The role of the Stratum Corneum lipids and enzymes in skin condition

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Abstract

The stratum corneum lipids, mainly ceramides, cholesterol and fatty acids, play the predominant role in maintaining the water barrier of the skin. In order to understand the biological variation in the composition of the barrier lipids, the alterations observed in winter xerosis, with the seasons, following UV damage and across the different bodysites were investigated.

Marked seasonal changes in stratum corneum lipid levels were observed, being at their lowest in winter, when susceptibility to dryness is increased. In particular, a reduction in ceramide 1 linoleate was observed. In winter skin xerosis, dryness was associated with a decline in the ceramide : fatty acid ratio. This suggests that both the overall stratum corneum lipid levels and the relative proportions of the different lipids may impact on barrier performance and susceptibility to dryness.

Although the stratum corneum lipids may contribute to corneocyte cohesion, the main cohesive force ensuring the integrity in the stratum corneum is provided by modified desmosomes called corneodesmosomes. These proteinaceous linkages are shown to be degraded during stratum corneum maturation by proteases such as ‘stratum corneum chymotryptic-like enzyme’ (SCCE). The role of lipids in modulating desquamation through influencing protease activity was investigated. Both cholesterol sulphate (elevated in recessive x-linked ichthyosis and following UV irradiation) and fatty
acids (elevated in cosmetic dryness) were found to inhibit SCCE activity. This highlights one mechanism through which the stratum corneum lipids can influence the desquamatory process.

Stratum corneum maturation includes changes in cornified cell envelope (CE) morphology, the highly crosslinked protein shell that encases the corneocytes. Transglutaminase 1 and 3 activity were identified within the stratum corneum, the levels of which increase during stratum corneum transit, alongside CE maturation. These effects on CE morphology may have an important structural role in maintaining the skin barrier.
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AMC 7-amido-4-methylcoumarin
Boc-phe-ser-arg N-tert-Butoxy-carbonyl-phenylalanine-serine-arginine
CE cornified cell envelope
DMSO dimethyl sulfoxide
Dsc1 desmocollin 1
Dsg1 desmoglein 1
EDTA ethylenediaminetetraacetic acid
EFA essential fatty acid
EFAD essential fatty acid deficiency
EM electron microscopy
FAME fatty acid methyl esters
FPLC fast protein liquid chromatography
GTP guanidine triphosphate
HCl hydrochloric acid
HPLC high performance liquid chromatography
HPTLC high performance thin layer chromatography
I11 Interleukin 1
Kda kilo dalton
LG lamella granules
MED minimal erythema dose
NaCl sodium chloride
PAGE polyacrylamide gel electrophoresis
PMSF phenylmethylsulfonyl fluoride
rSCCE chymotryptic-like enzyme
SD standard deviation
SDS sodium dodecyl sulphate
Suc-leu-leu-val-tyr succinyl-leucine-leucine-valine-tyrosine
TCA trichloroacetic acid
TEWL transepidermal water loss
TGase transglutaminase
UV ultraviolet radiation
V/v volume per volume
W/v weight per volume
CHAPTER 1

1. Introduction

1.1 The general structure of the skin

The skin forms the largest organ of the body, constituting a vast physical barrier between the organism and its environment. The skin of the average adult exceeds 2m² in area being more than 2mm thick in most places (Odland 1991). This organ can be divided into two main tissues (Figure 1.1). The outer stratified epidermis and the underlying dense fibroelastic tissue called the dermis. It is the lower layer which varies most in thickness over the different regions of the body. The dermis is highly vascularised, supporting an extensive neural network, specialised excretory and secretory glands and keratinized appendage structures such as hair and nail. Below the dermis is the subcutaneous tissue composed mainly of fatty connective tissue. Fibrous bands from the dermis traverse this layer forming attachments with the underlying skeletal components (Odland 1991).

The skin has a number of roles which are explained below:

• Barrier

The skin not only enables organisms to live within a dry environment, preventing excessive water loss, but it gives protection against physical, chemical and microbial attack (Odland 1991). The skin represents the interface between the body and a hostile environment, forming its first line of defence. Skin cells produce a range of biochemical markers which contribute to both cutaneous immune or
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inflammatory responses. Epidermal keratinocytes can produce cytokines (e.g. interleukin (IL): IL-1α, IL-6, IL-8) following appropriate stimulation, which can influence the function of lymphocytes and monocytes. In addition, these cytokines can influence the expression of cellular adhesion molecules, which help in the recruitment of immune cells. IL-1 has been shown to persist into the upper layer of the skin, the stratum corneum. It is proposed that the release of this pre-formed IL-1α may be important for triggering repair following skin disruption (wounding) (Kupper 1990).

- Thermoregulation

The vast array of capillaries at the skin surface, along with the sweating system help in thermoregulation. To reduce the body temperature during exercise or in response to a high external temperature, blood flow is enhanced at the surface and the production and hence evaporation of sweat is increased. The reverse is true as the body cools (Lenhardt et al 1999).

- Vitamin D synthesis

The skin is a major site of vitamin D synthesis in the body. UV light activates a vitamin D precursor which is then transported to the liver bound to Vitamin D binding protein (VDBP) in plasma. 25-hydroxilation occurs in the liver, followed by activation (1-hydroxylation) or inactivation (24-hydroxylation) by the kidney (SchmidtGayk et al 1997).
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- **Excretion**

  Sweat evaporation forms part of the skin's thermoregulation process. The presence of a range of organic compounds and salts makes it a good vehicle for excretion.

- **Others**

  The skin possesses a mass of nerve endings and receptors that respond to pain, temperature, pressure and touch. It may also account for 8-10% of the total body blood flow and therefore can act as a blood reservoir (Odland 1991).

![Figure 1.1 - A schematic diagram of the skin](image-url)
1.2 The epidermis

The epidermis forms a continuous covering over the whole body, being disrupted in its entirety only by the pores of glandular structures and hair follicles. This layer varies very little in thickness over most of the body, being between 75 and 150 µm, except on the palms and soles where its thickness may be 0.4-0.6mm. Structurally, the epidermis consists of an outermost anucleate, cornified cell layer, the stratum corneum and three main living inner cell layers. The principal cell type in this epithelia is termed the epidermal cell, or more commonly the keratinocyte. This term is derived from the group of filamentous proteins, the keratins, which make up the cytoskeleton (Odland 1991).

The epidermis also possesses a number of immigrant cell types, such as melanocytes, Langerhans and Merkel cells. The melanocytes which are produced from the embryonal neural crest produce the UV protective pigment, melanin. Langerhans are specialised monocytes making up 2-6% of adult epidermal cells, and are thought to help in T cell immune responses. The initial origin of the Merkel cells is unclear as they are slightly larger than the keratinocytes and possess keratin intermediate filaments. They are located in the lower epidermis attached by desmosomes to the basal keratinocytes, and appear associated with nerve fibres. These cells are thought to act as slow mechanoreceptors, aiding in the sensation of touch (Odland 1991).

In most regions of the body the epidermis can be split into 4 distinct layers, as illustrated in Figure 1.2. These layers are termed the stratum basale, spinosum,
granulosum and corneum (Fuchs 1990). Occasionally the stratum corneum is defined as 2 readily distinguished layers, the stratum compactum (1st few cell layers) and the stratum disjunctum.

**Figure 1.2 - A schematic diagram of the layers of the epidermis**

**Stratum basale**

This layer is composed of a single layer of cuboidal to columnar cells which form the germinative zone of the epidermis. The basal keratinocytes contain intermediate cytoplasmic filaments, made up of equal proportions of keratin K5 (58Kda) and K14 (50Kda) (Nelson and Sun 1983). These cells are attached together by desmosomes, forming
an anchorage for the intermediate filaments and interconnecting the cells in a three dimensional lattice. Each desmosome has a pair of dense plaques adjacent to, or on the cell membrane, composed of the proteins desmoplakins and plakoglobin. Within the cell these plaques are bound to cytoplasmic filaments, termed tonofilaments. The glycoproteins found within this cell junction are members of the cadherin family, desmocollins and desmogleins (Nelson and Sun 1983). These proteins play an important role in the cellular cohesion.

In this layer, cells which are committed to differentiation into stratum corneum cells or corneocytes, are then displaced outward and migrate upwards, taking on a polyhedral shape. The keratins produced by these more differentiated cells changes to keratin K1 (67Kda) and K10 (56.5Kda). These keratins become aggregated to form tonofilamentous bundles, forming the stratum spinosum (Eichner et al 1986), so named because of the spiky, stellate shape of the keratinocytes.

**Stratum spinosum**

This stratum consists of several layers of polyhedral cells, which contain keratin bundles, named tonofibrils. As the spinosum cells mature, they begin to flatten and form lysine and glutamine rich proteins such as involucrin (65Kda). These are layed down on the inner face of the plasma membrane, giving more structure to the cells. In the uppermost cell layers submicroscopic lamellar granules (100 x 300nm) begin to appear, these cells migrate upwards to form the stratum granulosum.
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Stratum granulosum

As the cells enter this layer the production of keratins eventually stops. Although the granulosum is only 1-2 cells thick, it plays a vital role in a number of key events. One of the most distinctive events in this layer is the appearance of the keratohyalin containing granules, which give this layer a granular appearance and hence its name (Holbrook 1989). These structures contain a histidine rich basic protein, profilaggrin (MWT, 350,000), which may have a role in bundling together the tonofilaments. Profilaggrin is processed to form filaggrin during the transition from the granulosum to the corneum. Filaggrin later breaks down to produce free amino-acids, urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA) natural sunscreens and moisturising factors (Scott et al 1982). Loricrin, the major component of the cornified cell envelope is also synthesised in this layer (Hohl et al 1991).

The stratum granulosum (and spinosum) are key sites for the synthesis of lipids such as the glucosylceramides, cholesterol and fatty acids. These lipids are packaged into vesicles termed, lamellar granules in the endoplasmic reticulum. The lamellar granules are 0.1-0.3µm ovoid organelles which contain, in addition to lipids, hydrolytic enzymes (Elias 1981). As the cells migrate upwards towards the stratum corneum the lamellar granules align themselves at the upper surface of the now flattened cells. The lipids and enzymes are then released or merge with the plasma at the granulosum / corneum interface (Odland and Holbrook 1981, Wertz et al 1986).
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Stratum corneum

The final event in epidermal differentiation is the formation of the stratum corneum (Holbrook 1989). This is a unique layer, being comprised of 15-20 layers of flat (0.5μm thick, 30-40μm wide), anucleated, non-viable cells, called corneocytes. These corneocytes are continually lost from the skin surface by a process termed desquamation. The epidermis is therefore able to renew itself in roughly 26-35 days. The stratum corneum turnover being 14 of these days.

The transition between the stratum granulosum and corneum is marked by a number of key events. One of the main ones is the degradation of all cellular organelles and disappearance of the nuclear envelope (Holbrook 1989). Although keratinization has been regarded as a specialised form of apoptosis or programmed cell death, the mechanism for this cellular transition at the molecular level is not yet well understood. Concomitant with this degradative stage is the appearance of a dense marginal band adjacent to the inner face of the plasma membrane (Odland 1991). The increased permeability of the cells to calcium activates epidermal transglutaminase, resulting in the crosslinking of envelope proteins (e.g. involucrin and loricrin) beneath the plasma membrane. This is initially laid down in the upper spinosum cells. This represents the initial formation of the cornified cell envelope which encases the final corneocyte.

The cell membrane of the keratinocyte is degraded by lipases (phospholipases) released from lamellar bodies, resulting in a progressive depletion of the polar lipids such as the phospholipids. Of the lipids released from
the lamellar bodies, the polar precursors are converted to the non-polar barrier lipids. So for example, the glucosyl ceramides are converted to the less polar ceramides by the action of glucosylcerebrosidases. The appearance of sphingolipids, in this case ceramides, which represent up to 50% of the stratum corneum lipids (Gray et al 1982) is a key requirement to producing the inert water barrier (Elias and Friend 1975, Elias and Menon 1991). The lipids which remain in the stratum corneum, ceramides, cholesterol and fatty acids, spontaneously form multilamellae sheets between the corneocytes.

The corneocytes of the stratum corneum are composed essentially of a network of extensively disulphide cross-linked keratin filaments encased with a highly cross-linked isopeptide bonded protein envelope, the cornified envelope (Hohl 1990). The overall integrity of the stratum corneum depends particularly upon intercellular protein linkages called desmosomes. In the stratum corneum these structures are modified and have been called corneodesmosomes (Allen 1975, Chapman and Walsh 1990). The modification appears to involve the plaque region which is an electron lucent zone found on the inside of the viable cell membrane, and electron dense in corneocytes. In addition, alterations in the desmocollin 1 (DSC1) and desmoglein 1 (DSG1) expression profile have been observed during differentiation (Arnemann et al 1994). These structures become crosslinked into the corneocyte structure through the action of transglutaminases.

The corneocytes have no biosynthetic capacity, although they contain a raft of enzymes e.g. phospholipases, ceramidases, sphingomyelinases and glucosylcerebrosidases.
along with proteases (Mao-Qiang et al 1996, Menon et al 1986, Holleran et al 1993, Jin et al 1994), which remain active following corneocyte formation. The cell envelopes which encase the corneocyte are coated with covalently bound lipids (Swartzendruber et al 1987). The origin of these bound lipids is unknown, although they may be derived from the lamellar granule membranes. This lipid coating may facilitate the interaction of the hydrophilic corneocyte proteins with the hydrophobic intercellular lipid lamellae. The overall morphology of the stratum corneum is likened to a brick wall with corneocytes (bricks) and intracellular lipid matrix (mortar) (Elias 1983). A schematic structure of the corneocyte is shown in Figure 1.3.

![Schematic structure of a corneocyte](image)

**Figure 1.3** A schematic structure of a corneocyte.

Although the stratum corneum has been considered a 'dead' tissue, there are a number of biochemical processes that are active. In the initial cell layers (stratum compactum), a majority of the corneodesmosomes are degraded by proteases, although enough remain to contribute to corneocyte cohesion (Chapman et al 1990).
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Filaggrin, is also rapidly degraded by proteases to form natural moisturising factors (NMFs) which bind water (Scott and Harding 1986).

During stratum corneum transit, the exact composition of the barrier lipids can alter as a result of enzymic degradation. For example, cholesterol sulphate is degraded in the surface layers by steroid sulphatase (Elias et al 1984). Such modifications to the intercellular lipid profile along with corneodesmosomal proteolysis allows a weakening of the cohesive forces between the corneocytes. The degradation of corneodesmosomes in particular is vital to the cell shedding at the skin surface, by a process known as desquamation (Figure 1.4). This process is essential to maintain stratum corneum thickness and prevent a build up of corneocytes and scaling.
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Corneodesmosomes

Loss of Cell (Desquamation)

Stratum corneum

Stratum granulosum

Figure 1.4 Schematic diagram of the desquamatory process.

1.3 Stratum corneum barrier lipids

The major intercellular lipids of the stratum corneum are ceramides (~50%), cholesterol (~20%) and fatty acids (~25%) (Elias 1983). Other lipids can also be present in lower amounts, such as glucosylceramides, cholesterol sulphate, triglycerides and phospholipids. The types and relative amounts of these lipids are quite different to those found in viable epidermal cells (Figure 1.5).
In addition to the free intercellular lipids, the stratum corneum also contains several classes of covalently bound lipid which form an external coating around the cornified envelope of the corneocytes. These lipids are composed of ceramide (A & B, ~78%), fatty acids (~12.7%) and ω-hydroxy acids (~9.4%) (Wertz et al 1989). Their precise role has not been determined although they do form an essential interface between the cornified cell envelope proteins, such as involucrin and loricrin, and the nonpolar intercellular lipids. The covalently bound lipids may also be important for maintaining the correct orientation of the intercellular lipid bilayers and contribute to corneocyte cohesion. Certainly if the
intercellular lipids are extracted, corneocytes adhere strongly together, due to the interaction of the covalently bound lipids on adjacent corneocytes (Chapman et al 1991). The covalently bound lipids are found at ten times greater levels in epidermal stratum corneum compared with oral (palatal and gingival) stratum corneum (Chang et al 1993). This observation supports their essential role in maintaining an effective barrier in a dry environment.

1.3.1 Ceramides

The ceramides are a class of lipids composed of a long chain sphingoid base (16-22C), which is most commonly sphingosine, but can be dihydrosphingosine, phytosphingosine or 6-hydroxy sphingosine, amide linked to the carboxylate head group of long chain non-hydroxy or alpha-hydroxy fatty acids (24-34C) (Wertz et al 1983, Robson et al 1994). More recently a new ceramide has been identified, constituting about 9% of the total ceramides, and was shown by NMR spectroscopy to be N-acyl-6-hydroxysphingosine (Stewart and Downing 1999). Of the seven main ceramide groups, ceramide 1 is the least polar, having a fatty acid esterified to the ω-hydroxylated amide-linked fatty acid (Figure 1.6) (Bowser et al 1985). The same can also be true of ceramide 6. Each of these ceramide groups has a corresponding glucosylceramide precursor, with the glucose attached to the sphingoid base (Gray et al 1978).
Figure 1.6 The structures of the stratum corneum ceramides
(Wertz et al 1983)
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Ceramide 1 is unique to skin, forming a repository of epidermal linoleic acid. As ceramide 1 has a very long carbon chain it was initially thought to have a cohesive function in the stratum corneum, spanning adjacent lipid bilayers acting as a "molecular rivet" (Downing et al 1987). However, this is now thought to be thermodynamically unfavourable. More recently, it has been shown to enhance stratum corneum flexibility at low environmental relative humidities (Rawlings et al 1992), and enhance lipid fluidity, preventing the crystallisation of other stratum corneum lipids at physiological temperatures (Oldroyd et al 1994). Recent X-ray studies have further emphasised the importance of ceramide 1 to the lipid organisation of the stratum corneum. Using defined lipid mixtures to examine the structure of lipid lamellae in vitro, diffraction patterns similar to those obtained in vivo rely on the addition of ceramide 1 (Bouwstra et al 1998).

In addition to the free ceramide species described above which form the lipid lamellae between the corneocytes, the stratum corneum also contains two long chain ceramides which are covalently bound to the corneocyte cornified envelope proteins (Wertz et al 1989, Marekoy and Steinert 1996). These two species, named ceramide A and B, make up some 80% of the total bound lipid. Ceramide A, the major component (53%) consists of C30-34 ω-hydroxy acids amide linked to sphingosine. The more polar Ceramide B contains the same hydroxy acids linked to the novel sphingoid base 6-hydroxy-4 sphingenine (Robson et al 1994).

Ceramide biosynthesis is triggered within the granular layer, beginning with the condensation of serine and palmitoyl CoA to form 3-Ketosphingenine. This is
catalysed by serine palmitoyl transferase and is the rate limiting step in sphingolipid biosynthesis. The next steps involve reductase dehydrogenase and ceramide synthase (Merill and Jones 1990). The ceramides are finally converted to glucosyl ceramides by specific glucosyltransferases, before being packaged into the lamellar granules (Braun et al 1970). Upon release at the stratum granulosum / corneum interface beta-glucocerebrosidase enzymes cleave off the sugar residues. The highest levels of this enzyme is observed in the lower stratum corneum (Takagi et al 1999).

The stratum corneum also contains ceramidase which cleaves the amide linked fatty acid off ceramide (Wertz and Downing 1990). This results in the release of free sphingosine, which is known to inhibit protein kinase C (PKC) and has been shown to inhibit cellular differentiation in a number of cell types (Merrill and Stevens 1989) and via topical application to skin (Gupta et al 1988). Spingosine also has an anti-microbial effect (Bibel et al 1991). It has been hypothesised that the release of sphingosine could serve as a differentiation regulator in skin (Wertz and Downing 1990b). However, it may not be bioavailable as it has been shown to be complexed with free cholesterol sulphate which is also present in low levels in the stratum corneum (Downing et al 1993).

1.3.2 Sterols and derivatives

The stratum corneum sterols consist almost exclusively of cholesterol, although cholesterol esters and diesters are found in the sebaceous lipid which coats the skin surface
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(Schurer and Elias 1991). The presence of cholesterol is essential for the skin's barrier performance. Barrier damage leads to an upregulation of cholesterol synthesis (Menon et al 1985). In addition to the free sterols, cholesterol can also be present in the covalently bound lipid fraction of palmoplantar corneum, clavus (Serizawa et al 1993).

Although cholesterol can be taken up by the skin from lipoproteins in the blood, the majority of the cholesterol in the stratum corneum is derived from de novo synthesis within the epidermis (Feingold et al 1986). The rate limiting step in this process is the formation of mevalonic acid by \( \beta \)-hydroxy-\( \beta \)-methyl glutaryl (HMG) CoA reductase. The 5 carbon mevalonate structure is then phosphorylated and three units are combined. Farnesylpyrophosphate along with two of these molecules are converted to squalene. The oxidation and cyclization of squalene to form lanosterol is irreversible. Finally lanosterol is converted to cholesterol.

1.3.3 Free fatty acids

Human sebaceous, or skin surface lipids contain high levels of free fatty acids, originating from the hydrolysis of sebaceous triglycerides (Clarys and Barel 1995). However, the stratum corneum contains epidermal derived fatty acids mainly of chain length C18-26 (Ansari et al 1970), reaching levels around 25% of the total Stratum corneum lipids (Gray and Yardley 1975). More recently it has been suggested that stratum corneum lipids from the deeper layers are more enriched in the
C24:0 & C26:0 fatty acids, when compared with the upper layers (Bronte et al 1997, Norlen et al 1998).

The stratum corneum fatty acids are mainly derived from epidermal phospholipid breakdown (Elias et al 1988). Fatty acid synthesis occurs in all the viable layers of the epidermis and is regulated by changes in the water and ion gradient across the stratum corneum (Grubauer et al 1987). As the skin both contains and requires essential fatty acids such as linoleic acid, these need to be obtained from the circulation.

1.3.4 Cholesterol sulphate

Cholesterol sulphate is found in epidermis in addition to cholesterol glucoside at approximately 3.5% (Lampe et al 1983). In fully keratinised tissues such as hoof, or hair, cholesterol sulphate is one of the major extractable lipids (Wertz and Downing 1984). Its presence in hoof which displays marked intercellular cohesion (Serizawa et al 1992), is supportive of its proposed role in intercorneocyte adhesion within the stratum corneum.

Unlike the other stratum corneum lipids, this lipid does not appear to be packaged in the lamellar granules (Grayson et al 1985). During the stratum granulosum / stratum corneum transition and during stratum corneum maturation, cholesterol sulphate is gradually degraded by the action of steroid sulphatase (Elias et al 1984). Analysis of desquamated corneocytes has shown that the levels of cholesterol sulphate are depleted, even relative to the surface stratum corneum layers (Ramasinghe et al 1986). The degradation of cholesterol sulphate towards
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the skin surface supports its role as a cohesive force within the stratum corneum. Its hydrolysis effectively reduces cellular cohesion and thus allows desquamation to take place. The mechanism by which cholesterol sulphate may influence cohesion is unproven, however, it has been suggested that it may influence desmosomal proteolysis, either indirectly through alterations in lipid phase behaviour or directly via inhibition of protease activity (Williams 1991). More recently, cholesterol sulphate has been shown to activate transcription of the transglutaminase 1 gene (Kawabe et al 1998). This enzyme is essential for corneocyte cell envelope formation.

1.4 Barrier regulation

Although the external environment is known to influence the biochemical events within the viable epidermis, the precise mechanism by which this occurs is not well understood. Murine barrier disruption by solvents and abrasion induces a wide range of epidermal events including induction of DNA synthesis (Proksch et al 1990), epidermal cytokine production (Wood et al 1992), lipid synthesis (Grubauer et al 1987), and lamellar granule release (Menon et al 1992). Some of these changes can be prevented by occlusion of the skin, suggesting the involvement of the water gradient as a signalling trigger (Grubauer et al 1989, Taljebini et al 1996). However, occlusion has been shown to have no effect on barrier recovery in humans using trans-epidermal water loss (TEWL) as a measure (Welzel et al 1996). Changes in the ion, particularly calcium gradient are thought to be key in the stimulation of barrier lipid synthesis in murine skin (Lee et al 1992)
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and lamellar body secretion (Menon et al 1994). The maintenance of an acidic pH is also thought to be important for barrier recovery, β-glucocerebrosidase requiring this for its activity (Mauro et al 1998).

All the stratum corneum lipids appear to be synthesised during barrier repair as highlighted using inhibitors to the key rate limiting enzymes (Feingold 1991). The barrier repair appears to occur in two phases in murine skin, an immediate repair phase (up to 4h) and a second longer repair phase (up to 24h). The synthesis of cholesterol and fatty acids increases almost immediately after barrier disruption, activation of the rate limiting acetyl-CoA carboxylase (fatty acid) and hydroxymethylglutaryl (HMG) CoA reductase (sterol) requiring only dephosphorylation. In contrast, the synthesis of glucosylceramides (ceramide precursors) is delayed until approximately 7 hours later, requiring transcription and translation of further enzyme (Holleran et al 1991). The magnitude of the repair response is modulated by the degree of barrier disruption, enhancement of HMG CoA reductase activity correlating well with the extent of damage (Proksch et al 1990). However the approach taken to disrupt the barrier does not appear to influence the repair response (Proksch et al 1990). In addition, low humidities can stimulate epidermal DNA synthesis in murine epidermis (Denda et al 1998).

Differences in the regulation of lipid biosynthetic enzymes have been highlighted recently with the discovery of the transcription factor, sterol regulatory element binding protein (SREBP). This is involved in the control of epidermal sterol and fatty acid synthesis.
but does not regulate the ceramide synthetic pathway (Harris et al 1997). SREBP is known to bind to response elements in the promoter regions of a number of lipid biosynthesis genes, enhancing gene transcription. These include HMG CoA reductase, acetyl coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) (Horton and Shimomura 1999). The role of SREBP in the barrier repair response has yet to be elucidated.

Damage to skin by a variety of environmental stimuli can elicit the production of a range of inflammatory mediators within the epidermis (Wood et al 1996). Lipid mediators such as the eicosanoids can be produced along with the water soluble peptide mediators such as the cytokines, interleukin 1α (IL-1α) and tumour necrosis factor α (TNFα). These mediators are known to induce leukocyte infiltration, through their induction of other chemotactic cytokines. The mechanism for the release of preformed IL1α from epidermal cytosolic stores is not well understood, although it is not affected by occlusion (Denda et al 1995). This suggests that the trigger for these factors and hence inflammation or epidermal hyperplasia, is not the water gradient.

The presence of high levels of IL-1α in the stratum corneum has led to the hypothesis that its release may represent a signal to the viable epidermis, triggering repair following skin disruption (wounding) (Kupper 1990). IL-1α may have a role in epidermal differentiation regulating the expression of cellular retinoic acid binding protein (CRABP II) and small proline rich proteins (Eller et al 1995). In addition IL-1α stimulates phospholipase A2 activity, enhancing release of
arachidonic acid from cell membranes, thus triggering the eicosanoid cascade (Homaidan et al 1999). The main lipid mediators produced in epidermis are the hydroxyeicosatetraenoic acids (12-HETE, 15-HETE), hydroxyoctadecadienoic acid (13-HODE) and prostaglandin E2. 13-HODE promotes skin differentiation, reversing the hyperproliferation caused by certain n-3 fatty acids (Miller and Ziboh 1990). Although much is known about the cellular signalling within the epidermis, our understanding of the regulation of barrier performance is still not well understood.

1.5 Stratum corneum cohesion

1.5.1 Corneodesmosomes and their degradation

The corneodesmosomes represent the main cohesive link within the stratum corneum. They consist of transmembrane glycoproteins, including desmoglein (dsg1) and desmocollin (dsc1) (Egelrud and Lundstrom 1989 & 1991) which are cross-linked into the cornified cell envelope via transglutaminases and span the intercellular space binding with the desmosomal proteins on adjacent cells. Since cells are continually being produced from the proliferating basal layer and undergoing differentiation, there is a need to degrade these cellular linkages, to allow the shedding of corneocytes from the skin surface. This ensures the thickness of the stratum corneum is maintained. This balanced process in which the peripheral corneocytes detach from adjacent cells and are released from the skin surface is termed desquamation. The degradation of a majority of the desmosomes occurs at the stratum compactum / stratum disjunctum interface (Skerrow et al 1989). However,
those remaining linkages are continually degraded during stratum corneum transit (Long et al. 1996). The desmosomes on the planar surfaces of the corneocytes appear to be degraded first, followed by those at the peripheral edge regions (Long et al. 1996). At these edges the cells are interdigitated, locking cells together.

The stratum corneum is a rich source of proteases, many of which are 'left over' from the autolytic process of its formation. The presence of proteases has been shown within the lamellar granules which are released at the granulosm / corneum interface along with the lipids (Menon et al. 1992). As there are numerous proteases within the stratum corneum it is a complex task to determine which one are responsible for the degradation of the desmosomal linkages. However, the most likely enzyme considered to be involved in this process is thought to be the serine protease 'stratum corneum chymotryptic enzyme' (SCCE).

SCCE is a human serine proteinase abundantly expressed within the human epidermis (Hansson et al. 1994) and hair follicles (Ekholm and Egelrud 1998). Analysis of recombinant SCCE (rSCCE) suggests it is produced with a 22-amino acid residue signal peptide and a propeptide of 7 amino acid residues. The SCCE propeptide is converted to the active form by tryptic digestion. The calculated molecular mass of unglycosylated active SCCE was 24.4 KDa. One N-glycosylation site is thought to be located near the C terminus (Hanson et al. 1994). Work on rSCCE has shown that it is an endoproteinase specific for amino acid residues with aromatic side chains in the P1 position (Skytt et al. 1995). SCCE was found to differ significantly from bovine chymotrypsin, human cathepsin
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G, and human mast cell chymases in regard to its inhibitor profile and substrate specificity (Egelrud 1993).

The role of SCCE in the terminal stages of epidermal turnover and desmosomonal degradation is supported by 1. its expression late within terminal differentiation (stratum granulosum) 2) an inhibition profile and a pH dependence similar to that of desmosomal (and dsg1) degradation in vitro and 3) localisation within the lamellar granules and intercellular spaces of the stratum corneum (Lundstrom and Egulrud 1991, Sondell et al 1994, Hansson et al 1994). In human oral mucosa SCCE was only localised within epithelial cells of the hard palate, which forms an orthokeratotic stratum corneum. No SCCE was localised to the epithelium of the lip or buccal mucosa, where the epithelium does not form a stratum corneum under non-pathological conditions (Sondell et al 1994). These observations support the link between SCCE and normal stratum corneum maturation.

The mechanisms by which desmosomonal degradation is controlled are currently unknown. One mechanism could be the proteolytic conversion of the inactive pro-SCCE to active SCCE (Hansson et al 1994), as both forms are found within the stratum corneum. However, the levels of active SCCE do not appear to alter with stratum corneum depth (Watkinson, Unilever Research, Unpublished observation). Alternatively regulation of SCCE may be influenced by the available 'free' water (water activity) of the stratum corneum. This has been shown previously to be responsible for filaggrin degradation to NMFs (Scott et al 1982). In vitro evidence is supportive of water activity regulating SCCE activity in
the superficial layers of the stratum corneum (Watkinson et al 1999).

Other proteases such as trypsin-like and cathepsin D-like enzymes have also been implicated in desquamation, yet for the most part the supporting evidence is poor (Suzuki et al 1992, Koyama et al 1993, Watkinson Unilever research, Unpublished observation). A trypsin-like protease is required for the activation of SCCE from its inactive pro-SCCE proform (Hanssen et al 1994, Nylander Lundqvist and Egulrud 1997). As desmosomal proteins are glycoproteins, the role of glycosidases in deglycating the proteins leaving them susceptible to proteolysis has been considered (Walsh and Chapman 1990). However, to date no specific desquamatory glycosidases have been identified in the stratum corneum.

1.5.2 Lipids and corneocyte cohesion

The lipids of the stratum corneum also contribute to the overall cohesion of this tissue. Evidence suggests that the lipids are modified during stratum corneum transit, leading to reduced cohesion in the superficial layers (Ranasinghe et al 1985, Bonte et al 1997, Elias and Menon 1991). Changes in the lipid composition at the skin surface are supported by an increase in lipid fluidity at the surface, compared with the deeper layers of the stratum corneum (Azimi et al 1992).
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1.6 The Stratum corneum cornified cell envelope

1.6.1 Cornified envelope structure

One of the main events in keratinocyte terminal differentiation is the production of a cell membrane associated cross-linked cornified cell envelope (CE) (Fuchs 1990, Matoltsy and Matoltsy 1965). This first appears at the stratum granulosum / corneum interface, as an electron dense band approx. 15nm thick (Hashimoto et al 1969). Under the differential interference contrast microscope (e.g. Nomarsky) the CEs show two distinct morphological forms (Rice and Green 1977, Marks et al 1983), the ratios of which vary according to depth in the stratum corneum (Figure 1.7). The majority of the cell envelopes at the base of the stratum corneum appear slightly smaller, with an irregular shape and ruffled surface. In comparison, most of those in the upper layers are larger and appear more flattened and polygonal with a relatively smooth surface (Reichert et al 1993). These two types of CE are termed fragile (CEf) and rigid or resilient (CEr) envelopes, respectively. The CE matures via a largely unknown process, although one possible mechanism is via the action of transglutaminases (TGases).

The initial process of CE formation is known to be catalysed by the calcium dependent transglutaminases (TGases) expressed by keratinocytes in the viable epidermis. Transglutaminases cross link precursor proteins such as involucrin and loricrin via $^{3}$-$\gamma$-glutamyl-$\varepsilon$-lysine isopeptide bonds (Rice and Green 1977). The structure is then further reinforced by multiple disulphide bonding between loricrin molecules and
loricrin and the intracellular cytokeratin filaments (Hohl 1990). Further transglutaminase (TGase) mediated crosslinking between the CE, desmosomal plaques and intermediate filaments leads to the formation of an essentially continuous protein backbone throughout the stratum corneum. This imparts a high degree of mechanical strength to the stratum corneum, helping it to withstand environmental insult.

Involucrin is a 65kDa rod-like protein (~45nm long and 1.5nm diam.) expressed within stratified epithelia early in the onset of terminal differentiation. Human involucrin has 37 repeats of 10 residues, three of which are glutamines and two are glutamates. The whole protein contains ~25% glutamine and ~20% glutamate residues. The repeat region is mostly α-helical in humans. CEs from human foreskin epidermis contained ~5% involucrin (Nemes and Steinert 1999). Loricrin is a CE protein expressed late in terminal differentiation. It contains three glycine rich domains which are thought to form flexible loops. These are interspersed with glutamine rich regions and flanked by lysine and glutamine rich amino and carboxy terminal domains (Nemes and Steinert 1999b).

Involucrin, the first envelope component identified, provides the early scaffolding upon which other components become organised. Proteins such as loricrin, cystatin α, pancornulins (SPR1 & 2), elafin and filaggrin have also been identified as envelope components (Reichert et al 1993). Cystatin α is initially crosslinked with involucrin, elafin and the SPRs then being crosslinked with loricrin. An elafin rich region is found in the ‘mid-region’ of the CE
Cystatin \( \alpha \) and elafin are proteolytic inhibitors, which may serve to protect the early envelope from unwanted proteolysis during stratum corneum formation. Alternatively they may provide protection against the invasion of microorganisms from the environment. Neither of the two inhibitors appear to effectively regulate the desquamatory protease stratum corneum chymotryptic like enzyme (SCCE) (Franzke and Christophers 1995). The SPRs (pancornulins) serve essentially as cross-bridging proteins among the loricrin, acting both as amine donors and acceptors. Crosslinks can also be formed through linker polyamines such as spermidine and spermine. Spermidine crosslinks are found at increased levels in psoriasis (Reichert et al 1993).

Although the effect of altering the CE protein composition on stratum corneum structure is currently unknown, the timing and order in which the proteins are laid down appears to be critical. This is supported by the precocious, early expression of involucrin and TGase in psoriasis resulting in the persistence of immature 'fragile' CEs at the skin surface (Reichert et al 1993).
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Figure 1.7  A schematic of the changes in cornified cell envelope morphology with stratum corneum maturation (S. Long, Unilever Research)
1.6.2 Transglutaminase biology

The transglutaminases (TGases) are a group of calcium dependent enzymes which catalyse the formation of a $\gamma$-glutamyl-$\varepsilon$-lysine isodipeptide bond between peptide bound glutamine and lysine residues (Figure 1.8). The TGases are involved in a wide range of physiological events including crosslinking of fibrin (Lorand 1986), epidermal cell envelope formation (Rice and Green 1979), immunologic phenomena (Auger et al 1993), coagulation of semen (Williams-Ashman et al 1972), neuronal differentiation (Maccioni and Seeds 1986), neurotransmitter release (Pastuszko and Ericinzka 1986) and the stabilisation of the cytoskeletal network of developing myotubules (Bersten et al 1983). Of the seven TGases identified in humans, only three are expressed during terminal differentiation in stratified squamous epithelia. These are the membrane associated TGase 1 or K (106KDa), soluble tissue type TGase 2 or C (80KDa) and the soluble pro-enzyme TGase 3 or E (77KDa). Of the three epidermal TGases, only TGase 1 and 3 are thought to be involved in CE formation, crosslinking CE proteins (Candi et al 1995).

In addition to the formation of a $\gamma$-glutamyl-$\varepsilon$-lysine isodipeptide bonds between peptide bound glutamine and lysine residues, TGases also catalyse the formation of a $\gamma$-glutamyl-amine bond between peptide bound glutamine and a primary amine group (Folk et al 1980) (Figure 1.9).
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Gamma glutamyl-lysine isodipeptide bond

Figure 1.8 A reaction scheme for the formation of a isodipeptide bond.

Pseudo-isodipeptide bonds

Figure 1.9 The formation of γ-glutamyl-amine bonds
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1.6.3 TGase 1

TGase 1 is the membrane associated anionic TGase (Thatcher and Rice 1985) which is initially expressed in the stratum granulosum as a 106 KDa protein (Kim et al 1995), acylated with myristic or palmitic acid and inserted into the membrane. Acylation and deacylation is thought to occur through the action of transacylases in the epidermis. The binding site being most probably a group of 5 cysteine residues at the amino terminus of the protein (Chakravarty and Rice 1989, Phillips et al 1993). In the viable epidermis, the binding of TGase 1 to the membrane is thought to be essential for its activation by calcium (Gibson et al 1996), and also for its conversion to a number of lower molecular weight forms (Kim et al 1996). TGase 1 forms can be released from the membrane with non-ionic detergent and by limited proteolysis (Chakravarty and Rice 1989, Phillips et al 1993). The activity of the soluble forms exists primarily as a soluble 67KDa form often complexed with a 33KDa protein, and the full length TGase1 enzyme of 106KDa. These highly processed soluble forms of TGase 1 account for 90% of the total TGase 1 & 2 activity within the epidermis and keratinocytes (Kim et al 1995). Of the 90% TGase 1 activity, 80% is due to the soluble TGase 1 and less than 10% is due to the membrane anchored forms.

Mechanistically, TGase 1 is thought to be responsible for the initial crosslinking of the CE protein involucrin (Sun et al 1976) forming the basic envelope scaffold (Rice and Green 1979). In the final stages of envelope formation, TGase 1 appears to be responsible for the crosslinking of the interchain links of the protein laid down later in CE formation, loricrin (Candi et al 1995). More recently the membrane-bound form of the TGase 1 enzyme has been shown
to form ester bonds between specific glutaminy1 residues of human involucrin and a synthetic analogue of epidermal specific omega-hydroxyceramides (Nemes et al 1999). This infers a role for TGase 1 in the formation of the covalently bound lipid layer encasing the corneocytes.

1.6.4 TGase 2

TGase 2 is a cytosoluble enzyme thought to be involved in the physiological process of cell death, apoptosis and exocytosis. This enzyme is the only human TGase whose activity has been shown to be regulated by GTP as well as calcium (Reichert et al 1993), the former decreasing and latter increasing activity. However, more recently GTP has been shown to inhibit recombinant murine TGase 3 (Hitomi et al 1999). Current views suggest that TGase 2 is not involved in CE formation under normal physiological conditions.

1.6.5 TGase 3

TGase 3 is a cationic TGase found in the stratum granulosum and corneum (Kim et al 1990). It is initially produced as an essentially inactive proform (77KDa), which can be activated by limited proteolysis, calcium, heat and organic solvents to the active 50KDa enzyme (Plishker et al 1978). This activation causes a change in overall morphology from an elongated to a more compact form, the 50 and 27KDa forms remaining non-covalently associated (Kim et al 1990). During envelope formation TGase 3 is thought to play an important role in the crosslinking of the intra-chain links of
loricrin, which account for a majority of the crosslinks found in CE in vivo (Candi et al 1995).

The mechanism of CE maturation and the precise changes that occur as corneocytes progress from a fragile to resilient morphology are currently unknown. However, research suggests that the conversion does not involve the incorporation of new proteins (Legrain et al 1991) or modification of the covalently bound lipids (Rogers, Unilever Research. Although it may involve further protein crosslinking by the action of TGase. The maturation process does coincide with the gradual degradation of desmosomes on the upper and lower surfaces of the corneocytes, prior to desquamation, suggesting that these processes are closely linked (Harding et al 1999).

1.7 Biochemical abnormalities in dry skin conditions

The vital importance of the stratum corneum lipids and specific enzymes in both barrier function and desquamation is supported by a number of severe dry skin disorders exhibiting lipid abnormalities. Examples of some of these are detailed below:

1.7.1 Recessive X-linked Ichthyosis (RXLI)

This is a genetic disorder which is associated with the retention of adherent rafts of corneocytes giving a characteristic scaling appearance (Long et al 1985). Biochemically it is characterised by a deficiency in the enzyme cholesterol sulphatase, which plays a key role in steroid metabolism, removing the sulphate groups from sterols and sulphated sterol hormones (Shapiro et al
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1978). Within the skin, this defect results in an accumulation of cholesterol sulphate, in particular within the stratum corneum (Long et al 1985).

The association between cholesterol sulphate and aberrant desquamation is supported by evidence that topical cholesterol sulphate induces scaling in mice (Maloney et al 1994). A number of mechanisms have been proposed for this. Cholesterol sulphate is a known stabiliser of membranes (Williams 1991) and the bridging of negatively charged cholesterol sulphate molecules by divalent cations has also been suggested to help maintain the lamellar structure and aid cohesion (Epstein et al 1981). Cholesterol sulphate also elevates the phase transition temperature of cholesterol / fatty acid mixtures, as unlike cholesterol it does not form a eutectic mixture when mixed with fatty acid (Rehfeld et al 1986). In stratum corneum the degradation of cholesterol sulphate at the skin surface is therefore proposed to enhance the fluidity of the lipid bilayers and reduce cellular cohesion. More recently it has been proposed that cholesterol sulphate may act by inhibiting stratum corneum protease activity (Sato et al 1998). The inhibition of acrosin, an acrosomal protease has been demonstrated previously (Bouthillier et al 1984). As a result of desquamatory protease inhibition, desmosomal proteolysis will be impeded, preventing intercorneocyte dyshesion and desquamation.

1.7.2 Atopic dermatitis

Atopic dermatitis is a common skin dryness disorder, characterised by rough scaling of the skin. Biochemically it is associated with decreases in stratum corneum ceramide levels in particular ceramide-1 linoleate
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(Yamamoto et al 1991). Historically, this reduction has been linked to reduced expression of the enzyme sphingomyelin acylase, which catalyses the conversion of sphingomyelin to ceramide (Murata et al 1996). However, recent research has shown that the presence of Pseudomonas aeruginosa in subjects with atopic dermatitis may influence the severity of the disorder. This microorganism produces and secretes the enzyme ceramidase, which is able to degrade ceramides (Okino et al 1998). The specificity of this enzyme for the different ceramides is unknown.

1.7.3 Psoriasis

Psoriasis is a hyperproliferative skin disorder characterised by red lesions surrounded by silvery scaling. In hyperproliferative conditions the turnover rate of the viable epidermis can change from 10-14 days to 4-5 days. As a result the stratum corneum is not formed correctly. A number of lipid abnormalities have been associated with this disorder including increased levels of free arachidonic acid (Hammarstrom et al 1975) and altered ceramide profiles in the stratum corneum. Ceramides 2, 3a, and 4 appear to increase and ceramides 3 and 5 decrease (Menon and Ghadially 1997).

Psoriasis also exhibits an abnormal CE morphology, with a retention of fragile CE at the skin surface. This is associated with desmosomal retention. These specific changes to the CE are thought to occur through the early (precocious) expression of involucrin and TGase 1 (Hohl et al 1986), leading to the subsequent incorrect assembly of the CE structure.
1.7.4 Essential fatty acid deficiency (EFAD)

The essential fatty acids, linoleic and linolenic acids, cannot be synthesised within warm blooded animals and therefore have to be obtained from the diet (Burr and Burr 1929). This is because mammals are unable to introduce double bonds at the n-9 and n-12 positions. The vital role of essential fatty acids, in particular linoleic acid in skin is highlighted in EFAD which exhibits faulty desquamation and severely impaired barrier function (Prottey 1977). Mechanistically it is the replacement of linoleate with oleate within ceramide 1 that appears to be the pivotal change leading to abnormalities in the ultrastructure of the stratum corneum lipids in this disorder (Hou et al 1991).

In addition to its key role in barrier function, linoleic acid is also required as a precursor for arachidonic acid (Duell et al 1988). Epidermal arachidonic acid is mainly esterified within membrane phospholipids at the sn-2 position, being released as required by phospholipase A2. Arachidonic acid itself is a precursor to the eicosanoids, a group of inflammatory and cellular mediators including the prostaglandins, thromboxanes and leukotrienes. These are essential for the regulation of inflammatory processes and cellular proliferation (Duell et al 1988).

1.7.5 Refsums disease

This is a metabolic disorder where the enzyme phytanic oxidase is deficient, resulting in an accumulation of the branched chain phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). In this disorder the epidermis is hyperproliferative and desmosomal structures persist in the upper stratum corneum (Williams 1991).
1.7.6 Lamellar ichthyosis

Lamellar ichthyosis is an autosomal recessive disease characterised by epidermal hyperproliferation, scaling and inflammation of the skin. In this condition the CEs within the upper stratum corneum retain their immature fragile morphology (Michel and Juhlin 1990). It is reported that there is reduced expression and activity of TGase 1 in this disorder, together with an accumulation of soluble involucrin and loricrin (Hohl et al 1993). Alterations in TGase are thought to be due to a defect in chromosome 14q, although this is not always the case in all subjects (Russell et al 1994, Huber et al 1995). Such defects in TGase 1 may result in irregularities in the formation of the early involucrin based scaffold of the CE and covalently bound lipid linkage. Thus the maturation process is unable to take place.

1.7.7 Lovastatin treatment

Cholesterol lowering drugs such as lovastatin can produce a scaling skin disorder upon topical application to the skin (Feingold 1991). In this condition, transepidermal water loss (TEWL) is increased and abnormal lamellar bodies are observed in the granular layer. Although lovastatin does inhibit HMG CoA reductase in skin, reducing cholesterol biosynthesis, cholesterol levels are quick to normalise again. However, lovastatin appears to increase fatty acid synthesis (Feingold et al 1991) which may lead to changes in lipid phase behaviour, accounting for the barrier disruption. This emphasises the importance of maintaining the defined ratios of the barrier lipids for optimal skin condition.
1.8 Aims of the Project

Our knowledge of stratum corneum lipid biochemistry and abnormalities in severe skin disorders has expanded over the last few decades. However, despite major advances, the precise changes that occur during the normal process of stratum corneum maturation, and the perturbation associated with mild xerosis are only just being determined. In particular, the precise involvement of the stratum corneum lipids in desquamation are still being discovered.

Many factors appear to influence dry skin formation, susceptibility to dryness being increased during the winter season, following UV damage, with increasing age, and varying according to bodysite. However, the reasons for the enhanced susceptibility have not been elucidated. A clearer understanding of the lipid composition and how it changes as a result of these factors will help in our development of dry skin treatments.

In recent years the importance of other biochemical factors such as the corneodesmosomal linkages, and proteases in determining corneocyte cohesion and ultimately desquamation have been highlighted. The precise events that occur during desmosomal degradation are still not fully characterised, although this process has been shown to be impeded in dry skin disorders (Chapter 3). As the desquamatory proteases must sit within an essentially lipid environment, the impact of the lipid composition on protease activity has been considered to be important. However, to date, although the role of lipids in barrier function is clear, little is known about the influence of lipids on protease activity in general and in particular SCCE, the main enzyme implicated in the desquamatory process. Understanding the influence of the
lipid environment on the activity of proteases such as SCCE, will help elucidate their involvement in desquamation.

Another key event in stratum corneum maturation is the maturation of the cornified cell envelope from a fragile to a rigid or resilient morphology. The process by which this occurs and the contribution to stratum corneum physiology are currently unknown. However, a lack of maturation is associated with a number of severe scaling pathological conditions. One hypothesis proposes that the transglutaminases which initially form the CE in the granular layer, continue to crosslink the CE within the stratum corneum, resulting in CE maturation. This research aims to confirm the presence and determine the relative levels of the TGases within the stratum corneum and establish any abnormalities observed in mild skin dryness.

The purpose of the work described in this thesis was to:

- Determine the role of the stratum corneum lipids in the susceptibility and formation of cosmetic skin dryness, and investigate the abnormalities in other key biochemical markers, such as desmosomal number.

- Investigate the influence of key lipids on the activity of the desquamatory protease SCCE. This will help to establish the association between lipid abnormalities and perturbed desmosomal degradation.
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- Determine the involvement of TGase enzymes in the maturation of the CE within the stratum corneum and investigate the changes that occur in cosmetic skin dryness.
2. Materials and methods

2.1 Stratum corneum sampling for lipids

The current techniques for stratum corneum lipid sampling often involve invasive techniques such as extraction from skin in vivo using organic solvents (Lavrijsen et al 1994), or the use of punch (Melnik et al 1989), shave (Fulmer and Kramer 1986) and cyanoacrylate (Kawashima et al 1990) biopsies. Although these approaches allow the measurement of lipids, they have a number of disadvantages. Direct solvent extracts are only really accurate for looking at lipid ratios within samples. They cannot take into account variabilities in extraction between samples. In the case of invasive biopsies, although lipid extracts can be normalised to the amount of material by weighing, they sample large depths of tissue, either whole epidermis or stratum corneum.

These techniques are useful for the measurement of stratum corneum lipid changes in relation to the permeability barrier, which has been demonstrated to involve the whole of the stratum corneum (Bowser and White 1985). However, they have inherent poor sensitivity when attempting to examine lipid changes in the more superficial corneum layers, which may be relevant to desquamation. This is highlighted by the evidence that levels of cholesterol sulphate are reduced prior to desquamation, as observed in shed corneocytes (Long et al 1985) and artificially removed corneocytes from in vitro model systems (Ranasinghe et al 1986).
To examine the factors influencing stratum corneum lipid levels and their involvement in maintaining both the barrier and normal desquamation, a new sampling methodology was required. To meet this aim, a tape stripping methodology was developed allowing the measurement of stratum corneum lipids to be quantified. In addition, purification and quantification methods were developed or optimised from original techniques.

2.2 Reagents and standards for stratum corneum lipid quantification

2.2.1 Lipid standards

Lipid standards were purchased from Sigma chemical company, Poole, Dorset, UK. The lipids used, along with their catalogue numbers are; cholesterol (cholest-5-enβ-ol, C8887), palmitic acid (P5917), N-stearoyl DL-dihydrosphingosine (OS-0254), triolein (T7140) and cholesterol sulphate (C9523). Fatty acid methyl esters (FAMES) were obtained from Nu-check-Prep, Inc. Elysian, USA).

In a number of the studies (where specified), ceramide 2 (N-hexadecanoyl sphingosine) was used as the ceramide standard. This was obtained from Quest International, Ashford, Kent, UK. This is a Di-C₁₆ variant of natural ceramide 2, with a charring response similar to N-stearoyl DL-dihydrosphingosine (which became unavailable). Ceramide 1 linoleate (N-ω-9,12-cis-cis-octadecenyloxy triacontanyl (C30) sphingosine) was supplied by Cookson
Chemicals, Southampton, UK. The purity of both being >90% by high performance thin layer chromatography (HPTLC).

### 2.2.2 Solvents/other lipid analysis reagents

All solvents used were supplied by BDH (Leicester, UK) and were HPLC grade. Pre-coated silica gel 60 HPTLC plates were used (10 x 20cm; Merck chromatography, Leicester, UK). Reagents for the protein assays; sodium dodecyl sulphate, 2-β-mercaptoethanol were supplied by Sigma (M 6250). BCA protein assay reagent was supplied by Pierce (Chester, UK).

### 2.3 Stratum Corneum Tape stripping methodology

Skin surface biopsies were taken from human skin using adhesive tape (Sellotape polyester 1601 electrical tape; Hadleigh Enterprises, Essex, UK). A 2 x 5cm piece of tape was applied to the test area of skin with gentle pressure (stroked 20 times), then carefully removed. In general eight consecutive tape strips were taken. The amount of stratum corneum present on each tape strip did visually vary, hence the amount of material was quantified using protein determination (Chapter 2.7). Tapes were stored at minus twenty degrees centigrade (-20°C) until ready to be analysed.

Each tape was placed in a tube containing HPLC grade methanol (5ml) and sonicated for 5 mins at 20°C using a Lucas Davies Ultrasonic bath to dissociate corneocytes from the tape. Methanol was evaporated and corneocytes
were dried at 40°C under oxygen free nitrogen using a sample concentrator (Techne Dri-block D8-3). Where necessary these were stored at -20°C until ready to be extracted.

2.4 Corneocyte intercellular lipid extraction

Lipids were extracted from the dry corneocytes using chloroform: methanol (2:1; 2ml) for 2 hours at room temperature. In all cases, the stratum corneum : solvent ratio (w/v) was equal to or less than 1mg/ml. The extract was transferred to a separate tube, ensuring the corneocytes remained in the original vial. Extracts were dried under nitrogen and dissolved in chloroform (200μL, containing the antioxidant butylated hydroxytoluene (BHT, 50mg/l). All extracts were stored frozen at -20°C.

2.5 Solid phase extraction chromatography

Solid phase extraction column chromatography is a convenient way to separate lipids based on their selective interactions with a chemically modified silica sorbent. In this case the modification with aminopropyl groups gives a more polar column packing with weak anion exchange characteristics. Using normal phase chromatography, solvents of increasing polarity are used to elute lipids from the columns. More polar lipids (e.g. fatty acids) are retained longer and are eluted last using polar solvents. In the case of fatty acids the inclusion of acid lowers the pH and allows their extraction from the aminopropyl bonded silica. In this research solid phase
extraction columns are used for two reasons: Firstly to remove contaminants originating from the glue of the adhesive tape; secondly to separate out the lipid classes so they could each be analysed at optimal levels, without wasting the sample.

The single column lipid separation procedure was developed from a multi-column isolation originally developed by Kaluzny (Kaluzny et al 1985). The modified method, requiring only one column for lipid separation, was as quantitative, yet far quicker than the original technique. The amino propyl bonded phase columns (100mg, 3ml, Anachem Ltd, Luton, UK) were washed with hexane (2ml) while under vacuum using a Vac-Elut System (Anachem, Ltd). The stratum corneum lipid extracts were applied to the columns in chloroform (200μl) and the columns washed with hexane (2ml) under vacuum. The stratum corneum lipids were then eluted using a gentle vacuum (5mmHG) by solvents in the following order:

- **Hexane : Ethylacetate 85:15**: 2ml was used to elute the neutral lipid fraction (e.g. cholesterol & triglycerides)

- **Chloroform : Isopropanol 2:1**: 2ml was used to elute the ceramides

- **2% Acetic acid in Methanol**: 2ml was used to elute the fatty acids

Following each elution, the vacuum was increased to 15mmHG to ensure all the solvent was removed. Eluates were dried as described above and stored frozen at -20°C. Extracts
were dissolved in chloroform : methanol (2:1; 100µl) in preparation for analysis.

To assess reproducibility and recovery of lipids from these columns, purified ceramides, cholesterol and palmitic acid of known concentrations were applied in pentuplicate, eluted and then assessed by high performance thin layer chromatography (HPTLC). The lipid recovery was found to be greater than 95% and the relative distribution of the ceramides after column separation, compared with the original sample were within the errors of estimation of the HPTLC procedure (Table 2.1, Appendix 1).

<table>
<thead>
<tr>
<th>Relative distribution of ceramides (µg)</th>
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<tr>
<td>Ceramide species</td>
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Table 2.1 The recovery of ceramides from the aminopropyl bonded columns.

A stratum corneum lipid extract was prepared in chloroform and equal volumes were loaded onto five amino-propyl bonded columns and eluted with the lipid separation solvents. The ceramide fraction was collected and analysed by HPTLC along with an equal
volume of extract without column separation. The data represents the value (n=1) or mean ± SD (n=5)

2.6 High performance thin layer chromatography (HPTLC)

HPTLC is a similar technique to TLC but uses a thinner layer of silica (~0.2mm) and a smaller mean grain size (7μm). Smaller volumes (>1μl) of samples can be loaded onto the HPTLC plates in narrow bands, using semiautomated loading systems. This gives a better resolution over shorter distances and a detection limit 10 times lower than TLC. The reproducibility of the HPTLC procedure was found to be good, giving an intraplate variation in ceramide levels of ~8% and an interplate variation of ~10% (Appendix 2).

Prior to sample loading, the HPTLC plates were first washed by development with the following solvents and dried at 37°C on a hot plate (Desaga Thermoplate S) after each step.

• Plate Wash
  1) Ethylacetate: Methanol (40:60)
  2) Chloroform: Ethylacetate: Diethyl Ether (30:20:50)

Samples and lipid standards in chloroform : methanol (2:1) were applied to the HPTLC plates using a Camag Linomat IV HPTLC sample loader. A sample (1-10μg) was loaded in a 4mm band, the origin being 5mm from the base of the plate, with an 8mm gap between samples. To enable the quantification of lipid bands, lipid standards were
applied to each plate at four concentrations. The standards were applied to the plates at 5, 10, 20 and 30μl loadings. These correspond to 0.5, 1.0, 2.0 and 3.0μg of cholesterol, cholesterol sulphate, triglyceride and ceramide; and 1.0, 2.0, 4.0 and 6.0μg palmitic acid. These levels represent the linear range when using the charring detection system. This was determined by producing a standard curve for each lipid (Data not shown).

2.6.1 Ceramide analysis:

The HPTLC methodology for ceramide analysis was performed essentially according to Wertz et al (1985), except 3 solvent runs were used instead of 2. This gave enhanced band separation (Figure 2.2). Briefly the plates were developed three times using chloroform: methanol: acetic acid (190:9:1) to 9.5cm, and were dried on a hot plate at 37°C between each development. After chromatographic separation, the lipids were visualised on the HPTLC plate by general degradative charring, and quantified by reflectance densitometric scanning, as described in 2.6.6. The ceramides were identified by their co-migration with ceramide standards, and rf values from the literature (Wertz and Downing 1983). The synthetic ceramide standard N-stearoyl DL-dihydrosphingosine was used to quantify the ceramides, as this was the only readily available ceramide at the time. Ceramide 1 was further identified by its change in chromatographic mobility following alkali hydrolysis and by its co-migration with chemically synthesised ceramide 1, the identity of which had been verified by mass spectrometry and proton-NMR.
Figure 2.1. A typical HPTLC separation of the stratum corneum ceramides. Lanes 1-3 represent the ceramide standard, N-stearoyl DL-dihydrosphingosine. Lanes 4-8 show the stratum corneum ceramides and lanes 9-11 the lipid extract from blank tapes. The ceramide bands were identified according to Wertz and Downing (1983). The individual bands represent ceramide 1, 2, 3, 3a, 4/5 then 6i & 6ii, counting from the most non-polar band, furthest away from the origin.

2.6.2 Fatty acid and cholesterol analysis

Neutral lipid and fatty acid eluates from the solid phase extraction columns (section 2.5) were loaded onto a HPTLC plate in chloroform : methanol (2:1) as described for ceramides (section 2.6.1). The initial separation of the fatty acids and cholesterol using solid phase extraction
columns allows for different loading volumes onto the HPTLC plates. As cholesterol levels are typically lower than those of fatty acids in the stratum corneum lipids, more of this eluate was loaded to ensure accurate quantification. This was achieved by ensuring the levels of each lipid fell well within the linear range on the standard curve.

The analysis of stratum corneum fatty acids and cholesterol in the initial studies were performed as described previously by Ponec (Ponec et al 1988). After loading the column purified samples (as previously described) the plates were developed with three different solvent systems:

- Chloroform: acetone: methanol (76:4:20); to 1.5 cm and then 3.0 cm from the base of the plate

- Chloroform: acetone: methanol (79:8.5:12.5); to 8.0 cm from the base of the plate.


Again, the plates were dried to 37°C between each run. In later studies, the fatty acids and cholesterol were separated using hexane/ethyl acetate 85:15 to 9.5 cm; then 70:30 to 9.5 cm. This system was developed to give enhanced separation of the lipids and tape residue. After chromatographic separation, the lipids were visualised on the HPTLC plate by general degradative charring, and quantified by reflectance densitometric scanning, as described in 2.6.6. The bands were identified by their
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co-migration with Sigma standards, used also as a standard curve.

2.6.3 Triglyceride analysis

The triglycerides were analysed using the method of Ponec (Ponec et al 1988). Although triglycerides are present in the neutral lipid column eluate, with cholesterol, they could not be analysed together due to tape contaminant interference. Samples of the neutral lipid column eluate were loaded onto the HPTLC plates as described and the plate developed with chloroform/methanol (90:10) to 1 cm from the base of the plate. The plate was then dried on a hot plate at 37°C and developed for a second time with chloroform / diethyl ether / ethyl acetate (80:4:16) to 9.5cm. After chromatographic separation, the triglyceride band was visualised on the HPTLC plate by general degradative charring. Triglyceride levels were quantified by reflectance densitometric scanning, as described in 2.6.6. The band was identified by its co-migration with the Sigma standard triolein, used also as a standard curve.
2.6.4 Cholesterol sulphate separation

Crude cholesterol sulphate purification:

As cholesterol sulphate is not very soluble in chloroform, a fraction of the lipid extracts was saved prior to separation on solid phase extraction columns. Typically this was 1/4 of the initial extract. The lipid extract was dried under N₂ and 1 ml of hexane was added and centrifuged at 20,000g at 4°C for 15 mins. The pellet was then reconstituted in chloroform/methanol 2:1 and warmed. This process removed neutral lipid components, which at the loading levels required, distort the cholesterol sulphate band on the HPTLC plates.

Quantification:

Cholesterol sulphate was separated and quantified using the HPTLC method of Downing et al (1993). Briefly, the extracts were loaded onto silica gel 60 HPTLC plates and the plates eluted twice with chloroform/methanol/ammonia (40:10:1) to 10 cm from the base of the plate. Plates were dried at 37°C between runs. This solvent system ensured dissociation of any cholesterol sulphate complexed with sphingosine, as demonstrated by Downing in the same paper. In addition it separated cholesterol sulphate from minor tape contaminants. After chromatographic separation, the lipids were visualised on the HPTLC plate by general degradative charring, and quantified by reflectance densitometric scanning, as described in 2.6.6. The bands were identified by their co-migration with Sigma standards, used also as a standard curve.
2.6.5 Analysis of lipid covalently bound to corneocyte proteins

After extracting the free intercellular lipid, the covalently bound lipids were hydrolysed and extracted from the corneocytes. These lipids are esterified onto the corneocyte cell envelope proteins, involucrin in particular (Chapter 1). To allow extraction, the ester linkages were initially hydrolysed using sodium hydroxide in methanol (1 ml, 0.4M NaOH) at 60°C for 2h. The methanol was cooled and acidified using hydrochloric acid (0.5M), and dried together with the corneocytes. The cooling and use of dilute acid ensures that ethyl esters of the fatty acids are not produced.

Covalently bound lipids were then extracted using chloroform/methanol (2:1, 2h) and then aspirated off into a separate vial and dried at 40°C under N₂. The lipids were then separated by HPTLC (Ponec et al 1988, Wertz et al 1989) as described for the earlier analysis of cholesterol and fatty acids. After chromatographic separation, the lipids were visualised on the HPTLC plate by general degradative charring, and quantified by reflectance densitometric scanning, as described in 2.6.6. The bands were identified by their co-migration with Sigma standards, and from their rf values quoted in the literature (Wertz et al 1989).

2.6.6 HPTLC staining and quantification procedure

Lipid bands on the HPTLC plates were visualised by staining and degradative charring. The reproducibility of
the staining is good as shown by the low interplate variations (2.6.1). This system was used as it gives the best contrast between the colour of the lipid bands and the plate background (Data not shown). The plates were initially dried at 120°C on a hot plate, removed and cooled, and stained with acidic copper sulphate solution (10% copper sulphate; 8% phosphoric acid; 15 ml) for 1 min. Excess acid was drained off, and the plate dried at 120°C for 1 min and then heated to 160°C for 20 min to char the lipids. Charring is a useful detection system for non-chromogenic groups. The charring procedure of heat in the presence of oxidising agents causes carbonisation of the lipid carbon chains. After cooling the lipid bands were quantified by reflectance densitometric scanning at 420nm using a Shimadzu CS-9000 flying spot densitometer (Shimadzu, Japan).

2.7 Determination of corneocyte protein levels

To assess the quantity of stratum corneum removed from subjects during the tape stripping, the level of extractable protein was determined using bicinchoninic acid (BCA) assay reagent. This is a highly specific reagent for the cuprous ion (Cu\textsuperscript{+}). The BCA assay combines this with the biuret reaction (protein reducing Cu\textsuperscript{2+} in an alkaline medium to produce Cu\textsuperscript{1+}).

Soluble proteins were extracted from dried corneocytes using 1ml of extract buffer (1% sodium dodecyl sulphate (SDS), 10mM sodium phosphate buffer and 20mM mercaptoethanol) at 60°C for 2h. After cooling, triplicate samples (50µl) were transferred to a microtitre plate (Flow laboratories, Essex, UK) and dried in a vented
oven for 24h at 85°C. The dried samples were dissolved in distilled water (50μl) and shaken for 1 hr. To this the BCA reagent was added (Reagents A:B; 50:1; 200μl). The microtitre plate was then heated at 37°C for 90min followed by 30min of shaking. Protein concentrations were determined by their absorbance at 540nm and comparison with a protein calibration curve of bovine gamma globulins (1-12μg) using a Multiscan Titertek apparatus (Flow Laboratories). The amount of protein present was shown to correlate well with the weight of corneocytes (Figure 2.3). The assay was also very reproducible giving a coefficient of variation for pentuplicate analyses of less than 6%.

Figure 2.2  Correlation between corneocyte weight and extractable protein. The SDS extractable protein was found to correlate well with corneocyte weight (n=3) by linear regression, r=0.97 P<0.05.
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2.8 Analysis of free and ceramide 1 esterified fatty acids

2.8.1 Sample preparation

**Free fatty acids:** After quantifying the free fatty acids by high performance thin layer chromatography (HPTLC), the remainder of the sample was dried under nitrogen (N\textsubscript{2}). Diazomethane (Synthesised by James C, Unilever Research) dissolved in ether (~200μl) was added to each extract at room temperature and effervescence was observed. The methylation of the fatty acids occurs within 5-10 seconds under these conditions. The samples were dried under N\textsubscript{2}. The reproducibility of producing FAMES was found to be good giving a coefficient of variation of 10%, as determined by HPTLC.

**Ceramide 1:** To isolate ceramide 1 a portion of the ceramide fractions was chromatographed as described in 2.6.1. To locate ceramide 1, CAMAG Test Dye III (Camag Scientific, Wilmington NC), which contains a component co-migrating with ceramide 1, was loaded on the end lanes of each plate. Silica gel, containing ceramide 1 was scraped from the plates and extracted in chloroform/methanol (2:1, 2h). Arachidonic acid (1μg) was added to the extraction solvent as an internal standard and the extract was filtered through a syringe filter (Gelman Acrodisc CR PTFE 0.45μm) to remove the silica gel, then dried under nitrogen. The fraction was hydrolysed in 1.2M sodium hydroxide in 95% methanol (500μl, 60°C, 1h) and then acidified with 0.5M hydrochloric acid (HCl). The methanol was evaporated and the residue dissolved in chloroform (200μl). Fatty acids were then purified using solid phase
2.8.2 Gas Chromatography

FAME samples were dissolved in hexane along with a methyl laurate internal standard (2\(\mu\)g). This standard was used to compare the retention times of the different GC runs, as a comparison. Loading volumes onto the GC were approximately 3\(\mu\)l. Mixed FAME standards, along with blank solvent and blank tape extracts were run on the GC daily to act as controls. FAMEs were separated using a silica capillary column cross linked with polyethylene glycol (SGE chromatography products, 25QC5/BP20-1.0, 25m long and 0.53 id). The inlet temperature was set at 250\(^\circ\)C and the helium flow rate at 8ml/min. The temperature programme was set at 150\(^\circ\)C for 2 minutes and programmed for a 4\(^\circ\)C rise per minute to a final temperature of 220\(^\circ\)C for 20 minutes. The detection system used was a flame ionisation detector, the response for the fatty acids detected having been shown to be identical. From the fatty acid peaks recorded on the GC run, the peak areas were determined as a measure of the level of each of the fatty acids present. Each peak area was expressed as a percentage of the total, so the final data represents the relative percentage profile of fatty acids in the sample.
2.9 Analysis of the stratum corneum proteases

2.9.1 Reagents used for protease experiments

The following chemicals were obtained from Sigma (Dorset, UK): Dimethyl sulphoxide (DMSO) (D5879, Sigma), Tris (T6791, Sigma), sodium chloride (S7653), Triton X-100 (X-100), SDS (L4509), glycerol (G7757), casein (C3335), chymotrypsin (C4129), trypsin (T8003), sodium azide (S2002), Coomassie brilliant blue (B5133), succinyl-leucine-leucine-valine-tyrosine-7-amido-4-methylcoumarin (suc-leu-leu-val-tyr-AMC) (S6510), monochloroacetic acid (40,292-3), Sodium acetate (S7670), N-tert-Butoxy-carbonyl-phenylalanine-serine-arginine-7-amido-4-methylcoumarin (Boc-Phe-Ser-Arg-AMC, B6388), Stearic acid (S4751), Sodium stearate (S3381), dioxane (D9553), sodium borate (S9640), glycine (G7403), Tween-20 (P7949), octanol (O4500). Ethanol, methanol, acetic acid and Immobilin P PVDF (polyvinylidene fluoride) were obtained from BDH (Leicester, UK). rSCCE was a gift from Dr. T. Egulrud and Symbicom (0.01|ig/jil), 2-20% SDS gradient gel (Biorad, UK). Acetic anhydride $^{14}$C, $^{14}$C methylated low molecular weight standards (Amersham, UK), squalene (S3626), Ready safe scintillation fluid (CAT 141349, Beckman, UK). Sephadex G25 (G25-125, Sigma, UK), Acrylamide Bis-2 (AO/0832, Camlab, Cambridge, UK), Acrylamide 40 (AO/0132, Camlab, Cambridge, UK), Pseudoceramide A (Lancaster Chemicals, Lancashire, UK), Sphingolipid E (propan-1,3-dione-1,3-bis[N-(2-hydroxyethyl)octadecylamine], Quest, Ashford, UK), ceramax (Quest, Ashford, UK), Sucrose ester S-270 ($\alpha$-D-glucopyranoside $\beta$-D-fructofuranosylacyl ester, Mitsubishi, Japan).
2.9.2 Protease extraction

The protease extraction was performed using a method developed by Watkinson (Unilever Research, Unpublished method). The corneocytes from 8 sequential skin surface tape strips were detached by sonication in methanol for 5 minutes (Kept on ice). After centrifugation at 2,000g for 1 min (4°C), the methanol was decanted off and the corneocytes dried under N2 and weighed. Dimethyl sulphoxide (DMSO, 5μl) was added to the corneocytes and the proteases were extracted using Tris-HCl buffer (1ml per 10mg corneocytes: 50 mM, pH 6.8) containing 1M sodium chloride and 0.1% Triton X-100 at 4°C overnight. Insoluble material was sedimented by centrifugation at 20,000g for 10 min and the supernatant was removed. Extracts were spin de-salted into sample buffer (Tris 3.03g (83.4mM) glycerol 100ml, SDS 2g (25mM), Water 300ml, pH 6.8 using HCl); briefly, Sephadex G25 columns (2ml) were pre-equilibrated with sample buffer and centrifuged dry (1500g for 5 min). Salt extracts (400μl) were applied to the columns and the desalted samples were collected on re-centrifugation and stored at -20°C until required.

2.9.3 Casein zymography

Protease activity was determined by the proteolysis of casein within casein impregnated gels following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (Horie et al 1984, Watkinson Unilever Research, Unpublished method). Briefly, 12% acrylamide gels, containing 0.2% (w/v) casein were prepared with a 4% stacking gel. Samples were then electrophoresed at 200V for approximately 1 h using a protean II system (Biorad)
along with molecular weight markers and standard proteases of bovine chymotrypsin (25kDa) and trypsin (23kDa). After fractionation, the gels were washed in 50mM Tris-HCl pH8 containing 2.5% (v/v) Triton X-100, followed by 50 mM Tris-HCl pH8. Incubations were performed at 37°C for 24h in 50 mM Tris-HCl pH8, 0.1% (w/v) sodium azide, 20% ethanol in the presence/absence of lipids, as indicated in individual experiments. Incubations were terminated by the immersion and staining of the gel in 0.1% (w/v) Coomassie brilliant blue (CBB) in 10% acetic acid, 40% (v/v) methanol. Quantification of the proteolytic activity was performed by scanning the gel using an Epson GT8000 scanner combined with the phoretix gel analysis software (Phoretix Int., Newcastle, UK).

### 2.9.4 Colorimetric stratum corneum chymotryptic-like enzyme activity assay

The effects of lipids on the activity of recombinant stratum corneum chymotryptic-like enzyme (rSCCE) was determined using a fluorescent substrate assay. 5μl of rSCCE was added to 50μl of 1mM substrate (suc-leu-leu-val-tyr-AMC) in reaction buffer (50mM Tris-HCl pH 7.8, 0.1% (v/v) Triton X100, 0.1% (w/v) sodium azide) in a multiwell plate (in triplicate). The optimal assay buffer and substrate type were as recommended by Watkinson (Unilever Research). The plate was incubated at 37°C, for 3h and the reaction stopped by the addition of 100 μl stop buffer (100 mM sodium monochloroacetic acid, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3). The protease activity was determined by the release of free aminocoumarin from the suc-leu-leu-val-tyr-AMC. The free
aminocoumarin being far more fluorescent. This release and hence the increased fluorescence, was shown to be linear over the 3h reaction period (Data not shown). The reproducibility of the assay was good, giving a coefficient of variation of 10%.

The reaction incubations were performed in the presence/absence of a range of lipids (according to individual experiments). Lipid solutions were prepared in 20% ethanol/assay buffer. The final concentration of ethanol in the assay was 10%, which did not interfere with the assay. The plate was assayed for fluorescence using a Millipore cytofluor™ 2350 fluorescence measurement system (Pharmacia, UK), with the emission set at 360nm and detection at 460nm. The results represent the final fluorescence minus the initial fluorescence.

2.9.5 Colorimetric chymotrypsin activity assay

The basis for the chymotrypsin assay is the same as that described for rSCCE in 2.9.4 and is based on a system initially developed by Watkinson (Unilever Research). Chymotrypsin (0.1µg) was added to 150µl assay reagent containing 50mM Tris-HCl, pH 7.8, 0.1% (v/v) Triton X100, 0.1% (w/v) sodium azide and 200µM suc-leu-leu-val-tyr-AMC in a multiwell plate. The plate was then incubated at 37°C for 30 minutes. After this point the reaction was stopped by the addition of 100µl stop buffer and fluorescence quantified as described (2.9.4). The reaction was performed in the presence/absence of lipid, solutions being prepared in 20% ethanol/assay buffer (final ethanol concentration 5%).
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2.9.6 Colorimetric trypsin activity assay

The basis for the trypsin assay is the same as that described for rSCCE in 2.9.3. The method for investigating the effects of lipids on trypsin activity is based on a system initially developed by Watkinson (Unilever research). Trypsin (0.1µg) was added to 150 µl assay reagent containing 50mM Tris-HCl, pH 7.8, 0.1% (v/v) Triton X100, 0.1% (w/v) sodium azide and 200µM Boc-phe-ser-arg-AMC in a multiwell plate. The plate was then incubated at 37°C for 1h, after this point the reaction was stopped by the addition of 100µl stop buffer and fluorescence quantified as described in section 2.9.4. The reaction was performed in the presence/absence of lipid, solutions being prepared in 20% ethanol/assay buffer (final ethanol concentration 5%).

2.10 Enzyme behaviour in lipid emulsions

2.10.1 Production of emulsions

A range of oil in water emulsions were produced containing the ratios of the lipid species as listed in Fig 2.4.

The emulsions were produced by heating the water and melting the fat phase (as shown in Table 2.4) at 80°C with continual stirring. The mix was homogenised (1800psi) with Multispec 500B homogeniser. The samples were assessed visually for signs of precipitation after 24h, and appeared stable. The level of sodium stearate used as an emulsifier was set at 0.3%, this has been shown previously to reduce the level of crystalline material
(Dunnet, Unilever Research). The pH was determined and drop size of the emulsions measured using a malvern mastersizer.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Percentage composition of emulsions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Pseudoceramide A</td>
<td>1</td>
</tr>
<tr>
<td>Sphingolipid E</td>
<td></td>
</tr>
<tr>
<td>Ceramex</td>
<td></td>
</tr>
<tr>
<td>Ceramide II</td>
<td></td>
</tr>
<tr>
<td>Sucrose ester S-270</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.7</td>
</tr>
<tr>
<td>Sodium stearate</td>
<td>0.3</td>
</tr>
<tr>
<td>Deionised water</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 2.2 The lipid ratios used in the formulation of the emulsions

2.10.2 Measurement of rSCCE activity in lipid emulsions

Each lipid emulsion (50μl) and a control sample of water only, were placed into eight wells in a multiwell plate. To four of the wells SCCE (0.5 μg in 50mM Tris-HCl pH 7.8, 0.1% (v/v) Triton X100, 0.1% (w/v) sodium azide) was added and to the other 4 wells the buffer alone was added. 50μl of 1mM suc-leu-leu-val-tyr-AMC in 50mM Tris-HCl buffer, pH 7.8, 0.1% (v/v) Triton X100, 0.1% (w/v) sodium azide was then added to all 8 wells. In addition a standard curve
for AMC was produced in all the liposomal systems as they were found to enhance the observed fluorescence. The plate was incubated at 37°C for 3h and at this point the reaction was stopped by the addition of 100μl stop buffer and fluorescence quantified as described (2.9.4). The SCCE activity was represented as ng AMC produced in 3h. The reproducibility of measuring rSCCE in the liposomal systems was less than the aqueous systems, giving a coefficient of variation of 20%.

2.10.3 Measurement of chymotrypsin and trypsin activity in lipid emulsions

The assay was performed generally as described above (2.10.2). However, the substrates and reaction conditions used were those as described in the aqueous colorimetric assay for chymotrypsin (2.9.5) and trypsin (2.9.6). The activity was represented as ng AMC produced in 30mins and 1h respectively. The reproducibility of measuring chymotrypsin and trypsin in the liposomal systems was good, giving a coefficient of variation of less than 7%.
2.11 Partitioning of rSCCE into an organic phase

2.11.1 Radiolabelling of rSCCE

rSCCE (640mg) was spin desalted using 2ml Sephadex G50 columns and sodium borate buffer (10 mM, pH 9.3). Briefly, 20ml of borate buffer was placed onto the column and spun in a centrifuge (500 rpm - 2 min, then 2000 rpm 5 min) into a tube. The waste collection tube was then emptied and the column spun again (2000 rpm 2 min). rSCCE (400µl, 0.4 mg/ml) was applied to each column and spun into an Eppendorf (500 rpm 2 min, 2000 rpm 5 min). To this eluate 200µl of dioxane containing 9MBq of acetic anhydride $^{14}$C (3.7-4.59 GBq/mmol) was added, vortexed and left on ice for 30 min. Labelled rSCCE was then purified using Sephadex G25 (pd10) columns, pre-washed with 25 ml sodium borate buffer. After sample loading, rSCCE was eluted with 3.5 ml buffer and 10µl glacial acetic acid was added to improve stability.

The radiolabelling of rSCCE was determined by separation on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli (Laemmli et al 1970), using a 2-20% SDS gradient gel (Biorad, UK) and $^{14}$C methylated low molecular weight standards (Amersham, UK). The protein bands were then blotted onto Immobilin P PVDF membrane by semi-dry electrophoretic transfer (30V, overnight), using a buffer of 39mM glycine, 48 mM Tris-HCl, 0.0375% SDS and 10% methanol. The PDVF membranes were washed in TBS-tween, dipped in methanol and dried on a hot plate at 60°C. The radioactivity in the membrane was detected by a Molecular Dynamics Phosphor imager (200µm sensitivity) after being exposed to the phosphor screen for 2 days. The bands were quantified.
using a flat bed scanner and Phoretix gel analysis software (Phoretix, UK). Incorporation being determined as 0.00013 MBq per mg rSCCE.

2.11.2 Solubility of rSCCE in nonpolar phases

SCCE is a protease working within the essentially nonpolar lipid environment of the stratum corneum. It is not known whether SCCE is localised in water pockets around the polar proteins of the stratum corneum or whether it is mobile within the nonpolar stratum corneum lipid matrix. 200µl of different buffers (Sodium citrate at pH 3 or 4; Sodium acetate at pH 5 or 6; and Tris/HCl at pH 7,8 or) containing rSCCE (0.016mg) were added to an Eppendorf along with 200µl octanol. Each pH experiment was analysed in triplicate. The solutions were shaken at room temperature, overnight and upon stopping the shaking the octanol and buffer phases separated and were pipetted into separate vials. 150µl of each of the phases were added to 5ml of scintillation fluid and the ^14C content measured using a scintillation counter.

There is evidence in the literature that enzymes are capable of being solubilised in organic/low water environments by ion pairing (Paradkar and Dordick 1994, Powers et al 1993, Bromberg and Kibanov 1994). Therefore the experiment was repeated at pH 3 and pH 6 with (and without) the addition of either palmitic acid, cholesterol sulphate or SDS to the aqueous phase. The lipids and SDS were added at a range of concentrations to give an rSSCE : added compound ratio of 1:33 or 1:133 or 1:666.
2.12 Measurement of transglutaminase activity

2.12.1 Reagents used in transglutaminase assay

The following chemicals were purchased from Sigma (Dorset, UK). Guinea pig liver transglutaminase 2 (T5398), ethylenediaminetetraacetic acid (EDTA, EDS), phenylmethylsulfonyl fluoride (PMSF, P7626), N,N-dimethyl casein (C9801), calcium chloride (C4901), dithiothreitol (D5545), trichloroacetic acid (T9159). Other chemicals include: sodium hydroxide (BDH, Dorset, UK), Hionic-fluor scintillant Hionic Fluor (CAT 6013319, Packard, UK), ¹⁴C Putrescine (Amersham Int, UK).

2.12.2 Transglutaminase sampling and extraction

Five sellotape strips (5x2cm²) were consecutively taken from the skin surface, or surface callus foot scrapings were collected, kept on ice and then frozen -70°C. After defrosting, the corneocytes were removed from the tape using hexane, spun down at 2500g for 5 minutes the hexane was removed. The corneocytes were then transferred to microfuge tubes in approximately 1ml of hexane and pelleted by centrifugation at 20000g for 5 min. The hexane was again removed and 1ml of fresh hexane added. This rinsing process was repeated twice more. The whole process was performed at 4°C. Transglutaminase (TGase) was then extracted from the corneocytes by resuspending the pellet (approx. 1mg) in 500 µl of soluble TGase extraction buffer (0.1M Tris-HCl, pH7.5, 150mM NaCl, 10mM EDTA, 1mM PMSF) and rotated for 1 hour at 4°C. The corneocytes were then pelleted by centrifugation at 20000g.
for 5 min, and the extract pipetted and stored frozen (-70°C) until being assayed for TGase activity. The extraction was then repeated with the addition of 1% triton X-100 to fresh corneocyte extract buffer. This will extract the buffer insoluble TGase. The corneocytes were pelleted by centrifugation at 20000g for 5 mins and the supernatant removed and stored frozen (-70°C) until being assayed for TGase activity.

2.12.3 Separation of TGase isoforms using anion exchange chromatography

TGase extracts (2.12.2) were concentrated 10 fold using Amicom columns (centricon-10). The concentrated extracts were then diluted (1:2) in the FPLC elution buffer (50mM sodium acetate buffer pH 6), containing 1mM PMSF and 10mM EDTA, and separated by FPLC (as described below). All samples were assayed for TGase activity within the same day.

Equipment:
The FPLC equipment used for the TGase separation was a Pharmacia SMART system, fitted with a Mono Q column 1.6/5mm (Pharmacia). The column was equilibrated with 50mM sodium acetate buffer pH6 containing 10mM EDTA. The fraction collector was set up with 45 250μl eppendorfs, and was filled with ice so all the collected samples were kept cool.

Separation:
Stratum corneum or callus concentrated extracts were fractionated on a Mono Q column. 50μl of the concentrated extracts were loaded onto the column and run using the
sodium acetate buffer (5 min), then on a gradient of 0-0.75M NaCl from 5-35min, at a flow rate of 100μl per min. The column was equilibrated with 1M NaCl for 10mins and back to 0% NaCl for 10mins. 100μl fractions were collected and immediately assayed for TGase activity.

2.12.4 Measurement of Transglutaminase activity

The TGase assay is based on the crosslinking of radiolabelled putrescine to the protein casein. TGase activity can be measured by the level of ^14C incorporation into precipitated protein. The more TGase activity, the more incorporation achieved. 150μl of TGase assay buffer was added to 100μl of sample extracts prepared in 2.12.1. Final concentrations were 0.1M Tris-HCl, pH7.5, 0.15M NaCl, 2mg/ml N,N-dimethyl casein, 10mM calcium chloride, 5mM dithiothreitol, 1% triton X-100 and 0.1μCi ^14C putrescine (100-124 mCi/mmole). In addition, blank extract buffer (no TGase) and 0.01U of guinea-pig liver TGase 2 were assayed as controls. The mixture was incubated for 60 minutes at 37°C, then the reaction was stopped by the addition of 200μl of ice cold 10% trichloroacetic acid (TCA) and 0.1% putrescine (unlabelled).

The assay buffer and substrate concentrations were based upon conditions extensively used in the literature (Reichert et al 1993). The linearity of the reaction was checked for TGase 2 (0-0.01U) and stratum corneum extracts and was found up to good up to 2 hours (Data not shown). A 1 hour incubation time was selected. TCA causes precipitation of the casein so it can be easily separated from the free labelled putrescine. The amount
of putrescine crosslinked casein was determined using two methods. For the dry skin study, TCA precipitates were blotted onto an Immobilon P membrane using a dot-blot apparatus, which basically has a 96 well format. The apparatus was disassembled and a piece of plate sealer was applied to the membrane, which was then removed and dried. This was then exposed for 1 week against a phosphor imaging screen (Molecular Dynamics). The amount of cross-linked protein retained on the membrane was then quantified using a Molecular Dynamics Phosphor Imager and the Phoretix gel analysis software (Phoretix, England). Statistical analysis of the data was performed using the SigmaStat software package. The data being presented as MEAN + S.D. arbitrary units per microgram of extracted protein (a.u./µg). Pre-blocking of the membrane with TBS / 5% tween had no effect on the results (Data not shown). The reproducibility of the assay was good giving a coefficient of variation of less than 8%.

For all the other experiments in chapter 7, the casein precipitates were spun down for 5mins at 12,000rpm, the supernatant was discarded and the casein pellet redissolved in 100µl of 0.5M sodium hydroxide. This process of precipitating the casein and dissolving was repeated twice more, by re-adding 200µl of TCA as described. For the final redissolving in sodium hydroxide 200µl was added. Once dissolved 150µl was placed into a small counting vial and 5ml of Hionic-fluor scintillant was added and the level of 14C counted in a scintillation counter. Using this quicker technique the coefficient of variation was less than 7%.
2.13 Analysis of desmosomal glycoproteins

2.13.1 Reagents specific for the desmosomal analysis

Urea (U5128), β-mercaptoethanol (M7154), iodoacetamide (I1149), manganese chloride (M9522), magnesium chloride (M8266), Conconavalin A (Con A, C2631), Sepharose (6B-100), α methylmannopyranoside (M6785), all from Sigma, UK. Other reagents used include: rabbit anti-bovine dsg1 antiserum (R882.2; a gift from Dr. T Egulrud, MillHill, London 1:100), mouse anti-rabbit IgG monoclonal antibody conjugated to biotin (1:1000; Zymed, UK), Streptavidin-horseradish peroxidase conjugate (1:1000; Amersham, UK), mouse anti-keratin monoclonal antibody (Clone K8.13; Sigma), sheep anti-mouse IgG-biotin (1:1000; Amersham).

2.13.1 Desmoglein 1 extraction

Corneocytes, isolated as described in 2.3, were extracted in 500μl of 50mM Tris-HCl buffer, pH9, 8M urea, 2% sodium dodecyl sulphate (SDS), 5% (v/v) β-mercaptoethanol and 2mM PMSF at 90°C. After 15mins, an equal volume of 0.5M iodoacetamide, in extract buffer, was added and re-incubated at 90°C for a further 15 mins. The extracts were microfuged (20,000g, 5mins) and the supernatant was diluted in 20 volumes of 50mM Tris-HCl buffer, pH7, containing 0.5M sodium chloride, 1mM magnesium chloride, 1 mM calcium chloride, 0.1% triton X-100 and 2mM PMSF (Con-A buffer). The diluted samples were loaded onto a Conconavalin A (Con A) Sepharose (200μl) column and washed with Con A buffer (3x10 ml). The bound proteins were eluted with 0.5M α methylmannopyranoside in Con A buffer
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(600μl). 10μg of casein was added to the eluates as a carrier protein before precipitation of the eluates with four volumes of chloroform : methanol (1:4) followed by three volumes of water; the precipitate was dried under nitrogen and reconstituted in sample buffer comprising 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 20mM dithiothreitol at 60°C, for 15min. (Long, Unilever Research, Unpublished method).

2.13.2 Desmoglein 1 analysis

The dsg1 extracts were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli (Laemmli et al 1970), in 8% polyacrylamide gels (crosslinking C=3.3%) with a 4% stacking gel (C=3.3%) and molecular weight standards. The protein bands were then blotted onto Immobilin P PVDF membrane by semi-dry electrophoretic transfer (30V, overnight), using a buffer of 39mM glycine, 48mM Tris-HCl, 0.0375% SDS and 10% methanol. The PDVF membranes were blocked overnight with 5% bovine serum albumin (BSA) in 50mM Tris-HCl, pH 7.5, containing 0.15 M sodium chloride, 2.5mM potassium chloride and 0.1% Tween-20 (TBS-T). Desmoglein 1 was detected with rabbit anti-bovine dsg1 antiserum (R882.2; a gift from Dr. T Egulrud, MillHill, 1:100) in TBS-tween (50mM Tris-HCl pH 7.5, 0.15M sodium chloride, 0.15% tween-20) with 0.1% BSA for 2h at room temperature (Long, Unilever Research, Unpublished method). The membranes were then rinsed three time (20min rinse) in TBS-tween. The membrane was reincubated with mouse anti-rabbit IgG monoclonal antibody conjugated to biotin (1:1000 in TBS-tween with 0.1% BSA; Zymed, UK), for 1h followed by washing as described above. The membrane was
then reincubated with Streptavidin-horseradish peroxidase conjugate (1:1000 in TBS-tween with 0.1% BSA; Amersham, UK) for 30min, followed by washing (as described). Keratin was detected with mouse anti-keratin monoclonal antibody (Clone K8.13; Sigma) at 1:50 to 1:200, as described above, except that the secondary antibody, sheep anti-mouse IgG-biotin (1:1000; Amersham) was used. The membrane was given a final wash (as described). The bands were visualised using the ECL chemiluminescence method. The detection system works by the horseradish peroxidase (conjugated to the secondary antibody), reacting with a chemiluminescent substrate (luminol) in the ECL reagents, to produce a light. This light can be detected by placing the blot against a light sensitive film. Bands on the resulting film are then quantified by scanning densitometry at 530nm using a Schimadzu CS-9000 dual wavelength spot scanning densitometer.

2.13.3 Desmocollin 1 analysis

Dscl was detected on the gels described in 2.13.2 using the α46/48 anti-dscl polyclonal antibody (directed against the extracellular N-terminal of dscl, a gift from Dr. I. King, NIMR, London) which was used at 1:200. The membranes were incubated with the primary antibody for 2 hrs., washed with 5x100mls of TBS-T followed by incubation with a 125I labelled goat anti rabbit IgG monoclonal antibody (Amersham, UK) at 1:1000 dilution for 1 hour. After washing and drying, the radioactivity on the membranes was detected by a Molecular Dynamics phosphor imager. Quantitation of dscl immunoreactivity was with the Phoretix gel analysis software (Phoretix Ltd., Newcastle, UK).
2.13.4 Desmocollin 1 immunofluorescence

The presence of dscl was probed using the \( \alpha-46/48 \) polyclonal antibody (a gift from Dr. I. King, NIMR, London) at 1:200 dilution. The dscl level was quantified using a FITC labelled secondary antibody at 1:50 dilution. Immunofluorescence was observed through a Leica DMRB microscope with UV light source and quantified by scanning the negatives into a Compaq P.C. using an Epson GT-8000 desktop scanner and analysing the images with Phoretix gel analysis software (Phoretix, Newcastle, U.K).

2.14 An in vitro desquamation assay

2.14.1 Preparation of porcine stratum corneum

Skin was removed from a pig (approx weight 25 kilograms) within 1h after death. The tissue was washed under running water and swabbed with Hibitane. Hair was removed from the skin with clippers and the skin was then dermatomed to obtain a thin layer of skin containing the epidermis and part of the dermis. Skin biopsies (6 mm) were punched out and placed onto agar (1%) in a petri dish. Aliquots of lipids (5μl) were dissolved in 20% ethanol/ 0.1M Tris-HCl, pH8, or chloroform/methanol; 2:1, applied to the skin biopsies and incubated (24h, 80% relative humidity, 37°C). Each biopsy was placed in an Eppendorf containing 100μl of SDS (2%), benzalkonium chloride (0.3%) and functionally desquamated corneocytes were then detached from the biopsies by sonication for 1min, using a Lucas Dawe Sonicleaner. The biopsies were then removed and the released cells were pelleted by centrifugation (13,000g for 5 min). Protein from these
corneocytes was extracted with 1 ml of 2% (w/v) SDS/ 50 mM β-mercaptoethanol overnight at 70°C, leaving the cornified cell envelopes. The resultant envelopes were then pelleted by centrifugation (20,000g, 5min) and 900μl of supernatant was removed and discarded. The remaining 100μl containing the envelopes was retained for assay.

2.14.2 Quantification of cornified envelopes

Cornified envelopes were quantified using a colorimetric assay based on Coomassie blue staining. A PVDF membrane (9 x 12 cm; Immobilon-P, Millipore) was wetted with methanol and blocked with Tris-HCl buffered saline (50mM Tris-HCl pH 7.5, 0.15M NaCl) containing 0.5% tween 20 (TBS-T) for at least 2 hours before assembly in the 96 well dot-blot apparatus (Biorad). The 100μl cornified envelope samples (prepared according to 2.14.1) were applied to the membrane and filtered under gravity. Once drained through, the wells were washed twice with 200 μl of TBS-T and finally a vacuum was applied to remove excess liquid. The membrane was dried at 70°C, stained with 0.1% (w/v) Coomassie blue in 20% (v/v) isopropanol/ 10% (v/v) acetic acid (30 min) and destained in methanol (30 min).

The membranes were analysed by electronic capture of the image with an Epson GT-8000 desktop scanner (400 d.p.i resolution) linked to a Compaq 486/66i computer using the Phoretix 1D gel analysis software package (Phoretix Int. Ltd., Newcastle). The data were converted from pixel values to optical density (O.D.) units by calibration with an O.D. Reflection Density Guide (Kodak). The volume (integrated peak height x spot area) of each spot was
determined. The cornified envelope staining was linear between 47-1520 envelopes (Data not shown). The coefficient of variation was found to be ~15%.

2.15 Electron microscopic analysis

Stratum corneum tape strippings were fixed for eight minutes in 0.1 M sodium cacodylate buffer pH 7.4, containing ruthenium tetroxide (0.2%), dehydrated through a series of alcohol solutions to propylene oxide and embedded in TAAB resin (TAAB Lab., Aldermaster, UK). Ultrathin sections were stained with lead citrate and viewed in a JEOL 100 CXII transmission electron microscope.

2.16 Transepidermal water loss measurements

Prior to tape stripping, transepidermal water loss (TEWL) measurements were made using a Servo-Med Evaporimeter. Subjects were equilibrated at 20 ± 1°C with a relative humidity between 35 - 45% for 30 minutes. During measurement the probe and study site were enclosed in a chamber to prevent any draughts and interference in the readings. All measurements were performed in triplicate, triplicate values typically having a ~5-10% variation. This technique was validated by Unilevers clinical assessment team.
2.17 Skin conductance

Subjects were equilibrated at 20 ± 1°C with a relative humidity between 35 - 45% for 30 minutes. Skin conductance was measured using the skin surface hygrometer, Skicon (IBS Ltd., Japan). Six readings were taken to get an overall value of skin hydration. Skin hydration and hence conductance being reduced in dry skin. This technique was validated by Unilevers clinical assessment team.

2.18 Erythema measurements

The Dia-stron erythema meter was used in addition to visual assessment to measure skin redness for determination of the subject MEDs following UV irradiation. The principle of this technique is to compare the reflection of red and green light from the skin. This technique correlates well with visual assessments (r=0.95) (Unilever internal report). This technique was validated by Unilevers clinical assessment team.
CHAPTER 3

3. Abnormalities in Stratum Corneum structure, lipid composition, and corneodesmosome degradation in skin xerosis

3.1 Introduction

Skin dryness is a common disorder (Chapter 1.7), being observed as the accumulation of visible scales or corneocyte clumps on the surface of the skin (Pierard et al 1987). The severity of dryness varies greatly (Chapter 1.7), being most severe in conditions such as the genetic ichthyoses affecting a minority of the population (Williams et al 1983, Williams et al 1991), to more mild dryness, often referred to as xerosis. Xerosis concerns a large proportion of the population, in particular during the winter season or in the elderly (Saint Leger et al 1989). Although the precise causes of skin xerosis are likely to be multifactorial, perturbed degradation of the corneocyte proteinaceous adhesive elements (corneodesmosomes) and impaired barrier function are thought to be key.

The stratum corneum lipids have been well studied in xerosis, although much is still unknown as to their precise role. Solvent (Imokawa et al 1986) or surfactant (Imokawa et al 1989) induced dry scaly skin has been shown to be associated with lipid depletion. In winter xerosis, fatty acid levels have been shown to be elevated, ceramides, cholesterol and cholesterol sulphate being refractory to change (Saint Leger et al 1989). This is in
total contrast to psoriasis, where free fatty acid levels have been shown to decrease by 46% and phytosphingosine containing ceramides to decrease by 27% (Motta et al 1994). A similar decline in ceramide levels has been demonstrated in atopic dermatitis (Imokawa et al 1991). Although, research by other groups suggests no difference in ceramide levels in atopic dermatitis (Yamamoto et al 1991), emphasising the lack of consensus to date. These are a few of many lipid abnormalities associated with skin dryness (Chapter 1.7), highlighting the importance of the stratum corneum lipids to skin condition.

Alterations in the biochemistry of the stratum corneum lipids is also likely to impact on their bilayer structure and phase behaviour (Naik et al 1995, Bonte et al 1997b). Perturbations in the barrier, including lipid depletion (Imokawa et al 1989), decreased hydration and extensibility (Leveque et al 1987) and increased erythema / trans-epidermal water loss (TEWL) (Grunewald et al 1995) have been demonstrated after surfactant and soap washing respectively. Although, surprisingly, no abnormalities in lipid structure were shown following surfactant use (Fartasch et al 1991).

The following study was designed to clarify the biochemical and morphological aberrations in stratum corneum lipids and corneodesmosomes in skin xerosis. These components were investigated in particular because of the lack of consensus on the role of lipids in xerosis and the increased recognition of the role of corneodesmosomes in stratum corneum cohesion and desquamation.
3.2 Panellist selection and sampling

3.2.1 Soap-induced winter xerosis (USA)

Caucasian female subjects (n=32) aged 30-40 were selected from a random population for this study. Panellist selection along with all other aspects of this study were cleared through Unilevers ethical and safety clearance committee. The study protocol was based on that originally described by Boisits et al (1989). To generate xerosis, all subjects refrained from using moisturisers and only used soap for cleansing three times per day for two weeks, as described previously (Boisits et al 1989). The soap cleansing involved rubbing soap on the backs of the hands for three periods of 1min on each occasion. At the end of the two weeks, the clinical severity of the xerosis was determined by expert assessors (employed by Unilever Research, USA) according to the criteria shown in table 3.1. This soap washing protocol is known to increase the dryness grade of the skin. Final dryness grades for the subjects were: grade 2 (n=8), grade 3 (n=12), grade 4 (n=12). A group of subjects (n=8) having grade 1 skin condition were selected at the same time for comparison.

At the end of the study skin tape strips were taken. 8 consecutive strips were taken for lipid analysis (Chapter 2.3). 5 consecutive sellotape strips were taken for electron microscope (EM) analysis of lipid and desmosomal structure (Chapter 2.15). These tapes were labelled tape 1, 2, 3 etc. according to their depth. So tape 1 represents the surface tape strip, and tape 5 is the
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deepest tape strip. Skin sampling was done by study staff at Unilever Research USA.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal skin</td>
</tr>
<tr>
<td>2</td>
<td>Mild xerosis characterised by small flakes of dry skin and whitening of dermatoglyphic triangles</td>
</tr>
<tr>
<td>3</td>
<td>Moderate xerosis, small dry flakes giving a light powdery appearance to the hand. Corners of dermatoglyphic triangles starting to uplift.</td>
</tr>
<tr>
<td>4</td>
<td>Well defined xerosis; the entire length of a number of dermatoglyphic triangles have uplifted to generate large, dry skin flakes. Roughness is very evident</td>
</tr>
</tbody>
</table>

Table 3.1 Criteria for classifying dry skin grades used in Skin Condition Assessment, by expert assessors in the USA.

3.2.2 Natural winter xerosis (UK)

Seventeen Caucasian female subjects were selected during the winter season in the UK. The selection was based on obtaining the most females with each dryness grade. Both hands of each panellist were visually assessed and graded for dryness (Table 3.2). The assessment of dryness was done by trained assessors (Unilever Research, UK) and the scale was slightly different to the USA, being optimised for milder dryness. Three trans-epidermal water loss measurements (TEWL) were then made on each hand (Chapter 2.16). Eight consecutive tape strips (3x2cm²) were then
taken from the knuckle area on each hand, these were combined ready for lipid analysis. The corresponding skin dryness grades for both hands were meant. The dryness grades were similar for the two hands on each subject.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Dryness Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ (0.5)</td>
<td>A marginal dryness that is not sufficiently positive to be graded as slight.</td>
</tr>
<tr>
<td>1</td>
<td>A slight dryness: Dullness, mattness to all site or powdery on part of site.</td>
</tr>
<tr>
<td>1+ (1.5)</td>
<td>A slight dryness that is considered higher than slight but is not distinct.</td>
</tr>
</tbody>
</table>

Table 3.2 Grading criteria for levels of dryness used in Skin Condition Assessment, by expert assessors in the UK.

3.3 Stratum corneum lipid and corneodesmosome structure in normal and xerotic skin (USA)

The alterations in lipid and corneodesmosome morphology, in the upper layers of the stratum corneum, prior to desquamation, were investigated in the soap induced winter xerosis study (USA). In normal skin, within the lower cell layers (tape 3 of 8 consecutive tape strips) the corneodesmosomes appeared as intact structures (Fig. 3.1) in direct contact with the multiple lamellae bilayer lipid structures. As the cells migrate towards the upper stratum corneum (tapes 2 and 1), the corneodesmosomes appeared to undergo degradation,
becoming vacuolated and surrounded by the intercellular lipids prior to their total degradation. The desmosomal number therefore decreases dramatically in the upper layers of normal skin. This is in total contrast to dry skin, where the corneodesmosomes persist intact into the upper layers (Compare tape 1 Fig. 3.2 with tape 1 Fig. 3.1a).

In addition to the alterations in desmosomal processing in dryness, abnormalities in lipid structure were also observed. In normal skin, the lipids appear as multilamellae bilayers (Fig. 3.3), with the exception of the surface layers which appear to lose structure, becoming amorphous. In severe dryness, normal intercellular lipid structures were found in the deep layers. However, the peripheral layers were found to lack a bilayer structure, the lipids appearing highly disorganised (Fig. 3.4). This was visually different from the change in lipid morphology observed in the surface layers of normal skin.
Fig. 3.1 Corneodesmosome degradation at the surface of normal skin (Grade 1) from the USA xerosis study. Degradation of corneodesmosomes towards the surface of the stratum corneum in 3 consecutive tape strips from one subject. a. 1st tape strip; corneodesmosome completely degraded. b. 2nd strip; corneodesmosome is partially degraded and encapsulated with lipid. c. 2nd strip; corneodesmosome vacuolation. d. 3rd strip; lipid envelopes in direct contact with corneodesmosome (x200,000).
Fig. 3.2 Corneodesmosome retention in the surface layers of subjects with severe xerosis (grade 4) from the USA xerosis study.

The pictures above represent surface tape strips (Tape 1) from two subjects (a and b) with severe dryness (grade 4). This demonstrates the corneodesmosome persistence in the outermost layers of the stratum corneum. The pictures are typical for subjects of this grade. (x200,000).
Fig. 3.3 Alteration to the lipid structure with depth in normal skin (Grade 1) from the USA xerosis study

Morphological changes in lipid lamellae towards the surface of the stratum corneum. The electron micrographs represent 3 consecutive tape strips from one subject. a. 1st tape strip; absence of lipid bilayers, amorphous lipid present, corneodesmosome completely degraded. b. 2nd tape strip; disruption of lipid lamellae structure visible c. 3rd strip; normal lipid lamellae clear (x200,000).
Fig. 3.4 Electron micrographs of tape strippings from subjects with severe xerosis (Grade 4) from the USA xerosis study. Aberration in lipid organisation towards the surface of the stratum corneum. a. First tape strip; disorganised lipid lamella. b. Second tape strip; disorganised lipid lamella. c. Third tape strip; normal lipid lamella. (X200,000).
3.4 Stratum Corneum lipid biochemistry and barrier function

3.4.1 Soap induced winter xerosis (USA)

Fig. 3.5 The decline in ceramide levels associated with soap-induced winter xerosis (USA). The data represent the combined lipid extracts from 8 tape strips of hand stratum corneum. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 8 tapes (Chapter 2). The results represent the mean ± SD for subjects: grade 1 (n=8), grade 2 (n=12), grade 3 (n=12) and grade 4 (n=12). Values that are significantly different from grade 1 by the Students t-test are shown as * P<0.05.
As shown in Fig. 3.5, in comparison with normal skin (grade 1), statistically significant decreases in the total levels of ceramides were seen in xerosis grades 3 & 4 (P<0.05), but not with grade 2. There were no significant changes in the levels of fatty acids or cholesterol. Although total ceramide levels were found to decrease, no significant differences were observed in the relative levels of the different ceramide species with increasing dryness grade (Table 3.3). Although there is a trend of decreasing ceramide 6i and 6ii with increased dryness grade (grade 4). When the stratum corneum lipids are expressed as a percentage of the total lipid, no differences were observed between dryness grades (data not shown). The ratios of ceramide, fatty acid and cholesterol are therefore maintained.

As alterations to the structure of the stratum corneum lipids in response to soap induced winter xerosis (USA) were observed with skin depth by E.M. (Figs. 3.3, 3.4), changes in the relative lipid percentages with depth were investigated. This was performed by comparing the lipids of the peripheral first tape strip with those tapes lower down. In normal skin (grade 1), very little difference was observed in the lipid ratios between the surface (tape 1, outer) and deep layers (tapes 2-8, inner) (Table 3.4). In contrast, in xerosis (grades 2-4), the relative ceramide levels appeared depleted in the surface layers, whereas fatty acids showed a tendency to increase (Table. 3.4). In grade 4 xerosis, cholesterol also showed a tendency to increase in the surface layers. The decrease in surface ceramide levels observed in xerosis was observed for all the ceramide species (data not shown). Hence the relative percentages
of the different ceramides did not significantly differ between the dryness grades (Table 3.3).

<table>
<thead>
<tr>
<th>Skin Xerosis grade</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceramide levels (relative percentage)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cer 1</td>
<td>10.6 ± 2.8</td>
<td>12.4 ± 3.6</td>
<td>15.6 ± 3.3</td>
<td>15.7 ± 8.4</td>
</tr>
<tr>
<td>Cer 2</td>
<td>20.2 ± 4.2</td>
<td>23.2 ± 3.8</td>
<td>24.6 ± 3.8</td>
<td>28.6 ± 6.1</td>
</tr>
<tr>
<td>Cer 3</td>
<td>18.0 ± 2.9</td>
<td>17.5 ± 3.2</td>
<td>17.7 ± 4.5</td>
<td>16.1 ± 4.5</td>
</tr>
<tr>
<td>Cer 4</td>
<td>10.6 ± 4.6</td>
<td>6.4 ± 1.5</td>
<td>6.7 ± 3.3</td>
<td>6.7 ± 2.7</td>
</tr>
<tr>
<td>Cer 5</td>
<td>21.8 ± 5.1</td>
<td>22.9 ± 1.5</td>
<td>20.9 ± 2.5</td>
<td>19.7 ± 5.4</td>
</tr>
<tr>
<td>Cer 6I</td>
<td>7.0 ± 3.5</td>
<td>6.0 ± 1.7</td>
<td>3.7 ± 2.2</td>
<td>2.4 ± 1.6</td>
</tr>
<tr>
<td>Cer 6ii</td>
<td>12.4 ± 3.1</td>
<td>11.7 ± 1.4</td>
<td>10.4 ± 2.6</td>
<td>9.5 ± 2.7</td>
</tr>
</tbody>
</table>

Table 3.3 Individual ceramide levels in healthy and dry stratum corneum (Soap-induced winter xerosis study (USA)). The data represent the combined lipid extracts from 8 tape strips of hand stratum corneum. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 8 tapes (Chapter 2). The results represent the mean ± SD for each ceramide represented as a percentage of the total ceramide. Subjects: grade 1 (n=8), grade 2 (n=12), grade 3 (n=12) and grade 4 (n=12). No values were significantly different from grade 1 by the Students t-test.
Chapter 3 - Skin xerosis

### Table 3.4

<table>
<thead>
<tr>
<th>Skin Xerosis grade (Depth)</th>
<th>Relative lipid levels (% of total lipids)</th>
<th>Ceramides</th>
<th>Fatty acids</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Inner)</td>
<td>56.8 ± 12.2</td>
<td>38.6 ± 12.6</td>
<td>4.7 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>(Outer)</td>
<td>52.6 ± 11.9</td>
<td>42.4 ± 11.9</td>
<td>5.1 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>2 (Inner)</td>
<td>58.6 ± 11.8*</td>
<td>35.9 ± 14.4</td>
<td>5.6 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>(Outer)</td>
<td>45.4 ± 15.1</td>
<td>45.5 ± 21.6</td>
<td>9.0 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>3 (Inner)</td>
<td>44.7 ± 20.8*</td>
<td>46.4 ± 18.7</td>
<td>6.0 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>(Outer)</td>
<td>34.7 ± 18.5</td>
<td>59.3 ± 18.5</td>
<td>6.1 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>4 (Inner)</td>
<td>45.5 ± 10.6*</td>
<td>49.9 ± 9.4</td>
<td>4.6 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>(Outer)</td>
<td>35.2 ± 14.1</td>
<td>57.7 ± 13.8</td>
<td>7.1 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

The relative lipid levels in healthy and dry stratum corneum of different depths (Soap-induced winter xerosis study (USA)). The data represent the lipid extracts from tape 1 (Outer) compared with tapes 2-8 (Inner) of hand stratum corneum. Lipid classes were column isolated, quantified and expressed as the percentage of the total lipid extracted (Chapter 2). The results represent the mean ± SD for subjects: grade 1 (n=8), grade 2 (n=12), grade 3 (n=12) and grade 4 (n=12). Inner values that are significantly different from the outer values of the same dryness grade, by the Students t-test, are shown as * P<0.05.

### 3.4.2 Natural winter xerosis (UK)

The degree of dryness induced in this study was found to be less than that induced by soap damage in the USA, the
grades obtained in this UK study being 0.5-1.5, compared with 1-4 in the USA. One of the main reasons for this is the severe winter conditions experienced in the USA, with far lower temperatures and humidities relative to the UK.

The mass and percentage levels of the stratum corneum lipids in natural winter dry skin (UK), i.e. that caused by lower seasonal temperatures and humidities, are shown in Fig. 3.6 - 3.7. As demonstrated in Fig. 3.6, there was very little difference observed in the levels of the stratum corneum lipids in subjects with very mild xerosis (grade 1.5) and normal skin condition (grade 0.5). Although the relative percentage of fatty acids and ceramides (% of total lipids) showed a tendency to increase and decrease respectively with increasing dryness grade. However this did not reach significance (Fig. 3.7). This is consistent with the aberrations noted in the soap induced winter xerosis (Section 3.4.1). The trend of the decline in relative levels of the ceramides was consistent for all the ceramide species and hence the relative percentage of each, as observed in the soap induced winter xerosis (USA), remained constant (Table 3.5). In the UK natural xerosis study the level of dryness was not sufficient to measure a significant shift in our biochemical lipid markers. The lower subject number due to a drop-out of subjects in the study has also made it more difficult to achieve a significant result.
**Fig. 3.6** The lipid levels in natural winter xerosis (UK) compared with normal skin. The data represent the combined lipid extracts from 8 tape strips off both hands. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 8 tapes (Chapter 2). The results represent the mean ± SD for subjects: grade 0.5 (n=9), grade 1 (n=6), grade 1.5 (n=2). None of the values were found to be significantly different from grade 0.5 by the Students t-test.
Fig. 3.7 Alterations in the relative lipid levels with increasing dryness grade (Natural winter xerosis, UK). The data represent the combined lipid extracts from 8 tape strips off both hands. Lipid classes were column isolated, quantified and represented as a percentage of the total lipid extract. The results represent the mean ± SD for subjects: grade 0.5 (n=9), grade 1 (n=6), grade 1.5 (n=2). None of the values were found to be significantly different from grade 0.5 by the Students t-test.
### Table 3.5 The relative percentage of ceramides in winter dry skin.

The data represent the combined lipid extracts from 8 tape strips off each hand. Lipid classes were column isolated, quantified and represented as a percentage of the total ceramides. The results represent the mean ± SD for subjects: Grade 1 (n=9); Grade 2 (n=6); Grade 3 (n=2). No values were significantly different from grade 0.5 by the Students t-test.
3.4.3 Barrier function

In the natural winter xerosis study, the barrier performance, as determined by TEWL was measured. No significant correlation was observed between TEWL and the levels of ceramides, fatty acids or cholesterol (Fig. 3.8). In other forms of dry skin, for example dandruff, ceramide levels have been shown to give a good inverse correlation with TEWL (Data not shown). However, in this study the level of dryness obtained was insufficient to significantly alter TEWL levels.
Fig. 3.8 The relationship between transepidermal water loss and stratum corneum lipid levels (Natural winter xerosis, UK). Prior to tape stripping, TEWL measurements were made on the hands of 15 subjects. The data above represent TEWL values plotted against ceramide, fatty acid and cholesterol levels. No correlation was observed by linear regression.
3.5 Stratum corneum desmoglein 1 analysis

In support of the electron micrographs from the USA dryness study showing the retention of corneodesmosomes in dry skin, the levels of the biochemical desmosomal marker desmoglein 1 were compared in normal (grade 1) and xerotic (grade 4) skin tape strippings from the USA dryness study. In support of the E.M. evidence, a statistically significant increase of ~7 fold, in desmoglein 1 was observed in xerotic compared with normal skin. This highlights the retention of corneodesmosomes in xerosis as suggested by the EM studies. Quantification of dsg1 levels by this technique provides a rapid, sensitive and robust methodology for evaluating perturbations in corneodesmosomal hydrolysis. This study emphasised that skin dryness is associated with abnormalities in the proteolytic degradation of the desmosomal linkages.
Fig 3.9 desmoglein 1 levels in the stratum corneum of normal skin and xerosis (Soap-induced winter xerosis, USA). After extraction from the corneocytes (Chapter 2.13), the levels of dsg 1 in tape 1 were determined by SDS PAGE and Western blotting. The levels were quantified using a phoretix software analysis package and the values are expressed, relative to keratin. The data represent the mean ± standard deviation for 8 subjects (normal skin, grade 1) and 12 subjects (xerosis, grade 4). A significant increase in dsg1 levels was found in skin with xerosis, as determined by a Students t-test, P<0.05.
3.6 Discussion

An understanding of the biochemistry and structure of the surface layers of the stratum corneum, where skin dryness manifests itself, is essential to understanding skin xerosis. In order to achieve this a sensitive tape stripping methodology was used, that has the distinct advantage of sampling the tissue layers in direct contact with the external environment. This technique, alongside electron microscopic, chromatographic and electrophoretic methods, has clearly demonstrated that xerosis is associated with abnormalities in lipid biochemistry and structure, along with desmosomal processing.

In normal healthy skin, the majority of the desmosomal linkages are degraded at the stratum compactum / disjunctum interface, as described in chapter 1. However, as shown by E.M., modified desmosomes, corneodesmosomes, do persist into the stratum corneum, where they continue to be degraded. Visualisation of the desmosomal linkages using monoclonal antibodies has illustrated that corneodesmosomes on the upper and lower faces of the corneocyte are degraded first during stratum corneum transit (Long et al 1996, Chapter 1). The corneodesmosomes at the edge of the interdigitating corneocytes are degraded in the superficial layers prior to desquamation.

The proteolytic digestion of the corneodesmosomes, by stratum corneum chymotryptic like enzyme and possibly other serine proteases (Chapter 5), appears to begin with the internal components. This is followed by a
widening of the intercellular space along with a progressive detachment from the corneocyte cell envelope (CE). The stratum corneum lipids appear to flow around the desmosomal remnants, separating them from the CE. Similar findings to this have been reported previously (Egulrud T. et al 1989 Fartasch 1996). The degradation of the desmosomal linkages is undoubtedly important for desquamation to occur normally as they are the main intercorneocyte linkage. The engulfing of the desmosomal remnants by the lipids most likely ensures that the gap is filled and the water barrier is maintained.

In soap induced winter xerosis, the proteolytic degradation of the corneodesmosomes is perturbed. As a result, intact corneodesmosomes persist into the surface skin layers, as demonstrated by E.M. and analysis of dsg1 levels. This retention of corneodesmosomes has been demonstrated previously in other skin scaling disorders (Williams et al 1991, Ghadially and Chong 1992) and is likely to lead to the clumping of surface squames characteristic of dryness conditions. It may also contribute to the decrease in skin elasticity observed in soap induced winter xerosis (Leveque et al 1987). Desmosomal degradation has been shown previously to greatly enhance the elasticity of isolated stratum corneum (Rawlings et al 1995). However, lipid alterations and natural moisturising factors will also contribute to this loss of elasticity (Horii et al 1989).

In normal healthy skin the stratum corneum lipids also alter in morphology and composition during stratum
corneum transit. In all but the surface layers of the stratum corneum, where the lipids appear more disorganised, the intercellular lipids can be clearly observed as multi-lipid bilayers. These findings are supported by infrared spectroscopic methods that suggest a higher fluidity and level of disorder in the lipids of the outer stratum corneum (Bommanan et al 1990). The xerotic stratum corneum also showed perturbations in lipid structure in this study, although the lipid structures were far more disorganised than normal skin, forming an amorphous mass. It is unlikely that changes in surface lipid were due to residual petrolatum from previously applied moisturisers (Ghadially et al 1992), as the changes were consistent for all subjects. In addition, subjects refrained from using moisturisers for at least one week prior to the start of the study. This lipid disruption is likely to be due to the enhanced relative levels of fatty acid, in the surface compared with the deeper layers. Certainly the lipid phase behaviour is dependent on the ratios of ceramide : fatty acid : cholesterol (Wartewig et al 1998, Neubert et al 1997). In addition, increased levels of oleic acid which accounts for approximately 26% of the free fatty acids within the stratum corneum (Chapter 4), will increase the fluidity of the lipid mix and can cause phase separations (Naik A et al 1995). This may explain the marked disruption of lipid bilayers in soap induced xerosis.

Soap induced winter xerosis and to a lesser degree mild natural winter xerosis were also associated with alterations in lipid composition. Xerotic skin had a significantly lower level of ceramides (species 1-6) and
marginally elevated fatty acid levels relative to normal stratum corneum. Similar decreases in ceramides have been found in atopic dermatitis (Imokawa et al 1991, Yammamoto et al 1991). In a more recent study, the levels of ceramides, in particular ceramide 1 and 3 decreased and cholesterol significantly increased in atopics compared with healthy subjects. The ceramide:cholesterol ratio was significantly lower with respect to normal skin (DiNardo et al 1998). Alterations in the relative proportions of the different ceramides were not observed in either the soap-induced xerosis (USA) or the natural xerosis (UK) studies. In addition, increased amounts of fatty acid have been associated with the severity of dry skin in xerosis (Saint Leger et al 1989).

There may be a number of possibilities accounting for the aberrations in stratum corneum lipid composition in dryness. The increase in fatty acids may have come from the breakdown of ceramides themselves by stratum corneum ceramidase (Wertz et al 1990) or from the soap itself. Recent research has shown the presence of Pseudomonas aeruginosa in subjects with atopic dermatitis, which produces a ceramidase (Okino N. et al 1998). This may account for the decline in ceramides in this condition. However, the role of ceramidase in soap induced winter xerosis remains unproven.

The decline in ceramide and increase in fatty acid levels may also be reflective of altered expression of the lipids within the granular layer. Both barrier damage and epidermal damage (through soap penetration) can trigger a number of cellular responses within the
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stratum granulosm (Chapter 1). In particular, the disruption of the permeability barrier results in increased cholesterol, fatty acid, and ceramide synthesis in the underlying epidermis. This occurs through an upregulation of transcription for enzymes involved in the synthesis of these lipids. i.e. HMG-CoA reductase, acetyl-CoA carboxylase, fatty acid synthase and serine palmitoyl transferase, for cholesterol, fatty acid, and ceramide synthesis respectively (Harris IR et al 1997). In addition, β-glucocerebrosidase, which converts glucoceramides into ceramides, is also upregulated in response to barrier damage (Holleran et al 1994). In skin xerosis, the disregulation of this process is likely to play a role in the lipid perturbations observed.

Alterations in the levels of the stratum corneum lipids are likely to lead to both alterations in the phase behaviour and barrier properties of the skin. Certainly, fatty acids themselves offer little resistance to water transport (Friberg et al 1989), suggesting the increased levels in xerosis may affect both the structure and functionality of the barrier. However, in the natural winter xerosis study (UK) no correlation was observed between ceramide, cholesterol or fatty acid levels with TEWL. The lipid levels of different bodysites has been shown previously to inversely correlate with their known permeabilities, reflecting barrier performance (Lampe et al 1993). In this study, the level of dryness achieved, and hence alterations in the lipid levels are insufficient to alter the barrier performance as measured by TEWL. In other skin conditions, such as dandruff, ceramide levels are an excellent determinant
of dryness grade and hence TEWL (Rogers and Harding, Unilever Research, unpublished observation).

The surface aberrations in lipid structure/composition and desmosomal degradation in xerosis, may potentially be linked. Alterations in the ratios of stratum corneum lipids may prevent normal desquamation occurring in a number of ways. As desmosomal degradation is a protease mediated event, the water barrier and water activity will have a marked effect on the proteolysis, as described in chapter 6. These factors are clearly influenced by the levels and ratios of the different stratum corneum lipids. In addition, changes in lipid composition and phase behaviour within the intercellular space may have a more direct effect on the desquamatory proteases. Fatty acids, which are elevated in xerosis, are known to inhibit a number of chymotryptic-like enzymes (Kido et al 1984, Chapter 6). The decline in extractable SCCE activity that is observed in dryness (Watkinson, Unilever research, Unpublished observation) may be due to a number of factors including altered enzyme expression, inhibition by soap, leaching of the enzyme or a disrupted lipid environment. However, the effects of lipid alterations on SCCE activity in vivo are difficult to determine due to a lack of specific probes.

Initially it was suggested that cell cohesion in the stratum corneum was dependent on the intercellular lipid cement. It is now clear that it is the corneodesmosomes, and not the lipids which play the key role in corneocyte cohesion. The aberrations in winter xerosis appear to manifest themselves visually in the
upper layers of the stratum corneum, with retention of corneodesmosomes and a collapse of the lipid bilayer structure. Although at a biochemical level, alterations in the ratios of the stratum corneum lipids are detected far deeper. These factors appear to contribute to the aberrant desquamation and scaling common in xerosis.
CHAPTER 4

4. The effects of acute UV irradiation and chronic UV irradiation on the stratum corneum (a preliminary study).

4.1 Introduction

Exposure of the human epidermis to ultra-violet radiation (UV) leads to numerous, well documented, changes in the physiological and biochemical features of the skin (Chouinard et al 1997, Pathak 1997). However, the effects of UV on skin condition and water barrier function appear contradictory, both causing and relieving skin dryness. Exposure of the skin to UV radiation can induce a group of skin diseases, the photodermatoses (Hawk, 1993) and chronic exposure to UV is associated with an impaired water barrier (Abe et al 1979) and skin dryness (Chatenay et al 1990). However, UV has proved beneficial in the treatment of dermatoses such as psoriasis and atopic dermatitis (Jekler and Larko 1991, Falk 1985). These beneficial effects are thought to be at least in part associated with an immune suppression following UV (Jones et al 1996). Although they may involve alterations in the levels and composition of the stratum corneum lipids.

UV irradiation may affect the levels and composition of the stratum corneum lipid by a number of routes. These include a reduction in lipid biosynthesis in the viable epidermis (Punnonen et al 1991, Black 1971), the disruption of lamellar granules by the peroxidation of membrane lipids (Punnonen et al 1991b) and alterations in the activities of processing enzymes. Although there have
been a number of investigations into the effects of UV irradiation on skin lipids in the literature, the data are inconclusive.

UV irradiation (in vitro) has been shown by numerous workers to have an effect on skin lipid levels. Cholesterol levels have been shown to be depleted after acute UV exposure of punch biopsies (Black 1971) and human post-mortem skin (Rauschkolb et al 1967). The reports concerning the effects of UV irradiation in vivo are quite different from the in vitro data. Depletions in sebaceous neutral lipids (Picardo et al 1991) and elevated cholesterol levels have been observed by others following UV irradiation (Ohkido et al 1974). After chronic UV irradiation (9x 1.5 MED) and sub erythemal doses of UV, both Lehmann and Wefers respectively showed elevations in all 'stratum corneum' lipids. However, the lipids extracted in this study were mainly of sebaceous origin, the ceramides representing only 4% (Wefers et al 1991, Lehmann et al 1991). Although these studies have enhanced our knowledge into the effects of UV on skin, the data are marred by both heavy sebum contamination and the solvent sampling techniques used. The work in this chapter aims to clarify the influence of UV on the composition of the barrier lipids.

4.2 Panellist selection

Panellist selection along with all other aspects of this study were included in a full study protocol submitted to Unilevers ethical and safety committee for clearance. For the acute UV study 14 caucasian men with no pigmentary lesions such as moles on the back were selected and for
the chronic UV study, 3 caucasian females with no moles on their lower volar forearm were recruited. All panellists refrained from using any moisturiser or any other skin product for seven days prior to the study commencing. In addition they had no natural or artificial UV exposure for a period of one month prior to the study commencing.

4.2.1 Pre-test MED (minimum erythemal dose) evaluation

The lamp system used in this study was a bank of 6 Helaire UVB fluorescent strip lamps (Sun Health Company). These are regularly used by dermatologists for photo-chemical therapeutic treatment of psoriasis. Their spectral output is restricted to mainly UVB above 300nm (Diffey and Parr 1987). For the acute study, four squares measuring 2cm\(^2\) of back skin below the waistline were exposed to a varied dose of UVB to establish a 'personal' minimal erythemal dose (MED) for each volunteer. One MED is defined as the lowest amount of UV required to achieve an observable redness (erythema) 24 hours after exposure. For the chronic study this was performed on a site outside the test area on the inner volar forearm. MED values were determined by visual and instrumental assessment using an erythema meter (Chapter 2). After determining the dose of UV required to give 1 MED the level required to give 2 MEDs for the acute study and 1.5 MEDs for the chronic study was determined.
4.2.2 Irradiation protocol

For the acute study, ten areas (2cm x 7cm) were marked out on the test area below the shoulder blades, five on each side of the spine. Five out of the ten sites were exposed to 2 MEDS of UV irradiation, the other five were covered and acted as control sites, these were randomly assigned. On five occasions, at five day intervals, the first being immediately after the 2 MED dose, each volunteer was assessed for dryness, erythema, and trans-epidermal water loss (TEWL). Finally the assessed sites were tape stripped.

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Layout of Sites on the Back.

For the chronic study, one area (12cm x 5cm) was marked out on one inner volar forearm and the other arm acted as a control. On days 1,3,5,8,10,12,15 and 17 from the study commencing the site was exposed to 1.5 MEDs of UVB radiation. On day 18 the test and control areas were
assessed for dryness, erythema, water content and finally tape stripped. Sello 1601 tapes (8 consecutive strips) were collected for lipid analysis. 8 consecutive strips of sellotape were also taken for cholesterol sulphate determination (Chapter 2).

4.3 The effects of acute UV irradiation on the stratum corneum lipids

Following one acute UVB irradiation dose (2MED) there were no effects observed (days 1-10) on the levels of the stratum corneum lipids, ceramides, fatty acids and cholesterol (Fig. 4.1). In addition, the percentage composition of the lipids (ceramides, fatty acids and cholesterol) was similar for both the UV irradiated sites and the control (Table 4.1). The lack of an immediate effect (24h) on the nature of the stratum corneum lipid levels suggests that the barrier lipids, due to their low degree of unsaturation, or cholesterol levels, are fairly resistant to peroxidation damage by UV (Lasch et al 1997).

After allowing the stratum corneum to turnover (15-19 days) there was a tendency of increased lipid levels in both the covered (sham irradiated) and the exposed UV irradiated site (Fig. 4.1). However, the relative percentage of the ceramides, fatty acids and cholesterol measured remained fairly constant (Table 4.1). The same was found to be true for the relative levels of the individual ceramides (Fig. 4.2). The relative levels of the individual fatty acids were only measured at day 10 following irradiation, and they showed no significant difference between the exposed UV irradiated and covered (sham irradiated) sites (Fig. 4.3).
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In terms of eliciting a UV effect, it appears that the level of UV given to the subjects was insufficient to produce any dramatic changes in the stratum corneum lipid levels. This level of UV dose was at the time, the maximum permitted by the Unilever ethical and safety clearance group.

The effects observed of boosted lipid levels in both the sham (covered) and UV irradiated sites, is similar to what others have found following UV irradiation. Acute exposure of human subjects to UV irradiation (3 MED) has shown that after 9-11 days the percentage of neutral lipids (sterol esters and triglycerides) were found to decrease, whilst the percentage of ceramides, free cholesterol and total lipid increased (Punnonen et al 1991b). The level of all species returned to normal after 20 days. In the Punnonen study the UV irradiation was mixed UVA and UVB and the dose given was far greater, possibly accounting for the difference in results. In addition, the lipids were extracted by direct solvent extraction of the skin, without normalising to the amount of tissue extracted. As UV is known to induce dryness and parakeratosis (Chatenay et al 1990) increased penetration of solvent and hence subsequent extraction of lipids from the irradiated sites is likely. This emphasises the need to normalise the lipid data, to allow comparison of UV irradiated sites with the non irradiated sites.

There could be a number of explanations for the tendency of lipid levels to increase in the control (sham irradiated) and exposed UV irradiated sites after 15-19 days in this study (Fig. 4.1). One possibility is that following UV irradiation, soluble cytoplasmic factors such
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as the inflammatory cytokines migrated from the irradiated to the control sites. Skin barrier damage by various means (UV, Acetone, tape stripping) has been shown to upregulate the epidermal lipid biosynthetic enzymes in mice (Elias 1991, Grubauer 1987) along with the formation and rapid release of lamella bodies (Feingold 1990, Menon 1985). This repair response may be mediated by inflammatory mediators (Chapter 1). For example, both interleukin 1 and tumour necrosis factor, are elevated following UV exposure (Kock 1990, Hirao et al 1996). The repair response is essential in reinforcing the barrier so its function is restored.

A recent report showed that following one dose of UVB (7.5 MED) to murine epidermis, the stratum corneum appeared normal (24 hours), but the lamellar granules were destroyed in the cells immediately beneath the stratum granulosum / stratum corneum interface (Holleran et al 1997). As the lamella granule deficient cells reach the stratum granulosum / corneum interface (72-96h) a reduction in barrier performance is observed, triggering a large increase in barrier lipid biosynthesis followed by lamella granule release. If a similar effect was observed in humans, these cells would take ~15days to reach the skin surface, giving the appearance of boosted stratum corneum lipid levels. However, the levels of UV in the Holleran mouse study were much higher, and hence this effect is unlikely in our acute UV study, where no difference in lipid levels was observed between the UV treated and control (sham irradiated) skin sites.
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It was initially surprising that the control (Sham irradiated) site followed a similar trend in terms of increased lipid levels at 15-19 days (Fig. 4.1) to the UV irradiated site. However, the stratum corneum lipid levels are known to be influenced by temperature, having been shown to be elevated in the summer season (Chapter 5). Thus, possibly the intense heat from the lamp increased the skin temperature and upregulated lipid biosynthesis in both test and control sites via enhancing lipid biosynthetic enzyme activity. The heat stimulation of enzyme activity may occur due to the heat itself, or like UV, via an inflammatory damage response (Chapter 1). Although the covered sites appeared red initially following the treatment due to the heat, this soon disappeared and no change in erythema was observed in the control sites.

In terms of effects on barrier function, TEWL showed a tendency to be reduced at both the 5 and 19 day time point in both UV irradiated and covered sites, although not significantly (Fig. 4.4). This may suggest an improvement in the barrier at these time points. However, although lipid levels were boosted at 19 days, no elevation in lipid levels was observed at 5 days to account for this difference. It therefore appears that the level of UV given was insufficient to cause any barrier damage, as measured by TEWL. A Friedman’s test was used to determine the UV treatment effect on levels of erythema (redness) at each time point during the study. There was a significant increase in erythema levels on days 1 and 5 in the UV irradiated site, which gradually decreased over the period of measurement to that of the control site.
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This mild ‘sunburn’ was not severe enough to detect measurable changes in the biochemical parameters measured.
Figure 4.1  The effects of acute UV on the stratum corneum lipid levels. The data represent the combined lipid extracts from 8 skin tape strips of back stratum corneum, following a dose of two MEDs on an exposed (a) and covered site (b), at days 1, 5, 10, 15 and 19 post irradiation. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 8 tapes (Chapter 2). The results represent the mean ± SD for 14 male subjects. No significant difference was shown between the exposed and covered sites, as determined using a Students t-test.
### Table 4.1 The effects of acute UV on the relative percentage of the stratum corneum lipids.

The table represents the mean ± SD of the relative percentages of the different lipids calculated for each volunteer (n=14). These results are calculated from the raw data used in Fig. 4.1. No significant difference was shown between the exposed and covered sites, as determined using a Students t-test.
Figure 4.2 The relative percentage of ceramides in the stratum corneum following acute UV irradiation. The data represent the mean ± SD of the relative percentages of the different ceramide species, relative to total ceramide, calculated for each volunteer, following a dose of two MEDs on an exposed (a) and covered site (b), at days 1, 5, 10, 15 and 19 post irradiation. These results are calculated from the raw data used in Fig. 4.1, n=14. No significant difference was observed between UV exposed and covered sites for any of the time points.
Figure 4.3 The relative percentage of the individual free fatty acids following acute UV irradiation. The data represent the profile of free fatty acids which were column isolated from the back skin tape strips at day 10 (Fig. 4.1). Half of the free fatty acids were converted to fatty acid methyl esters and separated and quantified by GC (Chapter 2). The results represent the mean ± SD for 14 subjects. No significant difference was found between UV exposed and covered sites as determined using a Students t-test.
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Figure 4.4 The effects of acute UV irradiation on Transepidermal water loss (TEWL). TEWL was measured in g/cm²/h at day 1, 5, 10, 15 and 19 following UV irradiation (Chapter 2). This was performed prior to tape stripping. The repeated measures analysis of variance indicated that there were no statistically significant time effect (Pr>F=0.3689) or treatment effect (Pr>F=0.2810) on TEWL.
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4.4 The effects of chronic UV irradiation on the stratum corneum lipids

As the acute UV irradiation appeared to have little effect on the stratum corneum lipids, a preliminary trial was performed to establish whether multiple doses of UV irradiation would give a bigger effect. In addition, the aim of the preliminary chronic study was to investigate which of the other key factors which are important for skin condition, actually change as a result of UV irradiation. In contrast to our acute study, heat was not a potential factor in our chronic study as the irradiation time on the inner volar forearm was only 8-10 minutes.

Following 18 days of chronic UV irradiation, there was a definite trend towards a reduction in barrier lipid levels of approximately 25-30% (Fig 4.5). However, these effects were not significantly different due to the low subject number. In contrast the levels of cholesterol sulphate virtually doubled after UV irradiation (Fig. 4.5). The most likely reason for the decline in lipid levels is the continual destruction of the lamella granules as a result of UV damage (Holleran et al 1997). This disruption would lead to abnormal processing and delivery of lipids to the stratum corneum (Chapter 1), potentially explaining the differences observed. In addition, UV may reduce the activity of the lipid biosynthetic enzymes directly as shown for other enzymes (Punnonen et al 1991b). Although barrier lipid levels showed a tendency to decrease and cholesterol sulphate increase, no significant changes were found in the relative percentages, although the relative levels of cholesterol sulphate doubled (Fig. 4.5b).
Figure 4.5 The effects of Chronic UV treatment on the stratum corneum lipid levels. The data represent the combined lipid extracts from 8 skin tape strips of forearm stratum corneum, from a chronic (18 day) UV irradiated (1.5 MED) and control forearm. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 8 tapes (Chapter 2). The results represent the lipid levels (a) and relative percentage (b), mean ± SD for 3 subjects. No significant difference was shown between the exposed and covered sites, as determined using a Students t-test.
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Chronic UV irradiation has been reported previously to decrease skin cholesterol levels (Black et al 1971) and sebaceous lipid levels (Picardo et al 1991). Elevations in all the stratum corneum lipids have been suggested following both chronic and suberythemal doses of UV irradiation, compared to no treatment (Wefers et al 1991, Lehmann et al 1991). However the lipids extracted in that study were mainly of sebaceous origin, the ceramides representing only 4%. The low stratum corneum lipid content was possibly due to the use of solvent turbines for extraction, which are likely to extract mainly skin surface lipids.

Although the stratum corneum lipid levels were reduced after chronic UV irradiation, including an ~30% drop in ceramide levels, no significant differences were observed in the relative amounts of the different ceramides (Fig. 4.6). In contrast to these data, changes in the relative amounts of ceramides and the appearance of extra ‘ceramide’ bands have been previously shown as a result of both chronic (9 X 1.5MED) and suberythemal UV irradiation (Wefers et al 1991, Lehmann et al 1991). However, this study was mainly looking at sebaceous lipids, which could have contained significant quantities of oxidised fatty acids, that co-migrate with the ceramides and confuse interpretations (Rogers, Unilever Research, Unpublished data). Surface fatty acids, unlike those in the stratum corneum, are not protected from oxidation by cholesterol, which is found only in low concentrations in sebum (Chapter 1).

In normal skin the degradation of cholesterol sulphate between the 'living' epidermis and the stratum corneum is catalysed by cholesterol sulphatase. The retention of
cholesterol sulphate after UV irradiation may therefore be due to the UV reducing cholesterol sulphatase activity, as shown for other enzymes (Punnonen et al 1991).

In the chronic UV study the levels of the covalently bound lipids were all significantly reduced as a result of UV irradiation (Fig. 4.7). The levels of ceramide B, hydroxy-acid and fatty acid being decreased by 40-60%. The origin of these lipids is currently unknown although potentially they could originate from the lamella granule membranes. The UV mediated destruction of lamella granules (Holleran et al 1997) and subsequent decline in covalently bound lipid levels, supports this hypothesis. These bound lipids have more recently been shown to be esterified to the corneocyte cell envelopes by the action of transglutaminases (Nemes et al 1999). Therefore UV may also effect covalently bound lipid levels by interfering with transglutaminase activity and this esterification process. Within the covalently bound lipid fraction of both UV irradiated and control skin lipid extracts, a lipid band that co-migrates and has the characteristic purple charring of cholesterol was identified. Although this has previously been shown and identified as cholesterol in clavus (Serizawa et al 1993) and pig stratum corneum, it is unreported in human stratum corneum. In this analysis the band was assigned to cholesterol, although a full structural investigation is warranted.
Figure 4.6 The relative percentage of ceramides in the stratum corneum following chronic UV irradiation. The data represent the mean ± SD of the relative percentages of the different ceramide species, relative to total ceramide, calculated for each volunteer. These results are calculated from the raw data used for Fig. 4.5a, n=3. No significant difference was observed between UV exposed and control sites.
Figure 4.7 The effects of Chronic UV treatment on the stratum corneum covalently bound lipid levels. The data represent the combined lipid extracts from 8 skin tape strips of forearm stratum corneum from a chronic UV irradiated and control forearm. Following the extraction of the intercellular lipids from the squames, the covalently bound lipids were isolated by corneocyte alkali hydrolysis and solvent extraction. The lipids being quantified by HPTLC, staining and scanning densitometry (Chapter 2). The results represent the mean ± SD for 3 subjects. Values that are significantly different from the control as measured by the Students t-test are shown as * P<0.05.
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The effects of UV on a number of other markers were also investigated. The activity of the extractable caseinolytic proteases (serine proteases) of the stratum corneum were measured (Fig. 4.8). No significant changes were observed in caseinolytic protease activity. However, as the levels of cholesterol sulphate, an inhibitor of caseinolytic proteases e.g. SCCE (Chapter 7) are elevated in chronic UV damaged skin, the activity in situ may be reduced. In addition, in this preliminary study, no difference in water content was observed, as measured using Skicon, and only a slight increase in dryness grade was observed on the UV irradiated site (Table 4.2). However, an increase in erythema was observed following each irradiation and a tanning effect was observed at the end of the study (data not shown). At a higher UV dose, the level of dryness would be expected to be greater. However, as the study required ethical / safety clearance, a greater UV dose was not permitted.
Figure 4.8 **The effects of Chronic UV treatment on the stratum corneum proteases.** SDS extracts of tape stripped UV irradiated and control human stratum corneum were fractionated on 12% acrylamide gels containing 0.2% casein. Molecular weight markers and standard proteases of 1ng bovine chymotrypsin (25KDa) and 1ng bovine trypsin (23KDa) were also fractionated. After appropriate washing, the gels were incubated in 50mM Tris/HCl pH 7.5 containing 0.1% sodium azide. Bands of proteolysis were visualised using Coomassie blue (a) and quantified (b) as described in chapter 2. The results (b) represent the mean + SD for 3 subjects. UV irradiated values were not significantly different from the control values as determined by the Students t-test.
Table 4.2 The effects of chronic UV irradiation on the water content and dryness grade. The skin was dryness graded and the water content was measured using the Skicon apparatus (Chapter 2). This was performed prior to tape stripping. The results represent the mean ± SD for 3 subjects. No significant difference was observed as measured by the Students t-test.

4.4 Discussion

This chapter investigates the effects of both an acute and chronic dose of UV on the stratum corneum, particularly the barrier lipids. These studies have provided indicators that may help to explain the differences observed in UV effects previously. Following acute UV irradiation, no significant UV induced changes in the barrier lipids were observed. There was a trend of an upregulation of lipid levels following the repair phase (15-19 days) although this may reflect natural variations. In this acute study, a sham irradiated site was used as a control in an attempt to distinguish UV effects from those caused by heat damage. As the same effects were observed
in both sites it is possible that the boost in lipid levels was caused by the intense heating. Certainly panellists reported a big heating effect and the sites appeared red immediately following sham irradiation. However, as both sites were only physically separated by ~2 inches on the back of panellists, it is possible that soluble cytoplasmic inflammatory mediators produced in the skin as a result of UV irradiation, influenced both sites. The upregulation of lipid biosynthetic enzymes and hence levels occurs following many forms of insult to the skin (e.g. UV, solvent damage). However, whatever the mechanism, the elevated lipid levels in this study help explain some of the beneficial effects of short bursts of UV in the treatment of dermatoses such as psoriasis and atopic dermatitis (Jekler and Larko 1991, Falk et al 1985). Although suppression of immune responses are also key in this beneficial effect (Jones et al 1996). Also, as the UV is administered along with emollients and topically applied steroids, the initial mild UV induced barrier disruption may enhance the penetration of the topical products.

In contrast, during prolonged (chronic) UV treatment the natural repair of the skin is impeded, hence the lipid levels appear to drop. This was true for the intercellular lipids, and particularly the covalently bound lipids, which showed a dramatic reduction. This was possibly due to the destruction of the lamella granules previously suggested in murine skin (Holleran et al 1997). However, as the effect was most severe for the covalently bound lipids, it is more likely that the process of esterification to the cornified cell envelope was impeded. This could occur by a UV induced reduction in the activity of transglutaminase enzymes, responsible for the formation
and maturation of the envelope (Chapter 7) and the esterification of the bound lipids.

The two fold increase in cholesterol sulphate levels following chronic UV irradiation could have an adverse effect on the skin via both influencing lipid phase behaviour and through inhibition of the desquamatory protease 'Stratum corneum chymotryptic-like enzyme', SCCE (Chapter 6). In this investigation (n=3) no differences were observed in the degree of desmosomal hydrolysis, however this requires further investigation using a larger subject number.

Following chronic UV treatment a decreased sensitivity to irritants has been reported (Lehmann et al 1991). This apparent decrease in skin sensitivity may relate to epidermal thickening (Chatenay et al 1990). Although it is more likely that UV cell damage prevents the normal response to irritants.

As Solar irradiation (UV) is known to induce dry scaly skin conditions, it must interfere with the process of desquamation. A key event of which is the digestion of the desmosomal linkages between the corneocytes (Chapter 1). Dry skin conditions are associated with a retention of corneodesmosomes in the upper stratum corneum (Chapter 3). Following chronic UV treatment, no change in the desmosomal marker dsg1 (data not shown) or caseinolytic protease activity (including SCCE) was seen. Previous reports have suggested that UV induced dryness is associated with a retention of dsg1 (Kitahara et al 1992). Thus a decline in SCCE activity responsible for the degradation of desmosomes and dsg1 may be expected.
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However this was a preliminary investigation (n=3) and a larger study will be required to make any conclusions on dsg1 levels or SCCE activity.
CHAPTER 5

5. Seasonal and bodysite fluctuations in the stratum corneum lipids

5.1 Introduction

It is well established that the condition of the skin shows both seasonal and anatomical site (bodysite) differences. The most susceptible areas of the body to atopic dermatitis and xerosis are in general, the face, neck, and extensor surfaces. The more common occurrence of these conditions in winter compared with summer is generally believed to reflect the influence of the harsher environmental conditions on skin condition. One hypothesis for this intrinsic variation is that the condition of the skin’s barrier or integrity of the barrier lipids fluctuates with both site and season. As a result the skin is more prone to damage at those times in the year when the barrier is not at its optimum.

The lipids of the stratum corneum, primarily ceramides, cholesterol and fatty acids, are essential for maintaining the functionality of the skin as an effective barrier to water loss (Elias et al 1975, Bowser et al 1985). Perturbations in barrier lipid levels have been demonstrated as a result of environmental challenges (Chapter 3). Surprisingly however, current literature suggests that the stratum corneum lipids do not alter with the seasons, despite the change in environmental challenge (Yoshikawa et al 1994). However, this study was performed in Japan, the study panellists being Japanese, who have elevated stratum corneum lipid levels relative to
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Caucasians (compare Yoshikawa 1994 and Chapter 3). This along with the differences in environmental challenges may in part explain this difference.

Seasonal variations in the diet may also influence the synthesis of the stratum corneum lipids. The epidermis produces most of the stratum corneum lipids by de novo synthesis within the viable cell layers. Only a small proportion of cholesterol is taken up from the blood (Elias et al 1991). However, the essential fatty acid linoleic acid cannot be synthesised and therefore has to be obtained from the diet (Burr et al 1929). In normal stratum corneum, ceramide 1 is a repository for the essential fatty acid, linoleic acid, although it can be found acylated to ceramide 2 (Abraham et al 1985) or esterified to the corneocyte envelope (Wertz et al 1989). The importance of the essential fatty acids for skin condition are well established from the study of essential fatty acid deficiency (EFAD) in animal models (Burr and Burr 1929). In EFAD, the replacement of linoleate with oleate in ceramide 1 is associated with dramatic perturbations in the ultrastructure of the stratum corneum lipids (Hou et al 1991), which is instrumental in causing the scaling and barrier abnormalities. Reductions in the levels of linoleate esterified to ceramide 1 have also been associated with atopic dermatitis (Yamamoto et al 1991) and acne (Wertz et al 1985). In addition, the less severe, but more common problem of skin xerosis has been associated with a reduction in linoleate in the saponifiable portion of the stratum corneum lipids (Brod et al 1988). Although the levels of ceramide 1 linoleate were not measured by Brod and co-workers, all ceramides are reduced in soap induced winter xerosis (Chapter 3).
As regions of the body are exposed to different environmental stresses such as the weather, soap washing and UV light, the barrier requirements, and hence barrier lipid levels may vary. This theory is supported by an investigation looking at the ceramide and sebum levels of different body sites. This demonstrated that both total ceramide and sebum levels are highest in the forehead, which is fairly exposed, followed by the chest, and lowest in the sole of the foot (Yoshikawa et al 1994). However, the levels of fatty acids and cholesterol were not determined in this investigation so the precise impact on the barrier function of the different bodysites is difficult to determine. Investigations into age related changes in the stratum corneum lipids have also reported bodysite differences. Ceramide levels decrease in forearm skin with age (Imokawa et al 1991), yet not in leg skin (Akimoto et al 1993). In addition to lipid differences between the bodysites, there are also differences in the rate of desquamation (Roberts et al 1980), skin thickness (Odland 1991) and barrier properties (Lampe et al 1993). In the Lampe study, extractable lipid weights from the abdomen, leg, face and sole were found to be inversely proportional to the known permeability of the bodysites (abdomen > leg > face > sole).

The stratum corneum also has lipids which are covalently bound to involucrin, a corneocyte cell envelope protein (Steinert et al 1996). The precise function of these lipids is as yet undetermined, although there are a number of theories including: The structuring of barrier lipids; sealing the natural moisturising factors within the corneocytes; and forming an interface between the more hydrophilic corneocytes and hydrophobic barrier lipids (Chapter 1). Their importance in barrier function is
supported by the fact that total levels of covalently bound lipid are ten times greater in epidermal compared with both palatal and gingival stratum corneum (Chang et al 1993). Both oral tissues have a markedly inferior barrier and disorganised intercellular lipids. However, considering the possible importance of these lipids, little is known about their natural variation, in particular changes with bodysite or season.

This chapter focuses on the body site and seasonal variations in the stratum corneum lipids, particularly the alterations in ceramides and ceramide 1 linoleate. It aims to relate differences in the stratum corneum lipids to the known differing susceptibilities of the various body sites to dryness, particularly during the winter months of the year. In addition, this work attempts to increase our understanding of the relationship between lipid composition and stratum corneum functionality.

5.2 Study Protocol

Panellist selection along with all other aspects of this study were included in a full study protocol submitted to Unilevers ethical and safety committee for clearance. 26 healthy female Caucasian volunteers (Unilever employees) took part in the seasonal studies, their ages ranging from 21-50 years. Panellists had not participated in any trial in the 7 days prior to the study or any trial using the same test sites for 28 days prior to this study. Subjects were excluded if they regularly took drugs or medication, or were knowingly pregnant. All panellists refrained from using moisturisers on the test site for 1 week prior to the study commencing. For the hand study the total number
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of panellists sampled was different for each season: summer (August) n=26; spring (April) n=5; winter (January) n=17. For both the face (n=6) and the leg (n=9) studies all the panellists were sampled in both summer and winter.

Volunteers were visually assessed for dryness according to UK criteria (Chapter 3), barrier integrity was measured by transepidermal water loss (TEWL) and stratum corneum tape strip samples were then taken (Chapter 2).

5.3 Bodysite and seasonal variations in the intercellular stratum corneum lipids

The seasonal and bodysite variations in the levels of the stratum corneum lipids are represented in Figures 5.1 - 5.3. A pronounced seasonal decline in lipid levels from summer to spring and winter was observed in all bodysites (Figures 5.1-5.3). Of all the bodysites the face, which represents the most exposed site, showed the largest decline with ceramide levels dropping by ~60%, fatty acids by ~75% and cholesterol by ~50%. Of the other two bodysites, the hand showed the next largest the leg (Figure 5.3), the levels of fatty acids are not significantly decreased in winter whereas the ceramide and cholesterol levels are. This may be due to the level of dryness being higher on the leg versus the other two sites. Skin dryness being associated with elevated levels of fatty acids (Chapter 3).
Figure 5.1  Lipid levels in hand stratum corneum in winter compared with summer and spring. The data represent the combined lipid extracts from 5 tape strips of hand stratum corneum. Lipid classes were isolated, quantified and normalised to the amount of detergent soluble protein on the 5 tapes (Chapter 2). In this study fatty acids and cholesterol were separated using the second hexane:ethyl acetate chromatography system (Chapter 2). The results represent the mean ± SD for subjects: summer (August) n=6, spring (April) n=5; winter (January) n=17. Values that are significantly different from summer as measured by the Students t-test are shown as * P<0.05.
Figure 5.2  Facial stratum corneum lipid levels in winter compared with summer. The data represent the combined lipid extracts from 5 tape strips of face stratum corneum, taken from the cheekbone area. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 5 tapes (Chapter 2). In this study the fatty acids and cholesterol were separated using the second hexane : ethyl acetate chromatography system (Chapter 2). The results represent the mean ± SD for 6 subjects. Values that are significantly different from summer as measured by the Students t-test are shown as * P<0.05.
Figure 5.3 The leg stratum corneum lipid levels in winter compared with summer. The data represent the combined lipid extracts from 5 tape strips of leg stratum corneum, taken from the outer calf. Lipid classes were column isolated, quantified and normalised to the amount of detergent soluble protein on the 5 tapes (Chapter 2). In this study the fatty acids and cholesterol were separated using the second hexane : ethyl acetate chromatography system (Chapter 2). The results represent the mean ± SD for 9 subjects. Values that are significantly different from summer as measured by the Students t-test are shown as * P<0.05.
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On comparing the bodysites for each season (Figure 5.1-5.3), the total lipid levels in the face appeared 7 fold and 4 fold higher than in the hand and leg respectively. This bodysite difference was significant for both fatty acids and cholesterol, giving ~10 fold higher levels in the face, as determined using a student's t-test (P<0.05). Although ceramide levels were ~2 fold higher in the face, this was not significantly different, due to marked intersubject variations in ceramide levels. For both seasons, the ratio of fatty acid : ceramide was higher in the face than the hand and leg. The increased levels of fatty acid on the face may be reflective of the increased density of sebaceous glands on this site (Blume et al 1991). Sebum triglycerides are hydrolysed to free fatty acids via lipases produced by skin microphlora such as P. acnes (Schaefer et al 1980). The altered fatty acid : ceramide ratio is reflective of what we observe in skin xerosis (Chapter 3). So although generally increased lipid levels may be expected to decrease skin permeability, the enhanced levels of fatty acid are more likely to compromise barrier effectiveness (Chapter 3), leaving facial skin more susceptible to dryness in any season.

The relative percentages of the different lipids for the different bodysites and seasons are represented in Table 5.1. The relative proportions of the major stratum corneum lipid levels are maintained throughout the different seasons. The bodysite variation in the relative proportions of lipids does appear to vary. The relative levels of ceramide are highest in the leg (52-61%), followed by the hand (42-48%) and face 30-37%. The relative levels of fatty acids are highest in the face (58-64%), followed by the hand (46-54%), then leg (30-
42%). The relative levels of cholesterol were higher in the face (6-7%) and leg (6-8%), compared with the hand (3-5%). These differences may reflect degree of exposure to the environment in addition to sebaceous secretion, as detailed above.

The relative percentages of the individual ceramides are shown in Figures. 5.4-5.6.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Relative percentage of lipids (%) of total lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td><strong>HAND</strong></td>
<td></td>
</tr>
<tr>
<td>Ceramides</td>
<td>48 ± 15</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4 ± 1</td>
</tr>
<tr>
<td><strong>FACE</strong></td>
<td></td>
</tr>
<tr>
<td>Ceramides</td>
<td>-</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
</tr>
<tr>
<td><strong>LEG</strong></td>
<td></td>
</tr>
<tr>
<td>Ceramides</td>
<td>-</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.1  The relative percentage of stratum corneum lipids in different bodysites in different seasons.

The table represents the mean ± SD of the relative percentages of the different lipids calculated for each volunteer. These results are calculated from the raw data used in Figures. 5.1-5.3. Hand: summer (August) n=26, spring (April) n=5; winter (January) n=17, Face: summer/winter n=6 , Leg: summer/winter n=9. A '-' represents no data available. No significant differences were observed as determined by Student’s t-test (P>0.05).
Figure 5.4 The relative percentage of hand ceramide levels in summer, winter and spring.

The data represent the mean ± SD of the relative percentages of the different ceramide species, relative to total ceramide, calculated for each volunteer. These results are calculated from the raw data used in Figure 5.1: summer (August) n=26, spring (April) n=5; winter (January) n=17. Values that are significantly different from summer as measured by the Students t-test are shown as * P<0.05.
Figure 5.5  The relative percentage of face ceramide levels in winter compared with summer.

The data represent the mean ± SD of the relative percentages of the different ceramide species, relative to total ceramide, calculated for each volunteer. These results are calculated from the raw data used in Figure 5.2, n=6. Values that are significantly different from summer as measured by the Students t-test are shown as * P<0.05 compared to summer.
Figure 5.6  The relative percentage of leg ceramide levels in winter compared with summer. The Data represent the mean ± SD of the relative percentages of the different ceramide species, relative to total ceramide, calculated for each volunteer. These results are calculated from the raw data used in Figure 5.3, n=9. Values were not significantly different from summer as measured by the students t-test.
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The main ceramide species present in the stratum corneum are the sphingosine containing ceramides 1, 2, 4/5 and the phytosphingosine containing ceramides 3 and to a lesser extent 6i and 6ii (Figures. 5.4-5.6). Although seasonal alterations in the relative percentages of ceramides were observed in this study, no consistent seasonal trend was observed for all bodysites. In the hand, ceramide 1 is highest in spring and summer, decreasing markedly in winter (Figure 5.4). This decline is concurrent with an increase in ceramide 3a, which co-migrates on high performance thin layer chromatography (HPTLC) plates with the hydrolysis product of ceramide 1 (data not shown). This suggests that there may be a seasonal alteration in ceramide 1 processing within the stratum corneum. However, as shown in Figures. 5.5 and 5.6, this is not observed in the face and leg, the face showing only a slight (non-significant) winter decrease in ceramide 1 and no increase in ceramide 3a (Figure 5.5).

Comparing the bodysites, the relative percentages of the different ceramides did not show any marked variation.

5.4 Bodysite variations in the covalently bound lipid levels of the stratum corneum (winter)

The bodysite variations in the levels of the covalently bound lipids which encase the corneocyte have not been investigated previously. The covalently bound lipids are mainly composed of two ceramide species named ceramide A and B, fatty acids and cholesterol (Chapter 1). In this study the bound lipid levels are shown to vary according to the bodysite. The levels of Ceramide B were three fold higher in the face compared with the hand and leg. The
levels of fatty acid, and total bound lipid were five and three fold higher in the face and hand respectively, compared with the leg (Figure 5.7). The relative proportions of the different bound lipids also varied with bodysite (Figure 5.8). The relative percentage of ceramide B in the hand was half the level found in both the face and leg, and the percentage of ceramide A in leg was almost double that of the other bodysites, at the expense of fatty acid which was reduced in the leg.

Both the face and the hand which have the highest levels of covalently bound lipids, are also known to have the smallest corneocyte size, this may be reflective of a higher stratum corneum turnover rate. Indeed the corneocyte size of face is much smaller than for example the arm (Rougier et al 1988). In addition, hand corneocytes have been shown to be far smaller than the arm and leg (Richardson, Unilever Research, unpublished observation). The decrease in size of the corneocytes will result in an increase in cell surface area. A corresponding increase in the covalently bound lipids would be required to ensure that a complete coverage is maintained.
Figure 5.7  Bodysite variations in covalently bound stratum corneum lipid levels in winter. The data represent the combined lipid extracts from 5 tape strips of stratum corneum, taken from the different bodysites. Following the extraction of the intercellular lipids from the squames, the covalently bound lipids were isolated by corneocyte alkali hydrolysis and solvent extraction. The lipids were quantified by HPTLC, staining and scanning densitometry (Chapter 2). The results represent the mean ± SD for 17 (Hand), 6 (Face) and 9 (Leg) subjects. Values that are significantly different from the face as measured by the Students t-test are shown as * P<0.05.
Figure 5.8 The percentage levels of the covalently bound lipids for the different bodysites. The data represent the mean ± SD amount of each class of bound lipid calculated as a percentage of total bound lipid for each volunteer. These results are calculated from the raw data used in Figure 5.7, n = 17 (Hand), 6 (Face) and 9 (Leg). Values that are significantly different from the face as measured by the students t-test are shown as * P<0.05.
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5.5 Seasonal variations in stratum corneum essential fatty acid status

Essential fatty acids such as linoleic acid are known to be important for good skin condition. However, despite their importance, the natural seasonal variation in the fatty acid profile of the stratum corneum has not been investigated previously. The relative percentages of the free fatty acids were measured in the leg in both the winter and summer season (Figure 5.9). The leg was chosen as the best bodysite to study as more corneocyte material was obtained from this bodysite compared with the face and hand.

Seasonal differences were observed in the profile of free fatty acids. The main fatty acids in the stratum corneum were oleic (18:1), stearic (18:0), palmitic (16:0) and lignoceric acid (24:0). Myristic acid showed the largest decrease in winter (~90%), followed by palmitic (~50%) and palmitoleic acids (60%). Lignoceric acid (C24:0) was shown to increase in winter (~30%). This alteration may be reflective of the increased contamination with sebum during the summer season, sebaceous lipids being rich in shorter chain (14 and 16 carbon) fatty acids.

When looking at the free fatty acids, no change was observed in the essential fatty acid status as determined by the levels of linoleic acid (C18:2). Although in the stratum corneum, linoleic acid is also found esterified to ceramide 1 as shown in Figure 5.10.
Figure 5.9  Seasonal changes in the free fatty acid profile in the Leg. The data represent the profile of free fatty acids which were column isolated from the leg tape strips (Figure 5.3). Half of the free fatty acids were converted to fatty acid methyl esters and separated and quantified by GC (Chapter 2). The results represent the mean ± SD for 9 subjects. Values that are significantly different summer, as measured by the Students t-test are shown as * P<0.05.
Figure 5.10 Seasonal changes in the ceramide 1 esterified fatty acid profile. The data represent the profile of the fatty acids which were esterified to ceramide 1 in the leg. After measuring the total amount of ceramide, the remainder of the ceramides were separated using high performance thin layer chromatography and the ceramide 1 band was scraped off the plate (Chapter 2). This was extracted from the silica and the hydrolysed fatty acids were converted to fatty acid methyl esters and separated and quantified by GC (Chapter 2). The results represent the mean ± SD for 9 subjects. Values that are significantly different from summer, as measured by the Students t-test are shown as * P<0.05.
The ceramide 1 esterified fatty acids do show a dramatic seasonal change in EFA status. Levels of 18:1 are markedly increased (~100%) in winter at the expense of declining levels of 17:0 (~90% decrease), 18:2 (~20% decrease) and 24:0 (~30% decrease). The change in the ratio of linoleate : oleate reverses from 1.74 in summer to 0.51 in winter, representing a 25% decrease in winter ceramide 1 linoleate levels. This alteration in the relative proportions of oleate (18:1) and linoleate (18:2) is similar to that observed in EFAD, yet less pronounced. Decreases in the levels of esterified saturated fatty acids, in particular the 24:0, along with increases in oleic acid levels lead to a greater degree of fatty acid unsaturation in winter compared to summer.

### 5.6 Seasonal variations in skin dryness

<table>
<thead>
<tr>
<th>Score</th>
<th>Winter</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Dryness Grade</td>
<td>0.5 - 2.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5.2 The dryness grades for the hand in winter and summer. Prior to tape stripping, the skin was assessed for visual dryness using the criteria set in the natural xerosis study (Chapter 3). The dryness grades on the backs of hands in winter and summer were compared using a Mann-Whitney U-test. The values shown represent the range and median for the dryness grades, Winter n=17, summer n=26. A significant seasonal difference was found at the 5% level.

Skin dryness was found to be marginally greater in winter.
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(~grade 1) as opposed to summer (~grade 0.5), as expected. The biochemical alterations in the barrier lipids may contribute to this.

5.7 Bodysite variations in Transepidermal water loss

The TEWL values for the different bodysites are represented in Figure 5.11. The mean TEWL value for the face was significantly greater (by ~20%) than the hand, leg and arm. Despite the large depletion in lipid levels in winter, there was no decrease in the water barrier function in winter as measured by transepidermal water loss (Data not shown).
Figure 5.11 Transepidermal water loss values for the different bodysites. TEWL was measured using a Servo-Med Evaporimeter. (Chapter 2). The results represent the mean ± SD for Hand (17), Face (6), Leg (9), Arm (9) subjects. Values that are significantly different from the hand, as measured by the Students t-test are shown as * P<0.05.
5.8 Discussion

This study has clearly shown that in Caucasian females, the levels of stratum corneum lipids are subject to dramatic seasonal variation reaching a minimum in winter. All the lipid species analysed were depleted in winter compared to summer, mirroring some of the ageing influences on stratum corneum lipid levels (Rogers et al. 1996). The seasonal alterations in the lipids were consistent for all the bodysites analysed. However, marked bodysite variations were observed in total lipid levels and fatty acids/ceramides, the face > hand = leg.

The circannual variation in lipid levels observed in this study supports the winter decline in skin surface lipids (Abe et al. 1989) observed by others. These trends are also consistent with the seasonal decreases in epidermal glucosylceramide levels (Nieminin et al. 1967). However, these studies are in marked contrast, to a mixed sex study performed by Yoshikawa et al. on Japanese subjects, where no seasonal changes in lipid levels were apparent (Yoshikawa et al. 1994). This may reflect the difference in seasons in Japan, in particular the winter decline in humidity may be less than for example the USA. Racial and gender differences may also contribute to the results found. Japanese skin has far higher stratum corneum lipid levels than Caucasians (compare Yoshikawa et al. 1994 with Chapter 3). Gender differences could also potentially account for the differences, female sex hormones influence skin thickness (Brincat et al. 1983, Shuster et al. 1975), keratinocyte proliferation (Urano et al. 1992), sphingolipid lipogenesis (Denda et al. 1993) and barrier development rate (Hanley et al. 1996). However, in other investigations little difference has been observed between
the stratum corneum lipid levels of males and females (Rogers, Unilever Research, unpublished observation).

Overall, the increased levels of facial fatty acids could potentially be derived from the sebaceous glands which have a higher density on this site. Although as the fatty acid profile was similar for winter and summer, this is unlikely. As the total lipid content of the stratum corneum correlates well with barrier function (Lampe et al 1993), we might expect the face, which has the highest lipid content, to have the lowest TEWL. Surprisingly, in this investigation the face had a significantly higher TEWL than the other bodysites in winter. This suggests that lipid composition and corneocyte size may be more important in maintaining the barrier than just total lipid levels. The high fatty acid levels and a small corneocyte size in the face influencing its TEWL.

Although the control mechanisms that regulate lipid biosynthesis are not fully understood, there are a number of possibilities accounting for the winter decline in lipid levels. Decreases in skin temperature (Abe et al 1989) may influence the overall biosynthetic capacity of the epidermis, leading to decreased synthesis in winter. The alteration in temperature may give rise to slight alterations in the phase behaviour of the lipids. In the summer heat, the risk from water loss and the fluidity of the lipids may be greater, hence the need for the increased lipid levels. The amount of lipid being inversely proportional to barrier permeability, as determined from the lipid weights of abdomen, leg, face and sole (Lampe et al 1993). Alternatively, the seasonal decrease in stratum corneum lipid levels may reflect circannual fluctuations in hormone levels.
Whatever the reason for the decline in lipid levels in winter, it is likely to leave the skin more prone to damage from soaps and surfactants etc. This may be further exacerbated by the winter decline in ceramide 1 linoleate in leg stratum corneum, as observed in aged skin (Rogers et al 1996). The decline in the EFA status of the skin in winter may be due to seasonal changes in the dietary intake of EFA. However, this remains unproven and it may be due to altered skin uptake of linoleate from the plasma. However, the overall weakening of the barrier in winter is clear, as demonstrated by the increased susceptibility of skin in winter to irritants (Basketter et al 1996). Although in our study no changes were observed in TEWL suggesting an intact barrier, the lipid alterations are likely to increase the susceptibility of the barrier to damage by irritants etc.

The alterations in essential fatty acid status are likely to cause some perturbations to the ultrastructure of the stratum corneum as observed in the extreme case of EFAD (Hou et al 1991). The decline in linoleate has been highlighted previously for atopic dermatitis (Yamamoto et al 1991), acne (Wertz et al 1985) and dry skin (Brod et al 1988). Ceramide 1 linoleate is also important for maintaining stratum corneum flexibility (Rawlings et al 1992) and bilayer fluidity (Oldroyd et al 1994). In addition, the marked elevation in esterified oleic acid will lead to a greater degree of fatty acid unsaturation, disrupting lipid packing and weakening the barrier (Chapter 3).

In addition to the alterations in total lipid levels and ceramide 1, seasonal variations were also observed in the
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'free' fatty acids, with increased levels of myristic, palmitic and palmitoleic acids in summer. Although it is believed that the changes in lipid levels are intrinsic to the stratum corneum, sebaceous lipid may contribute to the seasonal increase observed in summer, despite precautions taken to remove excess sebum (surface wiping). However, the contribution of sebaceous lipid was established as negligible using squalene as a marker, suggesting a true epidermal change.

Differences were also observed in the covalently bound lipids, total levels being 50% less in the legs than the other bodysites. A similar difference between the hand and leg has also been observed with ageing (Rogers et al 1996). The relative proportions of the covalently bound lipids in the different bodysites appear quite different. For the leg the main species present was ceramide A as observed previously (Wertz et al 1989). However, for the face and hand, the main species was the fatty acid. The precise implications of these differences are currently unknown, although the alterations in the bound lipid ratios may lead to subtle differences in lipid lamellae organisation. The enhanced covalently bound lipid levels of the face may reflect its exposed nature and greater need for protection.

Despite a marked seasonal related decline in lipid levels we found no alteration in barrier function (TEWL). The preservation of barrier function may in part be due to the stratum corneum maintaining the relative proportions of the main lipids present and hence the organisation of the lipid bilayers (Man et al 1993). It should also be emphasised that the effectiveness of the stratum corneum water barrier is dependent not only on its lipid content
and composition, but also on its overall morphology (Denda et al 1994, Potts et al 1991). Indeed, the increase in corneocyte size which accompanies exposure to winter conditions, may compensate for the lipid depletion (Herrmann et al 1983). Although water loss does not appear to alter in winter, the reduction in lipid levels may explain the increased incidence of xerosis (Chapter 3). This reduction in lipid levels may in turn reduce the water content of the stratum corneum, reducing the activity of the desquamatory proteases (Watkinson et al 1999). The reduction in lipid levels may also lead to a decline in the levels of the natural moisturising factors (Scott et al 1986), thus leaving the stratum corneum more prone to xerosis.

In summary the present study has demonstrated a seasonal related reduction in stratum corneum lipid levels probably reflecting decreased epidermal lipid biosynthesis. As the expression of skin xerosis is related to the levels and types of stratum corneum lipids, these changes are likely to contribute to the increased susceptibility of skin in winter to xerosis.
CHAPTER 6

6. The influence of lipids on the activity of stratum corneum chymotryptic like enzyme in vitro

6.1 Introduction

Stratum corneum lipid abnormalities, desmosomal retention and aberrant desquamation are characteristic features of a range of skin disorders including: xerosis (Chapter 3), recessive x-linked ichthyosis, psoriasis and atopic dermatitis (Chapter 1). The retention of corneodesmosomes in dry skin, as observed by electron microscopy (EM) (Chapter 3) or biochemically using an antibody to desmoglein 1 (dsg1) as a marker, suggest a defect in the normal protease mediated corneodesmosomal digestion event.

The regulation of the desquamatory process is still not well understood. Although the major enzyme thought to be responsible for the degradation of the corneodesmosome is a serine protease named stratum corneum chymotryptic enzyme (SCCE). This putative role is supported by:

1) Its expression late within terminal differentiation
2) An inhibition profile and a pH dependence similar to that of desmosomal degradation in vitro

Other proteases such as trypsin-like enzymes, involved in the initial activation of Pro-SCCE to SCCE, and cathepsin D-like enzymes have also been implicated in desquamation,
yet for the most part the supporting evidence for a direct role is poor (Suzuki et al 1992, Koyama et al 1993, Watkinson, Unilever Research, unpublished observation). Since the proteases responsible for desmosomal degradation reside within the lipid-rich intercellular environment of the stratum corneum (Lundstom and Egulrud 1991, Watkinson, Unilever Research, unpublished observation), it has been proposed that changes in the lipid matrix may influence desquamation. This may occur, not only through modulating lipid mediated intercellular cohesion but also importantly, through influencing the activity of desquamatory proteases. These lipid mediated effects on proteases may occur directly, or indirectly, through increased leaching of the proteases from the stratum corneum or influencing water activity (available ‘free’ water) or ion gradients.

The capability of SCCE to passively diffuse within the intercellular lipid space enabling it to facilitate corneodesmosomal hydrolysis is currently unknown. However, there does appear to be an original ‘targeted’ distribution of proteases, with the corneodesmosomes on the planar surfaces being degraded first. This may imply an inability of the proteases to move, or alternatively suggests corneodesmosomal micro-heterogeneity, with some corneodesmosomes being more susceptible than others to proteolysis. Certainly, alterations to the lipid components will influence the phase behaviour of the lipid environment, which could affect the mobility of these enzymes impeding desmosomal digestion. In this respect it is noteworthy that glycerol, either through alterations in water content or lipid bilayer phase behaviour, enhances desmosomal digestion (Rawlings et al 1995).
Although there appears to be a close association between lipid alterations and the impeded protease degradation of corneodesmosomes in dry skin, little evidence exists for a direct effect. However, a number of lipids previously shown to be associated with dry skin such as elevated fatty acid levels (Chapter 3) and increased cholesterol sulphate as seen in UV damage (Chapter 4) have been shown to inhibit various other proteases. Free fatty acids have been shown to inhibit trypsin (Lui et al 1990), neutral protease (Zalewska et al 1988), steroid 5a-reductase and chymotrypsin-type serine endopeptidases eg. chymase (Kido et al 1984). Cholesterol sulphate is a known inhibitor of the acrosomal protease acrosin within the male hamster and pig reproductive tract (Bouthillier et al 1984). In addition, phosphoglycerides inhibit chymase activity (Fukusen et al 1985) and negatively charged phospholipids inhibit ER-60 protease, a novel cysteine protease (Urade and Kido 1992).

This chapter investigates the influence of lipids on the activity of recombinant human SCCE (rSCCE) using in vitro model systems. This is the first investigation to look specifically at this enzyme, having major advantages over more crude protease extracts. In addition, the effects of the same lipids on desquamation in vitro are evaluated. The implications of the observations to the understanding of dry skin conditions are discussed.

6.2 The influence of lipids on rSCCE activity

6.2.1 Effects of lipid species on proteolysis

SCCE activity:

The influence of lipids on rSCCE activity were determined
in a fluorescence assay (Chapter 2.9.4). All fatty acid species ranging from a chain length of 12:0 to 18:3 had an inhibitory effect on SCCE activity (Fig. 6.1). The inhibition observed was found to be increase with decreasing chain length, with 12:0 (~75% inhibition) > 14:0 > 15:0 = 16:0 = 18:0 (~50% inhibition). The inhibition also increased with increasing unsaturation 18:3 (~60% inhibition) > 18:2 > 18:1 = 18:0. The effects of hydroxy acids are shown in Fig. 6.2.

The effects of hydroxyacids on rSCCE show that the C2 hydroxyacid enhances the rSCCE activity (Fig. 6.2). As the chain length increases from C4 to C6 and C12, rSSCE activity is significantly inhibited, being reduced to ~60% of the control. However, the C8 hydroxyacids appeared to have no effect on rSCCE activity.
Fig. 6.1 The inhibition of rSCCE by fatty acids. The data represent the amount of rSCCE activity in the presence of fatty acids (1mM), relative to control activity in the absence of fatty acids. This was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC (Chapter 2). The graph represents the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the fatty acid treatments (n=8) and controls (n=8) all values were found to be significantly different from the control, as measured by the Students t-test (* P<0.05).
**Fig. 6.2 The inhibition of rSCCE by hydroxyacids**

The data represent the amount of rSCCE activity in the presence of hydroxy acids (1mM), relative to control activity in the absence of hydroxy acids. Hydroxyacids tested were glycolic (C2), lactic (C3), butyric (C4), caproic (C6), mandelic (phenylacetic, C8), octanoic (C8), lauric (C12). rSSCE activity was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC (Chapter 2). The graph represents the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the hydroxyacid treatments (n=8) and controls (n=8), values that were found to be significantly different from the control, as measured by the Students t-test are shown as * P<0.05
The variation in the response of rSCCE to fatty acids could be due to a direct chemical interaction influenced by fatty acid chain length, degree of unsaturation and hence solubility in the ethanol/aqueous system. The inhibition appearing greatest for those fatty acids with the lowest melting points. Fatty acids can inhibit enzyme activity by their detergent-like action. Detergents such as sodium dodecyl sulphate inhibiting SCCE activity (Watkinson et al 1998). Although at the pH, temperature and fatty acid concentration used, the C12-15 fatty acids, which had the most inhibitory effect, were below their critical micelle concentrations (Cistola et al 1988). The fatty acids will be in their monomeric form (Ito et al 1987, Lin et al 1976), the formation of detergent like micelles will not be favoured (Parvin et al 1970, Cistola et al 1988).

The effects of other lipids on rSCCE activity are shown in Fig. 6.3. Sphingosine, dihydro-sphingosine, cholesterol sulphate and sodium stearate/laurate all inhibited SCCE by at least 60%. The phospholipid, fatty alcohols and glucosyl/galactosyl ceramides had no inhibitory effect (Fig. 6.3). Although phospholipids and glucosyl/galactosyl ceramides are not normally detectable in the stratum corneum, they were evaluated as they are found in the viable epidermis and may be retained in damaged hyperproliferative skin.
Fig. 6.3 The inhibition of rSCCE by lipids. The data represent the amount of rSCCE activity in the presence of lipids (1mM), relative to control activity in the absence of any lipid. This was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC (Chapter 2). The graph represents the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), values that are significantly different from the control, as measured by the Students t-test are shown as * P<0.05
The inhibition of SCCE by cholesterol sulphate may be important as accumulation of this lipid is strongly associated with scaly skin as demonstrated in recessive x-linked ichthyosis (Brown et al 1984) and chronic UV irradiation damage (Chapter 5). Within the stratum corneum some cholesterol sulphate is found complexed with sphingosine (Downing et al 1993), although this is reversible (pH dependent). Certainly rSCCE inhibition by cholesterol sulphate was not prevented or reduced by the addition of this sphingolipid or any other amines, acids and alcohols tested (Data not shown).

The inhibitory action of cholesterol sulphate on SCCE may explain why this lipid and enzyme are segregated during the process of differentiation in the skin. The latter being localised to the lamellar granules of the granular layer. The hydrolysis of cholesterol sulphate at the granulosm/corneum interface concurrent with SCCE release, may be an important protective mechanism, preventing early inhibition of the protease. However, the stratum corneum still contains roughly 2% cholesterol sulphate which is hydrolysed towards the outer stratum corneum (Ranasinghe et al 1986). This ongoing hydrolysis may release SCCE from inhibition, as previously shown for acrosin (Legault et al 1980). This would enhance desmosomal degradation in the upper cell layers and hence promote desquamation.

Of the SCCE inhibitory sphingolipids, sphingosine is present at low levels within the stratum corneum, although the physiological relevance of this is currently unknown. However, sphingosine can act as a second messenger inhibiting protein kinase C activity (Pushkareva et al 1993) and may have a role in the control of proliferation/differentiation. The inhibition of rSCCE by sodium stearate links the effects of excessive soap
washing and dry scaly skin. Although soap washing is also likely to have a marked leaching effect on proteases within the stratum corneum via its disruption of the barrier. As shown in Chapter 3, soap damage, particularly in low humidities, rapidly leads to skin scaling.

A dose response curve was constructed for the most effective and physiologically relevant SCCE inhibitors, cholesterol sulphate and fatty acids (e.g. palmitic acid (Fig. 6.4). As demonstrated, the inhibition of rSCCE occurred over the range of concentrations 0.5-2.5mM for Palmitic acid and 0.5-2mM for cholesterol sulphate. The maximum inhibition achieved was 87% for cholesterol sulphate (>2mM) and 71% for palmitic acid (>2.5mM).
Fig. 6.4 The dose response of rSCCE inhibition by cholesterol sulphate and palmitic acid.

The data represent the amount of rSCCE activity in the presence of cholesterol sulphate (○) or palmitic acid (●) (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mM), relative to a control (activity in the absence of any lipid). This was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC (Chapter 2). The graph represents the mean ± SD for 8 separate measurements.
The kinetics of this rSCCE inhibition was investigated by measuring the activity of SCCE over a range of substrate concentrations in the presence or absence of 3mM cholesterol sulphate or palmitic acid (Fig. 6.5). As shown, both lipids dramatically reduce the reaction rate at all the substrate concentrations tested. Palmitic gave an approximately 75% decrease in the reaction rate, whereas cholesterol sulphate gave an 80-90% decrease in reaction rate.

rSCCE was found to obey Michaelis-Menten kinetics, giving a hyperbolic curve when reaction velocity is plotted against increasing substrate concentration. An estimation of the kinetics of the inhibition of rSCCE by cholesterol sulphate suggests both a decrease in the Vmax and an increase in the KM (Table 6.1). Whereas palmitic acid decreased the Vmax only. It appears that both lipids act as non-competitive inhibitors, i.e. the inhibition can not be overcome by increasing the level of substrate. The effects of cholesterol sulphate on increasing the KM may be due to reversible binding within the active site, or irreversible binding. However, this will require confirmation.

The protease inhibitory effects of cholesterol sulphate have been reported for the acrosomal serine protease 'acrosin' (Bouthillier et al 1984). Inhibition of the serine proteases, trypsin and chymotrypsin by cholesterol sulphate has been demonstrated previously (Sato et al 1998). This has been shown to be a non-competitive effect (Iwamori et al 1997).
Fig. 6.5 The kinetics of the rSCCE inhibition.
The effect of substrate concentration on the reaction rate of rSCCE hydrolysis of SLLVY-AMC was determined in the presence of 3mM cholesterol sulphate ▲, 3mM palmitic acid ■ or in the absence of any lipid ○. The data represent the mean ± SD for 8 separate measurements.
Table 6.1 Estimations of the kinetics of the rSCCE inhibition. The effect of substrate concentration on the reaction rate of rSCCE hydrolysis of SLLVY-AMC was determined in the absence or presence of cholesterol sulphate and palmitic acid (3mM). The reaction was found to obey Michaelis-Menton kinetics, a Lineweaver-Burke plot was used to estimate the Km and Vmax.

The effect of fatty acid species (C12:0-C18:3) on chymotrypsin and trypsin were also investigated for comparison with rSCCE. As shown in Fig. 6.6, all the fatty acids tested, with the exception of myristic acid (14:0) and palmitoleic (16:1) inhibited chymotrypsin activity by at least 50%. The reason for the lack of inhibition by myristic and palmitoleic is currently unknown. In contrast, trypsin was inhibited by at least 90% by all except the longer chain saturated fatty acids, 16:0 and 18:0 that inhibited by ~80% and 45% respectively.
Fig. 6.6 The inhibition of chymotrypsin and trypsin by fatty acids. The data represent the amount of chymotrypsin activity (a) and trypsin activity (b) in the presence of fatty acids (1mM), relative to a control activity in the absence of fatty acids. This was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC (a) or Boc-Phe-Ser-Arg-AMC (b) (Chapter 2). The graphs represent the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), values that are significantly different from the control, as measured by the Students t-test are shown as * P<0.05.
There is a large difference in the fatty acid inhibitory profile between all three proteases tested (Fig. 6.1 and 6.6). The degree of sensitivity to fatty acid inhibition being greatest in trypsin > chymotrypsin > rSCCE. As all three types of enzymes are present within the stratum corneum, although only SCCE is localised to the intercellular space, the difference in sensitivity to fatty acids, which appear increased in dryness, may have some physiological significance. However, as the precise roles of tryp tic and chymotryptic like enzymes within the stratum corneum have not been fully elucidated, it is difficult to determine potential physiological effects. However, the proteolytic degradation of corneodesmosomes in dry skin may be impeded in part by the relatively higher levels of fatty acids.

The effects of the hydroxy acids and other lipid species were also tested as shown in Figs. 6.7 and 6.8. As shown in Fig. 6.7a, hydroxy caproic acid, phenyl lactic acid inhibited chymotrypsin activity. All the others had no effect (Fig. 6.7a). This is different to what was observed for rSCCE where inhibitory effects were observed with C4, C6 and C12 hydroxyacids, in comparison with the C6 and C8 in this case.

In contrast to both chymotrypsin (Fig. 6.7a) and rSCCE (Fig 6.2), none of the hydroxy acids tested had any effect on trypsin activity (Fig. 6.7b).
Fig. 6.7 The inhibition of chymotrypsin and trypsin by hydroxy acids. The data represents the amount of activity in the presence of hydroxy acids (1mM), relative to a control (no hydroxy acids). This was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC by chymotrypsin (a) or Boc-Phe-Ser-Arg-AMC by trypsin (b) (Chapter 2). The graphs represent the mean ± SD for 8 separate analyses. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), values that are significantly different from the control, as measured by the Students t-test are shown as * p<0.05.
Of the other lipids tested, those that were inhibitors of rSCCE were also inhibitors of chymotrypsin and trypsin, with the exception of the sphingolipid, sphingosine (Fig. 6.8). Cholesterol sulphate was the most potent, but sodium laurate and stearate also produced significant inhibition. Again, trypsin was more sensitive than chymotrypsin. This suggests specific differences between the proteases under test. The two main inhibitors of chymotrypsin and trypsin were cholesterol sulphate and palmitic acid as identified for rSCCE.
**Fig. 6.8 The inhibition of chymotrypsin and trypsin by lipids.** The data represent the amount of activity in the presence of lipids (1 mM), relative to a control (no lipids). This was determined by the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC by chymotrypsin (a) or Boc-Phe-Ser-Arg-AMC by trypsin (b) (Chapter 2). The graphs represent the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), values that are significantly different from the control, as measured by the Students t-test are shown as * P< 0.05
6.2.2 The influence of lipids on the stratum corneum caseinolytic proteases as measured by zymography

To confirm the effects of the main rSSCE inhibitory lipids on the different proteases of the stratum corneum, including SCCE, zymography was performed. Zymography is a useful technique which is essentially gel electrophoresis with an enzyme substrate in the gel. Following enzyme separation, substrate hydrolysis can be assessed. As demonstrated by Fig. 6.9, the caseinolytic proteases of the stratum corneum can be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). During incubation in optimal buffer, the separated enzymes degrade the casein in the gel around them. The area of proteolysis, which corresponds to the level of protease activity, is represented by clear zones of casein proteolysis. Eight such bands of differing molecular weights (ranging from 25-40KDa) were observed in total from stratum corneum extracts (Fig. 6.9). The presence of the different stratum corneum caseinolytic proteases have been studied previously (Watkinson, Unilever research, unpublished observation), a similar profile being observed. The SCCE band was identified according to its comigration with rSCCE, having a molecular weight of 25KDa. As shown in Fig. 6.9, both cholesterol sulphate and palmitic acid effectively inhibited all the caseinolytic proteases of the stratum corneum along with the chymotrypsin and trypsin standards. In the case of cholesterol sulphate, some reduction in general staining was also observed, but as shown in Fig. 6.10, the effects are still significant.
Fig. 6.9 The effects of lipids on the zymography of stratum corneum caseinolytic proteases. SDS extracts of tape stripped human stratum corneum were separated on 12% acrylamide gels containing 0.2% casein. Chymotrypsin (25KDa) and trypsin (23Kda) (A), Skin extracts (B) and Molecular weight markers (C) were run on the gel. After washing, the gels were incubated in the absence (Control) or presence of 3mM cholesterol sulphate or 3mM palmitic acid. Bands of proteolysis were detected after staining with Coomasie blue.
The data represent zones of proteolysis shown in Fig. 6.9, quantified by Phoretix gel analysis software. The results represent a duplicate analysis.

Although the gels were semi-quantified using phoretix gel analysis software (Fig. 6.10), this did not appear to give inhibition values for chymotrypsin and trypsin as observed by eye. The data suggest that the inhibition of rSCCE (~80%) by both lipids, is more marked than trypsin (~50%), or chymotrypsin for palmitic acid (~20%). These effects are different to those observed in aqueous assay buffer, possibly due to the different environment in the gel.
The type of substrate used in zymography does dramatically influence the proteases observed. The use of gelatin as a substrate showed only 2 bands of gelatinolysis (Watkinson 1999). Although it is preferable to work with the natural substrates, this is hindered by the ability identify the natural substrate and purify the appropriate proteins in sufficient quantities. The demonstration that cholesterol sulphate and fatty acids inhibit SCCE caseinolysis is clear.

6.2.3 Enzyme behaviour in liposomal type systems

In order to mimic the potential interaction of SCCE with the lipids in the stratum corneum in vitro, the activities of rSCCE, chymotrypsin and trypsin were determined in a range of liposomal type systems. As the enzymes in the stratum corneum may interact with lipids within bilayers, liposomal systems mimic these systems more closely than the simple aqueous systems previously investigated. The lipids in these systems did not all form liposomes, and some oil droplets were observed in the aqueous phase (similar to an oil in water emulsion).

Sphingolipids, particularly ceramides, comprise the major lipid group of the stratum corneum, playing an important role in maintaining the water barrier and condition of the skin. Decreased ceramide levels are associated with dryness (Chapter 3). As synthetic skin ceramides (eg. ceramide 2) are expensive to produce, ceramide analogues such as sphingolipid E are often used in skin dryness products. Due to their hydrophobic nature, the effects of these complex
sphingolipids were tested in a liposomal rather than the aqueous system. A liposomal 'lamella' lipid system being more applicable to the lamella like structures of the stratum corneum.

The activity of rSCCE was determined using an AMC labelled peptide, as used in the aqueous assay system. In the negative control assays, it was found that the presence of a lipids in a liposomal type system enhanced the observed fluorescence of the AMC end product. A standard curve of AMC was therefore run in parallel with each liposomal mixture tested. The effects of these systems on rSCCE, chymotrypsin and trypsin activity are shown in Table 6.2.
<table>
<thead>
<tr>
<th>Liposomal system</th>
<th>Enzyme activity (ng AMC produced)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rSCCE</td>
</tr>
<tr>
<td>Aqueous</td>
<td>68 ± 9</td>
</tr>
<tr>
<td>Pseudoceramide A</td>
<td>68 ± 20</td>
</tr>
<tr>
<td>Sphingolipid E</td>
<td>97 ± 20*</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>45 ± 12*</td>
</tr>
<tr>
<td>Ceramide 2</td>
<td>68 ± 12</td>
</tr>
<tr>
<td>Sucrose ester</td>
<td>89 ± 18</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>High Palmitic acid</td>
<td>78 ± 12</td>
</tr>
</tbody>
</table>

Table 6.2 Enzyme activity within liposomal type systems. The data represent the amount of rSCCE, Chymotrypsin or Trypsin activity in a range of liposomal systems (Chapter 2). Activity is represented as the release of aminomethylcoumarin (AMC) from a short peptide, SLLVY-AMC or Boc-Phe-Ser-Arg-AMC over 3 hours (Chapter 2). The table represents the mean ± SD for 8 separate measurements. Values found to be significantly different from the control, as measured by the Students t-test are shown as * P<0.05.
The activity of rSCCE along with chymotrypsin and trypsin for comparison, was shown to vary with the different liposomal systems (Table 6.2). The activity of rSCCE appeared enhanced (~30% increase) in the sphingolipid E based liposomes and inhibited in the phosphatidylcholine liposomes (~35% decrease). None of the others showed any effect. Chymotrypsin showed a similar trend with a significant decrease in activity in the phosphatidylcholine (~15% decrease) and ceramide 2 liposomes (~15% decrease). Trypsin activity was found to be enhanced by pseudoceramide A (~10% increase), sphingolipid E (~15% increase) and ceramide 2 (~10% increase) and decreased in the phosphatidylcholine based liposomes (~25% decrease).

In this model, it is the outer surface of the vesicle that essentially interacts with the aqueous phase/enzyme, although some internalisation of the enzyme or substrate may occur due to fusion of small vesicles. Neither the pH or vesicle size appear to explain the variation in the effects of the different liposomes (Table 6.3), although the physical properties of the membrane surface may play a role. The association of proteins, particularly positively charged ones like SCCE, with the polar head groups at the liposome surface may occur via electrostatic forces. The interaction of rSCCE with the liposomes will be further influenced by both the lipid packing and phase behaviour (gel/lamella phases) (Oldroyd et al 1994, Hsuan et al 1995), more fluid membranes give enhanced protein interactions. The factors which influence rSCCE or protease/lipid binding in vitro will alter its interaction with the substrate. This may also be a mechanism that operates in vivo.
Table. 6.3 The physical properties of the liposomal systems. The data represents the overall pH and the drop size as measured using a Malvern Mastersizer. The conditions were: presentation value-0506, small presentation device and stirrer speed 50%.

6.3 The influence of lipids on desquamation

Desquamation was determined in the presence and absence of topically applied lipids in an in vitro porcine skin model system (Chapter 2). This ex vivo process of desquamation has been shown to be associated with corneodesmosomal proteolysis. The model is therefore relevant to the in vivo situation. The influence of some of the main stratum corneum lipids on the process of desquamation are illustrated in Figs. 6.11 - 6.13.
Fig. 6.11  The effects of fatty acids on desquamation
The data represent the corneocyte release from incubated biopsies in the presence and absence (control) of topically applied fatty acids. Functionally desquamated corneocytes were quantified using a colorimetric assay based on Coomassie blue staining (Chapter 2). The graph represents the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), values found to be significantly different from the control, as measured by the Students t-test are shown as *P<0.05.
Fig. 6.12 The effects of sphingolipids on desquamation

The data represent the corneocyte release from incubated biopsies in the presence and absence of topically applied sphingolipids. Functionally desquamated corneocytes were quantified using a colorimetric assay based on Coomassie blue staining (Chapter 2). The graph represents the mean ± SD for 8 separate measurements. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), no significant difference was observed from the control, as measured by the Students t-test.
Fig. 6.13  The effects of lipids on desquamation

The data represent the corneocyte release from incubated biopsies in the presence and absence of topically applied lipids. Functionally desquamated corneocytes were quantified using a colorimetric assay based on Coomassie blue staining (Chapter 2). The graph represents the mean ± SD for 8 separate measurements. The CER/FA/CHOL mix is the ceramide II liposomes described in chapter 2. On comparison of the actual fluorescence values for the lipid treatments (n=8) and controls (n=8), values found to be significantly different from the control, as measured by the Students t-test are shown as *P<0.05.
Of the fatty acids tested (Fig. 6.11) there was a trend of decreasing inhibition of desquamation with increasing chain length. This roughly matches the inhibition profile of fatty acids and rSCCE, inhibition decreasing with increasing chain length. Lauric and palmitic acid significantly inhibited desquamation by ~60% and ~50% respectively. Stearic acid was found to have no effect, and this was true for the unsaturated C18 fatty acids, oleic and linoleic acids. Of the range of sphingolipids tested, no effect on desquamation was observed (Fig. 6.12). Of the other lipid species tested, phosphatidylcholine and cholesterol sulphate both inhibited desquamation by ~40%. The free and esterified cholesterol, triolein and stratum corneum lipid mix had no effect (Fig. 6.13).

A number of the inhibitory effects of lipids on desquamation correlate with the inhibitory effects on rSCCE activity. This supports a role for SCCE in this process. Cholesterol sulphate for example inhibits rSCCE activity by up to ~80% and desquamation by ~40%. This lipid is a known inducer of scaling in vivo, either by topical application or through enhanced levels in skin disorders such as recessive x-linked ichthyosis (Maloney et al 1984). In addition, the lack of effect on desquamation of any of the sphingolipids tested is consistent with the lack of effect on rSCCE. Although in this case, the lack of any effect may be indicative of the problems of testing the non-polar lipids in such systems, in particular the lack of penetration. In comparison with in vivo, the results may also be influenced by the fact that the lipids are topically applied rather than incorporated naturally into the stratum corneum lipid lamellae. The
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differences observed between the inhibition of rSCCE and desquamation highlight the ability for a number of factors to influence enzyme activity within the stratum corneum. Most notably, phosphatidylcholine had little effect on rSCCE activity, yet inhibited desquamation. In this case this may be due to a disruption of the lipid bilayers or interaction with the corneodesmosomes. In vivo, lipids influencing the water activity, gradient and barrier properties, with no direct effect on SCCE activity may have a marked effect on desquamation (Chapter 1).

6.4 The influence of lipids on the partitioning of rSCCE into an organic phase

The ability of radiolabelled rSCCE to partition into an organic phase from an aqueous phase was investigated. The influence of pH on partitioning was determined using a series of different buffers covering a range of pHs (pH 3-7), as the aqueous phase, and equal volumes of octanol as an organic phase. The % of added rSCCE that partitioned into the organic octanol phase is demonstrated in Fig. 6.14.

The partitioning of rSCCE into the organic octanol layer appeared to increase with decreasing pH (Fig. 6.14). This is because SCCE is a very basic protein which carries a strong positive charge at low pHs. There is evidence in the literature that enzymes are capable of being solubilised in organic/low water environments by ion pairing (Paradkar and Dordick 1994, Powers et al 1993, Bromberg and Kibanov 1994). However, rSCCE does not partition differently into an organic environment from an aqueous system, in the
presence of possible ion pairs such as cholesterol sulphate, SDS and palmitic acid, compared with unpaired (Fig. 6.15).

**Fig. 6.14** The effects of pH on rSCCE partitioning

The data represent the relative partitioning of rSCCE between a buffer and a organic (octanol) phase. The buffers were sodium citrate pH 3,4, sodium acetate 5,6 and Tris/HCl pH 7 each containing rSCCE (0.08Bq) (Chapter 2). The level of incorporation into each phase was determined the addition of scintillation fluid and scintillation counting of $^{14}$C. The graph represents the mean ± SD for 3 separate measurements.
The effects of ion pairs on rSCCE partitioning

The data represent the relative partitioning of rSCCE between a sodium citrate buffer (pH 3) and an organic (octanol) phase as described in Fig 6.14 in the presence or absence (control) of SDS, cholesterol sulphate (CHOL SO4) and palmitic acid (Chapter 2). The level of incorporation into each phase was determined by the addition of scintillation fluid and scintillation counting of $^{14}$C. The graph represents the mean ± SD for 3 repeats. No significant difference was found for any of the lipids tested by Students t-test.

The partitioning of rSCCE into lipids such as squalene and the ceramide II liposomes which have a similar composition to the stratum corneum lipids were also
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investigated, although little partitioning was observed (data not shown). pH appeared to have the most marked effect on partitioning into an organic phase, although the natural pH within micro-environments of the stratum corneum has not been fully evaluated.

6.5 Discussion

The role of the stratum corneum lipids in corneocyte cohesion and ultimately dyshesion has to date, not been fully established. Their role is supported by the association of lipid abnormalities with both common and inherited scaling disorders such as; xerosis (Chapter 3), atopic dermatitis (Imokawa et al 1991) psoriasis (Elias et al 1984) and recessive x-linked ichthyosis (Brown et al 1984). However, desmosomal degradation within the stratum corneum is well known now to be key for normal desquamation, desmosomal retention being associated with skin dryness (Chapter 3). This suggests a defect in the normal protease mediated desmosomal digestion event is involved. As the main protease thought to be involved in this process, 'Stratum corneum chymotryptic like enzyme' (SCCE) sits within the intercellular lipid environment (Lundstrom et al 1991), lipids may affect desquamation through influencing SCCE activity (directly or indirectly).

The availability of SCCE in a purified form through recombinant technology has, for the first time, allowed us to establish whether lipids can affect SCCE activity and therefore may have a role to play in regulating desquamation.

A wide range of lipid components were evaluated for their effects on rSCCE activity, along with two other
serine proteases, chymotrypsin and trypsin for comparison. Of particular relevance to dry skin, where fatty acid levels are elevated (Chapter 3), in vitro models showed fatty acids and their salts to be good inhibitors of rSCCE activity. In addition, fatty acids (C12:0, C16:0) were also found to consistently inhibit desquamation, using a simple in vitro desquamation assay. This effect decreased with increasing chain length, possibly being influenced by the degree of penetration achieved by the fatty acid. The enzyme kinetics suggests that this inhibition is non-competitive or irreversible. The fatty acid inhibition was not specific to rSCCE, as fatty acids (particularly C18 species) inhibited the other serine proteases tested, as has been previously observed for chymase (Kido et al 1984), chymotrypsin (Lui et al 1990, Dahlmann et al 1985, Bargonni et al 1960), neutral protease (Zalewska et al 1988) and for trypsin (Fukesen et al 1985). In vivo some SCCE inhibition and hence dryness, may occur as a result of soap deposition upon washing. Indeed, SCCE activity is reduced in surface layers in soap induced dryness in vivo (Chapter 3, Watkinson, Unilever Research, unpublished observation). However, this will also be influenced by the leaching of proteases from the stratum corneum.

Sterols make up almost 35% of the stratum corneum lipids and although found mainly as free cholesterol, they are also present as cholesterol sulphate/esters. Using in vitro models, rSCCE has been shown for the first time to be inhibited by cholesterol sulphate. Unlike cholesterol, increasing cholesterol sulphate levels in the stratum corneum by topical application also reduced desquamation in our in vitro model and has
been shown to induce scaling in mice in vivo (Maloney et al 1984). In humans, increased levels of cholesterol sulphate within the stratum corneum are strongly implicated in dry skin disorders. Most notably the genetic disorder, Recessive X-Linked Ichthyosis, where cholesterol sulphate retention produces severe hyperkeratosis (Brown et al 1984). A similar, yet less pronounced cholesterol sulphate retention is also observed following chronic UV irradiation (Chapter 4). These findings strongly support the hypothesis that cholesterol sulphate induces dryness by directly inhibiting the activity of the desquamatory protease SCCE.

In addition to its direct effect on SCCE, cholesterol sulphate may influence desquamation by altering the phase behaviour of the lipid intercellular space. Unlike cholesterol, it does not form a eutectic mixture when mixed with equimolar concentrations of fatty acids (Rehfeld et al 1986) and cannot form hydrogen bonds (Rehfeld et al 1988) altering the lipid phase behaviour and polarity of the environment. In addition, as a membrane stabiliser, its degradation towards the skin surface may result in lipid lamella disruption (Chapter 3), reduced cohesion and hence, further enhancing desquamation.

The behaviour of rSCCE, chymotrypsin and trypsin were studied further in a simple liposomal model designed to mimic the "lipid bilayer" environment of the stratum corneum. Phosphatidylcholine and sphingolipid based liposomal formulations were found to influence protease activity. Most notably, phosphatidylcholine liposomes consistently inhibited all three proteases, whereas some enhancement of rSCCE activity was observed
with the sphingolipid E type liposomes. This was not observed with either pseudoceramide A or the natural ceramide 2 containing liposomes. Liposomes formulated with increased levels of cholesterol sulphate also inhibited rSCCE activity compared to a control liposomal mix (data not shown), although those containing higher levels of fatty acids did not show an effect. It is possible that the electrostatic interactions between the lipids and rSCCE may influence its activity. Of potential relevance to skin in vivo, is the observation that the sphingolipid bases sphingosine and dihydrosphingosine both inhibited rSCCE activity. The production of these bases by the action of ceramidase in the stratum corneum may influence dry skin formation as shown by the presence of the ceramidase producing Pseudomonas aeruginosa on atopic skin (Okino et al 1998). Although an elevation in intrinsic ceramidase activity is not thought to accompany the decline in ceramide levels in atopic dermatitis (Jin et al 1994).

Non-stratum corneum lipids such as fatty alcohols and phosphatidyl choline (PC) were also tested for their ability to influence rSCCE activity and desquamation. Although little effect was observed on rSCCE activity by these lipids, PC consistently inhibited desquamation in vitro. In a liposomal form, ceramax (contains PC) gave inhibition of all the serine proteases tested, possibly due its liposome form, or its polar/'fluid' nature as previously discussed. Alternatively, other components in ceramax may influence activity. Cysteine protease ER60 is only inhibited by acidic phospholipids, not PC (Urade et al 1985).
The hydroxy (OH) acids are known enhancers of desquamation (Wang 1999) acting as plastisizers, enhancing both water content and flexibility. We have now demonstrated that they may also influence protease activity, short chain variants enhancing rSCCE activity and longer chain C12 and C14 variants inhibiting activity. This may reflect their true mechanism of action in relief from dry skin.

The interaction of rSCCE with the lipids of the stratum corneum, particularly the inhibitory effects of both cholesterol sulphate (CS) and fatty acids (FA) do appear to be closely linked to desquamation in vitro. In vivo, data associating both lipid abnormalities and aberrant desquamation, as shown by the increased CS in chronic UV damage and increased FA in soap induced/natural dryness, are also consistent with this in vitro evidence. Despite this association, the precise interaction of lipids with SCCE in vivo and the ability of SCCE to be both active and mobile within the lipid intercellular space in vivo is unproven.

SCCE is unusually resistant (compared to other serine proteases) to a reduction in water activity (Watkinson et al 1999) and is inactive in low water environments such as organic solvent, like many enzymes. It is unlikely therefore that SCCE would be active totally within the lipid lamellae which are low in water (Bouwstra et al 1991, 1992). The intercellular space as a whole, does contain some water possibly associated as pockets around the proteins and desmosomal units. SCCE may therefore interact with the lipids at the lipid/water interface, in a similar fashion to the liposomes. Activity may be influenced by electrostatic interactions along with the phase behaviour of the
lipid mix. Lipids may also influence SCCE activity and ultimately desquamation more indirectly by alterations in lipid phase behaviour and bilayer structure, influencing both water activity and water localisation.

Although SCCE would not be active within the hydrophobic lipid domain, we have speculated that some partitioning and movement of SCCE within the lipid environment may occur. The in vitro data suggests that some partitioning (10%) into the lipid phase may occur. It appears likely though that most SCCE would remain in aqueous micro-environments within the stratum corneum. As a result "SCCE-poor" regions between corneocytes may be formed during lamellar body secretion, these areas would remain more cohesive during stratum corneum maturation, due to a lack of corneodesmosomal degradation. This may explain the slower degradation of peripheral-edge corneodesmosomes.

In conclusion, this research has highlighted the potential for the major serine protease implicated in desmosomal degradation, SCCE, to be modulated by defined lipid species. If these observations can be extrapolated to the skin in situ, they indicate strongly that desquamatory protease activity is influenced by the nature of the lipid environment within the intercellular space of the stratum corneum. Potentially the sensitivity of SCCE to cholesterol sulphate inhibition suggests that steroid sulphatase activity could represent a subtle control mechanism for desquamation. The decline in cholesterol sulphate levels towards the corneum surface, releasing SCCE from inhibition, and potentiating desquamation.
7. The role of the transglutaminases in stratum corneum maturation and dryness

7.1 Introduction

In the final stages of epidermal differentiation, a cornified cell envelope (CE) is laid down encasing the corneocytes. The formation of this protein envelope being catalysed by transglutaminases (TGase) 1 and 3. During stratum corneum maturation the corneocyte appearance changes from an irregular morphology 'fragile CEs', to a more flattened / smooth appearance 'rigid CEs' (Chapter 1).

Although the role of TGases in CE formation is now undisputed, involvement in CE maturation, where the cells transform from a fragile to a rigid appearance remains debated. However, there is now strong supporting evidence for their role. Cultured keratinocytes which lack TGase 3 expression and activity, fail to produce a resilient CE (Park et al 1992). Also in the dry skin conditions, lamella ichthyosis and psoriasis, the retention of the fragile envelope morphology is associated with an absence of TGase 1 activity and precocious expression of TGase 1 respectively (Michel and Juhlin et al 1990). A similar precocious expression of TGase 1 is observed in acne (Reichert et al 1992) and dandruff (Rogers J, Unilever Research, Unpublished data). This emphasises the importance of both the overall TGase activity and timing of expression.
Chapter 7 - Transglutaminase

To date most of the research regarding the isolation and characterisation of TGases from skin has been done on intact epidermis or cell culture. This has allowed their individual functions in CE formation to be elucidated. However, little is known about the activity or roles of TGases present within the stratum corneum. Although, there is a suggestion from the patent literature that TGase-like activity does persist into the superficial layers of the stratum corneum (Richardson and Schilling 1993).

The main techniques used to investigate TGases include: The separation of large bulk skin/epidermal extracts by fast protein liquid chromatography (FPLC) and the localisation of TGase expression in epidermis via antibodies (Kim et al 1995, Rice et al 1990). The TGases are generally extracted from epidermis as two pools. A buffer extract for the soluble TGases (TGases 1, 2 & 3) and a Triton extract for the membrane bound TGase 1.

The aim of this research was to characterise the transglutaminases (1, 2 or 3) present within the stratum corneum and investigate any changes with dryness or during stratum corneum transit (depth) alongside CE morphology. This would then support the hypothesis that transglutaminases are responsible for the maturation in addition to the formation of the CE.

7.2 Dryness study protocol

Six healthy subjects were selected who had no current skin complaint or history of any skin disorder. Subjects did not use any skin care product on the test
site (inner volar forearm) for a period of 1 week prior to the study commencing, or during the study. The procedures used in this study had been cleared by Unilevers ethical and safety clearance committee.

Each panellist washed their 'treated' inner volar forearm with ivory soap (3x1min) 3 times a day and their control arm with water only for a period of 3 weeks. After this time mild dryness was observed on the soap treated arm when compared with the control arm. Stratum corneum samples were obtained by taking 24 consecutive tape strips (sellotape) from each arm. These were temporarily kept over ice during collection and then stored at -20°C.

Three separate extracts were prepared from the corneocytes (buffer soluble, detergent soluble and particulate) and assayed for TGase activity. 10% of the corneocytes were saved for CE preparation and image analysis. The levels of desmocollin 1 (dsc1) were investigated using immunofluorescence and Western blotting (Chapter 2). TGase forms present in the buffer and detergent soluble fractions, were determined using bulk TGase extracts from the inner volar forearms of healthy volunteers.

### 7.3 The role of TGases and CE morphology in stratum corneum maturation from healthy and dry skin

As illustrated in Fig. 7.1, three pools of TGase activity can be detected within the stratum corneum, a buffer soluble, Triton-X100 soluble (suggestive of "membrane" association) and a detergent insoluble, "particulate" form. This "particulate" activity could
not be released by further extraction of the cornified cell residue by high salt solutions (Data not shown). The precise nature of the particulate associated TGase activity is unclear, however it presumably reflects TGase which has cross-linked itself into the triton-insoluble corneocyte complex.

All three pools of TGase activity, were found to increase towards the skin surface, relative to the amount of corneocyte material removed (extractable protein), consistent with their proposed role in CE maturation. This trend was statistically significant for both the triton soluble and insoluble TGase pools. The enzyme present in the triton soluble pool behaved similarly to TGase 1 on anion exchange chromatography (Fig. 7.5) and the latter possibly representing TGase which has partially cross-linked itself to the protein CE. The gradient was observed in both the soap treated and control sites, the enhancement of TGase activity towards the stratum corneum surface being associated with increased proportions of rigid CE and increased number of γ-glutamyl lysine crosslinks (Harding et al 1999).

7.4 Changes in TGase and CE morphology in chronic soap induced dryness

Following the soap induced dryness protocol the total activity of stratum corneum TGases and particularly that of the triton-X100 soluble 'membrane bound' pool, were decreased in the soap treated compared with the control arm (Fig. 7.1). This decline in TGase activity is apparent in both surface cell layers and deeper within the stratum corneum. Although the decline in
activity in the superficial layers may simply reflect leaching due to the reduced barrier in dry skin, the reduction in the deeper layers is unlikely to be caused by a similar process. The reduction in TGase activity observed is associated with a significant increase in the proportion of fragile CE observed at the surface (Fig. 7.2).
Fig. 7.1 Changes in stratum corneum Transglutaminase activity in healthy and dry skin.

The data represent the amount of buffer / detergent soluble and detergent insoluble transglutaminase activity expressed per amount of detergent soluble protein. Transglutaminase activity was quantified by phosphoimager analysis of membrane blots of crosslinked C14 putrescine / casein precipitates (Chapter 2). The graphs represent the mean of 6 subjects. Values found to be significantly different from depth 21-24, as measured by the Students t-test are shown as *P<0.05. All TGase values for dry skin were significantly reduced compared with healthy skin using a the Students t-test (^P<0.05).
**Fig. 7.2** Cornified envelope morphology in healthy and dry skin. The data represent the percentage cornified envelopes showing a resilient (rigid / mature) morphology. The maturation state of the envelopes was determined visually via a Leitz DMRB microscope equipped with differential interference (Nomarsky) optics (Chapter 2). The graphs represent the mean ± SD of 5 subjects. Dry skin values were found to be significantly different from the healthy skin, as measured by the Students t-test, *P<0.04.*
7.5 Changes in dscl levels in soap damaged skin

As predicted the levels of dscl detected by Western blotting were found to increase in the soap induced dry skin compared with the control site (Fig. 7.3). Retention of desmosomal components was also assessed using dscl indirect immunofluorescence and again there was an increase in the levels of fluorescence on the dry site, compared to control although this was not significant (Fig. 7.4).
**Fig. 7.3** Desmocollin 1 (dscl) immunofluorescence in stratum corneum of healthy and dry skin. The data represent the amount of dscl present in protein extracts, pre-separated on Con-A columns. Column bound material, was eluted and separated using SDS-PAGE (7.5% gels), together with $^{14}$C labelled molecular weight standards covering the range 14-200 KDa. Proteins were blotted onto PDVF membranes (Immobilon-P, Millipore) and dscl was detected with the a46/48 anti-dscl polyclonal antibody (a gift from Dr. I. King, NIMR, London, 1:200 dilution), followed by incubation with a $^{125}$I labelled goat anti rabbit IgG monoclonal antibody (Amersham, 1:1000 dilution). The radioactivity on the membranes was detected by exposure to a phosphor screen and visualisation in a Molecular Dynamics phosphor imager. Quantification of dscl was performed using the Phoretix gel analysis software. Dscl levels were found to be significantly increased in dry relative to healthy skin, as measured by the Students t-test *P<0.05.
Fig. 7.4 dscl levels in healthy and dry skin as measured by indirect immunofluorescence.

The presence of dscl was probed using the \( \alpha-46/48 \) polyclonal antibody (a gift from Dr. I. King, NIMR, London) at 1:200 dilution. The dscl level was quantified using a FITC labelled secondary antibody at 1:50 dilution (Chapter 2). Immunofluorescence was observed through a Leica DMRB microscope with UV light source and quantified by scanning the negatives into a Compaq P.C. using an Epson GT-8000 desktop scanner and analysing the images with Phoretix gel analysis software (Phoretix, Newcastle, U.K.). The data represent the mean ± SD fluorescence per corneocyte (arbitrary units- pixels per corneocyte) for six subjects. The data sets (healthy and dry) which represent the mean ± SD for 6 subjects were not statistically significant from each other by Students t-test (P=0.34).
7.6 The separation of TGases from stratum corneum extracts

The separation of TGase forms using anion exchange chromatography has been reported previously for whole epidermis (Kim et al. 1995), but not stratum corneum. The FPLC mono Q chromatography used for the separation of stratum corneum TGases was optimised to maximise the separation and detection of the TGase activity forms observed. This technique allowed the detection of TGase from as little as 4mg of stratum corneum (8-16 tape strips).

As shown in Fig 7.5, the resolved buffer extract of the stratum corneum fractionated into two main peaks of TGase activity by FPLC mono Q chromatography, which make up the soluble TGase pool. The first TGase peak was in the column breakthrough, eluting in the sodium acetate buffer, and the second larger TGase peak eluted at ~0.1M NaCl. The detergent extract, which in epidermal tissue represents membrane binding of TGase, was found to contain one main peak of TGase activity. The peak of elution for this TGase was at 0.175M NaCl. The elution profiles of the soluble TGases within the stratum corneum are similar to those reported for epidermis. Epidermal TGase 3 has been shown to elute with buffer alone, while TGase 1 elutes at around 0.15M NaCl from mono Q anion exchange columns (Kim et al. 1995).

Epidermal TGase 2 has been shown to elute at 0.3M NaCl (Kim et al. 1995), in stratum corneum no TGase activity was observed in the 0.3M NaCl eluted fractions (~fraction 17). This suggests that TGase 2 may be absent from the stratum corneum.
Fig. 7.5 A typical separation of stratum corneum TGases. Buffer and triton x-100 (detergent) soluble transglutaminases were extracted from corneocytes from bulked tape strips of forearm skin. These extracts were concentrated using amicon-10 columns and fractionated by micro-FPLC using an anion exchange Mono Q column (1.6/5mm, Pharmacia) (Chapter 2). The system was run using 50mM sodium acetate buffer (pH6) (0-5min) and a 0-0.75M NaCl gradient (5-35min) at a flow rate of 100μl per min. Each fraction numbered 1-24, represents consecutive 100μl eluates collected after injection of a 50μl sample onto the column. Transglutaminase activity was determined for each fraction (Chapter 2)
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7.6.1 The separation of TGases from Callus, compared with normal stratum corneum

For comparison, healthy stratum corneum was compared with callus obtained from foot scrapings of healthy volunteers. Callus in contrast to normal stratum corneum exhibits a CE morphology similar to the immature 'fragile' morphology. It was therefore of interest to determine whether the TGase profile was different, possibly accounting for this difference. The total levels of TGase present in stratum corneum vs callus, were found to be fairly similar, as shown in Fig. 7.6. In addition, the percentage levels of the buffer soluble compared with the 'membrane bound' detergent soluble TGases were also similar (Fig. 7.7).

Callus, in common with normal stratum corneum, had two main peaks of TGase activity within the soluble TGase pool separable by mono Q FPLC (Fig. 7.8). This supports the presence of both TGase 1 and 3 in callus. In comparison with stratum corneum, callus had a much higher percentage of TGase 3, the major TGase in stratum corneum being TGase 1. However, the inter-subject variations of TGase 1 & 3 in callus are far greater than the stratum corneum. The physiological significance of this variation is currently unknown, although the extent of variability has been eluded to previously (Kim et al 1995).
Fig. 7.6 The levels of both buffer soluble and triton soluble TGase in callus vs stratum corneum. Bulk extracts of homogenised corneocytes and callus scrapings were prepared from five subjects. TGase activity was measured and expressed per mg of tissue. The data represents the mean activity (n=5) for stratum corneum and callus, no significant difference was observed, as measured by a Students t-test.
Fig. 7.7 The percentage levels of buffer soluble and triton soluble TGases in stratum corneum and callus. The data represent the individual levels of TGase in stratum corneum (SC) and callus, shown in Fig. 7.6, expressed as a percentage of the total TGase activity. The graph represents the mean + SD for 5 subjects. No significant difference was observed, as measured by a Students t-test.
Fig. 7.8 The percentages of the two buffer soluble TGases in stratum corneum and callus.

The data represent the levels of TGase activity eluting from a mono-Q anion exchange column in buffer (TGase 3) and at around 0.1M NaCl (TGase 1). The levels observed are expressed as a percentage of the total buffer soluble TGase activity. The graphs represent the means ± SD from five subjects.
The levels of particulate (non-extractable) TGase activity were also determined for callus and stratum corneum, using the same assay buffer used for the extractable TGases. As shown in Fig. 7.9, the TGase activity associated with the particulate fraction, like the extractable TGase activity, is similar for stratum corneum and callus. The particulate TGase, being associated with the corneocytes, is able to crosslink putrescine to the natural CE proteins and hence the activity is not directly comparable to the extractable TGases, where casein is used as the substrate. The effects of the presence of casein in the assay buffer for the particulate TGase was investigated. If casein is removed from the assay buffer, the apparent particulate TGase activity drops (Fig. 7.10). The increase in apparent TGase activity in the presence of casein was shown to be due to particulate TGase cross-linking putrescine to the casein in solution, and not into the squames themselves.

7.6.2 Confirming the absence of TGase 2 in Stratum Corneum

GTP is a known inhibitor of TGase 2 (Smethurst and Griffin 1996) having little effect on the other TGases. As shown in Fig. 7.11, the level of GTP required to give inhibition of TGase 2 is dependent on the calcium concentration. Inhibition of TGase 2 at a calcium concentration of 0.1mM requires 500μM GTP, whereas at higher calcium concentrations (10mM calcium) ~10mM GTP is required.
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The total TGase activity in stratum corneum buffer / triton extracts (SCTG) was resistant to inhibition by GTP (Fig. 7.12). There was some inhibition observed at high GTP concentrations (10mM) in the presence of low calcium concentrations (0.1mM), but the other three combinations of calcium and GTP which were shown to inhibit TGase 2 (Fig. 7.11), had no effect on SCTG (Fig. 7.12). This confirms its absence within the stratum corneum.
Fig. 7.9 The percentages of the main Transglutaminase forms in stratum corneum and callus.

Bulk extracts of homogenised corneocytes and callus scrapings were prepared, and TGase activity was measured (Chapter 2). The data is expressed as a % of the total TGase activity measured and represents the mean ± SD activity (n=5). No significant difference was observed between callus and stratum corneum, as measured by a Students t-test.
Fig. 7.10 The influence of casein on the relative percentages of particulate transglutaminase.

Particulate TGase activity was measured in dry stratum corneum and callus in the presence and absence of casein (Chapter 2). The data are expressed as a % of the total TGase activity measured and represents the mean ± SD activity (n=3). Significant differences in the presence, compared with the absence of casein measured by a Students t-test are shown * P<0.05.
Fig. 7.11 The inhibition of TGase 2 by GTP.
The data represent the activity of TGase 2 (0.01u) at a range of calcium concentrations (0, 0.1, 1, 10mM) and two GTP concentrations (500μM and 10mM). The level of TGase activity was determined by measuring the crosslinking of 14C putrescine to casein. The casein precipitates were re-dissolved in sodium hydroxide (aq) and total incorporation of radioactivity (DPM) determined by scintillation counting.
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Fig. 7.12 The inhibition of TGase 2 in stratum corneum extracts by GTP.

The data represent the activity of stratum corneum buffer/triton TGase extracts (SCTG) at two calcium concentrations (0.1 and 10mM) and two GTP concentrations (500μm and 10mM). These are represented as a mean percentage of the (calcium concentration 0.1 and 10mM; no GTP) for three determinations. The level of TGase activity was determined by measuring the crosslinking of ¹⁴C putrescine to casein. The casein precipitates were re-dissolved in sodium hydroxide (aq) and incorporation of radioactivity (DPM) determined by scintillation counting.
7.7 Texas red cadaverine labelling of corneocytes

As shown in Fig. 7.13, transglutaminase activity as measured by Texas-red cadaverine labelling, was detected within the CE of the corneocyte in the presence of DTT. This labelling was totally inhibited by cystamine, and was reduced, yet still present in the absence of the thiol, DTT (Data not shown).

Fig. 7.13. Texas Red Cadaverine labelling of cornified cell envelopes.

The picture represents the labelling of an SDS protein extracted corneocyte, following incubation in the presence of Texas red cadaverine in TGase assay buffer (Chapter 2). The crosslinked cadaverine was located within the cornified cell envelope.
The aim of this research was to investigate the hypothesis that cornified envelope maturation is mediated by TGase enzymes. Although the significance of this maturation is still not fully understood, the persistence of the fragile envelope morphology in a range of dry skin conditions suggests that an inability to complete CE maturation has a fundamentally detrimental effect on stratum corneum integrity. TGases may potentially be involved in CE maturation, by either the continued cross-linking of existing CE proteins, or by the cross-linking of "free" proteins within the cytosolic pool of the corneocyte. There is currently no evidence to support the addition of new proteins into the CE during its conversion from fragile to rigid CE (Reichert et al 1993), although this requires confirmation. Recent evidence suggests that concurrent with the demonstrated elevation in TGase activity towards the skin surface, the number of (γ-glutamyl) lysine isopeptide bonds also increases (Harding et al 1999). It now appears that TGase may also be involved in the crosslinking of the covalently bound lipid to the CE. The membrane-bound form of the TGase 1 enzyme forming ester bonds between specific glutaminyl residues of human involucrin and a synthetic analogue of epidermal specific omega-hydroxyceramides (Nemes et al 1999).

The finding that the enzyme activity increases during stratum corneum transit, in the absence of any capability to express more enzyme, is surprising, and has not been reported previously. The data in this chapter are supported by previous research which showed the first 16 stratum corneum tape stripped layers had a
significantly higher TGase activity compared with tapes 17-32 (Rogers J, Unilever Research, Unpublished observation). Although the in vivo significance of this observation remains to be established it does parallel the major conversion of fragile to rigid CEs and an increase in γ-glutamyl-lysine crosslink number (Harding et al 1999). This supports the hypothesis that TGases are responsible for CE maturation. One possibility for the elevated activity could be alterations in the TGase 1 isoforms. This enzyme is known to exist in a series of differing molecular weight variants (33-106KDa) in skin, each with varying specific activities (Kim et al 1995). The interconversion of these forms in epidermis occurs within the membrane (Chakravarty and Rice 1989, Phillips et al 1993) and by altering their ratios, TGase activity can be altered. This would be particularly relevant within the detergent soluble pool, which should extract 'membrane bound' TGase. However, other possibilities such as the presence of TGase inhibitors within the deep stratum corneum and its extracts cannot be discounted.

This research has partially characterised the TGases of the stratum corneum and shown the presence of soluble TGase 3 and 1 and detergent soluble TGase 1 like activity. Due to the lack of commercial antibodies confirmation of the identity and levels of TGase 1 and 3 protein could not be unequivocally confirmed. In viable cells the bound TGase 1 can be released from the membrane by acylases, proteases or detergents (Chakravarty and Rice 1989, Rice et al 1990) although only the latter has been shown to be effective in stratum corneum (data not shown). However, within the stratum corneum it is misleading to think of the detergent soluble enzyme as being membrane-bound in the
classical sense, since no plasma membrane is retained within this tissue. This fraction may represent TGase 1 that is associated with the covalently bound lipid, although this remains to be confirmed.

The epidermal forms of TGase 1 include a number of soluble processed forms of differing molecular weights (67/33KDa complex, 67Kda and 106Kda proteins) (Kim et al 1995). The activity of these different TGase 1 isoforms varies, with the 67/33KDa complex having 10X the activity of the 67KDa form and 1/3rd that of the 106KDa form (Kim et al 1995). The release of TGase 1 from the membrane via dispase gives a 100% increase in activity, most likely associated with a change in the types of isoforms present (Kim et al 1995). These highly processed soluble forms of TGase 1 account for 90% of the total TGase 1 & 2 activity within the epidermis and keratinocytes (Kim et al 1995), and appears to account for 90% the TGase activity in stratum corneum and 60% in callus. Although in epidermis approx. 10% of TGase 1 was found to be membrane bound, in stratum corneum and callus, this appeared to be 30% and 50% respectively.

The TGase 3 present in the stratum corneum is likely to be fully activated. Heat or dispase treatment, which are known to activate the TGase 3 pro-form (Kim et al 1990), did not give any enhancement of activity in crude TGase extracts or TGase 3 column fractions (Data not shown). In addition, it appears that no TGase 2 was detected in the smart eluates in the region of elution of the TGase 2 (0.3M NaCl). This was confirmed by the lack of inhibition of extractable TGase by GTP, at levels shown to inhibit a guinea pig liver TGase 2. As shown by Smethurst and Griffin previously for TGase 2, the inhibitory effect of GTP is dependant on the calcium
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concentration (Smethurst and Griffin 1996).

Although much is known about the multi-factorial problem of cosmetic skin dryness, including perturbed desmosomal digestion, a disrupted lipid environment and abnormal filaggrin processing (Chapter 3), much less is known about the relevance of altered TGase activity. This research has shown that soap induced dryness is associated not only with a retention of desmosomes (as measured by the probe dscl) and the persistence of more fragile CEs in surface layers, but also with a reduction in TGase activity. This decline appears to be most significant for the detergent soluble extract, which has been shown by anion exchange chromatography to consist of unprocessed TGase 1. This reduction may reflect an abnormality in TGase 1 processing to its more active forms, possibly as a result of an altered water gradient in dryness. However, whether the reduction in activity is due to a reduction in TGase transcription is not currently known, and the lack of availability of reasonable antibodies prevents this being tested. However, the link between decreased TGase activity and the persistence of a fragile envelope phenotype provides further evidence to support the importance of this enzyme class for envelope maturation.

Many biochemical processes within the stratum corneum appear to be compromised in dry skin, therefore it is difficult to define the exact effect of the altered CE morphology on skin condition. The CE, as well as having a structural role, is coated by a monolayer of covalently bound lipid (Swartzendruber et al 1987), which is implicated in the correct aligning of the lipid bilayers and the improved retention of natural moisturising factors within the corneocyte. As TGase
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appears to have a role in the esterification of these lipids to the CE, a reduction in TGase activity in dryness may contribute to barrier abnormalities. In addition, the alterations in CE morphology may also influence extracellular lipid lamellae alignment (hence barrier function) and NMF leakage, as well as directly influencing surface appearance and mechanical integrity.

Certainly, the current finding strengthens the link again, between desmosomal degradation and envelope maturation. The precise chronological relationship between these events however, remains to be established. Although, desmosomal degradation (by removing the "straight-jacket" from the envelope) may be a prerequisite for allowing subsequent changes in surface morphology which are then "made permanent" by TGase cross-linking activity. The maturation of the CE and alteration in the shape of the corneocyte to a flatter smoother shape is likely to be important to facilitate desquamation.

In conclusion the current data are supportive of the theory that the maturation of the CE is associated with continued cross-linking by TGase. Evidence includes the presence of TGase 1 and 3 within the stratum corneum and TGase activity levels and γ-glutamyl-lysine isopeptide crosslink number increasing concurrent with CE maturation. In further support of this hypothesis, cystamine (a known inhibitor of TGase activity) significantly reduces envelope maturation using a crude in vitro screen (Long, Unilever Research, unpublished observation). Hence, factors which enhance TGase activity and/or CE maturation may be of cosmetic skin condition benefit.
CHAPTER 8

8. Conclusions

The research in this thesis has highlighted the complex control of stratum corneum maturation and desquamation. The research has in particular confirmed the multifactorial nature of skin dryness, involving abnormalities in the stratum corneum lipids and enzymes.

In skin dryness brought on by soap washing in low humidity winter conditions, one of the main alterations in the barrier lipids appears to be a decrease in the ceramide : fatty acid ratio. This is accompanied by alterations to the lipid lamella structure, as observed by EM, the lipids form a disorganised amorphous lipid mass at the skin surface in dryness. As discussed in chapter 3, the ratios of the barrier lipids greatly influences the lipid phase behaviour. Fatty acids such as oleic acid, which can account for 22-30% of the free fatty acids in the stratum corneum are known skin permeability enhancers. Oleic acid is known to enhance the fluidity of the lipid mix and can cause phase separations. It is likely therefore that the increased fatty acid levels will give an inferior barrier performance enhancing water loss and permeability to irritants. As the relative levels of ceramides decrease in dryness, skin products that boost ceramide levels either through formulation of synthetic ceramides or their precursors are likely to give benefit in cosmetic xerosis. These approaches are now being pursued by numerous skin care companies.
Skin dryness is also characterised by an abnormal accumulation of corneocytes at the skin surface due to a failure within the desquamation process. This appears to be due to a lack of desmosomal proteolysis. The barrier lipid abnormalities may influence desmosomal retention by the effects of the water gradient on stratum corneum protease activity. This problem can be addressed by assisting barrier repair and desmosomal degradation through the topical application of moisturisers containing humectants such as glycerol.

Barrier lipid abnormalities are also likely to appear in the summer months following chronic UV exposure. In particular the covalently bound lipids were significantly reduced following UV treatment and cholesterol sulphate levels appeared to increase. As this study had a low subject number, this novel finding would be worthwhile confirming. Certainly elevated cholesterol sulphate in the stratum corneum is known to be associated with scaling. Lower levels of the covalently bound lipids which encase the corneocyte are likely to impair the barrier, although the precise implications of this are currently unknown. In the photodermatoses, UV treatment alongside the use of topical products may enhance delivery of actives through impairing the barrier.

As the susceptibility of the skin to dryness appears to differ with both the seasons and bodysite, stratum corneum lipid differences were investigated. The results demonstrate a reduction in stratum corneum lipid levels in winter, in particular ceramide 1.
linoleate, when susceptibility to dryness is increased. This decline was true for all the body sites studied. There appears to be a need to formulate moisturisers for the different seasons. In winter, we have demonstrated in further work that topical application of products rich in linoleic acid rich triglycerides will help boost the ceramide 1 linoleate back to normal levels and relieve dryness. Any occlusive moisturisers such as vaseline will also give extra protection which appears to be required during the winter months when barrier lipids are reduced.

Although much is now known about the role of lipids in barrier function, the mechanism by which they influence desquamation is essentially unknown. This research has demonstrated that a number of lipids associated with skin dryness, i.e. cholesterol sulphate (elevated in recessive x-linked ichthyosis) and fatty acids (elevated in cosmetic dryness) were found to inhibit the desquamatory protease, SCCE. This supports the involvement of lipid abnormalities in aberrant desquamation. It is likely that SCCE is not the only enzyme involved in the desquamatory process. The research continues to determine which other proteases or glycosidases could be involved in the degradation of the desmosomal glycoproteins.

The stratum corneum has been shown to contain numerous enzymes, and this research has demonstrated the presence of TGases 1 and 3. The activity of these enzymes is closely linked to the process of CE maturation. TGase activity was demonstrated to increase during the process of CE maturation, being
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associated with increased CE crosslink number. Also, in skin dryness when the immature CE’s persist, the activity of TGases were significantly reduced.

The modulation of stratum corneum proteases and TGases is a good target for relieving both cosmetic and more severe skin dryness. This could be achieved by topical application of enzymes or actives to boost their activity. The methods developed as part of this thesis would prove useful in the further screening of actives to influence the activities of these enzymes. In the case of TGase, in psoriasis, dandruff and acne where the levels of TGase are elevated, TGase inhibitors may also prove of benefit.

Overall, this research has expanded our depth of understanding of the factors that influence skin condition. It has helped identify new opportunities for the treatment of skin dryness and new targets for the investigation of future skin care actives.
References


Abraham W, Wertz P, Downing D. J. Lipid Res. 26: 761-766 1985


Allen TD. J. Ultrastruct. Res. 5: 94-105 1975

Ansari MNA, Nicolaides N, Fu HC. Lipids 5: 838-845 1970


References


Bommannan D, Potts RO, Guy RH. J. Invest. Dermatol. 95(4): 403-408 1990


Braun PE, Morrell P, Radin NS. J. Biol. Chem. 245: 335-341 1970


References

Eromberg LE, Klibanov AM. Proc. of the Nat. Acad. of Sci. of the USA. 91(1): 143-147 1994


Burr G, Burr M. J. Biol. Chem. 82: 345-367 1929


Candi E, Chung SI, Marekov LN, Steinert PM. J. Biol. Chem. 270: 26382-26390 1995

Chakravarty R, Rice R. J. Biol. Chem. 264(1) 625-629 1989

Chang f, Swartzendruber DC, Wertz PW, Squier CA. Biochimica et Biophysica Acta 1150:(1) 98-102 1993


References

Elias P.M. Int. J. of Dermatol. 20 1-19 1981

Elias P.M. J. Invest. Dermatol. 80 44s-49s 1983


Elias PM, Menon GK, Grayson S, Brown BE. J. Invest. Dermatol. 91: 3-10 1988


References


Gray GM. Yardley HJ. J. Lipid Res. 16: 434-440 1975


References


Hawk J. In: The environmental threat to skin. Ed. R. Marks, G. Plewig. 67-72 1993


Hohl D. Dermatologica 180: 201-211 1990

References


Homaidan FR, Zhao LM, Chakroun I, Martin CA, Burakoff R. Mediators of Inflammation 8(4-5): 189-197 1999

Horie N, Fukuyama K, Ito Y, Epstein WL. Comp. Biochem. Physiol. 77B: 349-353 1984


Horton JD, Shimomura I. Current Opinion in Lipidology 10: 143-150 1999


References

Dermatol. 87(6): 758-761 1986


Kim SY, Chung SI, Steinert PM. J. Biol. Chem. 270 18026-18035 1995
References


Kock A, Schwarz T, Kirnbauer R. J. Exp. Med. 172:(6) 1609-1614 1990


References


References


References


Nelson WG, Sun TT. J. Cell Biol. 97 (1) 244-251 1983


Nemes Z, Steinert PM. Experimental and Molecular Medicine 31(1) 5-19 1999b


References


Plishker MF, Thorpe JM, Goldsmith LA. Arch. Biochim. Biophys. 191: 49-58 1978


References


Rice RH, Green H. Cell 18: 681-694 1979


Scott IR, Harding CR. Dev. Biol. 115: 84-91 1986
References


Steinert PM, Kim SY, Chung SI, Marekov LN. J. Biol. Chem. 271(42): 26242-26250 1996


Sun TT, Green H. Cell 9: 511-521 1976


Thacher SM, Rice RH. Cell 40: 685-695 1985


Watkinson A; Rogers J; Harding C. J. Dermatol. Sci. 16(1): s200 1998


References

Werz P, Downing D. J. Lipid Res. 24: 753-765 1983

Werz P, Downing D. J. Lipid Res. 25: 1320-1323 1984


Werz PW, Downing DT. J. Invest. Dermatol. 94: 159-161 1990

Werz PW, Downing DT. FEBS Letts. 268: 110-112 1990b


Zalewska T, Strosznajder J, Kawashima S. Neurochemical Pathology 8: 79-89 1988
Appendix 1

<table>
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<th>Lipid</th>
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<td>Palmitic acid</td>
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Appendix 1: Column recovery of cholesterol and fatty acid

A lipid standard was prepared in chloroform and equal volumes were loaded onto five amino-propyl bonded columns and eluted with the lipid separation solvents (Chapter 2). The cholesterol and fatty acid fractions were collected and analysed by HPTLC along with an equal volume of standard without column separation (Chapter 2.5-2.6). The data represents the value (n=1) or mean ± SD (n=5). The percentage recoveries from the column were >95%.
Appendix 2

<table>
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<th>Intraplate variation</th>
<th>Interplate variation</th>
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</thead>
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<td>154.4 ± 22.0 (13.9)</td>
<td>185.5 ± 27.0 (14.7)</td>
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</tr>
<tr>
<td>2</td>
<td>511.0 ± 45.7 (8.9)</td>
<td>501.0 ± 61.0 (12.1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>606.0 ± 54.0 (8.9)</td>
<td>623.0 ± 62.0 (9.9)</td>
<td></td>
</tr>
<tr>
<td>4/5</td>
<td>682.0 ± 29.0 (4.2)</td>
<td>672.0 ± 42.0 (6.3)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>779.0 ± 21.0 (2.6)</td>
<td>728.0 ± 45.0 (6.2)</td>
<td></td>
</tr>
</tbody>
</table>

Appendix 2: The intra and interplate variation in ceramide levels

The data represents the mean ± SD for five ceramide determinations on one HPTLC plate (Intraplate variation) and duplicate determinations on five plates (Interplate variation). The HPTLC procedure was performed using the modified Ponec method described in Chapter 2.6.2. The reproducibility of the HPTLC procedure was found to be good, giving an intraplate variation in ceramide levels of ~8% and an interplate variation of ~10%.
EXTERNAL PUBLICATIONS


