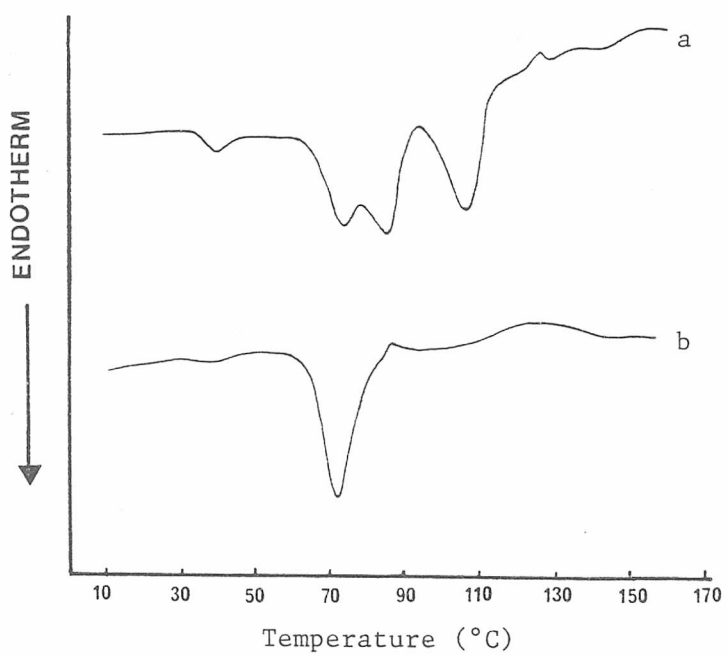



Figure 2.9 DSC thermograms of human stratum corneum obtained by Van Duzee (33). (a) First run, (b) second run.

stratum corneum and expose it and the extracted stratum corneum to DTA. The dry keratin showed no endotherms around 80°C when heated up to 100°C. However, the extracted stratum corneum still showing the T80 as well as T42 and T71, but at lower temperatures (Fig.2.7 and Table 2.7). The nature of this lowering in peak temperature is not understood. Further extraction with 0.005% subtilisin had no further effect on the transitions.

Table 2.7 The effect of extraction of water-soluble substances and keratin on the endothermic transitions of neonatal rat stratum corneum powder.

| Stage of Extraction | Endotherms °C | | | |
|---|---|---|---|---|
| Non-extracted | 42 | 70 | 79 | 110 |
| Water | 42 | 68.5 | 77 | 108 (s)* |
| 6M Urea | 41.5 | 68 | 75.5 | 110 (vs) |
| Tris-Urea-mercaptoethanol | 41 | 66 | 74 | — |
| Tris-Urea-meracptoethanol and Subtilisin | 40 | 66 | 74 | — |

* key (s) smaller than normal (vs) very much smaller than normal (——) no transition.

In this study, it was found that any substance which caused the loss of the T42 and T71 also caused the loss of T80, so that not only did C/M (2:1 v/v) eliminate T42, T71 and T80, so also did carbon tetrachloride and ether. The dried carbon tetrachloride and ether extracts gave endotherms at 38° and 66°C, and 39° and 67°C, respectively, similar to lipid extracted with C/M (2:1 v/v). Loss of T42, T71 and T80 was also observed with samples pre-treated with 2% SDS (see Chapter 6).

Removal of lipids with organic solvents has been reported to show no significant effect on the protein structure of stratum corneum as studied by IR and X-ray diffraction techniques (28,32,35). Also the isometric contraction study of Baden and Gifford (39) showed

that extraction of stratum corneum with C/M (3:1 v/v) had no effect on the inflection temperature at 82-86°C which they suggested was associated with α-keratin. Therefore, according to the literature, the irreversible endotherm at 80°C, which behaves similarly to the T85 of human stratum corneum (33), is unlikely to be due to the denaturation of α-keratin. Further supporting information can also be obtained from the IR, X-ray and Thermomechanical studies (29,31,36-38). Using IR spectroscopy Wildnauer et al. (29), reported that the denaturation of α-keratin to a β-structure can only take place at temperatures higher than 120°C in dry stratum corneum. X-ray diffraction of dry stratum corneum does not indicate significant structural changes in the protein even when heated at 157°C (31). Thermomechanical studies (36-38) also suggest that denaturation of protein takes place at temperatures higher than 150°C in the dry stratum corneum. In the work of van Duzee (33) there was no decisive evidence that the transition at 85°C was due to denaturation of α-keratin. Rather he based his conclusion on the work of Baden et al. (40), who had used skin immersed in water, i.e. fully hydrated, heating at 85°C was for 5 min. These conditions do not match those used by van Duzee who observed the T85 in both desiccated and relatively hydrated samples (0.2-0.6mg water/mg stratum corneum). Other reports (39,55,57) also suggest that low temperature denaturation of α-keratin requires stretching and heating in water. The low temperature denaturation under full hydration and loading has been explained (36) as being caused by swelling and a weakening of the attractive forces of the polypeptide chains of keratin, hence an earlier denaturation. Strong acids, alkalies and formic acid are known to cause denaturation of protein (28,62,70). Pretreatment of stratum corneum with 1M hydrochloric acid, 1M sod. hydroxide and 9M sulphuric

Table 2.8 The Effect of Pretreatment of Neonatal Rat Stratum Corneum with Acids and Alkalies on Endothermic Transition Temperatures.

| Pretreatment | Endotherm Temperatures °C |
|---|---|
| M. Hcl for 24 hours | 41 70 77 |
| M. NaOH for 24 hours | 40 69.5 75 |
| 9M. $H_2SO_4$ for 1 hour | 43 69 78 |
| 9M. $H_2SO_4$ after heating to 130°C | 40 67 74 |
| Formic acid for 1 hour | 40 59 70 |

acid showed some effect, not only on the T80, but also on the lipids at 42° and 71°C (Table 2.8). 1M sod. hydroxide had a more pronounced effect than the acids, causing the skin to disintegrate. 9M $H_2SO_4$ gave similar but more distinct endotherms and exotherms (on cooling) than normal stratum corneum and a more stable T80 endotherm, even after heating to 130°C. The mechanism of this stabilisation is not understood. Although formic acid caused disintegration of stratum corneum after a few minutes, the collected pieces revealed three endotherms but at the lower temperatures of 40°, 59° and 70°C. The T70 does not disappear when heated to around 100°C. However, rinsing with water following treatment with formic acid produced the endotherms at temperatures similar to normal stratum corneum, suggesting reversibility. The disintegration indicates a breaking of bonds in keratin, suggesting that there had been protein denaturation. Thus it appears that the T80 cannot be associated with keratin denaturation.

An alternative interpretation is required. The removal of T80 by organic extraction makes lipid melting a possibility. However only Bulgin and Vinson (30) have reported an irreversible lipid transition at around 80°C and their endotherm was very broad, from 77-88°C.

In this work, the irreversibility of T80 was found when stratum corneum was first heated up to 120-140°C. It was, therefore, decided to investigate the effect of lower heats on the 80°C transition. The effect of temperature on T80, and the two lipid endotherms at 42° and 71°C, are in Table 2.9 and depicted in Figures 2.10 and 2.11. From the table and figure it can be seen that T80

Table 2.9 The Effect of Heat on the Stability of the three Endotherms below 100°C in Neonatal Rat Stratum Corneum

| Heating up to (°C) | Endotherms on First Heating (°C) | | | Endotherms on Second Heating (°C) | | |
|---|---|---|---|---|---|---|
| 80 | 42 | 71.5 | 80 | 40 | 70 | 78 |
| 85 | 41.5 | 71 | 79 | 39 | 69.5 | 77.5 |
| 90 | 42 | 71.5 | 79.5 | 40 | 70 | 76 |
| 100 | 42 | 72 | 79.5 | 40 | 69.5 | 74 |
| 110 | 42 | 72 | 80 | 39 | 69 | 72.5 |
| 120 | 42.5 | 71.5 | 79.5 | 40 | 70 | — |
| 140 | 42 | 71.5 | 79 | 40 | 69 | — |

did not disappear when stratum corneum was heated from 80°C up to 110°C, but that both the transition temperature and the size of the peak were reduced gradually as the temperature increased, until it completely disappeared on heating the stratum corneum at 120°C and 140°C. The lipid endotherms at 42° and 71°C were also lowered to 39-40° and 69-70°C, respectively.

Table 2.9 also shows that the disappearance of T80 is not sudden. It was, therefore, important to investigate the effect of exposure time at a particular temperature on the T80 endotherm.

The endotherm temperatures of stratum corneum folded in a DTA crucible and covered for heating are given in Table 2.10. One hour treatment at 70°C has no effect on T80, although both T42 and T71 were reduced to 40° and 69.5°C. Heat treatment at 75°C for 1 and

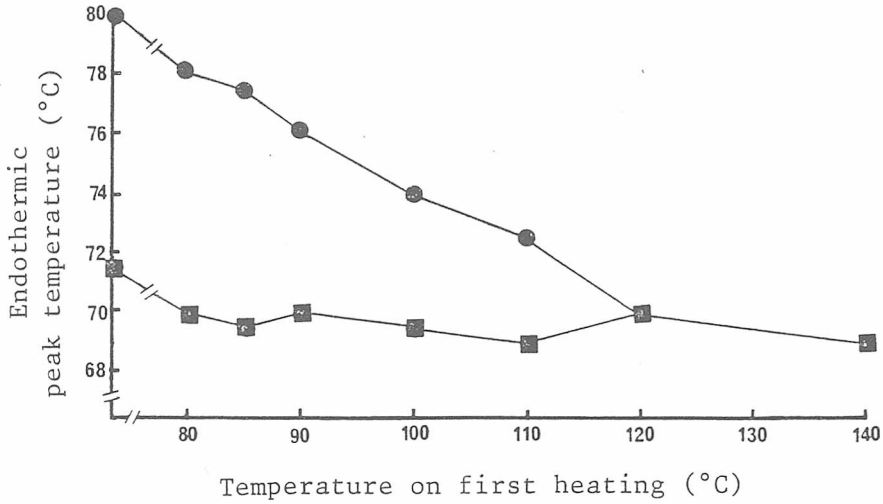



Figure 2.10 A plot of endothermic peak temperature, on second heating, against temperature reached during first heating of stratum corneum. (■) T71 endotherm, (●) T80 endotherm.

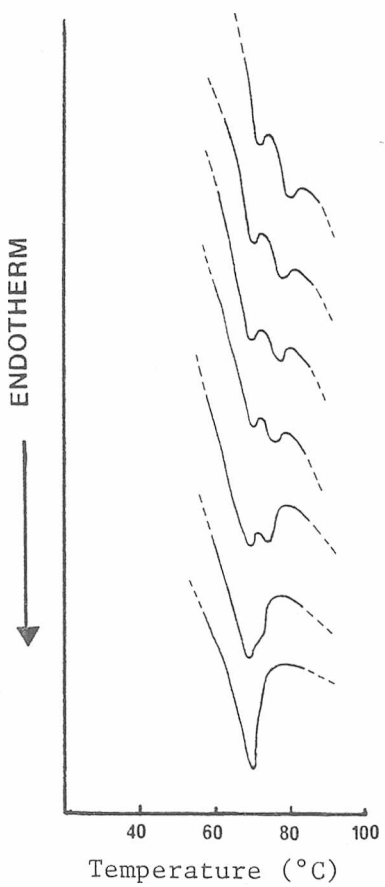



Figure 2.11 DTA thermograms of stratum corneum showing the sequence of reduction in both temperature and size of T80, from top (control) to bottom, (see Fig. 2.10).

Table 2.10 Effect of Exposure Time at a particular Temperature on the Lipid Endotherms of Folded Neonatal Rat Stratum Corneum.

| Exposure Temperature (°C) | Exposure Time (min) | Lipid Endotherms (°C) |
|---|---|---|
| Control | — | 42 71.5 79.5 |
| 70 | 30 and 60 | 40 69.5 80 |
| 75 | 60 | 40 70 75.5 80.5 |
| 75 | 120 | 39 70 76 81 |
| 80 | 1 | 41 70 77.5 |
| 80 | 5 | 40 69.5 75.5 |
| 80 | 10 | 40 70 74.5 |
| 80 | 15 | 41 69.5 74 |
| 80 | 30 | 40 70 73.5 |
| 80 | 45 | 39 69 71.5 |
| 80 | 60 | 40 70 — |
| 90 | 15 | 41 70 — |
| 100 | 5 | 40 69 — |

2 hours did not affect the T80 temperature but considerably reduced its size and produced an intermediate endotherm at 76°C (Fig. 2.12). Both the T76 and T80 were smaller than T70, the effect increasing with time.

Heat treatment at 80°C (Table 2.10 and Fig. 2.13), shows that the temperature of T80 was lowered with time. Complete loss of T80 occurred after 60 min. A similar effect was produced after 15 mins at 90°C and 5 mins at 100°C (Table 2.10).

Table 2.11 shows the effect of heat treatment with flat stratum corneum sheets on metal screens at 50% RH. The unfolded stratum corneum requires a longer exposure time for complete disappearance of T80. Thus heat treatment at 80°C requires 4-6 hours for complete disappearance of T80 from unfolded stratum corneum as compared to 1 hour for folded. However, heat treatment at

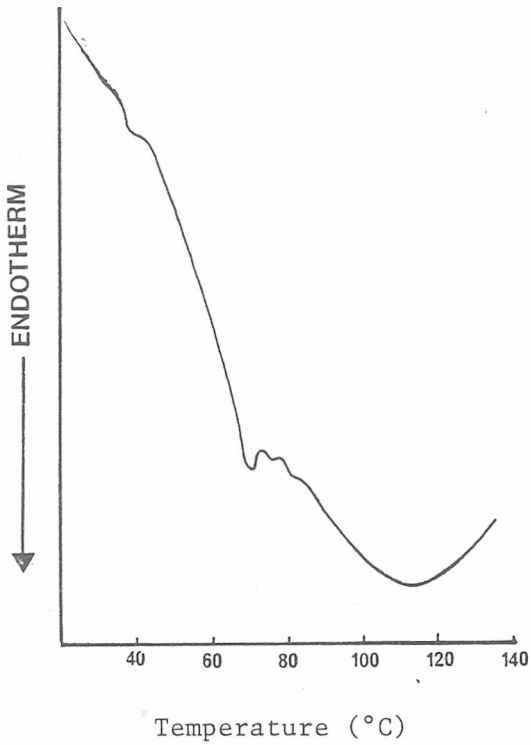



Figure 2.12 DTA thermograms of neonatal rat stratum corneum heated at 75°C for 2 hours prior to thermal analysis.

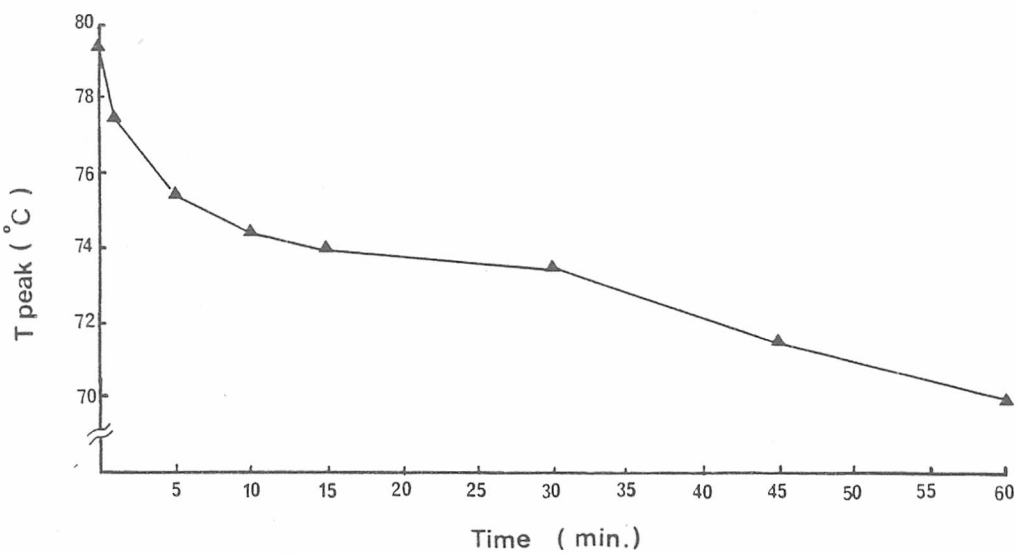



Figure 2.13 A plot showing the effect of time of exposure at 80°C prior to thermal analysis on the peak temperature of T80 endotherm.

Table 2.11 Effect of Exposure Time at a particular Temperature on the lipid Endotherms of Flat Sheets of Neonatal Rat Stratum Corneum at 50% RH.

| Exposure Temperature °C | Exposure Time (hr) | Lipid Endotherms (°C) | | |
|---|---|---|---|---|
| Control | — | 42 | 71.5 | 79.5 |
| 70 | 12 | 39 | 69 | 79 |
| 80 | 1 | 39 | 69 | 76 |
| 80 | 1.5 | 40 | 69.5 | 75 |
| 80 | 3 | 39 | 69 | 73 |
| 80 | 4 and 5 | 40 | 69 | 71-72 |
| 80 | 6 | 40 | 69 | — |
| 90 | ½ | 39.5 | 69.5 | 74 |
| 90 | 1 | 40 | 70 | — |
| 90 | 2 | 40 | 69 | — |

70°C again gave no effect on T80.

Further confirmation for this difference between folded and unfolded stratum corneum was made by splitting a stratum corneum sheet into two halves, one half being tightly folded between sheets of aluminium foil, or folded into a DTA crucible and covered with a lid. The other half was left unfolded. After 1 hour at 80°C there was complete disappearance of T80 with the folded samples, but samples left unfolded only gave a lowering of T80 to 75.5°C. Whilst there is a clear difference in the effect of heat on folded and unfolded skin, the explanation is not clear. It may be that the metal (foil or crucible) gave a better thermal contact than static air. It could also be that the pressure within the folded stratum corneum increased the mobility of the melted lipids and so caused a faster mixing of lipid molecules. It was noticed that, at the end of heat treatment, the skin had become soft, but hardened immediately on removal from the furnace. This may support the latter explanation.

It must be concluded, however, that extreme care is needed

in the interpretation of data on the effect of heat on stratum corneum transitions.

Table 2.9 shows that changes in endothermic temperature on heating were not restricted to T80, but occurred with the two lipid transitions at 42° and 71°C. Whilst they do not disappear, it suggests that the 80°C transition could also be lipid melting. To support this hypothesis, a cooling cycle was used to see whether there was an exotherm corresponding to T80. By heating up to 86-87°C prior to the cooling cycle, exothermic transitions at 35°, 63° and 68.5°C were obtained on cooling. When the same sample was reheated to 140°C, endotherms were observed at 40°, 69.5°, 75.5° and 106°C. A third heating cycle gave two endotherms at 40° and 69°C (Fig. 2.14). From this experiment it is clear that the three exotherms at 35°, 63° and 68.5°C do correspond to the endotherms at 40°, 69.5° and 75.5°C respectively, suggesting that all three are caused by crystallisation of supercooled lipids. Further evidence that T80 is due to lipid melting is presented in Chapter 4.

Thus it is suggested that the 80° transition is due to lipid melting. However the nature of the disappearance of T80 with temperature requires some explanation. Chemical degradation of lipid by heat may be discounted because the TLC work of Rehfeld and Elias (42) showed no essential difference between heat treated and untreated stratum corneum lipids. In this work a similar conclusion was reached (see Chapter 4).

Both heat treatment and lipid extraction caused the disappearance of T80, suggesting that a similar process may be involved. A possible explanation is that, once the T80 lipid melts, it interacts or mixes with other lipid components of the stratum corneum. These behave as in a eutectic-type system, leading to a

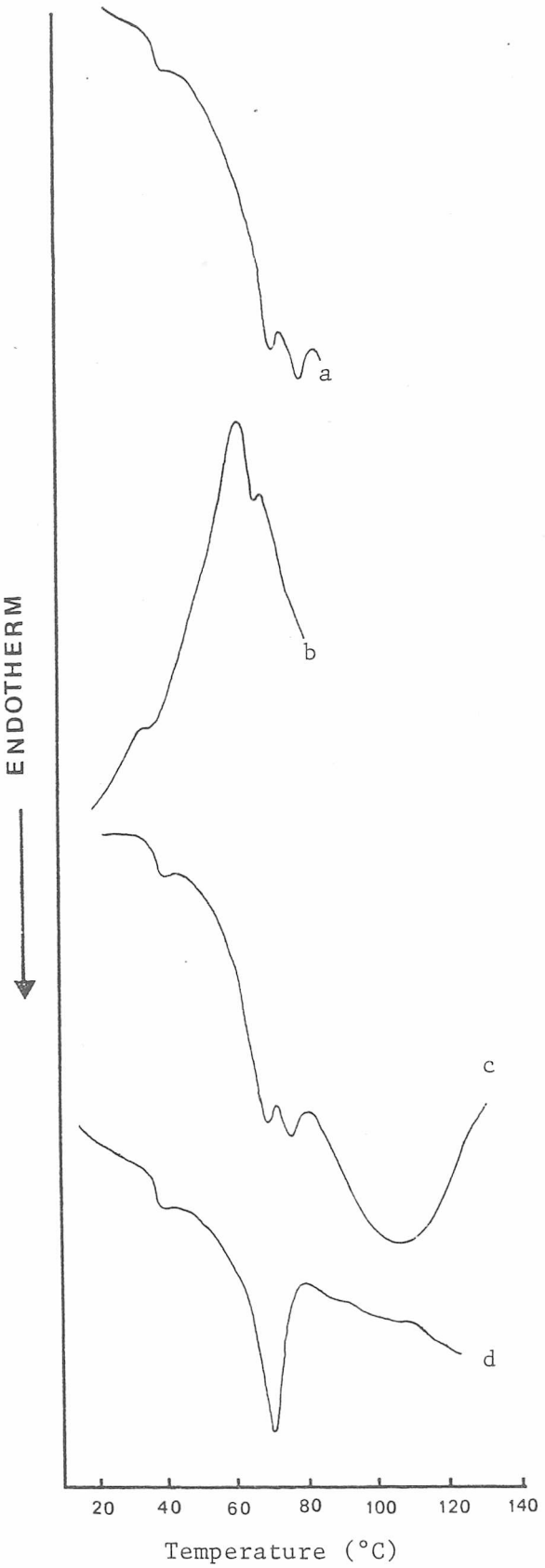



Figure 2.14 DTA thermograms of neonatal rat stratum corneum. (a) First heating up to 87°C, (b) cooling run (c) second heating run, (d) third heating run.

gradual reduction in melting temperature with time and temperature as the mixing proceeds. Eventually the transition temperature reaches about 70°C when the instrument cannot differentiate it from the original 71°C transition. Likewise, following organic extraction, the lipids mix throughly and so interfere with their mutual crystallisations. Such an explanation is supported by the X-ray work of Elias et al. (58) on neonatal mouse stratum corneum. They found a considerable difference between the X-ray reflections of in situ and extracted lipids and concluded that extraction of lipid caused alteration in the crystallisation of lipid components and that lipids in intact tissue may combine differently with each other and with protein. In chapter 4, TLC of extracted lipid has produced a lipid fraction showing (on DTA) an endotherm at 82°C, which probably corresponds to the 80°C transition.

Three lipid transitions were reported by Rehfeld et al. and Rehfeld and Elias (41,42) for both neonatal mouse and human stratum corneum, using DSC (Fig. 2.15). Human stratum corneum also showed three lipids, using X-ray technique, which melted at 40°C and two between 80-90°C (31).

From their data, it may be surmised that Rehfeld and Elias (42) did not exceed 80°C in their DSC studies on neonatal mouse stratum corneum, nor did they use a second heating cycle (Fig. 2.15). In the work of Wilkes et al. (31) neonatal rat stratum corneum gave a broad endotherm at about 80°C on first heating to 140°C. The second heating showed only a small lipid endotherm at 70°C (Fig. 2.8). They explained this as being either the crystalline lipid content being reduced considerably by the first heating, or that the initial endotherm was partially due to the loss of moisture. However, they failed to regenerate the endotherm with exposure to moisture. They

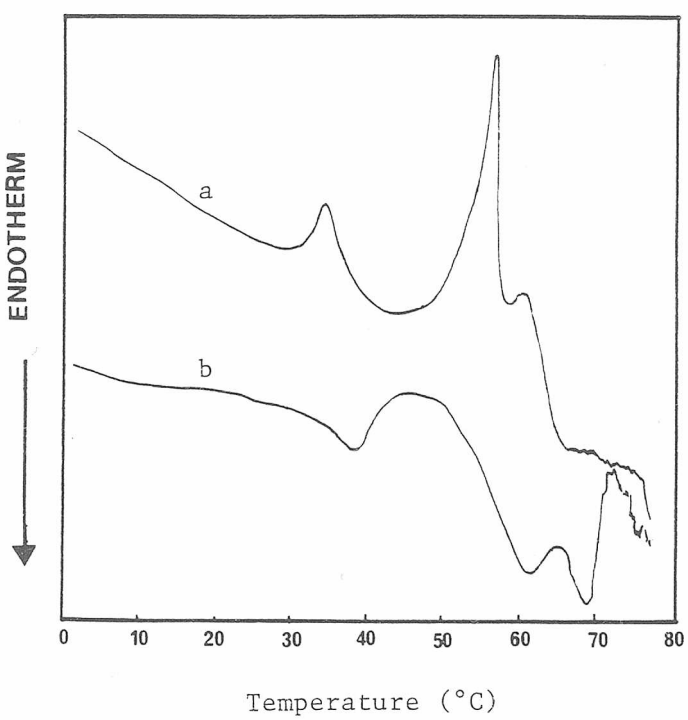



Figure 2.15 DSC thermograms of neonatal mouse stratum corneum obtained by Rehfeld and Elias (42). (a) cooling, (b) heating.

also found the same effect with human stratum corneum. It is, therefore, probable that they observed the same phenomenon but failed to recognise it.

To summarise, the endothermic transitions of neonatal rat stratum corneum are interpreted as being due to:

42°C Lipid melting

71°C Lipid melting

80°C Lipid melting

110°C Water desorption

## 6. Species/Regional Variation

Thermal analysis of stratum corneum by different workers (30,31,33,41,42) has produced different results probably due to factors such as species variation, instrumental variation (DTA or DSC), heating rate etc. By using identical experimental conditions in the present work, the effect of species variation should become clearer.

In this work, stratum corneum from different species was heated on DTA just above the appearance of a third endotherm, followed by immediate cooling, a second heating to 140°C and finally a third heating. Endothermic temperatures are presented in Table 2.12 and thermograms in Figures 2.14, 2.16, 2.17, 2.18. Table 2.13 shows additional work on two samples of human stratum corneum.

Table 2.12 Endothermic Peak Temperatures Obtained by DTA of Stratum Corneum from Different Species.

| Species | 1st Heating (°C) | Cooling (°C) | 2nd Heating (°C) | 3rd Heating (°C) |
|---|---|---|---|---|
| Neon. Rat | 42 71.5 79.5 | 35 63 68.5 | 40 69.5 75.5 106 | 40 69 |
| Neon. Mouse | 45 73 84 | 35 63 73 | 41 69 81 111 | 40 68 |
| Neon. Rabbit | 47 69 78 | 37 59.5 67.5 | 42 66.5 74.5 108 | 40.5 65.5 |
| Adult Human (Buttock) | 41 77 91 | — 64 76 | — 75 86 118 | — 70.5 |

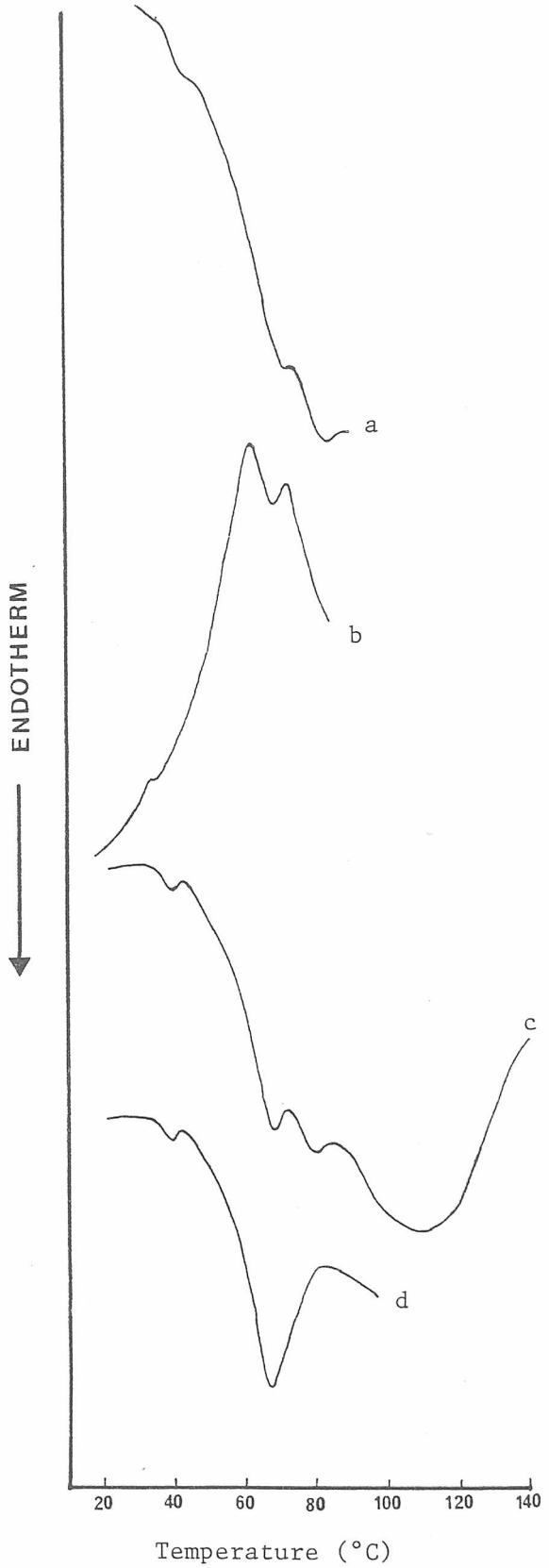



Figure 2.16 DTA thermograms of neonatal mouse stratum corneum. (a) First heating up to 91°C, (b) cooling run, (c) second heating run, (d) third heating run.

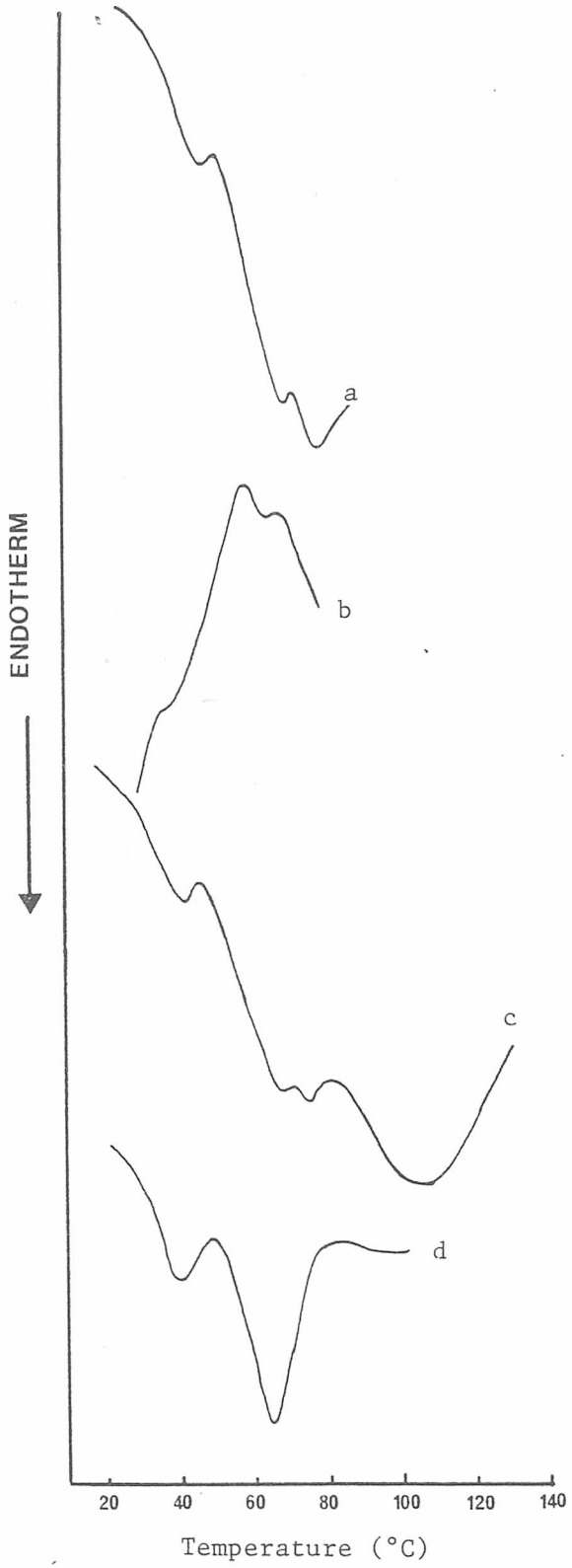



Figure 2.17 DTA thermograms of neonatal rabbit stratum corneum. (a) First heating up to 88°C, (b) cooling run, (c) second heating run, (d) third heating run.

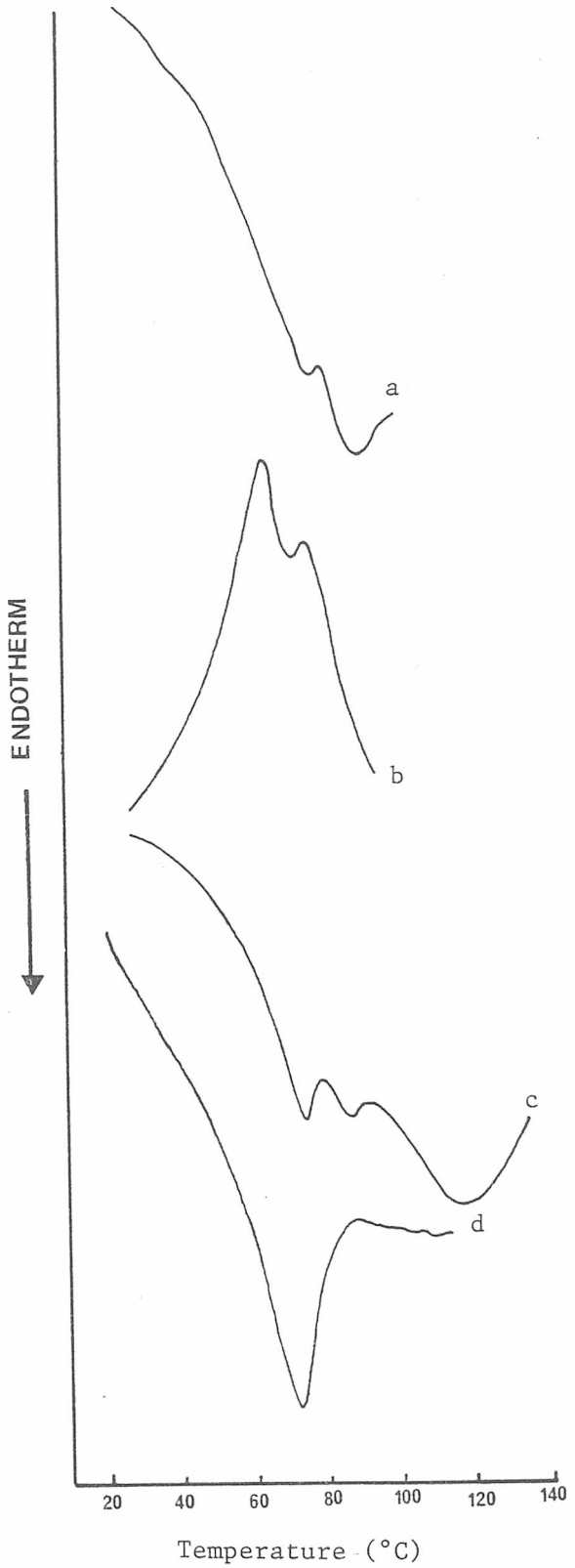



Figure 2.18 DTA thermograms of human (buttock) stratum corneum. (a) First heating up to 99°C, (b) cooling run, (c) second heating run, (d) third heating run.

Table 2.13 Endothermic Peak Temperatures obtained by DTA of Human Stratum Corneum, heated to 140°C, cooled and re-heated.

| Source of Stratum Corneum | First Heating | Second Heating |
|---|---|---|
| Arm | 42 75.5 87 115 | 70 |
| Plantar | — 74 (B)* 125 | 65 (SB) |

Key - B = Broad SB = Small and Broad

Four endothermic transitions were found with all four species. Also on the third heating of stratum corneum, the third lipid transition and the broad water transition were absent. All species showed a similar patterm of changes as a result of temperature cycling (Figs. 2.16, 2.17, 2.18). The appearance of exotherms on cooling and the pattern of reversibility of the third endotherm indicates the similarity of these species stratum corneum to that of neonatal rat discussed in detail earlier in this chapter.

Extraction with C/M (2:1 v/v) of mouse and adult human stratum corneum gave dry extracts having only two endotherms, at 38° and 68°C (mouse) and 41° (small) and 70° (human buttock). The extracted stratum corneum of both species gave only one endotherm, in excess of 100°C (Fig. 2.19). This broad peak above 100°C was regenerated by exposure to room humidity (50-60%) for 24 hours.

From this information, the four endotherms of mouse, rabbit and human stratum corneum were attributed to lipid melting and desorption of water as for rat stratum corneum. However, whilst the pattern of endotherms is the same, the actual temperatures of the lipid transitions vary. Neonatal rabbit stratum corneum gave the highest temperature for the first lipid endotherm, which was also the largest (Fig. 2.17). However, after heating to 140°C, whilst the peak was still large, the temperature was the same as for rat and mouse stratum corneum. Human stratum corneum (buttock and arm) did

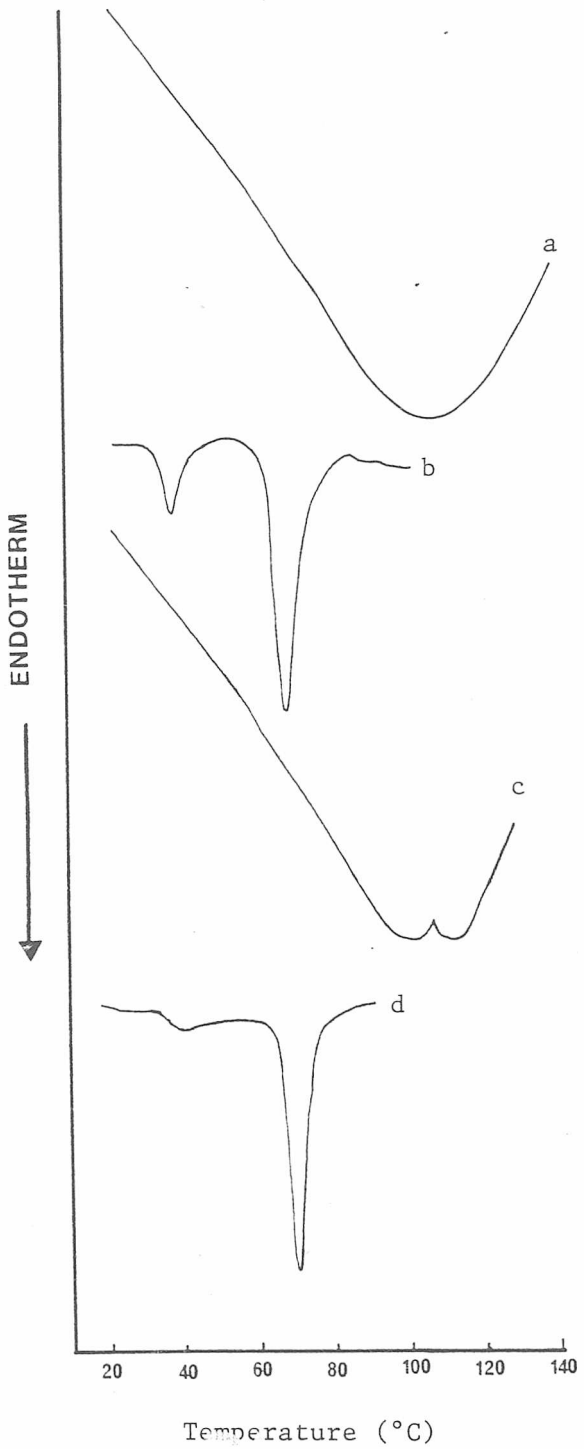



Figure 2.19 DTA thermograms of neonatal mouse and human stratum corneum extracted with C/M 2:1, and their dried extracts. (a,b) neonatal mouse stratum corneum and the dry extract, respectively, (c,d) human stratum corneum and the dry extract, respectively.

not show any transition around 40°C on the second or third heating and the transitions found on the first run were very small and difficult to distinguish. Wilkes and Wildnauer (32) in their dynamic mechanical studies also observed a lipid melt at around 40°C, which was less distinct in human than neonatal rat stratum corneum. Van Duzee (33) has also shown that the transition at 40°C is decreased considerably on second heating (Fig. 2.9). Wilkes et al. (31) made a similar observation in their work using X-ray diffraction and suggested that heated human stratum corneum did not show recrystallisation of the lipid melted at 40°C on cooling, that is, it was irreversible. The extracted lipid from human buttock stratum corneum showed the T41 to be very small (Fig. 2.19).

Table 2.12 shows that in all these species, as the melting temperatures of the second endotherm increases, so does that of the third endotherm both in the order rabbit < rat < mouse < human arm < human buttock. However, after heating to 140°C, apart from the T40, all four species gave a single transition at about 70°C. A detailed explanation for these differences is beyond the scope of the present work, although the literature does indicate that different species have the same lipid groups such as sterol esters, triglycerides, free fatty acids, free sterols, glycosphingolipids, ceramides and traces of phospholipid (20-22,71). The presence of phospholipid in stratum corneum has been doubted (22,72,73). Presumably, the particular lipids will vary between species, but be arranged in similar structures, giving the observed similarity of behaviour. Other work, using thermomechanical and dynamic mechanical properties has shown that human and neonatal rat stratum corneum behave similarly (28,29).

DTA of neonatal mouse stratum corneum gave three lipid endotherms

at 45°, 73° and 84°C. Rehfeld and Elias (42) used neonatal mouse stratum corneum (DSC) and found lower temperatures at 38.2°, 61.6° and 67.6°C. However, they used a heating rate of 0.1°C $min^{-1}$, which would produce lower transition temperatures (see Table 2.6).

Tables 2.12 and 2.13 show that the lipid endotherms of human buttock and arm stratum corneum are higher than those of neonatal mouse stratum corneum. However, Rehfeld et al. (41), using DSC, found human stratum corneum to have lipid endotherms at 33°, 55° and 68°C, which is lower than neonatal mouse stratum corneum (42). Thus the only similarity between the present work and that of Rehfeld and his coworkers (41,42) is the presence of three lipid endotherms in both species. However, the present work is in good agreement with Wilkes et al. (31) who found three lipid melts, by X-ray, in human stratum corneum at 40°C and two between 80-90°C, and in rat stratum corneum at 40° and about 80°C and on second heating at 40° and 70°C. Both Bulgin and Vinson (30) and Wilkes et al. (31) showed human stratum corneum giving higher lipid melting temperatures than neonatal rat stratum corneum, as was found in the present work. Van Duzee (33) reported the endotherms of human stratum corneum to be at 40°, 75°, 85° and 107°C, in close agreement with the present work.

It is probable that the melting temperatures of stratum corneum lipids affect skin permeability because the diffusion coefficient is inversely proportional to consistency (see page 63). Therefore, from the thermal analysis, one would expect penetration to decrease in the order rabbit > rat > mouse > human. This is in good agreement with the work of others (1,74,75).

Human plantar stratum corneum, unlike normal stratum corneum, gave a broad, less distinct transition at about 74°C and a

broad transition at about 125°C. The second heating gave only a small, broad transition at 65°C. The dry C/M (2:1 v/v) extract had a single transition at 54°C (Fig. 2.20). Whilst these differences between the thermal transitions of plantar and other human stratum corneum lipids is interesting, it is not surprising because it has been reported (73,76) that plantar lipids have inferior properties to those of other regions of the body. The DTA work of Bulgin and Vinson (30) also showed similar small, broad lipid transitions for psoriatic scales and human callus.

**7. Effect of DMSO on Stratum Corneum**

The effect of the aprotic solvent DMSO on skin permeability has been intensively investigated over the past two decades and is reviewed in Chapter 3. The effect of DMSO on the mechanical properties (60-62) and thermal shrinkage of skin (63) has also been investigated, but there have been no reports of the effect of DMSO on the thermal behaviour of skin using DTA or DSC.

Twenty four hour treatment of stratum corneum with DMSO, followed by rinsing with water and desiccation before DTA produced a thermogram in which the lipid transition at 71°C and 80°C had broadened, become less distinct and occurred at 69° and 77°C. After heating to 140°C, the second heating gave one very small, broad lipid endotherm at 67.5°C (Fig. 2.21). A DMSO extract was mixed with chloroform and water to give a final ratio chloroform : DMSO : water 2:1:1: (v/v/v). The dried chloroform layer (after centrifugation) gave two lipid endotherms at 37° and 66°C, akin to those obtained by C/M (2:1 v/v) extraction. The extracted stratum corneum still showed lipid endotherms, although they are very much smaller than normal. Thus DMSO has extracted some stratum corneum lipid, but not

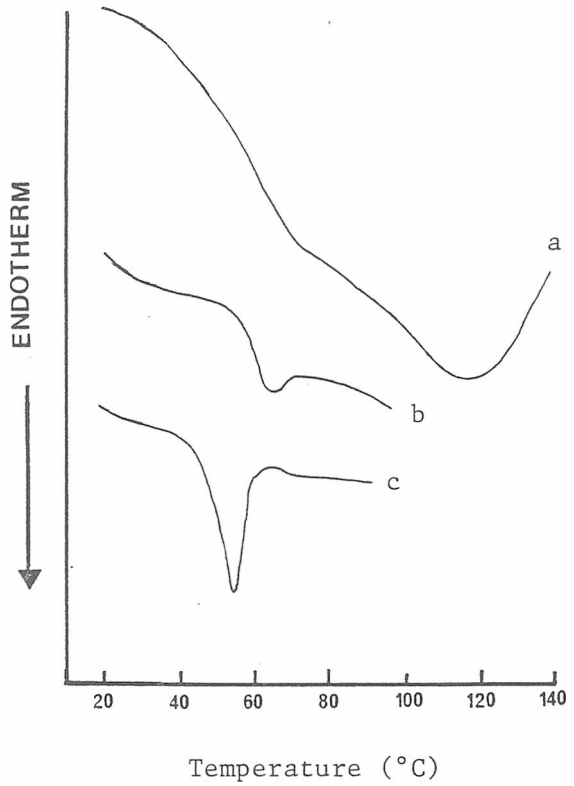



Figure 2.20 DTA thermograms of plantar stratum corneum. (a) First heating run, (b) second heating run, (c) dried C/M 2:1 extract.

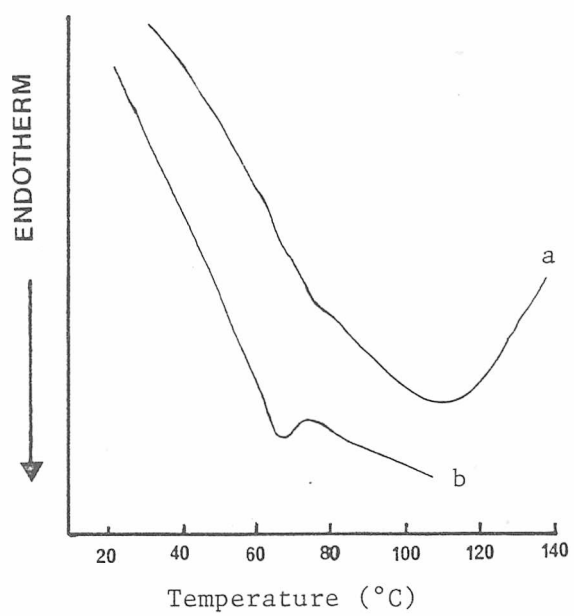



Figure 2.21 DTA thermograms of neonatal rat stratum corneum extracted with DMSO. (a) First run, (b) second run.

as efficiently as C/M (2:1 v/v).

When stratum corneum was exposed for 24 hours to DMSO vapour, either at 25° or 30°C, subsequent thermal analysis showed that the lipid transitions at 42°, 71° and 80°C were lowered to 40°, 59° and 65°C (Fig. 2.22). The second heating cycle gave only two lipid endotherms at 38° and 61°C. It was also noted that the disappearance of the upper lipid transition occurred more easily after treatment with DMSO. For example T80 disappeared after heating to 120°C, whilst the DMSO induced T65 was lost after heating to 82°C.

Stratum corneum treated for 4 hours with 50%, 70% and 90% DMSO prior to DTA gave reductions in the T80 to 77°, 75° and 72°C, respectively. The other lipid endotherms, normally at 42° and 71°C were indistinct probably due to the moisture present. Thus the effect of DMSO solutions on stratum corneum endotherms is concentration dependent.

In an experiment in which rat stratum corneum was pre-heated at 80°C for 6 hours before exposure to DMSO vapour for 24 hours, the lipid transition at 69°C was lowered to 60°C (Fig. 2.22).

Human buttock stratum corneum, exposed to DMSO vapour at 25°C for 24 hours, produced similar effects to those observed in rat stratum corneum. The lipid endotherms, normally at 77° and 91°C, were lowered to 68° and 82°C. This 9°C lowering for both endotherms, compares with the 13°C reduction in T71 and 15°C reduction in T80, seen with rat stratum corneum. This suggests that DMSO vapour has a similar effect on both human and neonatal rat stratum corneum and confirms that lipid endotherms at 71° and 80°C in rat stratum corneum and those at 77° and 91°C in human stratum corneum are closely related. Figure 2.23 shows that extracted lipids, human and rat, also showed a 10°C reduction in their melting temperature when mixed

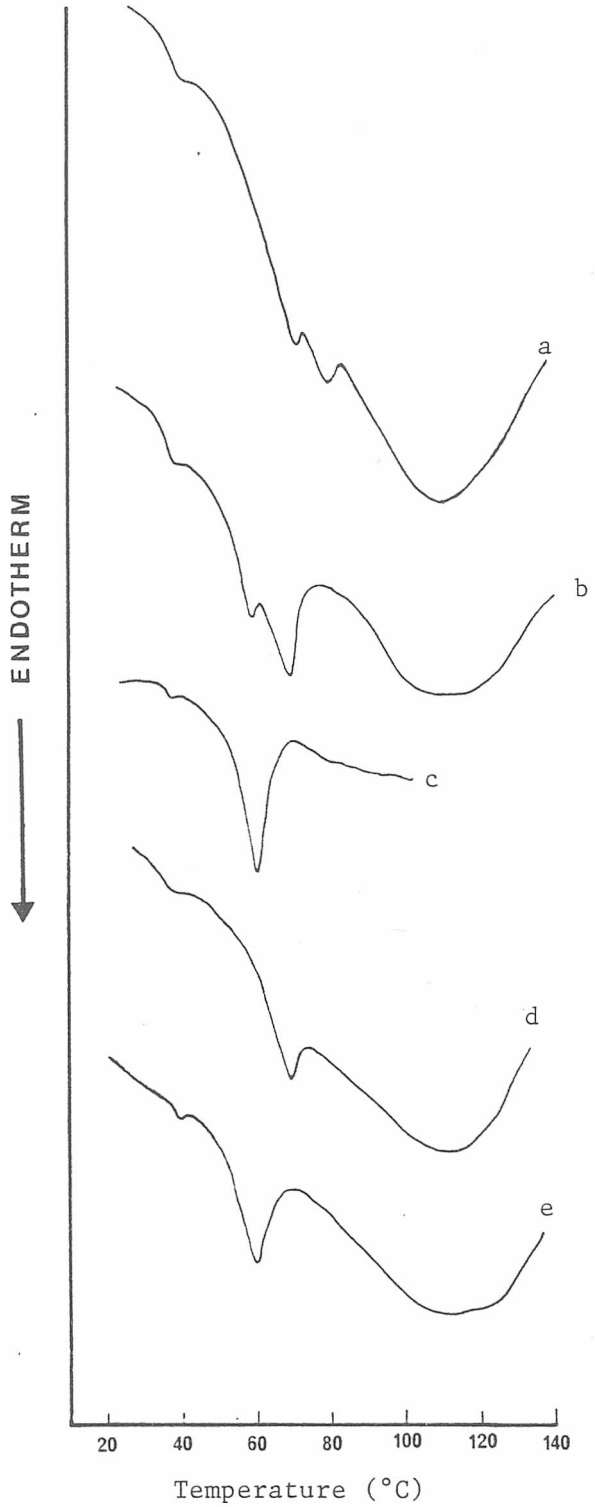



Figure 2.22 DTA thermograms of neonatal rat stratum corneum. (a) control, (b) exposed to DMSO vapour prior to DTA, (c) second heating run of b, (d) pre-heated at 80°C for 6 hours at 50% RH, (e) as d but followed by exposure to DMSO vapour prior to DTA.

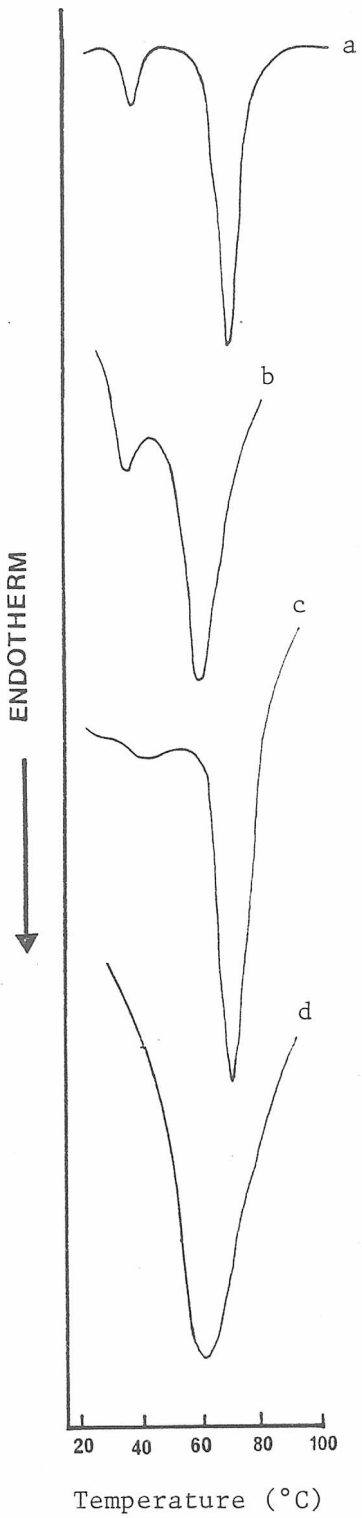



Figure 2.23 DTA thermograms of lipids extracted from neonatal rat (a,b) and human (c,d) stratum corneum. (a) Neonatal rat lipid (control), (b) neonatal rat lipid exposed to a small drop of DMSO prior to DTA, (c) human lipid (control), (d) human lipid exposed to a small drop of DMSO prior to DTA.

with a small drop of DMSO.

There is a similarity between the effect of DMSO vapour and 80 and 90% aqueous solutions (6 hours exposure, rinsed, desiccated), thermograms of which showed all the transitions to be similar to untreated stratum corneum. These results suggest that, unlike the treatment with 100% DMSO which extracted lipids, 80% and 90% DMSO and DMSO vapour probably do not extract lipid. Similarly, when stratum corneum, exposed for 24 hours to DMSO vapour was stored for 2 weeks over silica gel to allow evaporation of DMSO, subsequent thermal analysis showed the lipid transitions to be similar to the untreated stratum corneum (at 42°, 70° and 78°C). Thus the effect of DMSO vapour is reversible.

Removal of DMSO applied at 80% and 90% aqueous solutions also indicated that there was no noticeable damage to the stratum corneum lipids. This conclusion assumes that all the lipids are detected by the endotherms. This may not be the case (see Chapter 4). However, the lipid extraction with DMSO reported here is in good agreement with results obtained by other workers (77-80,105,106,208).

During these experiments it was observed that, at the end of the 24 hour treatment of stratum corneum with DMSO vapour, the skin had become soft. This softness was also observed with stratum corneum which had been extracted with C/M (2:1 v/v) before DMSO vapour treatment. Therefore, this softening is probably due to an interaction between stratum corneum protein and DMSO producing plasticisation. Unlike the stiffening produced by DMSO (liquid) extraction, the stratum corneum which had been exposed to DMSO vapour and left to dry over silica gel for two weeks showed the same physical appearance as the untreated stratum corneum. The interaction of DMSO with protein was observed from the effect on the thermal

shrinkage of collagen (63), the physical properties of keratin (60-62,81,82) and was found to be reversible.

From this data, the suggestion emerges that there is some interaction between DMSO and lipid molecules, which must be sufficiently weak to account for the reversibility. In view of the structure of DMSO (83,84) and lipids (85), hydrogen bonding is most probable. Hydrogen bonding between protein and DMSO has been reported (63,84).

Similar work on stratum corneum with DMAC vapour has also shown a reduction in lipid melting from 71° and 80°C to 63° and 72°C, suggesting a similar mechanism for this sorption promoter.

The significance of these conclusions will be discussed further in Chapter 6.

# CHAPTER THREE

# PERMEABILITY STUDIES

## Introduction

The literature review has been divided into a number of sections and sub-sections. There is a very large literature on skin permeability. Of necessity this review has had to omit many interesting and important aspects and concentrate on those aspects most relevant to the present work.

## 1. Anatomy and Physiology of the Skin

Two main tissue layers are usually recognised as constituting human skin. The outer layer is a thin stratified epithelium, the epidermis, which varies in thickness between 75 and 150μm over most of the body except on the palms and soles where it may be 0.4 - 0.6 mm thick. Underlying the epidermis is a dense fibroelastic connective tissue layer called the dermis. It makes up the bulk of the skin although it varies in thickness in different regions of the body. The connective tissue is composed of elastic collagen fibres (2,7). It is traversed by blood and lymphatic vessels, nerves and the epidermal appendages, eccrine and apocrine glands and the pilosebaceous units (4,6,9).

Beneath the skin is the subcutaneous tissue, or hypodermis, which is variably composed of loose areolar or fatty connective tissue displaying substantial regions and individual variation in thickness (5,7).

### The Epidermis

Even though it is the thinnest layer, the epidermis is a dynamic, multilayered structure originating in the basal layer

(stratum germinitivum). In this layer the cells are columnar in shape and contain the skin pigment, melanin, in the cytoplasm (5). As epidermal cells migrate upwards from the basal layer they lose their mitotic potential and begin to synthesize specific constituents such as fibrillar and amorphous proteins, keratohyalin and membrane-coating granules. Their surfaces become modified and finally their nuclei and cytoplasmic organelles are lost as the cells die to form the horny layer (stratum corneum). Looked at in more detail, it is found that the first layers outward from the basal layer, the spiny layer (stratum spinosum) has polyhedral shaped cells. Subsequent layers become progressively flattened and elongated before being termed the granular layer (stratum granulosum) which is 2-3 layers thick. The cells continue to flatten and at this stage contain clearly visible keratohyalin granules (8). In the palm and sole, there is next an additional, anatomically distinct zone, the stratum lucidum, which is a thin translucent layer, apparently devoid of cell walls (2,4).

The end of the epidermal keratinization process is the formation of the stratum corneum. Each thin polygonal cell is approximately 0.5 - 1.5μm thick, with a diameter ranging from 34μm on the forehead to 46μm on the thigh, and with an overall density of 1.5 $g.cm^{-3}$. Apart from the palms and soles, the dry stratum corneum has a thickness of around 10μm and is composed of some 10-15 layers organized in stacked columns. However, the stratum corneum of the palms and soles has up to 40 times more layers, in which the cells are randomly arranged (2). These differences have led Kligman (3) to suggest two types of horny layer, that of the palms and soles being adapted for weight bearing and friction and that of the rest of the body being adapted for flexibility, impermeability and fine sensory

discrimination.

The stratum corneum represents the most important interface between the internal and external environments of the organism, providing a tough protective covering and preventing the dehydration of the body, by regulating transepidermal water loss, and the passage of other materials (1,2).

Dead cells are continually being lost from the outer extremities of the stratum corneum. In normal healthy skin the dynamic process is in balance, with a time scale of about 28 days from basal layer to loss from the skin surface, with 14 days of this time as stratum corneum (reduced to 2 days in psoriasis) (2,5).

**2. Diffusion and Penetration**

The process of skin penetration may be described by a flux equation derived from Fick's First Law of Diffusion. This is normally expressed as:

$$Js = \frac{Q}{At} = Kp\ \Delta C_s \qquad \text{Eq. 3.1}$$

where Js is the flux (per unit time and unit area),

Q is the amount of solute penetrating in time t

Kp is the permeability coefficient

$\Delta C_s$ is the concentration gradient across the membrane

A is the area of the skin to which the drug is applied.

An alternative form of the equation is:

$$\frac{dQ}{dt} = \frac{D\ (Km)\ Cv}{h} \qquad \text{Eq. 3.2}$$

where $\frac{dQ}{dt}$ is the steady-state flux per unit area

D is the effective diffusion coefficient

Km is the partition coefficient of the drug between the skin and

the vehicle.

Cv is the drug concentration in the vehicle

h is the effective thickness of the stratum corneum.

When the steady-state diffusion plot is extrapolated it intercepts the time axis showing a lag time (L) which can be shown to be related to diffusion coefficient by the equation.

$$L = \frac{h^2}{6D} \qquad \text{Eq. 3.3}$$

However, both equations are limited to simple diffusion models and apply only to steady-state diffusion through the skin. Their limitations have been discussed, amongst others by Barry (2), Scheuplein (14,15) and Poulsen (16,17). Thus, assuming that the thickness of the stratum corneum is constant, the factors which affect the flux are the diffusion coefficient, partition coefficient and penetrant concentration.

**(a) Diffusion Coefficient**

The diffusion coefficient of a compound is a measure of the resistance of the stratum corneum barrier to the movement of that compound through it (16,17). It is strongly influenced by molecular volume of penetrant and the viscosity and temperature of the skin. If the diffusing molecule is spherical, and of comparable size to solvent molecules, the Stokes-Einstein equation gives an almost exact value for the diffusion coefficient.

$$D = \frac{kT}{6\pi\, r\eta} \qquad \text{Eq. 3.4}$$

where k is the Boltzmann constant

T is the temperature (°K)

r is the hydrodynamic radius of diffusant

$\eta$ is the viscosity of the diffusion medium.

Several workers have studied the effect of molecular size and structure on the diffusion coefficient (1,6,11, 86-91).

**(b) Concentration of Penetrant**

For passive diffusion, the flux of the penetrant is related to the concentration gradient (1,26). Thus the higher the concentration applied, the greater is the expected penetration rate (92), a relationship confirmed by many workers (93-98). However Fick's Law is applicable only to relatively low concentrations (1,99). It has been reported that with high concentrations the flux may not be as high as expected (100) and that it can show a reduction in penetration (101). According to Higuchi (102) the thermodynamic activity of the drug in the vehicle is of greater importance than the absolute concentration (see page 80). Whilst applicable in an ideal conditionsit has been shown that this approach has potential limitations (102,103). In other cases an increase in penetrant concentration may lead to skin damage (104). This could take the form of a change in viscosity of elements within the stratum corneum, which, according to the Stokes-Einstein equation (Eq.3.4),will alter diffusion rate (105-107).

There are a number of other factors, including pH, cosolvents, surface activity, micellisation and complexation which may alter the effective concentration of the solute and hence its flux. These have been reviewed by Barry (2).

**(c) Partition Coefficient**

The partition coefficient is the ratio of the equilibrium concentrations of a substance in two immiscible phases. It is useful

to view the skin/vehicle partition coefficient as an index of mutual affinity between solute and vehicle (108), a large value indicating that the vehicle has a poor affinity for the solute. Conversely, a low partition coefficient indicates a high degree of mutual interaction and reflects a tendency of the drug to remain in the vehicle (4,102,108). Several workers have suggested that, in order to obtain good skin permeability, penetrants should show good solubility in both oil and water and that slow penetration would be expected with those having limited solubility in either the aqueous or the organic phase (10,86,91). A maximum skin permeability would, therefore, be expected for compounds having a partition coefficient close to unity (86,91). However many exceptions are known which has led some workers to discount the need for a partition coefficient of unity for optimal skin penetration (4,109).

Another approach is to consider the skin/vehicle partition coefficients, which were found by Scheuplein (108) to be the only partition coefficient which gave good correlation with drug penetration rates of alcohols (up to C8).

## 3. Skin Transport

### I Routes of Penetration

There are two potential routes for molecules to penetrate the skin. These are through the appendages; hair follicles and sweat ducts, and the intact stratum corneum between the appendages (2,13,18). The hair follicles are filled with sebum, an oily secretion from the sebaceous glands, the ducts of which open into the upper portion of

the hair follicles. The sweat glands consist of a coiled tube in the dermis with a narrow duct passing through the epidermis to the skin surface (5).

**(a) Transappendageal Route**

The actual pathway of penetration via the hair follicle could be through the hair itself, through the outer root sheath of the hair into the viable cells of the follicle or through the sebum-filled canal into the sebaceous gland. The route for the sweat duct could be through either the lumen or the walls to below the epidermis and then through the thin ring of keratinized cells. Dense capillary networks closely envelope the bases of both sweat ducts and hair follicles so that most molecules reaching these highly permeable vascular regions would enter the systemic circulation (2).

These appendages are scattered throughout the skin in numbers which vary with body site, but they are comparatively few; their total cross-sectional area is probably between 0.1 and 1.0% of total skin area whilst the total volume available for transport, other than within the stratum corneum cells has been estimated by Blank and Scheuplein (99) at 0.01 to 0.1% of stratum corneum volume.

There is no clear evidence as to whether or not the sweat glands do play a role in the absorption process (10,12,23,27,110,111). It has been suggested that local anaesthetics (112) and histamine (96) are absorbed by this route although they penetrate poorly through the palm, which has numerous sweat gland ducts compared to other parts of the body.

The <u>in vitro</u>, but not the <u>in vivo</u>, studies of Wahlberg (113) suggest that hairy skin is more easily penetrated than non-hairy skin. Tregear (110), using Tri-n-butyl phosphate concludes that the

hair follicles of the pig are no more penetrable than the epidermis.

The transappendageal routes, sometimes called shunt diffusion, was shown by Scheuplein (114) to be dominant only in the initial, transient stage of diffusion before steady-state diffusion through the intact stratum corneum was established. Thus it appears that during the lag period of diffusion, a major part of the flux may occur via these shunts and that for slow moving molecules such as steroids the shunts may play a more significant role in the steady-state penetration process (99,115,116).

(b) Epidermal Route

i) Barrier Layer

Over several decades sufficient information has been accumulated for it is to be generally accepted that the principle barrier function of the skin resides almost entirely in the stratum corneum. This evidence has been reviewed by many workers including Kigman (3), Tregear, Idson (18,19) and Scheuplein and Blank (11). Therefore, once substances have penetrated the stratum corneum, there will be no further significant resistance towards drug penetration (19,99,117). However there is some evidence that the dermis may show some resistance to the penetration of steroids such as testosterone (due to protein binding) and highly non-polar substances. These aspects have been reviewed by Barry (2).

Data (reviewed by Tregear, 1 and Scheuplein and Blank, 11) from in vivo and in vitro studies have made it clear that skin penetration is by passive transport. This is consistent with the location of the barrier to diffusion being in the stratum corneum, which is composed of keratinized, metabolically inactive cells (14).

Naturally there has been much interest in the exact nature

of the barrier within the stratum corneum and here there is less unanimity of opinion. Amongst early workers, Berenson and Burch in 1951 (118) suggested that keratin acted as a framework for the lipids which acted as the barrier. In 1956, Treherne (90) suggested that the barrier was composed of alternate lipid and water layers. In 1971, Scheuplein and Blank (11) considered the intracellular keratin to be the likely barrier.

**ii) Intercellular or Transcellular**

Tregear (1) and Middleton (119) suggested that penetration can take place via an intercellular route. Scheuplein and Blank (11) agreed that diffusion between cells cannot be ruled out, but suggested that diffusion cannot be primarily intercellular. From permeability data they suggested that diffusion occurs through intercellular regions and across cell membranes alike, without discrimination, i.e., by a transcellular mechanism. It has been suggested that strongly polar and highly non-polar molecules diffuse by different pathways (99). Whilst polar molecules diffuse through bound, immobilized water, non-polar molecules dissolve in and diffuse through the non-aqueous lipid matrix between the protein filaments. The intercellular space was thought to be restricted to about 5% in dry stratum corneum and be as low as 1% in fully hydrated skin (11,108). However recent work has indicated that the intercellular volume may be as much as 20-30% of the total dry stratum corneum (20,21). Evidence suggests that the intercellular space may serve as a preferential transport pathway for certain lipid-soluble compounds (120-122).

### II Epidermal Reservoir

Malkinson and Ferguson (123) first postulated a skin depot, or reservoir, for topically applied materials such as hydrocortisone. Vickers (124), using steroids such as Triamcinolone and flucinolone acetonides, demonstrated the formation of a reservoir existing within the stratum corneum which could be enhanced by occlusion.

The reservoir effect for various steroids has been extensively studied by Barry and Woodford (125-128) who confirmed the importance of occlusion. Other workers have used a range of drugs (129-132) and shown a relationship between the reservoir and skin penetration (131,132). Reservoir formation may be caused by binding of the drug to components in the stratum corneum (24), a gradual accumulation because of a lower solubility in the more aqueous lower cell layers (133), or the precipitation of drug from solution (2,134). The clinical significance is still open to question(18).

## 4. Factors Affecting Percutaneous Absorption

Percutaneous absorption is that process whereby a chemical passes through the skin and enters the peripheral blood circulation. Skin itself is a complex dynamic organ which varies between species, individuals, sites around the body and with age, sex, disease. Apart from these physiological differences, the skin exerts physicochemical influences on any potential penetrant, which in turn may affect the skin or be affected by the vehicle used to administer it. That vehicle may also modify the behaviour of skin.

In the following review, physiological and physicochemical factors are used as major headings with further sub-headings as necessary.

### (a) Physiological Factors

When dealing with any biological system, variation is to be expected (100). This is true of both *in vitro* and *in vivo* experiments and the variation may be minor or major depending on the random choice of subjects. Many workers have reported large natural variation (70,93,100,135-137). Southwell *et al.* published useful data in which they differentiated between inter- and intra-subject variations (138).

#### (i) Age

Absorption through the skin of infants and the aged is generally considered to be greater than at other ages (92,139,140) although, in a review (23), it has been reported that in old age there is reduced penetration and it is a clinical observation that contact sensitisation reactions are less common amongst older patients.

Animal experiments indicate that some changes in skin permeability occur before birth, although these are relatively minor after birth (1). However recent literature (141,142) suggests that variation in permeability due to age difference may not exist.

#### (ii) Species Variation

*In vivo* experiments on human volunteers are the ideal way to study the penetration behaviour of drugs (143). Frequently this is not possible and in such situations it is necessary to use animal substitutes, either *in vivo* or *in vitro*. However, there are wide variations in the physical characteristics of skin between man and animals, which may affect the rate of penetration (144). For example the skin of rabbits, rats and mice lack sweat glands but abound in hair follicles (26). Rabbit skin shows faster absorption compared to other animals even though neither epidermal structure

nor appendageal frequency appears to differ significantly from species which are more resistant to penetration (145). The commonest experimental animals are rabbit, rat, and guinea pig (146).

A study of the literature produces many conflicting and confusing reports on the relative skin permeability of different species, although the usual order of increasing permeability is man, pig, guinea pig, rat, rabbit (1,74,147). The main conclusions are summarised in Table 3.1 and Figure 3.1.

Simple molecules such as n-alkanols penetrate the skin of man and hairless mouse at a similar rate (148). Bronauch et al. (149) studied the penetration of acetyl salicylic acid, benzoic acid and urea through pig, rat, hairless mouse, mouse and compared them with human skin. They found the rank order of penetration for the three compounds was not the same. However human and Rhesus monkey showed similar percutaneous absorption of hydrocortisone, testosterone and benzoic acid (150). The work of Bartek et al. (74) on the in vivo absorption of a range of materials produced a decreasing permeability order of rabbit, rat, pig and man (Fig.3.1).

McCreesh (135) whose ranked order is given in Table 3.1, explained the variation between species as being due to physiological factors such as degree of hairiness, the variability of growth pattern, differences in sebaceous mechanisms, sweating and tissue layer thickness. Dupuis et al. (132) have suggested that species variation arises from differences in number of cell layers and intercellular volume.

The permeability of different body sites also has relevance in relating other species to man (3,151).

Thus there are wide variations reported. Skins of some animals have been found to provide resistance to penetration similar

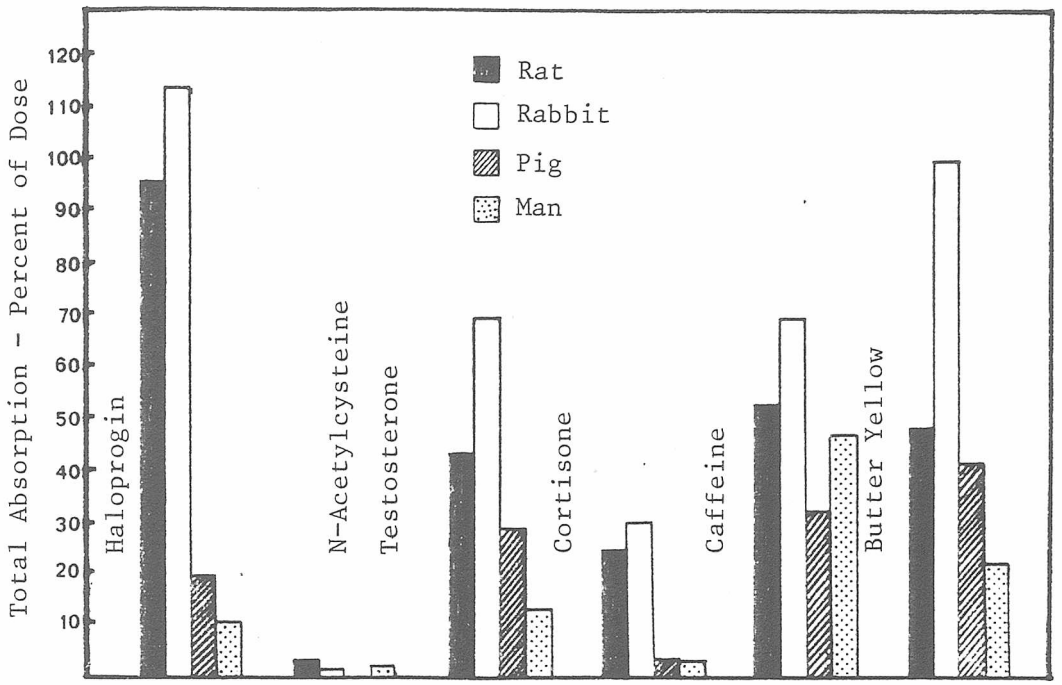



Figure 3.1 Total absorption of each test compound in rats, rabbits, pig and man. (Source: Bartek et al., 74)

Table 3.1 Skin permeability of different species as determined in vitro, ranked in decreasing order of permeability.

| Treagear (1) | Marzulli et al. (144) | McCreesh (135) |
|---|---|---|
| rabbit | mouse | rabbit |
| rat | guinea pig | rat |
| guinea pig | goat | guinea pig |
| man | rabbit | cat |
| | horse | goat |
| | cat | monkey |
| | dog | dog |
| | monkey | pig |
| | weanling pig | |
| | man | |
| | chimpanzee | |

Source: Wester and Maibach (146)

to that of human skin. These animals are Rhesus monkey (141,150, 152,153), hairless mouse (75,148) and pig (1,3,74,144,152). Most workers have found that the most permeable skin is that of rabbits (1,3,74,135,147,152). For more detailed information see Barry (2), Wester and Noonan (143) and Wester and Maibach (146).

**(iii) Variation between Body Sites**

It has been well documented that the rate of absorption through the skin varies from one anatomical site to another (135,154-157). For example Cronin and Stoughton (154) using ethylnicotinate, privine and histamine demonstrated the fastest absorption through the forehead, presternal and back areas when compared with limbs. In general the scrotum is found to be the most permeable site (3,100,156,157). Also postauricular and scalp regions were found to be amongst the highest permeable sites (155-159).

Attempts have been made to explain these variations. The early work of Blank (49) found a good inverse correlation between thickness of callus and diffusion of water. Other workers came to a similar conclusion (155). Thickness alone is not an adequate explanation for all the observed variations. In the case of such areas as the palms, which have a thick stratum corneum, penetration rates are high (1,48,160,161), although some reports contradict this (155-157).

An alternative suggestion was that the difference between sites may be related to the size and frequency of sweat glands and hair follicles (156,157), but others have concluded that such a conclusion is invalid (110). The variations have also been

attributed to number of cell layers (162), cell size (163,164), quantity of intercellular lipids (165,166) and quality of intercellular lipids (73,76).

**(iv) Circulatory Effects**

For most substances, the cutaneous circulation is sufficient to ensure prompt removal of molecules once they reach the dermis (1, 6), exceptions being very rapidly penetrating materials or those which cause vasoconstriction (167). Vasodilatation causes increased penetration (95,167).

**(v) Skin Metabolism**

Little is known about biotransformation of drugs applied topically with the exception of steroids (1,4). Normal skin is a metabolic site for steroids. Abnormalities in the metabolic processes are related to inflammation, acne, hirsutism and testicular feminization syndrome (168). Most work on drugs has been with the steroids (116, 168). Chaefer et al. (9), Barry (2) and Berliner (168) have reviewed the subject.

**(vi) Skin Condition**

The intact skin is an effective barrier to the penetration of a wide variety of substances, but it may be altered in various ways, such as physical damage, treatment with chemicals, disease state and diet (27). Thus skin condition is likely to be an important factor affecting penetration rate. When skin is not intact, penetration is greatly increased and the vehicle assumes greater importance i.e. the release rate of a drug from the topical vehicle provides the rate-limiting step in the diffusion process (2,169).

As reviewed by Barr (23), if the barrier is destroyed by trauma (eg. cuts) or eczema, substances will pass freely into the dermis. A 50% increase in the penetration of phenol and a 5-fold increase for strontium chloride was observed with abraded skin when compared to intact skin (170,171). There are many other literature examples of changes in permeability caused by damage to the skin using a variety of penetrants (111,112,123,136,154,161, 167,169,172-174).

After skin stripping, the barrier returns to near normal within 48-72 hours, not due to regeneration of the stratum corneum, but to the formation of a thin crust of dried intercellular fluid (173) or the formation of a temporary barrier by rapid conversion of granular cells into parakeratotic cells (175). Complete recovery requires two weeks (173,176), although this may be shortened by occlusion (177).

Strong acids and alkalis show an irreversible destructive effect on the stratum corneum, rendering the skin more permeable (15, 27). Barrier damage occurring above pH 10.5 may be due to extraction of keratin (70,178). Skin permeability is also significantly increased following extraction of stratum corneum lipids with organic solvents (79,97,98,105.106,179-181).

The skin barrier may be significantly altered by diseases such as psoriasis, neurodermatitis, exfoliative dermatitis and other infalmmatory skin disorders (3,182).

Essential fatty acid deficient diet has also been shown to affect the permeability of the skin (97,183-185).

(b) Physicochemical Factors

(i) Skin Hydration

A dry skin offers a more effective barrier to penetration of molecules than hydrated skin (137,186,187), because the mobility of water increases as the water content of stratum corneum increases with hydration, thus facilitating the diffusion of water-soluble penetrants (11). Normal stratum corneum contains 5-15% water (133) but on exposure to water the horny layer swells absorbing at least 4 times its dry weight of water (99,106). The resultant hydrated stratum corneum has an affinity for both water- and lipid-soluble compounds (11), thereby increasing the rate of passage of all substances which penetrate the skin, particularly water-soluble drugs (26,133). Nevertheless, the straum corneum remains a stable and extremely effective diffusion barrier, even after two weeks immersion in water at 27°C (106,108). Effect of hydration on the penetration of water and n-alkanols (188-191), steroids (94,136,192,193) and other materials have been reported (137,154,186,187,194).

Some hygroscopic water-soluble substances, called Natural Moisturising Factor (NMF) by Jacobi (47), have been held responsible for much of the water binding capacity of the horny layer. Their constituents have been reported (47,50-52,54) (see page 10). Detergents and sequential extraction with organic solvents and water remove the NMF, rendering the stratum corneum less easy to hydrate (35,47-53).

Woodford and Barry (128) attempted to increase the absorption of steroids by improving skin hydration by adding the NMF sodium-2-pyrrolidone-5-carboxylate to the vehicle. A reduction, rather than increase, was observed, probably due to a reduced thermodynamic activity, thus lowering the skin/vehicle partition coefficient of the penetrant.

Other hygroscopic materials do appear to improve hydration and permeability (195-197, reviewed by 2). However humectants increased the trans-epidermal water loss *in vitro* (198,199).

**(ii) Binding to the Skin**

It is reasonable to expect the skin, as a complex chemical structure, to interact with some chemcials. These interactions could range from weak, van der Waal-type attractions to strong chemical bonding (1,4,134). Many examples can be found in the literature (14,134,200-204). Increasing the number of polar groups increases binding (15,105) whilst addition of non-polar groups, such as $-CH_3$, increase the penetration rate (11,181).

**(iii) Effect of Temperature**

Temperature may have an effect on the passage of a material through the stratum corneum either directly, by altering diffusion rate, or indirectly by causing changes to skin structure or both. The latter normally only arise at high temperatures.

Tregear (1) reported that stratum corneum is a poor heat insulator and subjection of it to high temperature has been reported to alter permeability. Scalding or branding at 60°C for less than 1 minute was reported to have increased the penetration rate of water and low-molecular-weight alkanols (190,205,206). However, Blank and Scheuplein (133) reported that several hours exposure to that temperature produced little alteration in skin permeability. Polano *et al*. (207) also found no change in the penetration of methylnicotinate through skin heated at 60°C for periods up to 16 minutes. Exposure of guinea pig skin to hot water (60° and 70°C) for 45 seconds severely damaged the viable epidermal and dermal cells but no alteration

in the diffusion rate of water (97). Allenby et al. (208), whilst reporting the stability of the skin barrier when incubated in water at 65°C for 1 hour, also reported the onset of an irreversible structural change at 71.3°C. Behl and co-workers (209-211) observed an irreversible dramatic increase in skin permeability once heated at 80°C and above, and attributed to denaturation of proteins of stratum corneum. Cooper (212) has also reported an irreversible skin damage when heated in water at 100°C for 30 seconds. Under normal conditions, in temperate climates, stratum corneum is between 30° and 37°C (2,24). Under occlusion the temperature will increase (116,143) and may reach body temperature (192). The increased permeability observed following occlusion is, however, due to hydration rather than increased temperature (2,27,92,116,192). There will also be an increase in cutaneous circulation leading to increased drug absorption (27). In disease states, there may also be an increased surface temperature, but increased permeability is only likely to arise from disruption of the stratum corneum rather than temperature (2).

Several workers have studied the effect of temperature on the permeability of particular materials through the skin (97,170,179, 194,213-215). Blank et al. (105) have investigated the effect of temperature (over the range 5-50°C) on the transport of low-molecular-weight n-alkanols across the skin. The arrhenius plots for the polar alcohols ($C_2$-$C_5$) were linear with an activation energy of 16.5 ± 2.0 Kcal $mole^{-1}$. For the more non-polar alkanols ($C_6$-$C_8$) the arrhenius plots were non-linear, the activation energy decreasing to 10 ± 2 Kcal $mole^{-1}$ at temperatures above 25-30°C, suggesting that they penetrate via a lipid pathway which becomes less viscous at these temperatures. This is in good agreement with the work of Vinson et al. (97) who

observed deviation in the linearity of arrhenius plot for water permeability at above 40°C.

(iv) Effect of the Vehicle

In the past it was thought that vehicles acted as "carriers", carrying the drug all the way in any translocation (reviewed by 10). Malkinson, however, suggested that no vehicle can "carry" through barrier, a substance otherwise incapable of penetration.

The physiological availability of a topically applied drug depends on both the rate of release from the vehicle and the permeability through the skin. However, the former is of subsidiary importance if the material does not readily penetrate the skin (26). Also if a drug is entirely insoluble, the penetration depends on the particle size (10). It is also suggested that vehicles can affect solute permeation, even if the skin is not affected (216). The importance of vehicles and their effect on skin penetration has been reviewed (1,2,4,10,16,18,19,23,26).

Roberts and Anderson (217) investigated the penetration of phenol from different vehicles and found, for example, the penetration of phenol through excised skin was 5 times faster from light liquid paraffin than from water.

Blank (93,218) studied the effect of polar and non-polar vehicles on the penetration of low-molecular-weight alkanols. From an aqueous vehicle (saline), the penetration rate was found to increase as the molecular weight of the n-alkanol increased ($C_1$-$C_8$). The increase in chain length also led to reduced water solubility and an increased skin/vehicle partition coefficient (108). However, when a non-polar vehicle such as isopropylpalmitate was used, the penetration of alkanols was found to increase in the order octanol <

pentanol < propanol < ethanol. The flux of octanol and pentanol from olive or mineral oil was found to be lower than that of ethanol and propanol from the same vehicle (93). Blank (218) also showed that, whilst penetration of heptanol from water was 58 times faster than ethanol from water, ethanol showed 47 times higher penetration rate than heptanol when the vehicle was isopropylmyristate. This reduction in penetration of non-polar alkanols from non-polar vehicles was attributed to the unfavourable skin/vehicle partition coefficient (133). These studies show that penetration of polar alkanols is better from non-polar vehicles, whilst penetration of non-polar alkanols ($C_5$-$C_8$) is faster from water. Thus, depending on the vehicle used, a lipophilic solute may penetrate faster, slower or at the same rate as a comparative water-soluble solute (109). For example, pentanol penetrates 6 times faster than propanol from saline, but about 6 times slower than propanol from isopropylpalmitate (IPP). The flux of pentanol from saline was the same as that of propanol from IPP (93). Similar behaviour was observed with straight-chain alkanoic acids ($C_2$-$C_7$) by Liron and Cohen (219), who studied their penetration rates from the non-polar vehicle n-heptane. They found that the penetration decreased with increasing hydrophobic properties.

Higuchi (102) in his theoretical considerations, expressed the normal equation for diffusion rate (Eq.3.2) in terms of the thermodynamic activity of the penetrant in its vehicle.

$$\frac{dQ}{dt} = \frac{a}{\gamma} \frac{DA}{L} \qquad \text{Eq.3.5}$$

where a is the thermodynamic activity of the drug in its vehicle (product of drug concentration and its activity coefficient in the vehicle).

$\gamma$ is the effective activity coefficient of the agent in the

skin barrier phase.

It follows that, in order to obtain the maximum rate of penetration, the highest thermodynamic potential for the penetrating substances must be used. Because activities are important, for a given concentration of the penetrant, vechicles which have a lower affinity for the penetrant will produce faster penetration. Likewise, solutes held firmly by the vehicle will exhibit low activity coefficients and slow rates of penetration (102). Higuchi (102) also suggested that if a drug is present as a fine particle size suspension in different vehicles, it should produce equal thermodynamic activity and hence the same rate of penetration.

Woodford and Barry (103) measured the absorption of betamethasone valerate from different bases using the vasoconstrictor assay, and related the results to the thermodynamic activity. They concluded that the thermodynamic activity model applied only when formulations used the same solvents in slightly different ratios. This has also been found with the penetration of methylnicotinate from vehicles containing different ratios of water and glycerol (220).

Predictions on the basis of thermodynamic activity may not be valid with more complex formulations where other factors such as penetration enhancement by solvents in the formulation, irritancy, dehydration and binding of penetrant with components of the vehicle may occur. Glycols have been reported amongst those materials which form hydrogen bonds with penetrants (221) and cause slow penetration rates of solute (93,102,213,214,222). For example Barry and Woodford (103) postulated that PEG 400 formed hydrogen bonds with betamethasone valerate, reducing the thermodynamic activity of the drug and therefore decreasing its flux into the skin.

Information about drug-vehicle interaction has been

reviewed by Barry (2) and Katz and Poulsen (4), whilst the theoretical aspects of Higuchi's work on drug absorption from solutions and suspensions has also been reviewed (2,4,18,19).

(v) Surfactants

The role of surfactants in skin permeability has been reviewed by several workers (2,9,11,14,15,18,19,23-26). Stratum coerneum is an effective barrier to the penetration of surfactants (178 ,180,223-226), their penetration being indicated by their irritating effect on cells below the horny layer (178,227,228). Surfactants differ in their ability to penetrate and alter skin permeability, with effectiveness decreasing in the order anionic, cationic, non-ionic (180,227).

Anionic surfactants require a long time (2-6 hours) for penetration (106,226,227), but once penetrated, they increase the penetration of other compounds. Variations in rates of surfactant penetration are found with chain length (229,230) and type of anionic surfactant (224,230). Scheuplein and Ross (106) suggested that ionic surfactants bind strongly with α-protein, reducing the amount of bound water and causing a reversible denaturation and uncoiling of the filaments, accompanied by swelling of stratum corneum to increase skin permeability. Sprott (229) also explained the enhancing effect of surfactants in terms of protein-surfactant interactions.

Middleton (53) found that sodium lauryl sulphate extracted both lipid (including cholesterol) and water-soluble substances to a greater extent than sodium lauroylisothionate. Thus it appears that the loss of lipid from cell walls not only results in increased permeability, but allows the intra-cellcular water-soluble substances to escape. Increased skin permeability caused by

surfactants has also been attributed to lipid extraction (20,21).

Penetration of surfactants has also been found to vary inversely with pH from 6-10.8. Above pH 10.8, the damaging effect of the alkalinity itself overshadows the effect of pH change (180, 229). Lipid extraction was found to significantly increase the penetration of surfactants (178,180,225,229). Bettley (228) has concluded that neither pH nor surface tension are directly related to permeability.

**5. Sorption Promoters**

Materials which will interfere with the skin barrier have been called accelerants (208) sorption promotors (25) and penetration enhancers (2,4). The ideal properties of such a material for dermatological use have been enumerated by both Katz and Poulsen (4) and Barry (2). Prime amongst these are the need for safety in use and a reversible action. Whilst it is doubtful that an ideal material will be found, a number of compounds have been found which do enhance skin permeability, although it is often difficult to differentiate between a direct effect on the skin and a solvent effect (2). Roberts and Anderson (217) were able to do this by comparing results with excised rat skin and a polyethylene membrane using phenol as penetrant and DMSO as enhancer. They showed that DMSO has a direct effect on the barrier within rat stratum corneum.

In general it appears that penetration enhancers always affect the skin directly, by either reducing the resistance to diffusion, or altering or damaging the physicochemical nature of the barrier (2,4,106).

A solvent system such as chloroform/methanol removes stratum corneum lipids and thereby reduces the activation energy of

water penetration from 15.0 Kcal $mole^{-1}$ to 6.0-6.5 Kcal $mol^{-1}$ (105, 108). Similar changes were observed for propanol and heptanol (105). Delipidisation is, however, too drastic for normal use.

The mechanism of action of sorption promotors is obscure (4). They have been shown not to act as carriers, nor to act by creating a more favourable partition coefficient (231). A review of some of the most important penetration enhancers follows.

**(a) Dimethylsulphoxide**

DMSO is the lowest member of the group of sulphoxides with the general formula RSOR. It is a colourless, nearly odourless liquid which is a dipolar, aprotic solvent (ie. accepts protons) and is completely miscible with water and many organic solvents. It is also hygroscopic and mixes with water to produce considerable heat (2,4). The physicochemical properties of DMSO have been defined (84,232-234) and its history and toxicity have been reviewed (2,4,60, 235).

Both <u>in vitro</u> and <u>in vivo</u> studies have shown that DMSO acts as a penetration enhancer for a wide range of materials e.g. water (106,208,236-238), various synthetic dyes (60,239), organic phosphates (78), alkylsulphates (223,224) scopolamine (240), antibiotics (130), salicyclic acid (241), picrate ions (81,82,231), phenol (217), hexapyrronium bromide and naphazoline (242), griseofulvin (129,243) and various corticosteroids (129,242-244). Other groups, including local anaesthetics, antimetabolites, antiperspirants, antibacterials have been reviewed recently by Barry (2). However, DMSO does not allow the percutaneous absorption of macromolecules which are normally unable to penetrate the skin (239,245).

The literature indicates a concentration dependence of

the penetration enhancing effect of DMSO. Sweeney et al. (238) studied the effect of DMSO on water diffusion through excised skin of hairless mice and concluded that the concentration of DMSO is far more significant than the duration of exposure. To be effective, a concentration of greater than 60-70% is required. Sweeney et al. (238) and Allenby et al. (208) both reported that concentrations of DMSO in water below 50% are inactive. The in vivo work of Stoughton and Fritsch (242) is unusual in that they reported a 25-fold increase in absorption of hexapyrroniumbromide in 20% DMSO, a 5-fold increase in absorption of fluocinolone with 10-25% DMSO and a 25-fold increase in absorption of naphazoline with 50% DMSO. Akhter and Barry (246) using an open cell "in vivo mimic" experiment found a pronounced enhancing effect of DMSO on the penetration of Flurbiprofen with 20% DMSO, similar to that produced by 40-100% DMSO. This, could have been due to factors such as evaporation, permeation and depletion of vehicle. Most workers, however, have confirmed the need for concentrations greater than 60-70% (60-62,81,82,106,208,231,238-240, 247).

The literature is also conflicting on the possible reversibility of DMSO activity. Kligman (60) studied the effect of 24 hour treatment with 50, 90 and 100% DMSO on the penetration of fluorescein through human stratum corneum. After these treatments, rinsing with water for 3 hours showed that the effect was reversible. However Sweeney et al. (238) stated that the effect of DMSO on skin permeability is permanent. The in vitro work of Astley and Levine (237) suggested partial reversibility of human stratum corneum barrier capacity following treatment with DMSO. Partial reversibility of structural changes in stratum corneum, induced by 80% DMSO, has also been reported by Chandrasekaran et al. (240). The in vivo

work of Baker (236) on the effect of 100% DMSO, applied for 30 mins to the flexor aspect of the mid-forearm, on transepidermal water loss produced 8-, 11- and 17-fold increases in water loss on 3 subjects. The effect was reversed within 6½ hours.

Although DMSO has been extensively investigated, its mode of action as a sorption promotor has not been clearly elucidated. Various hypotheses have been postulated and are briefly reviewed.

According to the in vivo work of Creasey et al. (248) the accelerant effect of DMSO was not due to increased blood circulation, because there is no increase in clearance rate. The circulation can be increased without increasing the penetration rate (1) and the enhancement can be observed in vitro with isolated non-perfused skin preparation (78). Thus the effect must be associated with changes in the stratum corneum (248).

The polar nature of DMSO, its capacity to accept hydrogen bonds and its relatively small, compact structure, enable it to associate with water, proteins, carbohydrates, nucleic acid, ionic materials and other constituents of biological systems (233). DMSO forms an association complex with water in the molecular ratio 2:1 (water : DMSO) (233, 83), which corresponds to 67% DMSO (84). The hydrogen bond between DMSO and water is 1 1/3 times as strong as that between water molecules (234, 83), the latter being a more structured liquid than DMSO (233). Indeed Szmant (233) has suggested that DMSO stabilizes the ice-like water structure and may, therefore, change the equilibrium in a tissue from less structured to more structured water. A replacement of some water molecules associated with cellular constituents has been suggested as a possible mechanism of action of DMSO in skin. Rammler and Zaffaroni (84) suggested that the mode of action of DMSO was by altering the configuration of

the protein barrier by substituting for free or bound water, in addition to affecting other hydrogen bonded structures. They suggested that this would be reversible. Scheuplein and Ross (106) also suggested that the increased stratum corneum permeability arose from the capacity of DMSO to displace and substitute for bound water in the tissue, thus producing a looser structure. Since the hydration of cell constituents and the activity of water in general are not necessarily the same in the different stated of water (233). Thus DMSO may exert an indirect effect on biological systems due to the changes it causes in the structure of liquid water, by, for example, changes in the conformation and associations of proteins and other molecules. DMSO may also directly alter biological systems, without a profound change in their chemical identity, by affecting ion-pairing equilibrium or by specific solvation of hydrogen-bond donors (233).

Katz and Poulsen (4) suggested that DMSO causes swelling of the stratum corneum to open its dense, compact structure, so increasing its permeability. In addition, the formation of a continuous liquid phase of concentrated DMSO could attain relatively high concentration of drug in the stratum corneum because of the favourable solubility of many drugs in DMSO, thus leading to a further enhancement of percutaneous absorption. DMSO has been found to cause reversible swelling or unfolding of keratin (61,62,82). Although both the hair swelling and increased picrate ion penetration through the skin show a similar dependancy on DMSO concentration, the two processes do not appear to be completely alike, because the effect of DMSO on keratin is reversible, whilst that on skin permeability is irreversible. Rather, DMSO appears to alter the skin barrier through some combination of protein swelling and lipid

extraction (62,82). DMSO has been found to extract lipoproteins and nucleoproteins from stratum corneum, suggesting that this may contribute to DMSO action (80). The microscopical work of Montes et al. (249) showed that DMSO removed structural materials which have been identified as lipid and lipoprotein (77-79,105,106,208).

The exothermic reaction of DMSO with stratum corneum water could raise skin temperature. It has been suggested that this may increase diffusion (84), but the effect would be transient and, as discussed earlier, would not produce significant increases in penetration rate.

DMSO has also been reported to cause marked distortion and intercellular delamination of the stratum corneum accompanying high osmotic shock (240). Michaels et al. (250) also suggested that DMSO may operate by creating controlled disorder in organised lipid membranes of stratum corneum.

**(b) Dimethylacetamide and Dimethylformamide**

These two organic solvents appear to produce very similar permeability changes in the skin to those produced by DMSO, although neither compound has been as intensively studied (2,4).

The in vitro penetration of griseofulvin and hydrocortisone was better promoted from DMSO, DMAC and DMF than from ethanol, benzene and a cream base, but DMAC and DMF were less effective than DMSO (129, 243). DMAC established and maintained an effective concentration of hexachlorophene in stratum corneum (251).

Feldman and Maibach (244) studied the effect of DMAC and DMF on the penetration of testosterone and hydrocortisone and reported 2-3 fold increases in the penetration of both steroids in the presence of DMF. DMAC also showed an increase in the penetration

Methyldecylsulphoxide has also been found to increase the penetration of naproxen (147), urea and salicylic acid (212). Pyrrolidones have been studied intensively by Barry and co-workers (246,253-255, 257-262). N-methyl-2-pyrrolidone (NMP) and 2-pyrrolidone (2-P) were found to increase the bioavailability of steroids (253,254). Whilst NMP increased the penetration of mannitol (259), 2-P did not (262). 2-P retarded the penetration of caffeine and octanol but increased the penetration of methanol (255,257,258). However, irritant effects were apparent with both 2-P and NMP which may limit the clinical use of these materials (254). Barry (2) has reviewed the use of pyrrolidones.

1-dodecylaza cycloheptan-2-one (Azone) is a colourless, relatively odourless, non-toxic, water immiscible liquid producing minimal irritation on mucous membrane or human skin even when applied neat (263-265). The penetration by both hydrophobic and hydrophilic molecules is enhanced by azone, the effect being greatest with hydrophilic materials (265) and it is effective at concentrations as low as 1%. In general, however, concentrations of 2-10% appear to be appropriate for most formulations. For example, an 80-fold increase in the penetration of fluorouracil was observed when the drug was in an alcoholic solution containing 1.8% azone, higher concentrations giving lower enhancement. In contrast, the maximum enhancement of triamcinolone acetonide was found with 10%, indicating that the optimum concentration of azone varies with both the drug and the formulation (263-265). The enhancing effect of azone has also been investigated by other workers (254,266).

Tetrahydrofurfuryl alcohol (THFA) is moderately irritant to both skin and mucous membranes, but can enhance the percutaneous absorption of drugs such as fluocinolone (2). Sarkany et al. (252)

reported that incorporation of THFA into a suitable vehicle improved the percutaneous absorption of hydrocortisone to produce a vasoconstriction comparable with betamethasone-17-valerate and fluocinolone acetonide. The mechanism of action of THFA is not known, but it has been suggested that it may depend on its ability to dissolve lipids in the skin or its ability to produce vasoconstriction (252).

The evidence for the effect of solvents such as propylene glycol in enhancing skin permeation is conflicting (103,213,244,253, 254,266-268). However, in many instances, they appear to act as cosolvents to produce solutions of the drug at or near saturation, thereby maximising their thermodynamic activity (222,253). This can increase drug absorption even when the stratum corneum remains unaffected (2). On the other hand, some glycols such as PEG 300, PEG 400 and PEG 600 penetrate the skin poorly and may form hydrogen bonds with penetrants, thereby reducing the thermodynamic activity and penetration rate of the active ingredient (93,103,214).

Fatty acids and fatty alcohols have been shown to increase skin permeability (78, 106,208,215,254,266,269). Scheuplein and Ross (106) found that the permeability of water, butanol and butyric acid doubled in presence of concentrated solution of octanol. This effect was reversible when the octanol is washed out of the membrane. Saturated fatty acids such as formic, acetic, propionic, butyric, hexoic, decanoic and dodecanoic (78,208,215,269), the unsaturated fatty acid, oleic acid and polyunsaturated fatty acids, such as linoleic acid, have been shown to increase the skin permeability (254,266,269). The increased diffusivity of stratum corneum due to these fatty materials has been attributed to the plasticization effect on the stratum corneum lipids with increasing skin hydration and swelling (106). Cooper (269) however suggested, supported by DSC studies, that

the effect is due to an increased fluidization of the stratum corneum lipids caused by the solvent system containing fatty substances.

## Methods of Studying Percutaneous Absorption

Both *in vitro* and *in vivo* methods are used in absorption studies. The various methods have been reviewed by Idson (18,19), Katz and Poulsen (4), Wahlberg (92), Nugent and Wood (270) Schaefer *et al*. (9) and Barry (2).

### (1) In vitro Methods

*In vitro* methods using excised skin have both advantages and disadvantages. For instance, *in vitro* studies have been found useful for comparing the penetration rates of different materials (88,91,93, 178), and they enable the experimental environment to be controlled in order to gain a better understanding of the physicochemical factors which influence percutaneous absorption (105,106,209-212,229,271). The main objection to the use of excised skin is that it cannot accurately duplicate the full *in vivo* role of the skin, particularly with respect to blood flow and metabolism (144). For example, following topical application of some corticosteroids, their penetration may slow down due to their strong vasoconstriction effect, but this behaviour cannot be seen *in vitro* (2,18,19). Despite these limitations, some reasonable correlations between *in vitro* and *in vivo* data have been reported (144,214,256,272-275).

#### (a) Release Methods without a Membrane

In this method, two immiscible liquids, to represent the skin and vehicle, are placed in a container and each is stirred gently. It is usually used to estimate the release rate of a drug from a vehicle. Poulsen (271) and Poulson *et al*. (222) used isopropyl myristate as the receptor phase when evaluating the release of steroids

from gelled propylene glycol-water mixtures. Busse et al. (268) developed this model by using three layers, the top was an ointment containing the test substance, the middle layer of 15% aqueous alcohol represented the skin and finally a chloroform layer acted as a sink for the penetrant.

**(b) Diffusion Methods with a Membrane**

**(i) Simulated Skin Membranes**

Using this technique, the penetrant traverses a synthetic membrane via microscopic pores filled with the vehicle. Barry and El Eini (276) and Barry and Brace (277) have investigated the penetration of several steroids across a cellulose acetate membrane. Polydimethylsiloxane (silicone rubber) has also been used (278). In a different approach, Selkirk and Douglas (279) used a dialysis membrane, containing a proflavine cream, immersed in an aqueous sink. Filter membranes impregnated with vegetable oil has also been used for penetration studies (213).

Artificial membranes may be most useful in distinguishing between the physicochemical and biological effects of vehicles. For example Roberts and Anderson (217) compared the effect of different vehicles on the penetration of phenol across rat skin and a polyethylene film. They calculated the penetration ratios of skin to polyethylene permeability coefficient from different vehicles and found constant ratios from vehicles which do not interact with the skin and increased ratios for vehicles which do react with skin.

**(ii) Natural Skin Membranes**

In both animal and human, whole excised skin, epidermis and stratum corneum have been used. Stoughton (280) has reported that

skin can be subjected to extremes of heat and humidity and to various fluids without irreversibly altering its barrier properties. Either fresh or frozen skin may be used and various detailed preparation techniques have been reported.

Stratum corneum sheets of up to 15μ in thickness can be separated using cellulose-backed pressure-sensitive tape (155). Gilbert et al. (281) have isolated the epidermis by mechanical stretching of the whole skin. Kligman and Christophers (64) have reported three methods of separating the epidermis from the skin:

1) By treating the skin (in vivo) with 0.2% Cantharidin in acetone. After the formation of the blister, stratum corneum is obtained.

2) By exposing the skin to ammonia fumes for 30 min in a closed chamber.

3) Heat method: This is the most common method of separation. The skin is held at 60°C for 2 min in a water bath. The isolated epidermal sheet is then placed dermal side down on filter paper saturated with 0.0001% trypsin, containing 0.5% sodium bicarbonate (pH8-8.6) or phosphate buffer, and incubated overnight at 37°C. After incubation, the digested spidermis is then removed by firm rubbing with a moistoned cotton-bud, the stratum corneum is then rinsed with water and dried. When dry it can be kept indefinitely at room temperature.

Epidermal sheets can also be obtained by injecting 1-2 day old mice subcutaneously with exfoliative fractions obtained from the culture of certain phage group 2 staphylococci. Two hours later, on sacrificing the animals, the epidermis can be removed (282). These workers have also separated the epidermis by floating the skin, dermis side downwards on a solution of antibiotic and a partially purified fraction of staphylococcal exfoliatin for 4 hours at 37°C. Homogeneous sheets of stratum corneum and stratum granulosum could then be

peeled off.

Rietschel and Akers (283) have compared preparation techniques and shown that it does not affect the hygroscopicity of stratum corneum, although other work suggests that small histological changes may occur during separation and be of some significance in permeability studies (11). For permeability studies, generally the specimen of skin, trimmed to a suitable size, is mounted in a diffusion cell separating the donor and recipient phases. Passage of the penetrant through the skin is measured by either the loss of test substance from the donor phase or its appearance in the receptor phase, or by both methods simultaneously (24). Detailed design of diffusion cells varies and has been reviewed by Nugent and Wood (270) and Barry (2). A modified *in vitro* permeability technique may also be used to simulate the *in vivo* condition. This has been done by using similar diffusion cells in which the skin sheet is placed horizontally between the upper and the lower chamber of the cell. The latter contains the receptor phase and is immersed in a constant temperature bath (94,284). The donor chamber may be closed or left open, thus the epidermis is exposed to ambient condition or to a controlled humidity. The penetrant may be a fine solid deposited from a volatile solvent (usually acetone), a liquid, a semisolid (ointment, cream, paste, or gel), a film, or a drug device such as transdermal therapeutic system (TTS). This *in vivo* mimic method has been found useful to study the effect of accelerants on skin permeability(246,259-262,266).

(2) *In Vivo* Methods

Several methods have been carried out for *in vivo* measurements of drug absorption. Examples are:

(a) Observation of Physiological or Pharmacological Response

If the penetrant stimulates a measurable reaction this response may provide the basis for determining the penetration kinetics (2). Examples of response used include sweat secretion, pigmentation, sebaceous gland activity, vasodilatation, vasoconstriction, vascular permeability, epidermal proliferation, keratinization, change in blood pressure, altered pain threshold, production of convulsions, and red tear response (reviewed by Barry, 2). The most productive technique in terms of biopharmaceutical application is the vasoconstrictor or blanching response to topical steroids (103,125-128, 253,254,275).

(b) Analysis of Body Tissue or Fluids

Following topical application, analysis of urinary excretion of drugs is commonly used during in vivo studies (100,123,136,141,153, 156,167,170,186,244). Combined urinary and faecal analysis has also been reported (131,132,285). These approaches may suffer from general pharmacokinetic problems, which to some extent may be corrected by comparing data following percutaneous absorption with that following a single intravenous injection (144). Another way is to sacrifice the animal and assay the whole body for the drug (272).

(c) Surface Loss

In this method, the amount of drug remaining on the surface of the skin after a specific period of time of penetration is measured usually by determining the decrease in the radioactivity (174,272, 286). When it is possible to determine the rate of loss of a penetrant from an applied vehicle, the flux of the substance into the skin may be

obtained. However, concentration decrease would generally be small and so analytical techniques have to be very accurate and often such small changes could arise from vehicle evaporation or dilution with sweat or transepidermal water (2,272).

Other techniques have been used by some workers including measurement of transepidermal water loss (161,173), measurement of volatile substance in the expired air (187) and histological examinations (reviewed by ref. 2,270).

## Experimental Methods

### Diffusion Cells

Photographs of the diffusion cell are given in figure 3.2. Each half was identical, had a volume of six mls and gave 2 $cm^2$ exposed stratum corneum. They were made of pyrex glass. A single stratum corneum sheet was sealed between the two valves of the cell using vacuum grease. The complete cell was mounted on a magnetic stirrer to permit stirring of both chambers.

### Preparation of Penetrants

The vials, containing 500 µCi 1-$^{14}$C propanol and 250 µCi 1-$^{14}$C hexanol were diluted with cold n-propanol and n-hexanol respectively and used as a stock solution. The activity of n-propanol was found to be $1.7 \times 10^6$ dpm $\mu l^{-1}$ and for n-hexanol $1.3 \times 10^5$ dpm $\mu l^{-1}$.

In all experiments the concentration of penetrant was kept at about 3µl per ml solvent, equivalent to 2.3 µCi propanol and 0.2 µCi hexanol.

### Diffusion Experiment Procedure

All experiments were carried out at 22 ± 2°C. 1ml samples

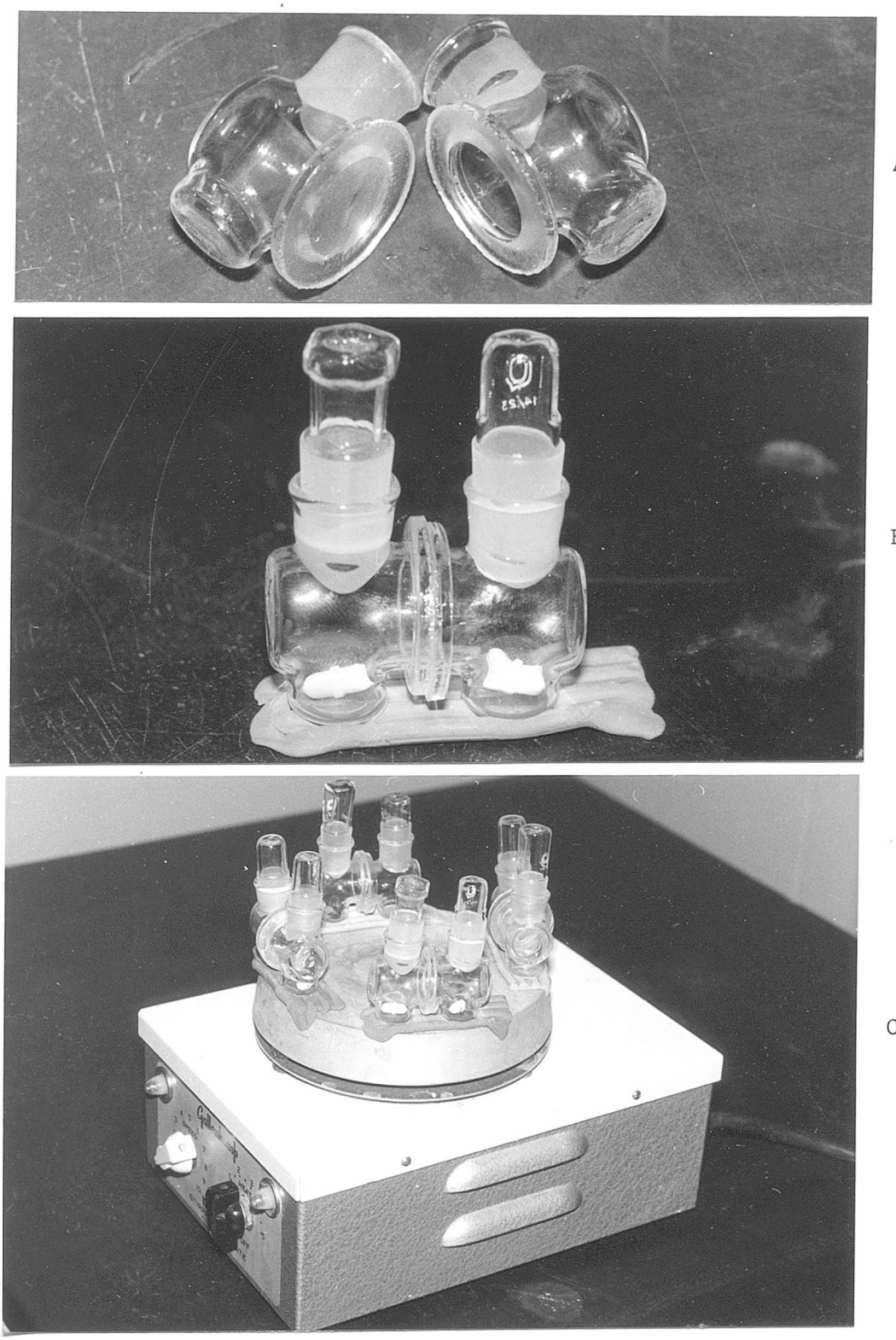



Figure 3.2 (A) Open diffusion cell with stratum corneum in place.

(B) Assembled diffusion cell, mounted on plasticine, showing magnetic stirrer followers.

(C) Four diffusion cells mounted on magnetic stirrer.

were collected hourly,or half hourly, from the receptor chamber and replaced with fresh receptor vehicle. Usually 4 replicates experiments were carried out. Each sample was added to 10 ml of scintillation fluid (Instagel) and the radioactivity determined using a liquid scintillation counter.

### Use of the Scintillation Counter

Instrument instruction manual procedures for constructing the quench curve were followed. 20µl of standard $^{14}C$ n-hexadecane (1 µCi ≡ 2.2 x $10^6$ dpm) was added to each of 10 vials which contained 10 ml of Instagel. 25µl, 50µl etc aliquots of reference quenching agent chloroform were added to the vials, shaken and counted. Using the Tri-Carb 2425, both the external standard ratio (AES) and counts per minute (cpm) were obtained. The percentage efficiency (%E) of each sample was calculated from the cpm and the standard dpm using the equation

$$\%E = \frac{\text{sample cpm}}{\text{standard dpm}} \times 100$$

Using the quench curve (%E vs AES ratio), the dpm of any sample can be calculated from its cpm and AES ratio (9). An example of the quench curve is given in Figure 3.3.

Using the Tri-Carb 460CD, the quench curve was stored in the apparatus, which automatically calculated the dpm of test samples.

### Calculation of Permeability Coefficient

From the activity of the penetrant, the dpm of each sample was converted into amount penetrated (µl) and cummulative penetration per unit area (µl $cm^{-2}$). A computer linear regression analysis was used to calculate the flux (µl $cm^{-2}$ $hr^{-1}$) from 3 hours onwards. The

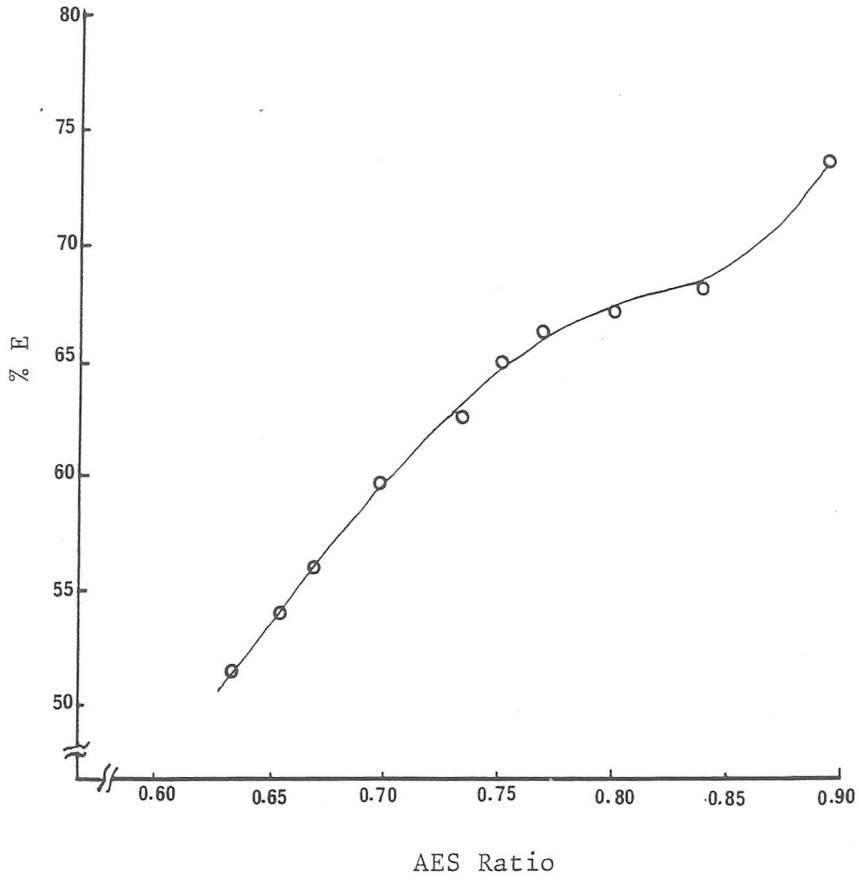



Figure 3.3 A quench curve obtained by plotting data for efficiency (%) against automatic external standard ratio.

permeability coefficient (Kp) was then calculated using the equation

$$Kp\ (cm\ hr^{-1}) = \frac{Flux}{\Delta CS}$$

where $\Delta CS$ the concentration of penetrant in the vehicle. The mean and standard deviation were calculated for each experiment.

**Penetration Studies**

For convenience, details of experimental variables are given under several headings.

**(a) Normal Stratum Corneum**

Table 3.2 details the experimental conditions used to investigate the effect of hydration and three vehicles on penetration rate. Hydration was carried out by filling both sides of the diffusion cell with distilled water which was stirred for 24 hours or 5 days (replaced each day).

Table 3.2 Penetration Conditions for Normal Stratum Corneum

| Type of Vehicle | Physical State of Stratum Corneum | Donor Phase | Receptor Phase |
|---|---|---|---|
| Aqueous | Dry | Water | Water |
| | 24 hour hydration | Water | Water |
| | 5 day hydration | Water | Water |
| | Dry | 66.7% PG | Water |
| Non-aqueous | Dry | LLP | LLP |
| | Dry | LLP | Water |
| | 24 hour hydration | LLP | LLP |

Key: PG - Propylene glycol
LLP - Light liquid paraffin

(b) Effect of Aprotic Solvents

(i) The effect of a concentration gradient across stratum corneum was investigated by using both 70% and 100% DMSO as donor phase only, receptor phase only and both phases. Water was used as the second phase as necessary. Following measurements with 100% DMSO, second runs were carried out using water as both phases.

(ii) Stratum corneum sheets were soaked for 6 hours in 90% DMSO, rinsed several times with distilled water and stored overnight in fresh distilled water. They were then dried, in the same way as during their preparation, before measuring hexanol permeability with water in both chambers.

(iii) Aqueous solutions of DMSO and DMAC were prepared as follows:

25,50,70,75,80,85,90% v/v DMSO

25,50,70,80,85,90,95% v/v DMAC

Each solution was used in both sides of the diffusion cell. After each experiment, the stratum corneum was washed in situ with several changes of distilled water, stirred overnight with water followed by a further rinse with fresh distilled water. Penetration was then measured using water as both donor and receptor phase.

(c) Heat Pre-treatment of Stratum Corneum

Dry stratum corneum was heated in an oven at 50% RH at 40°, 60°, 70°, 75°, 80°C, 85° and 90°C for time periods between 30 min and 12 hours. After each heat treatment samples were stored overnight at room temperature before penetration studies. Samples which had been treated for 6 hours were divided into two groups, the first was stored open to the atmosphere, the second group were suspended overnight over DMSO liquid in a closed container (see Fig. 2.1). Control samples were also exposed overnight to DMSO vapour. Water was used as receptor phase in permeability studies.

(d) Infrared Spectroscopy

Infrared spectra were obtained using a Perkin-Elmer 681 instrument with 0.01 mm cells and a scan speed of 6 mins. Spectra were obtained using 10% (of each material) in carbon tetrachloride solution.

## Results and Discussion

### 1. Effect of Hydration on Skin Permeability

Propanol and hexanol were used as model penetrants for their relative polar and non-polar properties. The effect of stratum corneum hydration on the permeability coefficient (Kp) of both is shown in Table 3.3.

Table 3.3 Permeability Coefficients (Kp) of Alkanols from Water.

| Initial hydration state of stratum corneum | Propanol | Hexanol |
|---|---|---|
| | Kp x $10^3$ cm $hr^{-1}$ | Kp x $10^3$ cm $hr^{-1}$ |
| Dry | 0.71 ± 0.21 | 10.3 ± 2.8 |
| 24 hour hydration | 0.88 ± 0.29 | 10.4 ± 1.0 |
| 5 day hydration | 1.15 ± 0.20 | 16.0 ± 5.5 |
| Dry (3 month storage) | --- | 9.8 ± 1.5 |

24 hour hydration did not produce a significant change in the permeability when compared with dry stratum corneum. This suggests that either hydration has no effect on penetration of alkanols or that the extent of hydration attained during the experiment was sufficient to produce the same effect as 24 hour hydration. The results in Table 3.3 indicate an increase in the penetration of both propanol and hexanol of about 50% after 5 days hydration. It has been suggested that such a small change is probably due to the "leaching" out of water-extractable components (27). It also suggests that the

stratum corneum barrier is stable even when extensively hydrated for several days or weeks (106,108). Similar studies on the effect of hydration on alkanol penetration was carried out by Behl et al. (188-191). They used hairless mouse skin and found little change in the permeation of polar substances such as water, methanol, ethanol with skin hydrated for up to 30 hours. The maximum increase in skin permeability caused by hydration, was found to be about 2-fold with butanol and hexanol (188). In further work, Behl et al. (191) investigated the effect of extended hydration on the penetration of alkanols using rat skin and found that the permeation of methanol increased 2.5 times after 3 or more days hydration, butanol about 25% and hexanol about 40% after 10 hours hydration. The latter declined to a permeability similar to that for initially dry skin. Their Kp for hexanol using adult rat skin was $9.4 \pm 1.0 \times 10^{-3}$ cm $hr^{-1}$, which is in good agreement with the value of $10.3 \pm 2.8 \times 10^{-3}$ cm $hr^{-1}$ obtained in the present work.

Such increases in penetration are minor compared with the effect of hydration due to occlusion on the in vivo absorption of corticosteroids which was found to range from 10- to 100-fold (94,100,136,192,193), or the 9-fold increase for the water-soluble glycol salicylate as a result of in vivo hydration (186). In general, the literature indicates that the effect of hydration is more pronounced in vivo than in vitro. A reason for this has been given by Scheuplein and Ross (94). Under normal in vivo conditions, the stratum corneum is very much less hydrated than the excised tissue routinely used in laboratory permeability studies. For example, when permeability is measured in a diffusion cell, the stratum corneum becomes well hydrated before steady-state flux measurements are taken, usually after 2-4 hours immersion.

In the present work, although stratum corneum was initially dry, the measurement of steady-state penetration was calculated from the third hour of the experiment. Thus, according to Scheuplein and Ross (94), the results obtained here are from hydrated and not dry stratum corneum.

To examine whether this is true or not, it is necessary to study penetration whilst maintaining the skin dry throughout the period of the experiment by using a non-aqueous vehicle. The vehicle chosen should not damage the skin, be immiscible with water (so that hydrated skin does not dehydrate with time) and be a solvent for the pentrants. Light liquid paraffin (LLP) was chosen. To maintain the stratum corneum dry throughout the experiment both donor and receptor phases were LLP. The effect of allowing gradual hydration was observed by using water as the receptor phase with LLP as the donor.

The results of these experiments are given in Table 3.4. They show that hydration does significantly increase the penetration of propanol, suggesting that hydrated stratum corneum has a higher penetrability to polar and non-polar substances (11) and, conversely, that dry skin is a more effective barrier (137,186,187).

Table 3.4 Permeability Coefficients (Kp) of alkanols from Light Liquid Paraffin (LLP)

| Initial state of hydration of stratum corneum | Donor Phase | Receptor Phase | Propanol | Hexanol |
|---|---|---|---|---|
| | | | Kp x $10^3$ (cm $hr^{-1}$) | Kp x $10^3$ (cm $hr^{-1}$) |
| Dry | LLP | LLP | 0.07 ± 0.04 | 0.44 ± 0.13 |
| Hydrated, 24 hours | LLP | LLP | 5.2 ± 0.8 | - - |
| Dry | LLP | Water | 6.1 ± 1.0 | 1.98 ± 0.59 |

Table 3.4, case 3, also shows that whilst initially dry, gradually hydrating stratum corneum shows an 87-fold increase in the

penetration of propanol, there is only a 4.5-fold increase for hexanol. Therefore, using the same conditions, the effect of hydration on propanol permeation is about 19 times that on hexanol. This is consistent with the conclusions of other workers, that the effect of hydration is more pronounced with more water-soluble materials (26, 133, 137,186,187).

In Figure 3.4, the penetration of propanol through hydrated, gradually hydrating and dry stratum corneum is shown. The permeability coefficient for the two hydrated systems are similar ($5.2 \pm 0.8 \times 10^{-3}$ and $6.1 \pm 0.1 \times 10^{-3}$ cm $hr^{-1}$), suggesting that hydration produced during the experiment gives an effect similar to 24 hr hydration. However, the lag time is extended from less than an hour for the constant hydration to 2 1/4 hours for the increasing hydration. According to Scheuplein and Ross (106), this extended lag period indicates a continuous change in penetration rate due to the changing hydration of the stratum corneum. Comparison of the gradually hydrating with the completely dry stratum corneum shows that, after 1 hour, there is no difference in total penetration, but from 2 hours the difference increases. Steady state was reached after 3 hours of gradual hydration, suggesting that such a time is required to produce sufficient hydration to permit optimal permeation of propanol. This is in good agreement with Scheupein and Ross (94).

Because water was only in contact with one side of the stratum corneum sheet, it is reasonable to assume that a shorter time would be required for effective hydration in the usual experimental situation of water in contact with both surfaces of the stratum corneum, it was found to be 75 min. For this reason it is likely that the actual effect of hydration may not be observed <u>in vitro</u> when both the donor and receptor phases are aqueous (94). It was for this

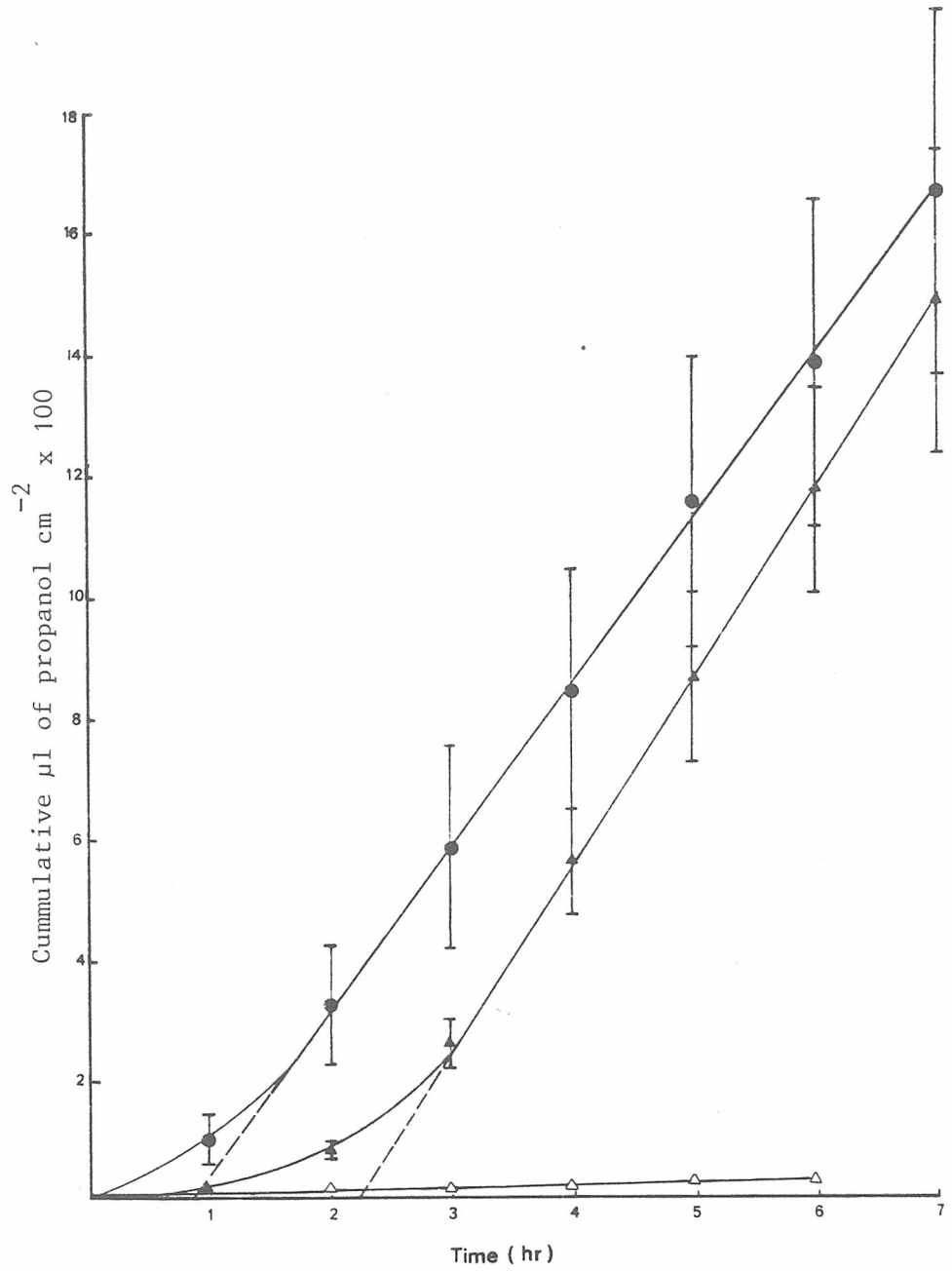



Figure 3.4 Plots of cummulative penetration data showing effect of hydration on the penetration of propanol.

(△) Dry stratum corneum (LLP donor phase, LLP receptor phase),

(▲) Dry stratum corneum (LLP donor phase, water receptor phase),

(●) 24 hour hydrated stratum corneum (LLP donor phase, LLP receptor phase).

reason that Scheuplein and Ross (94) studied the effect of skin hydration on the penetration of cortisone using an *in vitro* method in which they simulated *in vivo* conditions by using an aqueous receptor phase, but leaving the donor side open to an atmosphere (normal, desiccated or hydrated). Using this technique, they reported a 13-15-fold increase in the penetration of cortisone through hydrated skin compared with desiccated skin.

Although the skin can be desiccated to a certain extent using *in vitro* and *in vivo* techniques (94,186,284) they cannot prevent continuous outward diffusion of water from the dermal side of the stratum corneum (288). Thus, where such hydration is prevented, as in the present work with liquid paraffin both sides, a greater difference between desiccated and hydrated skin may be expected. This was found in the present work.

It has long been recognised that, materials penetrate better from vehicles which show poorer affinity for the penetrant because drug must release from the vehicle before it can penetrate the skin (10,102).

The effect of three vehicles on the penetration of alkanols can be seen in Table 3.5. The polar propanol penetrated more rapidly

Table 3.5 Permeability coefficeint (Kp) for Propanol and Hexanol from different vehicles, with water as receptor phase.

| | Permeability coefficient (Kp x $10^3$ cm $hr^{-1}$) from | | |
|---|---|---|---|
| | Water | 66.7% PG | LLP |
| Propanol | 0.71 ± 0.21 | 0.04 ± 0.01 | 6.1 ± 1.0 |
| Hexanol | 10.3 ± 2.8 | 1.63 ± 0.25 | 1.98 ± 0.59 |

from the non-polar light liquid paraffin, whilst the relatively non-polar hexanol penetrates more rapidly from distilled water. Both

alkanols gave poor penetration from 66.7% propylene glycol (PG). Blank (93,218) also found that polar alkanols penetrated more readily from non-polar vehicles, non-polar alkanols penetrated more readily from saline and the penetration of both polar and non-polar alkanols was significantly reduced from PEG600. It has been suggested that glycols form hydrogen bonds with penetrants, thus reducing their thermodynamic activity and penetration rate (102,103,213,214,221,222).

After thorough washing with water, stratum corneum used with the 66.7% propylene glycol gave a Kp for propanol of $1.10 \pm 0.27 \times 10^{-3}$ cm $hr^{-1}$ from water indicating that the glycol had no damaging effect on the stratum corneum. The effect of the glycol on the penetration of propanol and hexanol is similar to the effect of 70% DMSO on both alkanols (see later).

With dry stratum corneum and both sides of the diffusion cell containing LLP (Table 3.4), penetration of propanol was 6 times less than that of hexanol even though propanol should have a higher thermodynamic activity. An explanation for this may be found in the partition coefficient and the lack of adequately hydrated environment.

Propanol has a higher affinity for water than for LLP and therefore a low LLP/water partition coefficient. However when one considers the distribution of propanol between LLP and both hydrated and dry stratum corneum, one expects the hydrated stratum corneum/ LLP partition coefficient to be much higher than the dry stratum corneum / LLP partition coefficient because the propanol will be able to partition into the aqueous regions within the stratum corneum. Therefore, one would expect an increased penetration of propanol through hydrated skin because of the favourable partition into these hydrated regions. Other workers (11,99) have also suggested that polar substances penetrate via polar regions within the stratum

corneum and that non-polar materials pass via non-polar regions. In Table 3.4 and Figure 3.4, it can be seen that there is an 87-fold and 4.5-fold increase in the penetration of propanol and hexanol, respectively due to hydration taking place during the experiment. The difference between these changes is likely to be due to differences in the partition coefficients of the penetrants between LLP and dry and hydrated skin.

A simple statement which suggests that lipid-soluble substance penetrate faster than water-soluble ones has been criticised by Scheuplein (6,109) because it overlooks the effect of the vehicle. He pointed out that it is only correct when polar vehicles such as water is used, so that the skin/vehicle partition coefficient increases with increasing non-polarity of the penetrant.

In the present work, the results do not conflict with Scheuplein's view, but do emphasize the importance of skin hydration. When compared with propanol, using hydrated stratum corneum, hexanol was found to penetrate more rapidly from an aqueous vehicle and less readily from LLP. Similarly hexanol was found to penetrate faster than propanol from LLP providing stratum corneum was maintained dry throughout the experiment. Therefore it can be concluded that a lipid soluble substance may penetrate faster, slower or at a similar rate to a water-soluble substance depending on both the vehicle and the state of hydration of the stratum corneum.

## 2. Effect of DMSO and DMAC on Stratum Corneum Permeability

### i) DMSO

Table 3.6 shows the different permeability coefficients (Kp) of hexanol from water or 70% DMSO into water or DMSO. The maximum reduction was found when both sides contained 70% DMSO, whilst the presence of water as receptor gave a 70% increase in penetration,

Table 3.6 The effect of water and 70% DMSO on the penetration of hexanol through neonatal rat stratum corneum.

| Donor Phase | Receptor Phase | Kp x $10^3$ (cm $hr^{-1}$) |
|---|---|---|
| Water | Water | 10.3 ± 2.8 |
| 70% DMSO | 70% DMSO | 0.81 ± 0.18 |
| Water | 70% DMSO | 7.3 ± 0.5 |
| 70% DMSO | Water | 1.14 ± 0.23 |

perhaps due to dilution of the DMSO as water crossed the stratum corneum. Conversely, when water was used as the donor phase, the presence of DMSO in the receptor phase reduced the Kp. For any work looking at the effect of DMSO concentration on permeability, it is necessary to ensure that dilution does not occur. In this work, therefore, identical donor and receptor phases were used.

Table 3.7 and Figures 3.5 and 3.6, show the Kp data for propanol and hexanol in the presence of various concentrations of DMSO and, after its removal, in the presence of water. As DMSO

Table 3.7 The effect of various concentrations of DMSO on the penetration of propanol and hexanol in the presence of DMSO, and after its removal

| % DMSO (v/v) | Propanol Kp x $10^3$ (cm $hr^{-1}$) | | Hexanol Kp x $10^3$ (cm $hr^{-1}$) | |
|---|---|---|---|---|
| | 1st Run | 2nd Run | 1st Run | 2nd Run |
| 0 | 0.71 ± 0.21 | 0.88 ± 0.29 | 10.3 ± 2.8 | 10.4 ± 1.0 |
| 25 | 0.30 ± 0.07 | 0.56 ± 0.12 | 3.8 ± 2.5 | 9.60 ± 0.44 |
| 50 | 0.17 ± 0.03 | 0.60 ± 0.22 | 1.96 ± 0.96 | 16.1 ± 3.3 |
| 70 | 0.07 ± 0.02 | 0.57 ± 0.15 | 0.81 ± 0.18 | 16.8 ± 1.1 |
| 75 | 0.16 ± 0.02 | 0.63 ± 0.09 | 1.42 ± 0.11 | 14.4 ± 2.7 |
| 80 | 0.30 ± 0.06 | 0.80 ± 0.20 | 2.72 ± 0.40 | 19.2 ± 3.8 |
| 85 | 1.15 ± 0.35 | 2.13 ± 0.40 | 4.6 ± 1.0 | 21.4 ± 5.0 |
| 90 | 3.30 ± 0.9 | 8.5 ± 1.2 | 19.3 ± 4.5 | 62.2 ± 3.9 |

concentration increases, alkanol penetration decreases up to 70% DMSO, above which there is an increase in penetration with increase in DMSO

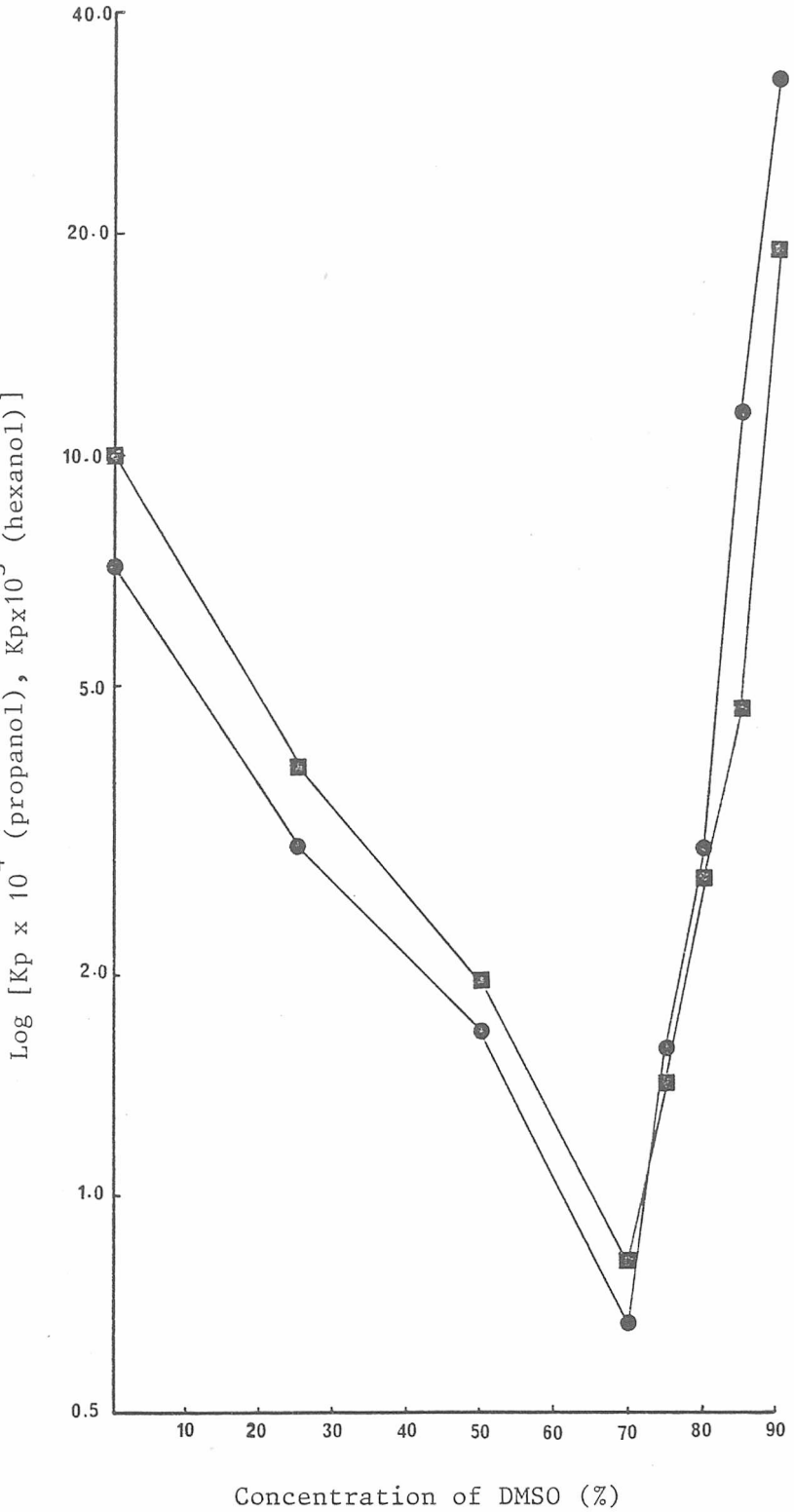



Figure 3.5 Graph of the effect of DMSO concentration on the log permeability coefficient (Kp) of propanol (●) and hexanol (■).

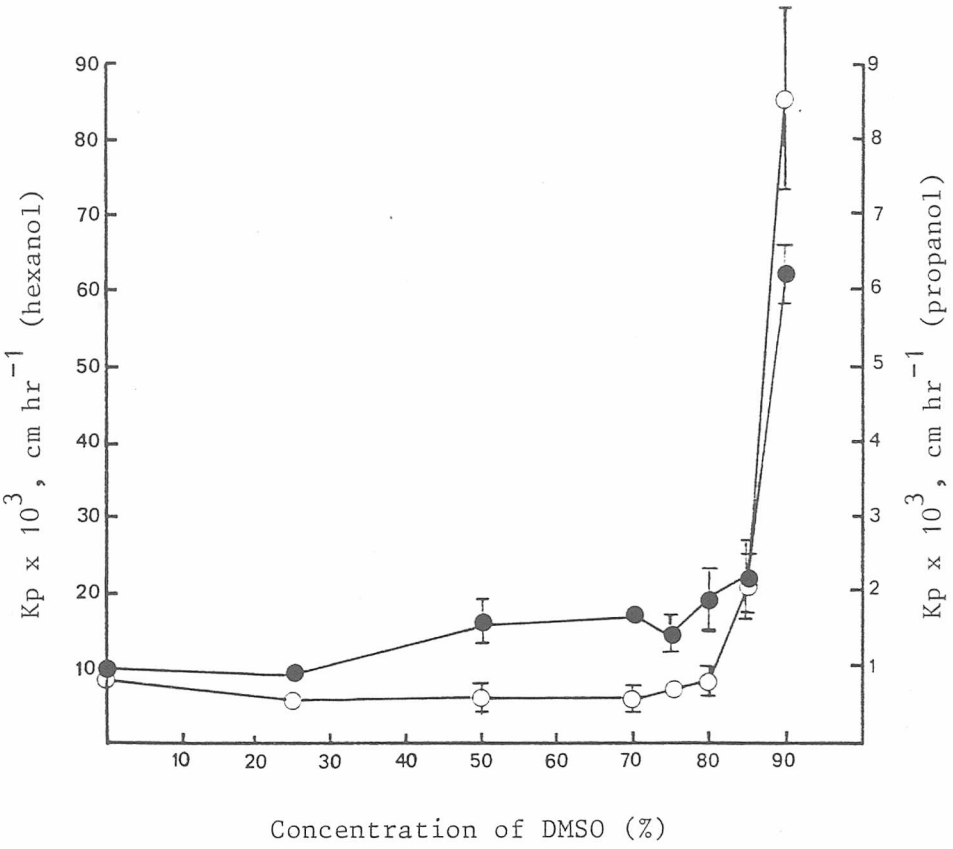



Figure 3.6 Permeability coefficient (Kp) of propanol (○) and hexanol (●), from water, after treatment of stratum corneum with various concentrations of DMSO for 6 hours.

concentration.

A. From 0 to 70% DMSO

From Table 3.7 it can be seen that the reduction of penetration (from 0-70% DMSO) is similar for both alkanols. For example the ratios of the Kp at 0% DMSO to that at 70% was found to be 10.1 and 12.7 for propanol and hexanol, respectively. This similarity suggests a similar mechanism for both reductions. Blank (personal communication) observed that the lipid-soluble alkanols penetrate more slowly from aqueous solutions of DMSO than from water and that DMSO is such a good solvent for these alkanols that they are not released to the skin, i.e., the partition coefficient strongly favours the solvent rather than the stratum corneum. The decrease in penetration rate of alkanols from aqueous solutions of DMSO (25-50%), as compared to their flux from water, has also been attributed to the effect of partition coefficient (224). The present work also suggests that the observed reduction in the penetration of alkanols is due to the higher affinity of alkanols for DMSO solution and hence a lower thermodynamic activity (102). This effect is not simply due to the increased solubility of alkanol in the vehicle because propanol is already miscible with water.

The strong binding of alkanols to DMSO was further investigated in the present work using infrared spectroscopy. The relevant portion of the spectra for systems involving hexanol and DMSO are shown in Figure 3.7.

Hexanol has a sharp absorption band at 3640 $cm^{-1}$ and a broad one at 3350 $cm^{-1}$ indicating free and bound hydroxyl groups, respectively (287). When DMSO was added there was a large reduction in the free hydroxyl band at 3640 $cm^{-1}$ which could be almost eliminated

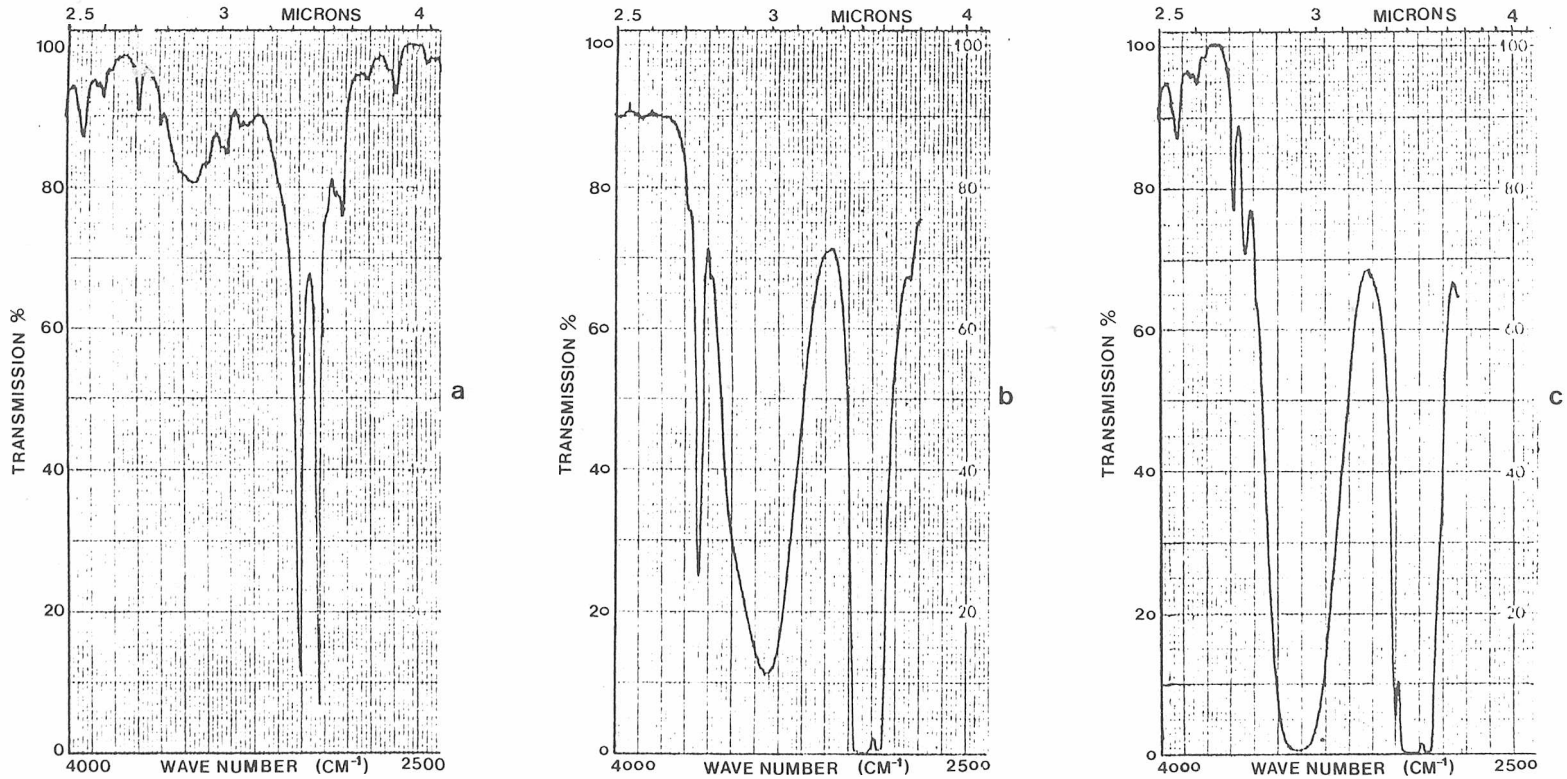



Figure 3.7 Infrared spectra of (a) DMSO, (b) hexanol, and (c) 1:2 (v/v) mixture of hexanol and DMSO, in carbon tetrachloride.

at 2:1 v/v DMSO - Hexanol. There was a corresponding increase in the size of the broad band at 3350 $cm^{-1}$ which also shifted to 3420 $cm^{-1}$. Similar results were found with propanol. These results suggest hydrogen-bonded complexation between the alkanol and DMSO molecules is taking place, as has been reported elsewhere (232). It is, therefore, reasonable to expect the alkanols to have higher affinity for solutions containing DMSO confirming the explanation for the reduction in Kp shown in Table 3.7 up to 70% DMSO on first run. Such a hydrogen-bonded complexation would cause a similar reduction in Kp irrespective of initial solubility.

On the second run, when water is the delivery phase, complete reversibility of the effect was observed. This indicates up to and including 70% DMSO there has been no residual effect on the stratum corneum (Fig. 3.6 and Table 3.7).

**B. From 75-90% DMSO**

Over this concentration range, the flux of alkanols increases with concentration (compared with flux from 70% DMSO). This suggests a reduction in the diffusional resistance of the stratum corneum barrier, although flux is not necessarily higher than from water because the solvent holds the alkanol (Blank, personal communication).

A similar observation and conclusion was reported by Roberts and Anderson (217) using phenol as penetrant. An approximate indication of the effect of DMSO on skin permeability may be obtained by comparing the Kp of an alkanol at a particular concentration of DMSO with the lowest Kp. For example the ratio of Kp at 90% DMSO to the lowest value (70%) shows a 47-fold and 23-fold increase in the penetration of propanol and hexanol, respectively. However, the

ratio assumes similar thermodynamic activity of the alkanols in both 70% and 90% DMSO. This is unlikely, but, because the thermodynamic activity at 90% is likely to be lower, the actual increase in skin permeability is greater than indicated by the ratio. It may be concluded that Figure 3.5 represents two opposed effects of DMSO, the drug-vehicle interaction discussed above and a skin-vehicle interaction which tends to decrease the diffusional resistance of the stratum corneum (78,217). To what extent these effects overlap each other can not be enumerated. Also, it has not been found possible to determine the concentration of DMSO which shows the beginning of increased skin permeability because of this overlap. This could be investigated further using both inert and biological membranes in the same manner as reported by Roberts and Anderson (217). However, results obtained with thermal analysis (page 55) of stratum corneum treated with 50% DMSO showed a reduction in a lipid endotherm, suggesting an interaction with stratum corneum lipids. It could be, therefore, that the increase in permeability could occur as low as 50% DMSO, but further evidence would be required for this.

The second run data (Table 3.7, Fig. 3.6) in which water was used both sidesof stratum corneum, showed no change in Kp up to pre-treatment with 80% DMSO. Thereafter a sharp increase in permeability was obtained. Thus the effect of DMSO was reversible below and irreversible above 80% DMSO.

Although the literature contains no report of work on alkanols covering such a range of concentration of DMSO, the results reported here are in general agreement with published works (60,82, 208,231,237,238). Sweeney et al. (238), using hairless mouse skin, investigated the effect of 30 min treatment with various concentrations of DMSO on the penetration of tritiated water. They observed

irreversible effects of DMSO at concentrations higher than 70%. Elfbaum and Laden (82,231) studied the effect of DMSO on the penetration of picrate ion and radiolabeled DMSO and reported increased skin permeability when concentrations higher than 60-70% DMSO were used, and an irreversible effect at about 80% DMSO.

Although the mode of action of DMSO is not fully understood (2), the literature contains a number of suggestions about its mechanism of action. These have been reviewed on page 84. In the present work the mechanism of action of DMSO will be discussed in detail in Chapter 6.

It has been suggested (81,224) that DMSO can increase skin hydration, causing swelling of stratum corneum and therefore increasing skin permeability. In the present work, this effect has been examined by comparing the penetration of hexanol through stratum corneum after 6 hr treatment with 90% DMSO (Table 3.7, second run), with stratum corneum treated identically but dried under room conditions to dehydrate it prior to permeability measurement. The latter treatment gave a Kp of $69.5 \pm 6.4 \times 10^{-3}$ cm $hr^{-1}$ which is similar to the former treatment ($62.2 \pm 3.9 \times 10^{-3}$ cm $hr^{-1}$). This result strongly indicates that the irreversible effect of 90% DMSO is not due to induced hydration or swelling of stratum corneum. The permanent effect of 90% DMSO reported here, disagrees with Kligman (60) who claimed that 90 and 100% DMSO have a reversible effect on skin permeability. A permanent effect of 100% DMSO has also been observed in the present work.

Stratum corneum treated for 24 hours with DMSO produced Kp values of $89.5 \pm 8.9 \times 10^{-3}$ cm $hr^{-1}$ and $194.5 \pm 18.5 \times 10^{-3}$ cm $hr^{-1}$ for propanol and hexanol, respectively. Thus the polar propanol was more dramatically affected by such treatment than the non-polar

hexanol. These Kp values are significantly lower than those obtained following treatment with C/M 2:1 (Kp propanol 258.4 ± 36.0 x $10^{-3}$ cm $hr^{-1}$, hexanol 404.6 ± 13.7 x $10^{-3}$ cm $hr^{-1}$). Thus, although DMSO can extract lipids (62,77-80,82,105,106,208), it is not as efficient in this respect as C/M 2:1 (105). This was also observed using thermal analysis (page 53), where stratum corneum extracted with C/M 2:1 did not show lipid endotherms, whilst that treated with DMSO showed a reduction in size but not complete removal of lipid endotherms. Dried extracts of both C/M 2:1 and DMSO showed lipid endotherms.

Table 3.8 shows the effect on penetration of propanol of having 100% DMSO in the receptor phase only and in both phases.

Table 3.8 The effect of a concentration gradient of DMSO on the penetration of Propanol through Neonatal Rat Stratum Corneum.

| Experiment* | Donor Phase | Receptor Phase | Permeability Coefficient Kp x $10^{3}$ (cm $hr^{-1}$) |
|---|---|---|---|
| First run | 100% DMSO | 100% DMSO | 91.4 ± 14.0 |
| Second run | Water | Water | 69.0 ± 8.0 |
| First run | Water | 100% DMSO | 28.4 ± 4.3 |
| Second run | Water | Water | 13.4 ± 4.6 |

* Stratum corneum washed with water for 16 hours between first and second run.

The presence of DMSO on both sides of the stratum corneum had a greater effect on skin permeability than when it was present in the receptor phase only. The former effect was similar to that observed with 24 hours pre-treatment with 100% DMSO. This suggests that when DMSO was placed on both sides of the stratum corneum, more lipid was extracted. The present work is in good agreement with Allenby et al. (78) who observed maximum effect of DMSO when placed on both sides of skin. However, it should be noted that, when DMSO was in

contact with one side of stratum corneum, water from the other side may penetrate the stratum corneum, dilute the DMSO and hence reduce its effect (237).

Elfbaum and Laden (231) studied the effect of 80% DMSO when placed on one side (the other side being buffer solution) and on both sides of skin. In both cases similar penetration enhancement of picrate ion was observed. Chandrasekaran et al. (240), however, reported greater increase in the penetration of Scopolamine when there was a concentration gradient of DMSO across the skin than when 80% DMSO was on both sides of skin. They explained the effect as being due to the high osmotic stresses produced within the stratum corneum so that as both water and DMSO were transported into the stratum corneum they caused swelling, distortion and intercellular delamination, resulting in a marked decrease in resistance to the transport of scopolamine.

This phenomenon is interesting, but was not observed by either Elfbaum and Laden (231) nor in this work (Table 3.8). It could be that the effect of a concentration gradient would be overshadowed at 100% DMSO by the lipid solvent effect. In Table 3.8, the first run data suggest that, although the affinity of propanol for DMSO should be higher than for water (and so have reduced thermodynamic activity) (102), the damage to the stratum corneum barrier has greatly increased its diffusivity to propanol. Thus the effect of the skin-vehicle interaction has dominated the effect of drug-vehicle interaction. Table 3.8 also shows that, after removal of the DMSO, there was a partial reversibility. This reversibility was less marked when DMSO was in both phases than when it was only in the receptor phase, supporting the view that lipid extraction was involved. This is in good agreement with other workers who observed

partial reversibility of 100% DMSO (106,236,237). This permanent effect of 100% DMSO (238) has been attributed to extraction of stratum corneum lipids (77-80,105,106). However, the observed partial reversibility does suggest that the entire effect of DMSO is more complex than the simple removal of some lipids (224).

ii) DMAC

The results of penetration study of hexanol using various concentrations of DMAC are presented in Table 3.9 and Figure 3.8.

Table 3.9 The effect of various concentrations of DMAC on the penetration of hexanol.

| % DMAC (v/v) | Kp x $10^3$ (cm $hr^{-1}$) | |
|---|---|---|
| | First Run | Second Run |
| 0 | 10.3 ± 2.8 | 10.4 ± 1.0 |
| 25 | 2.6 ± 0.9 | 10.9 ± 2.5 |
| 50 | 0.96 ± 0.32 | 11.7 ± 2.6 |
| 70 | 1.66 ± 0.42 | 8.8 ± 1.5 |
| 80 | 0.89 ± 0.16 | 11.4 ± 1.2 |
| 85 | 2.65 ± 0.43 | 33.4 ± 4.9 |
| 90 | 1.38 ± 0.68 | 66.0 ± 11.5 |
| 95 | 0.91 ± 0.43 | 90.4 ± 22.9 |

As with DMSO, penetration of hexanol is decreased as concentration of DMAC is increased. However this effect continues up to 95% DMAC, showing no increase in permeability at higher concentration. The second run, using water as delivery phase shows a similar effect to DMSO, the integrity of the skin barrier persisting up to 80% DMAC, above which there was an irreversible increase in skin permeability. The residual effects of 90% DMSO and DMAC on the penetration of hexanol were found to be similar. Thus, whilst the first run gave no indication of alteration to the skin barrier, the second run

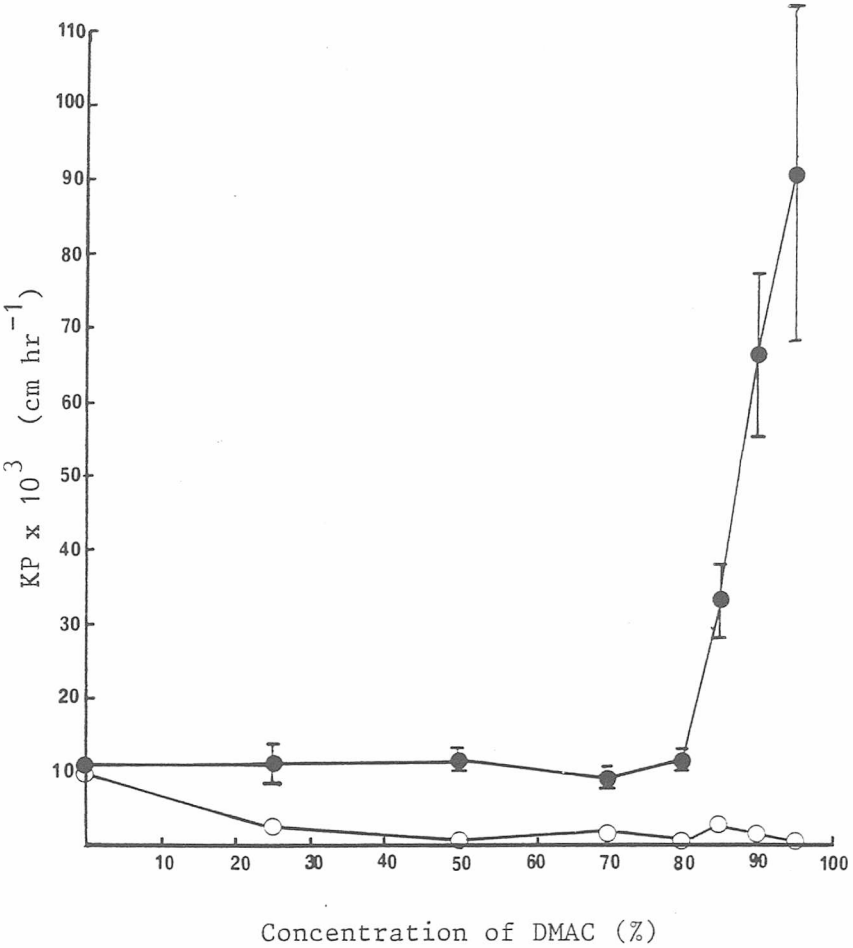



Figure 3.8 Plots of permeability coefficients of hexanol in the presence of DMAC (○), and from water following treatment with DMAC (●), against concentration.

indicates that it had occurred. It is suggested that the binding of hexanol to DMAC is stronger than to DMSO, thereby masking its enhancing effect. Therefore it is difficult to compare the enhancing effect of DMSO and DMAC on the penetration of hexanol in their presence. It has been reported that DMAC is less effective than DMSO (129,236,243). This was observed in this work using thermal analysis which showed a less effective reduction in the melting temperatures of stratum corneum lipids (see Chapter 2). This apparent discrepancy between the thermal analytical and permeability data has not been further investigated.

**3. Effect of Heat Treatment on Skin Permeability**

Results for the effect of heat treatment on permeability of stratum corneum to hexanol are shown in Table 3.10 and Figure 3.9. The latter shows three distinct regions

(i) below 70°C where there is very little increase in permeability over 12 hours heat treatment (1.9-fold at 60°C, 2.7-fold at 70°C).

(ii) at 75°C where there is a continuing increase with length of heat treatment and

(iii) above 80°C where there is a rapid increase followed by a levelling off with time.

Full thickness human (207) and hairless mouse (190,205,206) skin were found to give increased permeability when heated at 60°C for 30-60 seconds but no further increase when exposed up to 16 mins. Because of this agreement with the present work, it appears that heat treatment at 60°C produces similar effects on human, hairless mouse and neonatal rat skin.

In this work, stratum corneum exposed to 70°C showed permeability increased more than skin treated at 60°C and much lower than skin heated at 80°C. These observation are in agreement with the

Table 3.10 Permeability coefficients, Kp x $10^3$ (cm $hr^{-1}$) for hexanol, obtained using stratum corneum pre-heated at different temperatures for various periods of time.

| Temperature* (°C) | Time of Exposure (hr) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 1.5 | 2 | 3 | 4 | 5 | 6 | 8 | 12 |
| 40 | | | | 16.6 ± 2.7 | | 16.5 ± 2.4 | | 16.1 ± 0.6 | 15.4 ± 2.0 | 15.5 ± 1.7 |
| 60 | | | | 18.7 ± 2.0 | | 11.5 ± 1.8 | | 21.5 ± 3.0 | 23.9 ± 1.8 | 23.2 ± 2.5 |
| 70 | | | | 28.1 ± 3.8 | | 33.4 ± 2.0 | | 28.6 ± 4.6 | | 20.6 ± 0.9 |
| 75 | | 39.7 ± 10.0 | | 49.5 ± 18.0 | | 63.8 ± 7.0 | | 85.2 ± 14.2 | 78.6 ± 7.7 | 89.4 ± 12.8 |
| 80 | 34.7 ± 1.6 | | | 113.5 ± 10.4 | | 121.4 ± 9.3 | | 136.2 ± 12.1 | 138.8 ± 5.5 | 152.6 ± 4.7 |
| 85 | | 99.0 ± 18.8 | | 132.1 ± 7.0 | 136.2 ± 3.5 | | 140.4 ± 8.5 | 157.9 ± 13.5 | 155.1 ± 3.0 | 148.8 ± 4.5 |
| 90 | 65.7 ± 5.8 | 94.2 ± 11.6 | 106.2 ± 13.9 | 127.1 ± 21.0 | 131.0 ± 12.6 | 132.4 ± 11.5 | | 148.6 ± 12.5 | | 152.2 ± 24.0 |

* Control stratum corneum (22°C ± 2°C), Kp = 10.3 ± 2.8 x $10^{-3}$, cm $hr^{-1}$.

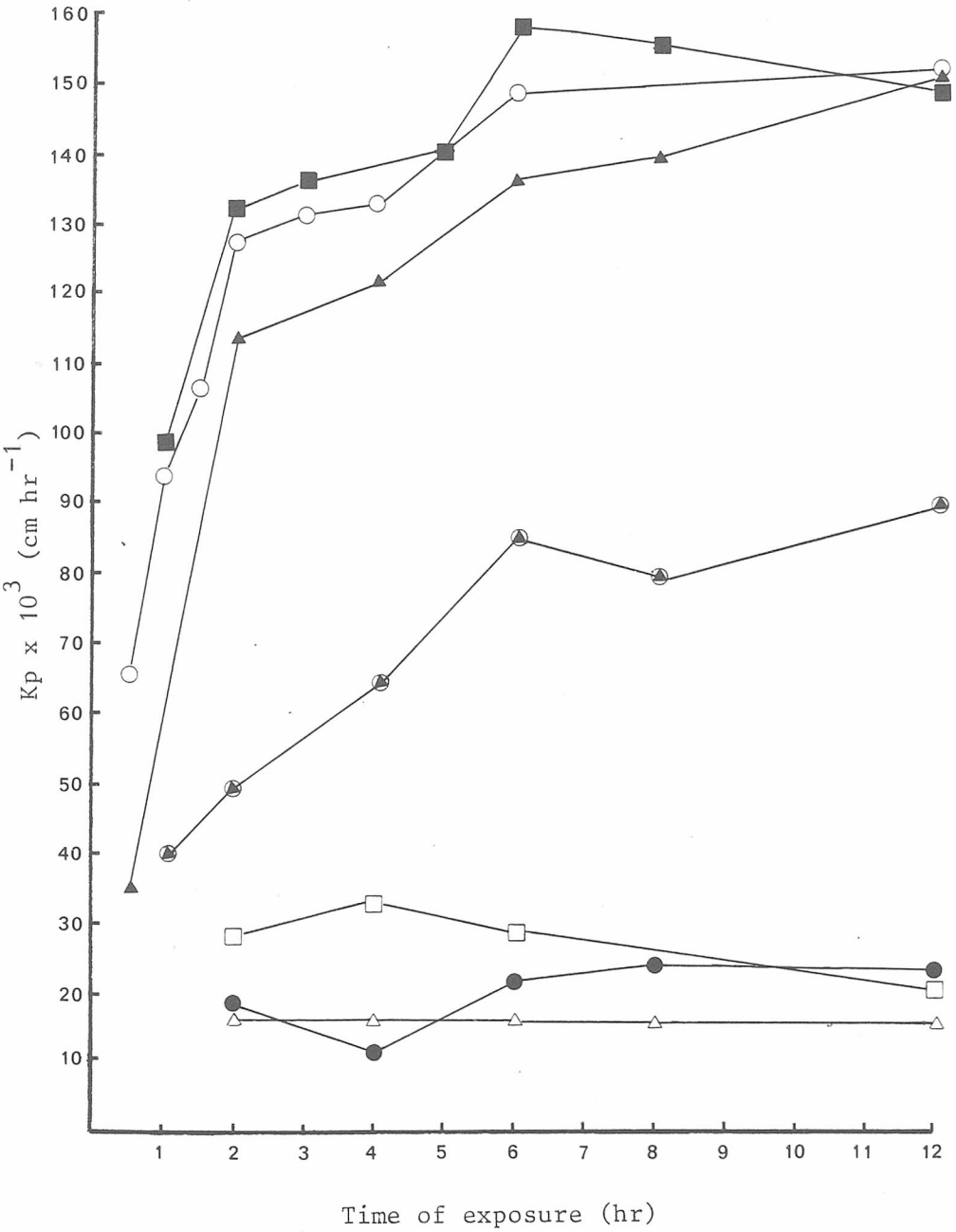



Figure 3.9 Plots of permeability coefficients (Kp) of hexanol as a function of time of exposure to elevated temperatures ( △ 40°, ● 60°, □ 70°, ◭ 75°, ▲ 80°, ■ 85°, ○ 90°C).

work of Behl and his co-workers (209-211) who reported that treatment of full-thickness, hairless mouse skin at 70°C produced a marginal effect on the permeability of water, phenol and n-alkanols, whilst heating above 80°C produced an exaggerated increase in skin permeability even after 1 minute. The early work of Allenby et al. (208) showed that one hour incubation of full thickness (epidermis and dermis) human skin in saline at 71.3°C and above, produced an irreversible increase in skin permeability as monitored by electrical impendence. Although Behl and his co-workers investigated the effect of temperatures up to 98°C (using a scalding technique) and up to 250°C (using a branding method), they exposed the skin to these temperatures for only 1 minute.

In the present work, the effect of both temperature and duration of heat exposure on stratum corneum permeability were investigated. Figure 3.9 shows that heat treatment at 80°C and above produced a large increase in skin permeability after 2 hours exposure and little further change when heated for up to 12 hours. This suggests that major damage to the permeability barrier occurred within 2 hours at these temperatures. Heat treatment at 75°C produced an approximately linear increase in skin permeability throughout 12 hours exposure. It seems likely that if exposure time was increased beyond 12 hours, a higher permeability might occur and eventually produce a maximum effect comparable with that obtained by heating at 80°, 85° or 90°C.

Figure 3.10 shows that there is an approximately linear increase in permeability with time of exposure for stratum corneum at 90°C and 75°C. This relationship suggests that the rate of increase in permeability i.e., rate of impairment of skin barrier, follows zero-order kinetics. The slope at 90°C was 8 times that at

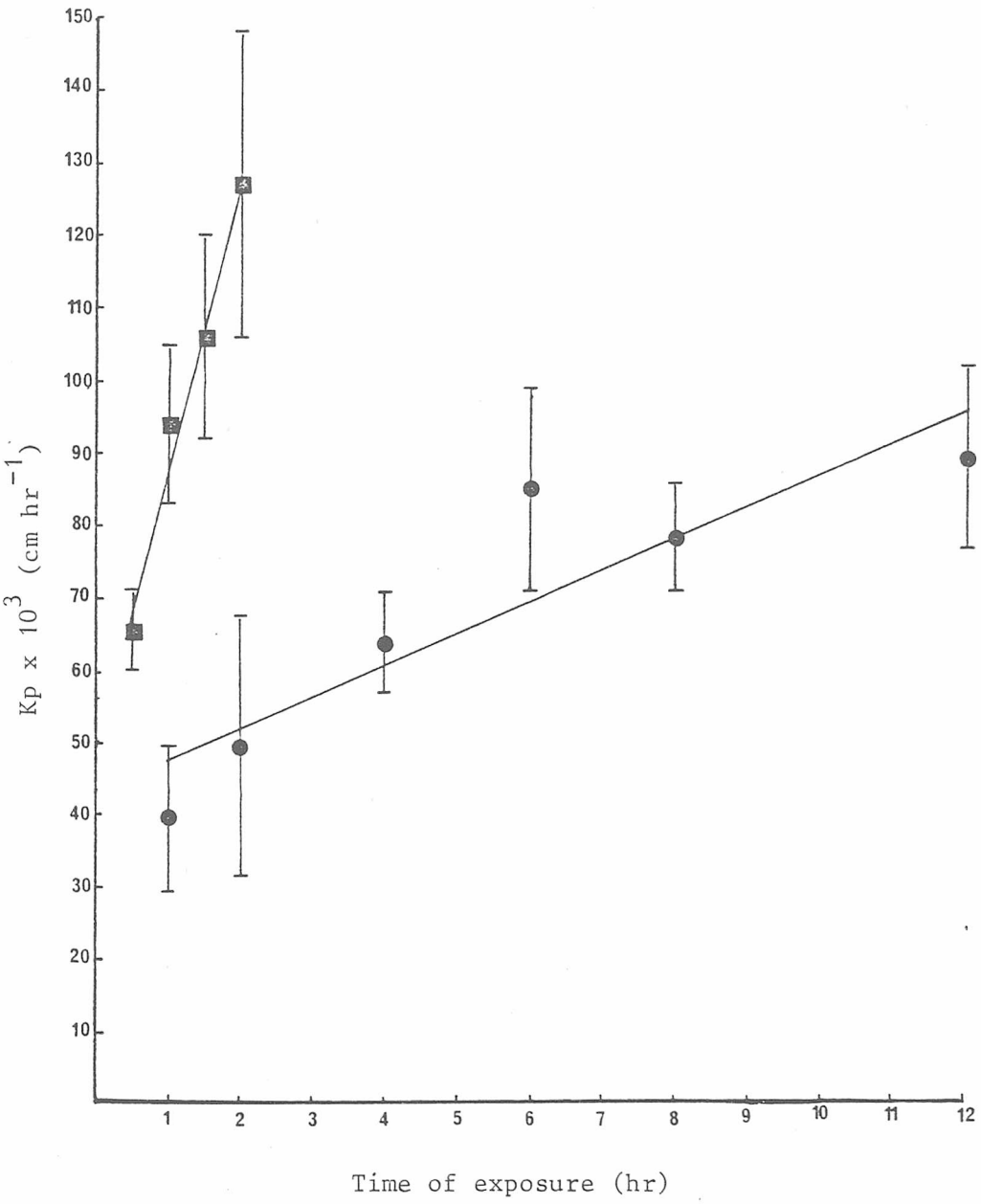



Figure 3.10 Plots pf permeability coefficients (Kp) of hexanol against time of exposure of stratum corneum at 75°C (●) and 90°C (■).

75°C showing that the rate of destruction of the barrier is dependent on both time and temperature of exposure.

These effects of heat exposure were only found at temperatures higher than 70°C. The mechanism of these heat effects has not been elucidated, although Behl et al. (211), without experimental evidence, have suggested that it involves protein denaturation.

Table 3.11 The effect of 6 hour heat treatment of stratum corneum on the penetration of propanol and hexanol.

| Temperature (°C) | Propanol Kp x $10^3$ (cm $hr^{-1}$) | Hexanol Kp x $10^3$ (cm $hr^{-1}$) |
|---|---|---|
| No heating | 0.71 ± 0.21 | 10.3 ± 2.8 |
| 40 | 0.68 ± 0.20 | 16.1 ± 0.6 |
| 60 | 0.61 ± 0.15 | 21.5 ± 3.0 |
| 70 | 1.00 ± 0.45 | 28.6 ± 4.6 |
| 75 | 7.7 ± 0.6 | 85.2 ± 14.2 |
| 80 | 12.0 ± 1.8 | 147.2 ± 11.3 |
| 85 | 11.7 ± 3.0 | 157.9 ± 13.5 |

Table 3.11 shows the permeability coefficients of stratum corneum heated for 6 hours, and stored at room temperature for 16 hours prior to permeability studies. The data is also shown in Figure 3.11.

Between 40° and 70°C, propanol shows no significant increase in permeability, whilst hexanol gives a small but steady increase. This difference between polar and non-polar alkanols is not understood. Behl et al. (190,205,206) have also observed that non-polar alkanols were more affected than polar alkanols, when stratum corneum was heated at 60°C. They concluded that the effect of heat treatment at 60°C is due to enhanced or induced hydration. However, in the present work, no water was available when the dry stratum corneum was heated at 60° or 70°C, indicating that the increase in penetration of hexanol

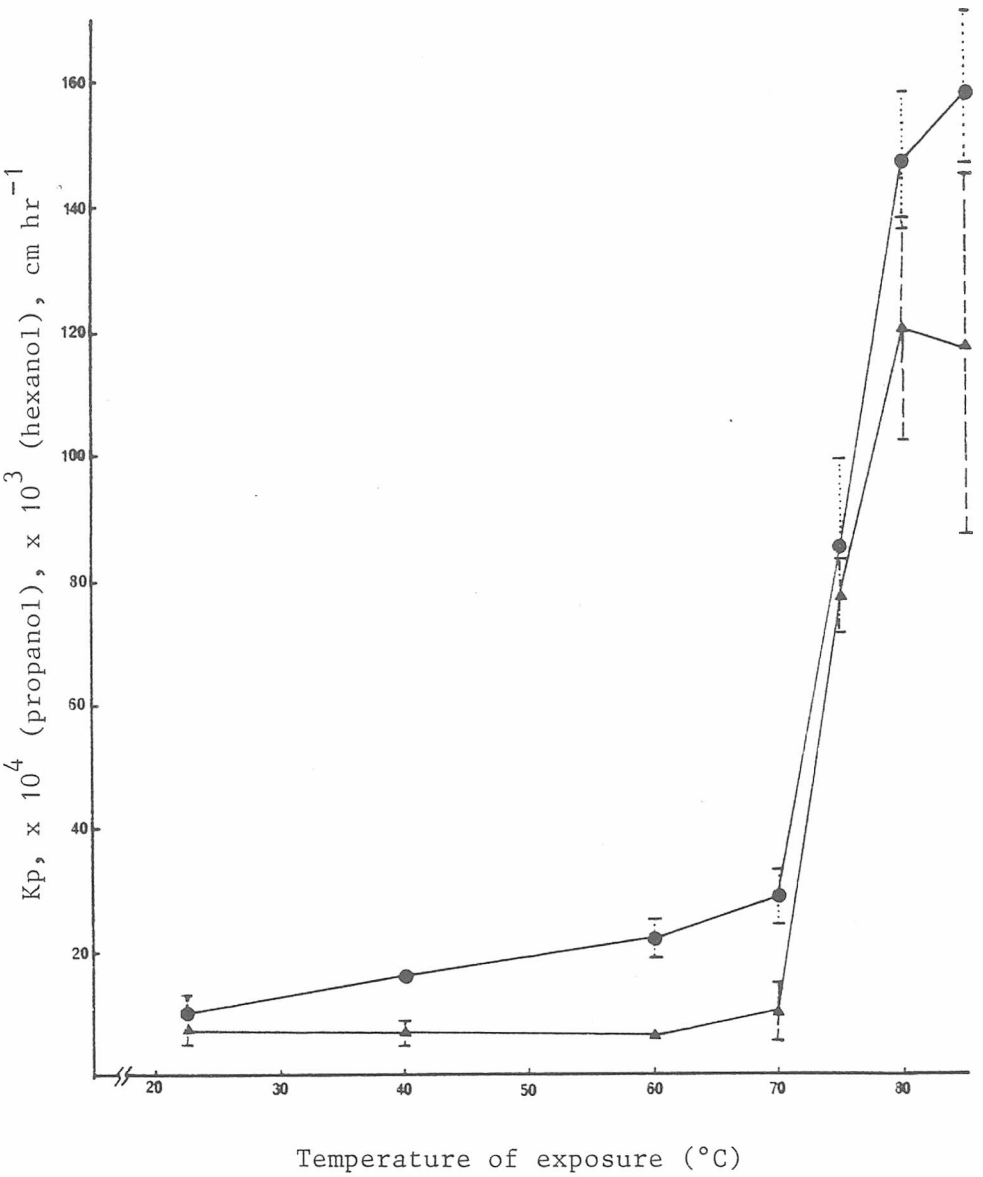



Figure 3.11 Plots of permeability coefficients (Kp) of propanol (▲) and hexanol (●) against the temperature of exposure of stratum corneum for 6 hours.

is due to a direct effect of heat on the stratum corneum and not hydration.

With both propanol and hexanol, there is a dramatic increase in skin permeability between 70° and 80°C, above which a plateau is reached. This suggests that maximum effect on skin permeability was reached in stratum corneum heated for 6 hours at 80°C. Comparison of permeability coefficients in normal skin with those in heat treated skin show that there is a 16-fold and 14-fold increase for propanol and hexanol (average of 80° to 90°C), respectively (Tables 3.10, 3.11). This observation suggests that, although the skin barrier has been damaged, the partition coefficient is still an important factor in penetration, there was no complete elimination of the barrier and that the increase in skin permeability was due to changes in diffusivity of the stratum corneum. It follows from this similarity in increase in permeability that, regardless of the polarity of the alkanol, the thermally damaged skin displays a similar increase in permeability. This is not in agreement with Behl and co-workers (209-211) who found that polar alkanol (and water) penetration was affected much more than non-polar alkanols when skin was heated at 80°C and higher. In the branding method, when skin was heated for 60 secs. at 200°C, the increase in the penetration of ethanol and butanol over hexanol was 8 and 5 times, respectively (210). With the scalding method (98°C for 60 secs) the comparable figures where 4 and 3 times (209). With the more non-polar octanol, neither the scalding nor branding techniques showed any significant increase in penetration, although 60°C did produce a significant increase in octanol penetration. Also when skin had been heated to 150°C the penetration of methanol was about 7 times that of phenol (211).

It is difficult to compare the present work with that of Behl

and co-workers because of the difference in species, their use of full skin (epidermis and dermis) which on heating may produce severe heat injuries, protein denaturation and cellular necrosis (27). It may also have been difficult for them to check for the intactness of the stratum corneum in the brittle skin following heat treatment. However, their work, which showed that a maximum increase in skin permeability can be reached in 1 minute, would indicate that scalding and branding techniques are far more drastic that the hot air treatment used in the present work.

From the observations reported here, it may be concluded that there is a dramatic change in the stratum corneum barrier between 70° and 80°C. The nature of this change must be of interest.

An approach to understanding the nature of these changes was made by investigating the effect of heat treatment using thermal analysis.

As discussed in Chapter 2, neonatal rat stratum corneum showed three lipid transitions at 42°, 71° and 80°C. It was found that the lipid transition at 80°C does not show any alteration or change in its temperature when stratum corneum was heated at 70°C for between 1 and 12 hours, although T71 was reduced by 1-2°C. This may explain the marginal increase in permeability when skin was heated at 70°C. However, when stratum corneum was heated to 80°C, even for only 60 secs. the T80 transition was reduced to 77.5°C. As the period of exposure increased, the transition temperature decreased, (77.5°→75.5°→74°...to 70°C) until it completely disappeared after 45 to 60 minutes exposure at this temperatures (see Table 2.10).

Complete elimination of T80 was also observed when stratum corneum was heated for 5 mins at 100°C or 15 mins at 90°C. The T71 transition was reduced by 1-2°C by these treatments. The 42°C

transition was also reduced to 39-40°C. Thus the elimination of the T80 transition was also found to be time and temperature related.

However, this effect was only found when stratum corneum was folded in the aluminium DTA crucibles. Unfolded skin in an oven at 50% R.H. required longer (1-2 hours at 90°C, 4-6 hours at 80°C) to produce the same effect. This did not arise from variation in the particular stratum corneum used, because a single skin was divided, half folded into a crucible, half flat, and both heated simultaneously in the DTA furnace at 80°C for one hour. Subsequent thermal analysis showed that, in the unfolded stratum corneum the lipid transition had moved from 80°C to 76°C, but the 80°C transition was completely eliminated from the folded stratum corneum. These differences may explain, to a certain extent, the more drastic effect of branding methods (210) compared with the present heat treatment. Effect of heat on folded and unfolded skin suggests that identical conditions of heating are necessary before there can be a reliable interpretation of the results following heat treatment for both thermal analysis and permeability study.

When stratum corneum (folded) was heated in the DTA at 75°C for 1 or 2 hours, there was no complete elimination of T80, but three lipid transitions were observed at 70°C, at 75-76° and at 80-81°C. This suggests that some, but not all the lipid responsible for T80, has produced an intermediate endotherm. If it is a solution process, it is apparently slow and requires a longer time to complete the disappearance of both the T80 and T75 transitions than heating at 80°C. This slow change explains why the permeability of skin treated at 75°C for 1 to 12 hours continues to increase and has not reached a maximum after 12 hours (Figs. 3.9, 3.10).

From these observations, it appears that the increase in skin

permeability when stratum corneum was exposed to temperatures above 70°C is due to changes in the normal lipid structures of the stratum corneum. As these take place a eutectic mixture of lower melting temperature and viscosity is produced. This disordering in lipid structure may increase the diffusivity of materials through the stratum corneum, suggesting that the normal lipid structure forms a significant part of the skin barrier (250). There is no evidence, however, from the present work to suggest that protein denaturation was responsible for increased skin permeability as has been suggested by Behl et al. (211).

When stratum corneum, which had been heated at 80°C for 6 hours was extracted overnight with C/M 2:1, the permeability coefficient for propanol was found to be 326.0 ± 13.3. x $10^{-3}$ cm $hr^{-1}$ and for hexanol 378.7 ± 11.6 x $10^{-3}$ cm $hr^{-1}$. Thus lipid extraction increased the penetration of propanol about 27 times over that of thermally damaged skin, but only 2.5 times for hexanol, producing similar penetration rates for propanol and hexanol in the absence of lipid (105). It follows that even the thermally damaged stratum corneum was still acting as a strong barrier, especially to the polar propanol.

Unheated stratum corneum, which had been extracted overnight with C/M 2:1 gave a Kp of 258.4 ± 36.0 x $10^{-3}$ cm $hr^{-1}$ for propanol and 404.6 ± 13.7 x $10^{-3}$ cm $hr^{-1}$ for hexanol. These results indicate that heat treatment prior to extraction with C/M solvent did not produce an additive effect. Thus it may be suggested that both heat and solvent treatment affected the same structures within stratum corneum.

The effect of two treatments (heat plus DMSO vapour) on the permeability of stratum corneum was also investigated.

Table 3.12 gives details of the permeability coefficients

of propanol and hexanol in stratum corneum which had been heated for 6 hours at various temperatures and exposed to DMSO vapour for 16 hours. The same data is shown in Figures 3.12 and 3.13 together with the Kp data in the absence of DMSO.

Table 3.12 The permeability coefficients of propanol and hexanol using stratum corneum preheated for 6 hours at various temperatures and exposed to DMSO vapour for 16 hours.

| Temperature °C | Propanol Kp x $10^3$ (cm $hr^{-1}$) | Ratio* | Hexanol Kp x $10^3$ (cm $hr^{-1}$) | Ratio* |
|---|---|---|---|---|
| No heating | 3.72 ± 0.37 | 5.2 | 50.3 ± 8.8 | 4.9 |
| 40 | 3.9 ± 0.6 | 5.7 | 48.6 ± 1.8 | 3.0 |
| 60 | 3.32 ± 0.47 | 5.4 | 70.3 ± 4.6 | 3.2 |
| 70 | 4.1 ± 0.8 | 4.1 | 88.9 ± 2.7 | 3.1 |
| 75 | 9.4 ± 0.8 | 1.2 | 130.7 ± 10.9 | 1.5 |
| 80 | 12.7 ± 0.8 | 1.0 | 146.8 ± 16.5 | 1.0 |
| 85 | 14.2 ± 1.0 | 1.2 | 147.9 ± 16.6 | 0.9 |

* Ratio of Kp with DMSO to Kp without DMSO (calculated from Tables 3.11 and 3.12).

Table 3.12 shows that both propanol and hexanol gave about 5-fold increase in their penetration when the unheated stratum corneum was exposed to DMSO vapour. With identical treatment, DTA of stratum corneum revealed lipid melting endotherms at lower temperatures (see page 56). This would suggest an interaction took place between DMSO and stratum corneum lipids which may explain the observed increase in skin permeability. However, both the donor and receptor phase contains water, therefore, penetration of water during the experiment will dilute DMSO within the stratum corneum. On prolonged exposure, DMSO concentration will be equalized to a very low level throughout the experimental system. Accordingly, the effect of DMSO on stratum corneum lipids may be lost and therefore, the observed change in skin permeability may be due to a permanent

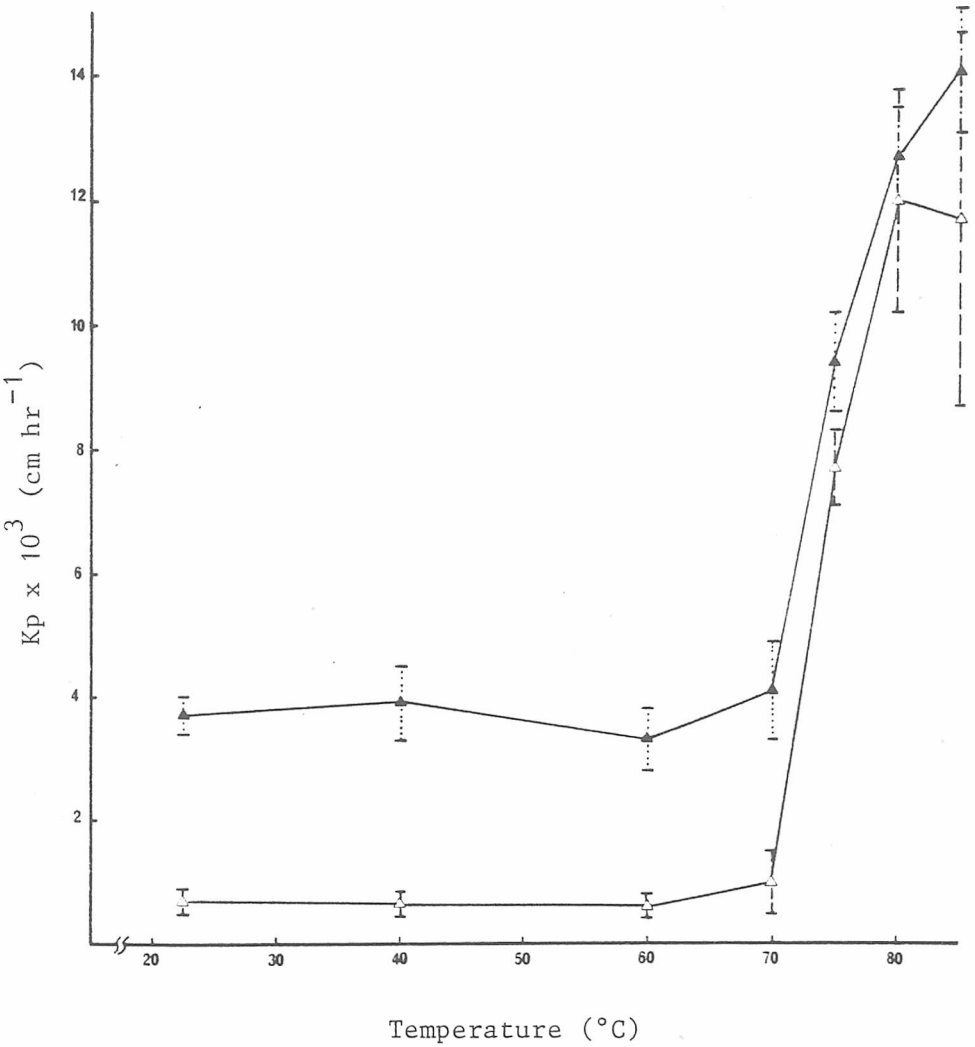



Figure 3.12 Plots of permeability coefficients (Kp) of propanol as a function of the temperature of exposure of stratum corneum to heat for 6 hours, (△) heat treatment alone, (▲) heat treatment plus exposure to DMSO vapour.

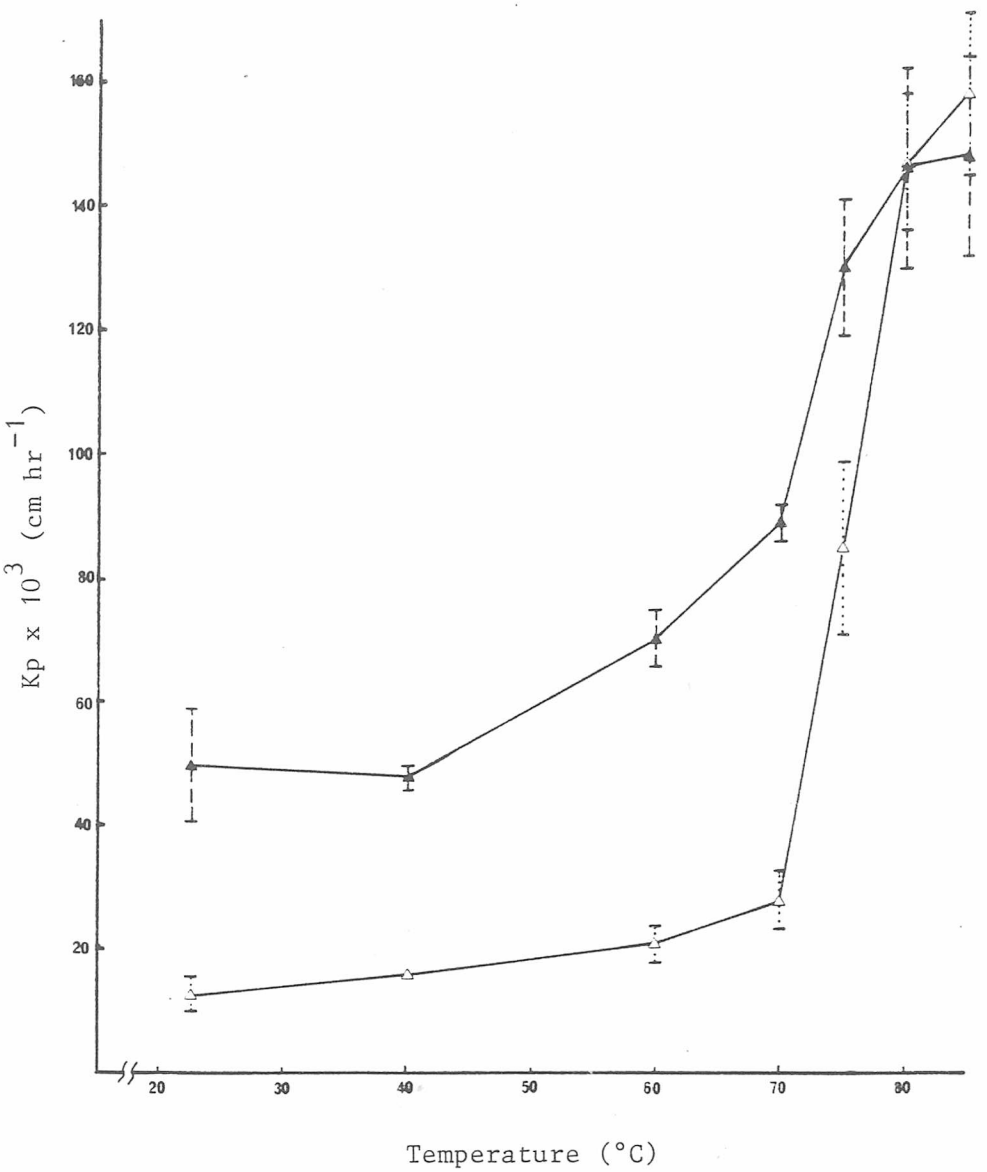



Figure 3.13 Plots of permeability coefficient (Kp) of hexanol as a function of the temperature of exposure of stratum corneum to heat for 6 hours, (△) heat treamtent alone, (▲) heat treatment plus exposure to DMSO vapour.

alteration in the barrier structure. Evidence to support this explanation was obtained by performing a second run of the same skin after overnight rinsing with water. The latter treatment gave a similar Kp for hexanol ($40.2 \pm 7.4 \times 10^{-3}$ cm $hr^{-1}$) to that obtained from the first run ($50.3 \pm 8.8 \times 10^{-3}$ cm $hr^{-1}$). It therefore seems probable that the DMSO had caused these irreversible changes prior to the first permeability determination. It is important to record that samples which had been treated with DMSO vapour and then exposed to room conditions for about two weeks prior to permeability study produced similar results to normal untreated samples (Kp of hexanol $13.0 \pm 3.9 \times 10^{-3}$ cm $hr^{-1}$).

Although results from Thermal Analysis (see page 58) suggest that the effect of DMSO vapour on lipid endotherms can be reversed, either by rinsing with water or prolonged evaporation of DMSO, permeability studies suggest that the reversible effect of DMSO vapour is only obtained when removal of DMSO with water was avoided. Removal of DMSO with water showed an irreversible effect on permeability indicating a permanent disruption of the stratum corneum barrier. Further explanation of this effect is beyond the scope of the present work. Scheuplein and Ross (106) have reported that the permanent increase in skin permeability seen after removal of DMSO with water can be reduced by removing DMSO with butanol prior to rinsing with water. Chandrasekaran et al. (240) also suggested that presence of a concentration gradient of DMSO produces high osmotic stresses within stratum corneum which may lead to swelling, distortion, and intercellular delamination of stratum corneum.

Exposure to DMSO vapour has further increased the permeability of preheated stratum corneum to both propanol and hexanol. This increase in penetration was almost constant up to heat treatment at

70°C, that is where there was no barrier damage. However between 70° and 80°C the difference between the permeability of heated and heat plus DMSO vapour treated stratum corneum was eliminated, so that at 80° and 85°C, DMSO vapour was observed to have no further effect on permeability. This strongly indicates that both heat treatment and DMSO were acting on the same or similar targets within the stratum corneum, namely the lipids.

It cannot, however, be concluded that both heat and DMSO have the same mechanism, because there is a difference in the Thermal Analysis results. Heat eliminates, progressively, the T80 transition, whilst DMSO vapour lowers the temperature of both the T71 and T80 transition to 59° and 65°C, respectively. The effect of heat is irreversible, that of DMSO vapour is reversible. A possible explanation for this difference has been suggested earlier. The concentration of DMSO within the stratum corneum, from the vapour, is sufficient to interact with the lipids, but is insufficient to cause extraction. The presence of these low concentrations of DMSO will increase the diffusivity of molecules by causing a permanent disorder of the lipid structure in the presence of water. However, where these structures have already been destroyed by heat treatment, no further effect is possible, because the concentration is not high enough to cause any extraction.

This explanation is also in agreement with the results obtained using aqueous solutions of DMSO up to 90% DMSO, which suggested an alteration in the lipid structure rather than extraction which only occurred with 100% DMSO.

### 4. Effect of Acidic and Alkaline Solutions

The effect of the pH of the vehicle on the rate of absorption

of acidic and basic drugs has been discussed by Higuchi (102) and its effect studied for a number of drugs (17,23,24,181,250).

The results shown in Table 3.13 indicate that strongly acidic

Table 3.13 The Effect of Hydrochloric Acid and Sodium Hydroxide on the Permeability of Neonatal Rat Stratum Corneum to Hexanol

| Experimental Status* | Donor Phase | Receptor Phase | Permeability Coefficient Kp x $10^3$ (cm $hr^{-1}$) |
|---|---|---|---|
| First Run | 0.1M HCl | 0.1M HCl | 16.2 ± 3.6 |
| Second Run | Water | Water | 15.1 ± 2.5 |
| First Run | 0.1M Na OH | 0.1M Na OH | 49.3 ± 0.2 |
| Second Run** | | | |

* Stratum corneum rinsed in water for 16 hours before second run.

** Stratum corneum ruptures.

solution produced only 50% increase in the penetration of hexanol, whilst the strongly alkaline solution produced a 4-fold increase, suggesting that the alkali damaged the barrier more than the acid.

These findings are in agreement with Matoltsy et al. (70) who investigated the effect of pH on the permeability of hairless mouse stratum corneum in vitro and found that the pH range of 4-10 had no effect on permeability. pH 1.5-4 was less than 2-fold higher and pH 11-12 gave a small but significant increase in permeability (4-fold at pH12). Similarly Allenby et al. (208) observed large changes in skin impedence when incubated in aqueous media at pH <3 or >9. Strong acids and alkalies have been reported to give an irreversible destructive effect on keratin (14,43,70,178).

The present results indicate that when alkali attacks the keratin the skin barrier integrity is only slightly affected. DTA showed that in stratum corneum exposed to 1M HCl and 1M Na OH, the lipid endotherms still showed, but with lower temperatures. The

more significant lowering was in stratum corneum which had been treated with 1M Na OH, which lost its physical strength and became difficult to handle.

# CHAPTER FOUR

# THIN-LAYER CHROMATOGRAPHY OF STRATUM CORNEUM LIPID

## Introduction

The Thermal Analysis of neonatal rat stratum corneum reported in Chapter 2 revealed three lipid endotherms at 42°, 71° and 80°C. The 80°C transition was found to disappear completely when stratum corneum was heated at 120-140°C by DTA. The disappearance was also found to be time and temperature dependant (see page 38). The T42 and T71 were also found to be lowered to 39-40 and 69-70°C, respectively, following heating. Neither the lipids responsible for these endotherms, nor the nature of the changes occurring at elevated temperatures are known. Accordingly, an attempt was made to identify the lipids responsible for these transitions and to investigate the nature of the effect of heat on them using thin-layer chromatography (TLC).

## Experimental Methods

### 1. Extraction of Stratum Corneum Lipid for TLC

Stratum corneum sheets were cut into small pieces (2-3mm) and shaken with C/M 2:1 in a stoppered conical flask for 5 minutes using a mechanical shaker. After standing for 1 hour and a further 5 minutes shake, the extract was drained through stainless steel mesh (120 mesh), to remove most of stratum corneum debris, followed by centrifugation at 3,000g for 10 minutes. The stratum corneum was extracted 3 times with fresh solvent, plus a further extraction with an overnight stand. The collected extracts were separated into two phases by adding water and methanol in which the overall solvent ratio was adjusted to chloroform : methanol : water (2:2:1.8 v/v/v) (289). The chlorform layer, containing the lipid, was separated in a separating funnel and transferred to a porcelain dish (the first 1-2ml were rejected), and evaporated under vacuum at 30°C. 3-4mg of lipid was used for DTA.

## 2. TLC of the Extracted Lipid

All TLC sheets were cleaned with chloroform : methanol (2:1) (C/M, 2:1) followed by petroleum ether : diethylether : acetic acid (80:20:1, v/v/v) using blank development. They were then heated at 110°C for 1½ hours before use.

The extracted lipid was dissolved in C/M 2:1. A spot of this solution was placed on a TLC sheet and sprayed with the reagent described by Kundu et al. (290). No blue colour was developed indicating the absence of phospholipids. Using silica gel G TLC sheets, stratum corneum lipid was developed using the solvent for neutral lipids; petroleum ether : diethylether : acetic acid (80:20:1, v/v/v). The lipid fractions were visualised under UV light after spraying with 0.25% aqueous 8-anilino-1-napthalene sulphonic acid (ANS). Individual spots were identified by co-chromatography with known standards (65). Identification of some lipid fractions was also supported by chemical tests.

## 3. Fractionation of Lipid for DTA

The C/M 2:1 solution of lipid was spread as a continuous horizontal line to within 1cm of each end of the TLC sheet. After fractionation as above, a width of 2cm was cut vertically from both edges. These strips were sprayed with ANS, re-attached to the sheet and visualised under UV light. Using the visualised lipid fractions as a guide, the unsprayed lipid fractions were located and marked by lines (Fig.4.1). Each lipid fraction was cut, scraped, transferred into a centrifuge tube and extracted with C/M 2:1, followed by centrifugation at 3,000g for 10 mins. The clear supernatant layer was evaporated to dryness on a watch glass under vacuum at 30°C. The extraction procedure was repeated 3-4 times. Fractionation of lipid was repeated until sufficient

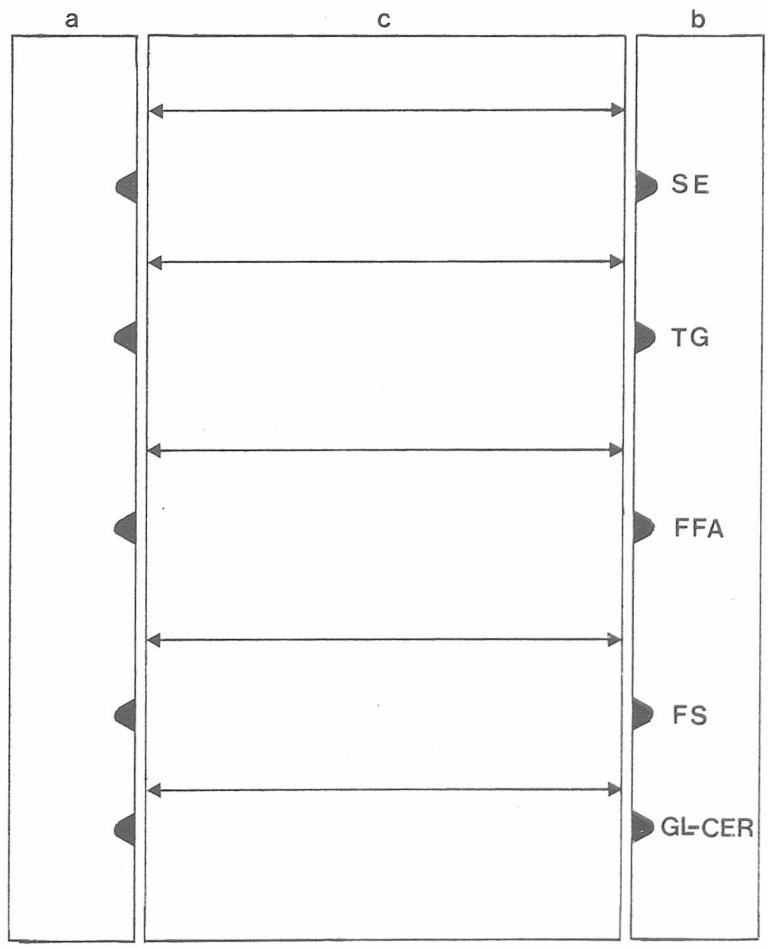



Figure 4.1 Diagram of TLC of neonatal rat stratum corneum lipid showing the re-attachment of the sprayed (with ANS) strips after lipid fractionation using petroleum ether : diethyl ether : acetic acid (80:20:1, v/v/v). (a) and (b) are strips removed from the original sheet and sprayed with ANS to visualize the lipid fractions under UV light, (c) unsprayed portion of the original sheet replaced between (a) and (b). Cuts were made along lines ↔ to separate lipid fractions for thermal analysis.
(SE) sterol esters, (TG) triglycerides, (FFA) free fatty acids, (FS) free sterols, (GL-CER) glycosphingolipids and ceramides.

amounts of lipid fractions were obtained. About 2mg of each lipid fraction was used for thermal analysis.

**4. Lipid Fractionation using other Solvents**

Using aluminium oxide TLC plates, lipid fractionation was performed using chloroform alone as a developing solvent. The lipid fractions were collected in the same way as above and used for DTA. Reagent cholesterol was used as a standard. Fractionation of lipid obtained from pre-heated stratum corneum (at 80°C, 50% RH for 6 hours) was also performed in the same way.

Following the fractionation with chloroform, the lipid remaining at the base line of TLC plate, was further fractionated using silica gel G TLC plates and the solvent system; benzene : acetone : acetic acid (80:20:1, v/v/v). The lipid fractions obtained were collected and used for thermal analysis in the same way as above.

**5. Thermal Analysis of mixed Lipids**

In general, lipids were mixed either physically, or by dissolving them in a few drops of C/M (2:1) and then evaporated to dryness at room condition, before thermal analysis. Materials and mixing proportions are detailed in the results and discussion section.

**Results and Discussion**

Following the identifications shown by Grayson and Elias (65), using the solvent system; petroleum ether : diethylether : acetic acid (80:20:1, v/v/v) lipid of neonatal rat stratum corneum was fractionated into glycosphingolipids and ceramides, free sterols, free fatty acids, triglycerides and sterol esters (Fig. 4.1). When these individual lipid fractions were thermally analysed, the

glycosphingolipids - ceramides, triglycerides, and sterol esters gave no endothermic transitions when heated up to 100°C. However endotherms were observed at 74.5°C for free fatty acids and at 43° (small) and 129°C (large) for free sterols (Fig. 4.2). Thermal analysis of reagent cholesterol gave endotherms similar to those obtained with free sterols fraction. These endotherms were found at 40° (small) and 149°C (large) (Fig. 4.3). This may suggest that the free sterols fraction is principally cholesterol (65). This was also confirmed using both Salkowski and Liebermann-Burchard tests for cholesterol (291). These results are in good agreement with those of Spier and Van Senden (44), who, using DTA, reported two endothermic transitions for cholesterol, at 40°C (small) and 150.8°C (large). The small endotherm at 40°C was attributed to a polymorphous transition related to a change in the packing of the terminal methyl groups of the aliphatic side chain of cholesterol (45).

When the free sterols and free fatty acid fractions were physically mixed (1:1 w/w) and heated up to 100°C, no change was observed in the peak temperature of the free fatty acids however the second run on DTA revealed a lowering in the melting temperature of free fatty acids from 74.5° to 68°C and loss of the melting endotherm (129°C) of the sterols (Fig. 4.2). Similar results were obtained when reagent cholesterol was mixed with the fatty acids fraction. Thus it seems most likely that, whilst the temperatures do not match exactly the endotherms seen on DTA of stratum corneum at 71° and 80°C are associated with melting of free fatty acids and that the T42 is associated with cholesterol.

In the experiment using aluminium oxide TLC plates and chloroform alone as a solvent, only free sterols were separated from stratum corneum lipids. This was identified by co-chromatography

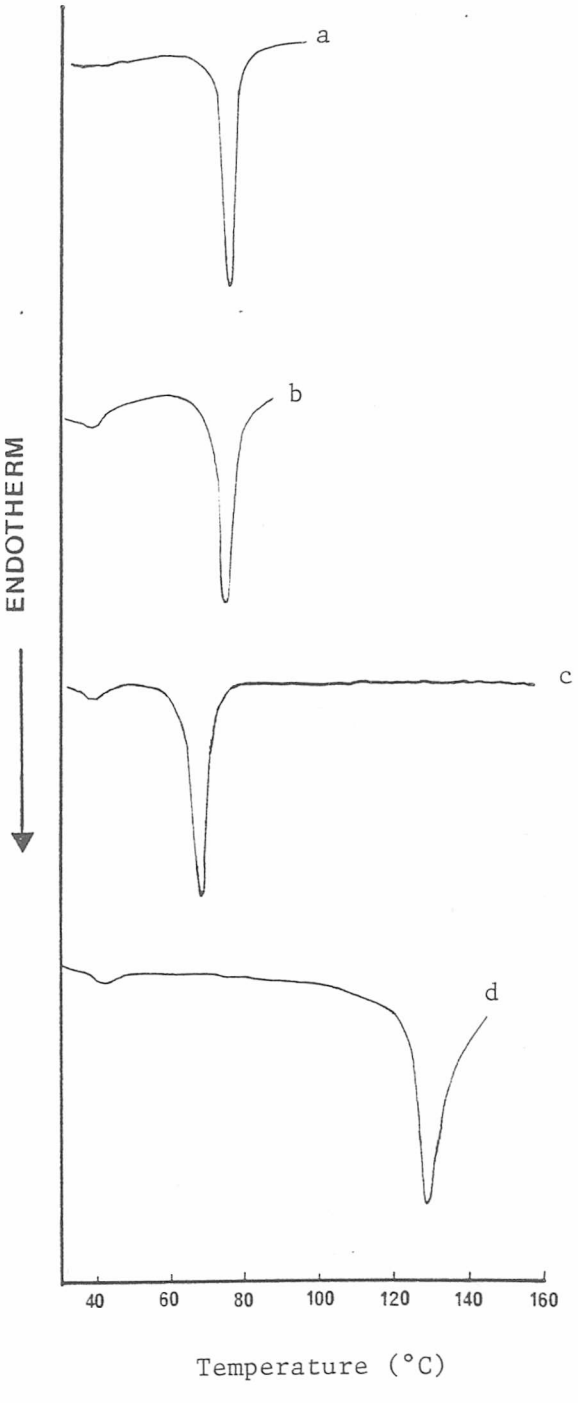



Figure 4.2 DTA thermograms of lipid fractions of stratum corneum obtained by TLC (see Fig. 4.1). (a) Free fatty acids, (b) Physical mixture of free fatty acids and free sterols (1:1, w/w), (c) second run of (b), (d) free sterols.

with standard cholesterol and by the Liebermann-Burchard test. Thermal analysis of this fraction revealed two endothermic transitions at 37° (small) and 134°C (large) (Fig. 4.3). DTA of the remaining lipid showed two endothermic transitions at 73° and 82°C (Fig. 4.3). These endotherms are very similar to those obtained with intact stratum corneum sheet (T71, T80) with the exception that, whilst in stratum corneum the T80 was irreversible, the fractionated lipid endotherm at 82°C was found to be reversible even when it was heated up to 150°C. However addition of an equal amount of reagent cholesterol to the sterol-free lipid, subsequent thermal analysis up to 100°C showed a large reduction in the size of T82 which then disappeared on second heating run (T73 was only reduced to 70°C) (Fig. 4.3). Similar results were obtained when the sterols fraction was used instead of reagent cholesterol. In the light of the similarity of behaviour of sterol-free lipid endotherms and those of stratum corneum sheet it seems probable that the two lipid transitions of stratum corneum and those of sterol-free lipid are caused by the same lipid species, namely free fatty acids. It may also suggest that the irreversibility of T80 (of stratum corneum sheet) is most probably due to an interaction of the free fatty acids responsible for T80 and cholesterol within the stratum corneum and not due to any thermal decomposition of lipids. This is consistent with the suggestion presented in chapter 2 which excluded thermal decomposition of lipids as an explanation for the disappearance of the 80°C transition. Further evidence for this was obtained by heating stratum corneum sheets at 80°C (at 50% RH) for 6 hours, to cause the loss of the T80 endotherm. The pre-heated skin was extracted with C/M 2:1 and fractionated in the same way (Chlororform, Aluminium oxide TLC plates). Two fractions of free sterols and sterol-free lipid were obtained.

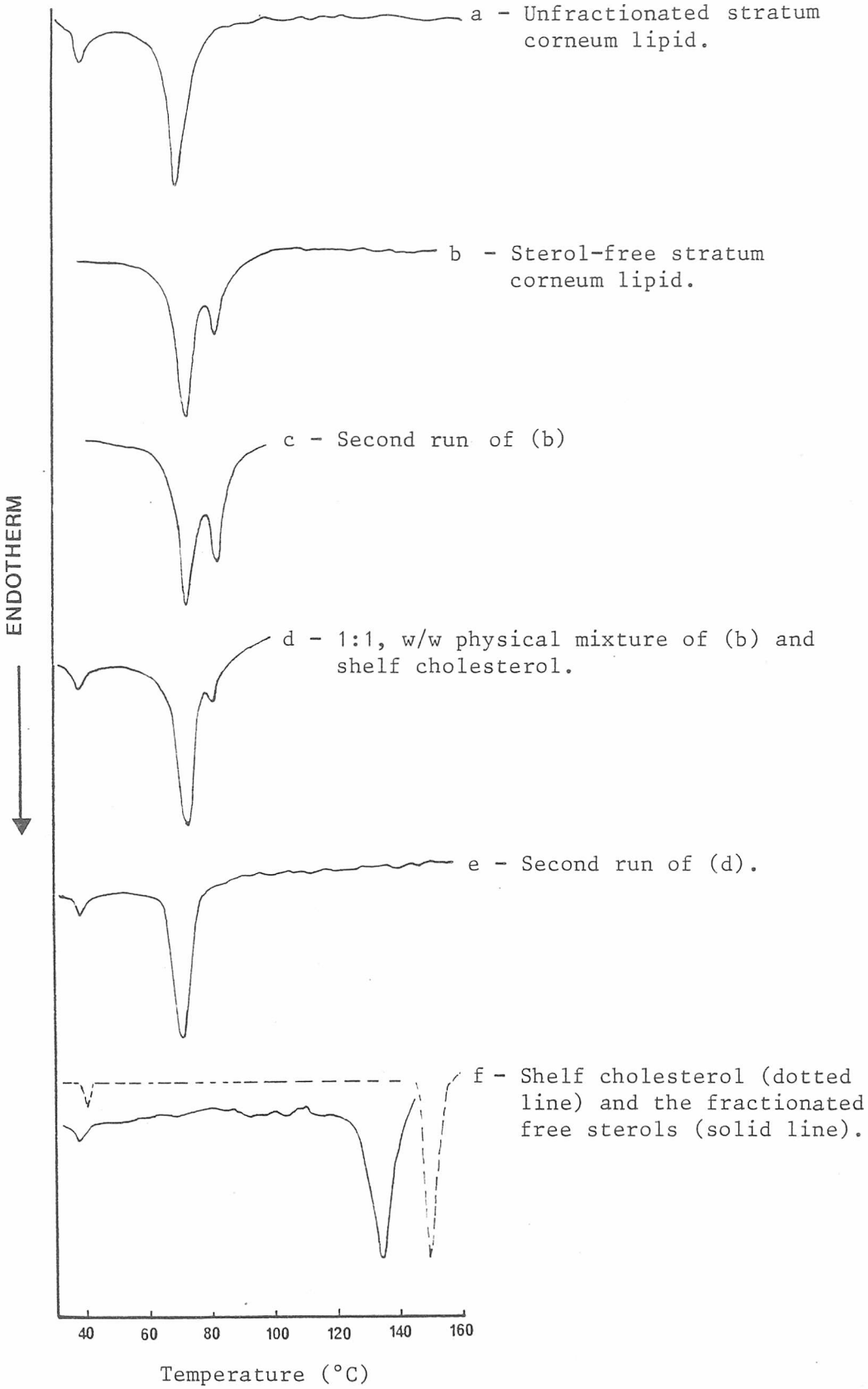



Figure 4.3 DTA thermograms of lipid fractions of stratum corneum. For preparation of lipid fractions see text (page 144).

Thermal analysis of the latter fraction showed two transitions (T73, T82). The fractions of lipid obtained are, therefore, identical before and after heat treatment. This work is in good agreement with Rehfeld and Elias (42) who observed no significant difference between TLC of heated and unheated lipids of neonatal mouse stratum corneum.

When sterol-free lipid was fractionated using silica gel G plates with benzene : acetone : acetic acid (80:20:1, v/v/v), six lipid fractions were obtained (Fig. 4.4). Thermal analysis of fractions 1, 2, 3 and 6 gave no distinct endotherms. However sharp endothermic transitions were found at 81° and 87°C for lipid fractions 4 and 5, respectively (Fig. 4.5). When these two lipid fractions were mixed together (1:1, w/w), dissolved in a few drops of C/M (2:1) and evaporated to dryness prior to DTA, a single endothermic transition was observed at 76°C. Using the same procedure, a mixture of lipid fractions 4 and 5 plus reagent cholesterol (1:1:1, w/w/w) was prepared for thermal analysis. Two endotherms were observed at 38° (due to cholesterol) and 70°C (Fig. 4.5). Likewise a physical mixture of the same materials (fraction 4, 5 and cholesterol, 1:1:1) gave a similar thermogram on the second heating run of DTA (Fi.g. 4.6). These results (Figs. 4.5, 4.6) are similar to those obtained with mixtures of cholesterol and free fatty acids (Fig. 4.2) or cholesterol and sterol—free lipid (Fig. 4.3) in which two endothermic transitions were observed at about 38° (small) and about 70°C (large) with the absence of the endothermic transition of cholesterol (149°C). In the light of this similarity, T81 and T87 found with lipid fractions 4 and 5, respectively (Fig. 4.5) are likely to be due to melting of free fatty acids. There are discrepancies in this work. For example the sterol-free lipid showed two reversible endotherms at 73° and 82°C and only single transition was obtained at 74.5°C for free fatty acids

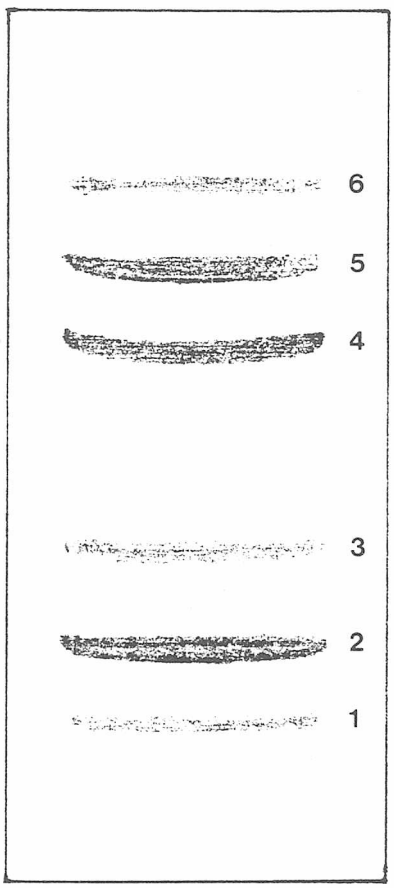



Figure 4.4 A diagram of TLC plate, showing fractionation of the sterol-free lipid of rat stratum corneum using silica gel G TLC sheets after developing with benzene : Acetone : Acetic acid (80:20:1, v/v/v).

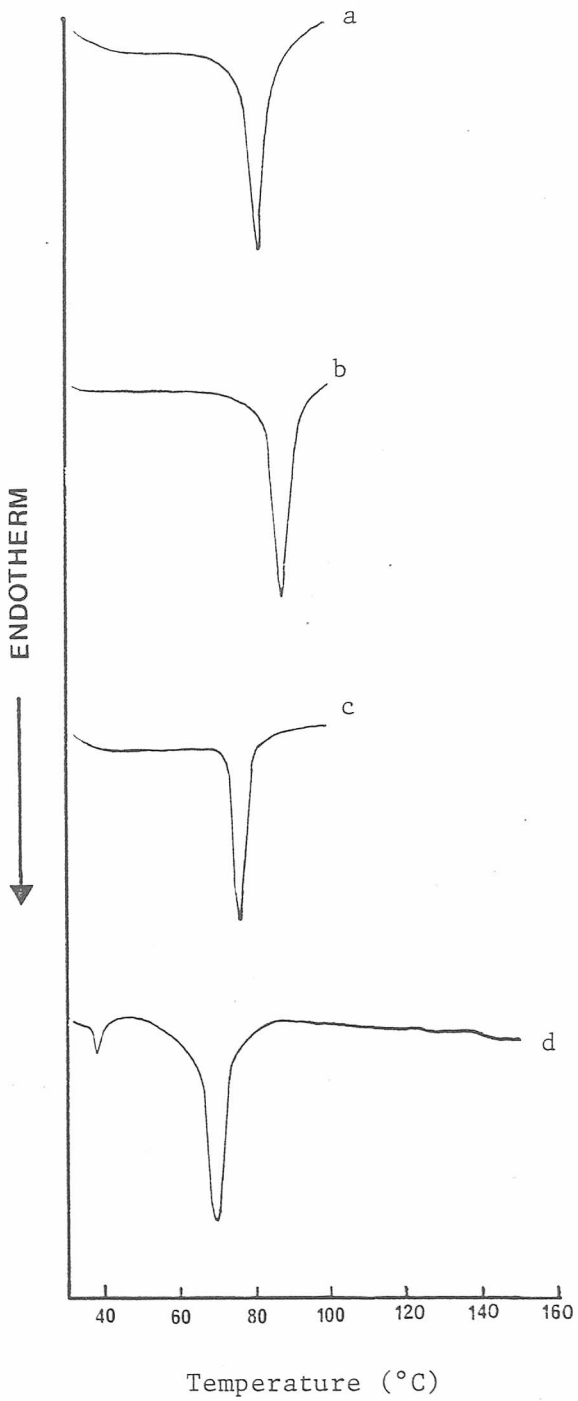



Figure 4.5 DTA thermograms of lipid fractions of stratum corneum (see Fig. 4.4). (a) Band 4, (b) band 5, (c) a mixture of band 4 and 5 (1:1 w/w) after dissolving in C/M, 2:1 and evaporation to dryness, (d) a mixture of band 4, 5 and shelf cholesterol (1:1:1, w/w/w) after dissolving in C/M, 2:1 and evaporation to dryness.

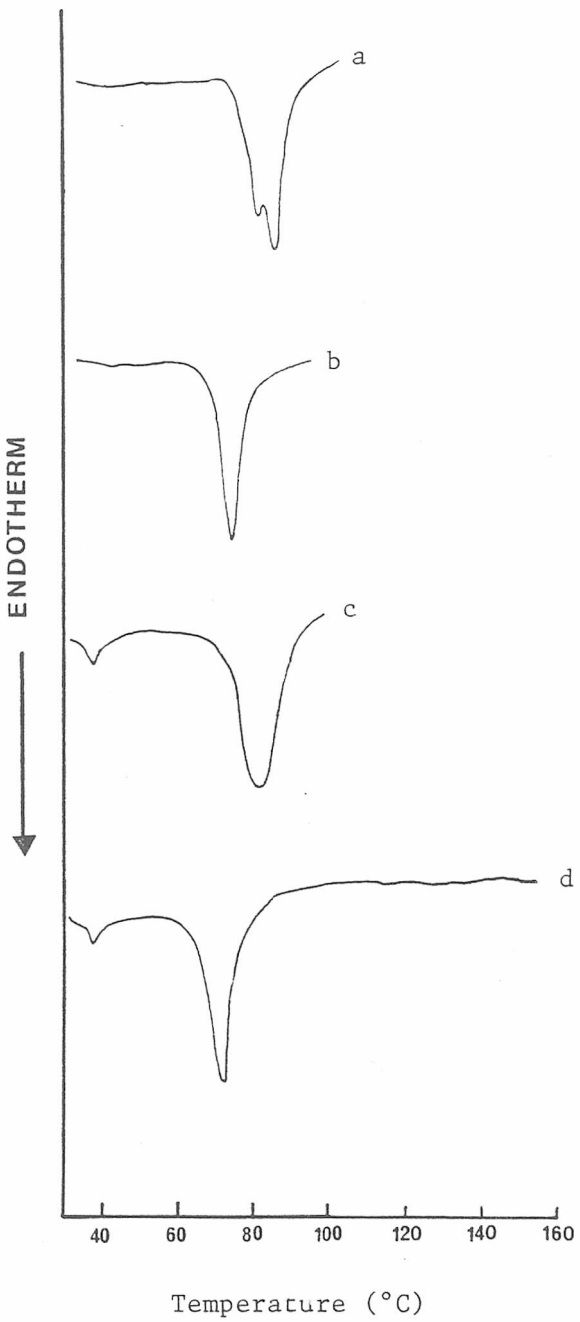



Figure 4.6 DTA thermograms of lipid fractions (see page 150). (a) 1:1 (w/w) physical mixture of band 4 and 5, (b) Second run of (a), (c) physical mixture of band 4, 5 and cholesterol (1:1:1, w/w/w), (d) Second run of (c).

fraction whilst there was a single endotherm at 76°C for the mixture of lipid fractions 4 and 5. These discrepancies are not understood, but no further investigation was made in this work.

An attempt was made to investigate possible changes in the melting temperatures of some reagents when mixed together prior to thermal analysis.

These reagents are decanoic acid (Capric), dodecanoic acid (Lauric) hexadecanoic acid (Palmitic) and octadecanoic acid (Stearic). When 1mg of each fatty acid was subjected to DTA,a single endothermic transition was obtained (Table 4.1). 2mg of 1:1 physical mixture (thoroughly blended using a glass mortar and pestle) of each fatty acid pair were then prepared. DTA of these mixtures revealed either two endothermic transitions at 32° and 54°C for Capric acid - Stearic acid, at 40° and 62°C for Lauric acid - Stearic acid, or a single transition at 60°C for Palmitic acid - Stearic acid mixture (Fig.4.7).

Table 4.1 Peak Temperatures for Endothermic Transitions of Fatty Acids.

| Fatty Acid | Peak Temperature (°C) |
|---|---|
| Capric acid | 35 |
| Lauric acid | 48 |
| Palmitic acid | 65 |
| Stearic acid | 72 |

When cholesterol was physically mixed with each fatty acid (1:1 w/w), the melting temperatures of the acids were again lowered and the melting transition of cholesterol (at 149°C) disappeared (Fig.4.8). These results show that mixing of fatty acids will cause a mutual lowering of melting point, and may explain the increasing melting point observed as better separation of lipid fractions was obtained (Fig. 4.5). This observation may indicate that a eutectic-type system is being

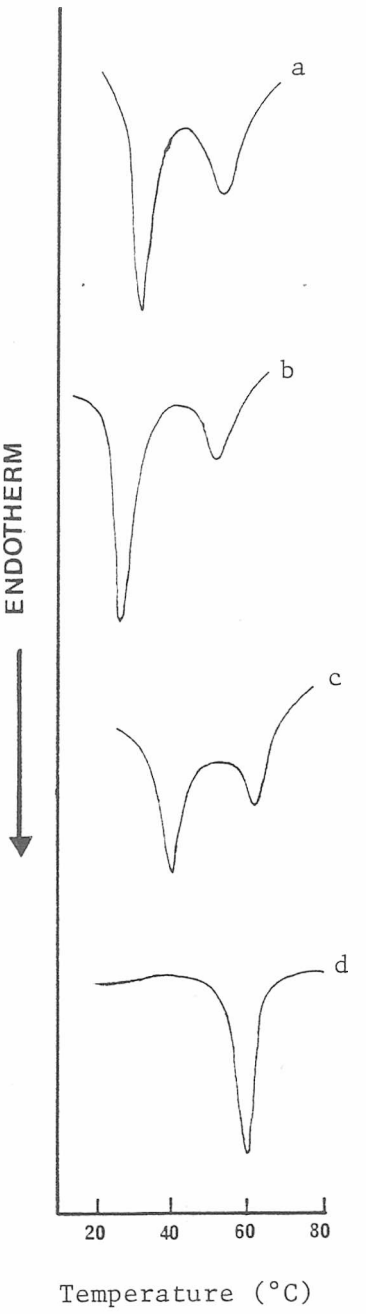



Figure 4.7 DTA thermograms of physically mixed fatty acids (1:1, w/w).
(a) Capric acid - stearic acid, (b) second run of (a), (c) lauric acid - stearic acid, (d) Palmitic acid - stearic acid.

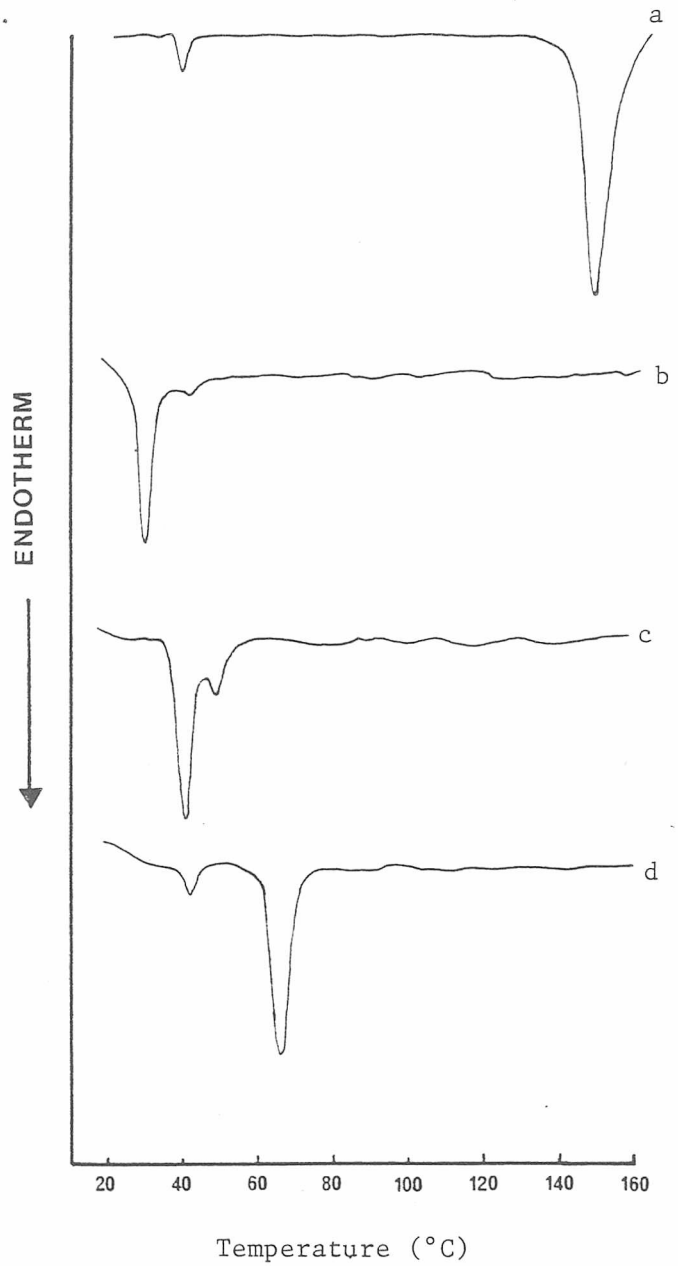



Figure 4.8 DTA thermograms of 1:1 w/w physical mixtures of fatty acids and cholesterol.
(a) Cholesterol alone, (b) capric acid - cholesterol, (c) lauric acid - cholesterol, (d) stearic acid - cholesterol.

formed. It also seems probable that cholesterol dissolves in the melted fatty acid, thus causing the loss of its endothermic transition at 149°C.

From the work presented in this chapter, it can be concluded that the lipid endothermic transitions at 71° and 80°C found with neonatal rat stratum corneum are due to free fatty acids and that at 42°C is due to cholesterol. However in chapter 2 it was observed that this transition varies with species from 41° (human skin) to 47°C (rabbits skin) and was found to be irreversible in human stratum corneum (see page 45). If the transition is due to cholesterol alone it is difficult to explain these observations.

The significance of these findings in relation to the nature of the skin permeability barrier is discussed in chapter 6.

# CHAPTER FIVE

## A POSSIBLE SCREEN TEST FOR SORPTION PROMOTERS

### Introduction

In chapter 3, it was established that increases in permeability coincided with changes in stratum corneum lipid transitions as detected by DTA. For example, stratum corneum which had been extracted with C/M 2:1 showed no lipid transitions on DTA and was extremely permeable. Also exposure to DMSO or DMAC gave reductions in the melting temperatures of the lipid. Permeability towards alkanols was increased after such treatment. When stratum corneum was exposed to heat at 75°C and higher, its permeability increased. DTA of pre-heated stratum corneum showed that this effect only occurred when the lipid transition at 80°C was destroyed or altered. Thus the lipid transition at 80°C has been shown to be a crucial indicator of the integrity of the stratum corneum barrier.

The possibility arises, therefore, that the DTA behaviour of the 80°C transition may be a suitable way of screening for substances which might have sorption promoter properties, in addition to using it to study the effects of known sorption promoters. In this chapter, results are presented for several substances of different chemical type which have been screened on the above basis.

The screening was carried out by treating stratum corneum with the substances, followed by Thermal Analysis to detect changes in the lipid transitions. Skin permeability measurement was carried out on similarly treated stratum corneum. The choice of substances to test was made on the basis of their ability to hydrogen bond and their lipid solubility because these are properties of DMSO. The safety of the materials was also considered during their selection, although some toxic substances, such as formic acid, were used.

## Experimental Methods

Stratum corneum sheets were treated in a number of ways prior to Thermal Analysis and penetration studies.

### (a) Neat Organic Liquids

The stratum corneum sheets were soaked in the liquid for a specified time. After soaking, the excess liquid was removed by pressing the sample between filter paper until no liquid was seen on the filter paper.

### (b) Solutions of Organic Materials

The sheets were placed in solutions of organic materials, or the solvent alone, for 24 hours at 30°C unless otherwise specified. They were dried as described above.

### (c) Organic Vapours

For most treatments the glass apparatus detailed previously was used (page 21). For decanol and dodecanol stratum corneum sheets were suspended in a closed glass vessel (volume about 150ml), containing the test liquid.

DTA and penetration studies with alkanols (from water unless stated to the contrary) were carried out as described previously. Details of the materials, conditions and concentrations used are given in Tables 5.1, 5.2, 5.3 and 5.4 with the results.

## Results and Discussion

The effect of organic materials on the endothermic transitions of stratum corneum are given in Table 5.1 and shown in Figure 5.1. Most organic substances used produced a reduction in the melting

Table 5.1 The Effect of Organic Substances on the Endothermic Transitions of Neonatal Rat Stratum Corneum.

| Substance | Exposure Conditions | Lipid Endotherms (First Run to 130)°C | Lipid Endotherms (Second Run)°C |
|---|---|---|---|
| Control | | 42 71 80 | 40 69 |
| Aliphatic Alcohols | | | |
| Decanol | Vapour, 30°C, 24 hrs | 42 62 70 | 41 — |
| Dodecanol | Vapour, 30°C, 24 hrs | (19) 42 62 72 | 40 — |
| Aliphatic Esters | | | |
| Ethylbutyrate | Liquid, 25°C, 24 hrs | No endotherms | broad 70 (S) |
| Ethylcinnamate | Vapour, 30°C, 24 hrs | 42 62 72 | — 59 |
| Isopropylmyristate | Liquid, 25°C, 24 hrs | 42 60 69 | 39 59 |
| Aliphatic Acids | | | |
| Formic Acid | Vapour, 25°C, 1 hr | 41 65 74 | 40 64 |
| Formic Acid | Vapour, 25°C, 2 hrs | 41 61 72 | NP |
| Formic Acid | Liquid, 25°C, 1 hr | 40 59 70 | NP |
| Acetic Acid | Vapour, 25°C, 1 hr | 42 63 73 | 40 63 |
| Acetic Acid | Liquid, 25°C, 1 hr | No endotherms | No endotherms |
| Butyric Acid | Vapour, 25°C, 24 hrs | 42 59 68 | 41 59 |
| Butyric Acid | Liquid, 25°C, 1 hr | No endotherms | No endotherms |
| Hexoic Acid | Liquid, 25°C, 2 hrs | — 47 66 | 44(L) 65(S) |
| Hexoic Acid | Liquid, 25°C, 24 hrs | No endotherms | No endotherms |
| Octoic Acid | Liquid, 25°C, 24 hrs | No endotherms | No endotherms |
| Decanoic Acid | Liquid, 33°C, 1 hr | (28(S)) No endotherms | 40 59 |
| Decanoic Acid | Liquid, 33°C, 24 hrs | (31(L)) No endotherms | No endotherms |
| Oleic Acid | Liquid, 25°C, 24 hrs | — 61 72 | 31 55 |
| Miscellaneous | | | |
| Acetone | Liquid, 25°C, 1 hr | 42(S) 71(S) 80(S) | 40(S) 69(S) |
| Azone | Liquid, 25°C, 24 hrs | — 51 67 | 42(L) |
| Propylene Glycol | Liquid, 25°C, 48 hrs | 42 64 73 | 40 64 |
| 1% dodecanoic Acid in PG/$H_2O$ 10:3 | Liquid, 30°C, 24 hrs | 40 46 63 68 | 42(L) 58(L) |

(S) small (L) large (NP) not performed

(—) no endotherms

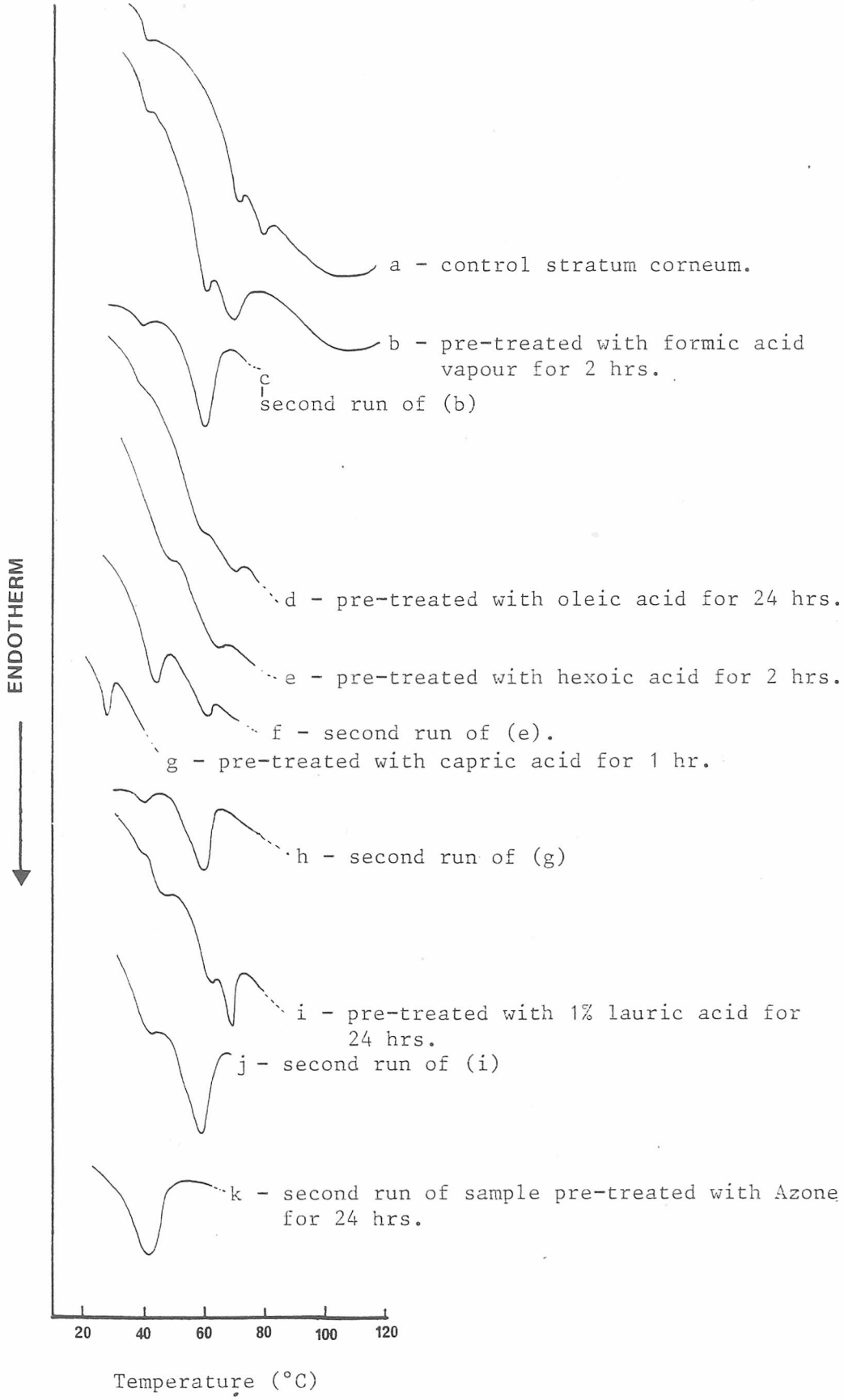



Figure 5.1 DTA Thermograms of stratum corneum pre-treated with various organic substances (see Table 5.1).

temperatures of stratum corneum lipid , which are normally at 71°C and 80°C. In some cases treatment resulted in the loss of the lipid endotherms,e.g., octoic acid. When stratum corneum was treated with both the vapour and liquid of the same substance, different results were observed. For example, whilst treatment with acetic (or butyric) acid vapour showed only a lowering in the lipid transition temperatures, treatment with liquid acetic (or butyric) acid eliminated the lipid transitions completely. The latter effect may be due to an extraction of lipids responsible for the transitions. Similar effect was also obtained when stratum corneum was exposed to formic acid vapour prior to DTA. However treatment with formic acid liquid caused disruption of stratum corneum but without elimination of the lipid transitions (Table 5.1).

Three of the materials used produced new endotherms within stratum corneum.Dodecanol, capric acid and lauric acid (in PG/$H_2O$) gave endotherms at 19°, 31° and 46°C respectively, and are probably due to the melting of residual reagent in stratum corneum (Table 5.1). Unlike the 1 hr pre-treated stratum corneum (with capric acid) which showed endotherms at 40° and 59°C, the second DTA run of 24 hr pre-treated stratum corneum did not show lipid transitions. From chapter 4, the explanation for this loss of the lipid transitions after 24 hour treatment is probably the formation of a eutectic mixture, in this case with a eutectic temperature below the melting point of capric acid. The one hour treatment did not eliminate the transition because insufficient capric acid had entered the stratum corneum. It is also possible to explain the 24 hour treatment in terms of extraction of lipids by capric acid, but this has not been investigated.

Unlike the water-insoluble fatty acids, the effect of water-

soluble carboxylic acids vapours on stratum corneum lipid endotherms can be reversed by rinsing with distilled water. The same is the case with DMSO vapour treatment, suggesting that the main effect of these materials is the same, reversible and, as has been proposed earlier, is produced by hydrogen bonding with the stratum corneum lipids.

The effect of these treatments on the permeability of stratum corneum was also studied. The results are presented in Table 5.2. All the substances, with the exception of propylene glycol, have significantly increased the penetration of hexanol. Stratum corneum exposed for 2 hours to formic acid vapour showed a 4-fold increase in the penetration of hexanol. This is consistent with the DTA result of identically treated stratum corneum. Together these suggest that the formic acid interacted with the stratum corneum lipids. According to the results obtained from DTA and permeability studies, both formic acid and DMSO vapour gave very similar effects on stratum corneum. This observation would suggest similar mechanisms are involved. There was a 6-fold and 8-fold increase in the penetration of hexanol using stratum corneum pre-treated for 1 hour with decanol and dodecanol, respectively. Scheuplein and Ross (106) reported that the penetration of water, butanol and butyric acid increased in the presence of a saturated solution of octanol. This effect was reversible when octanol was washed off and was explained as due to a plasticization of stratum corneum lipids by octanol. In the present work the effect of decanol and dodecanol appeared to be permanent, but otherwise is in good agreement with the work of Scheuplein and Ross. The difference in reversibility probably arises because both decanol and dodecanol have lower aqueous solubilities and so would not be washed out as easily as octanol.

Table 5.2 The effect of pre-treatment of Neonatal Rat Stratum Corneum with Organic Substances on the Permeability Coefficient of Hexanol.

| Substance | Exposure Conditions | Permeability Coefficient Kp x $10^3$ (cm $hr^{-1}$) |
|---|---|---|
| Control | — | 10.3 ± 2.8 |
| Aliphatic Alcohols | | |
| Decanol | Liquid, 30°C, 1 hour | 65.0 ± 20.0 |
| Dodecanol | Liquid, 30°C, 1 hour | 89.1 ± 10.5 |
| Aliphatic Esters | | |
| Ethyl butyrate | Liquid, 30°C, 1 hour | 68.3 ± 26.0 |
| Ethyl butyrate | Liquid, 30°C, 24 hours | 152.2 ± 12.8 |
| Ethyl cinnamate | Liquid, 30°C, 1 hour | 38.7 ± 8.3 |
| Ethyl cinnamate | Liquid, 30°C, 24 hours | 33.4 ± 2.4 |
| Isopropylmyristate | Liquid, 30°C, 24 hours | 41.2 ± 13.1 |
| Aliphatic acids | | |
| Formic acid | Vapour, 25°C, 2 hours | 42.7 ± 4.3 |
| Acetic acid | Liquid, 25°C, 1 hour | 344.4 ± 17.6 |
| Butyric acid | Liquid, 25°C, 1 hour | 330.0 ± 71.5 |
| Hexoic acid | Liquid, 25°C, 1 hour | 44.9 ± 9.8 |
| Octoic acid | Liquid, 25°C, 1 hour | 48.6 ± 11.2 |
| Decanoic acid | Liquid, 33°C, 1 hour | 80.9 ± 14.3 |
| Decanoic acid | Liquid, 33°C, 24 hours | 421.7 ± 26.7 |
| Oleic acid | Liquid, 25°C, 24 hours | 34.7 ± 5.2 |
| Miscellaneous | | |
| Acetone | Liquid, 25°C, 1 hour | 85.0 ± 20.0 |
| Diethyl ether | Liquid, 25°C, 1 hour | 277.6 ± 34.7 |
| Propylene glycol | Liquid, 25°C, 24 hours | 13.7 ± 1.5 |

Cooper (269) also found that the presence of a 0.25 mole fraction of butanol, hexanol, octanol and decanol in propylene glycol increased the penetration of salicylic acid, with decanol the most effective.

Stratum corneum pre-treated for 1 and 24 hours with ethylbutyrate showed a 6-fold and 15-fold increase in the penetration

of hexanol, respectively. Thermal Analysis indicated some extraction of the lipids. Ethylcinnamate has a much smaller effect than the butyrate. Although isopropylmyristate (IPM) is generally considered to be an inert vehicle, both Thermal Analysis and permeability measurements showed that IPM can interact with stratum corneum lipids and increase skin permeability to hexanol after 24 hour treatment.

Further examination of the data in Tables 5.1 and 5.2 indicates that there is a time factor in these effects. Pre-treatment with carboxylic acids for 1 hour showed that the acids of high mobility or volatility, such as acetic acid, are far more effective than those of higher molecular weight, such as capric acid. However, 24 hour pre-treatment with capric acid increased the penetration of hexanol to a level comparable with the effect of C/M 2:1 extraction (see page 133). This suggests that, although high molecular weight carboxylic acids are slower to give an effect, they are very effective on longer treatment.

Allenby et al. (208) investigated the effect of acetic, propionic, butyric and hexoic acids on the electrical impedance of human skin, and found that all these acids reduced the impedance, but that a faster effect was found with those of lower molecular weight. In an other work, Allenby et al. (78) also found that butyric acid significantly increased the penetration of tri-n-propylphosphate. Liron and Cohen (215) have shown that skin permeability increased with exposure to propionic or butyric acid. Results presented here are in good agreement with those of Allenby et al. (78, 208) and Liron and Cohen (215).

Twenty four hour pre-treatment of stratum corneum with oleic acid produced only a 3-fold increase in the penetration of hexanol. This small effect (compared with the other fatty acids) is consistent

with Thermal Analysis where 24 hour pre-treatment of stratum corneum with oleic acid did not eliminate the lipid transitions but lowered them from 71° and 80° to 61° and 72°C, respectively (Table 5.1). A similar magnitude of increase in skin permeability to Flurbiprofen was observed with skin treated with propylene glycol containing 7% oleic acid (266). Also 5% oleic acid (in propylene glycol) has improved the bioavailability of betamethazone 17-benzoate (254). 0.1M oleic acid in propylene glycol significantly increased the penetration of salicylic acid compared with its penetration from propylene glycol (269). Whilst the methods used by Bennett et al. (254), Akhter and Barry (266) and Cooper (269) were different to the method of the present work there is good agreement on the effect of oleic acid.

One hour pre-treatment of stratum corneum with diethylether and acetone increased the penetration of hexanol 27-fold and 8-fold, respectively. This is in good agreement with Matoltsy et al. (70), Scheuplein and Ross (106) and Sweeney and Downing (79) who concluded that ether is more effective as a lipid solvent than acetone because it produced a greater increase in the skin penetration of water. DTA of stratum corneum treated with acetone for 1 hour still showed lipid endotherms, but they were significantly smaller than the control (Table 5.1).

The effect of low concentration of carboxylic acids on permeability of stratum corneum to hexanol is shown in Table 5.3. The carboxylic acids decanoic (capric) dodecanoic (lauric) and tetradecanoic (myristic) acids were incorporated in vehicles containing mixtures of propylene glycol and water. For 5% lauric acid and 3% myristic acid, ethanol was also added to increase their solubility. Neat propylene glycol was avoided because it has been reported that

high molecular weight carboxylic acids, such as capric acid, give poor penetration from it (269). It has been reported that a minimum amount of propylene glycol, sufficient to produce reasonable solubility, was necessary for maximum release of corticosteroids (222). In this work, the maximum amount of water and the minimum amount of propylene glycol, to ensure both solubility of the carboxylic acid and optimal release, were used. By producing a near saturated solution, a higher thermodynamic activity may be obtained (102,103,253) and hence more of the carboxylic acid would partition into the stratum corneum.

Table 5.3 The effect of pre-treatment of Neonatal Rat Stratum Corneum with low concentrations of fatty acids on the penetration of Hexanol.

| Fatty acid concentration and Solvent Systems* | Permeability Coeff. Kp x $10^3$ (cm $hr^{-1}$) |
|---|---|
| Control | 10.3 ± 2.8 |
| Propylene glycol (PG) | 13.7 ± 1.5 |
| (PG : Ethanol : Water 13:6:1 v/v/v) | 15.6 ± 1.9 |
| Isopropyl myristate (IPM) | 41.2 ± 13.1 |
| 5% Cholesterol in IPM | 18.3 ± 1.5 |
| 5% Capric acid in IPM | 66.8 ± 14.4 |
| 1% Capric acid in 66.7% PG (v/v in water) | 53.6 ± 7.4 |
| 2% Capric acid in 66.7% PG | 162.0 ± 37.2 |
| 3% Capric acid in 66.7% PG | 287.8 ± 9.5 |
| 3% Capric acid in 66.7 PG (4 hr pre-treatment) | 264.5 ± 57.0 |
| 4% Capric acid in 66.7% PG | 334.7 ± 20.9 |
| 5% Capric acid in 66.7% PG | 407.2 ± 9.2 |
| 6% Capric acid in 66.7% PG | 404.7 ± 37.2 |
| 100% Capric acid (33°C) | 421.7 ± 26.7 |
| 1% Lauric acid in (PG : Water 10:3 v/v) | 41.6 ± 1.9 |
| 5% Lauric acid in (PG : Ethanol : Water 4:1:1 v/v/v) | 106.0 ± 17.6 |
| 3% Myristic acid in (PG : Ethanol : Water 13:6:1 v/v/v) | 45.9 ± 8.1 |

* Pre-treatment was at 30°C for 24 hours unless stated to the contrary.

When stratum corneum was pretreated with 1-6% w/v capric acid (in 66.7% PG), penetration of hexanol increased with capric acid concentration up to 5% (Fig. 5.2). This increasing effect of capric acid with concentration indicates that as more capric acid partitions into the stratum corneum so its interaction with the lipids increases, producing more damage to the skin barrier. Pre-treatment (24 hours) of stratum corneum with 5%, 6% and 100% capric acid, all produced extremely permeable stratum corneum similar to that pre-treated with C/M (2:1). This suggests that maximum increase in skin permeability can be achieved with capric acid at a concentration as low as 5%. DTA of stratum corneum pre-treated with 5% capric acid (24 hours) showed no lipid endotherms, i.e., similar effect to that of neat capric acid given in Table 5.1. As suggested earlier, the effect of capric acid on lipid transitions is probably due to a eutectic-type system is being formed with a eutectic temperature below that of capric acid. Alternatively, since the effect is similar to that of C/M (2:1) it could also be an extraction, or it may be some other process. Cooper (269) investigated the effect of fatty substances on skin permeability and suggested that fluidization of stratum corneum lipids was involved.

The time-course of the effect of 3% capric acid was investigated using 4 hour and 24 hour pre-treatment (Fig. 5.2). The effect of 4 hour pre-treatment was very close to that of 24 hour, suggesting that maximum effect can be reached within about 4 hours. However, it takes at least 30 mins to prepare samples for permeability after pre-treatment. During this time, penetration of capric acid into further layers of stratum corneum will have continued. A more accurate estimate of the time required to produce a maximum effect was obtained from the lag period when the hexanol was included in the 66.7% PG

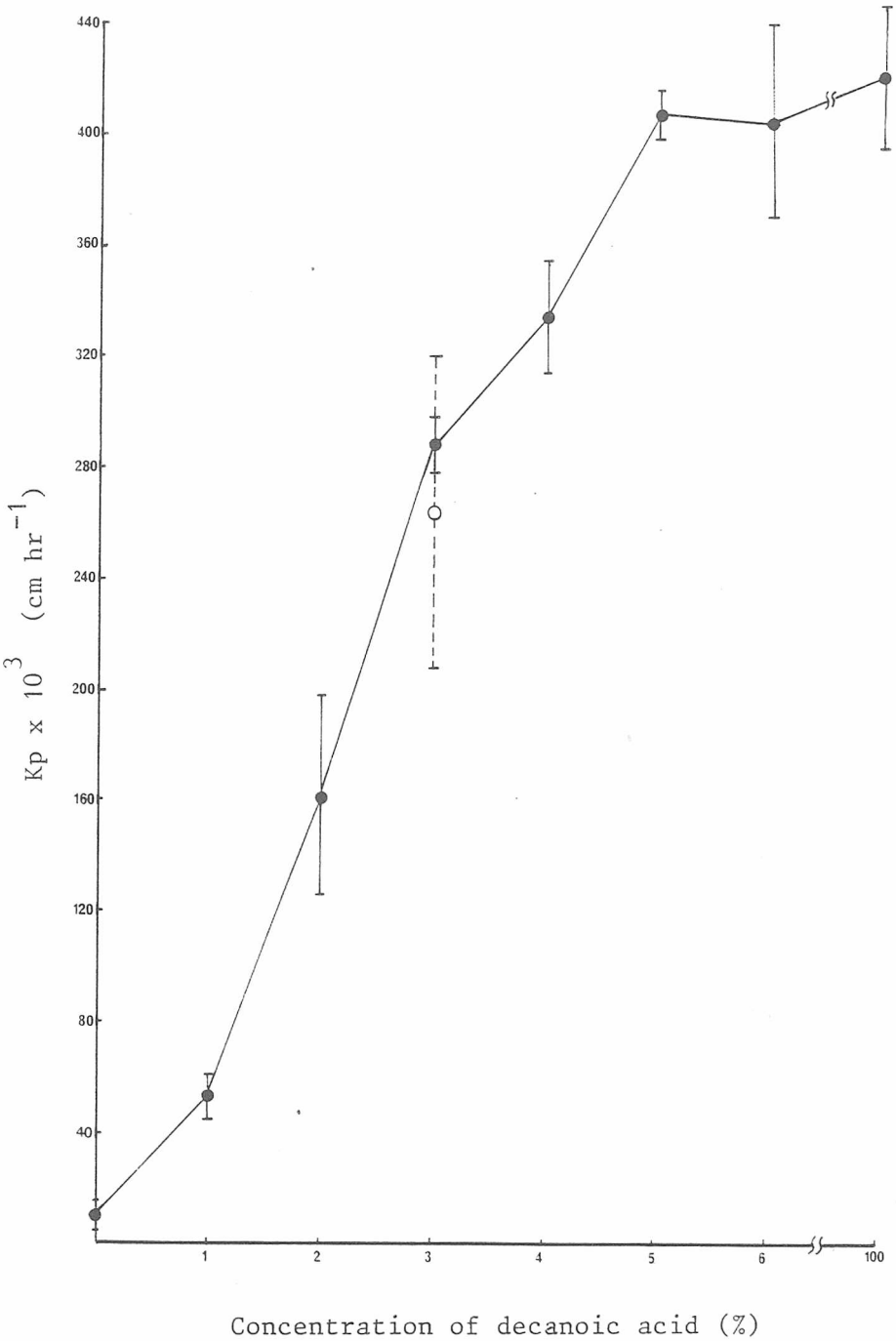



Figure 5.2 Plots of permeability coefficients of hexanol showing the effect of pre-treatment of stratum corneum, with various concentrations of decanoic acid (in 66.7% PG), for 24 hours (●) and 4 hours (○).

vehicle along with 2% capric acid (Fig.5.3). About 5 hours was required before steady-state penetration was achieved, producing a Kp of $58.3 \times 10^{-3}$ cm $hr^{-1}$. This was 2.7 times less than with pre-treatment alone (from water) and so was probably due to the effect of vehicle. Comparison of penetration rates from the PG vehicles with and without 2% capric acid, show a 35-fold difference in Kp (Fig.5.3). Cooper (269) using 1.7% capric acid found little effect on the penetration of salicylic acid. This difference may arise from his use of 100% PG as a vehicle.

Whilst no detailed study of the effect of the vehicle has been undertaken in this work, isopropylmyristate (IPM) was also used with capric acid (Table 5.3). IPM alone increased the penetration of hexanol 4-fold, but 5% capric acid in IPM produced only a 6-fold increase in skin permeability, as compared to the 40-fold increase from 66.7% PG. It is also similar to the effect of 1% capric acid in 66.7% PG. The difference is probably due to the higher affinity of capric acid for IPM, leading to a reduced thermodynamic activity and, therefore, partition coefficient (102).

The addition of 5% cholesterol to IPM produced less increase in the skin permeability than pre-treatment with the vehicle alone. This may be due to the cholesterol reducing the availability of the IPM molecules resulting in a lower concentration of IPM in the stratum corneum. It has been suggested (10) that the presence of cholesterol in a vehicle may add to the integrity of the skin barrier because it is naturally a part of the barrier.

Pre-treatment of stratum corneum with 1% and 5% lauric acid increased the penetration of hexanol 4-fold and 10-fold, respectively (Table 5.3). However, direct comparison is invalid because different solvent systems were used to obtain solutions. The effect of lauric acid on skin permeability was consistent with thermal analysis

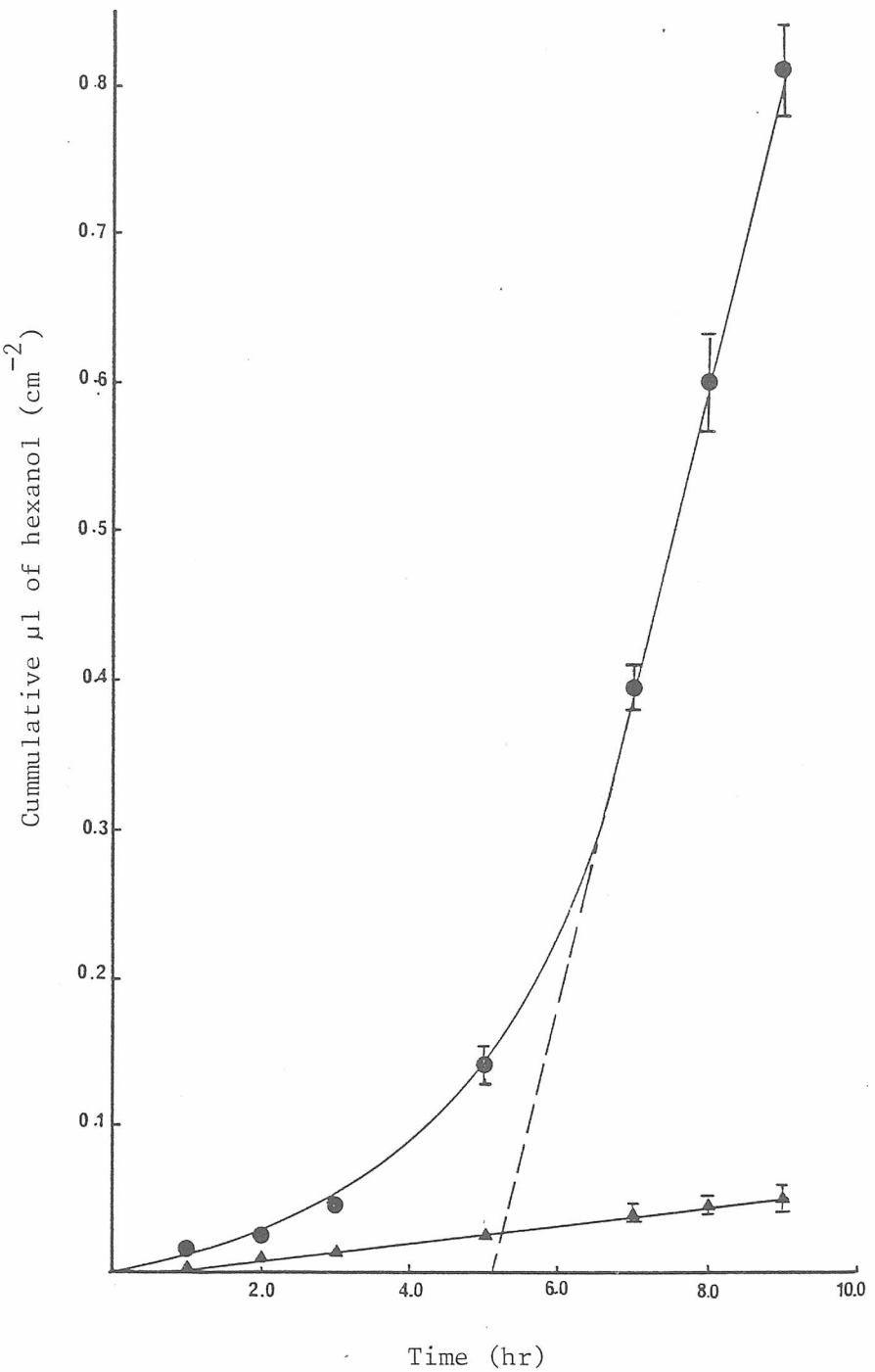



Figure 5.3 Plots of cummulative penetration data for hexanol from 66.7% PG with ( ● ) and without ( ▲ ) 2% decanoic acid.

results where treatment with 1% lauric acid lowered the lipid endotherms of stratum corneum from 71° and 80°C to 63° and 68°C, respectively (Table 5.1, Fig. 5.1).

When stratum corneum was treated with 3% myristic acid (in PG : Ethanol : Water 13:6:1 v/v/v), skin permeability increased 4.5-fold. However the vehicle alone produced a 1.5-fold increase in skin permeability suggesting that the increase due to myristic acid is probably about 3-fold (Table 5.3).

Because different vehicles were used, it is difficult to deduce the effect of carboxylic acid chain length on permeability, although from Table 5.3, it appears that as chain length of high molecular weight carboxylic acids increases, so the effect on skin permeability is decreased. Such a conclusion is in good agreement with thermal analysis results. Only one unsaturated acid, oleic acid, was used and it was less effective than any of the saturated acids.

The solvents used for the carboxylic acids cannot be considered to be ideal because other solvent systems were not investigated. The data in Table 5.3 emphasise that the choice of a suitable vehicle is very important for effectiveness of an accelerant. Bennett et al. (254) observed that oleic acid in propylene glycol improved steroid bioavailability, but not in dimethylisosorbide.

3% v/v Azone in a vehicle of PG : ethanol : water 10:1.2:3.5 v/v/v was also used. Water was incorporated into the solvent system to reduce the affinity of azone for the vehicle and so ensure better thermodynamic activity of azone. The permeability data (Table 5.4) show that penetration of propanol increased 2-fold, whilst that of hexanol was reduced. On the second run from distilled water, propanol and hexanol showed a 12-fold and 3-fold increase in penetration, respectively. The low permeability coefficients obtained on the

Table 5.4 The Effect of Azone and Acetic Acid on the Permeability of Neonatal Rat Stratum Corneum.

| Experimental Status* | Donor Phase | Receptor Phase | Permeability Coefficient Kp x $10^3$ ($cm\ hr^{-1}$) | |
|---|---|---|---|---|
| | | | Propanol | Hexanol |
| Control | Water | Water | 0.71 ± 0.21 | 10.3 ± 2.8 |
| First Run | 3% Azone | Water | 1.44 ± 0.19 | 4.16 ± 2.20 |
| Second Run | Water | Water | 8.9 ± 0.3 | 33.8 ± 11.6 |
| First Run | 25% Acetic Acid | 25% Acetic Acid | — | 55.6 ± 10.8 |
| Second Run | Water | Water | — | 36.1 ± 7.4 |
| First Run | 50% Acetic Acid | 50% Acetic Acid | — | 77.8 ± 7.4 |
| Second Run | Water | Water | — | 53.7 ± 4.2 |

* Stratum corneum washed with water for 16 hours before second run.

first run are probably due to the solvent effect of the vehicle, as discussed in chapter 3. Azone has been reported (265) to form complexes with some organic molecules.

Because azone is insoluble in water, rinsing the skin with water after the first run is unlikely to remove all the azone, particularly that which has partitioned into the stratum corneum. Thus the high values for permeability coefficient observed in the second run are likely to arise from the effect of azone still in the stratum corneum. Stoughton (265) said of azone that "penetration of both hydrophobic and hydrophilic molecules is enhanced, although more dramatic enhancements are usually seen with hydrophilic drugs." The present work confirms this statement.

DTA of stratum corneum, treated with azone, showed a lowering in the lipid endotherm temperatures, suggesting some interaction (see Table 5.1 and Fig. 5.1). This is also in agreement with the statement of Stoughton (265) that "Azone has, in common with most

of the N-alkyl lactams the ability to form non-covalent complexes with certain organic molecules." The enhancement effect of azone on the penetration of antibiotics, corticosteroids and other substances has been reported by Stoughton (263-265). 2% Azone in PG has also been found to enhance the penetration of flurbiprofen (in vitro) and betamethasone 17-benzoate (in vivo) (254,266).

As shown in Table 5.4, the penetration of hexanol increased 5-fold and 7-fold in the presence of 25% and 50% acetic acid, respectively. However, replacement of acetic acid solution with distilled water showed about a 30% reduction in the penetration. This suggests that the effect of both 25% and 50% acetic acid is partially reversible. Treatment of stratum corneum with undiluted acetic acid produced a 34-fold increase in the penetration of hexanol, presumably by lipid extraction (Table 5.2).

From this present work, it appears that the 80°C lipid transition is a sensitive indicator of the activity of a sorption promoter and that thermal analysis may provide a rapid screen test for potential new sorption promoters.

# CHAPTER SIX

# THE SKIN BARRIER AND THE ACTION OF SORPTION PROMOTERS

Two matters, the nature of the skin barrier and the mode of action of sorption promoters may now be discussed. They will be looked at separately.

## A. Skin Permeability Barrier

The site and nature of diffusional resistance to drug penetration within the stratum corneum is the subject of disagreement between investigators. Some agree that the intracellular keratin matrix of stratum corneum provides the main diffusional resistance to penetration (1,2,11-13,18,19,56,97,292), whilst others think it is the lipid of stratum corneum (20-22,65,73,98,121,250,293). In both cases, physico-chemical models have been produced to explain the selective semi-permeability of the skin (11,22,250,269,294-296).

In the present work, the results have strongly indicated the direct involvement of the lipid of the stratum corneum in the barrier function. From their intensive work on stratum corneum lipids (using morphological, histological and biochemical studies) Elias and co-workers (20-22,58,65,121,122,166,282,293,294) have concluded that the intercellular lipid is the principle barrier to penetration of drugs. Elias et al. (165) presented five reasons why the keratin-phospholipid complex model suggested by other workers (11, 19,56,292) is inadequate.

1. Histochemical studies show that the stratum corneum cell is devoid of lipid.

2. Because most of the lipid present is highly non-polar, there is no candidate to serve as a "sleeve" or matrix for intracellular keratin filaments.

3. The intercellular volume fraction is much larger than previously recognised and it is probably expandable either by solvent/detergent or by inspissation of non-polar vehicles, carriers or permeators.

4. There is experimental evidence that almost all the stratum corneum lipid lies between the cells of the stratum corneum (58,65).

5. There is now direct evidence that epicutaneously applied substances permeate via intercellular spaces.

Elias et al. (165) have also shown that anatomic sites of high permeability, such as the soles of the feet, have a low lipid weight per cent, whereas those regions of low permeability, such as the face, showed a high weight per cent, thus confirming the important role of lipids in permeability.

Futher evidence for the involvement of stratum corneum lipid in the skin barrier has been reviewed in detail by Elias and co-workers (20-22,295). This includes the deposition of abnormal lipids in the intercellular space in essential fatty acid difficient animal skin and other skin disorders. Penetration of water-soluble tracers was found to be via the intercellular space containing these abnormal lipids.

The results obtained in the present work using thermal analysis and permeability studies also provide some evidence to support the conclusions of Elias and his collaborators.

1. Extraction of the intracellular keratin did not affect the usual pattern of lipid endothermic transitions (page 30). This may suggest that the lipids responsible for these endotherms are not complexed with the keratin and are located intercellularly (58,65).

2. There is a strong relationship between thermally damaged lipid and increased permeability of stratum corneum (page 123). As was discussed earlier, a major change in the permeability of stratum corneum only occurs when the crystalline lipid which metlts at 80°C is

altered by heat. Furthermore, alteration of the lipid transition, detected by DTA, and the increased permeability of stratum corneum are dependent on both temperature and time of exposure to the elevated temperature.

3. Both polar and non-polar alkanols showed a similar magnitude of increase in their penetration rate through thermally damaged skin (page 128) indicating that the diffusivity of the barrier has altered and the partition coefficient concept still holds. This suggests that the resistance to penetration of both alkanols, irrespective of their polarity, arises in their diffusion in and/or across the intercellular lipid. This observation contradicts the conclusion of Scheuplein and Blank (11,13) who suggested that water-soluble molecules can enter and diffuse through aqueous channels of the tissue without passing through a lipid barrier. If the increase in the permeability of stratum corneum following heat treatment was due to the denaturation of intracellular keratin, which is unlikely, the Scheuplein and Blank model predicts that water-soluble molecules would be affected to a greater extent than non-polar ones. This was not observed in the present work.

However the observations reported above (and page 130) do agree with the two-phase or two-compartment models of stratum corneum proposed by Michaels et al. (250) and Elias and co-workers (22, 295). These models depict the keratinized cells imbedded in continuous lipid bilayers. Therefore,

> "for any permeating species there are only two routes for penetration of the barrier: one requires alternate transit through protein and lipid phases and the other transit solely through the continuous lipid phase. Within each phase, the permeating species is assumed to have a characteristic solubility (proportional to its local thermodynamic activity) and diffusivity"
>
> (250)

If this model is correct, one would expect the water-soluble propanol to prefer the alternate transit through lipid and protein (aqueous) phases. Thus, in order to pass from one cell layer to the next, propanol must also cross the lipid barrier before re-entering the hydrated proteinaceous region of the stratum corneum. In thermally damaged skin, the diffusion rate within the aqueous region is unlikely to have been affected, the observed increase in skin permeability being due to the altered diffusivity of the disordered lipid. Michaels et al. (250) have suggested that temperature can cause reversible and irreversible microstructural changes in the lipid phase of the tissue, which, in turn, alter penetration rates. Elias et al. (294)have developed a more detailed structural model for intercellular space, suggesting alternate lipid bilayers and hydrophilic aqueous environments. According to this model, penetration of both water-soluble and lipid-soluble molecules may take place mainly via this intercellular route (119-122), although it would not mean that a substance could not reach the intracellular region of the stratum corneum (98). For example, the swelling of stratum corneum with water is a good indication of hydrated intracellular keratin (11,99). However, on continuous hydration of stratum corneum, such as during penetration experiments, the two models combined (22,294) suggest that both intercellular and intracellular regions contain aqueous environments. In the present work, propanol showed an 87-fold difference in penetration between dry and hydrated stratum corneum (page 105) indicating the great importance of an aqueous environment for its transport within stratum corneum. With the relatively non-polar hexanol, which diffuses through lipid (11), only a 4-fold increase in the penetration on hydration was observed (page 105) Thus, in addition to penetrating through the lipid phase, hexanol may

also penetrate through a more aqueous region, unless the water of hydration has had a direct effect on the lipid barrier.

4. If the keratin matrix had been the main diffusional resistance, heat would have increased skin permeability by denaturation of keratin. If this model is correct, dissolving the keratin with alkali (page 139) would damage the so-called lipid-keratin complex (11,19,292) as effectively as denaturation with heat and so produce a similar increase in permeability. However, a much smaller increase in the penetration of hexanol was observed from strong alkaline vehicle than with heat pre-treatment (page 139). DTA (page 35) indicated that treatment with sodium hydroxide solution caused some alteration in the lipid endothermic transitions of stratum corneum, further suggesting the involvement of lipids in the skin barrier. Unlike lipid solvents, reagents which attack keratin have been found to have little effect on skin permeability (70,118,179).

5. It has been suggested that strong detergents, such as sodium dodecyl sulphate (SDS) increase skin permeability by denaturation of keratin (11,106,229). However, the evidence suggests that the main effect of SDS is on the intercellular lipids. For example, following treatment with SDS, penetration of water-soluble tracers (ferritin and horseradish-peroxidase) was found to take place only via the intercellular pathway (20,21,122). In the present study, stratum corneum soaked for 4 hours in 2% SDS, did not give any distinct lipid endotherms on thermal analysis (page 33). However, the second heating run did show the 70°C lipid transition, but very small in comparison to a control (Fig. 6.1). This observation suggests that SDS has an effect on stratum corneum lipid. Stratum corneum which had been treated with 2% SDS showed swelling and was easily ruptured, suggesting some denaturation of structural proteins. However, because of the link

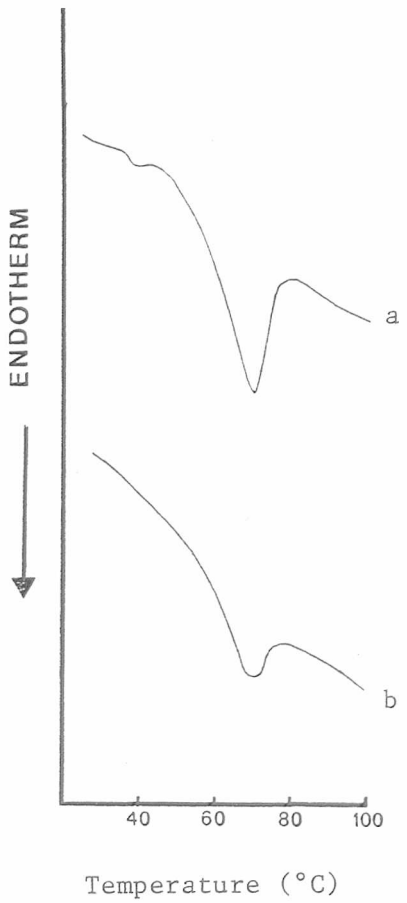



Figure 6.1 DTA thermograms of neonatal rat stratum corneum.
(a) Pre-heated up to 125°C, (b) pre-treated with 2% SDS for 4 hrs, rinsed with water, desiccated and pre-heated up to 130°C.

between lipid endotherms and skin permeability established in this work, the effect of SDS on stratum corneum lipid is probably the prime mechanism by which it increases skin permeability. Scheuplein and Ross (106), whilst acknowledging that SDS extracts skin lipid, suggested that denaturation of keratin is more important and held it to be responsible for the increased skin permeability. Evidence against the direct involvement of keratin denaturation in skin permeability may be obtained from the work of Wood and Bettley (297). They studied the effect of various detergents on epidermal protein denaturation and correlated the results with changes in epidermal permeability, but did not obtain a complete correlation. Some detergents, of high denaturing activity, showed minimal effect on permeability whilst other surfactants, of low denaturing activity, produced a greater effect on skin permeability. In their work, using IR spectroscopy, Park and Baddiel (35) have concluded that removal of lipids from stratum corneum can take place following treatment with SDS. Middleton (53) has shown that surfactants which extract more lipid from stratum corneum also have a greater effect on the water binding capacity of stratum corneum. He suggested that the effect of surfactants on skin permeability is due to extraction of some lipids, mainly cholesterol, and that the effectiveness of detergents on skin permeability is determined by their ability to extract lipids.

6. Whilst it is generally recognised that different anatomical sites and species show some differences in skin permeability, attribution of these differences to the number of hair follicles, number of sweat glands, number of cell layers or thickness of stratum corneum have been unsuccessful (3,110,113,149,156,165,298-300). In the present work it has been suggested (page 45) that these differences in skin permeability are due to variation in lipid structure. This was

concluded from the differences in the peak temperatures of the lipid transitions between both species and anatomic sites (page 45). There is an inverse relationship between melting temperatures of stratum corneum lipid and the skin permeability of the different species. This relationship may be explained by the higher melting temperature lipid molecules being more closely packed, leading to a greater barrier stability and hence impermeability (85,301). This is in line with the inverse relationship between diffusion coefficient (D) and viscosity (consistency) of the skin barrier phase (see equation 3.4, page 63). It also seems reasonable to attribute the appearance of lipid transitions at higher temperatures to the presence of more longer-chain, saturated fatty acids. The latter, free fatty acids, are responsible for lipid transitions of stratum corneum (see chapter 4), and may be able to form a more stable hydrophobic barrier (294,301). Plantar stratum corneum is known to provide least resistance to penetration (1,3,11). It also showed a small lipid transition at a lower temperature than human buttock stratum corneum which is amongst the least permeable regions of human body (298). Recent studies (165,166,299) have established a correlation between skin permeability and quantity of lipid in stratum corneum. The anatomical sites which show low permeability contain the highest concentration of lipid. It was, therefore, concluded that quantitative, but not qualitative, variations were responsible for the variations in skin permeability. Smith et al. (73,76) studied the effect of lipid on the aggregation and permeability of human stratum corneum by fragmentation and grinding of the stratum corneum, from both calf and plantar regions, before lipid extraction. The lipid-depleted cells were re-combined with the extracted lipid. The effectiveness of the barrier function of the stratum corneum sheets

was directly proportional to the amount of lipid added. They also observed a higher water permeability when calf cells were reaggregated with plantar lipid than with an equal amount of calf lipid. They concluded, therefore, that the importance of lipids in skin permeability is both qualitative and quantitative. Although this work has been accepted by some workers (22), it is not known whether the reaggregation experiments produce lipid arrangements within stratum corneum matching the original, because the extracted lipids may crystallise differently *in vitro* (58). Certainly Vinson *et al.* (97) reported a dramatic rise in skin permeability when chloroform-methanol was applied to the skin surface and evaporated without removal of the lipid. This suggests an altered structure of lipid barrier after it has been dissolved in the organic solvent. Thermal analysis (chapter 2) has shown a change in the thermal behaviour of lipid when extracted from stratum corneum with C/M (2:1) and other organic solvents. After evaporation to dryness, the third lipid transition (normally at 80°C) was missing (page 28). In this work it has been established that changes in T80 indicate an alteration of the lipid barrier causing an increase in skin permeability. This may explain the result obtained by Vinson *et al.*

Qualitative differences in the lipid composition of stratum corneum from different body sites have been excluded by Elias and his collaborators (20-22, 165, 166). Their conclusion was based on lipid fractionation using Thin-Layer Chromatography. However the qualitative difference detected by DTA in this work is not necessarily observed using TLC because the latter technique is unable to detect variation in the structural arrangement of lipid components. Therefore, for a better understanding of the lipid barrier, it is necessary to develop techniques to study lipids *in situ*.

7. In the present work, all substances which increased skin permeability were found to interact with stratum corneum lipid as indicated by their altering the size and temperature of the lipid endotherms at 71° and 80°C (see chapters 2,3,5).

From the above discussion, it is concluded that the diffusional resistance of stratum corneum is provided by highly organised intercellular lipids.

**B. Mechanism of Action of Sorption Promoters**

In the present work various sorption promoters have been examined for their ability to enhance skin permeability. Whilst these accelerants have different physico-chemical properties, e.g., liquids and solids, volatile and non-volatile, water-soluble and lipid-soluble, they also have the common features of a potential hydrogen bonding capacity and the ability to dissolve in lipids. Their effect on stratum corneum was investigated using both thermal analysis, to examine the effect on stratum corneum lipids, and permeability studies.

When water-soluble accelerants, such as DMSO, were used in an aqueous vehicle, it was observed that high concentrations were required for the accelerant to produce an effect on skin permeability. With lipid-soluble accelerants, such as capric acid, in aqueous vehicles, low concentrations were sufficient to produce a pronounced increase in skin permeability. This difference may be explained in terms of the skin/vehicle partition coefficient. Although no measurements of partition coefficient were made in this work, there is evidence to support the above suggestion. After pre-treating stratum corneum with various concentrations of DMSO (50,70 and 90%), thermal analysis revealed a reduction in the peak temperatures of the

lipid endotherms (page 55). This reduction was directly proportional to the concentration of DMSO, indicating that more DMSO has partitioned into the stratum corneum lipid as concentration increased. Also, comparison of the effect of 5% capric acid in 66.7% propylene glycol and in isopropyl myristate (IPM) on skin permeability, showed that the effect of capric acid was reduced 6 times when incorporated in IPM (page 166). Unlike DMSO, which binds strongly with water (83,84,233, 234) to give a low skin/vehicle partition coefficient, capric acid has a lower affinity for aqueous vehicle (and a higher affinity for IPM) and, therefore, has a high skin/vehicle partition coefficient. The converse is true with IPM. Cooper (212) investigated the partitioning of decylmethylsulphoxide ($C_{10}$MSO) into human stratum corneum and found a 25-fold increase in the amount of $C_{10}$MSO partitioned into stratum corneum when the concentration was raised from 0.1 to 1.0% $C_{10}$MSO in aqueous vehicle. Sekura and Scala (256) studied the penetration rates of alkylmethylsulphoxides and their effect on the penetration of sodium nicotinate and found that their effect increased with alkyl chain length up to decyl ($C_{10}$). This may be attributed to their affinity for the aqueous vehicle being reduced with the increasing chain length. Addition of a hydroxyl group to $C_{10}$MSO reduced its effect on the penetration of sodium nicotinate, probably because its aqueous solubility was increased. This indicates the significance of partition coefficient and may explain why a higher concentration of DMSO than of $C_{10}$MSO is required to produce an equal effect on skin permeability. Cooper (212), based on his studies using DSC and permeability studies, concluded that $C_{10}$MSO interacted with stratum corneum proteins, altering transport by opening keratin-controlled polar pathways. This explanation is not in agreement with the general effect of accelerants presented in this thesis. Although

$C_{10}$MSO has not been investigated in this work, its reported behaviour is consistent with an interaction with stratum corneum lipid being the mechanism of action.

The behaviour of the penetrant has also to be considered. If a penetrant has a higher affinity for the accelerant/vehicle system than for the stratum corneum, an increase in the diffusivity of the barrier may not be observed. This has been demonstrated when alkanols were incorporated in solutions of DMSO, DMAC, capric acid and Azone (pages 111,121,169,172). The effect of an accelerant is better observed if the stratum corneum has been pre-treated with the accelerant, especially if it is hydrophobic, and the penetration of the alkanol is then determined from water. However, in this case a water-insoluble sorption promoter, which has already reacted with the stratum corneum lipid barrier, may also increase the skin/vehicle partition coefficient of penetrants, and hence increase penetration rate. For example, as reported on page 172, the enhancing effect of azone for propanol was 4 times that for hexanol. This difference may be due to the increased skin/vehicle partition coefficient for propanol. Hexanol, already having a high partition coefficient (11), is probably little affected by azone.

In his study on the effect of $C_{10}$MSO on the partitioning of salicylic acid into stratum corneum, Cooper (212) found no increase in the penetration of undissociated salicylic acid but a significant increase when ionised in an alkaline pH. Thus it appears that polar molecules are more affected by a sorption promoter than non-polar molecules.

The present work has again demonstrated the importance of the vehicle used for an accelerant. Fatty acids, esters and alcohols have long been incorporated in pharmaceutical formulations (25) but,

probably because of their high affinity for the bases, little or no enhancing effect has been obtained. This effect of the vehicle in enhancing the effect of sorption promoters has already been discussed. It can be concluded that, to obtain the optimum effect of an accelerant, it must be treated as a penetrant, i.e., a vehicle should be used which will produce the highest thermodynamic activity of the accelerant, so that more of its molecules partition into and interact with the lipid barrier phase of the stratum corneum.

From the results obtained by TLC (chapter 4), it has been concluded that the lipid transitions, observed in neonatal stratum corneum sheets at 71° and 80°C, were due to free fatty acids. Shelf stearic acid showed a reduction in peak temperature from 72° to 64°C when exposed to DMSO vapour overnight (for methods see page 21). This suggests that the alteration of lipid endothermic temperatures, observed following the treatment of stratum corneum with penetration enhancer, indicate an interaction between fatty acids and the accelerant. However, some lipid species such as sterol esters, triglycerides, and sphingolipids did not show any thermal transitions (see chapter 4). These lipids, also, may have interacted with the accelerants.

The effect of sorption promoters on cholesterol was studied using both DTA and IR spectroscopy. As shown in Figure 6.2, thermal analysis of shelf cholesterol previously exposed to DMSO vapour for 24 hours produced endothermic transitions at 91°, 121° and a small one at about 140°C and no transition at 40°C. A second heating run showed that the 91°C transition had almost disappeared and a small transition at 40°C has appeared whilst T121 and T140 are unaffected. The nature of these transitions and the irreversibility of the T91°C have not been investigated further. The melting transition of cholesterol at 149°C was also lowered to 122°C when cholesterol was blended with azone in

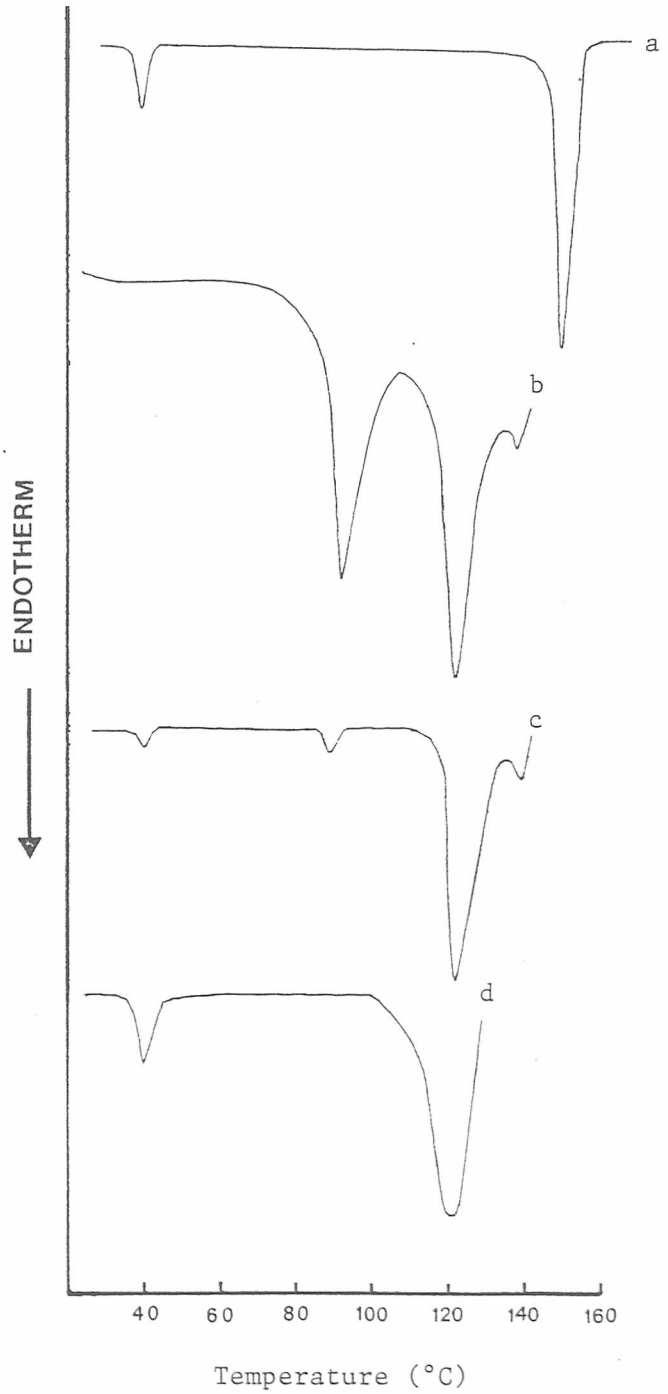



Figure 6.2 DTA thermograms of cholesterol.
(a) Untreated cholesterol, (b) exposed to DMSO vapour for 24 hrs prior to DTA, (c) second run of (b), (d) mixed with azone in the ratio 2:1. w/w.

the ratio 2:1 (w/w). Therefore, although the number of endotherms for DMSO-treated cholesterol and azone-treated cholesterol are different, both systems showed a major reduction in the melting temperature of cholesterol. Addition of capric acid to cholesterol resulted in the absence of the melting transition (149°C)(page 155). These effects are consistent with their effect on skin permeability reported in chapters 3 and 5 (pages 111,166,172).

The IR spectra (for methods see page 103) of cholesterol, azone, and capric acid are shown in Figure 6.3 and those of mixtures of cholesterol-DMSO, cholesterol-azone and cholesterol-capric acid in Figure 6.4. Cholesterol shows two absorption bands at $3620cm^{-1}$ (sharp) and $3400cm^{-1}$ (broad), indicating free and bound hydroxyl groups, respectively (similar to those observed in hexanol reported on page 115). A significant reduction in the size of the sharp transition was observed in the presence of DMSO, azone and capric acid (Fig. 6.4). These changes suggest that hydrogen bonding is taking place between cholesterol and these sorption promoters, although non-polar bonds are not excluded. This finding is in good agreement with the earlier suggestion (page 59) that the lipid-DMSO interaction was through hydrogen bonding. From the information obtained in the present work, it appears that sorption promoters interact with free fatty acids and free cholesterol in stratum corneum lipids. However, other lipid species, such as ceramides and sphingolipids (85) also have a hydrogen bonding capacity. Unfortunately, DTA of these lipids did not produce any transitions (page 145). The DSC work of Rehfeld et al. (41) also failed in this respect. Despite this, the possibility of an effect of an accelerant upon them cannot be excluded. It has been suggested (22,85,301) that both sphingolipids and cholesterol sulphate are important for the efficiency of the skin

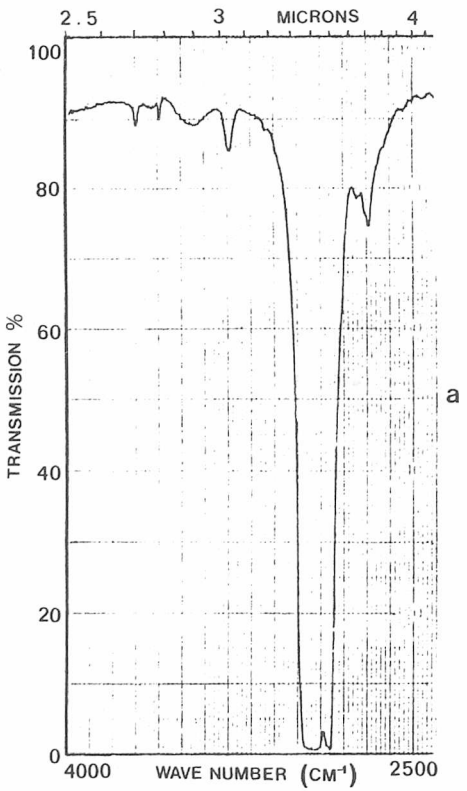
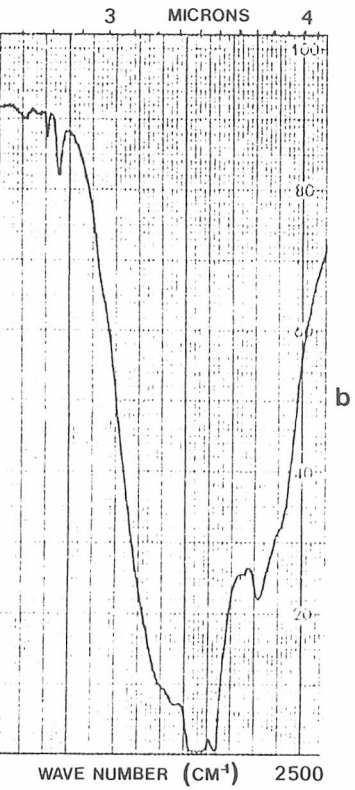
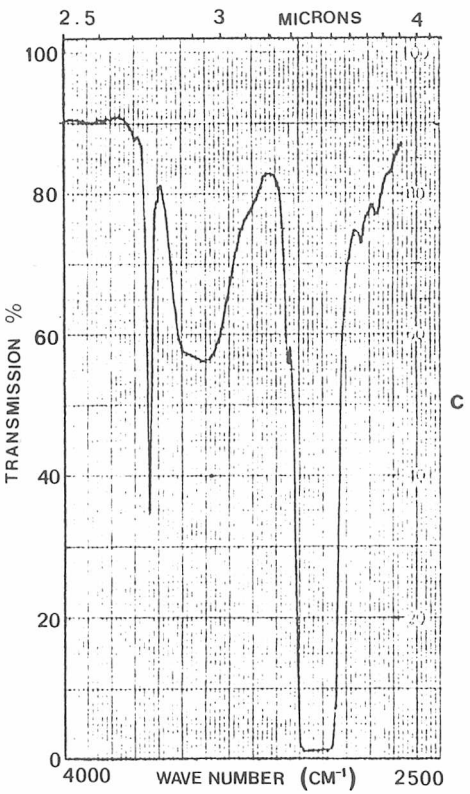



Figure 6.3 Infrared spectra of (a) azone,




(b) decanoic acid, and (c) Cholesterol, in carbon tetrachloride.

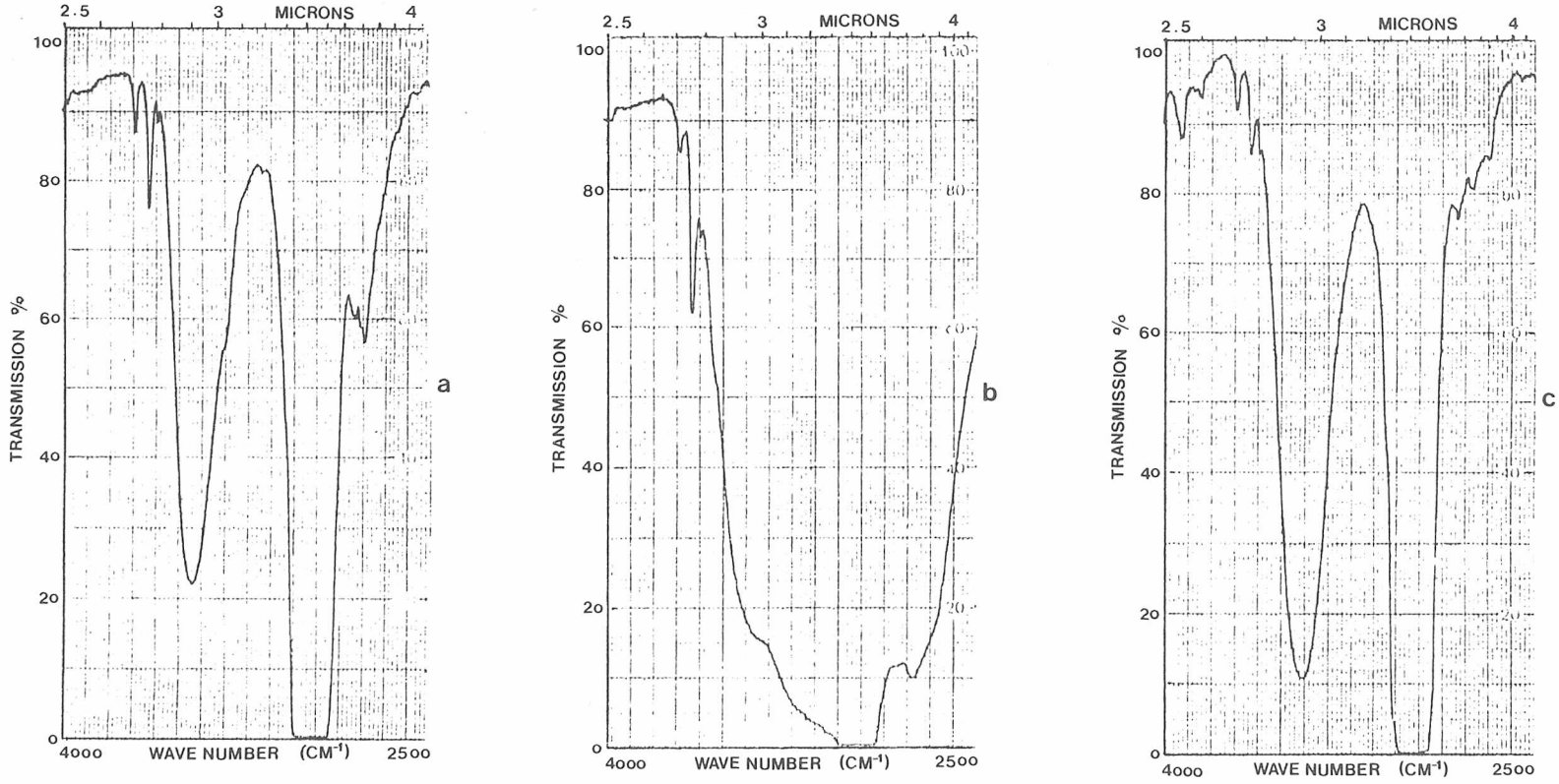



Figure 6.4 Infrared spectra of (a) 2:1 (v/w) mixture of azone and cholesterol, (b) 1:1 (w/w) mixture of decanoic acid and cholesterol, and (c) 1:1 (v/w) mixture of DMSO and cholesterol, in carbon tetrachloride.

permeability barrier. According to the results obtained in this work and from the literature (22,65,71,85,166,282,294,295,301), it may be suggested that all lipid species found in stratum corneum lipid play a role in the stability and impermeability of the lipid barrier. Any disturbance, of any lipid species may alter the integrity of the barrier, thereby leading to increased skin permeability.

In this work, DTA has been found to be a useful tool in studying the effect of factors which affect skin permeability and in particular the effect of sorption promoters. It may provide a useful *in vitro* screening test for potential penetration enhancers. The effect of accelerants has been attributed to their interaction with stratum corneum lipids. The exact nature of this effect is not known, but may be by altering the physico-chemical properties of the lipid barrier. This may include disordering of organized lipid membranes (250) including breaking inter-lipid hydrogen bonds, reducing the consistency of the lipid barrier (fluidization) (269), increasing skin/vehicle partition coefficient of penetrants, and extracting some lipid components from stratum corneum (78). Depending upon the physico-chemical properties of the penetration enhancer, one or more of these mechanisms may assume greater importance.

# CHAPTER SEVEN

## CONCLUSION

From results obtained in this study, the following main conclusions may be drawn:

1. Thermal analysis of neonatal rat stratum corneum shows three lipid endothermic transitions at 42°, 71° and 80°C and a broad endothermic transition at 110°C, the latter associated with desorption of water. Moisture content of stratum corneum has an effect on the resolution of the transitions, the dryer the sample the more distinct are the transitions.

2. Whilst sample weight and type of instrument (DTA or DSC) have no effect on the peak temperatures of the transitions, heating rate ($°C\ min^{-1}$) of stratum corneum does have an effect.

3. The lipid transitions at 42° and 71°C are reversible even when heated up to 140°C, although both transitions show reduction in their peak temperatures. The broad transition at 110°C is lost on heating but can be regenerated by exposure to water or moisture (50-60% RH) for 24 hours. T80 is irreversible, its loss being dependent on, (a) temperature of exposure, (b) time of exposure, and (c) method of preparing samples for heat treatment, i.e., folded or unfolded stratum corneum.

4. Transition at 42°C is associated with cholesterol, both T71 and T80 are associated with melting of free fatty acids fraction of stratum corneum lipid. The disappearance of T80, from heat-treated stratum corneum (and its absence from lipid extracts), is most probably due to an interaction of free fatty acids and cholesterol producing a eutectic-type mixture of lower melting temperature.

5. With the exception of human plantar, stratum corneum obtained from different animal species and human body sites are of similar

chemical structure and order because they show very similar patterns of DTA thermograms, although with varying endothermic temperatures. For each skin type, the third lipid endothermic transition was observed at 78°, 80°, 84°, 87° and 91°C for stratum corneum of neonatal rabbits, neonatal rats, neonatal mice, human (arm), and human (buttock), respectively, and in each case was irreversible.

6. Using *in vitro* methods, the effect of hydration on the permeability of stratum corneum to alkanols was found to be greater when using hydrophobic, non-aqueous vehicles such as light liquid paraffin (LLP). The penetration of the polar alkanol, propanol, was affected to a greater extent by hydration of stratum corneum than was that of the nonpolar alkanol hexanol.

7. Penetration of hexanol is mainly though the continuous intercellular lipid, whilst propanol diffuses alternately through intercellular lipid and aqueous regions of stratum corneum.

8. The vehicle is of great importance in the penetration of alkanols. The penetration rate of both propanol and hexanol is slower when there is a penetrant-vehicle interaction, e.g., both penetrants with aqueous solutions of propylene glycol, DMSO and DMAC. Otherwise, the affinity of the penetrant for the vehicle affects penetration. Thus, for example, with hydrated stratum corneum, propanol penetrates faster from LLP than from water and the converse is true with hexanol.

9. When alkanols penetrate from aqueous solutions of DMSO, two opposing mechanisms are involved, (a) a penetrant-vehicle interaction which reduces the rate of penetrant release from the vehicle, and thereby slows the penetration rate, and (b) a skin-vehicle interaction, which reduces the diffusional resistance of the skin barrier, thus increasing the penetration rate. The balance of the two effects

determines the overall effect on penetration rate of alkanols. The effect of DMSO on the permeability of stratum corneum is reversible below and irreversible above 80% DMSO. The effect of DMSO on both lipid endothermic transitions and stratum corneum permeability increases with concentration, i.e., depends on the amount of DMSO partitioned into the lipid phase of stratum corneum. 100% DMSO in addition to its effect on lipid physico-chemical properties, also extracts lipid from stratum corneum. Pre-treatment of stratum corneum with aqueous solutions of DMAC produces similar effects to DMSO which are also irreversible at concentrations above 80% DMAC.

10. Stratum corneum lipid is highly organised and provides the diffusional resistance to drug penetration. Heating above 70°C, causes alterations in the organised lipid structures, the effect increasing with time of exposure.

11. Pre-heating stratum corneum above 70°C causes an increase in permeability. Exposure of stratum corneum to DMSO vapour also increases permeability. The use of both pre-heating (above 75°C) and exposure to DMSO vapour gives a permeability equal to that of heat treatment alone, indicating a common site of effect. Changes in the T80 endotherm indicates this site to be stratum corneum lipid.

12. The various sorption promoters increase skin permeability by their interaction with the lipid barrier of stratum corneum.

13. Changes in the T80 endotherm are always associated with increases in permeability. The possibility that lipid transitions of stratum corneum may form the basis of a screening test for potential sorption promoters has been explored with encouraging results.

14. Choice of vehicles for sorption promoters is very important for

their action. In general, for optimum effect, vehicles which provide higher skin/vehicle partition coefficient (higher thermodynamic activity) for sorption promoters are required.

15. The difference in skin permeability between species and anatomic sites are probably due to a qualitative difference between the lipid structures of stratum corneum. Free fatty acids in the lipid barrier are candidates for these variations.

16. DTA has been found to be a useful tool for investigation of factors affecting transdermal permeation, in particular sorption promoters. It may be used as a rapid in vitro screening test for sorption promoters.

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# Appendix

## Materials

**A - Radiolabelled Chemicals**

[1- $^{14}$C] n-propanol (Radiochemical Centre, Amersham),

[1- $^{14}$C] n-hexanol (ICN Pharmaceuticals, Inc.),

[$^{14}$C] n-hexadecane (Radiochemical Centre, Amersham).

**B - Other Chemicals**

1. Normal alkanols

   Methanol, ethanol (Fisons Scientific Equipment), decanol (Hopkin and Williams Ltd., England), dodecanol (BDH Chemicals Ltd.).

2. Esters

   Ethyl butyrate, ethyl cinnamate (BDH Chemicals Ltd.), isopropyl myristate (Sigma Chemical Company).

3. Carboxylic acids

   Formic acid (90%), butyric acid, hexoic acid, octoic acid, decanoic acid, tetradecanoic acid, hexadecanoic acid, octadecanoic acid (BDH Chemicals Ltd.), acetic acid, dodecanoic acid, oleic acid (Fisons Scientific Equipment).

4. Miscellaneous chemicals

   Acetone, cholesterol, tris (hydroxy methyl methylamine) (BDH Chemicals Ltd.), benzene, chloroform, dimethyl acetamide, dimethyl sulphoxide, propylene glycol, petroleum ether (60-80°C), trypsin (Fisons Scientific Equipment), diethyl ether (Koch-Light Laboratories Ltd.), hydrochloric acid, sodium hydroxide (May and Baker, England), light liquid paraffin, sodium dodecyl sulphate,urea (Thornton and Ross Ltd., England), 2-mercaptoethanol, protease VIII (subtilisin), 8-aniline-1-naphthalene sulphonic acid (Sigma Chemical Company), insta-gel

scintillation liquid (Packard Instrument Ltd., England) azone (a sample provided by Nelson Research, California, USA).

C - Source of skin

1. Human skin

   Obtained from a forearm of a female aged 16 years, buttock of a female aged 22 years and plantars of adults.

2. Mice skin

   Obtained from neonatal mice (albino outbred, OLAC MF1), OLAC 76 Ltd., England.

3. Rabbits skin

   Obtained from neonatal rabbits (Offspring of $F_1$, New Zealand/ California Cross), Rowett Institute of Technology, Aberdeen,U.K.

4. Rats skin

   Obtained from neonatal rats (albino outbred, spraque dawley), School of Pharmacy, RGIT, Aberdeen.

D - Apparatus

1. Differential Thermal Analyser (DTA) (Stanton Redcroft Model 671B and BD9 recorder), Stanton Redcroft, England.
2. Differential Scanning Calorimeter (DSC), (Du Pont Model 900 with DTA/DSC Cell), Du Pont (U.K.) Ltd.
3. Infrared spectrophotometer (Perkin-Elmer 681), England.
4. Thermogravimetric apparatus (Home-made apparatus), School of Chemistry, RGIT, Aberdeen.
5. Diffusion Cells (Manufactured by Norlab Instruments, Aberdeen).
6. Liquid scintillation counters (Packard Tri-Carb 460D and 2425), Packard, Instruments Ltd., England.
7. Injection Humidity Oven (Vindon Scientific Ltd., England).
8. Thin-layer pre-coated plastic sheets (silica gel G and aluminium oxide) (Polygram), Macherey-Nagel, Germany.

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