THE SKIN BARRIER

TAPE STRIPPING STUDIES

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SUMMARY

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BACKGROUND

The skin, being an exposed organ, is an easy site to access for research. However, standard interventions, such as a biopsy, leave the skin scarred and are logistically cumbersome. Therefore, non-invasive methods are preferable providing reliable *in vivo* data can be obtained. The study of the skin barrier is particularly suited to non-invasive techniques, as the ability of the skin to allow molecules to permeate is a dynamic process best seen in living tissue. A widely utilized method is the application of adhesive tapes to the skin. These tapes, when removed, take with them a small amount of stratum corneum that is adherent: this can be studied further, as can the remaining skin at the stripping site. Typical studies performed in this way include studies of "drugs reservoirs" in the stratum corneum (where lipophilic compounds accumulate), studies of the skin barrier to water and other substances, studies of transdermal drug delivery and studies of wound healing and stratum corneum physiology.

There is no standardized method for tape stripping, resulting in confusion over technique and difficulty in interpreting other workers' data. Recently there have been attempts to improve the methodology. One such improvement by this laboratory has been the development of a method to quantify the exact amount of stratum corneum on each tape. This information is expected to advance the use of the tape stripping model considerably particularly because other variables can now be measured against stratum corneum mass and depth. It is the aim of this thesis to take that work forward.

AIMS

The aim of this thesis is to build on a new model of stratum corneum tape stripping and demonstrate its usefulness as a method of studying the stratum corneum, both physiologically and pharmacologically. The thesis is organized through a series of logical studies. In the first study, the method itself is applied *in vivo* to human volunteers. Building on the previous work, the study explores a variety of factors, which have not previously been addressed. For example, does it make a difference which tape is used to strip the skin? Simultaneously, a large amount of data regarding skin sites and transepidermal water loss is also gathered, with the aim to use the model to quantify water kinetics in the stratum corneum with respect to mass and depth.

In the second study, the aim is to apply the model to skin pharmacology. Using a well-known drug, salicylic acid (SA), the study aims to assess whether the model is useful in quantifying clinical efficacy. As the drug is thought to make stratum

corneum shed, the study aims to demonstrate that increase is demonstrable through stratum corneum protein quantification on tape strips.

The third study returns to the area of skin physiology, partially because the second study raised some interesting questions about skin pH and the pH/pKa of topical drugs. This study aims to use the quantitative tape stripping method to create precise profiles of skin pH within the human stratum corneum *in vivo*. This is particularly of interest to two main disciplines – the study of metabolic processes within the stratum corneum and to the study of drug delivery through the stratum corneum. The aim is to define accurate pH profiles, to enhance our knowledge of pH in the stratum corneum and to show that the quantitative tape stripping model is a useful tool in this area of research.

METHODS

In this thesis, the methods are generally consistent for the three studies, although there are variations specific to each project, detailed later. All three studies involved the same tape stripping model, and were carried out on humans *in vivo* with ethical approval. Tapes were placed on the skin and a 10kPa pressure was applied. Following this, the tape was gently removed and stored in a borosilicate scintillation vial for further analysis. Non-invasive bioengineering studies were performed on subjects before, during and after tape stripping in order to quantify transepidermal water loss, pH and skin color as appropriate for the study concerned. Subsequently, the protein on each tape was extracted using a strong alkali and then quantified using a spectrophotometer. These protein extractions

allowed a calculation of the mass of stratum corneum on each tape and therefore the depth of stratum corneum that had been stripped and measured. By comparing these results to the clinical and bioengineering measurements, an understanding of skin function was obtained. In study 2, another specific tape stripping technique known as squamometry was also used. This is a method by which the morphology of the stratum corneum sheets is assessed under a microscope to detect irritancy of topical products.

RESULTS

Study 1: Each type of tape successfully stripped the stratum corneum, but the rayon tape did not induce SC barrier disruption. Neither the type of tape nor the site stripped significantly influenced the mass of SC removed. Water kinetic parameters did not differ significantly for the tapes that did induce barrier disruption. Individual variation in barrier disruption to water following tape stripping was demonstrated.

Study 2: Tape stripping combined with protein analysis was sensitive in detecting keratolytic effect of SA within hours of application. Squamometry was not useful in detecting skin irritation in comparison to visual assessment and bioengineering chromametry. Importantly, whereas the pH of the preparations only minimally influenced efficacy, local dermatotoxicity was significantly increased at acidic pH. This indicates that the quest to increase the amount of free, non-dissociated SA is, in fact, counterproductive as the more acidic preparations resulted in skin irritation and barrier disruption.

Study 3: A pH profile with respect to depth was successfully created using the stratum corneum tape stripping method. The acid mantle was identified as a distinct zone of acidity in the superficial stratum corneum localized to a median depth of 1.8μm in the volar forearm and 1.4μm on the upper arm. Additionally, a new zone of stable pH was identified deeper in the stratum corneum.

CONTRIBUTION OF THE RESULTS TO THE EXISTING FIELD

Each of the studies performed has advanced the existing database of knowledge in this field. Study 1 has demonstrated that a variety of tapes can be successfully and generally equivalently used to study skin water kinetics. It has provided a database of information on tape stripping for future investigators and re-calculated water kinetics using the new quantitative data. In addition, this study has identified that a proportion of the population do not display increased transepidermal water loss despite significant barrier damage. This implies that the sheer mass of stratum corneum alone cannot explain the skin barrier to water, but rather there must be other factors such as the proportion of different types of lipids that must be responsible for this.

Study 2 has, for the first time, demonstrated a quantitative method to assess the efficacy of keratolytic drugs. In addition, it has refuted a commonly held premise that acids applied to the skin must be at a pH near their pKa in order to retain their efficacy. In fact, this thesis shows that such practice results in increased skin

irritation but no increase in efficacy, compared to less acidic preparations of the same acid. This data will allow those who formulate such drugs to make less irritating preparations.

Study 3 has, for the first time, localized the acid mantle *in vivo* in human skin and also confirmed that this acid mantle is distinct from skin surface acidity. Also, this study has discovered a zone in the stratum corneum where pH is almost stable for 1μm, consistently between subjects, suggesting that common metabolic processes are taking place at this site. This information is of use to those who formulate drugs for transdermal delivery, to the study of skin metabolism and to the study of diseases with altered skin pH, such as atopic dermatitis.

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Chapter 1

OVERVIEW OF THE STRUCTURE AND FUNCTION OF HUMAN SKIN

Issues Relevant to Skin Barrier Formation and Maintenance

INTRODUCTION

The skin barrier can be perceived as the interface between the body and its environment. The structural components of the skin are discussed in this section in order to understand their role in the formation and maintenance of the skin barrier and to demonstrate the structures that must be considered in the transdermal delivery of xenobiotics.

Human skin can be divided into histological subunits, which reflect skin function. Broadly, the skin consists of the epidermis and dermis, which form the basis of a broad division of the skin. The epidermis consists of several cell layers, which rest upon a basement membrane, superficial to the dermis. The outermost cell layer of the epidermis is the stratum corneum, which forms the actual barrier interface between the body and the environment. This non-viable outer layer arises by the differentiation and upward migration of the epidermal layers below it, from the stratum basale, which rests on the basement membrane. As the keratinocytes migrate upwards from the basal layer, they differentiate, giving rise

to distinct layers: the stratum spinosum and stratum granulosum, and finally terminal differentiation to form the stratum corneum (cornification). Underlying the stratum basale is the basement membrane, which in turn overlies the dermis. The dermis is the support structure upon which the epidermis lies. It is composed of a variety of cells and their products, including: fibroblasts, collagen, elastin, glycosaminoglycans, hyaluronic acid and ground substance. As the dermis is vascular, substances which penetrate through the epidermis have access to the systemic circulation. As such, these substances have penetrated the skin barrier. Therefore, the dermis is not discussed further in this thesis.

In addition to the histological subdivisions of the skin, certain cell layers, for example the stratum corneum, can also be considered to have biochemical subdivisions that relate directly to functions. These include the arrangement and concentration of molecules such as lipids and proteins, which are discussed in the next chapter.

THE EPIDERMIS

The epidermis consists of a stratified squamous epithelium resting on a basement membrane that anchors it to the dermis. This layer covers the entire outer surface of the body, varying in thickness and appendages at different sites. The predominant cell type is the keratinocyte (80%) but other specialised cells are present including melanocytes which produced melanin pigment, Langerhans

cells which perform immune surveillance and Merkel cells, which may act as a mechanoreceptor for touch. Nerve endings permeate into the viable epidermis with membrane-to-membrane apposition with keratinocytes (1). In pathological states, other cell types such as T-lymphocytes or neutrophil polymorphs may also infiltrate into the epidermis.

Keratinocytes are named for their unusual richness in keratin proteins, which can comprise up to two-thirds of the dry weight of the cell. Keratins are a family of water insoluble alpha-helical proteins ranging in size from 40 to 70kDa and are found in all epithelial cells. These proteins are structural cytoskeleton components, also known as intermediate filaments because their 8-10nm diameter lies between that of actin (6nm) and microtubules (23nm). Organised into bundles, they extend from the nuclear envelope to the desmosomes and hemi-desmosomes at the plasma membrane. Approximately 30 different keratin polypeptides have been identified in humans; 20 in epithelial tissue and 10 in hair. These can be divided into two groups, type I and type II, based on their isoelectric point and other properties. Type I keratins (K10-20) are the acidic type, with an isoelectric point <5.5, whereas type II keratins (K1-9) are the basic-neutral with an isoelectric point >6.5.

Keratins assemble into filaments within cells and also *in vitro* as obligate hetero-polymers, consisting of and acidic and basic pair. The type of keratin expressed depends on the tissue, its health and stage of differentiation. In the human epidermis, K5 and K14 are expressed in the basal layer, whilst K1 and K10 are

expressed increasingly as cells migrate and differentiate upwards until they reach the stratum corneum.

Differentiation of keratinocytes to form the skin barrier takes place from the basal layer upwards. The basal cell population can be subdivided into groups: the stem cell population comprises approximately 10% of basal cells and have the ability to divide giving rise to further basal cells or another cell type known as transit-amplifying cells, which make up 50% of the basal cells. This latter group of cells has a limited ability for further mitoses before entering a differentiation pathway, when they are termed committed cells. The remaining 40% of cells found in the basal layer are already committed to differentiation.

Proliferation of keratinocytes in the basal layer is associated with their upward migration, forming a constant turnover of cells within the epidermis, although migration can occur even when proliferation is blocked (2). As the cells migrate upwards and differentiate, they lose their basal characteristics and begin to develop specialised structures to allow them to form into the corneocytes that constitute the skin barrier.

Basal keratinocytes express K5 and K14, which provide cytoskeleton with sufficient flexibility to permit cell division and migration. As they become committed to entering a differentiation pathway, they begin to express involucrin and K10. It is estimated that keratinocyte transit time once detaching from the basal layer is 14 days to reach the stratum corneum; a further 14 days are required to transit through the stratum corneum and desquamate.

The histological layer above the basal layer is the stratum spinosum, named for histological appearance of "spines" between the cells under light microscopy. These structures are the desmosome junctions between the keratinocytes, which become visible because of artefactual cell shrinkage during tissue processing. In comparison to the basal cells, which are cuboidal in shape, these cells are more polyhedral. They contain large amounts of keratin filaments, which are organised concentrically around the nucleus and inserted peripherally into desmosomes. Although K5/K14 still persist within the spinous layer following synthesis in the basal layer, K1/K10 are newly synthesised in the spinous cells. These latter keratins are characteristic of the epidermal type differentiation pattern. In the more superficial aspects of the spinous layer, the upwardly migrating cells begin to alter, developing a more flattened appearance and forming intracellular organelles that are characteristic of the next layer.

This next layer of migration is the granular cell layer, where the cells can be recognised by characteristic basophilic cytoplasmic structures termed "keratohyaline granules." The granules are composed of proteins such as profilaggrin, loricrin and a cysteine rich protein related to cystatin-A, in addition to keratins K1/K10, acting as a repository for these future stratum corneum components. Although these molecules are stored together, they have different fates. Loricrin and cystatin A are substrates for transglutaminase activity and are constituents of the cornified envelope, whilst profilaggrin is an important intracellular protein.

Profilaggrin is a histadine-rich polyphosphorylated high molecular mass protein (>400kDa), which is composed of tandem repeats of filaggrin. This is released from the granules after dephosphorylation and it is subsequently processed into monomer filaggrin units. The monomers are thought to promote aggregation of keratin intermediate filaments into macrofibrils, which form the bulk of protein in corneocytes (keratinocytes of the stratum corneum).

Another granular organelle is also seen in the upper stratum spinosum and stratum granulosum. Measuring approximately 0.1μm by 0.5μm in dimension these are termed "lamellar bodies." The granules consist of one to several stacks of lamellar disks surrounded by a unit bounding membrane. The internal disks of the lamellar granules are thought to be either flattened lipid vesicles or a membranous sheet folded in an accordion like manner. The contents of the granules include glycoproteins, glycolipids, phospholipids, free sterols and several acid hydrolases. Whilst they form in the peripheral cytoplasm of the granular cells, they migrate to the apical membrane in the upper granular layer where they bind via their membrane to the plasma membrane. As the membranes fuse, the contents of the lamellar granule are exocytosed into the extracellular space. The released acid hydrolases (including proteases, lipases, acid phosphatases and glycosidases) act on the released polar lipids converting them into mature barrier lipid. This processing is associated with remodelling: edge-to-edge fusion of the flattened lipid vesicles and physical rearrangement into bilayers.

Another structure gaining importance as keratinocytes migrate through the granular cell layer is the cornified envelope. The layer can be identified in the

upper spinous layers by electron microscopy as a thin electron dense band underlying the apical plasma membrane. The band thickens as the keratinocytes migrate through the granular layer, reflecting the deposition of proteins cross-linked by transglutaminases. Proteins that make up this envelope include loricrin, involucrin, cystatin-A (keratolinin), filaggrin linker segment peptide and others. As the lamellar bodies are exocytosed, a ω-hydroxyceramide from the released vesicle becomes covalently attached to the cornified envelope and the plasma membrane disappears as phospholipids are degraded.

The final layer of upward migration is the stratum corneum, which is the end-point of differentiation for keratinocytes. The cells in this layer are known as corneocytes, which is a useful distinction from other keratinocytes as these cells are non-viable and have particular characteristics. Histologically, there is an abrupt transition from the stratum granulosum to the stratum corneum. In order to make the transition, the granulocytes must undergo programmed self-destruction. Whilst some of the metabolic changes seen are very rapid and are immediately localised to this area, other take more time and begin within the stratum granulosum and extend into the initial layers of the stratum corneum, known as the stratum compactum. Therefore, this area may best be considered as a transition zone with a gradient of maturation. Intracellular changes seen in this transition zone include destruction of the nuclear envelope, sub-cellular organelles and the plasma membrane, driven by several degradative enzymes. Some of the morphological changes are characteristic of apoptosis including internucleosomal fragmentation of deoxyribonucleic acid (DNA) and activation of caspases.

Extracellular processes include modifications to the lipids exocytosed from lamellar bodies and adjustments to the cornified envelope.

The corneocytes are flatter, larger cells than their preceding keratinocytes, stacked overlapping each other in approximately 15 layers, measuring approximately 40μm by 0.5μm (3). . They consist of bundled keratins surrounded by a cornified envelope of cross-linked proteins, which are in turn bound covalently to lipid. The corneocytes are interconnected to each other by corneodesmosomes. The intercellular space is filled with lipid, primarily derived from lamellar bodies, but also from sebaceous secretions. The lipid layers form a tortuous contiguous path throughout the stratum corneum and may play a significant part of barrier function, discussed later. However, the stratum corneum is not homogeneous and can be split into different domains. The stratum compactum is the innermost area, comprising approximately the initial 3 to 4 layers of corneocytes, whereas the stratum disjunctum represents the outermost 3 to 4 layers at the skin surface. There is variation in the thickness, density, water content, amino acid and lipid content between these layers, perhaps reflecting maturation and functional differences. For example, the stratum compactum has a higher density of corneodesmosomes than the stratum disjunctum, where desquamation occurs.

Desquamation is the final event for epidermal cells, resulting in the shedding of corneocytes from the body into the environment. The thickness of the stratum corneum results from a balance between cornification and desquamation.

Chapter 2

THE SKIN BARRIER

INTRODUCTION

The skin barrier can be considered as a physical structure, which is the product of keratinocyte differentiation as outlined above. However, it should also be considered a dynamic structure, which is constantly responding to its environment. In this chapter, the physical components of barrier integrity will be discussed, followed by the physiological responses to environmental challenges.

STRUCTURAL ELEMENTS OF THE SKIN BARRIER

Stratum corneum

The stratum corneum is the interface between the skin and the external environment and therefore is the first barrier between external agents and the human body, and the last barrier between internal agents and the environment. The underlying layers of epidermis support the stratum corneum structurally and provide a new population of cells to replace those that are shed. The stratum corneum, being devoid of nucleated cells, was previously considered "dead" and

therefore a passive rather than an active component of the skin barrier. However, recent work detailed below, provides evidence of biochemical activity within the stratum corneum that is important in barrier formation and maintenance. More correctly, this skin layer should be considered "non-viable," recognising its molecular functions despite its inability to replicate.

Cellular and Molecular Organisation of the Barrier

The physical structure of the skin barrier can be considered in both cellular and extracellular terms. Neither of these components in isolation can provide the barrier properties seen *in vivo* in humans, and the two domains interact to develop and maintain the barrier. Although the components shall be discussed in isolation for simplicity, the relationship of the components must be remembered.

As detailed above, the epidermis consists mainly of keratinocytes, which differentiate and migrate upwards to the skin surface. During this period of migration, their metabolic activity alters, leading to the production of the molecules required for barrier formation. The lipids manufactured are secreted in lamellar granules, to contribute to extracellular component of the barrier. However, in addition to metabolic changes, the keratinocytes' physical structure also alters throughout differentiation and migration, leading to the formation of a specialised cell called the corneocyte.

Corneocytes

The corneocyte is a terminally differentiated keratinocyte with particular physical properties that allow it to function as the outermost layer of the skin barrier, the stratum corneum. These are flat polyhedral cells, approximately 40μm in diameter and 0.5μm thick (3). The cells are keratin filled, and bound by a protein envelope. Keratin is a hydrophilic material that can bind large amounts of water. Within the cells, keratin can be seen to be in fibrous and amorphous components. The fibrils, 8nm in diameter, span the inside of the corneocyte forming an internal reinforcement, which ensures that the plane of the skin remains unchanged even after extensive hydration. The fibrils are arranged in the plane of the cell, with almost no fibrils providing vertical reinforcement. As a result, the cells can swell up to 25% in the vertical direction but less than 5% in the horizontal plane. This ensures a minimal roughness of skin even at maximal swelling, thus minimizing the risks of mechanical stress causing surface breaks on wet skin. (4)

The principal keratins in the stratum corneum are K1 and K10, which are synthesized in the suprabasal layers. Keratins can be present in both α-helix and β-pleated sheet conformation, the former being more prevalent in human callus ("soft" keratin). Also present is the protein filaggrin, but it has a limited half-life. It is degraded, giving rise to low-molecular-weight compounds with water binding capacity.

The cornified envelope which surrounds the corneocytes represents an interface between the proteinaceous intra-cellular environment and the predominately lipid inter-cellular domain. This structure is discussed in more detail below.

Desmosomes join the corneocytes together, acting as "rivets," that prevent relative movement between the cells in the plane of the skin. This prevents shearing forces from disrupting the stacked lipid lamellae in the extracellular spaces. Again, these structures are discussed below in the next section, which focuses on proteins.

Protein Composition of the Skin Barrier

Research on the skin barrier has predominantly been focussed on its lipid component, but, it is becoming increasingly apparent that the protein components of the epidermis and stratum corneum are functionally involved in barrier integrity and homeostasis.

The Cornified Envelope

The cornified envelope can the divided into two parts: a thick inner layer, composed of protein, adjacent to the cytoplasm and a thin outer layer composed of lipid which is exterior to the protein part. The rigid protein structure, approximately 15nm thick, is formed by transglutaminases active within the stratum corneum. Isopeptide bonds form between γ-carboxyl groups of peptide bound glutamine and ε-amine groups of peptide bound lysine. Additionally, disulphide bonds form and the resultant structure is highly insoluble and resistant to chemical attack.

Desmosomes

Cell adhesion in the human body is maintained by adherens junctions, which bridge the gap between cells. Specialized forms of this cell adhesion complex are found in the epidermis: the hemi-desmosome, which anchors cells in the basal layer to the basement membrane, and desmosomes, which anchor the epidermal cells to each other. In the stratum corneum, there are some differences in the electron-microscopic appearances of the desmosome, which is therefore termed a corneodesmosome. Understanding the function of the desmosomes is important not only in understanding how protein components of the skin help maintain the barrier integrity, but also helps us to understand the processes involved when the barrier desquamates or is damaged by physical or chemical trauma.

Desmosomes consist of several proteins, which are inter-related to form a transmembrane complex. There are two transmembrane proteins which each have extracellular and intracellular domains. These proteins are termed desmogleins and desmocollins and are related to the cadherin family of calcium dependent cell adhesion molecules. The extracellular domains of the desmogleins and desmocollins interact with the respective desmogleins and desmocollins of the desmosome on the neighbouring cell forming a bridge between the cells. As these intercellular bridges are anchored on the cytoplasmic side (via anchoring proteins) to the keratin intermediate protein filaments, the result is a very strong junction complex.

Recently, it has been demonstrated that some blistering diseases can be explained by an understanding of desmosome function. For example, pemphigus foliaceus

and pemphigus vulgaris are characterised by the presence of circulating immunoglobulin G antibodies to desmoglein proteins. In pemphigus foliaceus, the antibodies are specific for desmoglein 1, resulting in blistering of the superficial epidermis. In pemphigus vulgaris, the antibodies target desmoglein 3, which is found in the deep epidermis, resulting in deeper, tense blisters in contrast to the fragile superficial blisters of pemphigus foliaceus. Similarly, exfoliative toxin, which mediates blister formation in staphylococcal scalded skin syndrome, has been shown to degrade desmoglein 1 (5).

Additionally, there is evidence that desmosomal proteins play a role in normal physiological desquamation. The degradation of desmoglein 1 only occurs in the outermost layers of the stratum corneum and is associated with corneocyte shedding. *In vitro* studies of dissociation of corneocytes demonstrate that cohesive corneocytes stain positively for intact desmoglein 1 whilst cells that had dissociated do not. Rather, the dissociated cells are associated with desmoglein 1 breakdown products. (6, 7).

Enzymes

Several enzymatic processes take place in the stratum corneum, and more processes and enzymes are being identified. Hydrolases are secreted into the intercellular space from lamellar granules, and are activated by the acidic pH in the superficial stratum corneum (8). Some of these hydrolases are proteases, which have been shown to be involved in desquamation, such as chymotrypsin, trypsin and cathespin. Recently, the enzyme cathepsin D, which is a protease, has been localised by immunofluorescence to the lipid envelopes of plantar squamous

cells, in a distribution similar to that of the desmosomes, implying that this enzyme plays a role in desquamation (9).

Cholesterol sulfate is an important inhibitor of proteases within the skin, and its accumulation because of steroid sulfatase deficiency is seen in X-linked ichthyosis. This might delay the degradation of desmosomal proteins, resulting in the clinical appearance of ichthyotic skin. The role of cholesterol in the stratum corneum is discussed in the next section on lipids, however this example demonstrates that the protein and lipid components of the skin cannot be considered in isolation, but rather are likely to work in equilibrium.

Lipid Composition of the Skin Barrier

The stratum corneum differs from other human lipid domains in its lipid composition and in its stacked arrangement. Given that one major function of the barrier is water retention, it is assumed that a major component of the barrier are the lipid layers, as these are hydrophobic and are highly organized.

Lipids that can form biological membranes are characterised by a hydrophilic head group and a hydrophobic part, usually a carbon chain. Thermodynamically, energy is required to keep the hydrophobic region dissolved in a water solution, so the molecules tend to aggregate into micelles or bilayers. In this way, they form a hydrophobic compartment away from water, whilst the hydrophilic head groups face the water. The ability of lipids to form stable membrane barrier aggregates depends on variety of factors, including the temperature, length of the

hydrophobic chain and the degree of unsaturation. Therefore, the understanding the lipid composition of the epidermis and stratum corneum sheds light on its barrier properties.

It has been demonstrated that significant lipid metabolism takes place in the epidermis. During epidermal differentiation, the nature of the lipids expressed alters in different layers, perhaps reflecting local functional requirements. This has been demonstrated using thin layer chromatography on lipids extracted from human abdominal skin at different levels in the epidermis (10).

In the basal layers of the epidermis, the predominant lipids are phospholipids, neutral lipids and free sterols. These lipids are functionally useful for common biological membranes. As differentiation occurs, the synthesis of lipids alters and the relative proportions vary leading to the formation of the specialised lipid layers of the epidermis and stratum corneum. In the granular layer, there are significant proportions of ceramides, glucosylceramides and free fatty acids in addition to increased cholesterol and phospholipids. However, in the stratum corneum, the phospholipids are completely degraded and the glucosylceramides are deglycosylated, with the result that ceramides, cholesterol and fatty acids are the dominant lipids of the stratum corneum.

Cholesterol

Whilst basal keratinocytes express the plasma membrane associated low-density lipoprotein (LDL) receptor, differentiating keratinocytes, which have moved from

the basal lamina, do not. As a result, the majority of cholesterol present in the epidermis must be synthesised *de novo*.

Several lines of evidence support epidermal synthesis as the main source of cholesterol, rather than a systemic source. Cholesterol synthesis occurs in the epidermis; barrier disruption leads to increased epidermal but not dermal cholesterol synthesis, a change which can be blocked by occlusion, suggesting that it is related to barrier function; and epidermal cholesterol synthesis is independent of serum levels, except in cases of prolonged fasting (11).

Cholesterol is formed from acetate, via the synthesis of ꞵ-hydroxymethylglutaryl CoA (HMG CoA) from three acetyl CoAs. The rate-determining step is the NADPH-dependent reduction of HMG CoA catalysed by HMG CoA reductase to yield mevalonate CoA. Therefore, cholesterol synthesis is dependent on the stability of HMG-CoA reductase mRNA and the rate of enzyme turnover. Most of the biosynthetic steps occur in the cytosol, although the rate-determining step occurs in the endoplasmic reticulum.

As mentioned earlier, barrier perturbation with solvent or by tape stripping results in an increase in sterol synthesis that is associated with the epidermis and not the dermis (12). The application of HMG-CoA reductase inhibitors reduces the recovery rate of the skin barrier after acetone treatment. These inhibitors can also cause barrier perturbation when applied topically, which can be prevented with co-administration of cholesterol. (13)

Although cholesterol accounts for approximately 30% of the stratum corneum lipid composition, its function remains unclear. When abnormally high amounts of cholesterol sulfate are present, defective desquamation occurs, seen in X-linked ichthyosis as thickened, scaly stratum corneum. In this condition, there is a deficiency of the enzyme sterol sulfatase, which catalyses the conversion of cholesterol sulfate to cholesterol. There are several possible mechanisms by which cholesterol sulfate may play a role in the control of desquamation. There is evidence of a delayed degradation of desmosomes in X-linked ichthyosis which may be the result of protease inhibition by cholesterol sulfate. Certainly, pancreatic proteases can be inhibited by cholesterol sulphate and, when applied directly to the skin, it can lead to an ichthyosis like hyperkeratosis (14). Also, it is possible that cholesterol sulfate inhibits desquamation by acting as a substrate modifier or by altering the extracellular physico-chemical environment to conditions unfavourable for desmosomal degradation.

Although both cholesterol sulfate and cholesterol are not concentrated in lamellar bodies, they are delivered to the intercellular spaces of the stratum corneum, and are present in increased concentrations in the granular layer. The ceramides are rod like or cylindrical in shape, which allows the formation of highly ordered gel phase membrane domains, which are less permeable than the liquid crystalline domains seen in other biological membranes.

Cholesterol, unlike cylindrically shaped ceramides and fatty acids, has the ability to stiffen or fluidize membranes depending on the proportion of cholesterol and other lipids present. In the stratum corneum, cholesterol may function to add

fluidity to what would otherwise be very rigid membranes, perhaps resulting in an inter-digitated system of microscopic gel phase domains in equilibrium with liquid crystalline domains. (15)

Ceramides

Ceramides comprise 50% of the total lipid mass of the stratum corneum and are structurally heterogeneous. Ceramide biosynthesis originates from the production of a sphingoid base from the condensation of serine and palmitoyl CoA. The principal sphingoid precursors are sphingosine and phytosphingosine. Additionally, in humans a unique 6-hydroxysphingosine base is also found. The final step is the addition of an acyl chain via its CoA derivative. The fatty acyl chains tend to be long chain hydroxyacids, ranging from 16-carbon (ceramide 5) to 34-carbon (ceramide 1) molecules. The different classes of ceramides found in the epidermis reflect the different sphingoid bases and fatty acyl CoA substrates that combine to form the ceramide. At physiological pH, the long chain ceramides, in the presence of cholesterol and fatty acids, have been shown to have the capacity to form lamellar lipid structures, similarly to phospholipids. The long chain length suggests close crystalline packing at normal skin temperatures.

Ceramide 1 is structurally unusual. It consists of a long carbon ω-hydroxyacid, ranging from 30- to 34-carbons, amide linked to sphingosine and dihydrosphingosine bases. The long carbon chain is ester-linked to linoleic acid, an essential fatty acid. It has been proposed that ceramide 1 is the primary source of lipid bound to the cornified envelope and it may serve as a "molecular rivet," linking together the intercellular lipid lamellae. Ceramide 1 may also play a key

role in the structural lamellar arrangement of lipids into their 13-nm broad-narrow-broad lamellar organisation.

Free fatty acids

The remaining major component of the stratum corneum lipids are free fatty acids, accounting for approximately 9% of lipids in the human stratum corneum. Similarly to the fatty acids bound to ceramides, they are predominately of long chain length, ranging from 16- to 28-carbon atoms. Approximately 7% are unsaturated. These characteristics are quite distinct from the fatty acid content of serum and sebum, suggesting that *de novo* synthesis must take place in the epidermis. Fatty acid synthesis, of which the main precursor is acetate, results in the formation of palmitic acid (C16) and stearic acid (C18). The rate-determining step is the conversion of acetyl CoA into malonyl CoA, catalyzed by the biotin dependent acetyl CoA carboxylase. In order to lengthen the chain further, fatty acid elongation systems localized to mitochondria or endoplasmic reticulum exist. However, the skin does not have the ability to synthesis essential fatty acids (linoleic and linolenic acid) which must be obtained from dietary sources, nor can it catalyse the synthesis of arachidonic acid from linoleic acid. Therefore, transport systems must be in place to deliver these molecules to the epidermis: keratinocytes have a novel fatty acid transport protein which may be involved in fatty acid uptake (16).

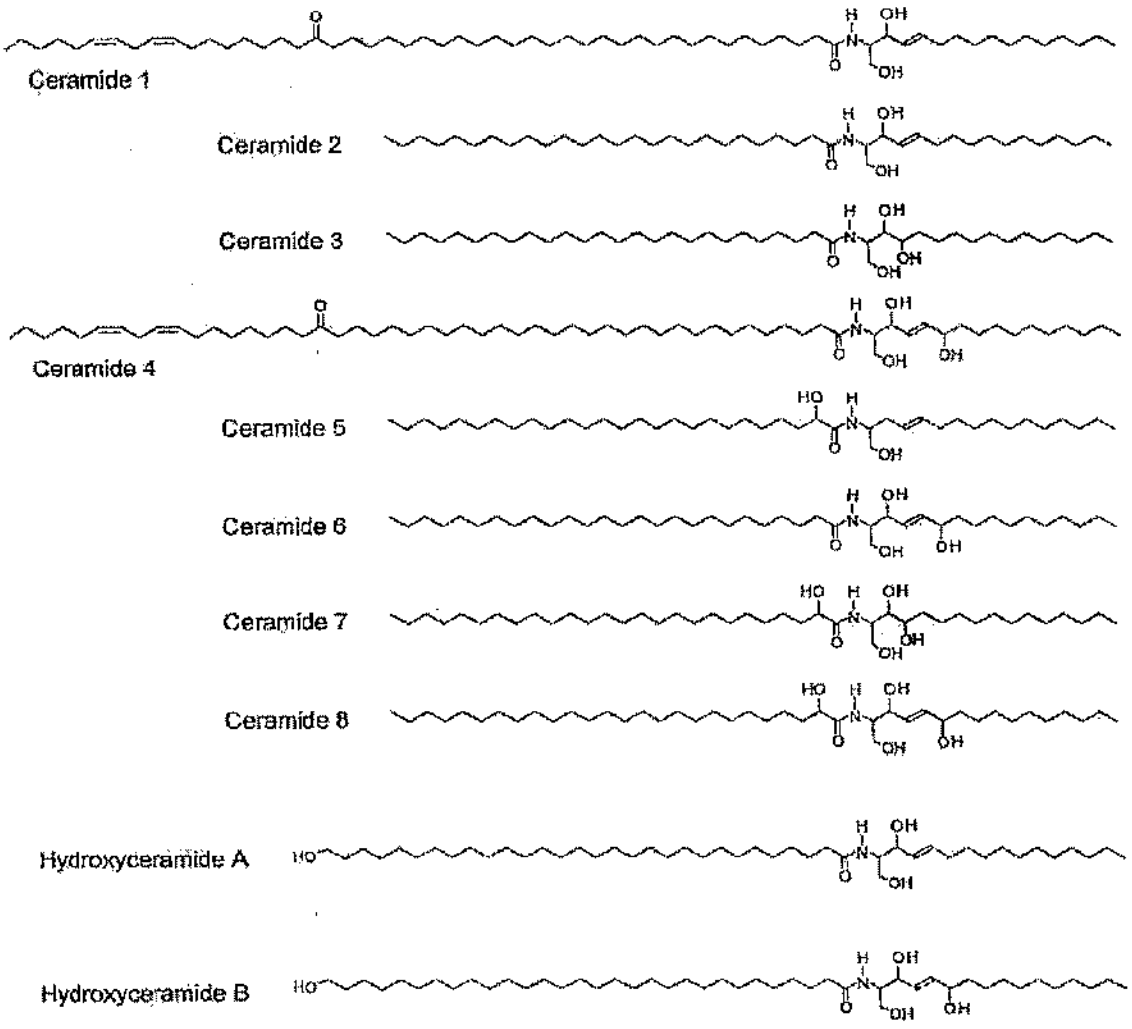


Figure 1: Structure of Ceramides in Human Skin (17)

WATER BINDING CAPACITY

The stratum corneum needs to maintain hydration in order to remain supple. In several skin diseases and in normal ageing the skin becomes dry, which may affect the function of the skin barrier. As transepidermal water loss is widely used as a surrogate marker of skin barrier function, it is important to understand how the skin handles water in order to understand barrier perturbation and to understand the movement of water soluble molecules within the barrier.

A significant decrease in water content is seen between the viable epidermis and the stratum corneum. The water gradient, measured by electron spin resonance microscopy, (18) falls sharply from a water content of 70% in the stratum granulosum and spinosum to 15%-20% in the outer stratum corneum. The molecular basis for this reduction in water content is not fully understood, but is thought to be related to a progressive reduction in water binding capacity in the more superficial layers of the skin. This may be because there are changes in the composition of keratinocytes as proteins, nucleic acids and phospholipids undergo degradation during the transition from the granular layer to the stratum corneum. As these molecules break down, there may be fewer chemical groups able to bind water.

Compounds that are able to bind water within the stratum corneum include corneodesmosomes, proteins, amino acids, natural moisturizing factors and phospholipids. Primarily, water is thought to be bound within the inner compartment of corneocytes as this domain is relatively more polar than the intercellular lipid domain. This is consistent with the fact that exogenous removal of lipids with organic solvents only results in a partial reduction of water content of the stratum corneum.

Nevertheless, water can be found within the lipid domain of the stratum corneum. The extraction of stratum corneum lipids by organic solvents results in the loss of the intercellular lamellae observed by electron microscopy. An approximate 13% decrease in water content from 33% to 20% is also noticed, which can be reversed by the application of topical stratum corneum lipids. Topical application of these

lipids also reverses the structural changes. Fractionation of stratum corneum lipids demonstrates that they vary in their ability to restore water content, with ceramides being the most effective (19).

After lipid extraction, further compounds can be extracted from stratum corneum sheets using water. These water soluble low molecular weight polar compounds are known as "natural moisturizing factors" (NMF) because their hydrophilic nature implies a role in water retention. The NMF primarily consist of amino acids or their derivatives, including pyrrolidone carboxylic acid and urocanic acid, together with lactic acid, urea, citrate, sugars and other molecules and ions (see Table 1).

NMF are present within corneocytes, and may represent 20-30% of the dry weight of the stratum corneum. Particular NMF compounds such as pyrrolidone carboxylic acid and lactic acid salts are hygroscopic, which means that they attract and absorb atmospheric water, dissolving in their own water of hydration. Thus, they behave as humectants, without which the stratum corneum cannot absorb significant amounts of water unless the relative humidity is 100%.

The origin of the lactic acid and urea components of the NMF remain poorly documented, but the amino acid derivatives are thought to be entirely derived from fillagrin. Fillagrin is rapidly broken down in the first two or three layers of the stratum corneum, except for a small proportion that is incorporated into the cornified envelope. The breakdown of fillagrin (which is derived from profillagrin by dephosphorylation and limited proteolysis) involves two main

steps: deimination by peptidyl deiminase, followed by complete hydrolysis to free amino acids. Two of these amino acids, histadine and glutamine, are metabolised to urocanic acid and pyrrolidone carboxylic acid respectively. The former is protective against ultraviolet light and the latter is a potent humectant.

Table 1: The Chemical Composition of Natural Moisturizing Factors (20)

Chemical	Percentage composition
Free amino acids	40%
Pyrrolidone carboxylic acid	12%
Lactate	12%
Sugars, organic acids, peptides, unidentified materials	8.5%
Urea	7%
Chloride	6%
Sodium	5%
Potassium	4%
Ammonia, uric acid, glucosamine, creatine	1.5%
Calcium	1.5%
Magnesium	1.5%
Phosphate	0.5%
Citrate, formate	0.5%

DESQUAMATION

Desquamation is the shedding of corneocytes. This is a controlled process, in which the factors responsible for stratum corneum cohesion are broken down. Diseases in which desquamation is impaired, result in thickened skin, for example, X-linked ichthyosis and psoriasis. In normal health, different topographical areas of the skin have different thicknesses, and rates of turnover: the stratum corneum over the heel is significantly thicker than elsewhere on the body, representing local functional adaptation. Thus, a complex and little understood homeostatic process exists between stratum corneum renewal and desquamation allowing local control of skin thickness. Desquamation must involve the destruction of intercellular molecular bridges, the desmosomes, leading to the detachment of individual corneocytes. Although this appears to be a primarily protein metabolic process, there is evidence to suggest that changes in intercellular lipid composition in the outer stratum corneum may also create an environment which promotes desquamation.

Proteases in the stratum corneum are thought to play a role in desquamation, although their substrates remain to be confirmed. Exogenous factors can also influence the rate of desquamation. For example, exposure to ultra-violet light can reduce desquamation resulting in thickened skin (which is also partly explained by UV induced epidermal hyperplasia), whilst exposure to moisturizers and humidity may increase the rate of desquamation.

Chapter 4

THE SKIN AS A METABOLIC BARRIER

Cutaneous Metabolism of Topically Applied Substances

Adapted from

Bashir, S.J., Maibach H.I. (1999) Cutaneous Metabolism of Xenobiotics. In,

Percutaneous Absorption 3rd Ed. Marcel Dekker, New York. pp 65 - 80

INTRODUCTION

Metabolic activity within the skin can lead to the degradation of compounds which are topically applied, leading to a "metabolic barrier." Understanding the skin's metabolic behaviour provides insight into the more advanced methods by which the skin deals with compounds that have penetrated the physical barrier. Practically, this is particularly important in the development of topical drug delivery systems. It is not sufficient that a drug or pro-drug be able to penetrate the stratum corneum, but the compound must be able to survive the enzymatic processes that take place within the epidermis in order that a useful amount actually reach the systemic circulation via dermal blood vessels. In the future, sophisticated gene therapies that may target genodermatoses will also need to survive this metabolism. It is clear, therefore, that the application of substances to

human skin is widespread. Although many preparations are placed on the skin on the assumption that the skin is biologically inert, this chapter demonstrates that many exogenous compounds are metabolized in skin (xenobiotic metabolism). This chapter reviews the cutaneous enzymes that are capable of metabolizing cutaneous xenobiotics, and some of the factors regulating their activity. Recent work documenting the metabolism of commonly prescribed drugs and the metabolism of environmental agents on the skin is reviewed. Further, the role of cutaneous xenobiotic metabolism in the production of toxic metabolites, irritants, and allergens is discussed, in addition to the implication of cutaneous metabolism for transdermal drug delivery in healthy and damaged skin (

Figure 2).

XENOBIOTIC METABOLISING ENZYMES

These are enzymes participating in the metabolism of foreign compounds. They metabolize substrates that are predominantly lipophilic (and thus penetrate the skin well) into substances that are hydrophilic and less active and can then be excreted in the urine via the kidney.

There are two distinct metabolic steps in this process. The first step is known as the phase I reaction and introduces a polar reactive group into a molecule, which renders the molecule suitable for further metabolism as part of the phase II reaction.

Phase I reactions include metabolism by cytochrome P-450-dependent monooxygenases, which have been demonstrated in skin (21). These enzymes add a single oxygen atom from a molecule of 0_2 to a carbon atom, resulting in the formation of an -OH group on the substrate (hydroxylation) and one molecule of water, H_2O.

Subsequently, these metabolites formed by the phase I reaction undergo further metabolism, known as phase II reactions. These are conjugation reactions, which render the substrate more hydrophilic, allowing renal excretion. Metabolites can be conjugated with substances such as glucuronic acid, sulfur, or glutathione, resulting in the production of easily excretable products.

PHASE I METABOLISM: CYTOCHROME P-450 MONOOXYGENASES

The cytochrome P-450 monooxygenase enzymes are microsomal enzymes demonstrated in the liver and other organs including skin (22). They play an important role in the phase I metabolism of both exogenous and endogenous compounds such as fatty acids, prostaglandins, leukotrienes, and steroid hormones, and it is has been suggested that many dermatological topical drugs are suitable substrates for this enzyme (23).

Cytochrome P-450 enzymes are cofactor-dependent enzymes: they require energy from an external source such as NADPH to catalyze the reaction. This is in

contrast to cofactor-independent reactions, which require only the enzyme to catalyze the reaction.

Cytochrome P-450 exists in both prokaryotes and eukaryotes. In eukaryotes, the enzyme is mainly located in the membranes of the endoplasmic reticulum and the mitochondria. The structure of cytochrome P-450 is a protoporphyrin ring that contains a centrally placed Fe3+ and a polypeptide chain of approximately 45,000 to 55,000 kD (24)

The substrate to be metabolized binds to the protein moiety of the cytochrome P-450, inducing a conformational change. This triggers the necessary cofactor NADPH -P-450 reductase, which donates an electron to the cytochrome P-450; the Fe3+ is reduced to Fe2+. The reduced cytochrome P-450-substrate complex may now bind to a molecule of oxygen. Another electron is donated from NADPH-P-450 reductase; the oxygen molecule is split into two oxygen atoms, with one binding to the substrate, which is then released from the enzyme as a hydroxylated product. The second oxygen atom is released as water (

Figure 3.) (24).

Evidence for the existence of cutaneous cytochrome P-450 was initially obtained from the study of the carcinogenic effects of polycyclic aromatic hydrocarbons on the skin. The carcinogenic consequences of cutaneous metabolism are discussed later. Other more recent studies have demonstrated that cytochrome P-450 metabolizes topically applied medications in a fashion similar to the metabolism of systemic medications by the liver.

For example, recent work using the model drug theophylline has demonstrated the relevance of cytochrome P-450 in the context of therapeutic drugs. Theophylline, which, in the liver is metabolized by monooxygenases to the metabolites 1, 3-dimethyluric acid, 3-methylxanthine, and 1-methyluric acid, was studied using a percutaneous penetration/metabolism model (25). This in vitro flow-through system demonstrated the same theophylline metabolites as would be found in liver metabolism, demonstrating that cutaneous cytochrome P-450-dependent enzymes in the skin had metabolized the xenobiotic.

Many isoenzymes of cytochrome P-450 exist (Table 2), and there are many genes that encode for them. No particular isoenzyme has unique substrate specificity; rather, there is an overlap of substrates. The isoenzymes are categorized by their amino acid similarities into families, named with the root CYP followed by the family number, a capital letter denoting the sub-family, and a number identifying the particular form.

The family CYP1 has been implicated in xenobiotic metabolism and the families CYP2 and CYP3 in the metabolism of both xenobiotics and steroids. The CYP1A1 is a well-studied member of the cytochrome P-450 family, and is expressed in the skin (26). The CYPs, including CYP1A1, are normally expressed at a low level in the skin; however, their activity can be induced by a variety of agents, discussed later in this chapter.

PHASE II METABOLISM

Much of the literature on cutaneous metabolism of xenobiotics focuses on phase I reactions, especially on the role of cytochrome P-450 enzymes. However, phase I reactions are only part of the metabolic process. Following the phase I reaction, the metabolite must be conjugated to facilitate its elimination.

Transferases

Transferase activities in the skin can be as high as 10% of that of liver. In comparison, the relative activity of cytochrome P-450 in skin may be only 1-5% of the liver's (27). For example, glutathione-S-transferase, an important metabolic enzyme, is present in skin (28). One group has shown that the cutaneous metabolism of nitroglycerin (GTN) to 1,2- GDN (glyceryl di-nitrate) and 1,3-GDN is heavily dependent on the presence of glutathione, which is a cofactor for the transferase enzyme (29) They exposed GTN to skin homogenates with and without the glutathione cofactor to determine its role in cutaneous metabolism. In the tissue with the cofactor, 30% of the GTN was metabolized within 2 h, whereas only 5% of the GTN was metabolized in the tissue without glutathione.

Glycine conjugation is another mechanism of metabolism in the skin, which occurs in both human and rat keratinocytes (30). The metabolic pathway involves the activation of the carboxylic acid group with coenzyme A (CoA) in an ATP-dependent reaction. This is followed by the reaction of the S-CoA derivative with the glycine molecule catalyzed by a mitochondrial acetyltransferase. The

resulting glycine conjugation renders the metabolite more polar than the parent compound, and it can then be excreted renally (Figure 4). Another drug that is the substrate of transferases is benzoic acid, used topically for tinea infections. When it is administered systemically, it is excreted as hippuric acid in urine. Using cultured human and rat keratinocytes, the ability of these cells to metabolize benzoic acid into hippuric acid has been demonstrated (30), albeit to a smaller extent than hepatocytes.

The foregoing studies demonstrate that transferase activity may play a significant role in the metabolism of topically applied compounds.

EXAMPLES OF XENOBIOTIC METABOLISM

This section outlines the metabolism of common or important substances which come into contact with skin (see Table 3 for a summary).

Corticosteroids

Topical corticosteroids are extensively prescribed for dermatological conditions. The metabolism of betamethasone 17-valerate (B-17) in the living skin equivalent (LSE) model has been studied (31). The betamethasone 17-valerate was initially isomerised to betamethasone 21-valerate (B-21), before it was hydrolyzed to the more polar betamethasone. The rate of conversion of B-17 to B-21 was the same

with or without skin homogenate, suggesting that the initial isomerization step was not enzyme dependent but possibly a passive chemical degradation.

Taking this study further, the rates of metabolism of the two isomers B-17 and B-21 were compared (32). When the B-17 isomer was applied to the LSE, half of the drug was left unchanged. In contrast, when B-21 was applied to the LSE, almost all of the drug was metabolized. Thus, the esterases that are responsible for this second, enzyme-dependent step demonstrate preference for the B-21 isomer.

In the human setting, it has been subsequently demonstrated that that B-17 isomer is metabolized to form the B-21 isomer and betamethasone in both human skin in vivo and the LSE model (33). This work showed that the B-17 isomer accumulated in the human skin. This was possibly because the B-21 isomer was metabolized faster than the B-17 isomer, which would be consistent with isomeric preference shown in vitro.

These studies have therefore shown that corticosteroids are metabolized in human skin. Further, this metabolism may involve a passive step of chemical degradation as well as active enzyme-dependent metabolism. Importantly, the isomeric structure of the topical agent may influence the rate of metabolism within the skin. Therefore, different isomers of the same compound may be more or less suitable than one another for topical application. This must be considered in the study of any agent to which the skin is exposed.

Beta-Adrenoceptor Antagonists

Propanolol is a widely prescribed, highly lipophilic beta-adrenoceptor antagonist. As it is lipophilic, having a partition coefficient of 5.39 at pH 7.0, the topical route of administration may theoretically achieve a steady drug release and plasma concentration. Oral propanolol is subject to first-pass metabolism, leading to variable absorption and low systemic bioavailability.

In the study of the percutaneous absorption and metabolism of propanolol in vitro, using intact human skin and microsomal preparations, it was found that between 10.4% and 36.6% of the drug was absorbed, but only 4.1% to 16.1% of the drug penetrated the skin (34). Some propanolol was retained in the skin, and metabolites of propanolol were found. Naphthoxyacetic acid, 4-hydroxypropanolol, and N-desisopropyl propanolol were formed by intact human skin. The concentration of these metabolites was lower compared with hepatic metabolism, suggesting less enzymatic activity in the skin compared to the liver. These metabolites were also formed by the skin microsomes, albeit in a greater concentration than in intact skin, perhaps because of the greater surface area that the microsomes (everted endoplasmic reticulum) have to react with drugs. The microsomal preparations biotransformed propanolol to norpropanolol, which was not noted in intact skin.

Taking this further, studies have been performed using human, LSE, and keratinocyte models (33). Propanolol was shown to accumulate in human skin, which may be responsible for its irritant or toxic effects. Its accumulation may be explained by the differences between the metabolism of the drug in skin and liver,

although it could not be attributed to the degree of enzyme activity, as the enzyme saturation points in the metabolism of propanolol in liver and skin were similarly high. However, the difference in metabolism may lie in the stereoisomeric structure. Using racemic propanolol, they demonstrated that the S-enantiomer was eliminated more efficiently by the skin than the R-enantiomer. This is in contrast to hepatocytes, which are more efficient at removing the R-enantiomer (35) Therefore, the irritation caused by the topical application of propanolol may be the result of accumulation of the R-enantiomer (36).

These studies therefore suggest that propanolol is metabolized by human skin, and that its metabolism may be stereoselective. This metabolism and the retention of propanolol in the skin may explain both the low plasma concentration and irritant dermatitis after topical application.

Topical Nitrates

The metabolism of nitroglycerin has been studied using intact skin and homogenates from hairless mice (29). In the homogenate study, GTN was incubated with homogenized tissue. After 2 h of incubation, 30% of the GTN had been metabolized to the breakdown products 1, 2- and 1, 3-GDN. This metabolism was shown to be heavily dependent on the presence of glutathione (see earlier). Using the intact skin model, the investigators compared the extent of metabolism using different formulations of the GTN: a 1-mg/ml aqueous solution, a 2% ointment, and a transdermal delivery system. The percentage of metabolites

formed was greatest with the aqueous solution (61 %), followed by the patch (49%), and least of all with the ointment (35%). This difference is thought to be the explained by the greater transdermal flux with the patch and ointment compared to the solution: The smaller the flux, the greater the relative level of skin metabolism.

Theophylline

Theophylline is a xanthine derivative that is used as a bronchodilator. This drug has a narrow therapeutic index at which optimal bronchodilation is maintained with minimal adverse effects occurring. Considering this, topical administration may give theoretical advantage over the oral route, as the latter results in variable plasma concentrations and is subject to altered absorption with the presence or absence of food in the gastrointestinal tract. Using both human skin samples and its microsomes, it has been demonstrated that theophylline was metabolized to produce 1,3-dimethyl uric acid, 3-methyl uric acid, and 3-methylxanthine in the skin samples (25). These metabolites of theophylline are produced via cytochrome P-450-dependent metabolism in the liver, and the authors proposed that a similar mechanism may occur in skin (Figure 5).

METABOLISM OF ENVIRONMENTAL XENOBIOTICS

An important consideration in this subject is the metabolism by the skin of compounds it is exposed to in the environment. The skin barrier is constantly exposed to environmental compounds, which may be both natural and manmade. This section addresses the effects of their metabolism.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are produced by the incomplete combustion of fossil fuels and other organic matter. Their potential role in human carcinogenesis is suggested by their presence in the environment and the carcinogenicity of their metabolites.

Cutaneous metabolism of PAH is capable of forming carcinogenic metabolites (reviewed elsewhere(37)). Studies with model compounds such as benzo[a]pyrene have demonstrated that cutaneous metabolism of PAHs can lead to the formation of phenols, quinones, dihydrodiols, and the reactive diol epoxides. The diol epoxides are thought responsible for the carcinogenic effect, binding covalently to macromolecules. Covalent binding with DNA correlates well with the tumorigenicity of the metabolites of benz[a]pyrene (38).

PAHs are present in crude coal tar, which is extensively used in dermatological practice, particularly in the treatment of psoriasis and some dermatitis. The exposure of crude coal tar to the human hair follicle results in the induction of

aromatic hydrocarbon hydroxylase, which is a cytochrome P-450-dependent enzyme (27). This results in benz[a]pyrene derivatives which bind to DNA.

These studies of PAHs therefore exemplify the potentially hazardous nature of the cutaneous metabolism of environmental xenobiotics. Another group of compounds with potential to cause harm are pesticides.

Pesticides

Skin is the most important route of exposure to such agents and topical exposure could result in systemic absorption. Whether the metabolic activity of the skin barrier serves to toxify or detoxify topical compounds remains to be determined, but skin metabolism of pesticides does occur.

Previously this laboratory has investigated the cutaneous metabolism of an environmental pesticide, 2-chloro-2,6-diethyl-N-(butoxymethyl) acetanilide (butachlor), on human skin in vitro (39). In this study, the butachlor was metabolized to 4-hydroxybutachlor and was NADPH dependent, implying that the metabolism may be dependent on monooxygenases in the skin. The 4-hydroxybutachlor metabolite was noted to accumulate in skin. Cysteine- and glutathione-conjugated metabolites were also found. The formation of glutathione conjugates is consistent with the known presence of glutathione in human skin (28). Although the significance of these metabolites is not yet known, their formation and accumulation in the skin may be potentially hazardous.

The cutaneous metabolism of another widely used herbicide, atrazine, also was investigated in this laboratory (40). The metabolites 2-chloro-4-ethyl- amino-6-amino-s- triazine (desisopropylatrazine) and 2-chloro-4,6-diamino-s-triazine were found in the receptor fluid and the skin supernates. An additional metabolite (2-chloro-4-amino-6-isopropylamino-s-triazine) was found in the skin supernates. This study again showed that metabolites of an environmental agent can be produced in the skin, further reinforcing the need for the detailed study of skin metabolism as a possible source of pathology (Table 2).

FACTORS AFFECTING CUTANEOUS METABOLISM

The factors that influence the metabolism of cutaneous xenobiotics can be dynamic or static. Dynamic metabolism may vary according to the physiological and pathological condition of the skin. In contrast, static factors may be related to the structure of the skin at a particular site.

Dynamic Factors

The dynamic response of enzymes to inductive and inhibitory stimuli could be an important factor in determining the extent of metabolism within the skin. Also, in the case of isoenzymes, such as the cytochrome P-450 family, which particular isoenzymes are induced and in what proportions must also be considered.

Enzyme Induction

The induction of enzymes that metabolize xenobiotics may increase the rate and/or amount of metabolites produced. Some xenobiotics may induce enzymes for which they themselves are substrates, or may induce enzymes that act on other exogenous or endogenous substrates.

For example, a 10-fold increase in the activity of the enzyme aryl hydrocarbon hydroxylase can be seen in skin homogenates from rats pre- treated with 3-methychloranthene (41). Aryl hydrocarbon hydroxylase is a cytochrome P-450-dependent enzyme associated with the expression of CYP1A1 (23). Further studies have shown that topically applied polycyclic hydrocarbons, coal tar, and petroleum derivatives are also effective in the induction of aryl hydrocarbon hydroxylase in human skin (37).

Another study of enzyme induction looked at the effect of topically applied dexamethasone on the induction of cutaneous cytochrome P-450 isoenzymes in murine skin (42). The induction of cytochromes 1A1, 2B1, 2E, and 3A was seen, in addition to induction of the monoxygenase enzymes catalyzed by these CYPs. The group further employed immunohistochemistry to localize the expression of the CYP2B1 isoenzyme within the epidermis. This particular isoenzyme was investigated as it was involved in the greatest enzyme induction. The isoenzyme was localized to the suprabasal layer of the epidermis and the cells of the hair follicle.

That dexamethasone can induce several isoenzymes of cytochrome P- 450 is a significant finding because the cytochrome P-450 monooxygenases are not substrate specific. Therefore, if one substrate induces a series of enzymes, other xenobiotics that are applied to the skin, either intentionally or unintentionally, may be metabolized at an increased rate. For example, if one came in contact with benzo[a]pyrene while using topical corticosteroids for atopic dermatitis, the metabolism of carcinogenic metabolites could be increased.

Enzyme Inhibition

In contrast to induction, the inhibition of enzymes must also be considered. Inhibition of the cutaneous metabolism of xenobiotics has several theoretical advantages. For example, selectively inhibiting an enzyme may increase the overall percutaneous absorption of a particular medication. The imidazole antifungal agents, widely prescribed in dermatological practice, are potent inhibitors of the microsomal P-450-dependent monooxygenases. In skin, they inhibit the activity of aryl hydrocarbon hydroxylase and epoxide hydrolase activity (37). Also, imidazoles induce glutathione-s-transferase activity and inhibit the cutaneous metabolism, macromolecular binding, and carcinogenicity of topically applied benzo[a]pyrene in cultured mouse keratinocytes (43). Plant phenols also inhibit the monooxygenase metabolism of benzo[a]pyrene in vitro (44). These studies suggest that inhibitors of xenometabolizing enzymes may be useful in the prevention of polycyclic hydrocarbon skin malignancies.

BARRIER DISRUPTION AND CUTANEOUS XENOBIOTIC METABOLISM

Several studies have attempted to study the metabolism of xenobiotics following disruption of the skin barrier. One study used both heating and tape stripping methods to damage barrier function (45) whilst investigating the effect of skin condition *in vitro* on the cutaneous metabolism of nitroglycerin. Full- thickness excised skin from hairless mice was placed in a plastic bag prior to immersion in boiling water for 10 min. Heating the skin disrupted its barrier function, a fact that can be inferred from the increased total nitrate flux across the heated skin compared to controls. The skin did continue to metabolize the GTN; however, compared to control skin, the heated skin showed a preference for the formation of 1,3-GDN rather than 1,2-GTN. The heated tissue continued to metabolize the nitroglycerin at a steady rate during the 10-h experiment, whereas the control specimen's metabolism decreased with time. This suggests that the altered metabolism may be the result of non-enzymatic metabolism of the drug. In the same study, the authors also damaged the skin barrier using adhesive tape stripping. The greater the number of sequential tape strips, the more damaged the skin barrier, with greater flux of nitrates and less metabolic activity.

However, another study demonstrated that freezing human skin did not alter its metabolic capacity (46). Investigating the metabolism of 8-methoxypsoralen (8-MOP) on human skin in vitro, it was demonstrated that the skin barrier had been perturbed, as there was a greater flux of 8-MOP in the frozen specimen compared to the control. However, the metabolic capacity of the skin remained constant.

Different insults to the human skin barrier may alter the metabolism of xenobiotics in different ways. These studies demonstrate that further investigation of skin barrier function in cutaneous metabolism is necessary. As products for topical use become increasingly popular, their use on damaged skin must be investigated.

CONSEQUENCES OF CUTANEOUS XENOBIOTIC METABOLISM

For any drug metabolized in the skin, the potentially toxic nature of any metabolite must be considered. For example, a metabolite may be irritant, allergenic, or even carcinogenic, either locally or systemically. The pre-carcinogen benzo[a]pyrene was described earlier as an example of this, as was the metabolism of propanolol.

The ability of the enzymes responsible for xenobiotic metabolism to be induced or inhibited may affect the rate and extent of metabolism of any compound on the skin. This may affect the efficacy of drugs applied topically for either local or systemic administration. Indeed, there is potential for topical formulations to include inhibitors of enzymes to enhance drug delivery.

Metabolism of the drug at the cutaneous level constitutes "first-pass metabolism," which may result in sub-therapeutic doses reaching the systemic circulation. Indeed, the metabolic activity of the enzymes may be dynamic rather than static;

this implies that the under different physiological and pathological conditions, variable doses of the drug may be delivered through the skin, perhaps resulting in toxicity or decreased effectiveness. Particular regard must therefore be paid to drugs of narrow therapeutic index. In conclusion, this chapter has demonstrated that cutaneous metabolism is relevant in the application of any topical agent to the skin and that the skin has a metabolic barrier to topical substances.

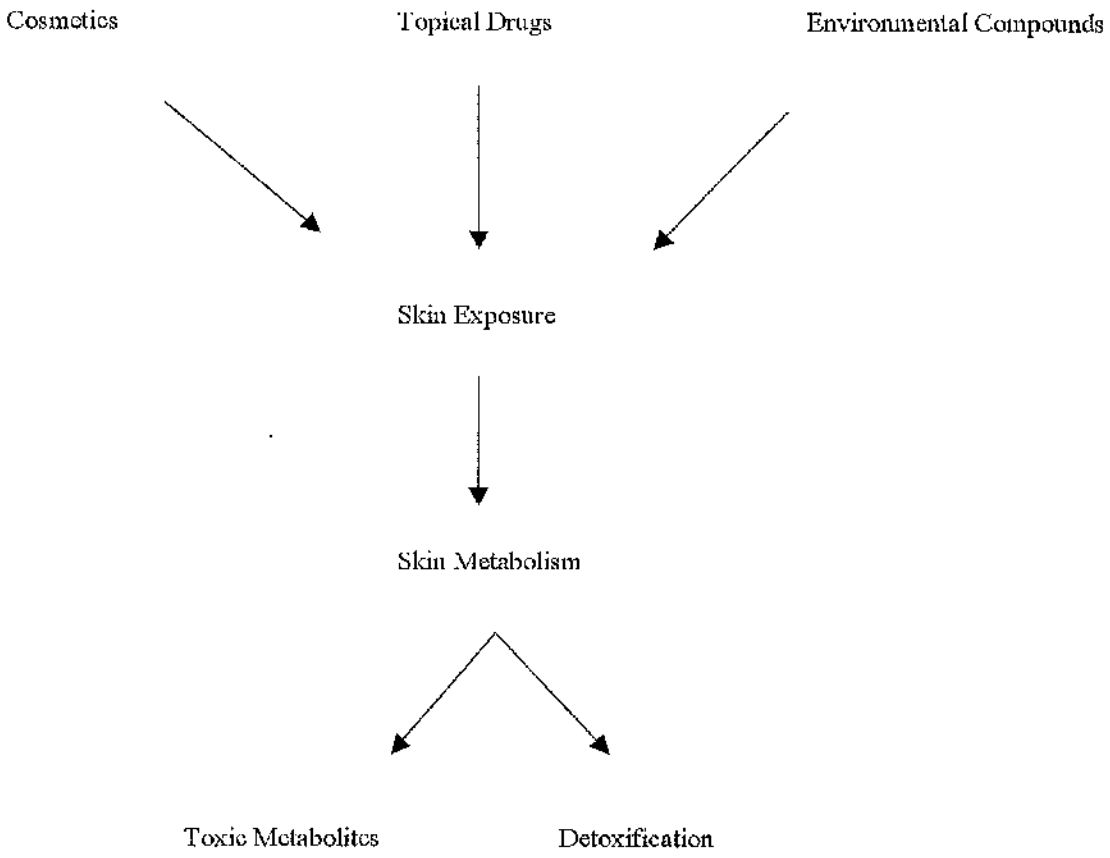
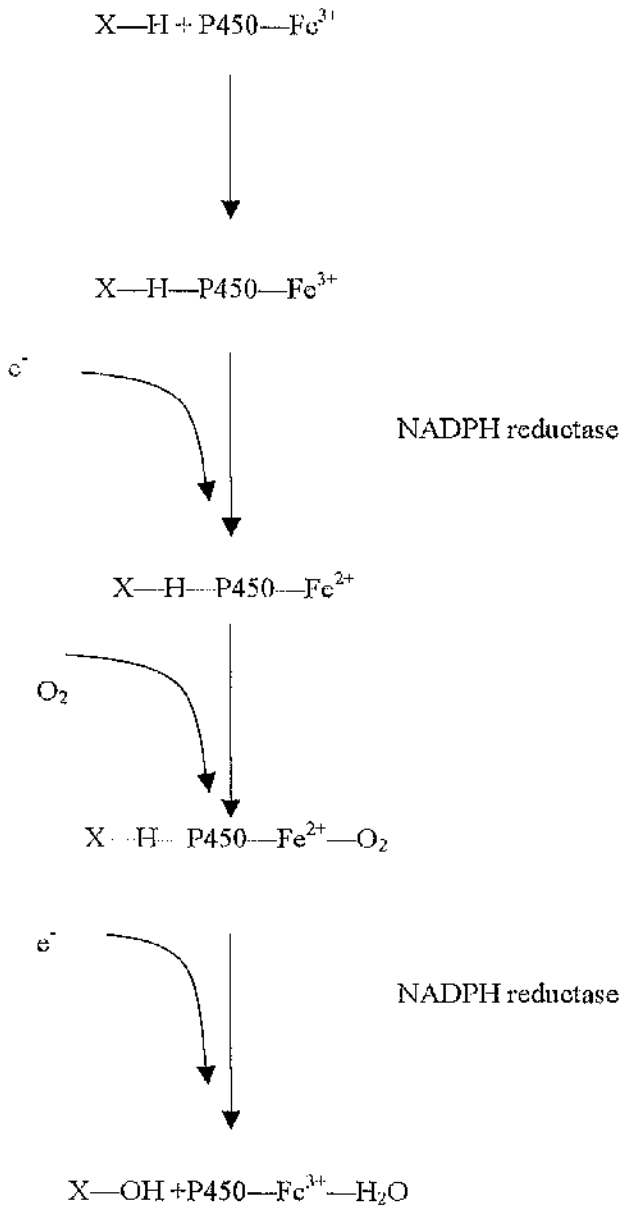


Figure 2: Metabolism of Xenobiotics



Key:
X = substrate
P450 = cytochrome P-450 enzyme

Figure 3: Mechanism of action of cytochrome P- 450

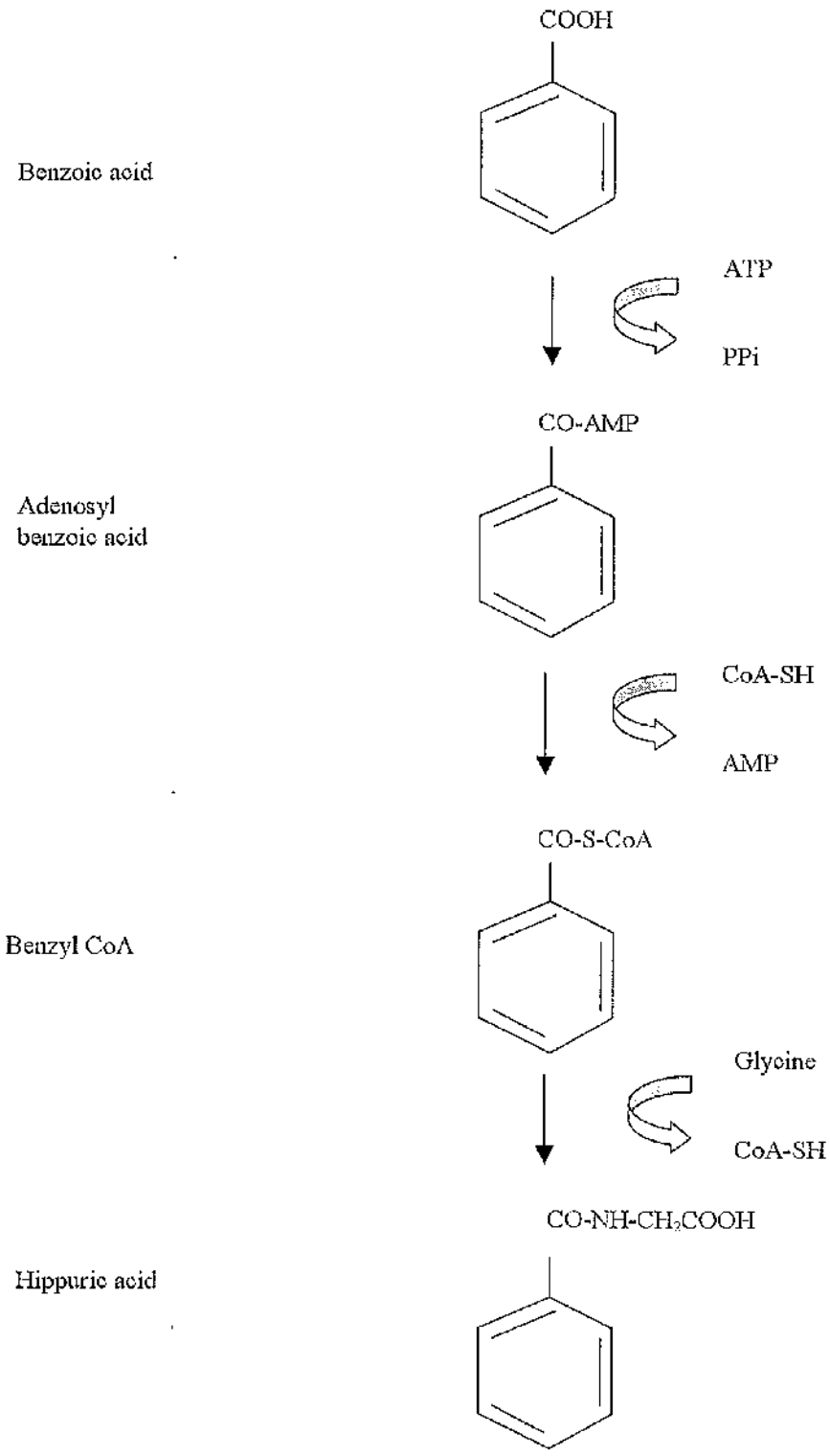


Figure 4: Metabolism of benzoic acid: an example of a conjugation reaction

Figure 5: Pathways of theophylline metabolism

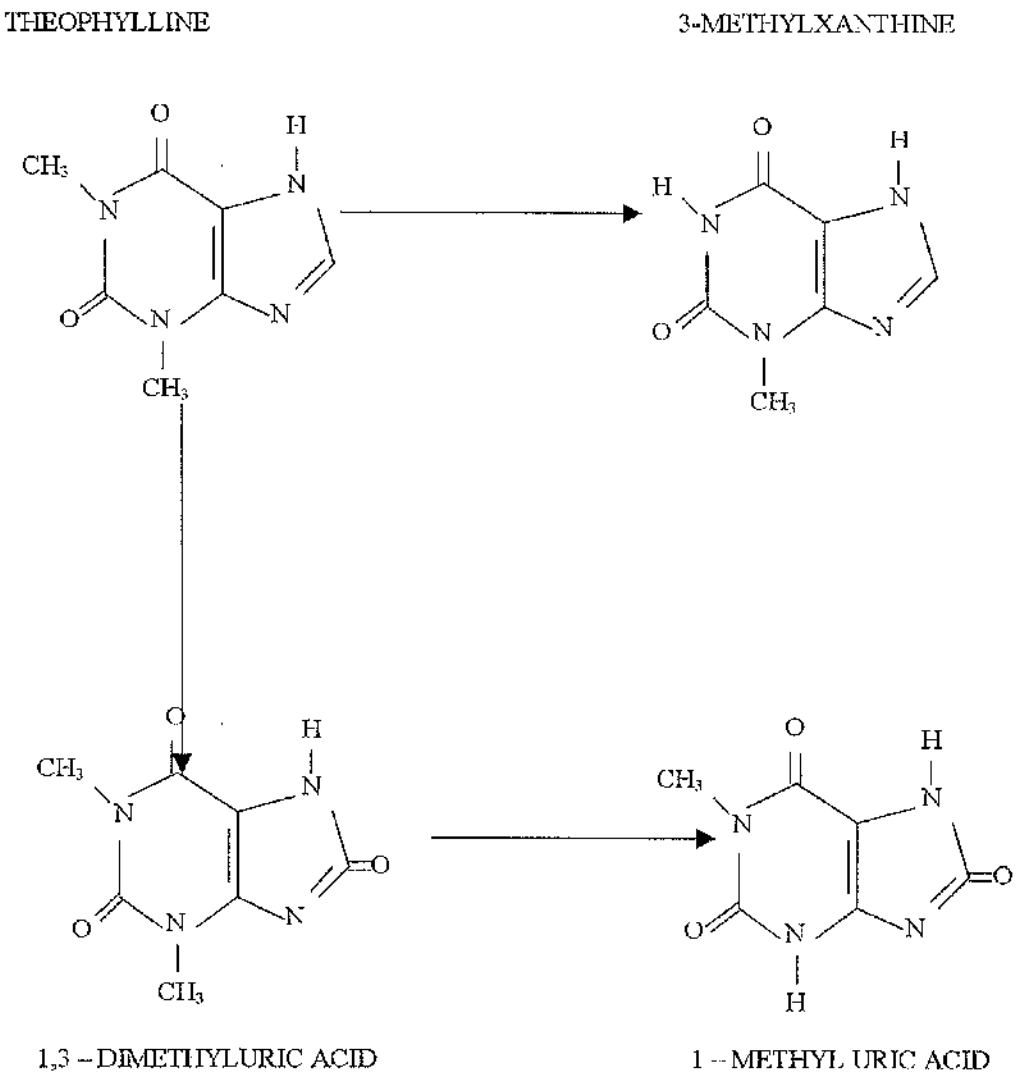


Table 2: Cytochrome P-450 Isomers Determined in Mammals and their Functions

Isomer	Function
CYP 1	Metabolism of xenobiotics
CYP 2	Metabolism of xenobiotics and steroids
CYP 3	Metabolism of xenobiotics and steroids
CYP 4	Fatty acid ω and ω-1 hydroxylation
CYP 5	Thromboxane synthetase
CYP 7	Cholesterol 7 α-hydroxylase
CYP 11	Steroid 11 β-hydroxylase
CYP 17	Steroid 17 β-hydroxylase
CYP 19	Aromatase
CYP 21	Steroid 21-hydroxylase
CYP 24	Vitamin D-25 hydroxylase
CYP 27	Cholesterol 27-hydroxylase

Table 3: Examples of Some Xenobiotics and their Metabolites

Compound	Major Metabolites	Comment
Betamethasone 17-valerate	Betamethasone 21-valerate	Chemical degradation
	Betamethasone	Active metabolism
Propanolol	Naphthoxyacetic acid	Produced by intact skin
	4-hydroxy propanolol	Produced by intact skin
	N-desisopropylpropanolol	Produced by intact skin
	Norpropanolol	Only produced by microsomes
Nitroglycerin	1,2-glyeryldinitrate	
	1,3-glyceryldinitrate	
Theophylline	1,3-dimethyluric acid	
	3-methyluric acid	
	3-methyl xanthene	
Polycyclic aromatic hydrocarbons	Phenols	
	Quininones	
	Dihydrodiols	
	Diol epoxides	Carcinogenic
Butachlor	4-hydroxybutachlor	
	Cysteine conjugates	
	Glutathione conjugates	
Atrazine	Desisopropylatrazine	
	2-chloro-4,6-diamina-s-atrazine	

Chapter 5

DYNAMIC QUANTIFICATION OF SKIN FUNCTION

Adapted from "Cosmetic Efficacy" S.J. Bashir, H.I. Maibach

SKIN HYDRATION AND MOISTURE

Assessment of dry skin is well established in cosmetic practice. Whilst clinical assessment of dry skin is effective, there are a variety of instrumental methods which assist the investigator. However, these methods are an indirect quantification of skin hydration: there is no single instrumental method but, rather, a range of modalities should be employed.

The choice of instruments available reflects the signs of dry skin: dryness, scaling, roughness and diminution of flexibility. Methods available include measurement of electrical properties, spectroscopic methods, evaluation of barrier integrity to water, magnetic resonance imaging , skin surface topography and scaling of the skin surface. This section reviews the use of bioengineering instruments in assessing dry skin (47) (48) (49)).

There is no data at present to confirm that dry skin is actually linked to a diminution of skin water content; rather, the positive pharmacologic effects of moisturizing to relieve dry skin have been repeatedly demonstrated (48). The water content of the skin is influenced by several parameters (see Table 4).

Electrical Methods for Determining Skin Water Content

These measurements are based on the principle that water is a major contributor to the electrical properties of the skin. Therefore, the total impedance of the skin (Z), resistance (R) and capacitance (C) are related in a model, which depicts the skin as a resistor and capacitor in parallel (50).

Dry stratum corneum is typically a weak medium of electrical conduction, whose electrical properties change greatly upon hydration. The electrical properties of the skin are not solely mediated by water, but also by proteins, ions, glycerine and other chemicals (48). Devices commonly utilized to measure the hydration of the skin based on its electrical properties are listed in Table 5 and are described below.

a) Corneometer ® CM 825

This device measures capacitance of the skin in arbitrary units. The measuring probe consists of an interdigital grid of gold covered electrodes, covered by a low dielectric vitrified material that is 20μm thick. Therefore, there is no direct galvanic contact between the electrodes and the skin. Within the probe head is a spring

mechanism that ensures the application of a constant pressure (1.6 N/m^2) when the probe is placed on the skin. The electrical field present in the upper epidermis is a function of the dielectric material covering the electrodes and the capacitance of the skin in contact with the electrode. The total capacitance is changed by changes in the dielectric constant of the skin surface. Data output is expressed in arbitrary capacitance units (ACU) that read from approximately 30-40 in dry skin to 120 in very hydrated skin.

b) Nova Dermal Phase Meter, DPM 9003

This device measures the impedance-based capacitance properties of the skin (capacitance reactance). Unlike the Corneometer ® detailed above, there is actual galvanic electrical contact between the skin and the two concentric brass ring electrodes. This device also applies less pressure to the skin during measurement ($0.6N/m^2$). Data is expressed as arbitrary capacitance-reactance units ranging from 90-999, which are related to the hydration of the horny layer.

c) Skin Surface Hygrometer, Skicon-200

The measuring principle of this device is skin conductance (reciprocal of resistance) in μSiemens (μS). Its measurement of skin hydration reflects the superficial part of the stratum corneum, with a penetration depth of less than 20μm.

The above and other electrical based hydration methods are reviewed in detail elsewhere (48, 49).

Infrared Spectroscopy

The premise of this approach is that the infrared absorbance of water yields a characteristic spectrum that can be uniquely identified. Fourier-transformed infrared spectroscopy can be utilized to assess the efficacy of a test preparation. Post application, increase in absorption at specific wavelengths may indicate increased water content in the stratum corneum. However, there are several drawbacks: interference by other absorbing compounds in the stratum corneum is possible; absorption bands of keratin, the major stratum corneum protein, change upon hydration; and the instrument measures only the outer layers of the stratum corneum (48, 49). Whilst it is an exciting principle, it is not yet suited for widespread efficacy testing.

Transepidermal Water Loss

The rate of evaporation of water through the skin into the atmosphere can be measured utilizing a variety of instruments including the Tewameter ® (Courage & Khazaka, Cologne) and the Evaporimeter ® (ServoMed, Sweden).

The relationship between this flux of water through the skin and water content in the skin is complex. Normal values of transepidermal water loss (TEWL) have been demonstrated despite a wide range of hydration values determined by electrical methods. Also, increased TEWL may be seen where the hydration remains normal.

TEWL measurements reflect the skin's function as a barrier to water movement through it. For moisturizers which attenuate water movement by covering the skin with an occlusive layer of oils, emulsions, fats and lipogels, a decrease in TEWL may be seen. This decrease may be accompanied by a slow increase in skin hydration (51) (52). Therefore, TEWL may be utilized to document the short-term occlusive effects of moisturizing agents.

A Clinical Trial Example

De Paepe et al(53) investigated the efficiency of hydrating body lotions and protective creams, utilizing transepidermal water loss and skin hydration as markers. Firstly, they measured the efficacy of five commercially available hydrating lotions (oil/water emulsions) in a blind fashion. The lotions were applied to the inner forearm and the transepidermal water loss was measured with a Tewameter ® (Courage & Khazaka, Cologne, Germany) pre-application and 1, 2 and 3 hours post application. Skin hydration was measured with a Corneometer CM820. This was followed by a longer application phase where the subjects applied the lotions twice daily for fourteen days, with TEWL and capacitance measurements performed on 12 hours after the final application. Utilizing this method, statistical differences were seen in the ability of each test product to reduce TEWL and increase capacitance. Certain products were statistically more efficacious than others in both the short term and long term studies, decreasing TEWL by as much as 30% and increasing hydration by the same degree.

SKIN COLOR MEASUREMENT

Objective, reproducible measurement of skin color is of considerable value. Human visual perception of skin is dependent on light scattered from it that reaches our retina, for central nervous system processing. This sophisticated process is dependent on several factors: the wavelengths of light incident on the skin, the angles of reflectance, the degree of light absorption and the individual's perception of what he or she sees. Therefore, objective measurement of skin color does not replicate human perception; rather, it provides a standard reference for quantification.

The constituent elements that determine skin color can be classified as chromophores or scatterers (54). (See Table 6). Chromophores absorb light whereas scatterers are structures which have a different index of refraction from the medium in which they are embedded (e.g. cell membrane or stratum corneum-air interface). The concentration and distribution of these two components varies throughout the skin leading to different optical properties at different sites. A detailed review of the physical events responsible for skin color is provided elsewhere (54).

In order to resemble daylight, skin surface colorimeters utilize heterogeneous wavelengths that lie within the visual spectrum. Tristimulus colorimeters emit the wavelengths 700, 564.1 and 435.8 nm, which correspond to red (R), green (G) and

blue (B)(55). This RGB color measurement system is one of many: for skin color measurements, the $L^*a^*b^*$ system (Cielab) is commonly utilized. Tristimulus values can readily be converted to Cielab values, which are more useful in the determination of color differences. The Cielab values plot skin color three dimensionally as coordinate values, which can be utilized to determine a difference in color (ΔE) according to the equation:

Equation 1

$$\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

In dermatologic evaluation of skin color, investigators may consider the overall color or focus on the parameter of interest. For example, in the investigation of skin irritation, the a^* value may be of prime interest, as it parallels erythema scores (56).

Applications of Skin Color Measurement

Whilst there are obvious advantages to the measurement of skin color, from the treatment of diseases such as vitiligo to the potential benefits of skin color measurements in cosmetology, there is little published work outside the area of irritant dermatitis.

The use of erythema as measure of skin irritation is established in contact dermatitis research models. Standard clinical scales use faint redness of the skin as an early sign of irritation that becomes progressively stronger as the irritation worsens. The use of color analysis in this setting is becoming more established (56), with the red values taken to correspond to erythema scores. As reflectance meters are able to detect redness that cannot be seen by the investigator, their sensitivity is useful to detect skin irritation which is not visible to the naked eye. This "subclinical irritation" is the focus of increasing research because of the potential to detect irritants at a much lower threshold than can be done in present standard clinical irritation tests. How this data will be interpreted with respect to population exposure and product safety is not yet clear, and these bioengineering methods have not yet been embraced by regulatory bodies. Nevertheless, the detection of erythema by skin bioengineering remains a powerful research tool. Outwith skin irritation, one example that highlights the importance of objective skin color measurement comes from a Japanese plastic surgeon who utilized this method to chose skin for grafting (57). In this study of 40 subjects, the skin color of the cheek was compared to the pectoral area, abdomen, scapula, back, upper arm, lateral upper arm, volar forearm, anterior and lateral thigh, peroneal area and dorsum of the foot. The aim was to find which of these skin sites corresponded in color most closely to the cheek, to select skin for grafting. Utilizing the Minolta CR-300 Chromameter (Osaka, Japan) as their tristimulus light source and detector, this group obtained ΔE and L* a* b* values for each of the skin sites. They

concluded that cheek was most similar in color to the dorsum of the foot and the flexor forearm utilizing the E* value. The reliability of skin colorimetry has been well determined and methodologic studies have determined strategies to minimize variation (58) (59). This technology can be readily utilized in many aspects of dermatological science and medicine.

CONCLUSION

Cutaneous bioengineering is an advancing field. The correct understanding of the limitations and uses of devices that measure skin function is essential if correct conclusions are to be drawn from the data provided. In this thesis, we have chosen three instruments: a tristimulus chromameter; a transepidermal water loss meter (evaporimeter) and a glass electrode pH meter. These devices have been selected because their previous use allows comparison to previously published work, they provided data that is reproducible and their probe sizes correspond well to the tape strips and test sites.

Table 4: Factors Affecting Skin Water Content

Factor	Comment
Water diffusion from viable epidermis	This water is normally retained by the stratum corneum barrier (e.g. lipids)
Fixed water in the horny layer	Water in this form is bound to the natural moisturizing factors (NMF) in the stratum corneum (e.g. urea, pyrrolidone carboxylic acid, urea, amino acids, lactic acid)
Equilibrium between horny layer water content and ambient air	In a simple physical equilibrium, the movement of water from the skin to the atmosphere (or vice versa) depends on a concentration gradient, that depends on the value of external humidity.
Moisturizing agents	These may hydrate the skin by forming an occlusive layer of oils and fats on the skin surface and/or by fixing water in the stratum corneum with humectants.

Table 5: Bioengineering Measurements of Skin Hydration

Device	*Measurement*
Corneometer® CM 825	Measurement of electrical capacitance in arbitrary units
(Courage & Khazaka, Cologne)	Constant application pressure of 1.6N/m^2
	Measures entire stratum corneum and upper viable epidermis. (depth 20-40μm)
Nova Dermal Phase Meter ®	Measures capacitance-reactance in arbitrary units
	Constant application pressure of 0.6 N/m^2
	Correlates well with Corneometer® and Hygrometer®
Skin Surface Hygrometer, Skicon-200	Measures skin conductance
	Data is from superficial stratum corneum
Infrared Spectroscopy	Measures water content based on specific absorption bands
	Superficial indication of stratum corneum water content
	Currently a research tool
Transepidermal water loss	Measures barrier function to water
	Does not always correlate with electrical hydration measurements
	May be useful to measure short term occlusive effects of moisturizers

Table 6: Chromophores and Scatterers in the Skin

Skin layer	**Chromophore**	**Scatterer**
Stratum corneum	Melanin	Melanin
Viable Epidermis	Melanin	Melanin
		Cellular structures
Dermis	Melanin	Collagen
	Hemoglobin	Melanin in macrophages
	Bilirubin	Erythrocytes
	others	others

Chapter 6

STRATUM CORNEUM TAPE STRIPPNG

INTRODUCTION

As the skin is readily accessible, compared to other human organs, it is an ideal organ for research. However, sampling the skin can result in scarring, which is an undesirable adverse effect that can deter potential research volunteers. As a result non-invasive methods of studying and sampling the skin are sought.

Classically, sampling the skin involved taking a biopsy, such as a punch biopsy or a shave biopsy, or raising a bleb with a vacuum to form a suction blister. These methods are painful, may involve contact with bodily fluids that carry infection and may need suturing or wound care. As an alternative, methods to sample the stratum corneum without inflicting such damage to the skin have been developed.

Early in these studies, slides with "superglue" cyano-acrylates were applied to the skin and peeled off, to give a sample of epidermal skin. However, this procedure was difficult to control and the sampling was not reproducible. A more controlled method of removing the outer layers of the skin is to apply an adhesive tape and gently peel it off (60).

When the tape is applied to the skin and then removed, it takes with it a fine layer of stratum corneum. This process is a minute direct physical disruption of the skin barrier, which leaves the human skin sufficiently intact for this to be a suitable *in vivo* method. The main advantage of the technique is that it can serve two purposes simultaneously. Both the skin removed by the adhesive tape and the remaining skin on the research subject are amenable to study and measurement. Therefore, with one intervention, the barrier has been disrupted and the skin has been sampled.

Sequential tape stripping over the same site allows several layers of the stratum corneum to be sampled. With this method, depth profiles can be created within the stratum corneum allowing more detailed study. For example, in the case of a topical drug, the concentration profile with respect to depth in the stratum corneum may give an idea of its penetration kinetics into the skin and even into the systemic circulation.

Unfortunately, the tape stripping method, employed by many laboratories and pharmaceutical firms, has not been standardized. This perhaps reflects the varied uses to which tape stripping has been applied. Many of these applications seem to have generated mini-protocols of their own.

For example, tape stripping has been used a method to study wound healing. The premise is that a superficial epidermal wound can be created by sequentially tape stripping over the same site. Once the wound has been created, healing can be studied e.g. by measuring transepidermal water loss or later taking biopsies to

look for mRNA expression. In some of these studies, the skin has been sequentially tape stripped until the skin glistens, a point which represents the depth of the dermis. Therefore, no regard was given to the manner in which the tapes were stripped, whether pressure was applied, whether the tapes are handled with forceps etc. Other investigators performing the same type of barrier studies sequentially tape stripped the skin until a transepidermal water loss measurement of 30 $gm^{-2}h^{-1}$ was reached (61), which was thought to indicate significant barrier disruption.

Investigators studying other parameters, such as the presence of a pesticide in the upper layers of the stratum corneum (the "reservoir" effect) may strip only five or ten times over a site to gain enough tissue for analysis.

As a result of these and other varied practices, the method of tape stripping has been not been standardised, although the value of standardisation is increasingly recognized.

PRACTICAL APPLICATIONS OF TAPE STRIPPING AND THEIR DRAWBACKS

A common application of stratum corneum tape stripping is to quantify percutaneous penetration of a compound. In these studies, a topical preparation is placed on the skin, perhaps under occlusion, for a short period and then removed. The test area is then sequentially tape stripped: each tape is rapidly weighed to

assess the mass of stratum corneum on the tape and then the tape can be analyzed for the presence and concentration of the topical drug, for example by infra-red spectroscopy (62) or high performance liquid chromatography after solvent extraction. The data generated is the mass of skin stripped and concentration of the test material, allowing the penetration kinetics of the drug into the skin to be calculated.

Whilst this method is in common practice, there are several pitfalls. Firstly, the tapes must be weighed immediately after tape stripping because water evaporates from the stratum corneum sampled. Therefore, any delay in weighing leads to a lighter tape. Weighing tapes is a manually difficult and laborious procedure, which requires much practice.

Secondly, there is considerable potential error in weighing tapes. In addition to the evaporation problem, many balances are not sensitive enough to distinguish between the mass on different tapes. As the readings are very small (micrograms), vibration error is also pronounced. This can be caused by other laboratory equipment or even floor vibration from footsteps. To reduce vibration, the balances are housed in quiet areas, and placed on a marble slab. This limitation means that portability is difficult and studies must be conducted in or near a specially created environment.

Thirdly, in this model of drug delivery, not only the stratum corneum and the tape is being weighed, but also the drug and its vehicle. The rate at which these evaporate also influences the results obtained. Ideally, a more reproducible

method of measuring the amount of stratum corneum on each tape would liberate investigators from the drawbacks of weighing tapes and allow a more accurate profile of percutaneous penetration.

In order to overcome these and other standardization errors, this laboratory has been researching possible improvements to the model. In order to create a more reproducible method, a standard operating procedure has been developed which is employed throughout this thesis (detailed later). In brief, the handling of the tapes, the degree of pressure applied to the skin, the method of stripping from the skin and storage of the tapes has been made uniform. This thesis also combines other non-invasive methods of analyzing the skin in combination with the tape stripping, to allow the skin barrier to be assessed in other ways.

In order to overcome the dilemma of weighing the tapes, a method of quantifying the protein content on the tapes as a function of stratum corneum mass was developed in this laboratory (63). The purpose of this thesis is to develop the model further and assess its usefulness in common tape stripping applications.

Chapter 7

AIMS OF THE THESIS

Using the Tape Stripping Model to Investigate Dynamic Barrier Function

DEVELOPMENT OF THE MODEL

Previous studies of the skin barrier, percutaneous drug delivery, skin physiology and skin microbiology have employed the adhesive tape stripping method. Progress in our laboratory has enabled the precise quantification of the mass of skin removed on each tape using spectrophotometry (63). This information allows the tape stripper to create a map of the functional changes in skin, following layer-by-layer disruption of the skin barrier.

This thesis represents the ongoing work of the laboratory in the development of the new method of tape stripping. The purpose of this thesis is to further develop our understanding of this model, and test its applicability to dynamic physiological and pharmacological study of the stratum corneum barrier function.

Study 1: Physical and Physiological Effects of Tape Stripping

This is a detailed study of the experimental model, with the aims of standardizing the technique whilst obtaining physiological data on normal human volunteers.

Several different tapes have previously been used for tape stripping and the three most common are investigated. This will allow other investigators to have a reference for a variety of tapes and their properties when used for tape stripping. Also, it may influence the choice of tape for future tape stripping studies.

To explore new physiological areas, more tape strips will be performed than have previously been undertaken, allowing us to gather data from deeper in the epidermis. As skin function can be assessed in a variety of modalities, non-invasive bioengineering will be used to assess transepidermal water loss to study the skin barrier function. The data generated on water loss will allow the calculation of water kinetics in the skin, updating our understanding of the diffusion of water through the skin barrier with more accurate quantification of the mass of stratum corneum stripped. The data will be of value both to dermatopharmacologists studying drug penetration and to skin researchers studying normal skin function.

Study 2: Cutaneous Bioassay of Salicylic Acid as a Keratolytic

This study aims to apply the model in a more pharmacological setting. Recent issues in dermatology have surrounded the use of keratolytics. These have been traditionally used to treated genetic keratodermas, acquired keratoderma, eczema, psoriasis, acne and warts. More recently, keratolytic drugs have been used in cosmetic dermatology for rejuvenation techniques (chemical peels). However, the efficacy of these drugs has been difficult to quantify. The aim of this study is to

investigate the efficacy of one keratolytic, salicylic acid, in relation to pH of its formulation, which is another "hot topic." In addition to gaining efficacy data, the study aims to validate the model in a practical setting, and to obtain further physiological data.

Study 3: pH Profiles in the Stratum Corneum: Localization of the Acid Mantle

The results of the previous study have highlighted the importance of increasing our understanding of stratum corneum pH profiles. The pH of the stratum corneum is important to maintenance of the barrier itself, and influences the physico-chemical properties of compounds placed on the skin such as drugs, cosmetics and environmental chemicals. As the previous study demonstrated, the pH of topically applied preparations does not necessarily influence efficacy as predicted by the chemistry alone. Therefore, the aim of this study is to explore the pH environment of the stratum corneum using the protein assay method to "map" pH profiles with respect to depth, which has never previously been done in this precise way.

Chapter 8

STUDY 1

PHYSICAL AND PHYSIOLOGICAL EFFECTS OF STRATUM CORNEUM TAPE STRIPPING

Published

Bashir, S.J., Chew, A-L, Anigbogu, A.N.C., Dreher, F, Maibach, H.I. Physical and Physiological Effects of Stratum Corneum Tape Stripping. *Skin Res Technol.* 2001 Feb;7(1):40-8

ABSTRACT

Background: Tape stripping of the human stratum corneum has been performed to measure stratum corneum mass, barrier function, drug reservoir and percutaneous penetration. However, the technique itself requires further development to facilitate interpretation. **Methods:** In this study we quantified stratum corneum (SC) tape stripping and water kinetic parameters utilizing three types of adhesive tapes, in an *in vivo* randomized clinical trial. Stratum corneum was tape stripped and the mass of SC removed by each tape was quantified utilizing a protein assay. Transepidermal water loss (TEWL) was measured and barrier disruption and SC water kinetics calculated. Three commonly utilized acrylate adhesive tapes were utilized and a comparison made between them. **Results:** Each type of tape successfully stripped the stratum corneum, but the rayon tape did not induce SC barrier disruption. Neither the type of tape nor the site stripped significantly influenced the mass of SC removed. Water kinetic parameters did not differ significantly for the tapes that did induce barrier disruption. Individual variation in barrier disruption to water following tape stripping was demonstrated. **Conclusion:** The tapes utilized removed a similar amount of SC. The tapes have a different propensity to cause barrier disruption. Some individuals do not demonstrate increased TEWL despite an equivalent mass of SC being removed compared to those who do show a response.

INTRODUCTION

Adhesive tape stripping (ATS) studies of human and animal stratum corneum, have been used to calculate percutaneous absorption profiles (62) (64) (65) and to disrupt the stratum corneum (SC) barrier (66). This laboratory (63) improved the method by quantifying the amount of stratum corneum removed by each strip utilizing a colorimetric protein assay. This study utilizes this method to determine the amount of SC removed at two skin sites, utilizing tapes with different physico-chemical properties. Further, we relate physiological responses following ATS to the thickness of SC removed.

MATERIALS AND METHODS

Subject Recruitment & Preparation

Six Caucasian subjects, four male and two female, median age 34 years (range 30-37) were recruited. Subjects were instructed not to apply any topical drugs or cosmetics and to avoid deliberate sun exposure to the test sites. Each subject's skin was gently clipped of hair if necessary at the dorsal and volar forearm test sites. 15 minutes were allowed for acclimatization prior to baseline TEWL and chromametric measurements.

Subjects who were currently, or within 14 days, involved in other trials were excluded, as were pregnant females and subjects receiving any oral medications. Informed consent was obtained for all subjects, and ethical approval was provided by the Committee on Human Research, University of California, San Francisco.

Tape Preparation & Acrylate System

Three commercial adhesive tapes were utilized in this study: D-squame® (CuDerm, Dallas, Texas), Transpore ® (3M, St. Paul, MN, batch no. 2002-12 AP), and Micropore ® (3M St Paul, MN, batch no. 2001-08 AN.).

A description of the adhesive system on the 3M tapes was provided by the manufacturer. Data was not available for the CuDerm manufactured tape. The polyethylene copolymer tape (Transpore ®) consisted of : iso-octyl acrylate and methyl acrylic acid polymer; colophony resin (which was a source of residual protein on the tapes); ethylene vinyl acetate (EVA) polyethylene copolymer (tape). The rayon tape (Micropore ®) consisted of non-woven rayon, with an iso-octyl acrylate and acrylic acid copolymer. This tape did not contain resin, so the source of protein detected on this tape (see results section) is unexplained.

The proprietary tapes (D-squame ®) were supplied pre-cut into disc shapes, whilst the others were supplied on a standard roll of tape. Therefore, prior to commencement of the study, the polyethylene (Transpore ®) and rayon (Micropore ®) tapes were cut to size using a custom made tape cutter. This was

performed under clean conditions and the discs were mounted for storage on the glossy side of paper sheets that had previously held adhesive Finn Chambers ® (Epitest, Helsinki).

Test Sites

Six test sites were chosen; three on the proximal volar forearm and three on the proximal dorsal forearm. The test sites on each aspect were as closely opposed as possible to reduce variation within the dorsal and volar test areas. Each tape was randomly assigned to both a dorsal and volar site in each subject to avoid the confounding effect of any variation between sites on each aspect of the forearm. The sites were outlined precisely with a marker pen to ensure that the tapes were consistently applied over the same site.

Tape Stripping

Each site was tape stripped 40 times with one type of the adhesive tapes, using a fresh tape for each strip. The tape was applied to the test site with forceps and pressed onto the skin with a standardized 10kPa pressure for 2 seconds. The pressure was then removed and the tape was peeled from the skin unidirectionally. Tapes were then placed, adhesive side upwards, into borosilicate glass scintillation vials and refrigerated for subsequent analysis. Tapes were handled with gloved hands (non-powdered latex) at all times to prevent proteinaceous contamination.

Transepidermal Water Loss Measurements

Transepidermal water loss (TEWL) was measured at baseline (immediately prior to the onset of tape stripping at each site) and following every 10 strips at each site. TEWL was delayed for 1.5 minutes after each of the 10 strips to allow the SC water diffusion to equilibrate. Room temperature and humidity were recorded whilst TEWL measurements were made (22-24°C, relative humidity 55-64%). Measurements were made with an evaporimeter (Tewameter ®, Courage & Khazaka, Cologne, Germany). The TEWL was read in the plateau phase, between 20 seconds and 1 minute of recording. Measurements with a standard error of the mean less than 0.1 or as close as possible to this were taken to be the TEWL value.

Protein Colorimetric Assay

Protein assay was performed according to the method of Dreher et al (63). Refrigerated scintillation vials containing the tapes were left for one hour to reach room temperature prior to assay. 1ml of 1 M NaOH was added to each vial, which was then placed in a mechanical shaker for two hours, to allow the protein on each tape to disperse. 1ml of 1M HCl was then added to the vials to neutralize the basic solution. Colorimetric protein assay was then performed utilizing the Bio-Rad DC Protein Assay ® (Bio-Rad Laboratories, Hercules, CA) which is a modification of the Lowry assay (67) (68). Absorbance at wavelength (λ) 750 nm was performed

utilizing the Hitachi U-2001 Spectrophotometer (Hitachi Instruments Inc, San Jose, CA, USA).

Standard Dispersion Curve

Epidermal membranes were prepared by the heat dispersion method (69). Excess subcutaneous fat was removed from the skin, which was then immersed in water at 60°C for 45s. The epidermis was gently teased off the underlying dermis taking care not to unduly stretch the membranes.

The epidermis was floated overnight on an aqueous solution of trypsin 0.0001% w/v at 20 +/- 1°C. The digested epidermal cells were removed by swabbing. The sheets of stratum corneum were rinsed in water and left to dry. The membranes were then rinsed in cold acetone for 10s to remove contaminants, such as sebaceous lipids and subcutaneous fat, which adhere to the surface of the membranes during preparation. The clean stratum corneum was stored in an aqueous evacuated desiccator until required (70).

A fixed mass of this SC was weighed and dispersed in a 1 M NaOH solution, utilizing sonication and stirring. From this stock solution, 10 serial dilutions were made. 1ml of each dilution was assayed in an identical manner to the adhesive tapes, to yield a standard curve of known protein concentrations against UV absorption. Regression analysis was performed to determine the equation of the best-fit straight line. This line did not pass through the origin, as a 1 M NaOH

solution with no SC still had some color value after assay. The equation yielded was:

$$Absorbance = 0.019\,[SC]\ (\mu g/2ml) + 0.076$$

Equation 2

Therefore, the amount of SC in each sample was given by the equation:

$$SC\ (\mu g) = [(Absorbance - 0.076) / 0.019] \times 2$$

Equation 3

The absorbency values obtained by spectrophotometry were converted into protein mass using Equation 3 (R^2 for Equation 2 = 0.99).

Control Measurements

Unused samples of each type of tape were randomly selected from the batch utilized for tape stripping. They were handled and analyzed in exactly the same manner as the tapes that had been utilized. In total, six tapes of each type were analyzed for protein content, and the mean value taken. This mean value was subtracted from the mass of protein found on the stripping samples.

Determination of Water Kinetics in the Stratum Corneum

Utilizing Fick's First Law of Diffusion, the insensible loss of water across the SC can be described mathematically:

$$Js \propto (C_1 - C_2) / H$$

Equation 4

Where: Js is the steady state flux across the membrane (TEWL); $C_1 - C_2$ is the difference in concentration of water across the membrane, also written as ΔC; H is the thickness of the membrane (SC)

Equation 4 can be rewritten as:

$$TEWL \propto \Delta C / H$$

OR,

$$TEWL = D \cdot \Delta C / H$$

Equation 5

Where D is the average membrane diffusion coefficient (cm^2/s). However, as the SC is not an inert membrane, but rather has an affinity for water, a partition coefficient (***Km***) must also be added to the equation:

$$TEWL = Km \cdot D \cdot \Delta C / H$$

Equation 6

According to this adaptation of the Fick principle, TEWL is dependent on the thickness of the SC, which is the membrane across which water loss is being measured. If the thickness of the stratum corneum (*H*) were reduced, it would be expected that TEWL would rise. Therefore, following tape stripping, the equation can be further adapted:

$$(TEWL)_x = Km \cdot D \cdot \Delta C / H - x$$

Equation 7

Where $(TEWL)_x$ corresponds to the water loss following removal of x μm of stratum corneum, in this case by adhesive tape stripping. This equation can be converted to a linear form, by inverting it.

$$(TEWL)_x^{-1} = [\, H / (Km \cdot D \cdot \Delta C) \,] - [\, x / (Km \cdot D \cdot \Delta C) \,]$$

Equation 8

Plotting this equation allows the calculation of *D* from the gradient, and *H* can be determined from the abscissa intercept. Therefore, from the combination of TEWL measurements, and quantification of the mass of stratum corneum removed by adhesive tape stripping, it is possible to determine the actual thickness of the stratum corneum being stripped, and the diffusion coefficient of

water through the SC. ΔC has been previously defined as 1g/cm^3 and Km has been previously defined as 0.162 (71).

The permeability coefficient (K_p) was also determined from the following equation:

$$Kp = D \cdot Km / H$$

Equation 9

Determination of SC thickness

The thickness of the stratum corneum removed was calculated utilizing the following equation:

$$x_i = m_i / F \cdot \rho$$

Equation 10

where x_i is the thickness, m_i is the cumulative mass of SC removed by i consecutive tape strips, F is the area of the tape strips (3.8 cm^2) and ρ is the SC density, set to $\rho = 1$ g/cm^3. (72).

Statistical Analysis

The SC mass data was analyzed with the one way ANOVA test on SigmaStat (SPSS Inc, Chicago, IL, USA). The data was expressed as cumulative sums of 10, 20, 30 or 40 sequential tape strips. The TEWL data was analyzed with the

repeated measures one way ANOVA, to take into account variation in response to the previous tape strips. If the ANOVA F value suggested significance, a pair-wise comparison was performed with the Tukey Test. $\alpha = 0.05$ for all tests.

In some cases, highlighted in the results section, data was not normally distributed. For this data, the Kruskal-Wallis One Way Analysis of Variance on Ranks was utilized.

RESULTS

Analysis of Water Kinetics following Stratum Corneum Tape Stripping

Mean water diffusion coefficients, stratum corneum thickness and permeability were calculated by tape and by site. The results are shown in Table 7, Table 8 and Table 9. No significant difference was found in the kinetic parameters between the tape types. P values are shown in the tables.

Measured Mass & Thickness Stripped

Using the colorimetric method, it was possible to measure the amount of protein on each tape and convert this into a mass, using the standard dispersion curve. The mass of stratum corneum removed by each of the tapes is shown in detail (Table 10). As the mass stripped by one tape may depend on the mass stripped by the previous tape, the data are presented as cumulative values. There was no difference in the mass removed by the tapes, although the paper tape consistently removed less SC than the other tapes. Also, there was no statistical difference in the amount of SC removed between the dorsal and volar sites, although for the first 10 strips, the P values were close to significance.

The mass of SC stripped by each tape progressively declined in an exponential manner with sequential stripping (Figure 6).

Pearson's Correlation r^2 for Tapes

The mass of SC removed by all tapes strongly correlated with each other. R^2 value was >0.98 for all tapes and sites.

TEWL Measurements

Transepidermal water loss was measured at all sites prior to tape stripping. There was no significant difference between the test sites at baseline, indicating the skin barrier was intact and comparable. On the dorsal forearm the baseline TEWL was 10.3 $gm^{-2}h^{-1}$ (±SD 2.0) for the proprietary tape; for the polyethylene tape the baseline was 8.9 $gm^{-2}h^{-1}$ (± SD 1.2) and for the rayon tape the baseline was 9.4 $gm^{-2}h^{-1}$ (=SD 1.7). Similar values were seen on the volar forearm (Table 11).

TEWL increased significantly for the proprietary and polyethylene tapes at both dorsal and volar sites, but not for the rayon tape. However, TEWL did not increase until the deeper layers of the SC had been reached (see Table 11, Figure 7 & Figure 8).

DISCUSSION

Tape Stripping

Tape stripping is a method with great potential, which requires refinement. Surber *et al* (73), in their review, describe the method as standardized but we believe many factors remain to be investigated. In our detailed study, we performed forty tape strips at each site. Previous reports describe six (64), ten (65), fifteen (74), twenty (63) and thirty-five tape strips (75) with mixed reports of the depth achieved. Equally variable are the types and sizes of tapes utilized. Another presumed source of variation is the pressure applied to the strip prior to stripping, and the peeling force applied for removal. Many papers do not describe any method to control these factors, which may increase inter-subject and inter-investigator variation. We have attempted to improve standardization, quantifying the mass and thickness of stratum corneum removed by the tapes utilized. This data provides clear guidance regarding how much SC they have stripped and its physiological effect, whether in the measurement of barrier function, percutaneous absorption or the bioequivalence method.

We quantified the mass of SC removed for each tape and at each site. This comprehensive analysis has provided several insights into the tape stripping method. Firstly, with the tapes utilized, there was no statistical difference in the mass of SC removed by either tape type or stripping site. However, there was a

trend between the tapes, in which the proprietary tape stripped the most, and the rayon tape stripped the least.

Tape stripping was performed on the dorsal and volar forearm of each subject, with three types of tape. There was no significant difference in the amount of SC removed by any of the tapes utilized, although there was a difference in the degree of barrier disruption to water. There was no significant difference in the amount of SC removed between the dorsal and the ventral site for each tape.

As the stratum corneum is progressively stripped, the amount of SC that is removed with each strip decreases (Figure 6). This is presumably because of an increased cohesiveness of the SC in the deeper layers. Considering that the stripping was performed sequentially on the same site, the mass of SC removed by each strip depends on the mass of SC removed by the previous strip. The mass of SC removed is an interaction between the adhesive force of the stripping and the cohesive force of the SC. As one strips the SC, the adhesive properties of the tape remain constant but the cohesive properties of the SC increase. Therefore, it is theoretically possible that a greater difference between the mass removed by the type of tapes may be demonstrated in the deeper layers of the SC. Although this was not readily apparent in this study, this may influence the SC mass removed by other tapes.

It is unlikely that analysis of an individual strip can accurately predict how much SC had been removed previously to that strip, or how much SC will be removed by a subsequent strip. This is because the mass of SC on any given tape is

influenced by the mass of SC on the preceding tape strips. On this basis, the SC removed by all 40 strips for each site was quantified individually. The data was analyzed as a cumulative sum of all 10, 20, 30 or 40 strips, so that this source of variation could be eliminated.

In conclusion, for the tapes and sites tested, neither the site nor the type of tape significantly influenced the mass of SC removed by the tapes.

Water Loss

The polyethylene and proprietary tapes were able to induce a statistically significant increase in the TEWL, whilst the rayon tape was not. On the dorsal forearm, 20 strips of Polyethylene (1805.9 μg SC) or 30 strips of Proprietary (2655.0 μg) were necessary to induce a significant increase in TEWL ($p<0.05$). 20 strips of Proprietary (1421.7 μg) yielded a p value suggesting that significance was being approached in this region. Notably, even 40 strips of Rayon did not yield a significantly increased TEWL despite removing a mean mass of 1894.9 μg.

Importantly, the statistical significance in water loss existed despite a lack of statistical significance in the mass/thickness of SC removed. This discrepancy requires further investigation. We can regard the response to tape stripping to be a property of the tape, a property of the subject and the combination of both (assuming environmental elements are constant). If we regard this difference to be

a property of the tape, it is possible that the adhesive system on the tapes is a source of variation in the barrier disruptive properties, beyond obvious mechanical trauma. Considering that the amounts of SC removed by adhesive tape stripping are statistically similar, this explanation is plausible and requires further follow up. It is possible that whilst the tapes have equal adhesive properties for the cellular SC, the extracellular components of the SC barrier mechanism, i.e. free fatty acids, ceramides and lipids, are not removed equally by the tapes. If the rayon tape does not bind to and remove these components in the same manner as the other two tapes, then this may explain the difference in barrier disruption.

On the other hand, although there is no statistical difference in the amount of SC removed, there is nevertheless the possibility that there may be a physical point in the SC beyond which stripping must occur in order for TEWL to rise. Plotting the TEWL against thickness removed (Figure 7 & Figure 8) demonstrates that approximately 5-7 μm was removed by both Polyethylene and Proprietary before TEWL was significantly elevated. Rayon, however, did not reach this critical depth even after 40 tape strips. (The lack of statistical significance in the SC mass stripped arises for the degree of variation within and between the groups. It is possible that a true difference in the amount of SC stripped does exist, and would be discovered if more volunteers were tested).

This finding is in accordance with previous work, where TEWL was demonstrated not to rise until deeper in the stratum corneum. Kalia *et al* (15) demonstrated that TEWL began to rise after 6-8 μm of SC had been stripped in their study of three

subjects. Although their study did not employ statistical methods or the novel SC colorimetric assay, their results are in accordance with the detailed data presented here. Additionally, the Scotch no. 845 Book Tape was utilized (3M, St. Paul, MN) by that group. In our study, we utilized three types of tapes, a more accurate quantification method and statistical analysis. The interim conclusion must be that Proprietary and Polyethylene demonstrated a depth-related increase in TEWL. This suggests that the structural elements responsible for SC barrier to water may lie at a particular physical point in the SC rather than the homogeneously distributed barrier that is currently perceived. The rayon tape, however, did not demonstrate any increase in TEWL: whether this is depth related or adhesive related remains unclear at present.

Currently, SC tape stripping is a widely utilized method to induce SC barrier disruption for research studies. Our work in this paper highlights that the choice of tape influences the degree of barrier disruption, as does the number of tape strips. In studies where only a few tape strips are performed, it is possible that the barrier to water was not actually significantly disrupted, as indicated by TEWL changes. However, the relationship of TEWL to barrier disruption to other substances is less clear. Therefore, investigators must be aware when inducing barrier disruption, and in comparing barrier data.

Water kinetics

Previously, SC tape stripping has been utilized to quantify water kinetics in the SC (74) (75). However, the data presented above provides a deeper analysis of the subject than previously published. As with the above publications, we calculated kinetic parameters of D, H, and K_p from TEWL data and measurement of stratum corneum removed. We believe the SC colorimetric assay (63) has enhanced the use of Fickian kinetics in the stratum corneum as with accurate quantification of the SC thickness.

In the discussion of water kinetics with reference to the tapes studied, we focus mainly on the Polyethylene and Proprietary tapes, as these induced a statistically significant rise in TEWL.

The calculation and practical application of water kinetics is more complex than has been apparent from previous studies. In Pirot et al (74) and Schwindt et al (75) the assumption was made that the SC, despite having diverse structural components, was a homogeneous barrier to water diffusion. The Pirot study employed 15 adhesive tape strips and, utilizing the Fickian principles described above, calculated mean D, H, and K_p, values of 4.4×10^{9}, 12.6 μm and 2.1×10^{9} respectively. These values, based on relatively few tape strips, differ to some degree from the values presented here. For example, Table 8 indicates D for the ventral and dorsal forearms: 1.9 & 2.6 x 10^{9} for the proprietary tape and 1.2 & 1.99 x 10^{9} for the polyethylene tape respectively. These are more akin to the values presented by the Schwindt study, probably because her study had tape stripped up to 35 times, giving a TEWL of 50 g/m^2/hr. The prolonged tape

stripping gives a more encompassing view of the SC kinetic parameters. In reality, the kinetic values probably do vary throughout the SC, Pirot's paper reflecting perhaps the more superficial kinetics, and our work reflecting a more global picture of the SC. Whilst the SC is considered a homogeneous barrier to water for practical purposes, the significance of this simplification remains unclear.

Difference in calculated SC thickness between sites

Schwindt et al (75) demonstrated a difference in the SC thickness at different skin sites utilizing the Fick principle. They found that the volar forearm had a median SC thickness of 13.5 μm (utilizing Scotch Book Tape) whilst we found a median thickness of 12.6 μm with the Proprietary tape and 7.9 μm with the Polyethylene tape. On the dorsal forearm (not studied by Schwindt), H was more consistent between the two tapes (16.4 μm for both tapes). These figures are consistent with the physical thickness of the SC, and are not consistent with an excessively tortuous course of water diffusion through the SC. Calculated D values were also consistent with Schwindt et al. In vitro work on cadaveric abdominal skin, not utilizing the ATS method, has also yielded similar data for D, and H. In that case, the thickness of the SC was calculated from the dry weight of the SC and its density. Interestingly, there is reasonable agreement between their calculated thickness of dry SC, and our *in vivo* calculation.

The H value from equation (63) has been utilized previously to calculate the SC total thickness of the stratum corneum (74) (75). However, we have demonstrated

that the choice of tape utilized can alter the *H* value. Clearly, the rayon tape could not have simply been stripping thicker SC than the other two tapes, as the sites were carefully randomized. Therefore, one can assume that the rayon tape did not strip the components of the SC that are responsible for barrier maintenance in the same proportions or quantity as the Proprietary and Polyethylene. As discussed above, the amount of SC removed was statistically similar, suggesting the explanation may lie in the tape adhesive's affinity for particular SC components. Interestingly, identification of the SC components which the more disruptive tapes have a greater affinity for, may identify the components most responsible for barrier integrity to water. This, however, remains to be determined.

The *H* value can be utilized to reflect the *relative* potential for a tape to cause barrier disruption to water. Whilst we have demonstrated that, for the tapes tested, the mass and thickness of SC removed is similar, the barrier disruption to water is different. Therefore, it should be possible to create an index of relative disruption, to allow for comparison between studies utilizing this common technique. As the *H* value is supposed to be the distance across which the water must diffuse, it is probably the best marker of relative barrier disruption. A simple ratio of, for example, $H_{(\text{Rayon})} : H_{(\text{Proprietary})}$ gives a useful quantification of their relative effects on water diffusivity i.e. their *barrier equivalence*.

$$\textit{Barrier equivalence} = H \text{ (tape 1)} / H \text{ (tape 2)} \qquad (7)$$

where H is the calculated SC thickness (μm) for each of the tapes in question. For the example above (Proprietary and Rayon), the equivalence can be determined as follows:

$$\begin{aligned} \textit{Barrier equivalence} &= H_{(Rayon)} / H_{(Proprietary)} \\ &= 25.9 / 16.4 \\ &= 1.58 \end{aligned}$$

Therefore, the SC barrier integrity to water following tape stripping by the rayon tape is approximately one and a half times greater than after tape stripping by the proprietary tape, although the amount of SC removed is approximately similar. This quantification of the relative ability of adhesive tapes to disrupt the SC barrier to water is important in the comparative assessment of studies utilizing different tapes.

Permeation Pathways Across the SC

The route by which substances diffuse through the SC, and the location of barriers to such diffusion, has been a source of debate. The calculated H values for the Polyethylene and Proprietary tape presented above suggest the thickness of the SC in the sites tested to be less than 16μm. This calculation depends on the movement

of water through the SC, suggesting that the SC barrier to water is 16μm thick. This barrier distance is consistent with Schwindt *et al* (75).

Interindividual Variation

Importantly, this study highlights the variation of barrier disruption to water in tape stripped individuals. Tape stripping is increasingly used to induce barrier disruption prior to assessing permeability or irritation *in vivo* or *in vitro*. However, we have demonstrated that, for some individuals, the SC barrier to water is not disrupted by 40 adhesive tape strips, regardless of the tape utilized.

Figure 9 demonstrates the variation in TEWL response to standardized SC tape stripping between the six subjects, for one type of tape. Interestingly, if one subject's SC water barrier was not disrupted by the proprietary tape, it would also not be disrupted by the polyethylene tape, or *vice versa*. To add further weight, if the SC barrier was not disrupted at one site in an individual, it would not be disrupted at any of the other test sites in that individual. These findings suggest that the variation in SC barrier disruption to water is a function of the individual, rather than the tape, investigator or environment. It is possible that these individuals are expressing an accelerated response to ATS injury, producing inflammatory exudates which may have a physical effect on water loss.

In van der Valk's work (61) subject variation to TEWL was also found. This group, rather than performing a fixed number of tape strips and measuring TEWL, stripped the skin until the TEWL was $30g/m^2/s$. The number of tape strips

required to do this varied from the early 20s to late 30s in the seven subjects selected. However, in their early study, the water loss was not correlated with the mass of SC stripped, either by weight or assay.

This is considered to be a real finding rather than an artefact, as it was present at all sites and for all tapes for the individual concerned. Firstly, in this study, two individuals were studied on each day, with the same equipment and environment. Therefore, mechanical error is extremely unlikely. Secondly, this finding implies that the Fick principle adapted to water kinetics cannot be utilized in all individuals for mathematical modelling following ATS. It is unlikely that the assumptions of this principle in application to skin are entirely true, or can be relied upon sufficiently to allow a generalized rather than individualistic approach to SC kinetics.

Conclusions

This study has demonstrated, utilizing a standardized reproducible method, several insights into the tape stripping method of stratum corneum study. It is possible to accurately determine the SC mass stripped, and utilize this data to calculate depth and water kinetics for a variety of tapes and skin sites. Further, the SC is not a uniform barrier to TEWL, which rises significantly once the deeper layers of the SC have been reached. It has also raised questions regarding individual variation in TEWL responses to ATS, and the application of Fickian kinetics to the SC.This data is relevant to the study of topical drug kinetics and bioequivalence, stratum corneum reservoir and stratum corneum barrier function.

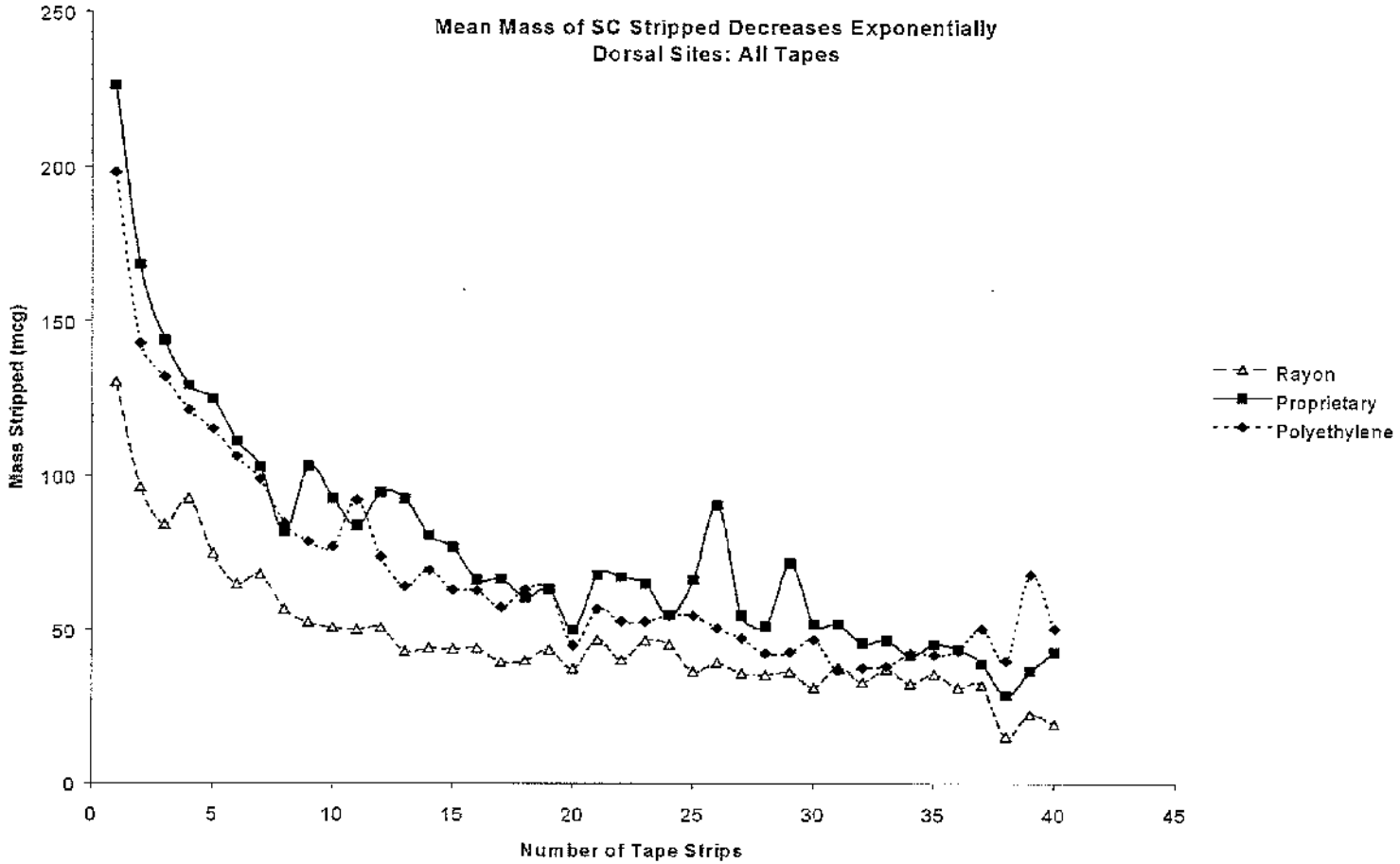


Figure 6: The mean mass of SC stripped declines in an exponential manner rather than a linear manner reflecting the more cohesive deeper stratum corneum

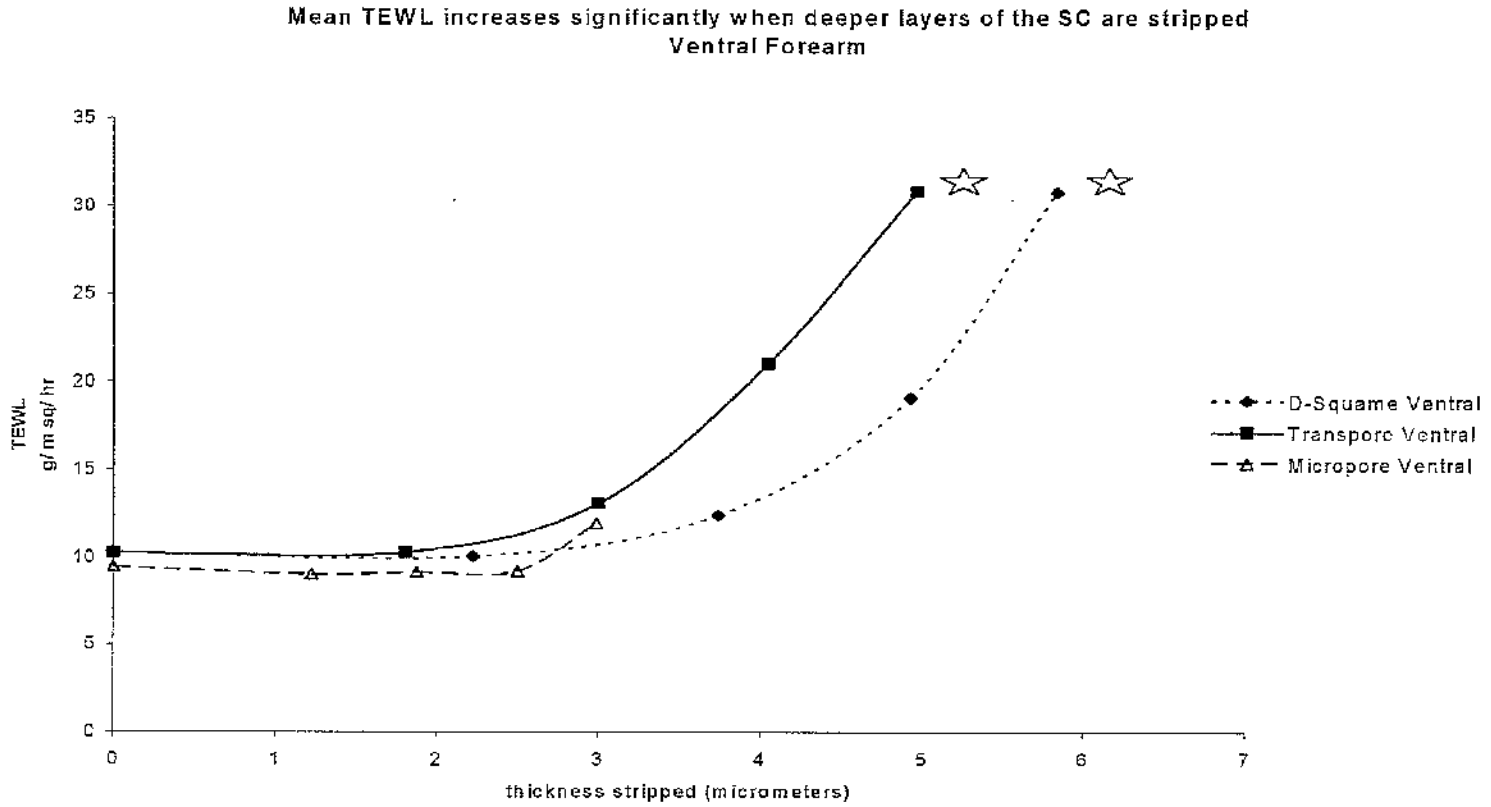


Figure 7: TEWL rises as the deeper layers of the SC are reached by tape stripping. ☆ indicates statistical significance ($P<0.05$).

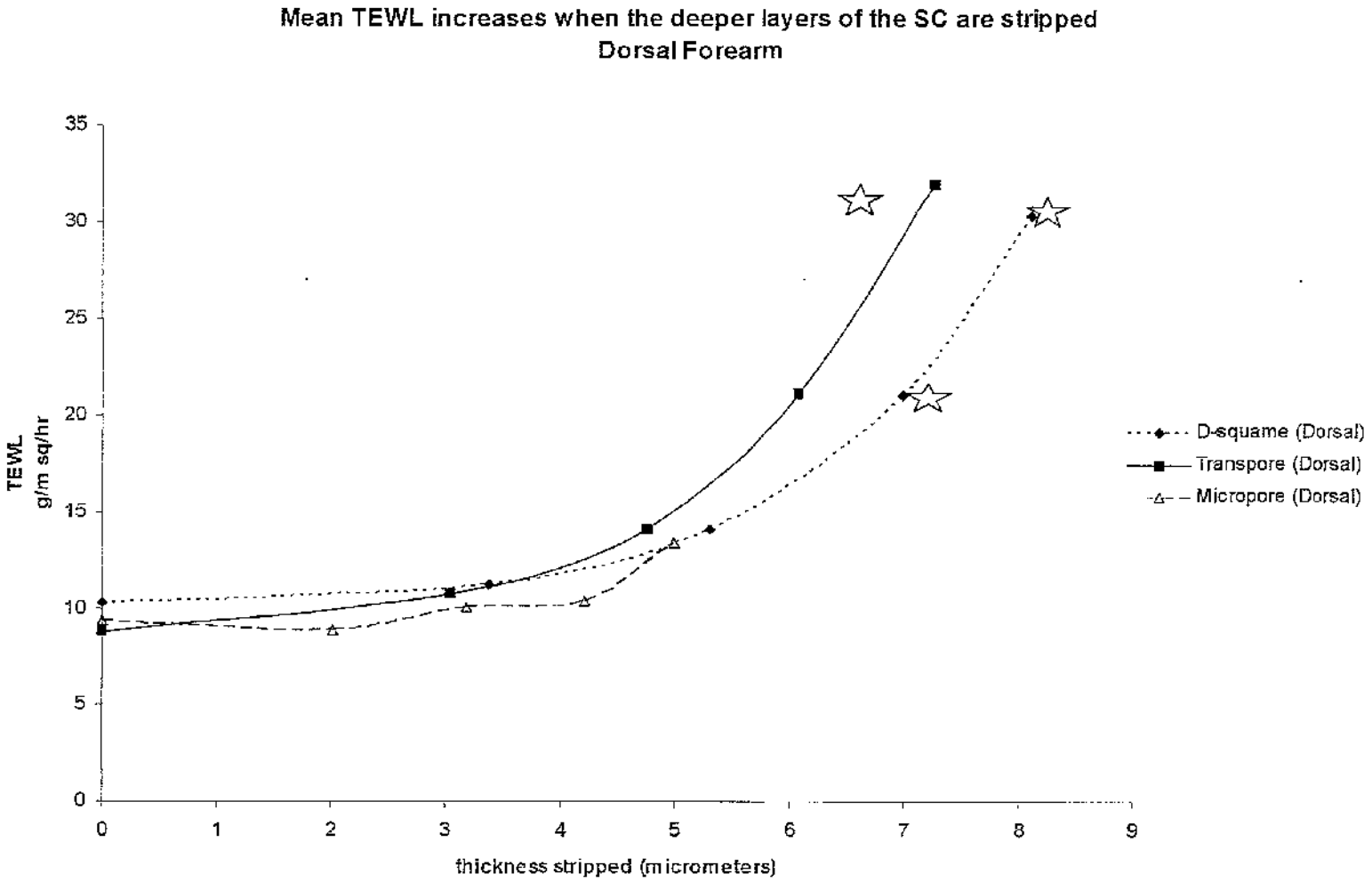


Figure 8: TEWL rises as the deeper layers of the SC are reached by tape stripping. ☆ indicates statistical significance ($P<0.05$).

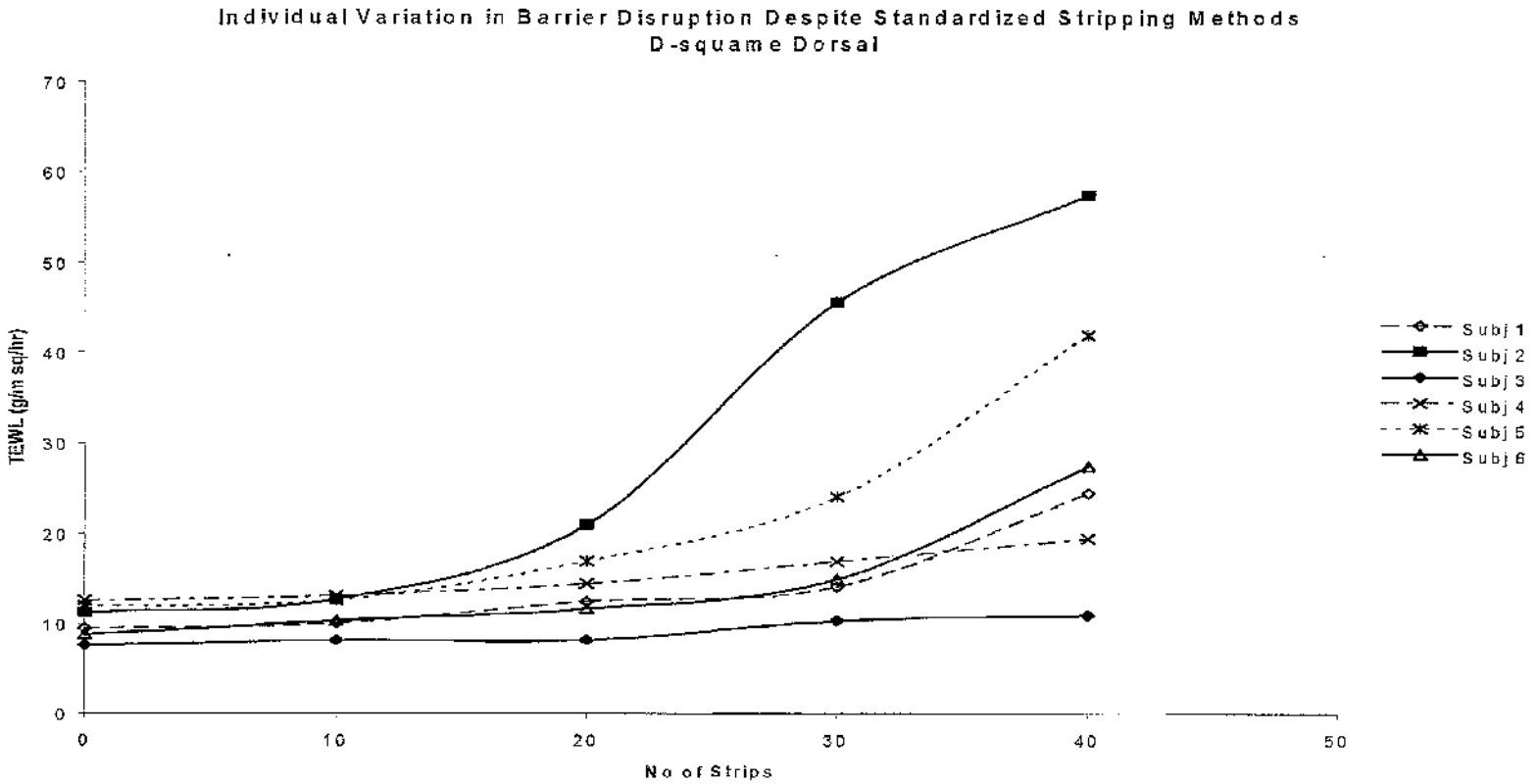


Figure 9: TEWL responses to adhesive tape stripping are variable, despite a standardized stripping technique, and similar thickness of SC being removed.

Parameter	Tape (Dorsal)	N	Mean	Std Dev	SEM
H	Proprietary	6	16.407	5.364	2.190
H	Polyethylene	6	16.435	8.479	3.461
H	Rayon	6	25.894	11.410	4.658
	Tape (Ventral)	N	Median	25%	75%
H	Proprietary	6	12.627	8.863	22.767
H	Polyethylene	6	7.460	3.211	11.864
H	Rayon	6	32.774	6.242	44.957

Table 7: Calculated SC Thickness (*H*). Dorsal: No significant difference between tapes (P = 0.131, One Way ANOVA). Ventral: No significant difference between tapes: (P = 0.169, Kruskal-Wallis One Way ANOVA on Ranks (normality test failed).

	Tape (Dorsal)	N	Mean	Std Dev	SEM
D	Proprietary	6	2.68E-09	7.05E-10	2.88E-10
D	Polyethylene	6	2.5E-09	1.09E-09	4.47E-10
D	Rayon	6	4.03E-09	1.72E-09	7.03E-10
	Tape (Ventral)	N	Median	25%	75%
D	Proprietary	6	1.99E-09	1.48E-09	4.32E-09
D	Polyethylene	6	1.2E-09	3.13E-10	2.15E-09
D	Rayon	6	5.38E-09	6.8E-10	7.85E-09

Table 8: Diffusivity of Water Following Tape Stripping *Dorsal*: No significant difference between tapes. (P = 0.101, One Way ANOVA) *Ventral*: No significant difference between tapes. (P = 0.236, Kruskal-Wallis One Way ANOVA on Ranks, as normality testing failed).

	Site	Tape	N	Mean	Std Dev	SEM
K_p	Dorsal	Proprietary	6	2.77E-011	6.22E-012	2.54E-012
K_p	Dorsal	Polyethylene	6	2.53E-011	5.55E-012	2.26E-012
K_p	Dorsal	Rayon	6	2.55E-011	4.97E-012	2.03E-012
K_p	Ventral	Proprietary	6	2.57E-011	4.95E-012	2.02E-012
K_p	Ventral	Polyethylene	6	2.57E-011	5.19E-012	2.12E-012
K_p	Ventral	Rayon	6	2.19E-011	6.47E-012	2.64E-012

Table 9: Permeability coefficient K_p (cm / s) remains unchanged between tapes Dorsal: No significant difference between the tapes. (P = 0.715, One Way ANOVA) Ventral: No significant difference between the tapes. (P = 0.461).

No of Strips	N	Tape (Dorsal)	Mean (µg)	Std Dev	SEM	Diff. Between Tapes	Tape (Ventral)	Mean (µg)	Std Dev	SEM	Diff. Between Tapes	Difference Between Sites
10	6	Proprietary	1281.6	359.9	146.9	No	Proprietary	844.5	328.4	134.1	No	P=0.05
		Polyethylene	1152.6	478.8	195.5	P=0.09	Polyethylene	684.2	302.2	123.4	P=0.08	P=0.07
		Rayon	769.5	319.0	130.3		Rayon	465.1	145.6	59.5		P=0.06
20	6	Proprietary	2015.1	573.1	234.0	No	Proprietary	1421.7	570.4	232.9	No	P=0.10
		Polyethylene	1806.0	853.3	348.3	P=0.14	Polyethylene	1140.2	534.7	218.3	P=0.07	P=0.14
		Rayon	1205.5	609.4	248.8		Rayon	712.1	309.5	126.3		P=0.11
30	6	Proprietary	2655.0	721.5	294.6	No	Proprietary	1870.8	775.9	316.8	No	P=0.10
		Polyethylene	2306.8	1122.5	458.3	P=0.18	Polyethylene	1537.7	734.6	299.9	P=0.08	P=0.19
		Rayon	1598.0	963.8	393.5		Rayon	949.1	443.5	181.1		P=0.19
40	6	Proprietary	3076.6	842.7	344.0	No	Proprietary	2216.0	952.0	388.7	No	P=0.13
		Polyethylene	2755.8	1393.5	568.9	P=0.23	Polyethylene	1885.3	864.5	352.9	P=0.23	P=0.22
		Rayon	1894.9	1218.2	497.3		Rayon	1134.4	529.9	216.3		P=0.240 ++

Table 10: Mean Mass of Stratum Corneum Removed By Adhesive Tape Stripping. There is no significant difference in the amount of SC removed by the different types of tape, or by the same tape at different sites. All tests are with ANOVA, except the comparison marked ++, which was an ANOVA for Ranks.

No. of Strips	Transepidermal Water Loss ($g/m^2/hr$)		
	Proprietary	Polyethylene	Rayon
Dorsal			
Baseline	10.3 (±2.0)	8.8 (±1.2)	9.4 (± 1.7)
10	11.2 (±2.0)	10.8 (±2.1)	8.9 (±1.0)
20	14.2 (±4.5)	*14.1 (±5.1)	10.1 (±2.0)
30	*21.0 (±12.8)	*21.1 (±16.4)	10.4 (±1.8)
40	*30.3 (±17.0)	*32.0 (±21.1)	13.4 (±5.4)
Ventral			
Baseline	10.3 (±3.0)	10.3 (±2.7)	9.5 (±1.9)
10	10.1 (±2.4)	10.3 (±2.0)	9.0 (±(1.6)
20	12.4 (±4.2)	13.1 (±3.3)	9.2 (±1.9)
30	19.1 (±11.0)	21.0 (±12.5)	9.2 (±2.3)
40	*30.8 (±21.0)	*30.8 (±19.2)	11.9 (±2.7)

Table 11: Mean TEWL ±SD readings following tape stripping for each tape and site. (n=6). * indicates TEWL is significantly different from baseline (repeated measures ANOVA).

Chapter 9

STUDY 2

CUTANEOUS BIOASSAY OF SALICYLIC ACID AS A KERATOLYTIC

A Novel Approach with Quantitative Tape Stripping

Published

Bashir, S.J., Dreher, F, Chew A.L., Zhai H., Levin C., Stern, R., Maibach, H. I. Cutaneous bioassay of salicylic acid as a keratolytic. Int. J. Pharm 2005

ABSTRACT

Keratolytic efficacy of topical preparations containing salicylic acid was studied in humans utilizing adhesive tape stripping and quantifying SC removal by protein analysis. In combination with tape stripping, squamometry was used to evaluate the influence of salicylic acid on skin surface scaliness and desquamation. Furthermore, skin barrier perturbation and skin irritancy was recorded and related to the dermatopharmacological effect of the preparations. In contrast to squamometry, tape stripping combined with protein analysis was sensitive in detecting keratolytic effect of salicylic acid within hours of application. Importantly, whereas the pH of the preparations only minimally influenced efficacy, local dermatotoxicity was significantly increased at acidic pH. This indicates that the quest to increase the amount of free, non-dissociated SA is, in fact, counterproductive as the more acidic preparations resulted in skin irritation and barrier disruption.

INTRODUCTION

Salicylic acid (SA, $pK_a \approx 3$ (76), representing a pseudo β-hydroxy acid, is widely used as a peeling or keratolytic agent to treat callus, keratosis or warts (77). SA is further applied in the treatment of acne, psoriasis and photoaging in various concentrations depending on the desired amount of keratolysis. Its keratolytic mechanism is not fully elucidated, but SA's dermatopharmacological effect may be related to its impact on the stratum corneum (SC) structure affecting intercorneocyte cohesion and desquamation. Although various methods including sequential adhesive tape stripping (78) (79) have been employed to assess the keratolytic effect of SA, they are limited or need further development to accurately quantify its dermatopharmacological activity.

SC tape stripping is a minimally invasive technique to sequentially remove SC by repeated application of appropriate adhesive tapes (60). This technique can be used to investigate SC cohesion *in vivo* by quantifying the amount of SC removed (80). Today, weighing with precision balances is the most used method to determine the amount of SC removed on a tape strip. However, weighing may be biased by water absorption or desorption onto or from the tape (81). Furthermore, after topical product application such as keratolytics, SC weighing is only reliable to some extent since the tape strips may not only contain SC but also applied vehicle and solute. As

an alternative to weighing, a simple and detergent compatible colorimetric method based on a commercially available total protein assay was described (63). Briefly, the assay is performed by immersing SC holding tapes in a sodium hydroxide solution in order to extract the soluble SC protein fraction. The SC protein containing solution is then neutralized with hydrochloric acid because the assay is not compatible with strong alkaline conditions. This quantification method makes it possible to determine accurately and reproducibly as little as a few µg SC adhering to a single tape strip. Furthermore, the uptake of most product ingredients including water into the SC after topical product application does not interfere with this SC quantification method.

The study aimed to better understand the keratolytic effect of SA as a function of its formulation pH utilizing adhesive tape stripping and quantifying SC removal by the protein assay. In combination with tape stripping assessing SC cohesion progressively from the skin surface to the bottom layers, squamometry was used to evaluate the influence of SA on skin surface scaliness and desquamation. Furthermore, skin barrier perturbation and skin irritancy was recorded and related to the dermatopharmacological effect of the salicylic acid preparations.

MATERIALS AND METHODS

Study design

The study was randomized, double blind and placebo controlled. Investigators and subjects remained blind to nature of the treatment solutions until the statistical analysis was complete. The test preparations were coded. It was not possible to be blind to the open and occlusion controls. The test sites were randomized. Additionally, the arm designated for tape stripping or squamometry was chosen at random. The study was performed in November in San Francisco (CA).

Human subjects

6 healthy adult subjects (3 male and 3 female) aged between 20 to 40 years were enrolled. Subjects receiving topical medications, utilizing topical cosmetics at the test sites or those receiving anti-inflammatory medications were excluded, as well as volunteers with a history suggestive of allergy to adhesive tape. Subjects were required to acclimatize for 30 minutes in the clinic prior to baseline bioengineering measurements. Subjects with hirsute forearms had the hair gently clipped at least 30 minutes prior to baseline readings. Written informed consent was obtained for all subjects and the study was approved by the Committee of Human Research, University of California, San Francisco.

Test preparations and treatment

The test preparations were (A) aqueous vehicle control of pH 7.4, (B) 2% SA aqueous solution of pH 3.3, (C) 2% SA aqueous solution of pH 6.95, and (D) 2% SA aqueous solution of pH 3.3. (D). All preparations contained at least 10 w% specially denatured ethanol (SD alcohol 40). They further contained 20 w% propylene glycol; preparation (D) contained in addition 1 w% menthol. An untreated skin site (E) and an untreated, but occluded site (F) served as additional controls. 0.2 ml of the test preparations were pipetted onto a 25 mm plastic chamber (Hilltop Research, Cincinnati, Ohio) immediately prior to patch application and applied to the skin on pre-marked test sites secured with Scanpor® tape (Norgesplaster, Norway). The occlusion site (F) consisted of the test chamber with no test preparation, secured in the same manner as the other sites. The untreated, unoccluded skin site (E) was left open. The volar forearms were utilized as the test site. Both forearms were treated identically but one was randomly selected for sequential tape stripping and the other for squamometry. Bioengineering measurements and visual scoring were performed on the forearm designated for tape stripping. In addition to randomizing the arm for measurements, individual test sites were also chosen at random to eliminate the possibility of the treatment effect being dependent on skin site.

Skin irritation

Skin irritation of the test sites was assessed visually according to the following scale: (0) normal skin and no erythema, (1) macular erythema, (2) erythema or edema, (3) vesiculation, and (4) ulceration or erosion. The skin was assessed prior to application of patches and one hour after their removal. In addition, skin color was measured with the Minolta Chromameter CR-300 (Minolta, Japan). The a*-value was utilized as a measure of erythema due to treatment (82). Erythema was also evaluated during tape stripping after 10 and 20 strips. As with the transepidermal water loss (TEWL) readings, the measurements were performed 1 hour after removal of occlusive patches. Each measurement was performed 3 times and the mean was recorded.

Tape stripping

A proprietary adhesive tape (D-Squame® 2.20 cm diameter, CuDerm, TX) was placed on the pre-marked test sites and tape stripping was performed as described earlier in this thesis. 20 tape strips were taken from each test site and from the control sites. Immediately after tape stripping, individual tapes were placed into a borosilicate glass vial (Fischerbrand scintillation vial, Philadelphia, PA) and refrigerated until further use.

Protein assay

The protein assay was performed on each of the tape strips according to the method described earlier in this thesis. The spectrophotometric absorbance was compared to a standard dispersion curve derived from stratum corneum sheets to give the mass of protein on each tape (as described in Study 1). The amount of SC removed by tape stripping was calculated as follows: µg SC ml-1 1M NaOH = (OD750nm - 0.0257) (0.0015)-1.

Squamometry

Squamometry was performed according to the method of Piérard (83). One tape disc (D-Squame, 22 mm) was applied to the skin at each test site. A 10 kPa pressure was then applied to the tape disc for 5 seconds using a cylindrical weight system, after which the tape was gently removed utilizing forceps. Thereafter, each tape disc was stained with 1 to 2 drops of a solution of toluidine blue and basic fuchsin (Polychrome Multiple Stain; Delasco, Council Bluffs, IA) for 30 seconds. The tape discs were rinsed with deionized water, dried on a slide warmer and finally mounted onto a glass slide. The stained tape discs were evaluated by colorimetry as well as by microscopy. The colorimetric evaluation was performed using a Minolta Chromameter CR 300 (Minolta, Osaka, Japan). The tapes were mounted on a glass slide, which was then placed onto a white reference plate. Three measurements in the Lab-space were performed. The averaged

parameters L*, a* and b* were used to calculate the squamometry index $C^* = (a^{*2} + b^{*2})^{1/2}$ as well as the colorimetric index of mildness CIM = L* - C*. The microscopic evaluation of the stained discs was performed at 100-times magnification using the 5-point scales for intercorneocyte cohesion and cell staining. The scales for intercorneocyte cohesion were: (0) large sheet, (1) large clusters and few isolated cells, (2) small clusters and many isolated cells, (3) clusters in disruption and most cells isolated, and (4) all cells isolated and many cases of lysis. The scales for amount and distribution of dye within the cells were: (0) no staining, (1) staining between cells or slight staining, (3) moderate, but uniform amount of dye in cells, and (4) important staining in all cells, often with grains.

Transepidermal water loss

Transepidermal water loss (TEWL) represents a measure for skin barrier integrity and was measured utilizing an evaporimeter (Tewameter® TM210; Courage-Khazaka, Germany; Acaderm, Menlo Park, CA). TEWL readings were taken before treatment (baseline value) and then 1 hour after removal of the occlusive patches to allow for deconvolution from occlusive super-hydration. TEWL was also recorded after 10 and 20 tape strips at each test and control site. At least 2 minutes were allowed to elapse between removing the preceding tape and measuring TEWL to allow for redistribution of water within the stratum corneum. Room temperature and

humidity were monitored and ranged between 19°C and 21°C and between 50 and 60% relative humidity during the study.

Data analysis and statistics

Statistical analysis was performed utilizing SigmaStat 2.03 (SPSS Inc., Chicago, IL). For non-parametric data, such as clinical scoring, data was analyzed utilizing the analysis of variance (ANOVA) for ranks combined with Dunn's pair-wise comparison. Parametric data such as a*-values were analyzed with the repeated measures one-way ANOVA combined with the Tukey or Bonferroni pairwise comparison. Statistical significance was accepted when $p < 0.05$. TEWL was analyzed utilizing the one-way repeated measures analysis of variance with S-N-K test for multiple pair-wise comparisons.

RESULTS

Skin irritation

In order to assess the local dermatotoxicity of the preparations, skin irritation was evaluated visually and with the help of a chromameter after product application (Fig.1). As assessed visually, neither vehicle (A), nor occlusion (F) induced erythema or edema in any of the subjects; the clinical scores were 0 (25^{th} percentile = 0, 75^{th} percentile = 0). The salicylic acid pH 3.3 solution (B) induced confluent macular erythema in 3 subjects

as well as edema in 3 subjects, giving a median score of 1.5 (25^{th} percentile = 1, 75^{th} percentile = 2). The salicylic acid pH 6.95 solution (C) was less irritating with a median score of 0.25 (25^{th} percentile = 0, 75^{th} percentile = 0.5). The salicylic acid pH 3.3 solution containing no propylene glycol but menthol (D) irritated all subjects with a median score of 1 (25^{th} percentile = 1, 75^{th} percentile = 1.75). The occlusion control test site and the untreated, unoccluded test sites both yielded a median of 0 (25^{th} and 75^{th} percentiles = 0). Preparations B and D were significantly (ANOVA on ranks, S-N-K pair-wise comparison) more irritating than the vehicle (A), the occluded control (F) and the untreated control site (E). The differences between preparations C and B, or respectively, D did not reach significance. Changes in chromameter a*-values were expressed as differences (a*) between baseline values and after treatment (Fig.1). For some subjects, the a*-value decreased post-treatment, even after allowing one hour for the skin to recover after occlusion. Only treatment with salicylic acid pH 3.3 solution (B) resulted in a significantly increased a*-value as compared to baseline measurement. Otherwise, treatment with preparation D resulted in a slightly increased, but not statistically significant higher a*-value as compared to baseline measurement ($p = 0.06$). When comparing chroma a*-values between treatments, preparation B resulted in a significantly (ANOVA on ranks, S-N-K pair-wise comparison) greater a* than observed after treatment with vehicle (A), the salicylic acid pH 6.95 solution (C) as

well as for the occluded control (F) and the untreated control site (E), respectively.

Transepidermal water loss (TEWL)

Skin water barrier integrity after treatment and during tape stripping was evaluated by TEWL (Fig. 2). TEWL increased significantly after treatment with the salicylic acid pH 3.3 solution (B) and the salicylic acid pH 3.3 solution containing no propylene glycol but menthol (D) as compared to treatment with the salicylic acid pH 6.95 solution (C), the vehicle (A) as well as the occluded (F) and the untreated control site (E), respectively. The increase in TEWL after treatment was 2.3 ± 1.1 $gm^{-2}h^{-1}$ (mean ± SD) for preparation A, 5.3 ± 1.9 $gm^{-2}h^{-1}$ for preparation B, 2.4 ± 1.6 $gm^{-2}h^{-1}$ for preparation C, and 5.1 ± 3.1 $gm^{-2}h^{-1}$ for preparation D. The unoccluded control (E) site's TEWL increased by 0.75 ± 1.1 $gm^{-2}h^{-1}$ and the occlusion control (F) site's TEWL increased by 1.0 ± 1.1 $gm^{-2}h^{-1}$. Following 10 or 20 tape strips, skin sites treated with either the salicylic acid pH 3.3 solution (B) or the salicylic acid pH 3.3 solution containing no propylene glycol but menthol (D) showed a significantly increased TEWL as compared to the vehicle (A), the occluded (E) as well as the untreated control (F) sites, respectively. Furthermore, the increase in TEWL was significantly greater after 10 and 20 tape strips at the site where the salicylic acid pH 3.3 solution (B) was applied as compared to the salicylic acid pH 6.95 solution

(C). Whereas the increase in TEWL was significantly greater for the salicylic acid pH 3.3 solution containing no propylene glycol but menthol (D) as compared to preparation C after 10 tape strips, no such difference was found after 20 strips.

Squamometry

Microscopic analysis of the tapes did not reveal significant differences in the intercorneocyte cohesion or cell staining (no data shown). Further, there was no significant difference in the squamometry index (C*) as well as the colorimetric index of mildness (CIM) between the treatments (no data shown).

Mass removed after tape stripping

The cumulative mass sum of SC removed by sequential tape stripping after each treatment is shown in Figure 3. After 10 and 20 tape strips, significantly (one-way ANOVA for repeated measures, with S-N-K pair-wise comparisons) more SC was removed by tape stripping in test sites treated with salicylic acid preparations (C) and (D) as compared to the vehicle (A) and with the open (F) and occluded control (E) sites. Each of the effective treatments (C, D) stripped more than double the amount of SC than the vehicle (A) or the control sites. Treatment with the salicylic acid pH 3.3 solution (B) resulted also in an increased amount of SC removed after 10 and 20 tape strips as compared to vehicle (A) and the other control

sites (E, F). However, this difference was only significant after 20 tape strips. Treatments with the salicylic acid pH 6.95 solution (C) and the salicylic acid pH 3.3 solution containing no propylene glycol but menthol (D) removed significantly more SC in the first 10 strips as compared to treatment with the salicylic acid pH 3.3 solution (B). After 20 strips, however, similar and not statistically different SC amounts were removed for all preparations containing salicylic acid (B, C and D). Moreover, there was no statistical difference in the mass of SC stripped between any of the control sites including vehicle (A) and occlusion control (E).

DISCUSSION

The study describes an accurate and easy procedure to assess the efficacy of keratolytics and their formulation dependency in humans *in vivo*. Utilizing the protein assay to accurately quantify SC removal after tape stripping in combination with cutaneous biometrics represents a significant progress in the quantitative assessment and evaluation for keratolytic and desquamative therapies.

Previously, varieties of simple to more complex methods have been employed to determine the efficacy of SA in humans. Davies and Marks (84) were unable to determine histological differences between skin treated with SA compared to vehicle on regular light microscopy but found differences between the two sites on scanning electron microscopy. They identified irregular lamellae of loose squames, irregularity in scale apposition and gaps of about 3 to 10 μm between individual squames after SA treatment. However, without any means to objectively quantify the degree of desquamation, they only stated that the SC appeared to separate more easily when performing cryostat preparations. Nevertheless, their work with the scanning electron microscope led them to conclude that SA was likely to work by dissolving intracellular cement. Another method to quantify and further elucidate the effect of SA on normal skin utilized forced desquamation cell counts (85). In this study, subjects were treated

with 6% SA in 70% ethanol either for 8 h or twice daily for 10 days. In both cases, 70% ethanol was used as a vehicle control. In the 8 h study, an increased number of corneocytes shed by forced desquamation in the SA treated skin was seen in comparison to control sites, although no statistical analysis was presented. In the 10-day study, the SC was stained with two dyes (silver nitrate, dansyl chloride) in order to quantify corneocyte loss by determining the change in stain density with time. This method did not reveal a statistical difference between the control and treated skin for either stain, although it was noted that the rate of stain loss for silver nitrate was greater in SA treated skin as compared to vehicle control. However, the 10-day study did clearly demonstrate a significantly reduced number of SC cell layers in SA treated sites compared to vehicle treated sites as assessed by histology. In another study also employing silver nitrate to quantify the keratolytic activity of SA, Nook (86) described the method as being able to detect changes only after a prolonged study period following product application, but that large standard deviations led to difficult product comparisons. Summarizing, all these studies suggest that visual assessment of the keratolytic activity, whether by microscopy or by observing staining patterns on the skin surface, may take several days for detecting an effect.

In the present tape-stripping study, we utilized the volar forearm for treatment, as the facial skin is not the ideal test site because of the resultant erythema and possible hyper- or hypo-pigmentation following tape stripping. Topical administration of SA has been studied on the volar

forearm, demonstrating that plasma concentration peaks between 5 hours and 10 hours post administration depending on the formulation in humans (87). On this basis, the six-hour application period was selected. In comparison to previous studies (85) (88), our study identified clear statistical differences in the amount of SC removed already after a six hours treatment with SA. This suggests that sequential tape stripping is sufficiently sensitive to detect early desquamative effects confirming the results of a comparable study by Lodén (2% SA dissolved in a hydro-alcoholic solution) (78), where SA's keratolytic effect could be demonstrated after 6h, but not after 3h of occlusive application. Lodén and coworkers assessed SC removal semi-quantitatively by measuring the light transmission through the tape, whereas SC removal was determined with the protein assay in the present study. In contrast to weighing, and eventually also to the former quantification method, the protein assay enables to quantitatively determine the SC mass on a tape independent of the SC's unbound water content. This factor is particularly relevant when the preparations are studied under occlusive conditions as done in these studies.

Otherwise, the present squamometry data helped to further understand the mode of action of SA as a desquamative active ingredient. Squamometry allows visualizing the superficial arrangement of corneocytes on the tape-strip by low power light microscopy, in addition to assessing the dye uptake of damaged corneocytes under higher magnification. We

anticipated that, at least after a single short-term application, keratolytic treated sites would demonstrate a more scattered arrangement of corneocytes on the tape as compared to vehicle treated sites which would demonstrate a more intact corneocytes sheet on the tape surface. In fact, there was no significant difference in the arrangement of corneocytes on the tape, all sites demonstrated a relatively intact sheet independent of treatment. The sensitivity of this method seems therefore not sufficient to detect a keratolytic effect after a short treatment period. As shown in a separate study, squamometry allowed detecting an altered intercorneocyte cohesion only after a prolonged and repetitive application of two weeks and more (88). The authors of this study indicated that squamometry represents not a direct measure for the desquaming effect of SA and other keratolytics since lost corneocytes are not accounted by this method. The observation that the keratolytic effect of SA was not detectable by squamometry after a short-term application appears to be consistent with the findings of previous studies reporting difficulties to observe a keratolytic effect of SA using dyes such as silver nitrate(85) (86).

As for most solutes, SA penetrates across skin better in its neutral form compared to its ionized form (89, 90). On the other hand, the skin permeability of salicylate may be facilitated depending on the presence and properties of counter-ions (91, 92). Since SC is the target tissue for keratolytic activity, formulations optimized to deliver SA in this skin compartment presumably show most therapeutic efficacy. As a

consequence, a SA containing formulation designed for optimal systemic absorption may not necessarily show highest keratolytic efficacy. The fact that preparation C (pH 6.95), where SA is mostly present as salicylate, showed a comparable keratolytic activity to preparations B and D (pH 3.3, approximately 65% present as salicylate (without taking into account the influence of the formulation ingredients on the dissociation constant of SA)) corroborates this hypothesis, which was already postulated before by others (93). This suggests that the uptake of SA into the SC was sufficient from the neutral preparation C to produce a pronounced keratolytic effect.

The skin water barrier integrity in relation to SA treatment was further studied by TEWL. Different formulations of 2% SA affected the skin barrier differently, following a pattern related to their pH. However, TEWL represented no predictive measure for a keratolytic effect after SA treatment. In the two treatments with acidic pH preparations (B and D), the TEWL increased significantly. However, for treatment C, which was similarly effective as a keratolytic preparation, there was no significant rise in TEWL as compared to baseline. This suggests that SA may significantly decrease SC cohesion by only minimally disrupting skin barrier to water diffusion. Since corneodesmosomes are believed to be the major component providing SC cohesion (94) (95), our results indicate that SA mainly affects their structure and seems not significantly to perturb the SC lipid composition and organization, which is mainly responsible for SC's excellent barrier properties.

In addition to monitoring skin water barrier function by TEWL, we also documented skin erythema after SA application. At least for SA, we demonstrated that acute skin irritation must not be a necessary side effect for a keratolytic effect. This observation contrasts studies alluding keratolytic efficacy of alpha-hydroxy acids with some dermatotoxicity (96). Hence, the preparation containing 2% salicylic acid at neutral pH (C), induced only a slight TEWL change, was minimally irritant, whilst retaining its ability to be as keratolytic as similar preparations at acidic pH (B and D). This demonstrates that effective desquamation can be achieved in the absence of any clinical signs of cutaneous irritation. These findings suggest that the keratolytic effect of SA is not simply the result of a non-specific skin irritant effect, but is rather a real pharmacological effect.

Concluding, using tape stripping in combination with a protein assay to accurately quantify SC removal substantiated earlier reports that SA acts as a significantly keratolytic. This experimental model appeared sensitive in detecting efficacy of SA preparations within hours of application. Importantly, whereas the pH of the SA containing preparation only minimally influenced the keratolytic efficacy, the dermatotoxicity was significantly increased at acidic pH instead. In light of this, our data indicates that the quest to increase the amount of free, non-dissociated SA is, in fact, counterproductive as the more acidic preparations resulted in skin irritation and barrier disruption. These properties are especially undesirable as keratolytic products containing hydroxy acids are frequently

used on the face to improve the condition of lesional skin and enhance cosmetic appearance. Hence, a 19th century drug has allowed new insights in the 21st century; much still remains to be understood.

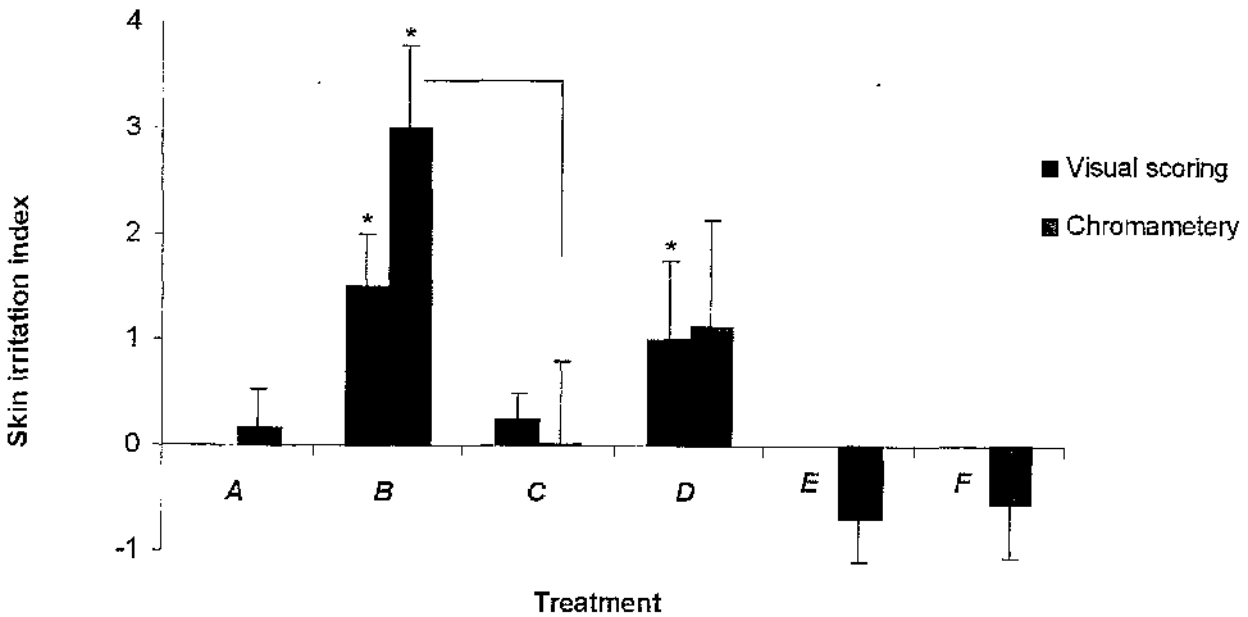


Figure 10 Skin irritation, evaluated visually (median and 75th percentile of irritation score) and by Chromameter (mean ± SEM of Δa*-value) Significant differences between SA-treatments and baseline as well as between treatments and vehicle (A), occlusion (F) or, respectively, untreated control site (E) are indicated (*). Significant differences between SA-containing preparations are further indicated.

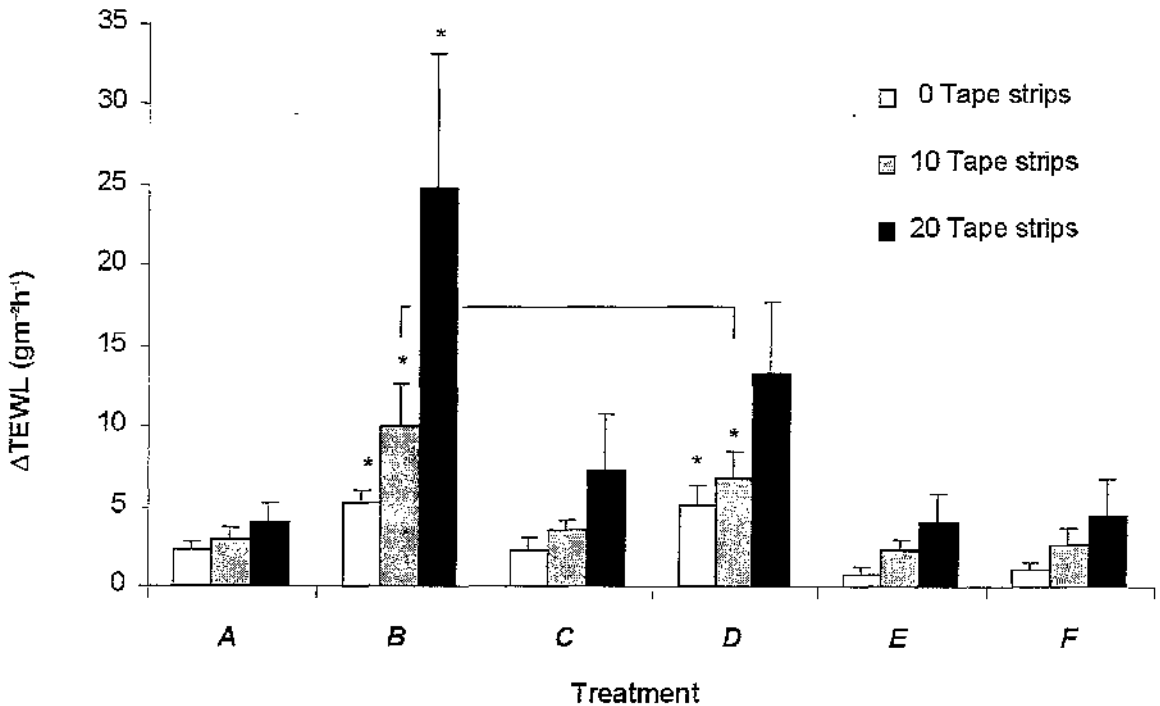


Figure 11: Skin barrier integrity as assessed by TEWL. Differences in TEWL (ΔTEWL, mean ± SEM) between treatment and corresponding baseline value are given. Significant differences between preparations (B) or (D) and preparation (C), vehicle (A), occlusion (F) or, respectively, untreated control site (E) are indicated (*). Significant differences between preparations (B) and (D) are further indicated.

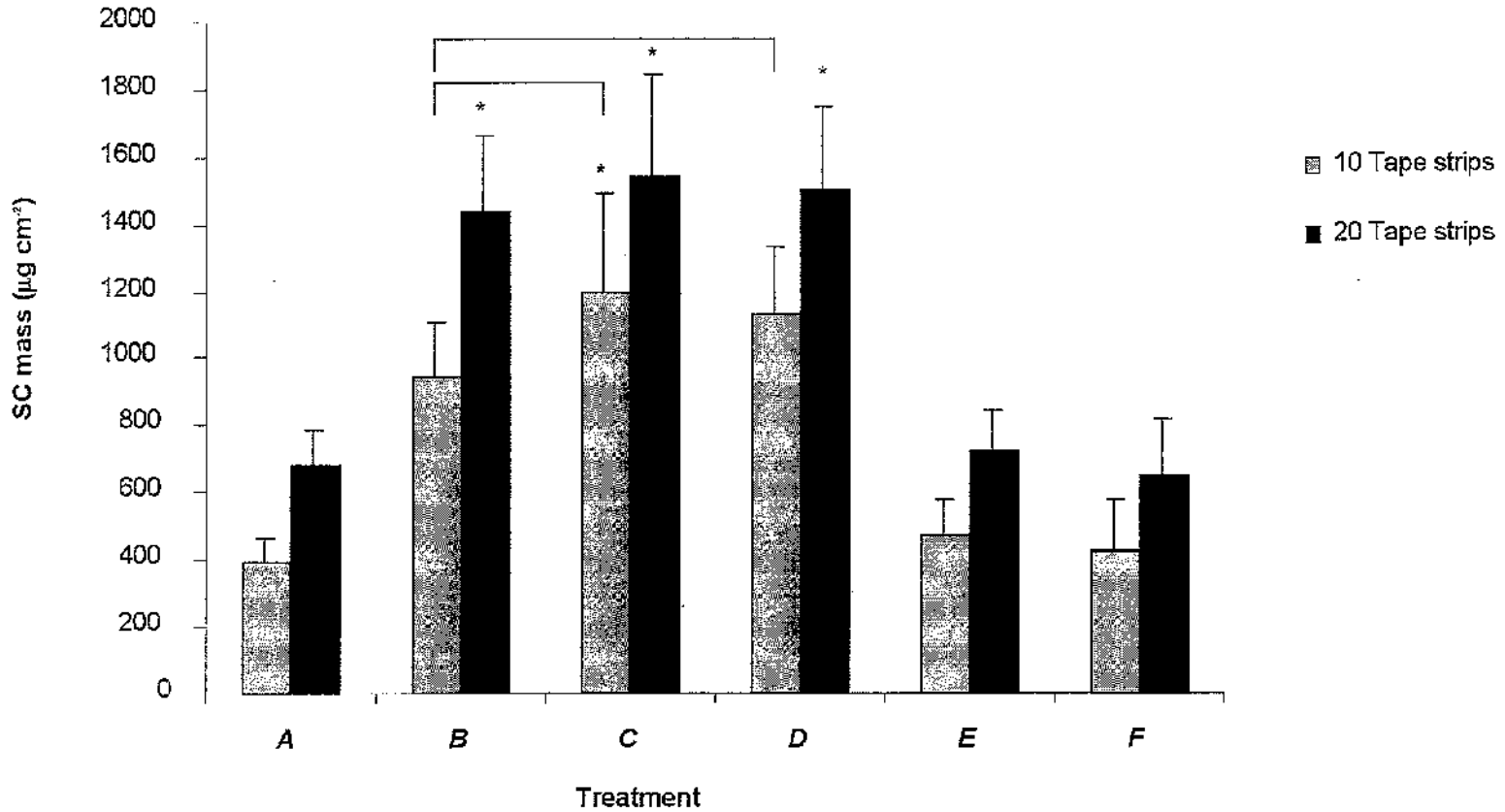


Figure 12: Mass (µg cm^{-2}, mean ± SEM) of SC removed per treatment. Significant differences between SA-treatments and vehicle (A), occlusion (F) or, respectively, untreated control site (E) are indicated (*). Significant differences between SA-containing preparations are further indicated.

Chapter 10

Study 3

pH PROFILES IN HUMAN STRATUM CORNEUM

&

LOCALISATION OF THE ACID MANTLE

INTRODUCTION

The human stratum corneum forms the interface between the human body and the external environment. As molecules enter the skin from the outside world, they become subject to a new environment, the environment of the stratum corneum with its complex pH gradients. Rather than considering the skin barrier as a structure composed of a mass of lipids and proteins, we can consider the barrier in a physico-chemical environment with a complex pH gradient. This gradient challenges penetrating molecules to retain their original molecular charge and structure as they pass into the skin, and such factors have been shown to influence the flux of molecules across the skin barrier (97).

Tape stripping has been successfully utilized to identify the pH gradient within the skin (98) but methods have been inconsistent and lack the detailed precision offered by the protein assay application of tape stripping. We aim to assess the pH profile of the skin with respect to depth within the skin and also with respect to time, forming a dynamic quantification of the pH barrier *in vivo*.

MATERIALS AND METHODS

Subjects and Ethical Approval

Subjects with no history of skin disease were recruited to the study. Subjects were over 18 years of age, in good physical health and had no history of skin disease. Ethical approval was granted by the University of California, San Francisco Committee on Human Research and all subjects provided written informed consent. Subjects were asked to refrain from using soaps and cosmetics on the test sites for 24 hours prior to the study. Hair was gently clipped from subjects with hirsute forearms 24 hours before measurements were taken. All subjects enrolled completed the study.

Tape stripping

Tape stripping was performed according to the standardized method employed in the previous studies in this thesis. Test sites were divided into two zones: the volar forearm and the upper arm, in each subject. A template was applied to the skin to mark test sites so that they were consistent from subject to subject.

Transepidermal water loss

Transepidermal water loss was measured at baseline and 15 minutes after the final tape strip with an evaporimeter (Tewameter, Courage & Khazaka, Cologne, Germany). The method was consistent other studies in this thesis.

pH measurement

pH was measured with a pH meter (Courage and Khazaka, Cologne, Germany) at baseline and after every 5 strips. The pH meter was calibrated to the standard solutions provided by the manufacturers. Before each measurement, the skin was cleansed with water-moistened tissues (Kimwipes, Kimberley-Clark, Roswell, Georgia). The tip of the pH probe was cleansed with water (high performance liquid chromatography grade) prior to each set of measurements and was wet when applied to the skin. The pH was correlated to the depth in the stratum corneum utilizing the protein assay data, which quantified the amount of protein removed on each tape strip. 3 pH measurements were taken at each time point and the mean reading was calculated. The time points for each set of 5 strips were: immediately, 1 minute and 2 minutes following tape stripping.

Data Analysis

The data demonstrated stratum corneum pH profiles for each individual. The mean and standard deviation for the study population were calculated and expressed graphically. Statistical comparisons were performed for surface pH and pH at particular depths within the stratum corneum in order to compare the upper and lower arm sites and also the different time points. However, much of the value of the data is within the shape of the curve generated, exploring how pH changes with depth within the skin. The inter-individual variation in such data is lost when the means are calculated, and therefore some data is presented as raw values and graphs.

Comparisons of pH were performed using the one way repeated measures analysis of variance, with subsequent multiple pairwise comparison (Tukey Test).

Determination of SC thickness

The mass of protein on each tape strip was quantified using the quantitative spectrophotometric protein assay method described earlier. The thickness of the stratum corneum removed was calculated utilizing the following equation:

$$x_i = m_i / F \cdot \rho$$

Equation 1

where x_i is the thickness, m_i is the cumulative mass of SC removed by i consecutive tape strips. F is the area of the tape strips (3.8 cm^2) and ρ is the SC density, set to ρ = 1 g/cm^3.

RESULTS

Surface pH of Human Skin

pH was measured on the skin surface at each site prior to tape stripping, to give the resting surface pH. The mean pH on the volar forearm was 5.4 (±SD 0.9) and 5.5 (± SD 0.9) on the upper arm at baseline. The pH ranges were normally distributed. There was no significant difference between the baseline pH on the volar forearm and upper arm.

pH profiles

pH profiles were obtained through sequential measurement of pH after each set of five tape strips. In each subject, there was an acidic zone in the superficial stratum corneum, leading to a more neutral zone in the deeper stratum corneum. These pH variations are represented graphically with respect to depth within the stratum corneum for each subject. The initial gradient in the superficial stratum corneum progressed towards an acidic pH in

all subjects, except those whose surface pH was the most acidic of the study population. In these subjects, the superficial stratum corneum pH gradient was relatively flat in following tape stripping (see Figure 13 to Figure 18).

Localisation of the acid mantle

The most acidic pH was identified in each subject and the mean was calculated. On the volar forearm, the mean most acidic pH was 4.8 (±SD 0.8) and on the upper arm the pH was 5.0 (±SD 0.2). The pH of the acid mantle was significantly different from the surface pH (P=0.002). On both the volar forearm and upper arm, the most acidic zone was within 20 tape strips, the median being 10 strips (IQR 5, 10). Represented as depth within the stratum corneum, the most acidic point was at a median depth of 1.8µm (IQR 1.1, 2.5) on the volar forearm and 1.4µm (IQR 1.36, 1.79) on the upper arm, from the surface of the stratum corneum. There was no difference in the depth of the most acidic point between the upper arm and volar forearm sites.

Further zones within the stratum corneum

Whilst the pH profiles for each individual were non-linear and often fluctuating, a general trend in the shape of the curves suggested a point of inflection mid-way through tape stripping. This can be seen clearly in Figure 13, the graph of mean pH. As the pH begins to rise after the point of maximum acidity, the curve plateaus momentarily at 25 strips, which

corresponds to a mean depth of 4.6μm (±SD 0.8) on the volar forearm. The mean pH at this point was 5.44 (±SD 0.35) and immediately afterwards the pH environment remained almost constant for the next 5 strips with a pH of 5.46 (±SD 0.18), thereafter becoming more acidic at depth of 5.6μm (±SD 1.0). This suggests that there is a zone of constant pH at this point, for approximately 1μm, within the limits of our method.

A similar zone was also seen on the upper arm, in the region of 30 to 35 tape strips, or 5.3μm to 5.9μm with a pH of 5.46 (±SD 0.22) to 5.43 (±SD 0.22).

pH response to injury

pH was measured immediately after tape stripping, then at 1 minute and 2 minutes following tape stripping every five strips. This allowed the response of the stratum corneum to injury to be measured in terms of pH. As seen in Figure 13, the mean pH at 1 and 2 minutes post tape stripping closely resembled the pH measured immediately after tape stripping. However, when considering the data for each individual, wide variations in pH could be seen in the 1 and 2 minute readings, as shown in Figure 16. As each reading presented is a mean value from three measurements with very little variation (data not shown), it is likely that the pH changes are a real phenomenon rather than simply variation in measurements.

DISCUSSION

Origin of skin surface pH

It is well documented that the skin has an acidic surface and that there is layer of acidity within the stratum corneum, termed the "acid mantle." Somewhat confusingly, some earlier references consider the acid mantle to be a surface film, composed of sweat, sebum and other compounds. However, surface pH is increasingly considered to be distinct from the pH found within layers of the stratum corneum, which is also acidic in the superficial but more neutral in deeper layers. The data presented shows that there is an acidic zone in the superficial stratum corneum which has a mean pH that is significantly more acidic than the surface pH ($p<0.002$). This provides strong evidence that the acid mantle should indeed be considered a sub-surface phenomenon and this thesis refers to the acid mantle as a zone of acidity within the stratum corneum, distinct from superficial surface pH.

The fact that the outer layers of the human body are acidic may serve a variety of biological roles: for example, the acidity may prevent microbial invasion, or perhaps facilitate the activity of stratum corneum enzymes. Further, the acidic nature of the outer layers may help encourage barrier recovery in damaged or traumatised skin. The acidic layer may be important in facilitating skin barrier

repair following injury (99). Whilst the origin of the acid mantle is unknown, several processes have been thought to contribute (see Table 12).

As pH is a logarithmic function of hydrogen ion concentration, the molecules that contribute hydrogen ions to the stratum corneum and regulate its pH homeostasis are the subject of ongoing research. This study, in addition to previous work, identified the outer stratum corneum as the most acidic zone. This acidity is likely to reflect the metabolic processes taken place in the stratum corneum if one excludes sebum and sweat as the source of the acid mantle in the upper stratum corneum. In attempting to understand which molecules are responsible for creating this environment, it is important to understand the biochemical process taking place at this site. Unfortunately, it has only been relatively recently recognised that biochemical processes take place within the stratum corneum and that these have a functional significance.

The amino acid breakdown products of the stratum corneum protein fillagrin, have been proposed as a major contributor to the acid mantle (100). This theory arose from the study of pH in patients with two differing types of ichthyosis: X-linked recessive and autosomal dominant. Ichthyosis vulgaris patients have a significantly higher skin surface pH than patients with X-linked recessive ichthyosis and also healthy controls. This difference in acidity compared to controls was attributed to the deficiency of fillagrin which is a

hallmark of ichthyosis vulgaris. Fillagrin breakdown products include urocanic acid and pyrrolidone carboxylic acid, which are thought to contribute to the acid mantle. Further, X-linked ichthyosis patients have significantly lower surface and superficial stratum corneum pH that both normal controls and ichthyosis vulgaris patients. This suggests that the deficiency of steroid sulfatase seen in this condition leads to acidification via the accumulation of the acidic lipid cholesterol sulphate.

In addition to filaggrin breakdown products and acid lipids, other molecules that are thought to contribute to stratum corneum acidity include free fatty acids and alphahydroxy acids. The pH of human skin *in vivo* has been shown to correlate with lactate and potassium levels in stratum corneum tape strips (101). A variety of other amino acids and protein breakdown products are present in the stratum corneum, and their individual contributions to the pH of the skin have not yet been characterised. Caution must be exercised in the interpretation of studies that focus on individual molecules. Whilst such work does provide valuable insight, the stratum corneum is a complex biomolecular environment which must be considered as a whole.

pH Gradients within the stratum corneum

A gradient of pH within the stratum corneum has previously been identified *in vivo* in humans. In the first study determining a vertical gradient, two methods were used to remove layers of stratum corneum: adhesive tape stripping and cyano-acyrlate stripping (98). Although their method of stripping was not as standardized as the model in this thesis, their data generated a curve of pH against the number of strips. The data presented in this study is in agreement with that work, although much more detail regarding the pH profile has been obtained.

Previous work has shown the general shape of the stratum corneum pH gradient curve: it is a sigmoidal curve with inflection towards increased acidity in the superficial stratum corneum and then towards neutrality with increasing depth.

However, there are some interesting nuances to this data demonstrated by this thesis, which have not previously been discussed in the literature. These are: the variation in surface pH between subjects; the variation in the shape of the curve between subjects; that there can be quite sharp differences in pH separated by a distance of micrometers, such as an island of relatively neutral pH within a region of acidity.

Figure 13 shows the pH gradient, plotted against the number of tape strips, which presents the data in a form that can be readily compared with previously published work. However, the use of the protein assay to quantify the mass of protein on each tape, with a subsequent conversion to the thickness of skin stripped, provides data for Figure 15, which is a plot against pH against stratum corneum depth from the surface. This graph shows a rapid decline in pH from the surface to the most acidic point, a few micrometers in to the stratum corneum. As tape stripping progressed, fluctuations in pH could be seen, with a general trend towards neutrality as the depth of stripping nears the dermis. This profiling provides new insights into the metabolic activity within the stratum corneum. Figure 16 shows the wide changes in pH that occur within a few microns of each other, suggest that there are micro-domains of acidity within the human stratum corneum. As the figure shows, the pH alters with time as well as with depth, suggesting that within 1 or 2 minutes of tape stripping and, therefore, exposure to the environment, there are large changes in hydrogen ion concentrations at that site. It remains to be determined whether this reflects an enzyme mediated metabolic process or whether this reflects a more passive degradative process as a response to epidermal injury. It has been shown that barrier recovery is delayed in the presence of a neutral or alkaline pH and accelerated in the presence of an acidic pH after acetone barrier disruption in mice (99). It may be that the alterations in pH seen

immediately after tape stripping in our subjects represent an endogenous attempt to "normalise" pH to create an environment that facilitates would healing.

When the data is analysed as a population mean (Figure 13) this fine detail of pH fluctuation is largely lost as the data averages out. Nevertheless, there remains a non-linear rise in pH to alkaline even when the data is considered as a mean. This suggests that there are definite zones of variation in pH which are broadly similar between individuals, in addition to the fine variation which we can seen within each subject.

Figure 13 shows that there is a point of inflection in the pH curve. This has not previously been reported, perhaps because there had been no standardized tape stripping method with mass/depth quantification. The point of inflection lay at a depth of 4.6μm to 5.6μm where the pH remained almost constant between 5.44 and 5.46 respectively on the volar forearm. The fact that the standard deviation at this point is very low (SD of pH 5.46 = 0.18 at 5.6μm) suggests that this a true finding, common amongst our population. In fact, this is point at which standard deviation of pH measurement was the lowest. The shape of the curve was similar on the upper arm, including the pH plateau seen on the volar forearm, although at slightly deeper level within in the stratum corneum.

As in previous studies, variation in surface pH was noted. In subjects who had a very acidic surface pH, and therefore had an upward sloping curve (see Figure 17), the most acidic point was considered to be at the 5th tape strip, rather than the surface pH because of possible surface contamination.

Variations in pH: endogenous factors

This laboratory has previously demonstrated (102) that racial differences in pH and TEWL between white and black skin types follow tape stripping. In that study, black African-Americans demonstrated significantly greater increases in transepidermal water loss (TEWL) and significantly greater acidity in the superficial layers of the stratum corneum. Whilst the origin of the racial difference was unclear, the relationship between TEWL and pH was postulated. Recalling that the outer stratum corneum is predominately a lipid medium, the expression of H^- ion concentration as pH is likely to be related to the water content of the skin at the point at which pH is measured. Therefore, as tape stripping causes water barrier disruption, the increased presence of water in the outer stratum corneum may allow the expression to H^+ ions in a more aqueous medium to be measured as an acidification. Combining our earlier study on tape stripping effects and skin physiology (*Study* 1), this appears to be consistent with our data. However, we know that the transepidermal water loss continues to rise with subsequent tape stripping, but

the pH becomes more alkaline rather than more acidic. Thus, it cannot be suggested that water movement alone or the expression of H^+ in an aqueous environment can explain the pH changes. It is possible that the metabolically active living cells in the deeper epidermis contribute more to the pH in basal stratum corneum environment than the non-living corneocytes themselves. Nevertheless, the data presented in this thesis demonstrate that there are changes in pH occurring within 1 or 2 minutes of tape stripping, even in the superficial stratum corneum. This suggests that there may be pH regulatory processes present even in the non-viable areas of the skin.

In order to eliminate racial variation within our study, all the subjects were Caucasian. Despite this, variation between the individual pH profiles between the volunteers remained. Molecular studies of the skin have provided some further insights in to the origin and distribution of acidic elements. An Na^+/H^+ antiporter (NHE1) has been shown to play a role in the acidification of the stratum corneum of mice (103). Knockout mice deficient in this protein are unable to acidify the stratum corneum, when the pH is compared to the granular layer. This study is particularly interesting as it demonstrated that a novel method of assessing pH, fluorescence lifetime imaging microscopy, was able to detect pH differences that standard glass electrodes could not. Using this increased sensitivity, areas of acidity were found to be localised within the stratum corneum in micro-domains. As the stratum corneum became more

acidic towards the surface, the number of these acidic domains increased, rather than increased acidity within the domain itself. Whilst this study was carried out in mice, it may be that such domains are present in human stratum corneum, perhaps explaining the sudden fluctuations in pH seen in our tape stripping method. Further work, characterising the molecules present on the tapes at these zones of variation, may help identify sources of pH at different levels within the stratum corneum.

Atopic dermatitis, a disease characterised by epidermal barrier perturbation with lichenified, hyperkeratotic, dry and fissured skin also shows alteration in surface pH. As the skin is dry with high transepidermal water loss, the application of emollients to improve hydration is a key feature of therapy. However, it has been shown that alteration in pH can be found in the skin of these patients, which may impair barrier recovery and healing. The pH of the skin is less acidic in areas of active dermatitis, gradually becoming more acidic in perilesional skin and reaching normal pH on uninvolved skin, compared to matched controls (104). This finding has been repeated in other studies, but the mechanism of the pH changes in atopic dermatitis remains elusive.

CONCLUSION

In conclusion, this study demonstrates that the acid mantle is, in fact, a zone of acidity in the upper stratum corneum that is distinct from the surface pH. This strongly suggests that surface components are not responsible for the maintenance of this acidity. Rather, the acid mantle is likely to be generated from within the stratum corneum itself. This study demonstrates that it is possible to precisely localize the acid mantle in each subject.

Additionally, the tape stripping model has been successful in developing pH gradients against stratum corneum depth in each subject. Using this data, a zone of relatively constant pH has been identified in the stratum corneum, with very little variation between subjects. This zone, which has not previously been reported, is seen as a point of inflection in the pH profile, which represents approximately 1μm of constant pH

As stated above, pH is the negative logarithm to the base 10 of the activity of hydrogen ions in an aqueous solution. By definition therefore, *in vivo* studies are not measuring the actual pH of the skin (which is not an aqueous solution). Rather, the use of a pH probe measures the pH of the extractable water-soluble contents of the skin (105). This important distinction tells us that apparent pH measured is a reflection of the biochemical make up of the skin, and that, as the pH changes with depth, so must the biochemical environment. Measuring

skin pH, therefore, is an indirect marker of metabolic activity within the stratum corneum. This poses a question: is the purpose of this metabolic activity to create a suitable pH for skin function, or is the pH a simple by-product of these processes?

Table 12: Stratum Corneum Acidification

Mechanisms which may contribute to the acid mantle
Surface microbial metabolism
Sebum derived fatty acids
Outward proton flux
Insertion of proton pumps
Hydrolysis of phospholipids to free fatty acids
Bulk hydrolysis in outer epidermis
Hydrolysis of specific proteins to amino acids

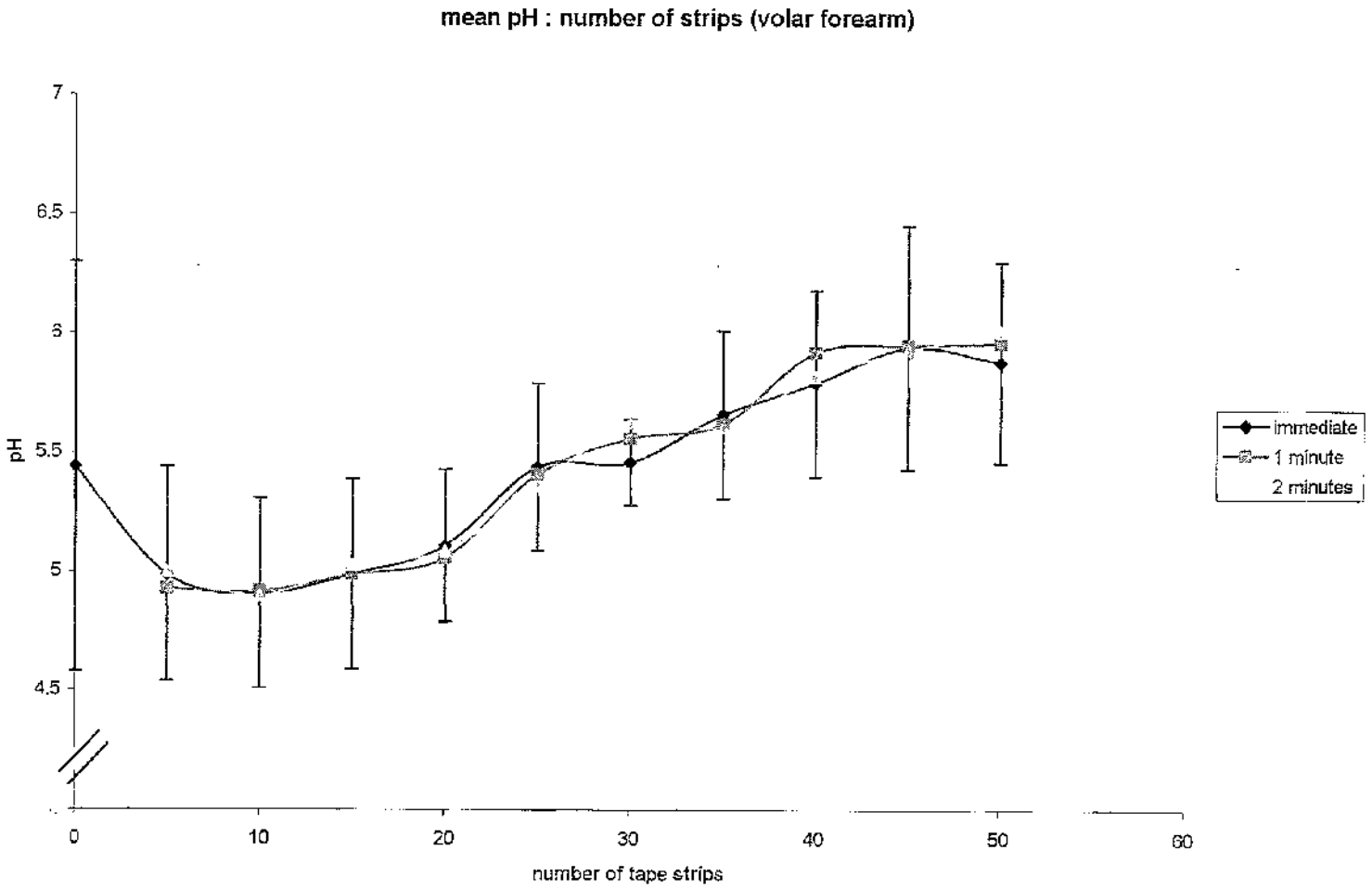


Figure 13: Mean pH against number of tape strips, for all subjects (volar forearm)

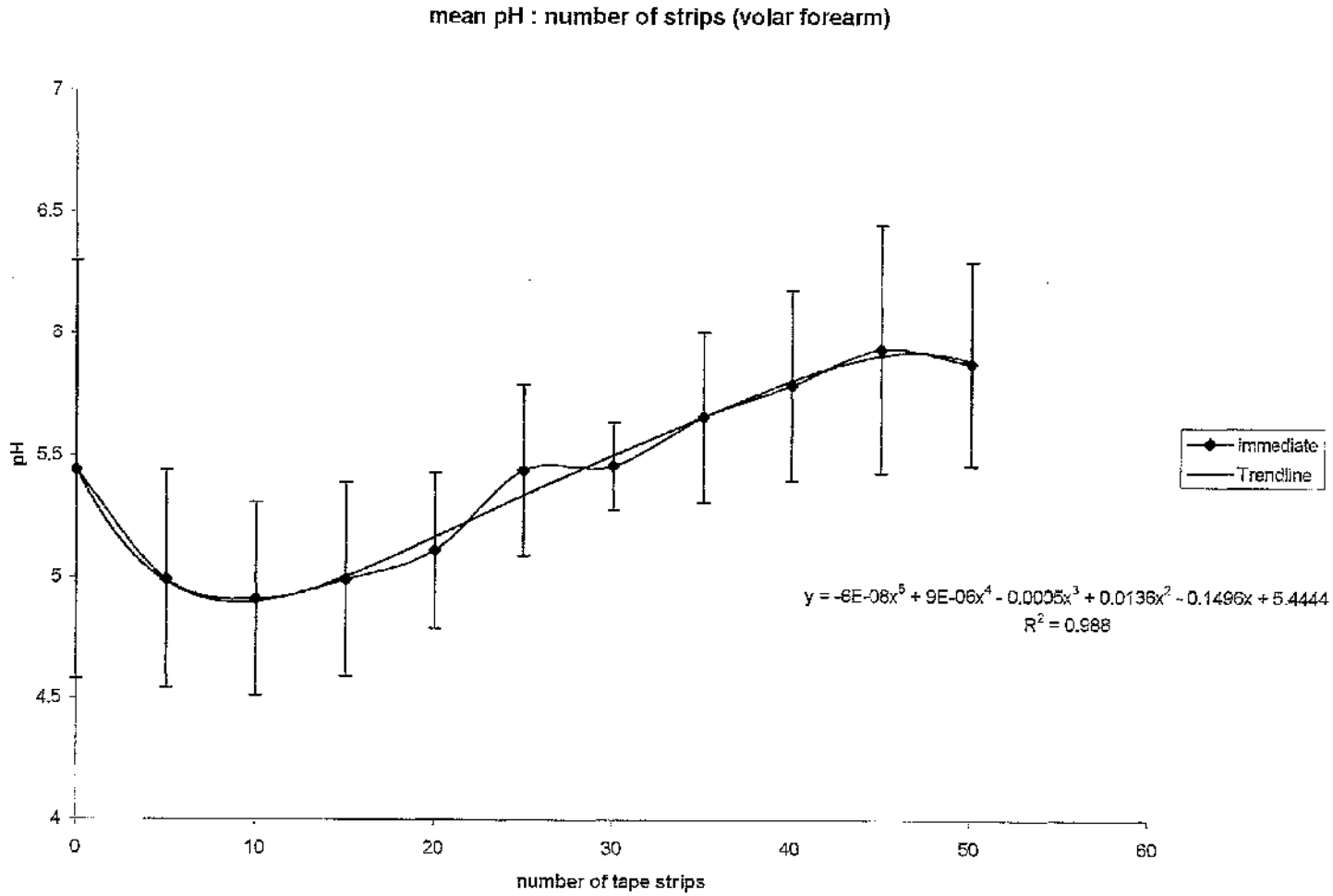


Figure 14: A 5th order polynomial regression trendline has been added. The equation given has a correlation coefficient of 0.99

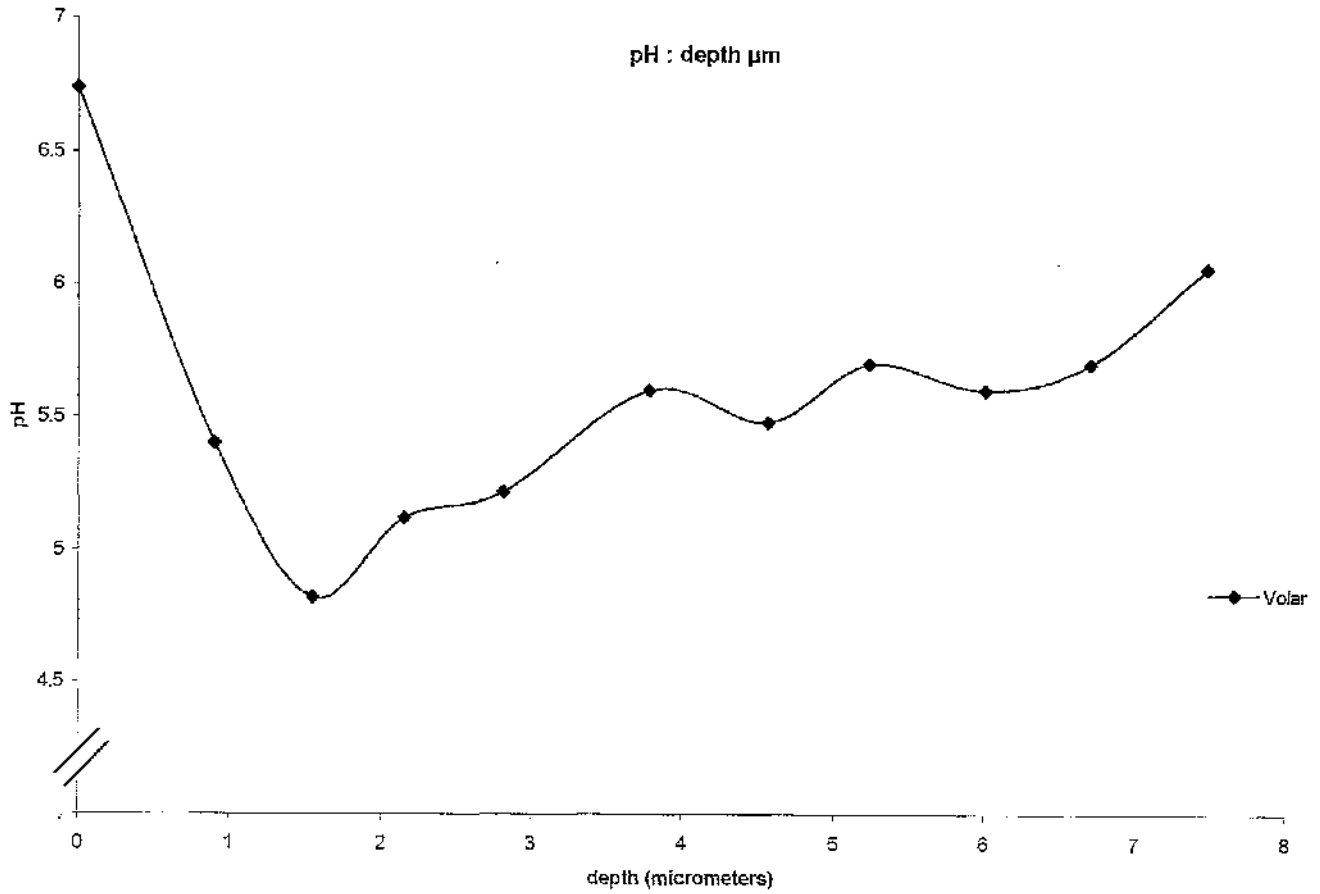


Figure 15: pH against calculated depth in the stratum corneum. The graph shows the initial pH drop to maximum acidity, followed by a tortuous rise in pH with depth (volar forearm subject 1).

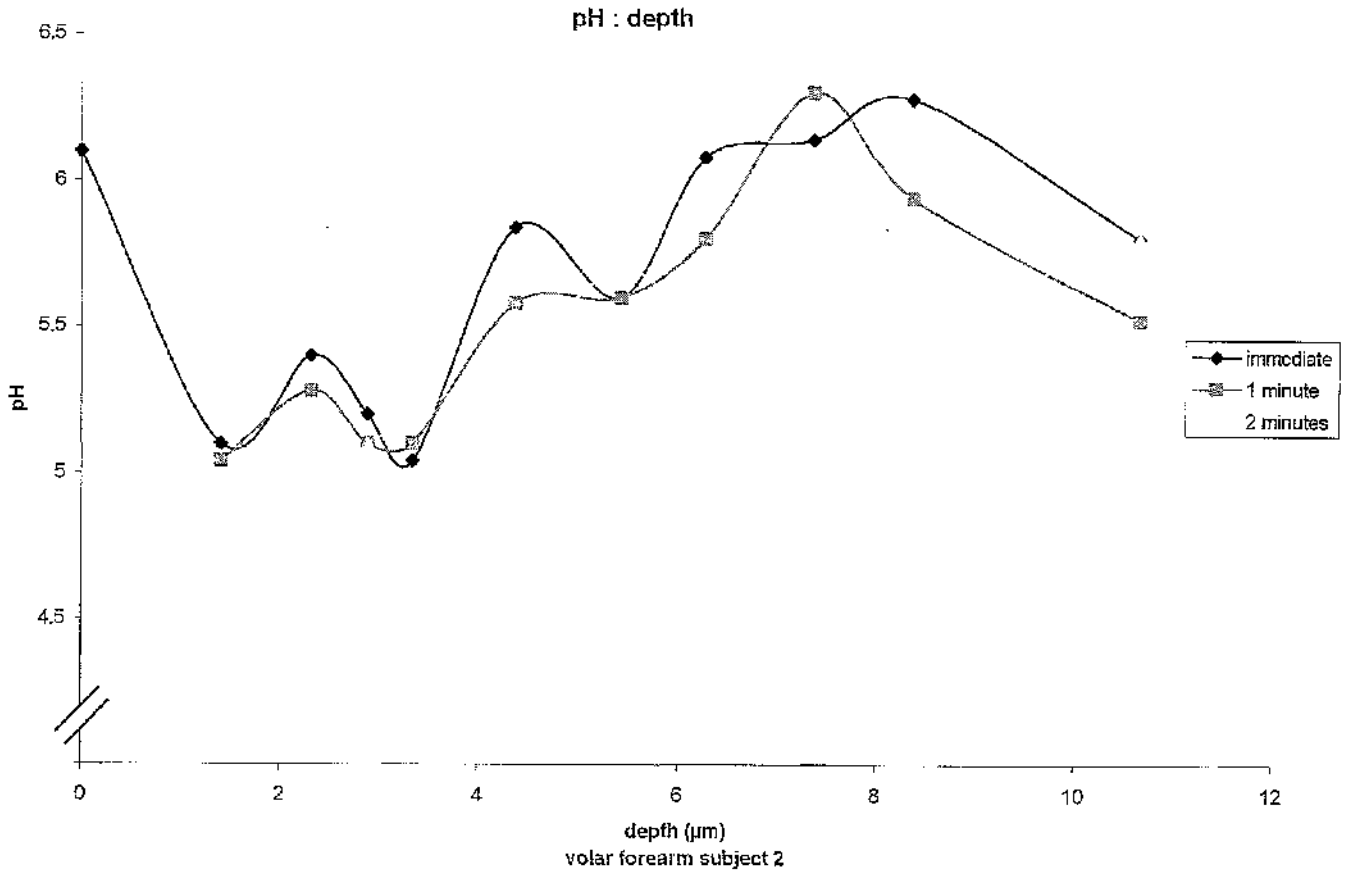


Figure 16: Wide variations in pH occur within a few micrometers of each other in the stratum corneum (subject 2, volar forearm)

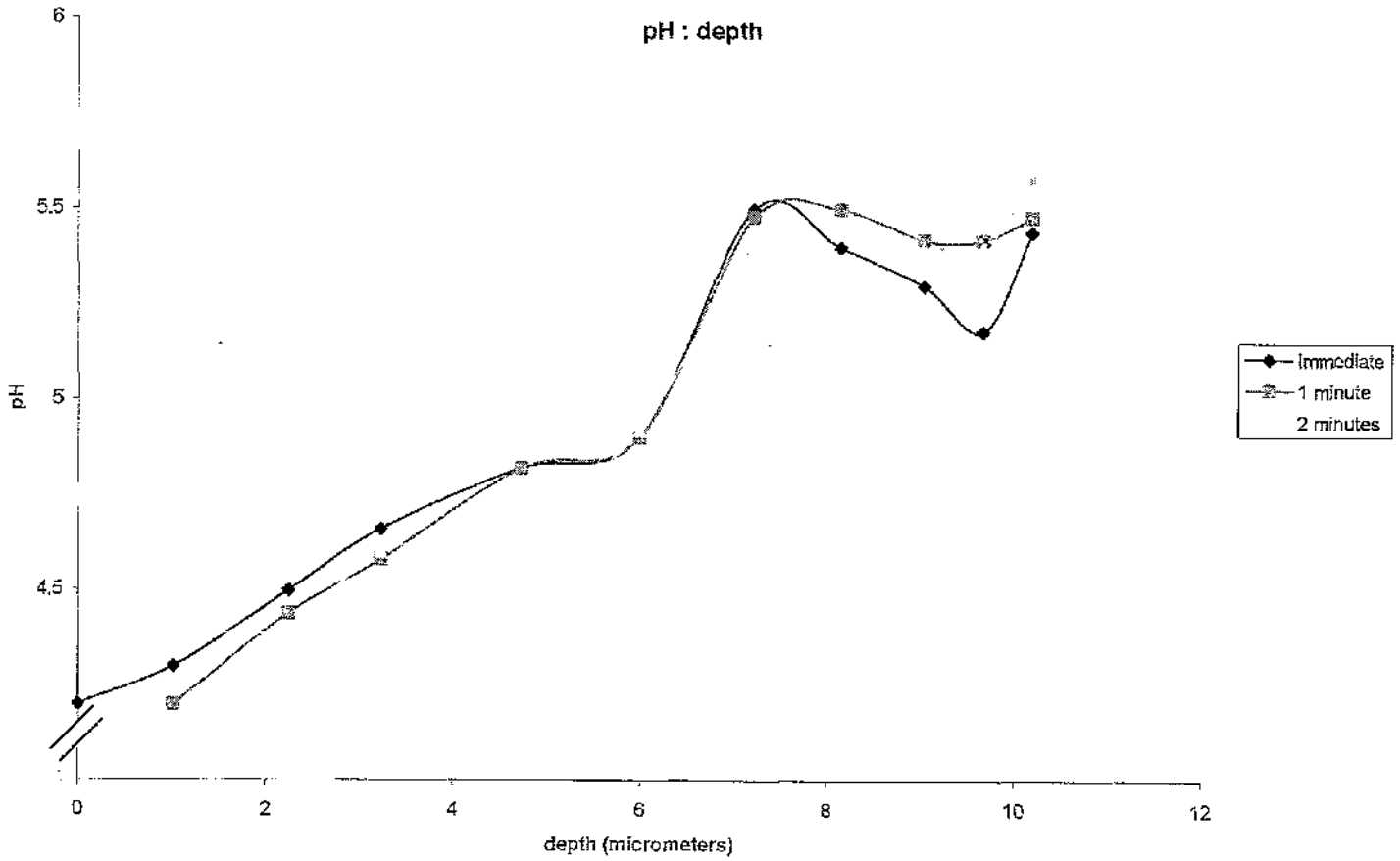


Figure 17: Baseline pH in this subject is more acidic, so the slope of the first segment of the curve is upwards rather than downwards. (subject 4 volar forearm)

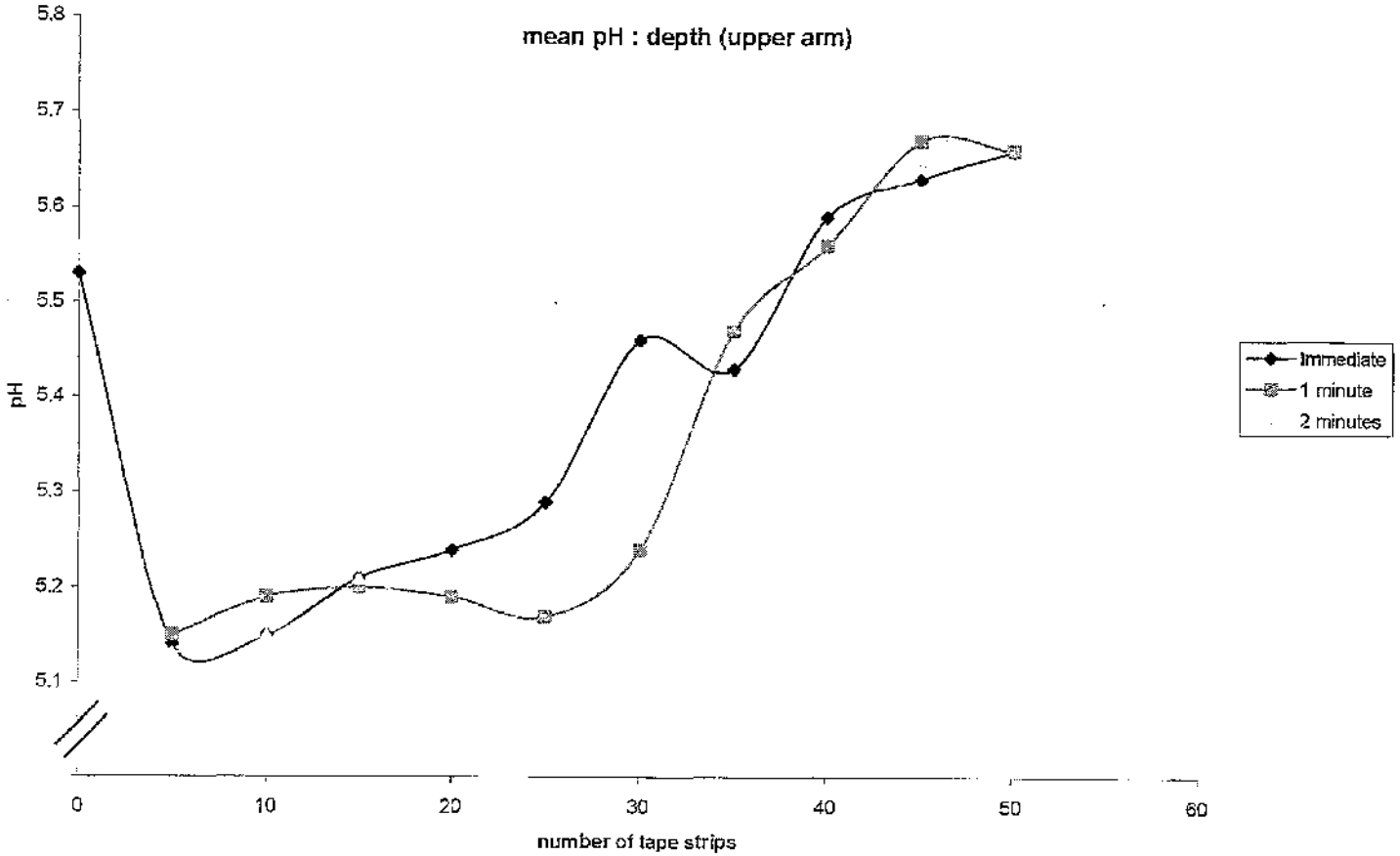


Figure 18: pH profiles in the upper arm are similar to those in the volar forearm (mean for all subjects)

Chapter 11

UNIFYING OVERVIEW AND CONCLUSION

CRITIQUE OF THE METHODS

The purpose of this thesis was to improve the present practice of stratum corneum tape stripping and to demonstrate the usefulness of reliable quantitative tape stripping data in the study of skin physiology and pharmacology. In the process, several limitations of the method came to light.

Vehicle Interference

In study 2, the study of the keratolytic properties of salicylic acid, it became obvious that the test vehicle could be a limiting factor in tape stripping. When several test keratolytics were developed, such as salicylic acid in petrolatum or retinoic acid in commercially available creams, it became clear that the model needed modification. This is because the tape needs to stick efficiently to the skin in order for the stratum corneum to be stripped off. If greasy vehicles are used, the tapes simply do not stick. As a result, the study was performed with a volatile liquid solvent base, which evaporates sufficiently to allow adhesive tape stripping. In the future, it could be possible to modify the dosing schedule to allow substances in

these bases to be tested. The most straightforward way to do this would be to allow a period of >24 hours after application before tape stripping commenced. It is important to solve this problem as many topical drugs are delivered in greasy bases rather than in volatile solvents.

Mass of protein

The spectrophotometric method of analyzing the tapes was highly efficient. The tapes could be stored for analysis at a convenient time and large numbers of samples could be processed in a few hours (far more efficiently than weighing). However, in the study of keratolysis, a pilot study with high concentrations of salicylic acid (6%, data not shown) caused the skin to desquamate in thick sheets on the tape strips. The incubation of the tapes in 1M NaOH was not sufficient to dissolve these large stratum corneum samples. Whilst it may be possible to dissolve the sheets in a higher molarity of NaOH, the HCl required to neutralize that solution would also be of higher molarity. The large number of ions involved would unfortunately inhibit the protein assay, leading to inaccuracy. Therefore, the method as published would need significant modification to work on large sheets of stratum corneum. One possible method would be to perform serial dilutions on the resultant solution, but such dilutions do increase the possibility of error and are undesirable. Therefore, we restricted the model to the test concentrations of salicylic acid shown in

study 2. As a result, an accurate method to quantify keratolysis has been presented.

Transepidermal water loss

Transepidermal water loss and its measurement are the subjects of a large number of studies. In study 1, new insights into the effect of tape stripping on transepidermal water loss have been presented. Prior to this work, many assumptions had been made regarding TEWL and barrier integrity. The fact that some subjects did not have a TEWL rise despite a significant mass being removed is very important. It suggests that the barrier mass alone is not sufficient to keep water in. Rather, it is likely that the molecular composition is more important. This raises some important questions – for example, which lipids are present (or stripped) at the point at which TEWL starts to rise? What features of the outer layers of the stratum corneum allow it to undergo repetitive damage several times before the TEWL rises?

Before attempting to answer these questions, investigators must be aware that the methods available to measure TEWL are subject to limitations. The major limitation that was apparent in this thesis is that the standard deviation of TEWL rises markedly when the TEWL is very high. This represents not only variation between subjects, but also limitations of the

device. At high TEWL values, the probe head becomes saturated and readings begin to fluctuate wildly. On intact skin, the device usually takes a few seconds to provided a stable reading with a low standard error. At high values, is sometimes impossible to wait for a low standard error but rather the investigator must closely watch the digital printout and decide when the TEWL has reached a plateau. Of course, this means that measurements at the extremes of barrier disruption become more subjective. Fortunately, at baseline and less extreme barrier disruption, measurements of TEWL are extremely reliable and this non-invasive measurement provides valuable information.

pH Measurement

The measurement of pH with a glass electrode is an established method. In this study, we encountered some minor difficulties which were easily overcome. The most important challenge was that the tapes themselves appeared to interfere with the pH measurement. In pilot studies (data not shown) the test site was slightly more acidic after the tape was removed, and this pH seemed to remain constant despite sequential stripping. However, this residue was easily removed with water, and once the site had been cleaned, the pH was akin to previously published studies. The published literature does not mention this difficulty, which may simply

reflect the type of tape used in this study. Once this problem was overcome, the measurement of pH profiles was relatively straightforward.

CONCLUSION

This thesis has achieved its aims. From the data, a clearly usable model of quantitative stratum corneum tape stripping has been presented. The data in the first study has been used to provide insight into the tape stripping model itself and the behaviour the skin barrier in response to tape stripping. This work has demonstrated that depth and water kinetics can be calculated with the new method. The quantitative methods employed have shown that the skin is not a uniform barrier to water, and that water loss rises significantly once the deeper layers have been reached. The failure of barrier disruption in some subjects in spite of tape stripping has been demonstrated and published for the first time.

Further, the work of this thesis has shown that the model can provide new and useful information in skin pharmacology and physiology. For the first time, a quantitative assay of keratolytics has been proposed and published, providing a method to assess efficacy of a whole class of commonly used drugs.

In the study of pH, the work of this thesis has precisely localized the acid mantle in human stratum corneum *in vivo*, and provided a detailed profile of the pH gradients within the skin

There is much work still to be done in each of these areas, and as bioengineering technology advances, the data presented here will surely be refined further. For those interested in stratum corneum biology, this thesis has provided not only reliable methodology but also raised questions for the future.

ABBREVIATIONS

° C: Celsius

µS: micro-Siemens

1 M: 1-molar

8-MOP: 8-methoxypsoralen

ANOVA: Analysis of variance

ATS: Adhesive tape stripping

CoA: Co-enzyme A

DNA: Deoxyribonucleic Acid

GTN: glyceryl trinitrate

GDN: glyceryl dinitrate

HCl: Hydrochloric acid

LSE: Living skin equivalent

NADPH: Nicotinamide adenine dinucleotide phosphate

NaOH: Sodium hydroxide

N/m^2: Newton/meter-squared

OD750: Optical density at 750nm wavelength

PAH: Polycyclic aromatic hydrocarbons

SA: salicylic acid

SC: stratum corneum

SEM: Standard error of the mean

TEWL: Transepidermal water loss

GLOSSARY

Antiporter: An integral membrane transport protein which transports two solutes across a membrane in opposite directions.

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