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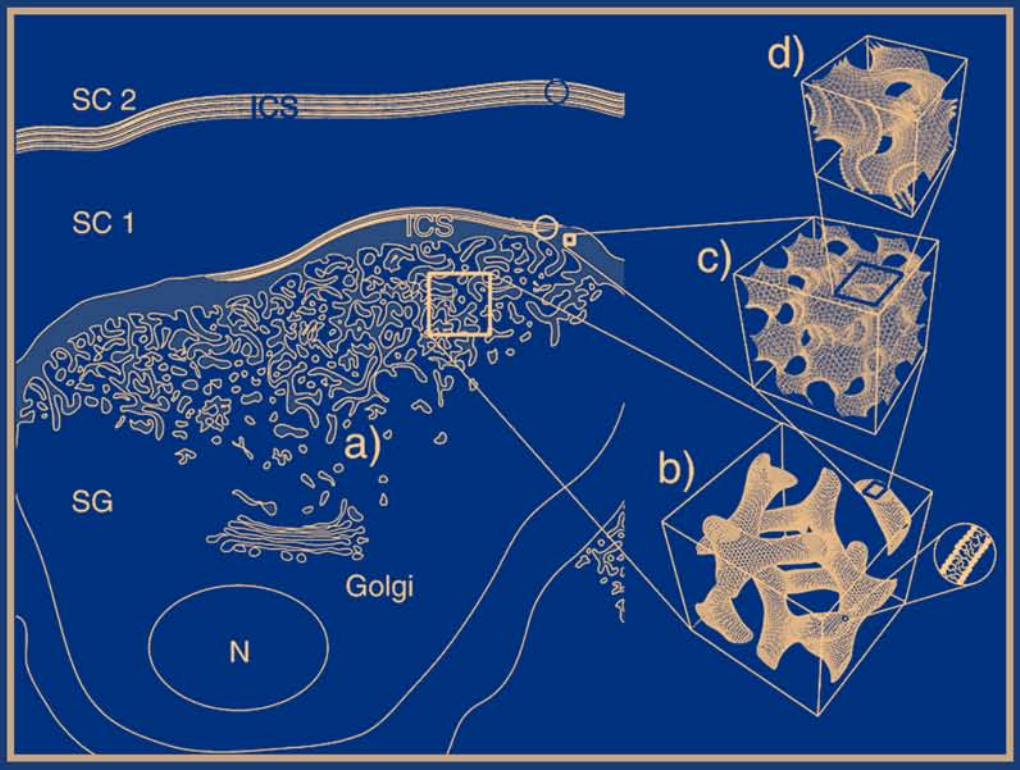
DRY SKIN and MOISTURIZERS

Chemistry and Function



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Preface

In the five years since the first edition of this book was published, there has been an explosion in new information relating to the nature of dry skin and its treatment. Investigators from various disciplines, including dermatologists, pharmacists, chemists, biochemists, molecular biologists, physiologists, pharmacologists, and even psychologists have advanced our knowledge tremendously. We now understand that the stratum corneum has a surprisingly large number of functions in maintaining the physiologic stability and homeostasis of the skin and mind. This second revised and updated edition of *Dry Skin and Moisturizers* is therefore timely and most welcomed.

Our quality of life is influenced not only by the appearance and tactile feel of the skin surface, but also by the sensorial feelings inside the skin. The generic term "dry skin" denotes a diverse family of conditions with a variety of pathologies preceding each one of them. We also recognize the complex interactions between topically applied substances and the skin.

The chapters in this volume provide information to the health care professionals who would like to increase their knowledge about dry skin disorders and learn more about the effects of constituents in moisturizing creams and pharmaceutical preparations. Moreover, the pharmaceutical and cosmetic industry will find information for an improved tailoring of moisturizers according to the underlying skin abnormality. Regulatory and safety issues are also covered. Furthermore, experienced researchers will get inspiring and fruitful ideas due to the easily accessible information about neighboring research areas, which will promote new discoveries. Readers who wish to go deeper into topics in which they have a special interest are able to do so via the numerous references provided.

We are indebted to the 60 renowned international specialists who have contributed excellent chapters for the book. We sincerely hope that you will enjoy reading the book as much as we have enjoyed planning and receiving the chapters from these authors. Suggestions for improvements for coming editions are appreciated.

We also wish to express our appreciation to Ms Erica Dery and Ms Helena Redshaw at CRC Press for accelerating the editorial process.

Marie Lodén
Howard I. Maibach

Editors

Marie Lodén, Pharm., Dr. Med. Sc., Assoc. Professor, is head of Research and Development for the dermatological company ACO Hud AB, in Stockholm, Sweden. She obtained her pharmacist degree in 1980 from Uppsala University, received her doctoral degree in Medical Science in 1995, and began her dermatological research with chemical warfare agents at the National Defense Research Institute. She assumed her present position in 1992. Dr. Lodén was appointed associate professor in experimental dermatology at Uppsala University in 2005.

Dr. Lodén is a member of several national and international societies such as the International Society for Bioengineering and the Skin, and acts in the Committee of the European Group of Efficacy Measurements of Cosmetics and Other Topical Products (EEMCO) and the Scandinavian Society of Cosmetic Chemists (SCANCOS). She is also a member of the editorial board of the *International Journal of Cosmetic Science*.

Dr. Lodén's major research interests are skin barrier function, contact dermatitis, skin care, biophysical measurements, and toxicology. She has published almost 70 original papers and book chapters.

Howard Maibach, M.D., is a professor of dermatology at the University of California, San Francisco and has been a long-term contributor to experimental research in dermatopharmacology and to clinical research on contact dermatitis, contact urticaria, and other skin conditions.

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Contents

1 Introduction: Perspectives and Prospects

Albert Kligman

In the five years since the first edition was published, advances in our knowledge of the nature of dry skin and its treatment have been nothing short of stunning. An enormous amount of new information has been produced by an international confederation of investigators from diverse disciplines, encompassing dermatologists, biochemists, molecular biologists, geneticists, anatomists, physiologists, pharmacologists, immunologists, and even psychologists.

More than 350 scientific papers on dry skin have been published in the last five years. Dry skin is a popular topic in scores of women's magazines giving rise to a thriving industry whose annual sales in the United States alone are about 40 billion dollars, equal to the budget of the National Institutes of Health. More than 90% of women apply moisturizers to their face and hands every day for most of their lives. Nonetheless, authors of dermatology give short shrift to the subject, relegating it to a minor condition among the panoply of dermatologic disorders. Academicians frequently take the position that dry skin disorders are trivial, banal problems, amounting to nothing more than cosmetic nuisances that are disagreeable but not important to health and well-being. This disparaging assessment is simply wrong, stemming from neglect of the subject in the training of medical professionals. Dry skin, especially as the population ages, is becoming ever more serious and worthy of more attention by biomedical professionals. For example, few doctors are aware that the dry, fragile, cracked skin of the hospitalized, immobilized elderly is an early warning sign of impending pressure ulcers which are enormously distressful to patients and an enormous burden on the health care system, worsening steadily as the population continues to age. Moreover, dry, rough, fissured skin is a sure sign of advanced malnutrition, presaging death and disease among the elderly with limited access to the healthcare system. My informal inquiries among diabetologists reveal that few are aware that dry, cracked, fragile skin, which is easily torn, is exceedingly common in insulin dependent patients, presaging dreaded infections of the foot, which may necessitate amputation. Few physicians are sufficiently informed to know which moisturizers among hundreds are most efficacious for treatment and prevention of dry skin syndromes. Treatment is often left to nurses who use whatever moisturizers are available in the supply closets.

Likewise, my experience shows that general practitioners are in no better position than their patients regarding selection of the most effective moisturizers for treating the common everyday dry skin disorders, such as winter xerosis. It should be emphasized that the prevalence of dry skin among the elderly in northern climates approaches 100%. Leaving aside appearance, rough feel, and various discomforts, the most important sensorial component of xerosis is persistent itching which can ruin the quality of life.

It should be appreciated that the generic term "dry skin" denotes a diverse family of conditions ranging from relatively benign disorders such as winter xerosis to devastating, life threatening

hereditary icthyotic states. Dry skin is not a diagnosis any more than the term "rash" to describe inflammatory disorders. A variety of pathologies precede the development of the different varieties of dry skin. Proper classification and an understanding of the pathogenesis of dry skin disorders is the key to proper treatment. This is no easy task.

The commonest way to treat dry skin is through the use of "moisturizers." So, what in fact is a moisturizer and what is dry skin? In my 1978 paper that described the regression method for assessing the efficacy of moisturizers, I professed an operational definition, which has received general acceptance.[1] "A moisturizer is a topical product, which is effective in relieving the signs and symptoms of dry skin." This definition was intended to be useful rather than definitive since very little was known about the anatomy, physiology, and biochemistry of dry skin syndromes. We now know that this definition was an oversimplification of what has turned out to be an exceedingly complex problem, which still continues to puzzle us. Happily, basic research has brought us a great deal of light where formerly dark clouds hovered over nearly every aspect of the subject.

Pierard was not merely being mischievous when he asked "is dry skin really dry" suggesting that, as is often the case in scientific inquiry, the obvious conceals more than it reveals.[2]

The manifestations of dry skin are expressed clinically in the form of scales, a result of faulty desquamation in which corneocytes are shed in clusters rather than as single cells. This immediately directs attention to the outermost portion of the stratum corneum where shedding takes place. However, this focus on surface scaling is misleading and misguided. Merely spraying water on the surface or applying any kind of oil, swells and hydrates the corneocytes so that they do not reflect and scatter light, filling up the empty spaces between loose scales, which immediately obliterate the signs of dry skin. As I earlier noted, even one day of unseemly warm, wet weather in Philadelphia will completely eliminate the appearance of dry skin. Obviously, the underlying pathologic factors leading to scaling are still present. Grading the efficacy of moisturizers based on the visible reduction of scales yields spurious results. To the naked eye every moisturizer works well, enabling hundreds of marginal products to compete in the market place. Sometimes harmful ones are touted as the most effective.

It is now recognized that scaling is simply a common pathway of a variety of disorders related to abnormal epidermal differentiation and cornification. The underlying pathologies however may be very different involving a variety of causal mechanisms. As a result, we are now obliged to look at the dry skin problem at a much deeper layer, using new sophisticated methods to characterize the abnormalities of epidermal differentiation, a tightly regulated process that can go awry from a variety of endogenous and exogenous causes. Methods are now at hand to characterize and quantify abnormal mechanisms.[3]

Dermatology in general has been the beneficiary, usually unacknowledged, of the great advances in our understanding of the biologic significance of the stratum corneum in health and disease. Until recently, the stratum corneum was depicted as having one function, namely acting as a "barrier" to the inward and outward diffusion of endogenous and exogenous substances. The term "barrier," short-hand for the stratum corneum, was always thought of as a passive inert structure, dead as a door nail, little more than a plastic wrapping, comparable to Saran Wrap (my description). Largely owing to the energetic and creative researches of Peter Elias and his academic group in San Francisco, we now understand that the stratum corneum is very much alive metabolically and has a surprising number of functions in maintaining the physiologic stability and homeostasis of normal skin.[4] One of these is worth citing: the stratum corneum is a very lively signaling or semiotic device, rapidly responding to mechanical and chemical stresses and insults inaugurating a series of events aimed at repair and restoration to a normal status. Environmental changes, especially dry, cold weather bring forth a variety of adaptive responses to prevent dehydration.[5] Other functions of the stratum corneum include: (1) a reservoir for various endogenous metabolites, cytokines, and enzymes, (2) a depot for topical drugs such as corticosteroids and anesthetic agents, (3) source of antimicrobials (defensins and carthelicidins).[5] The important contributions of many other scientists located in industry and academia are well-represented in this text.

Homage is also due to the basic investigations of Anthony Rawlings and his coworkers of Unilever regarding the principles of formulating moisturizers and their mechanisms of action right down to the molecular level, again emphasizing the multiplicity of events that have to be synchronized to produce a normal stratum corneum.[6]

The modern period began with Irwin Blank at Harvard who at mid-century laid down the foundation for all future work.[7] He placed a piece of dried callus in various hydrophilic oils, basic ingredients of all moisturizers. The callus remained hard and brittle even after months. Brief immersion in water immediately made the callus soft and elastic. After exposure to soaps, the callus could no longer absorb as much water and became less soft and elastic. These beautifully simple experiments shaped the ways moisturizers were to be formulated thereafter.

Special honors are also due to S. Jacobi, who like Irwin Blank was not a clinician but a Ph.D. scientist. He discovered the famous "natural moisturizing factor," made up of low molecular weight, water-soluble substances, dominantly amino acids, which are chiefly responsible for the ability of the stratum corneum to absorb water and to hold onto it in the face of a hostile dry environment.[8] He went on to show that an extract of the natural moisturizing factor could ameliorate dry skin.

Incidentally, the first important clinical paper on dry skin was written by Louis Duhring, the first professor of dermatology of the University of Pennsylvania School of Medicine, under the interesting title of "pruritus hiemalis," otherwise known as winter xerosis.[9] He found that in susceptible persons cold, dry winter weather in Philadelphia invariably caused reoccurrence of winter xerosis. I cite this informative and eminently readable paper because Duhring emphasized certain features of dry skin that receive little attention, namely purely subjective sensorial symptoms, notably itching (hence the term pruritus in his title) and also other discomforts such as stinging and tightness. Intractable itching is a maddening sensation, antagonistic to sleep, leading to compulsive scratching, which in turn causes further damage to the skin, inaugurating the well-known itch-scratch cycle. We now know that scratching and other forces that disrupt the horny layer initiate the release of a cascade of epidermal proinflammatory cytokines and chemokines, which perpetuate the pathologic process. Scratching not only increases permeability to damaging substances but also promotes colonization and infection by pathogenic microbes such as *S. aureus* and hemolytic streptococci, which in older, immunocompromised persons may go unnoticed in the absence of fever and evaporation but which nonetheless cause malaise and fatigue.

It cannot be said too often that even the ordinary forms of dry skin are not trivial problems about which members of the health profession should be better informed. The chapters in this volume convey all the information which clinicians might want to know.

The marketplace for new and better moisturizers is being driven by a new breed of consumer, mainly women, who are better educated, more demanding, more critical, with more disposable income to boot. We already see the effects in the availability of a plenitude of supra-moisturizers which are designed to accomplish a great deal more than keeping the skin soft, smooth, and moist.

Multiple ingredients are being added to the moisturizer matrix to produce the universal formulation that covers all needs, an impossibility of course. Toward this ideal we already find offerings of products that include sunscreens, antioxidants, vitamins, minerals, phytoestrogens, bioflavenoids, inflammation inhibitors, hormones, enzymes, and everything else. Of course, the claims for many of these products are enormously exaggerated, unsubstantiated, untested, often fictional, aimed only to increase sales in a frenetic, competitive, unregulated marketplace, catering to a credulous susceptible public looking for the newest and the best. I know of one French mega-moisturizer which has 83 "active" ingredients, including 6 sunscreens, 5 free-radical scavengers, 7 macro and micro-minerals, 5 vitamins (including some that have no chemical existence), 9 emollients, 3 hormones, and a vast assortment of ancient Chinese and Indian herbs which have a special appeal to the Green Movement who seem to believe that "naturals" are safer and superior to synthetics. Needless to say, major manufacturers of skin care products who have long been in business in Europe, Japan, and the United States eschew these pseudoscientific concoctions. The notion of multifunctional products is basically sound and the compositions are scientifically certified.

Progress toward the creation of the complete "ideal" moisturizer is already in evidence. New delivery systems give promise of enhanced efficacy, based on liposomes, niosomes, transferosomes, methods of bypassing the horny layer barrier by physical means (electroporation, sound waves, etc.). Standing on the wings is the fantastic possibility of nanotechnology, in which the tiniest solid particles of oils and emollients can be directly delivered to the visible epidermis. Along with these creative stirrings are entirely new approaches to enhance effectiveness. Elias' group has proposed the use of physiologic lipids, analogous to those comprising the bilaminar membrane of the intercellular spaces of the horny layer, which give the horny layer its barrier properties.[10] These are actually prodrugs that incorporate into the lamellar bodies of the epidermis to be subsequently extruded by exocytosis into the newly forming stratum corneum, at and just above the granular layer.[11] In a similar vein, Rawlings' group has proposed what he calls lipid modulation as a way to make moisturizers more effective.[11] The idea here is to incorporate ceramide-dominant lipids in moisturizers to correct for their absence or lack in virtually all dry skin disorders.

Japanese scientists are also experimenting with these so-called "skin-identical lipids" to enhance efficacy.

This second edition covers the problem of dry skin in a comprehensive, complete, systematic way, incorporating the voluminous amount of new knowledge that has so recently been acquired. All the major players in this consistently enlarging field are represented here along with many other contributors who are enriching our knowledge of the subject.

This new volume is certain to become a standard reference text for a varied readership with different interests who will be able to locate within this expanded new text the specific information being sought.

The editors themselves are major players in this field who with a wealth of personal experimental experience, have produced a one of a kind volume that merits great praise as a scholarly practical treatise for those who want a reference source that includes everything that is worth knowing about the subject of dry skin and its treatment.

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Part I

The Skin

Biochemistry and Function

2 The Skin as a Barrier

*Magnus Lindberg and Bo Forslind**

CONTENTS

2.1 INTRODUCTION

This chapter will deal with the stratum corneum barrier with a special focus on structure–function relationships. For this reason our approach has been to describe some details of the epidermal physiology that have a bearing on upholding the barrier function. We see it as important that skin barrier function is regarded as part of the dynamic processes of cellular transformation during the differentiation of epidermal keratinocytes, hence dependent on the status of the skin.

It is taken for granted that the skin barrier prevents foreign material from entering the system. But, a deeper insight into the barrier function of the integument makes it clear that the primary function of the barrier is to prevent water loss, and the barrier toward environmental factors is only of secondary importance, albeit very important.[1] The water homeostasis is absolutely necessary for normal physiology, and the role of the kidneys is to maintain that homeostasis. Therefore, the integument should represent a water-impermeable "bag." However, we have to account for the perspiratio insensibilis, which obviously has its origin in the need for a hydration of the corneocytes. Water acts as a plasticizer on the corneocyte keratin, giving the cells the necessary elastic properties. If deprived of water, a dry skin is prone to crack open at mechanical stress. Since the relative humidity of the environment varies enormously, the corneocytes have to be hydrated from a permanent water source, the body. The fact that the perspiratio insensibilis is markedly constant reveals that this water leakage is not a defect in the barrier, but an inbuilt factor with a required function.

* Deceased author.

2.2 THE CORNEOCYTES CONSTITUTE A SCAFFOLD FOR THE BARRIER LIPIDS

The entire horny layer, the stratum corneum, can be regarded as the outer barrier of the skin. Although at a closer look there is a differentiation in lipid structure and composition across stratum corneum. The horny layer is continuously exposed to contact with the environment and suffers from the effects of chemical and physical agents, which will cause a continuous loss of material. We can assume that the daily loss of material over the entire body surface ($\sim$1.8 m^2) corresponds to a "film," the thickness of which is at least that of a corneocyte. Assuming that the surface of a corneocyte is $\sim$1000 μm^2, this surface "film" corresponds roughly to $1.8 * 109$ cells. The thickness of a corneocyte is $\sim$0.3 μm, and with a specific weight of 0.75 kg m^{-3} (= protein) these data can be used to calculate a daily loss of about 40 mg of horny cells, most likely an underestimation. Thus, the total amount of material in this turnover is not negligible. This continuous renewal of cells is a prerequisite for keeping the thickness of stratum corneum approximately constant and thus the barrier intact in all its aspects. It has been demonstrated that the control of barrier homeostasis is under strict control. The transepidermal water loss (TEWL) and the Ca^{2+} distribution appear to be important signals controlling the mechanisms involved in the homeostasis of stratum corneum[2–4] such as up-regulation of lipid synthesis. Other important factors are the distribution of sodium and potassium within epidermis and the pH-gradient across stratum corneum.

Through autoradiographic investigations it has been shown that a corneocyte stems from 1 of about 20 basal cells under the projected area of a corneocyte.[5,6] This is the so-called proliferative unit (Figure 2.1). The cells on the basal lamina communicate via gap junctions, and through this means a regulation of cell division is possible within the proliferative unit controlling the progeny

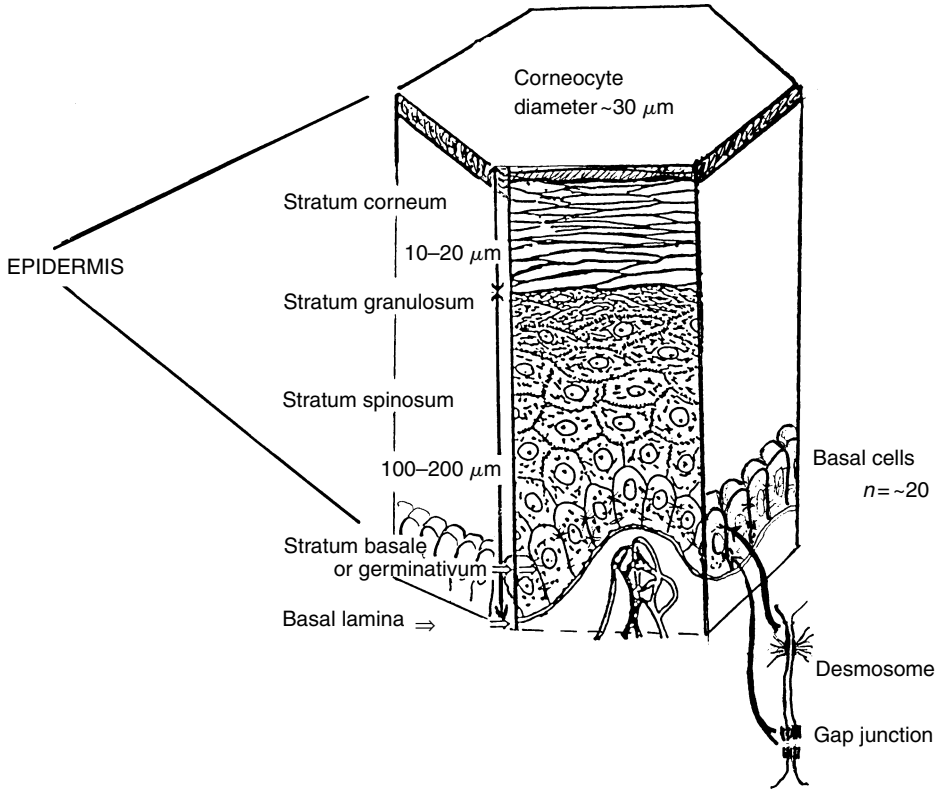

FIGURE 2.1 The proliferative unit as deduced from Potten.[5,6]

travel from the stratum basale to the stratum corneum at a pace that ensures a smooth surface.[7] An additional controlling mechanism may be the shift in the Na/K ratio that occurs as the cells move into the stratum spinosum.[8] Thus, higher than normal Na and lower than normal K concentrations within the cell of the upper stratum will effectively hinder the cell to enter the cell division cycle.

2.3 CORNEOCYTE STRUCTURE

A corneocyte can be described as a very flat cell, about 30 μm in diameter and ~0.3 μm thick, filled with keratin inside a protein envelope. Keratin is a highly hydrophilic material that can bind substantial amounts of water, and we discern a fibrous component as well as an amorphous one. The fibrils, 8 nm in diameter, span the inside of the corneocyte and thus constitute an internal reinforcement ensuring that the cell form in the plane of the skin remains virtually unchanged even at long exposures to water. This is achieved by an orientation of the fibrils in the plane of the cell (Figure 2.2[a]). In the vertical dimension there are virtually no reinforcement fibrils, and thus the cells have more freedom to swell in this direction. Norlén et al.[9] have actually shown that the swelling is less than 5% in the horizontal dimension, but can be more than 25% in the vertical dimension. This ensures a minimal roughness of the skin surface even at maximal swelling, thus minimizing the risk of surface breaks at mechanical stress on wet skin. The conspicuously thicker stratum corneum

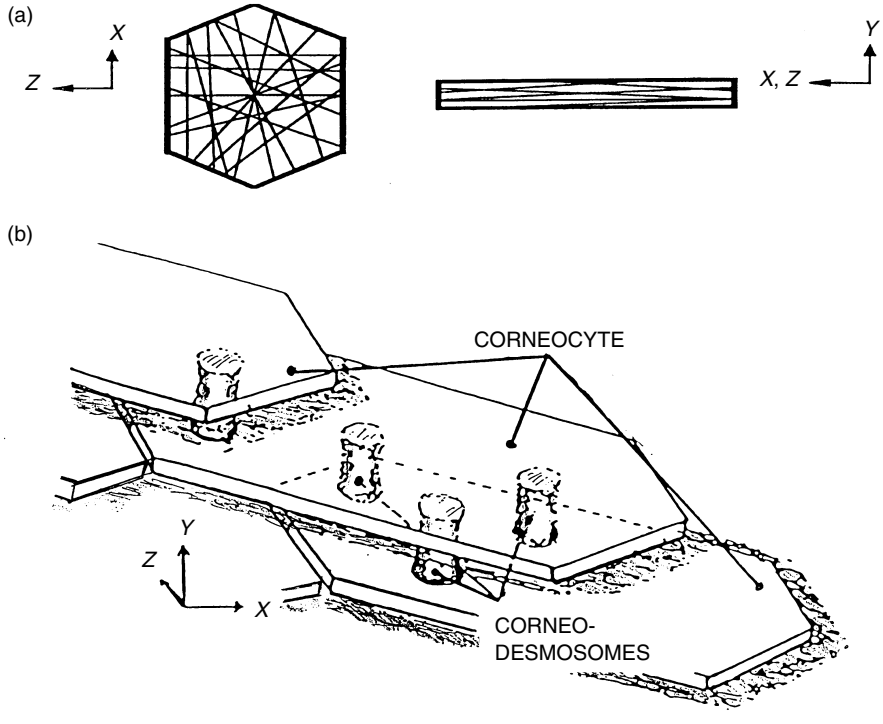

FIGURE 2.2 (a) The corneocyte is a flat, hexagonal-like structure with a surface area of about 1000 μm^2 and a thickness of 0.3 μm. A protein envelope encloses a cell compartment containing only fibrous and amorphous keratin. The keratin fibrils inside the cell are randomly oriented in the plane of the cell and constitute an internal reinforcement, which ensures that the cell form in the plane of the skin is preserved within very narrow limits. (b) The classic view of corneocytes coupled to each other through protein "rivets," corneodesmosomes. This arrangement makes a mechanically rigid scaffold. The lipid bilayers, which are separated by thin water sheaths and are mechanically very soft, are protected from sliding relative to each other and being directly exposed to mechanical shear that would break up the structure.

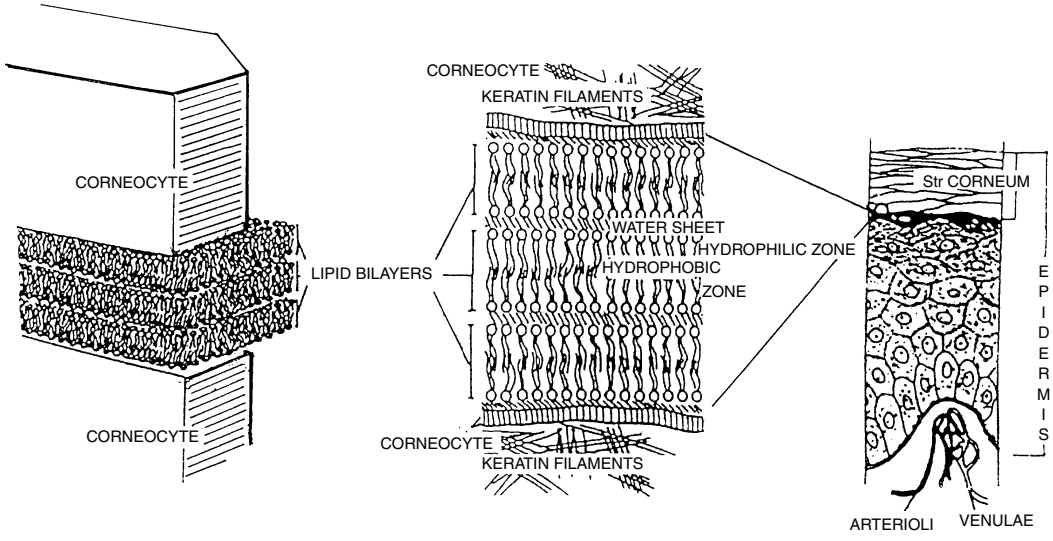

FIGURE 2.3 Stacked bilayers of lipids are inserted into the extracellular space of the corneocyte scaffold to form the lipid barrier of the skin.

of the palms and foot soles do indeed become wrinkled at maximal swelling, but here a conspicuous thickness of the stratum corneum compensates for this roughness.

The classical view of stratum corneum includes the presence of desmosome rivets ("corneosomes"). The corneocytes are mutually joined by these desmosome rivets that effectively hinder the cells to move in relation to each other in the plane of the skin (Figure 2.2[b]). This prevents shearing forces from disrupting the stacked bilamellar lipid structures in the extracellular space (Figure 2.3). The desmosome "rivets" also prevent this space from being increased due to mechanical forces imposed on the skin. Today it is known that there is an ongoing, partly pH-dependent, enzyme activity in stratum corneum including both lipases and proteases, which are involved in the process of corneocyte desquamation.[10] The activity of these proteolytic enzymes, such as the stratum corneum tryptic enzyme,[11] is necessary for degrading protein structures allowing for the desquamation of corneocytes. A new view of the ultrastructure of the skin and especially stratum corneum structures has presently been published.[12,13] By using a new method with instant freezing of a tissue sample allowing for a complete vitrification, it has become possible to produce skin sections for low temperature cryotransmission electron microscopy. The newly obtained structural information on the protein–lipid interaction in stratum corneum suggests that the classical view of the desmosome rivets has to be reevaluated (Lars Norlén, pers. comm.).

2.4 THE HYDROPHILIC AND THE HYDROPHOBIC PATHWAYS THROUGH THE SKIN BARRIER

Looking at the barrier in more detail, we find that it can be described as composed of two main components. Interspersed between the corneocytes we find the "hydrophobic" (water-repellent) substance, the barrier lipids. The keratinized corneocytes containing fibrous and amorphous proteins represent a "hydrophilic" (water-attracting) component. Neutral lipids (fatty acids, cholesterol) and ceramides dominate the lipid phase, and it is mainly these lipids that are responsible for the control and limitation of water transport through the skin.[14] Visualization of the penetration pathway through the skin by tracer methods has demonstrated that the extracellular pathway is likely to be the only route through the barrier for substances other than water.[15] Water diffusion through the keratinocytes

is not expected to occur freely due to the fact that keratin will adsorb water. The bound water is likely to take on a certain degree of structured organization; hence the amount of freely diffusible water will be comparatively small. Consequently, the water transport through the keratinocytes will be impeded. Norlén et al.[16] have shown that water permeation through lipid-extracted stratum corneum membranes is only about three times higher than through a nonextracted stratum corneum membrane.

2.5 THE PHYSICAL STATE OF THE LIPIDS DETERMINES THE PROPERTIES OF A LIPID MEMBRANE OR BARRIER

Lipids that can form biological membranes are characterized by a hydrophilic head group and a hydrophobic part, usually a carbon chain (cf. fatty acids versus cholesterol). From physical, thermodynamic considerations it can be shown that it takes a lot of energy to keep the hydrophobic part of a lipid dissolved in a water solution.[17] For this reason lipids tend to aggregate in micelles or bilayers. This means that they form a hydrophobic compartment [or phase], which encloses the carbon chains that separate them from water. The hydrophilic head groups face the water and thus constitute a border between a hydrophobic phase and water (Figure 2.4). A number of factors determine how stable such aggregates are.[18] These include temperature, the length of the hydrophobic carbon chain, their degree of unsaturation [double bonds], the temperature, the water content, the presence of divalent ions, etc.

Temperature is in general an important factor for lipid membrane configuration. It has been demonstrated that lipid membranes exist in two main physical states: one extremely close-packed, the crystalline state (Figure 2.4[a]), and the other, the liquid crystalline state (see Figure 2.4[b]). In the latter state the structure is more open, and the lipid units are free to diffuse in the plane of the membrane. This actually allows water molecules to pass right through the membrane. The transition between these two main states is determined by the so-called transition temperature, and this is in turn dependent on the particular properties of the lipids forming the membrane[17] (Figure 2.4[c]). Lipids with short chains and lipids that are unsaturated have their transition temperature at lower temperatures than long-chain and saturated lipids. Biological membranes (bilayers) are generally complex mixtures of different lipid species, and the transition temperature for such a structure is expected to vary with the actual proportions of the lipid components. This also means that the transition occurs within a comparatively broad temperature interval compared to the corresponding sharply defined interval of a single lipid species.[8]

As a generalization, we may be allowed to state that the transition temperature for cell membranes in biological living systems is found between 0 and 40°C and the chain lengths are between 16 and 18 carbons. This is in conspicuous contrast to the lipids of the stratum corneum barrier where chain lengths up to and over 30 carbons have been demonstrated.[14,19] From such facts we expect the transition temperature of the skin barrier lipids to be around 40°C, and this has also been substantiated in a number of investigations.[20–22] This means that under normal conditions with a skin temperature about 30°C, the barrier will essentially be impermeable to water.

Straight carbon chains can be housed in comparatively small volumes and allow van der Waal's forces to act and cause a close packing (Figure 2.4[a]). The van der Waal's forces are not effective if the distance between the atoms is several atoms in diameter.[18] Double bonds tend to create kinks on the carbon chains, preventing them from close apposition with neighbor chains, which is a prerequisite for allowing the weak van der Waal's forces to contribute to a close packing of the chains. Thus, kinked carbon chains hinder close packing of the lipid chains and promote a liquid crystalline state of the bilayer where the lipid units are allowed to diffuse in the plane of the bilayer[23] (see Figure 2.4[b]). A cell membrane is actually this kind of structure with a very rapid diffusion of lipids within the membrane and therefore allows almost free passage of water in both directions over the membrane. The important message here is that the cell membrane is not a water barrier.

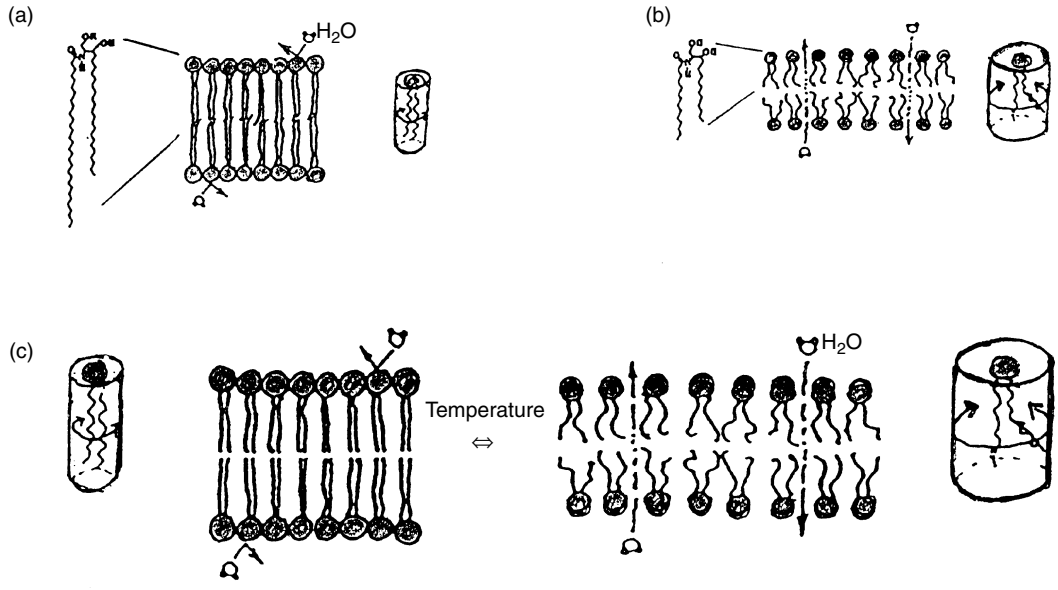

FIGURE 2.4 (a) Long, saturated carbon chains can attract each other through van der Waal's forces, and this causes a tight, close-packed crystalline structure that is impermeable to water. Straight (saturated) carbon chains demand less space than kinked (unsaturated) chains. Saturated long aliphatic chains ($C > 20$ carbons) tend to pack close at skin temperatures (26 to 32°C). When associated with water, there may still be a freedom of rotation along the carbon chain axis and the structure is sometimes denoted gel phase. (b) Short carbon chains and carbon chains with a double bond form liquid crystalline structures, where the chains of the bilayer show high degrees of freedom to diffuse in the plane of the bilayer. The liquid crystalline state thus becomes favored if one of the carbon chains is unsaturated. (c) The transition temperature of bilamellar lipid structures. Long, saturated carbon chains (left), tightly close packed form a crystalline structure that is impermeable to water. Short carbon chains (right) form liquid crystalline structures where the chains of the bilayer show high degrees of freedom to diffuse in the plane of the bilayer. The transition between these two states is dependent on temperature, chain length, and degree of unsaturation of the chain. If the temperature is lowered, the thermal movements of the chains decrease and van der Waal's attraction forces become operative; the structure becomes crystalline and impermeable to water. Thus, the transition between these two states depends on the parameters of temperature, chain length, and degree of unsaturation of the chain. Saturated, long aliphatic chains ($C > 20$ carbons) tend to pack close at skin temperatures (26 to 32°C).

This is in sharp contrast to the conditions in stratum corneum where the lipid membranes are almost impermeable to water. As a consequence of these facts, we expect the bulk of lipids that form the skin barrier to be in a crystalline (gel) state, that is, to have long carbon chains ($C > 20$:0) to comply with the physical requirement that the transition temperature should be higher than normal skin temperature (>35°C). A physiological mixture of ceramides, free fatty acids (FFA), and cholesterol is indeed needed for a normal barrier function.

2.6 THE CERAMIDES OF THE HUMAN SKIN BARRIER

At physiological pH the long-chain ceramides of the horny layer barrier in the presence of cholesterol and fatty acids have been shown to have equal capacity to form lamellar lipid structures as have phospholipids.[24,25] The chain length of the ceramides is to a great extent longer than 18 carbons, even up to 34 carbons in one of the chains, and this suggests close packing of the crystalline type at normal skin temperatures.

Several classes of ceramides have been described in human skin.[14] Today it is considered that the ceramides are essential for the barrier properties. It has been suggested that the lower amount of ceramides found in stratum corneum in atopic dermatitis[26,27] explains the increased TEWL seen in dry atopic skin. In this context it is of special interest to note that part of the long-chain ceramides of the horny layer are covalently bound to the proteins forming the corneocyte envelope.[25] This suggests that such lipids constitute anchors of the hydrophobic phase to the corneocytes and thereby add to the cohesion of the cells of the horny layer.

2.7 FREE FATTY ACIDS AND CHOLESTEROL

The recent data of Norlén et al.[16,28] demonstrate that the FFA retrieved from stripped lower arm skin (and therefore essentially uncontaminated by sebum lipids) are all saturated and long-chain species ($C > 20$). This harmonizes with lipid data from epidermal cysts, which are virtually free from triglycerides of sebum origin.[29] Furthermore, the ceramides of the barrier lipids are all long-chain species and therefore also comply with the requirement set up for a water-impermeable barrier.

The third class of lipids found in stratum corneum extracts is represented by cholesterol and cholesteryl esters. The actual role of cholesterol remains enigmatic, and no clear reason for its role in the barrier function has been proposed so far. However, it is possible that contrary to what is the role in cell membranes where cholesterol increases close packing of phospholipids, it can act as kind of a detergent in lipid bilayers of long-chain, saturated lipids.[30,31] This would allow some fraction of the barrier to be in a liquid crystalline state, hence water permeable in spite of the fact that not only ceramides, but also fatty acids found in the barrier are saturated, long-chain species.[28,32]

2.8 LIPID GRADIENTS WITHIN STRATUM CORNEUM

Some data indicates that there is a change in composition and arrangement of the lipids during the transition through stratum corneum.[30,31] This can in part depend on the presence of lipids from sebum secretion. Together with the decrease in water across stratum corneum it is possible that there is a rearrangement of the lipid structure, which in turn can be of importance for the desquamation process.

2.9 STRUCTURE OF STRATUM CORNEUM — BARRIER MODELS

2.9.1 The Brick and Mortar Model

In 1975, Michaels et al.[33] presented a conceptual model of the arrangement of corneocytes and lipids in stratum corneum. They envisaged stratum corneum as a brick and mortar structure with the keratin filled corneocytes as bricks and the intercellular lipids as mortar. This model was further explored by Elias and co-worker.[34–37] This model does not per se include a structure–function perspective on the barrier but has had a tremendous impact on the research on stratum corneum and its composition, function, and the regulation of homeostasis.

2.9.2 The Domain Mosaic Model

In 1994 Forslind presented a more structure–function orientated model, the domain mosaic model.[38] With the background given previously, the requirements on the stratum corneum barrier can be summarized as follows: the barrier should be watertight but still allow a small, controlled amount of water to leak from the system in order to keep the corneocyte keratin hydrated.

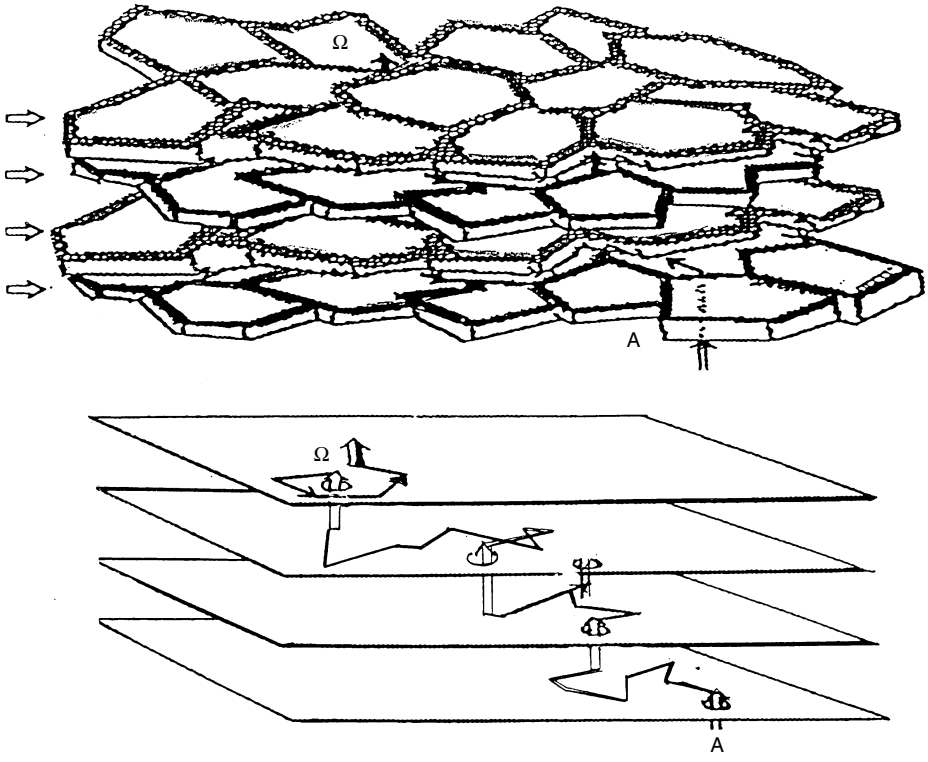

FIGURE 2.5 The stacked bilayers of the skin barrier are envisioned as composed of crystalline domains separated by fringes of lipids in the liquid crystalline state.[38] The fringe zones may actually oscillate in a very small time scale between a liquid crystalline state and a crystalline (gel) state. Such a tentative idea would mean that the barrier is open just temporarily at a certain location since penetration must occur in the liquid crystalline areas. Thus, the action of a penetration enhancer would be to "stabilize" a liquid crystalline state or transform it into another type of structure, for example, a cubic phase.

From these requirements we may infer a structure where the bulk of the intercellular lipids exist in the crystalline, close-packed state in stacked bilayer structures (Figure 2.5) due to the large amounts of long-chain saturated species. However, circumstantial evidence, for example, TEWL, indicates that a fraction of the lipid compartment should be in the liquid crystalline state, but as yet we do not know the composition of this fraction. Again the role of cholesterol may be crucial, as mentioned earlier.

Accepting that the bulk of barrier lipids are in the watertight crystalline state we may depict the bilayers as composed of crystalline domains separated by lipids in the liquid crystalline state.[38,39] The cross section of a domain can tentatively be assumed to be of the same size as the cross section of a lamellar granule, the structure from which the lipids are extruded into the extracellular space of the stratum corneum, that is, ~200 nm. Several bilayers are stacked on top of each other and separated by a thin film of water adherent to the hydrophilic head groups (Figure 2.3). Since it is unlikely that the crystalline domains are exactly uniform in size and form, we do not expect the fluid crystalline interdomain areas to overlap precisely. A water molecule leaving the body via the stratum corneum on a downhill diffusion gradient will therefore have to suffer a tortuous, meandering way through the lipid barrier.[40,41] (Figure 2.5). In the water sheath separating the bilayers, the water molecule will diffuse randomly until it finds a "hole-in-the-roof," that is, a liquid crystalline phase through which it can tunnel into the next, overlaying water sheath. Considering the fact that it, in addition to a number of water molecules, will have to circumvent water-saturated corneocytes, shows us that the path out to the environment will be extremely long, hence the actual low value of the TEWL.

2.9.3 The Single Gel Model and the Sandwich Model

Models of stratum corneum have been further developed. During the past few years two major and substantially different models have been proposed, the Single gel phase model by Norlén (for reviews, cf.[30,42]) and the Sandwich model by Pilgram and Bouwstra (for reviews, cf.[31,43]). The basic concept of the Single gel model is that the lipids forming the lipid phase in stratum corneum are present in one, continues gel phase without phase separation.[44] The model also includes a new view on the formation of the lipid phase of stratum corneum.[45] Based on ultrastructural analysis of serial sections and freeze sections of vitrified skin biopsies[12] it is postulated that the lipid phase is formed as a continuous tubular system within the upper part of the epidermal keratinocytes, also continuous with the cell membrane. At the interface between stratum granulosum and stratum corneum the tubular structures are unfolded into the intercellular space. This model of formation contradicts the classical view of lamellar bodies with preformed lipid membranes in the keratinocytes, the Landmann model.[46] It is postulated that this model would be compatible with lowest energy cost for producing the lipid phase of stratum corneum. In the Sandwich model the lipids of stratum corneum are proposed to be arranged in membranes with alternating crystalline and liquid crystalline phases. The importance of cholesterol sulphate, pH, and calcium ions has been highlighted in this model.[47,48] Pros and cons for these models have been discussed extensively.[30,31,49,50]

2.10 PROPERTIES OF THE LAMELLAR BARRIER — EFFECTS OF PENETRATION ENHANCERS

Based on the concept of the domain mosaic model and the Fick model for downhill gradients over a barrier, Engström[51,52] has presented arguments to show that only a fraction of the total lipid mass of the barrier has to be involved in structural changes that will open up or prevent barrier passage. These ideas were more extensively presented in a sequel publication which demonstrated that enhancement factors for barrier penetration of the order of 100 could easily be obtained for substances with partition coefficients far from one.[40] This is true even if the fraction of the extracellular bilayer that has undergone structural transformation, for example, to a hexagonal or cubic phase, is small, that is, 1 to 10% (Figure 2.6). It is to be noted that the structural transformations, for example, conversion of a lamellar phase into a hexagonal phase, a bicontinuous cubic phase, or a sponge phase, are expected to occur only in the liquid crystalline phase regions between the crystalline domains, hence only a very small part of the total barrier is involved in the process.

It must be realized that structural changes of these kinds are local phenomena. This reasoning implies that a penetration enhancer introduced into the lipid barrier is expected to diffuse in the liquid crystalline phase and exert its structure transformation effects more or less exclusively there. Within a relatively short time it will also be diluted through this diffusion process and then the bilayer structure will be restored and the normal barrier function will be regained.

A problem that is rarely taken into account is related to the fact that the water concentration shows a conspicuous gradient within the stratum corneum thickness. These factors are likely to influence the physical state of lipids in bilayer formations, and therefore we expect lipid barrier structure to vary within the thickness of the stratum corneum.[53]

2.11 CONCLUSIONS — BARRIER PENETRATION IN A FUNCTIONAL PERSPECTIVE

In the past, barrier research for the most part had the character of “black box” descriptions of the dynamics of substance penetration through the skin. Today, skin barrier research is oriented toward an understanding of the molecular structures of penetrants and the lipid bilayers including

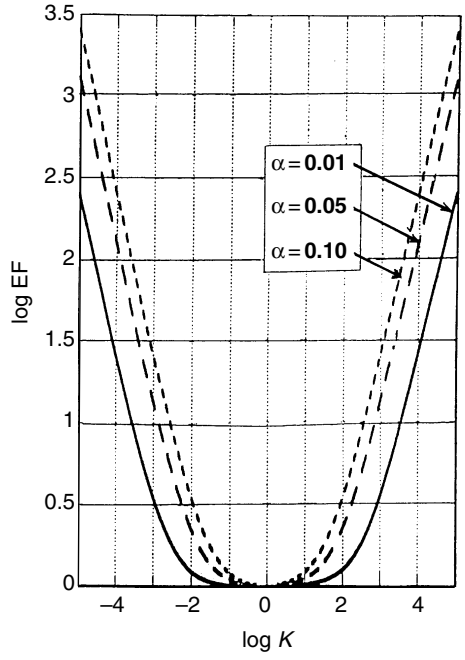

FIGURE 2.6 The enhancement factor EF (log EF) plotted versus the partition coefficient (log K) demonstrates that small changes in the fraction a of the liquid crystalline phase of the barrier that undergoes a structural transformation from lamellar to cubic, hexagonal, etc. phase causes vast changes in the EF.[51]

processes and structural events occurring at penetration. Our knowledge of the actual lipid barrier structure(s) and its detailed function is starting to emerge. Biophysical techniques such as x-ray diffraction, NMR, and FTIR have confirmed that a large part of the barrier lipids are in a crystalline state.[31,43,54–56] This is supported by lipid analyses of stripped human skin extracted *in vivo*, which has demonstrated that the FFA and the ceramides are long-chain species ($C > 22$) and hence should pack in crystalline (gel) structures at skin temperature. The role of cholesterol remains enigmatic, but is likely to influence the structural organization of the FFA and ceramides. New structural evidences contributing to a broader understanding of the organization and function of stratum corneum[12,44–45] is now published. These findings are to some extent contra dictionary to the structural data obtained by other techniques.

The lipid bilayers of the stratum corneum not only constitute a barrier, but may also function as a pool from which substances can slowly penetrate into the system on a downhill gradient. The actual effect of solvents and detergents on barrier lipid structure is not known in any satisfactory detail. Likewise, we are only starting to understand how different moisturizers might influence the structure and function of the barrier. We still lack an understanding of how the composition of the ceramide, FFA, and cholesterol influences the defect barrier in some pathological disorders, for example, dry atopic skin.

The unique character and the particular composition of the human skin barrier lipids call for investigations on human skin, possibly pig skin, and to a great extent preclude rodents as models for barrier function in penetration studies.

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3 Epidermal Lipids and Formation of the Barrier of the Skin

Philip W. Wertz

CONTENTS

3.1 LIPIDS IN THE EPIDERMIS

3.1.1 Introduction

The evolution of life in the relatively dry terrestrial environment required the development of a waterproof integument.[1] In the terrestrial vertebrates, the stratum corneum provides the primary barrier to water loss. The barrier function of the stratum corneum depends upon a unique mixture of lipids that form lamellar structures in the intercellular spaces.[2–5] This generally consists of ceramides, cholesterol, and long chain fatty acids.

In human epidermis, the lipid end products of differentiation consist of cholesterol, 22- through 28-carbon straight chain saturated fatty acids, and nine different series of ceramides.[5,6] The building blocks from which the ceramides are composed include sphingosine, phytosphingosine, and 6-hydroxysphingosine as the base components and normal saturated fatty acids, α-hydroxyacids, and ω-hydroxyacids as the amide-linked fatty acids. In addition, the ω-hydroxyacid-containing ceramides bear ester-linked linoleate on the ω-hydroxyl group. All nine possible combinations of base–acid pairings are formed.[6] Representative structures are shown in Figure 3.1.

In addition to the free lipids found in the intercellular spaces of the stratum corneum ω-hydroxyceramides, ω-hydroxyacids, and fatty acids are covalently attached to the outer surface of the cornified envelope.[7–9] The hydroxyceramides and hydroxyacids are thought to be attached through ester-linkages involving glutamic or aspartic acid side chains,[10] while the fatty acids are thought to be attached through formation of ester linkages with serine or threonine hydroxyl groups. Evidence has been presented indicating that transglutaminase 1 may be responsible for attachment of the hydroxyceramides to the envelope.[11] Representative structures of the covalently bound lipids are presented in Figure 3.2.

The literature is replete with the use of chromatographic fraction numbers to indicate ceramide structural types. This can be very confusing because different laboratories have achieved different degrees of separation and because, even with the best resolution achieved, there is at least one fraction

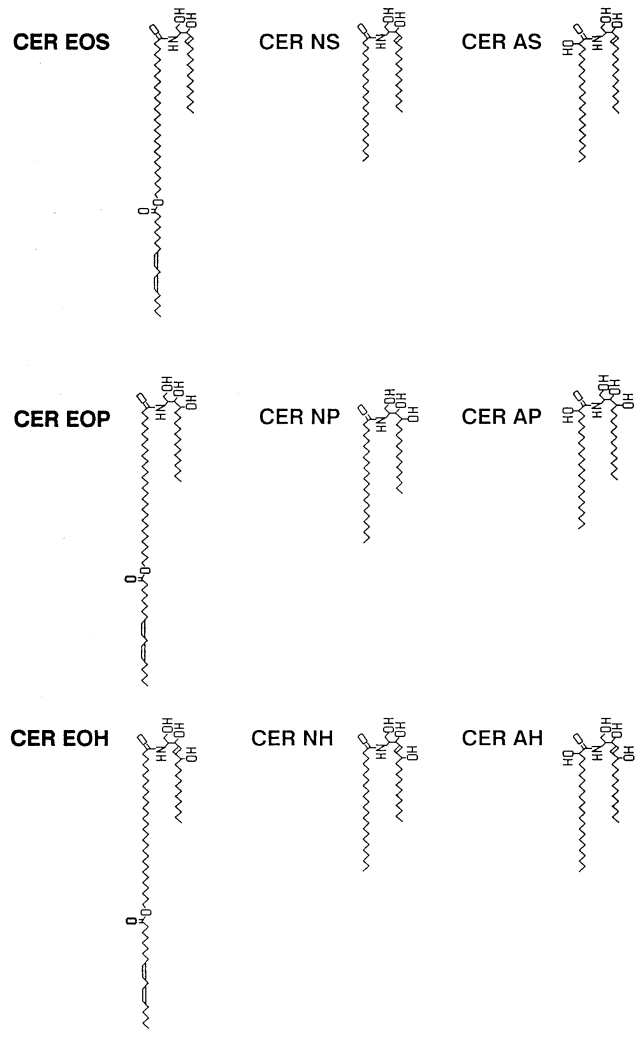

FIGURE 3.1 Representative structures of human stratum corneum ceramides.

that contains two structural types of ceramides. A solution to this problem is a nomenclature system in which the long chain base and amide-linked fatty acids are designated by single letters[12]: S for sphingosine, P for phytosphingosine, H for 6-hydroxysphingosine, N for normal fatty acid, A for α-hydroxyacid, and O for ω-hydroxyacid. The presence of an ester-linked fatty acid is indicated by a prefix E. Thus the acylceramide in which ω-hydroxyacid is amide-linked to sphingosine and linoleate is ester-linked to the ω-hydroxyl group would be designated as ceramide EOS. Similarly, the ceramide consisting of normal fatty acids amide-linked to phytosphingosine would be ceramide NP. This nomenclature is used in Figure 3.1 and Figure 3.2.

Both x-ray diffraction studies and investigations using transmission electron microscopy have indicated that the intercellular lipids are organized into 13 nm trilaminar structures.[13–15] The formation of these trilaminar units seems to require ceramide EOS,[16,17] although to a lesser extent supplementation of lipid mixtures with synthetic EOP can promote self assembly.[18] The possible role of ceramide EOH has not been studied directly; however, it is clear that the natural proportion of ceramide EOH[6] is probably insufficient to promote self assembly of 13 nm units. A transmission electron micrograph of the intercellular lipid lamellae is shown in Figure 3.3. Controversy exists regarding details of the organizational state of the intercellular lipids.[19–21]

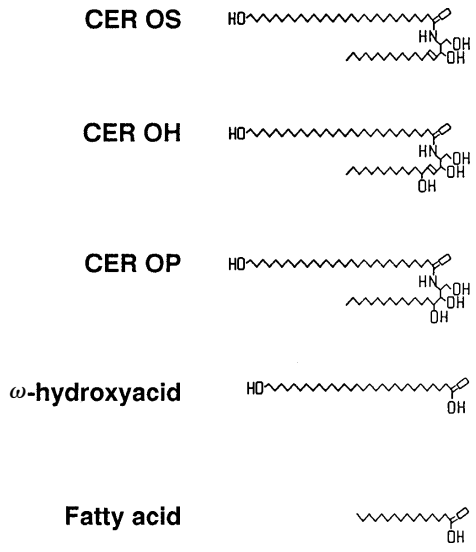

FIGURE 3.2 Representative structures of covalently bound lipids from human stratum corneum.

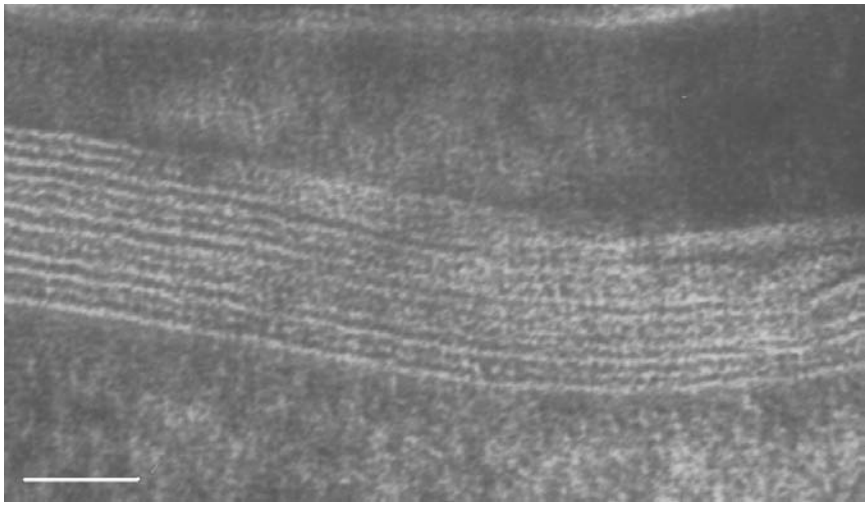

FIGURE 3.3 Intercellular lipid lamellae in the stratum corneum. Bar equals 30 nm.

Interactions between water and the polar head groups of lipid molecules are necessary for the formation of lamellar phases; however, it appears that there is no free water associated with the 13 nm trilaminar units. This is supported by the observation that this periodicity does not increase with increasing stratum corneum water content.[22] There is likely water hydrogen bonded to the polar regions of the lamellae. In contrast, the minor short periodicity swells from 5.8 to 6.6 nm as the water content of stratum corneum increases from 12 to 50%.[23] This suggests that the lipid lamellae are simple individual bilayers and free water molecules can exist between adjacent bilayers, thus causing the increase in the lamellar spacing.

3.1.2 Carbon Sources

Linoleic acid is the parent essential fatty acid of the ω-6 series, and as such, must be obtained in the diet.[24] It is primarily derived from vegetables and nontropical vegetable oils. When linoleate was

injected intradermally into porcine skin, it was initially taken up into a small, rapidly turning over pool of triglycerides.[25] It was rapidly transferred to phosphoglycerides, then to an acylglucosylceramide (glycosylated version of ceramide EOS), and finally to ceramide EOS. Basal keratinocytes have low density lipoprotein (LDL) receptors and can thereby derive cholesterol from the circulation; however, once keratinocytes move upward and out of the basal layer, the LDL receptors are internalized and degraded.[26] Except for linoleate and lipid internalized via basal cell LDL receptors, it is thought that most of the remaining carbon for epidermal lipid synthesis is derived from circulating acetate.[27] Although cultured keratinocytes have been shown to incorporate carbon from glucose into lipids, when radiolabelled glucose was injected intradermally, only the glycerol moiety of the phosphoglycerides became labeled.[27]

3.1.3 Energy Production

Adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are required to support lipid biosynthesis. Basal keratinocytes have functional mitochondria and are thought to produce energy by β-oxidation of fatty acids.[28,29] The enzymes necessary for glycolysis are also present, but this is much less efficient than β-oxidation and the mitochondrial system. However, as cells move upward metabolism becomes increasingly more anaerobic until in the granular layer energy is produced entirely by anaerobic glycolysis with reduction of pyruvate to lactate. In fact, the mitochondria are degraded. The degradation of mitochondria and other internal membranous organelles would result in the release of calcium previously sequestered by these structures. The generation of the calcium gradient is one of the factors driving differentiation.[30]

3.1.4 Major Biosynthetic Pathways

All the major biosynthetic pathways use acetyl-CoA as the basic building block, and in each pathway the rate limiting enzyme is regulated by phosphorylation with the phosphorylated enzyme being active. In the biosynthesis of cholesterol, the rate limiting step is catalyzed by hydroxymethylglutaryl-CoA (HMG-CoA) reductase. Initially, three molecules of acetyl-CoA are condensed to produce β-HMG-CoA. HMG-CoA reductase then uses two NADPH molecules to reduce HMG-CoA to mevalonate-CoA. The remaining steps in cholesterol biosynthesis are numerous and well-documented.

The rate limiting step in fatty acid synthesis is catalyzed by acetyl-CoA carboxylase to produce malonyl-CoA at the expense of one ATP.[31] Malonate and acetate are transferred from CoA to acyl carrier protein in the cytosolic fatty acid synthetase complex, where chain extension leads to the production of palmitate. Palmitate can then be transferred back to CoA, and the chain can be extended two carbons at a time through the action of a fatty acid elongase system located in the endoplasmic reticulum. The ω-hydroxylation that produces the ω-hydroxyacids of the acylceramides is thought to be mediated by a cytochrome p450 just when the fatty acid is long enough to span the endoplasmic reticular membrane.

The rate limiting step for all sphingolipid biosynthesis is serine palomitoyl transferase, which condenses palmitoyl-CoA with serine to produce 3-ketodihydrosphingosine.[32] The keto group is rapidly reduced, and the resulting dihydrosphingosine group is N-acylated to produce a simple ceramide. The 4,5-*trans* double bond can then be introduced in the base component, and various positions can be hydroxylated to produce α-hydroxyacids, phytosphingosines, and 6-hydroxysphingosines. These hydroxylation reactions require vitamin C.[33]

3.2 LAMELLAR GRANULES

Much of the lipid that accumulates with keratinization is packaged in small organelles called lamellar granules.[4,34] These small organelles have also been called Odland bodies, keratinosomes, membrane

coating granules, lamellar bodies, and cementsomes. They are derived from the Golgi apparatus and are generally round to ovoid in shape and about 0.2 μm in diameter. They consist of a unit bounding membrane surrounding one or several internal stacks of lipidic disks. They are lipid rich, and therefore, have a low buoyant density. This property has been exploited to isolate lamellar granules from rodent and porcine epidermis.[35–37] They are particularly rich in glycolipids, especially the glucosylated analogue of ceramide EOS, and phospholipids, and contain a relatively high proportion of cholesterol. They contain little ceramide or free fatty acids. It has been suggested that glucosylceramide EOS may be involved in assembly of the internal lamellae of the lamellar granules. More recently, it has been suggested that a large portion of the lamellar granule-associated glucosylceramide EOS is actually in the bounding membrane.[5] This pool of glucosylceramide EOS would be introduced to the cell periphery when the bounding membrane of the organelle fuses with the cell plasma membrane, and could be the precursor of the covalently bound hydroxyceramide on the cornified envelope.

3.3 CATABOLISM

In addition to delivering lipids to the intercellular space between the granular layer and the stratum corneum, lamellar granules also deliver a battery of hydrolytic enzymes that convert the initially extruded phospholipid- and glycolipid-rich lipid mixture into the fatty acids and ceramides of the stratum corneum intercellular spaces.[38,39] In rodent epidermis, some of this lipid processing continues in the intercellular spaces of the stratum corneum; however, with porcine and human epidermis conversion to the mature barrier lipids is completed at the stratum granulosum–stratum corneum interface. The enzymes that mediate this transformation are mainly acid hydrolases and include a glucocerebrosidase to convert glucosylceramides to ceramides, and acid sphingomyelinase to convert sphingomyelin into ceramides and a battery of phospholipases to release fatty acids from phosphoglycerides.

3.4 COMPOSITION

The literature regarding the composition of human stratum corneum lipids has recently been reviewed.[40] In general, there is a great deal of variation among the published compositions. Some of this probably reflects differences in the analytical methods that were used; however, much of the variation reflects failures to recognize contaminants including sebaceous lipids, subcutaneous fat, and environmental hydrocarbons. When the known contaminants are factored out, it is apparent that the main stratum corneum lipids are ceramides, cholesterol, and free fatty acids in the ratio of 50:27:12 by weight. When the average molecular weights are taken into consideration, these major components are present in roughly 1:1:1 molar proportions. Mixtures of ceramides:cholesterol and fatty acids in a 1:1:1 molar ratio have been used by a number of investigators to approximate stratum corneum lipids for studies of physical properties.[41–43]

The remaining 11% of the stratum corneum lipid mass consists mainly of cholesterol sulfate and cholesterol esters.[44] The cholesterol sulfate has been implicated in regulation of the desquamation process. It has been shown that cholesterol sulfate inhibits serine proteases of the types that degrade desmosomal proteins leading ultimately to cell shedding. A sterol sulfatase must act on cholesterol sulfate to make the proteolytic degradation of the desmosomes possible. The degradation of cholesterol sulfate in association with desquamation has been demonstrated both with an organ culture model and with human skin *in vivo*. Cholesterol esters have long been cited as a hallmark of keratinization; however, these liquid phase lipids are probably not found within the intercellular lamellae. Late in the keratinization process, oleate is transferred to cholesterol to produce cholesterol oleate. This cholesterol ester is not accommodated well by membranes, and it has been suggested that it phase separates into isolated pockets within the intercellular space. The cholesterol ester deposits are

thought to be reflected in amorphous pockets within the intercellular spaces in transmission electron micrographs. The transfer of oleate to cholesterol and subsequent phase separation of cholesterol oleate may provide a mechanism for keeping oleic acid, a well-known permeability enhancer, out of the lamellar domains that provide the barrier function.

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4 Lipid Structures in the Permeability Barrier

Lars Norlén

CONTENTS

4.1 INTRODUCTION

The physical state and molecular organization of the stratum corneum intercellular lipid matrix largely determines the hydration-level of the stratum corneum and thus, indirectly, the mechanical properties and appearance of the skin. A better understanding of stratum corneum lipid organization may thus aid the development of more efficient cosmetic formulations.

Notwithstanding the spectacular progress made during the last 25 years in the field of stratum corneum research, the structure and dynamics of the lipid matrix of stratum corneum intercellular space is still largely undetermined. This may partly be due to the compositional as well as functional complexity of the stratum corneum intercellular lipid matrix and partly due to the technical difficulties involved in this research field. For example, uncontaminated compositional skin lipid data are difficult to obtain (Wertz and Norlén, 2002). Furthermore, the skin separates two very different compartments (i.e., inside and outside the organism) and consequently several pronounced gradients are present over the stratum corneum. The endogenous stratum corneum thus represents an open (i.e., out of equilibrium) system, and is therefore difficult to model *in vitro*. Also, *ex vivo* techniques, such as conventional electron microscopy and x-ray diffraction, usually requires extensive skin sample preparation which may alter the endogenous lipid organization of the stratum corneum intercellular space. Although everyone knows that sample preparation for conventional electron microscopy may yield but a poor representation of reality, few may be aware of how profoundly different the molecular organization as well as higher order structure of endogenous skin may be with respect to its representations in conventional electron micrographs (Figure 4.1). Using

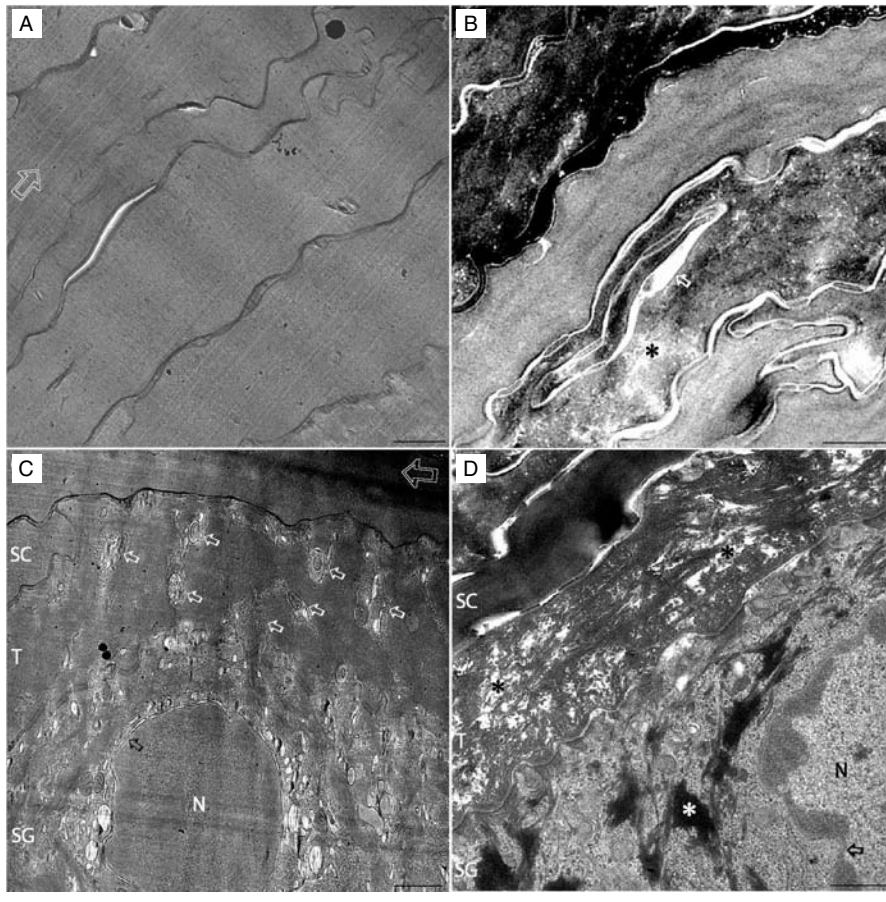

FIGURE 4.1 Conventional sample preparation for electron microscopy results in important losses of epidermal biomaterial. Low magnification transmission electron micrographs of human epidermis at the interzone between viable and cornified cell layers (A, B: lowermost stratum corneum; C, D: uppermost stratum granulosum). (A, C): cryo-electron micrographs of vitreous sections of native epidermis. (B, D): conventional electron micrographs of resin embedded sections. In the vitreous cryo-fixed epidermis (A, C) cellular as well as intercellular space appears densely packed with organic material, while in the conventionally fixed epidermis (B, D) the distribution of biomaterial is characteristically inhomogeneous. Loss of biomaterial appears to have taken place in (B, D), both in the cytoplasmic (black asterix) and intercellular (white arrow) space. Large portions of the biomass of the viable cells appear as aggregated, heavily stained clusters, so-called keratohyalin granules (D, white asterix). Furthermore, the rich variety of cytoplasmic organelles and multigranular structures present in the stratum corneum/stratum granulosum transition (T) cells of native epidermis (C) (white arrows) are replaced by empty space in resin-embedded samples (D) (black asterix). Inner and outer nuclear envelopes and nuclear pores are clearly distinguished in the native cryo-fixed nonstained specimen (C) (black arrow) while they are difficult to distinguish in the conventionally-fixed stained specimen (D) (black arrow). Electron dense single-spot in (A) and double-spot in (C) correspond to surface ice contamination. SG: uppermost stratum granulosum cell; T: transition cell; SC: lowermost stratum corneum cell; N: nucleus; open white double-arrow (A, C): section cutting direction. Section thicknesses ~100 nm (A, C), ~50 nm (B, D). Scale bars 500 nm (A–D). A–D adapted from Norlén and Al-Amoudi (2004). With permission from the Blackwell Science Publications.

conventional tissue embedding methods for electron microscopic observation, the skin is chemically fixed during minutes and subsequently dehydrated by organic solvent, with important loss and precipitation of biomaterial. Methods such as freeze-substitution or low-temperature embedding are less destructive, but the skin is still dry and the use of staining means that it is the local ability to bind stain, rather than the biomaterial per se, which is observed. More precisely, the contrast in conventional epoxy-sections is ascribed to the difference between the inherent electron scattering properties of individual biological elements plus the additional electron scattering of osmium, uranium, and lead, and the epoxy embedding medium itself (Kondo, 1995).

To better understand the structure, function, and dynamics of the endogenous lipid matrix of the stratum corneum intercellular space some general principles of lipid phase behavior, dynamics, and structural organization may represent a useful starting point. Further follows a short overview of some basic physico-chemical principles that may be of relevance for stratum corneum lipid research, followed by a presentation of the new technique cryo-transmission electron microscopy of fully hydrated vitreous skin sections and how this technique recently has been applied to the study of the structural organization and formation of the lipid matrix of the stratum corneum intercellular space.

4.2 LIPIDS AND LIPID ORGANIZATION

4.2.1 Lipid Classification

The term lipid can be defined as fatty acids, their derivatives, and substances related biosynthetically or functionally to these compounds. This definition encompasses cholesterol and bile acids, but does not include other steroids, fat-soluble vitamins, carotenoids, or terpenes (Christie, 1987, p. 42).

From a physical point of view lipids can further be subdivided into nonpolar and polar lipids. Fatty acids, triacylglycerols, diacylglycerols, sterols, and their esters are relatively nonpolar, while monoacylglycerols, phospholipids, galactosylglycerolipids, and sphingolipids belong to the group of polar lipids (Larsson, 1994, pp. 1–3).

4.2.2 Lipid Self-Assembly

Lipids with a hydrophilic (headgroup) and a hydrophobic end (hydrocarbon chain) are termed amphiphilic. The degree of hydrophilicity of molecular groups is important for the understanding of interactions and associations of amphiphilic molecules. These molecules can self-associate or self-assemble into larger three-dimensional (3D) aggregates such as micelles, bilayers, or biological membranes when certain conditions are fulfilled. Such aggregates are fluid-like since the forces that hold these aggregates together are weak screened electrostatic-, van der Waals-, hydrophobic-, and hydrogen-bonding interactions (Israelachvili, 1992, p. 341; Larsson, 1994, p. 47; Hyde et al., 1997). A change in solvent conditions, such as pH or electrolyte concentration, will not only affect the interactions between the aggregates but also the intermolecular forces within each aggregate with resulting changes of structure, shape, and size.

A difference in the cohesive energies between the molecules in the aggregated and the dispersed states is a prerequisite for aggregates to be formed. The monomer concentration at which aggregation formation starts is termed the critical aggregation concentration (CAC) or critical micelle concentration (CMC) (cf. Israelachvili, 1992, pp. 348–352). The major forces involved in self-assembly of lipids are the hydrophobic attraction at the hydrocarbon/water interface and the hydrophilic, steric, or ionic repulsion of the headgroups. The former tending to decrease and the latter to increase the interfacial headgroup area per molecule. To understand the phase-behavior of a system, the complex separate force contributions do not need to be known in detail since one can expect the first term in any energy expansion to be inversely proportional to the surface area occupied per headgroup. The headgroup surface area at which the total free energy per molecule in a system is at a minimum

is termed the optimal surface area, defined at the hydrocarbon/water interface. This implies that, to a first approximation, the interaction energy between lipids has a minimum at a certain headgroup area, a_0. In fact, self-assembly of lipid aggregates can largely be quantitatively understood from geometrical considerations. The packing properties of amphiphilic molecules depend on their optimal headgroup area, a_0, hydrocarbon chain volume, v, and the critical chain length, l_c (maximum effective hydrocarbon chain length). Once these three parameters are determined one can to a good approximation estimate into which structures the molecules can self-assembly. Entropy will then favor the structure with the smallest aggregation number. The dimensionless critical packing parameter, cpp $= v/(a_0 l_c)$, can be used to determine whether the lipids will form bilayers ($\frac{1}{2} < v/(a_0 l_c) < 1$), spherical micelles ($\frac{1}{3} < v/(a_0 l_c) < \frac{1}{2}$), or reversed structures ($v/(a_0 l_c) > 1$). Each of these structures will exist as the smallest aggregate in which all the lipids have minimum free energy (cf. Israelachvili, 1992, pp. 368–371).

As indicated above, the lipid structures formed can to a large extent be modified by the ion-concentration, pH, and temperature of their environment as well as by the degree of unsaturation of the hydrocarbon chains. For anionic headgroups the headgroup area can be decreased by increasing the salt concentration, particularly of divalent ions like Ca^{2+}, or by lowering the pH. This also has the effect of condensing the hydrocarbon chains. Introduction of branched chains, particularly of *cis* double bonds, reduces the critical chain length and consequently increases the critical packing parameter. This will ultimately favor reversed structures. An increase in temperature reduces the critical chain length due to increased hydrocarbon chain motion. However, it can also alter the optimal headgroup area in both directions depending on the specific headgroup characteristics (cf. Israelachvili, 1992, p. 380).

4.2.3 Lipid Phase Behavior

4.2.3.1 Solid State

In the solid state the translational motion of the molecules is slow and the molecules are arranged with long-range orientational and positional order. However, for compounds with long hydrocarbon chains the molecules may rotate in their lattice sites at the same time as they maintain full positional order, forming so-called "plastic crystals" (Evans and Wennerström, 1994, p. 412). The stability of these "plastic" crystalline phases (α-forms) increases with chain length and with the presence of impurities (e.g., broad chain-length distributions) (Larsson, 1994, p. 27).

Lipid molecules have a unique property in that they often can be packed in different ways in the solid state, that is, they exhibit polymorphism, although there is only one best packing mode. The main mechanisms behind polymorphism are variations in the tilt of molecules of the bilayer and variations in hydrocarbon chain packing (Larsson, 1994, p. 11). The best way to describe the chain packing is to use the subcell corresponding to the smallest repetition unit within the unit cell (Hernquist, 1984, p. 14). The main polymorphic forms are the α-, β'-, and β-form. A melt crystallizes into an α-form upon cooling. Using x-ray diffraction, α-forms show one strong diffraction line at 4.15 Å. This corresponds to the hexagonal chain packing, which expresses rotational disorder along the hydrocarbon chain axes. When an α-form is stored there is usually an irreversible phase transition into a crystal form with fixed hydrocarbon chain planes (i.e., this crystal form has a higher melting point than corresponding α-forms). An orthorhombic chain packing may then be formed, where every second hydrocarbon chain plane is perpendicular to the others. This crystal form is called a β'-form. It is characterized by two diffraction lines at 3.8 and 4.2 Å. (Hernquist, 1984, pp. 16–18, 24–31; Small, 1986, pp. 98–101; Larsson, 1994, pp. 13–14, 27).

Crystals of most polar lipids can swell in the presence of water. The corresponding phases, gel-phases, with lamellarly packed lipid, and water layers, are sometimes thermodynamically stable (Larsson, 1994, p. 41). Also, the hydrocarbon chain packing of gel-phases usually show some axial rotational disorder. The alkyl chain cross-sectional area is close to 20 $Å^2$ in a plane perpendicular

to the direction of the chain (Evans and Wennerström, 1994, p. 247). If the headgroup area and the cross-sectional area of the alkyl chain is similar, a lamellar L_β-phase with chains perpendicular to the bilayer surface is formed. If the headgroup area is dissimilar to the cross-sectional alkyl-chain area a lamellar $L_{\beta'}$-phase with tilted chains or a rippled $P_{\beta'}$-phase results (Evans and Wennerström, 1994, p. 247).

4.2.3.2 Liquid State

The transition from the crystalline to the liquid state is accompanied by absorption of heat, a loss of long-range order, and an increase in molecular volume. However, many long chain lipids show only small volume changes (10–20%) during the transition from solid to liquid, which indicates that some short-range order should remain in the liquid state (Small, 1986, pp. 56–57).

4.2.3.3 Liquid Crystalline State

When a lipid molecule in the crystal state is heated it may pass through intermediate states called mesophases or liquid crystals (Friedel, 1922) instead of melting directly into an isotropic liquid. These liquid crystals are characterized by residual long-range order, but lack of short-range order (Small, 1986, p. 49). Since they have both some degree of order and of fluidity they possess the characteristics of both liquids and of crystalline solids. In the liquid crystal the hydrocarbon chains are "melted" and consequently there is solely a crystalline periodicity in the direction corresponding to the bilayer thickness. This is because the forces between the polar headgroups are stronger than the van der Waals interaction between the hydrocarbon chains. Consequently, when energy is added to the system, the thermal motions will overcome the forces between the hydrocarbon chains but not the forces in the polar headgroup sheets, resulting in hydrocarbon chain disorder but unchanged bilayer structure (Larsson, 1994, p. 47).

The change from a crystalline into a liquid crystalline state can be brought about by changes in, for example, temperature or pressure. Furthermore, some molecules may be induced to form liquid crystals by the addition of a solvent such as water. This behavior is in reality a liquid crystalline formation in a two component system and is called solvent-induced liquid crystal formation or lyotropic mesomorphism (Small, 1986, p. 49).

Liquid crystals can be in the smectic, nematic, or isotropic states. In the smectic liquid crystalline state there is a long-range order in the direction of the long axis of the molecules. These molecules may be in single- or bilayer conformation, have molecular axis normal or tilted to the plane of the layer, and frozen or melted chains. In the nematic liquid crystalline state the molecules are aligned side by side but not in specific layers. The isotropic liquid crystalline state is more or less a liquid state, but where clusters with short-range order persist (Small, 1986, pp. 49–51).

As described above, different phases and geometries can be formed by self-assembly of liquid crystalline amphiphilic molecules depending on their optimal headgroup area, a_0, hydrocarbon chain volume, v, and the critical chain length, l_c (maximum effective hydrocarbon chain length) (Israelachvili, 1992, p. 370). To form a lamellar phase (L_α) a cross-sectional area of 28 to 32 Å^2 is required (Larsson, 1994, p. 48). By increasing the temperature and/or decreasing the relative amount of water and/or decreasing the optimal headgroup area a reversed hexagonal phase (H_{II}) and eventually a reversed micellar phase (L_2) may be induced. In contrast, by decreasing the temperature and/or increasing the relative amount of polar solvent and/or increasing the optimal headgroup area a normal hexagonal phase (H_I) and eventually a normal micellar phase (L_1) is formed. Other phase geometries may appear between the L_1, H_I, L_α, H_{II}, and L_2-phases. These are most commonly the descrete (I_1, I_2) and bicontinuous (V_1, V_2) cubic phases (Figure 4.2) (Larsson, 1989). The reversed bicontinuous cubic phases (V_2) represent infinite periodical minimal surfaces (IPMS) (Larsson, 1994, pp. 50–55) (cf. Section 4.2.4).

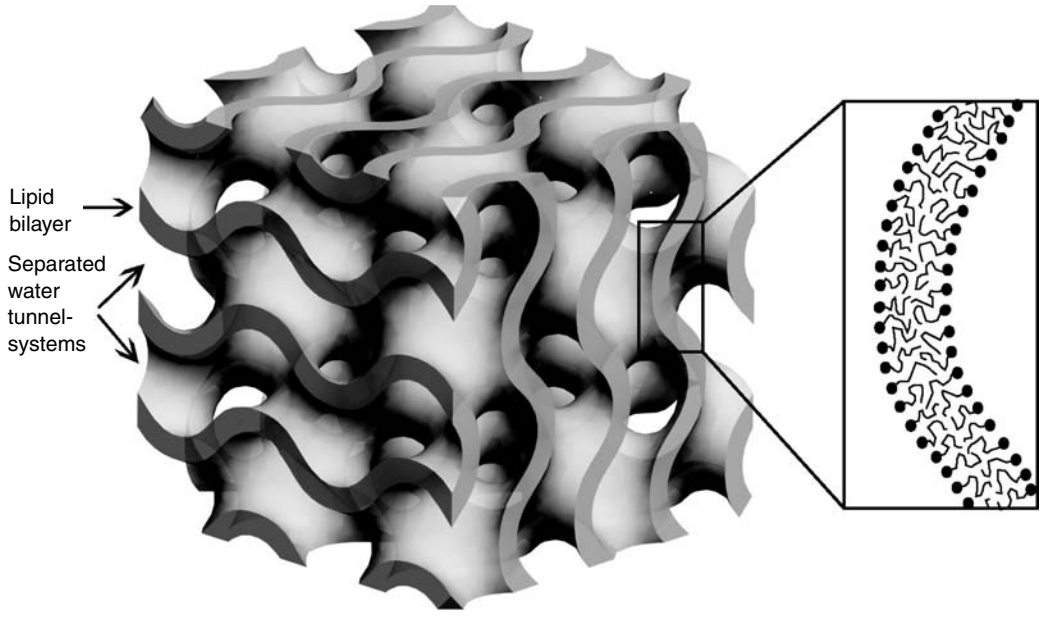

FIGURE 4.2 Schematic illustration of $2 \times 2 \times 2$ unit cells of a lipid/water phase with gyroid cubic symmetry. In reversed bicontinuous cubic phases the lipid bilayer membrane separates two intertwined water-filled subvolumes resembling 3D arrays of interconnected tunnels. Black box (right) represents an enlargement of a part of the folded liquid crystalline lipid bilayer membrane structure.

4.2.4 Liquid Crystalline Lipid/Water Phases with Cubic Symmetry

A central issue in the field of lipid self-assembly is the structure of liquid crystalline mesophases denoted bicontinuous cubic phases (Figure 4.2). Cubic lipid/water phases were detected by Luzatti et al. and Fontell in the 1960s although they were believed to be rare in comparison with the classical lamellar, hexagonal, and micellar mesophases. It is now clear that these phases are ubiquitous in lipid systems (Hyde et al., 1997). Further a number of cubic phases can occur in the same system as the temperature or solvent concentration/composition is varied. It is surely no coincidence that the symmetries of these lipid/water phases are precisely those of low genus three-periodic minimal mathematical surfaces with cubic symmetry. The simplest three-periodic hyperbolic (i.e., saddle shaped, or, more specifically, with negative average Gaussian curvature) surfaces are IPMS of the primitive (P), gyroid (G), and diamond (D) types. For these surfaces the mean curvature is constant and everywhere identically zero, just like for a flat surface. In fact, geometrical analysis indicates that reversed (bilayer) bicontinuous cubic phases are only to be found in lipid/water systems that also form lamellar phases readily (i.e., where the average molecular shape is close to cylindrical ($v/a\mathrm{l} \sim 1$)). This close geometrical resemblance between the lamellar and the bicontinuous cubic phases is further emphasized by the low enthalpy difference between these two phases ($\sim$0.5 kJ/mol lipid) (Engström et al., 1992) as compared to the enthalpy difference between the lamellar and reversed hexagonal phases ($\sim$5 to 10 kJ/mol lipid) (Seddon et al., 1983). These facts strongly suggest that hyperbolic mesophases with cubic symmetry may be presented in biological cells, which typically express lamellar membrane bilayer morphologies. In fact, most biological membranes contain at least one lipid species that can form a cubic and/or hexagonal phase (Lindblom and Rilfors, 1989).

Recently it was shown that biological membranes with cubic symmetry are indeed present in many living systems, and, as suspected on theoretical grounds (cf. earlier), often closely associated

with lamellar membrane morphologies (Landh, 1996; Hyde et al., 1997). There are, however, several differences between the hitherto identified cellular membranes with cubic symmetry and cubic lipid/water equilibrium phases (Bouligand, 1990; Landh, 1996, pp. 171–173). Perhaps the most striking difference is that the observed periodicities in biological membrane systems with cubic symmetry studied so far by conventional electron microscopy are much larger (unit cell size ~50 to 2000 nm) than for corresponding cubic lipid/water equilibrium phases (unit cell size ~10 to 30 nm). Other differences are that reversed bicontinuous cubic lipid/water phases usually are balanced (i.e., the two subvolumes separated by the lipid bilayer are of equal size; Figure 4.2) and constituted by a single bilayer leaflet, which often is not the case for hitherto identified biological membranes with cubic symmetry (Landh, 1996). In other words, in the nonequilibrium situation *in vivo* it is not clear whether lipid composition (i.e., average molecular shape) can be directly related to membrane geometry. However, using cryo-electron microscopy on vitreous sections of native human skin the existence of cubic-like cellular membrane morphologies of dimensions corresponding to those of cubic lipid/water equilibrium phases space was recently indicated (Al-Amoudi et al., 2004). Such membrane structures may be of central importance for the formation of the lipid matrix of the stratum corneum extracellular space as well as for the formation of the stratum corneum keratin network (Norlén and Al-Amoudi, 2004).

4.3 CRYO-TRANSMISSION ELECTRON MICROSCOPY OF VITREOUS SKIN SECTIONS

Water is a major constituent of skin. However, most of our knowledge of skin ultrastructure has been gained from observation on dehydrated epidermis (e.g., using conventional electron microscopy on dehydrated skin samples or x-ray diffraction on isolated stratum corneum). It is possible, therefore, that the deleterious effects of conventional specimen preparation may not have been fully acknowledged in the present perception of epidermal cellular/molecular organization. This may be particularly true for biological liquid crystalline structures such as lipid membranes as water activity represents a major factor determining lipid phase behavior and structural organization (Guldbrand et al., 1982; Small, 1986; Israelachvili, 1992; Evans and Wennerström, 1994; Larsson, 1994).

Cryo-electron microscopy of vitreous sections of freshly taken, fully hydrated, noncryoprotected, nonstained (i.e., native) skin samples has several major advantages over conventional electron microscopy of chemically fixed specimens. These are: (i) no loss of biomaterial (including water); (ii) infinite preparational reproducibility (in the case of successful complete tissue vitrification); (iii) biostructures may be preserved down to atomic resolution; (iv) the optical density of the recorded image is directly related to the local density of the biological material of the sample; (v) immobilization of skin can be achieved within seconds after sample acquisition; (vi) total tissue fixation time is in the millisecond range. These advantages should, however, be weighed against possible pressure-induced artifacts, cutting-induced deformations, and electron beam damage. Furthermore, even a millisecond cryo-fixation time is long compared with the characteristic time of many dynamical biological processes. Another drawback is that section handling and microscopy is demanding, rendering serial sectioning and 3D-reconstruction more difficult. Low electron dose computerized cryo-electron tomography on vitreous skin sections may, however, in future largely overcome this limitation.

Recently, it was reported that a freshly-taken, fully hydrated full thickness human epidermis can be completely vitrified, directly, without the use of cryoprotectants or any other pretreatment (Norlén et al., 2003). As mentioned above, successful tissue vitrification has the potential to preserve biostructures down to atomic resolution. Consequently, the native ultrastructure of epidermal biomolecular complexes could now therefore, theoretically, be observed at subnanometer resolution *in situ*.

Due to optimization of the cryo-sectioning method (i.e., minimized section thickness) and microscopy technique (i.e., minimized electron dose), native epidermal ultrastructural *in situ* data, with a resolution of a few nanometer, has recently been presented of the skin barrier lipid organization and its formation process (including desmosomal differentiation) (Al-Amoudi et al., 2005) as well as on the structural organization and formation of the corneocyte cell matrix (i.e., the keratin intermediate filament network) (Norlén and Al-Amoudi, 2004). These high-resolution cryo-electron microscopic data are closer to the biological reality, more detailed and differ from those obtained by conventional electron microscopy of resin embedded epidermis. Notably they indicate (a) that the skin barrier formation process may evolve via a lamellar "unfolding" of a small lattice parameter lipid "phase" with cubic-like symmetry with subsequent "crystallization," or "condensation," (including lamellar reorganization) of the epidermal intercellular lipid matrix and b) that the multilamellar lipid organization of the stratum corneum intercellular space may be more complex than earlier thought (Al-Amoudi et al., 2005) (Figure 4.3). Notably, "lamellar body discs" (cf. Landmann, 1986) at the interface between stratum granulosum and stratum corneum, as well as "13 nm lamellar repeats" (cf. Bouwstra et al., 2000; McIntosh, 2003; Hill and Wertz, 2003) of the stratum corneum intercellular space are conspicuous by their apparent absence in cryo-electron micrographs of vitreous skin sections (Norlén et al., 2003; Al-Amoudi et al., 2005).

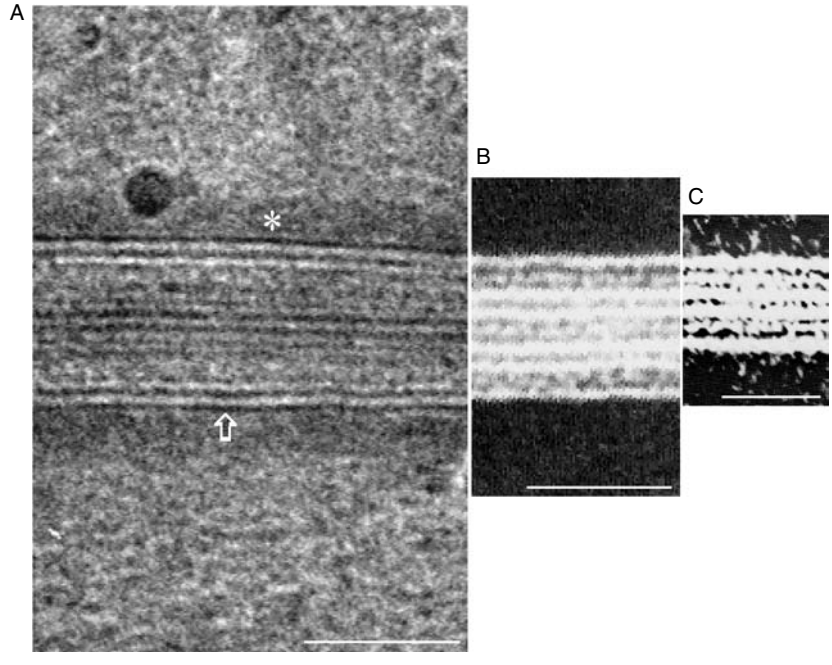

FIGURE 4.3 High magnification transmission electron micrographs of multilamellar membrane structures in the intercellular space of the cornified part of human epidermis. (A): cryo-electron micrograph of vitreous section. (B, C): conventional electron micrographs of resin embedded sections. The cell plasma membranes appear as 3.8 nm wide bilayers in (A) (open white arrow). A 16 nm broad zone of electron dense material, the cornified cell envelope (white asterix), is directly apposed to the cytoplasmic side of the bilayer plasma membranes in the native sample (A) (open white arrow). Scale bar 50 nm (A). Scale bars 25 nm (B, C) adapted from measures given in Swartzendruber et al. (1989). (A) reprinted from Norlén (2003). With permission from Blackwell Science Publications. (B, C) reprinted from Swartzendruber et al. (1989). With permission from Blackwell Science Publications.

4.4 LIPID ORGANIZATION OF THE STRATUM CORNEUM INTERCELLULAR SPACE — A SINGLE AND COHERENT MULTILAMELLAR "DRM"?

Water homeostasis is a strict requirement for normal physiological function. The most important task of the human skin is thus to create a watertight enclosure of the body to prevent water loss. It is the intercellular lipid matrix of the outermost keratinized horny layer of the skin (possibly together with recently reported claudin-based tight-junctions; Furuse et al., 2002) that represents the skin barrier proper as once this lipid matrix (composed foremostly of saturated long chain ceramides (∼50% wt/wt) and cholesterol (∼30% wt/wt) (Wertz and Norlén, 2002)) has been removed, substances diffuse freely into or out of the body system (Blank, 1952; Breathnach et al., 1973; Elias and Friend, 1975). At the same time the intercellular lipid matrix ensures that the stratum corneum remains hydrated and thus the skin surface appears healthy and smooth.

The strong gradients in, for example, water concentration, present over the stratum corneum *in vivo* suggests that the structure and function of the intercellular lipid matrix cannot be fully understood unless the processes involved in its formation are considered. The conventional view of the formation of the stratum corneum intercellular lipid matrix is essentially that "lamellar bodies" (i.e., discrete spherical lipid bilayer vesicles), containing in their turn "lamellar disks" (i.e., discrete flattened lipid bilayer vesicles), bud off from the *trans*-Golgi network and diffuse toward the plasma membrane of the differentiating stratum granulosum cells (i.e., topmost viable epidermal cells facing stratum corneum). After fusion of the limiting membrane of the "lamellar bodies" with the plasma membrane of the stratum granulosum transition cell, the lamellar body lipid content is thought to be discharged into the intercellular space where the "lamellar discs" merge into intercellular lamellar sheets via a second fusion process (Landmann, 1986). However, cryo-electron microscopic indications of the existence of "lamellar bodies" and "lamellar body discs" in native skin is lacking (Norlén et al., 2003; Al-Amoudi et al., 2005). Quite differently, the skin barrier formation process may instead take place as a lamellar "unfolding" (or "phase transition") of a small lattice parameter liquid crystalline lipid "phase" with cubic-like symmetry with subsequent "crystallization" or "condensation" (including possible lamellar reorganization) of the intercellular lipid matrix (Norlén, 2001a). Furthermore, the idea of a direct close-packing (crystallization or condensation), without marked sorting of lipid species during the close-packing process, of such a liquid crystalline structure into a single multilamellar gel-like structure (Norlén, 2001b, 2002), reduces to a minimum introduction of unknown features when trying to explain the formation, structure, and function of the lipid matrix of the stratum corneum intercellular space.

Cholesterol may represent a key entity for the proper formation, molecular packing, and function of the lipid matrix of the stratum corneum intercellular space. Generally, in the presence of cholesterol the movements of *liquid* crystalline lipid chains are strongly reduced with resulting diminished distances between the hydrocarbon chains and thus increased van der Waals interaction. Consequently, liquid hydrocarbon chains seem to be "condensed" toward the cholesterol skeleton, however, without crystallizing and without loosing all mobility. For saturated, *crystallized* lipids, a competition arises for the hydrocarbon chains (e.g., those of skin ceramides and free fatty acids) between cholesterol and the crystalline aggregate. Cholesterol may consequently "steal" hydrocarbon chains from the crystalline aggregate by offering these a more favorable van der Waals interaction. In such an aggregate with cholesterol, the saturated hydrocarbon chains cannot be in all-*trans* conformation and are thus, by definition, liquid crystalline (i.e., they cannot give rise to crystalline wide-angle reflections in x-ray experiments). From a crystallographic point of view, the single gel-phase predicted in the single gel-phase model (Norlén, 2001b) may thus *in cholesterol-rich regions* be an unusually close-packed *liquid crystalline* structure. Consequently, the endogenous lipid organization of the stratum corneum intercellular space may resemble that of nonionic detergent resistant membrane fragments (DRMs) isolated from a variety of eukaryotic cells. These, like the skin barrier lipid matrix, are composed of a mixture of saturated long acyl-chain sphingolipids and cholesterol and, likewise, may

exist as a *liquid ordered* structure (i.e., a "gel phase"; Ahmed et al., 1997; Brown and London, 1997; Brown, 1998; Ge et al., 1999; Xu and London, 2000).

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5 Particle Probes and Skin Physiology

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CONTENTS

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5.1 INTRODUCTION

The condition of the skin, whether it is normal, dry, eczematous, etc., reflects its physiology/pathophysiology. Only during the past three decades has it been possible to probe the physiology of human skin, and this has been achieved through the means of particle probes. The electron and the proton probe both rely on the production of secondary x-ray quanta emission, which allows identification as well as quantification of elements. Since there exists no well-established model to substitute for human skin in experimental approaches to clinically normal and pathological skin conditions, this chapter is devoted to the study of element and particularly trace element distributions in normal and pathological human skin. It is interesting to note that the development of modern medicine from the moment of the discovery of x-rays has been closely linked to the development of physics. Almost immediately after his discovery of x-rays in December 1895, Konrad Röntgen made an image of his left hand carrying a finger ring. From a historical point of view, this can truly be regarded as the first clinical x-ray image. It is a fact that this image had a tremendous impact on the contemporary medical body, and the clinical applications of the method were greeted with great enthusiasm among medical doctors. It became obvious that the density of the material was related to the degree of x-ray absorption, that is, the bones were seen easily against the background of soft tissue. In clinical practice this resulted in the invention of contrast media which allowed, for example, the intestinal system to be imaged with a fair amount of detail.

Early on, x-rays were used for structure determination, and Bragg, father and son, are justly regarded as portal figures in this basic research application of the "mysterious rays." The development of this and other analytical methods based on x-ray techniques has had a pronounced impact on modern biology. This chapter will briefly outline the history of x-ray absorption in biological research and then concentrate on the application of micro probes with special reference to the proton probe in experimental dermatology. References to results from other techniques will, however, be included.

5.2 THE BEGINNING — QUANTITATIVE MICRORADIOGRAPHY

The UV-absorption method, with which quantitative determination of DNA in tissue sections can be done, provided the inspiration for the development of x-ray spectrographic methods at the end of the 1940s. The quantitative x-ray analysis methods were developed to provide quantitative elementary analysis on a histochemical and cytochemical scale, that is, quantitative elemental analysis of tissues *in situ* and at a subcellular level. Engström[1] formulated how the problem could be attacked in the following way:

1. *Alternative 1*: Quantitative analysis of the element in question in a very small piece of tissue, microdrop, or something similar; the localization of the element in question in the tissue being obtained in the preparation of the analysis object
2. *Alternative 2*: Quantitative determinations of the element in question, which has a relatively low atomic number, within a cell or a very small area in a microscopic section of a tissue, but retaining the structure; resulting in the analysis being directly correlated to the cytological structure

The second alternative was developed in Engström's thesis, "Quantitative micro- and histochemical elementary analysis by roentgen absorption spectrography," which he published in 1946, and his method became known as quantitative microradiography.[1] A satisfactory resolution was granted by fine-grain Lippmann emulsions, available in the 1930s, and this film material allowed a resolution in the 10-μm range.

This spectrographic method that allowed chemical elementary analysis of single mammalian cells was based on the selective absorption of monochromatic x-rays measured directly in the spectrometer

or by photometry of the x-ray photographic image of the cell/tissue. By exposing the same object for x-rays on each side of the absorption edge for the element to be determined, quantitative data were obtained, for example, concentrations when the mass of the exposed area/volume was determined by a "white" radiation exposure. In early studies, ^{15}P and ^{20}Ca were determined in 10-μm bone sections within an area of $10 * 10$ μm, and the amounts determined were of the order of 10^{-9} to 10^{-12} g. The error of the analysis was estimated to be 5 to 10%. The photographic recording provided a precise localization of the area measured, and grain density can be determined by photometry, out of which quantitative data can be calculated. Engström's method was further developed and refined by the work of Lindström[2] (Figure 5.1). He expanded the theoretical basis for x-ray absorption spectrophotometry and constructed an x-ray spectrophotometer with a bent crystal that produced high intensity monochromatic x-rays of varying wavelengths. The basis provided by the work of Engström and Lindström is presently put to good use in fully-automatic microradiographic systems and standard tools in, for example, dental research.

5.3 INERT PREPARATION — CRYO-METHODS FOR ELEMENTAL ANALYSIS OF TISSUE SAMPLES

The content of a cell can be regarded as a gel in which ions are free to move at appreciable speed with minor restrictions. The study of the physiology of a cell in a particular phase of its activity must be done on a sample where all ionic movements have been instantaneously arrested. Chemical fixation relies on the diffusion of the fixing agent into the cell and its contents, and will obviously perturb the particular conditions sought. If the tissue temperature can be instantaneously lowered to produce vitreous ice, this would be an ideal preparative choice.[3–6] However, the heat conductive properties of organic material are far from excellent, and, therefore, we expect a gradient of temperature to move down into a tissue block exposed to a freezing medium. It has been shown that the depth to which a complete momentary freezing will reach is only about 50 to 100 μm. Further down in the tissue a temperature gradient will cause ice crystals to form, and these ice crystals not only disrupt the morphology of the cell, but also create redistribution of movable ions in a freezing-out process. Therefore, only a surface portion of a cryo-fixed tissue block is suitable.

After the subsequent sectioning of only the outer part of the frozen tissue block, we should ideally have a tissue section with a vitrified cellular gel containing all ions in their "natural" morphological positions. However, cryo-sectioning is actually a process of shearing. The shearing process may actually cause a rise in the temperature of the section surface unless precautions against this are taken. Samples aimed for high resolution analysis require that sectioning be performed preferably in a temperature-controlled chamber at an ambient temperature of lower than -100°C. Also, the knife temperature must be controlled and kept very close to this temperature if very thin sections ($<$200 nm) are desired.[4] This is especially the case for x-ray microanalysis (EMP) in the scanning transmission electron microscope (STEM).

5.4 ENERGY DISPERSIVE X-RAY MICROANALYSIS IN THE ELECTRON MICROSCOPE

The original electron probes were wavelength dispersive, utilizing a crystal spectrometer for analyzing the particular characteristic x-ray emission from an element sought for. It was realized in the 1960s that the scanning electron microscope (SEM) actually represented an analysis system, that is, had a potential of being a versatile analysis instrument. In addition to the secondary electrons used for imaging, the electron beam of an SEM produces a number of signals, for example, back-scattered electrons, x-rays, cadluminiscent light, Auger electrons, electric current, etc.

In the early 1970s when the energy dispersive detectors of semiconductor origin were commercially introduced, they actually revolutionized elemental analysis in the electron microscope. X-ray

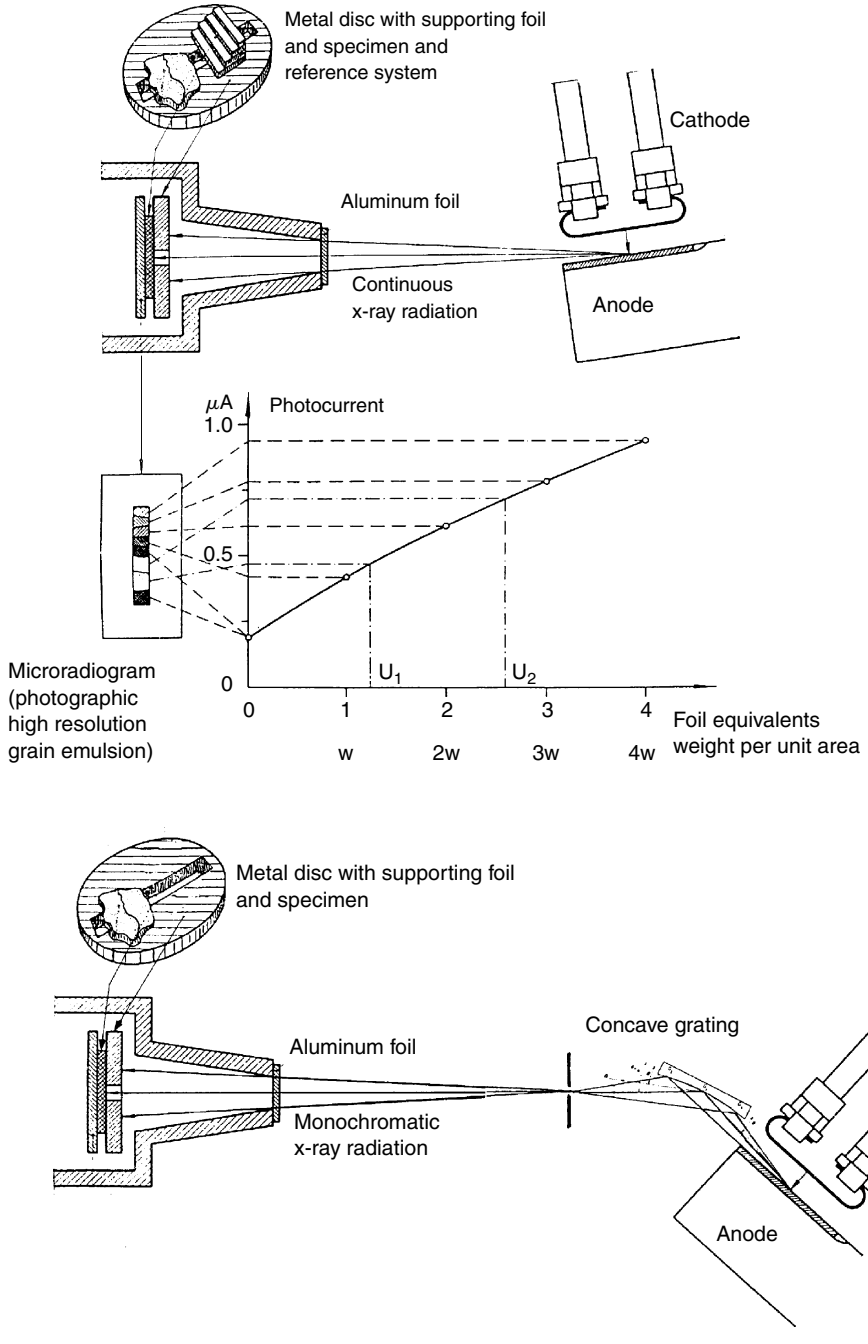

FIGURE 5.1 Top: The principle of microradiography — an x-ray absorption technique for quantitative assessment of dry weight (mass). Bottom: In addition to the mass information, a specified element can be quantitatively assessed by using two monochromatic radiation wavelengths on each side of an absorption edge for the element. (Adapted from Lindström, B., *Acta Radiologica Suppl.* 125, 206, 1955.)

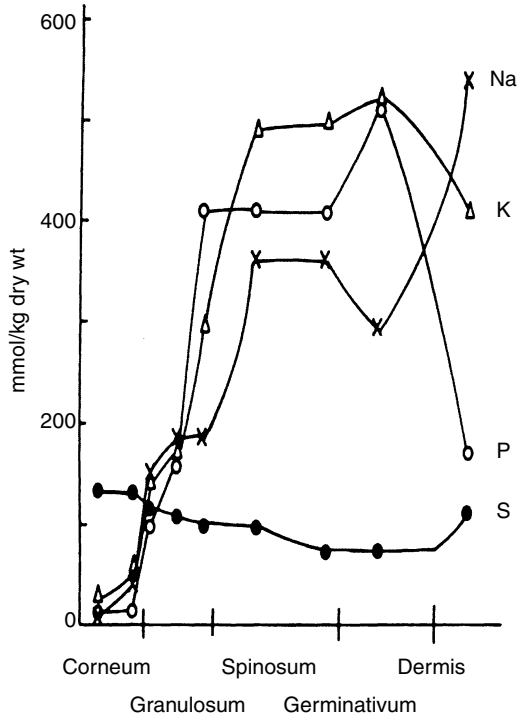

FIGURE 5.2 STEM x-ray spectrum from a skin sample.[48] Note that there is a conspicuous shift in the Na/K ratio moving from the dermis into the basal cell layer. Again, moving into the stratum spinosum, Na increases and K is lowered, a ratio shift, which suggests that the spinosum cells are incapable of entering the mitosis cycle.

microanalysis (XRMA) almost immediately found numerous applications in medical and biological sciences. The energy-dispersive detectors allowed a simultaneous recording of "all" elements in the irradiated volume. Cytochemical methods are hampered by the obvious drawback of not allowing multielement analysis in the same section. The particle probes present a great advantage because virtually all elements of physiological interest can be measured simultaneously within one and the same volume. Consequently, comparisons of the relative contents and formation of elemental ratios, for example, Na/K, that provide sensitive markers for cellular function[7] (Figure 5.2), are often more sensitive indicators of a physiological change than the absolute amounts of an ion (i.e., an element). The additional fact that the electron beam could scan a surface area of the object meant that elemental mapping now in principle was possible. However, it is clear for what was hinted previously that the XRMA technique requires inert preparation in order to minimize ion flux during the preparation and analysis. In the past three decades, cryo-fixation and cryo-sectioning methods, as well as freeze-drying techniques, have consequently been the focus for preparation technique development.[3,5,8]

5.5 PROTON PROBE ANALYSIS

The use of particles heavier than electrons, and especially proton-induced x-ray emission analysis, was developed under the supervison of Professor Sven Johansson at Lund University, Sweden, during the 1970s.[9] Generally referred to as PIXE (particle- or proton-induced x-ray emission) analysis, it has proven to be a sensitive trace element technique. The initial response among medical researchers was a cautious one, most likely due to the fact that the problems of specimen preparation initially were

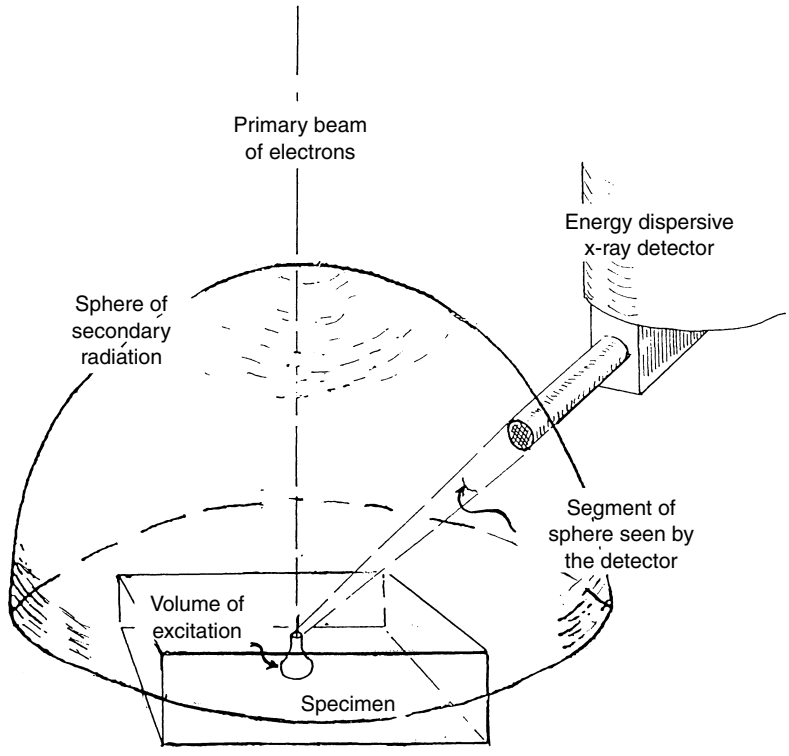

FIGURE 5.3 Sphere of secondary radiation. The detector "sees" only a fraction of the total number of emitted x-ray quanta. (Adapted from Lindström, B., *Acta Radiologica Suppl.* 125, 206, 1955.)

not fully appreciated by the physicists or even among biologists. Hence, the interpretation of results obtained from ill-prepared specimens were difficult, if not impossible, to make. Today technical and preparative problems are well-acknowledged and proton probe analysis is used in medical or biological applications by several research groups around the world.

5.6 DETECTION OF THE X-RAY SIGNAL

In particle probe analysis systems, x-rays are generated from the elements due to an excitation caused by the impinging particles, whether they are electrons or protons, and these secondary x-rays are emitted in all directions. However, the detector can only cover a small part of the sphere of secondary radiation (Figure 5.3), even if the geometry of the experimental setup allows the detector to come very close to the object, which will increase the spatial angle from which the detector "sees" the volume of analysis. Here we see a factor which influences markedly the sensitivity of the analysis method.

The x-rays generated represent quanta of energy. Since characteristic x-ray quanta represent "fingerprints" of the atom they are originating from, a detector that can sort quanta according to its energy will allow identification of elements present in the excited specimen volume. The number of quanta recorded will be proportional to the amount of that particular element present (Figure 5.4). The energy-dispersive system is a fast detector system that uses a signal processor, which transforms the incoming x-ray quanta into electric pulses, subsequently fed into a multichannel analyzer that recognizes the different energies. The energy resolution of the system depends on the channel width that is usually set to 10 to 20 eV (Figure 5.4). The information collected in such a multichannel analyzer can be presented as an energy spectrum, which presents the relative intensities of the x-ray

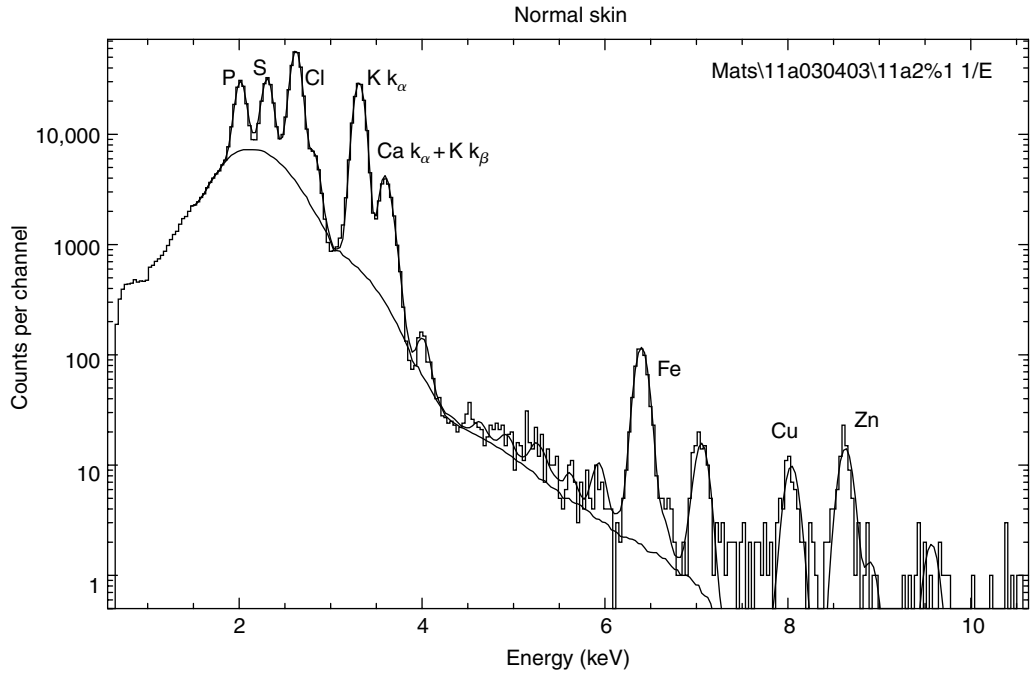

FIGURE 5.4 An x-ray spectrum from a normal skin sample obtained by PIXE-analysis. Detectable peaks are seen above the continuous background (Bremsstrahlung) radiation. Note that the Na peak is not detectable (due to absorption in the sample and the detector window).

signals from the object. As is generally the rule for spectrographic techniques, calibration of the system is done for absolute quantification.

5.7 COMPARISONS BETWEEN ELECTRON AND PROTON PROBES

From what was given earlier, it is clear that PIXE analysis of tissue physiology requires cryo-methods for tissue preparation. But are the data obtained with PIXE compatible with those given by the EMP (XRMA)?

Using standards for biological quantitation as specimens, we have compared XRMA and PIXE[10] and obtained identical results — a correlation coefficient of 0.996 between the methods was obtained for elements such as ^{16}S and ^{28}Ni. Hence, as analysis techniques these methods are fully complementary.

But how well do the XRMA and PIXE compare in practice? The advantages and disadvantages of the two techniques are summarized in Table 5.1. It can be recognized that even if EMP and PIXE are fully compatible for a number of physiologically important elements, there are some notable differences in two important aspects: their sensitivity (e.g., to trace elements) and their spatial resolution.

5.7.1 Sensitivity

The PIXE analysis has a sensitivity that allows analysis of most of the physiologically important elements down to 1 ppm level with the notable exception of Na. The characteristic x-ray quanta from Na have a low energy and are, therefore, to a great extent, suffering self-absorption within the

TABLE 5.1
The Advantages and Disadvantages of XRMA and PIXE

XRMA		PIXE	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
High spatial resolution, 0.2 μm (200 nm)	Need for very thin *cryo-sections (<200 nm), cumbersome preparation*	*High sensitivity, <1 ppm* Allows trace element analysis, for example, Ca, Fe, Zn	Comparatively *low spatial resolution* $\leq$5 μm, which can be improved with loss of sensitivity
Sensitivity ~200 ppm	Absolute quantitation is not a straightforward procedure, appropriate *standards* and some approximations of *correction factors* are always involved in the practical application	Sensitivity 1 ppm	Need for rather cumbersome *cryo-preparation*
		Low thermic load on specimen due to scanning data acquisition	Thick samples require *correction factors*
			Quantitation of thin samples is straightforward
Simultaneous recording of elements within a specified volume that allows formation of elemental ratios, which can be used as sensitive monitors of physiological balance and unbalance in cells and tissues, for example, Na/K	Rapid *burnout of the organic scaffold* causes registration of higher than normal contents of elements	*Simultaneous recording* of elements within a specified volume that allows formation of elemental ratios, which can be used as sensitive monitors of physiological balance and unbalance in cells and tissues, for example, Ca/K, Ca/Zn, Fe/Zn	*Very long acquisition* times may result in *burnout of the organic scaffold*, causing a virtual higher than normal contents of elements
	Mapping, even of small areas, requires very long analysis time		
Mass determination by background absorption		*Mass determination* by back scattered protons or STIM, light element detection by nuclear complementary techniques, e.g., back scattering (C,N,O), photon tagged nuclear reaction analysis, pNRA for detection of B, Li, Na	
Elemental mapping to depict distributions of elements over the mass distribution image or a secondary electron image		*Elemental mapping* to depict distributions of elements over tissue section is a routine	

specimen, and also attenuation by the detector window. However, Na can be quantified concomitantly with energy detection via g-detectors by utilizing the nuclear reaction occurring as a result of proton capture by the Na nucleus.[11]

The heavy particles in the probe used in PIXE analysis are not as easily retarded as electrons by biological materials, and this results in a negligible background production allowing even weak secondary x-rays to be detected, hence the high sensitivity of 1 ppm.

The XRMA can generally be said to analyze elements down to contents of 200 ppm and is therefore essentially insensitive to elements such as Ca, Fe, and Zn occurring at low concentrations in the tissue and thus are denoted trace elements in biological tissues. The reason for this insensitivity is the fact that the light electrons impinging on the section are subject to multiple scattering and retardation, effects that produce a significant background of continuous radiation in which the weak trace element signals are buried.

5.7.2 Spatial Resolution

In EMP the cross section of the electron probe is often of the order of 2 nm, and with a section thickness approximately 100 nm a resolution at the subcellular level can be obtained. Considering the fact that physiologically interesting elements generally are freely dispersed in the cytosol, it is clear that local variations in concentration in biological tissues are to be expected. Therefore, analysis data are retrieved from sets of spots in regions of the tissue deemed to be representative of the structure under investigation.

In order to get reasonable acquisition times for data in PIXE-analysis, the sections used are generally 15 μm or thicker. This thickness causes an overlap of the secondary x-ray emission information from cellular structures in the depth of the section precluding a subcellular resolution at analysis (Figure 5.5). Furthermore, as the width of the PIXE probe is $\leq$5 μm, this represents another factor that diminishes the spatial resolution of PIXE. Thus, PIXE analysis superposes data from the intra- and extracellular compartments in the tissue during analysis due to the comparatively low spatial resolution of the measuring system and on the thickness of the sections ($\sim$15 μm or more). These facts relate to the considerable smoothing of curves describing the elemental and mass distribution over the cellular layers of a differentiated epidermis (Figure 5.6). The effect is perhaps most conspicuous in the very narrow stratum corneum region (a total width in a section of approximately 10 μm) where the mass curve is rather wide in these experiments. In general terms, this means that generally analysis within a defined cell compartment is not possible, and data are generally referred to as originating from a defined morphological entity, for example, a stratum of an epithelium, a special structure in the brain, etc.

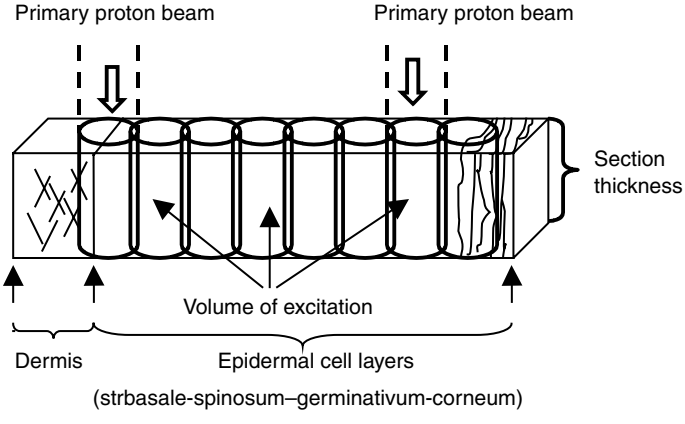

FIGURE 5.5 The secondary x-ray information will emerge from cellular structures in the depth of the section within volume of the proton beam volume of excitation. In addition, a probe diameter of 5 μm will result in lateral overlap of cellular compartments. Hence, the spatial resolution of the proton probe is restricted to strata rather than single cells. The resolution can be improved by diminishing the probe diameter ($<$2 μm) and the section thickness ($<$6 μm) at the cost of a substantial increase in acquisition time.

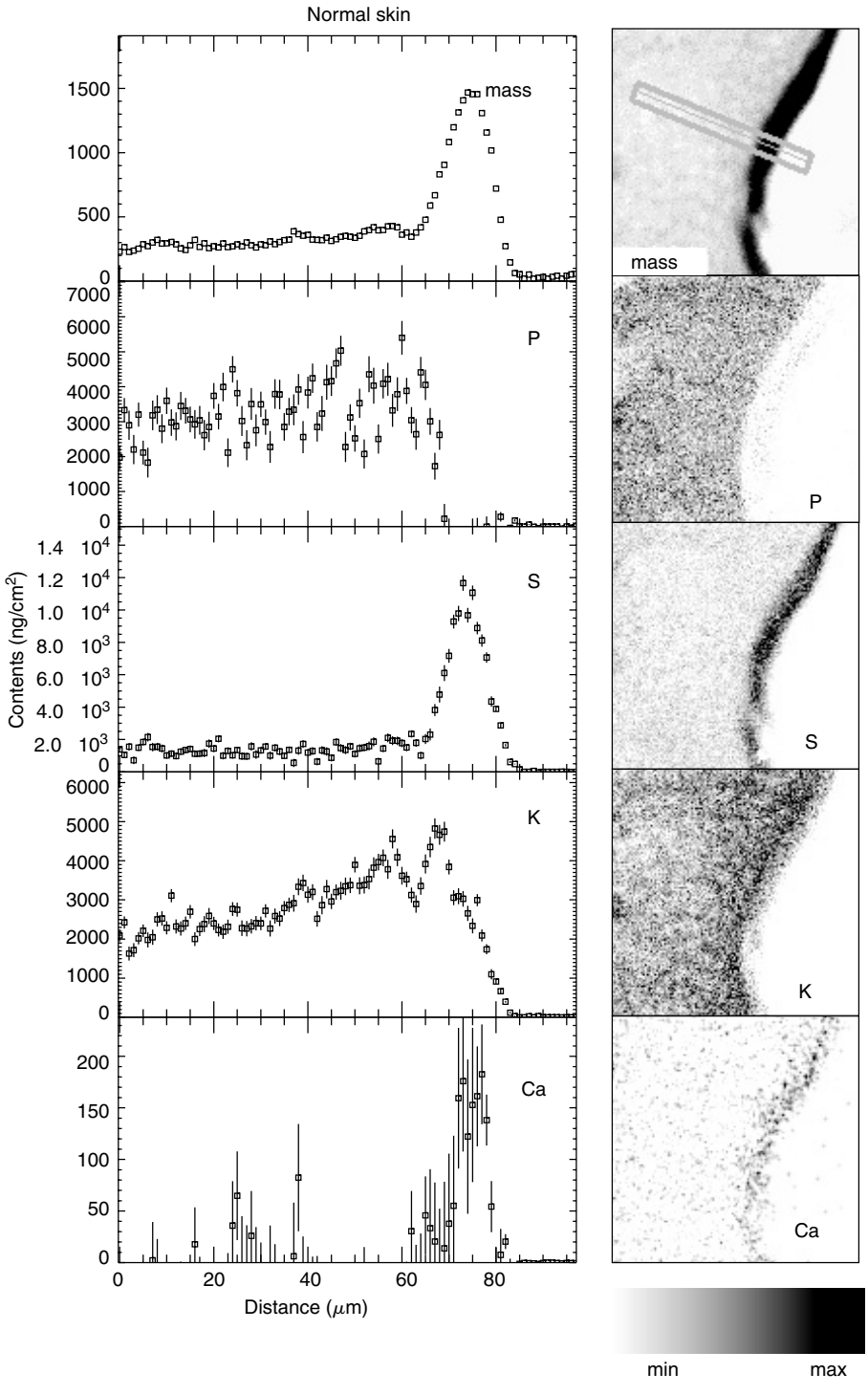

FIGURE 5.6 Proton probe analysis of a normal skin sample. The left panel demonstrates mass, P, S, K, and Ca distributions. To the right, the top picture represents a "vertical" scan across epidermis perpendicular to the skin surface (stratum corneum to the right; the basal cell layer to the left). The pixel mapping for P, S, K, and Ca are given in the right panel with the corresponding profiles in the left panel. Distance given in the left panel indicating the dermal–epidermal border at 0 and the outer part of stratum corneum at 80.

5.7.3 Limitations of PIXE Analysis

The number of specimens investigated in a PIXE study may appear small in comparison with the corresponding numbers used in different types of light microscopic investigations or biochemical studies. The typical acquisition time for a pixel map from a single section is generally about 30 to 45 min when trace elements are analyzed. Cost/benefit aspects of running an experiment will obviously require optimization of data acquisition for evaluation. To assess a number of data that would allow statistical analysis by the algorithm used, the time allowed has not been sufficient so far to quantify such elements as Mg, Cu, Ni, and Se.

5.8 ELEMENTAL MAPPING

In PIXE applications spot analysis was initially the dominating analysis method, but during the 1980s scanning procedures were developed, which allowed pixel mapping of the specimen. With a probe size of 5 μm, a tissue surface of $200 * 200$ μm can be covered by the pixel map in an acquisition time of approximately 30 to 45 min, that is, within this time period a representative area of the tissue section can be analyzed for all elements and mass (Figure 5.6). A further important advantage in PIXE mapping is the markedly diminished thermal load on the volumes of analysis that results in more reliable data when the influence of burnout of the organic matrix material was minimized.

From pixel maps obtained by the scanning proton probe, cross-section profiles of elemental distributions can be extracted (Figure 5.6). For elements present in trace amounts, long acquisition times are needed as mentioned. A corresponding map obtained with the EMP would require at least a five times longer acquisition time, which is the reason why elemental mapping for elements present in low concentrations has not been favored in XRMA.

5.8.1 Pixel Maps Provide Information on the Dynamics of Tissue Activity

Analysis of PIXE pixel maps reveals as one conspicuous feature the variation in the distribution of elements and trace elements seen between different strata of the skin as well as between different sections. This is found both in the normal and pathological skin. Such variations in the distribution of elements, and especially trace elements, indicate that there are obvious differences in the detailed cellular physiology of the differentiating keratinocytes. Such findings also harmonize with our previous experience from electron microscopic studies of irritant contact reactions.[12–14] These studies show that the correlation between the morphological image and the quantitative elemental data is not a direct one. Although morphologically similar, certain cells reveal their different stage of differentiation in the patterns of elemental distributions as suggested by the elemental maps. Combining other techniques (e.g., immunological, molecular, genetic) with elemental analysis should provide an additional detailed insight into the keratinization process programed to develop a complete stratum corneum with a functional barrier.

5.8.2 Multivariate Analysis Identifies Co-Variations of Elements and Strata

A recent development of proton probe analysis involves multidimensional statistical analysis of all data extracted from mass and elemental maps. The SIMCA™ program, which essentially analyzes

covariations of factors, allows not only comparisons of elemental levels within strata of an epidermal cross section, but it also allows comparisons between strata, elements, individuals, etc.[15]

5.9 TRACE ELEMENT ANALYSIS IS POSSIBLE WITH THE PROTON PROBES

In the initial studies of trace elements of human skin cross sections, a Ca profile evolved which was increasing from the basal, germinative level of the epidermis toward the horny layer. The drastic concentration drop of Ca concentration down to threshold values at the border of the stratum germinativum and the stratum corneum was a particularly interesting feature.[15–17] Only a few years later this finding could be correlated to the fact that a Ca concentration >0.1 mmol was essential if a fully cornified stratum corneum was to be obtained in cell culture. This relationship between Ca and terminal differentiation of the epidermal cells was verified in a PIXE study of epidermal cell cultures.[18]

5.9.1 The Ca^{2+} Signal

The influence of trace elements on the normal and abnormal physiology of the skin has not yet reached a full understanding. The role of calcium for epidermal differentiation has been demonstrated in a series of elegant studies by Elias and coworkers[19–21] using different techniques such as ion capture cytochemistry at transmission electron microscopic resolution. In the normal murine skin the most prominent localization of Ca^{2+} is noted in the upper stratum granulosum and the dermis, whereas the basal region is virtually free from Ca^{2+} precipitates. When the barrier was broken by acetone treatment, a redistribution of Ca^{2+} took place with a conspicuous accumulation of precipitates in the extracellular space of the stratum corneum and loss of the stratum granulosum localization. Treating the barrier-disrupted skin with iso-osmolar sucrose containing Ca^{2+} replenished the epidermal Ca^{2+} reservoir. However, the secretion of lamellar bodies was impeded and hence barrier recovery. Conversely, treatment with iso-osmolar sucrose only lead to barrier repair through lamellar body secretion in the absence of the normal Ca^{2+} gradient. The conclusion of the authors was that loss of the Ca^{2+} reservoir is an important signal for restoration of barrier function after damage. It has also been postulated that the elemental gradient across epidermis is crucial for the maintenance of a normal barrier function (stratum corneum).[22,23] In spite of these investigations there is a need for fully quantitative data to support these findings, and such quantitative data can be obtained using PIXE analysis.

5.9.2 Ca^{2+} and Programmed Cell Death or Apoptosis

The importance of Ca^{2+} as a signal for various cell functions is now established. A further aspect of the Ca^{2+} function is its property to promote "programmed cell death" (or apoptosis). Zn^{2+} has been shown to inhibit this effect.[24] As of new, it is not clear what role such a phenomenon plays in the sequel of cellular differentiation of the epidermis. This process can be regarded as a kind of programed cell death, which involves the complete dissolution of nucleic acid material in the stratum granulosum.[25] One indication on the importance of increased Ca levels in this cellular stratum is related to the finding that a full differentiation of the epidermis does not occur in tissue culture unless the Ca content equals at least 0.1 mM.[26] One may speculate that these findings suggest an explanation to the sporadic occurrence of parakeratotic cells in the stratum corneum in the paralesional psoriatic epidermis where unusually high zinc levels are recorded in the stratum granulosum zone. Whether this effect is directly coupled to an increased cellular activity in the germinative pool may be a matter of speculation, but the actual high levels of iron (Fe) compared to normal skin suggest such

an increased activity. Further detailed analyses, including particle probe studies, are required before this question can be settled.

The skin dependence on appropriate availability of Zn^{2+} for normal function is a subject that has not been completely resolved.[27,28] However, it is conceivable that these problems can at least partially be solved using particle probe analysis.

5.9.3 Iron and Zinc

Iron has a high peak value in the basal cell region, drops to values less than half the peak value in the uppermost epidermal layers, and is not detectable in the stratum corneum region.[16] Zinc (Zn), which is represented in the dermis by concentrations below or just at the detection level of the system, shows a comparatively stable level over the Malpighian epidermis and disappears coincidentally with Fe in the stratum corneum region.[15,16] So far, the PIXE data obtained from normal skin suggest an approximate ratio of Zn content in epidermis/dermis of 3:1 to be compared with a 6:1 ratio given by neutron activation analysis.[29] Copper (Cu) is just barely detectable in the Malpighian layers, and generally no quantitation is possible within the acquisition times used in most of our experiments.

5.9.4 Mass and Elemental Distributions of the Epidermis — PIXE Data

The mass distribution curves reach a peak in the stratum corneum region, flatten out in the basal cell region, and then rise again in the dermis in full agreement with the previous XRMA data. The S distribution curves follow the mass curves in concert. Around 30 to 50 μm below the maximum mass peak, at a region corresponding to the stratum spinosum, we find the P distribution peak. Chlorine (Cl) has a weak minimum approximately where the P distribution has its peak. In the vicinity of stratum corneum, which is virtually free from Cl, there is a conspicuous drop in the Cl content. K reaches its highest levels in stratum granulosum and drops to nil in the stratum corneum.

5.9.5 Recycling of Diffusible Ions

Summing up the data on the distribution of physiologically important diffuable elements, it is interesting to note that at the border between the viable epidermis and the stratum corneum their contents are close to or below the detection limit of the particle probes with the particular exception of calcium (see Chapter 8). The disappearance of the diffusible ions can be understood if we consider the fact that the water content is roughly constant over the epidermal cross section, finally dropping to low values within the stratum corneum.[21,30,31] The mass content of the cells increase continuously on the passage from the cells of the basal layer to the final fully cornified corneocyte. Since keratin binds water, we can see that the amount of free water available for the freely diffusible ions decreases with increasing mass. This creates a downhill gradient directed toward the dermis. Therefore, the recycling of freely diffusible ions requires no special energy-consuming mechanism.

5.9.6 Local Variations of Element and Trace Element Distributions

There are slight, but obvious variations in the elemental distribution patterns from one section to another, although a general trend can clearly be discerned. The Fe and Zn distributions have their centers of gravity in the stratum spinosum/stratum granulosum area, but Zn is more clearly confined to the basal layer.

5.9.7 Horizontal Elemental Distributions

The element distribution curves have generally been extracted from pixel "channels," which cover the cross section from stratum corneum down into the papillary dermis. When data are retrieved so as to represent pixel channels that cover a specified stratum horizontally, it can be seen that the mass distribution along the basal lamina of normal skin varies somewhat along the horizontal scan. However, it remains approximately constant at the upper level (the level of the stratum spinosum/granulosum). This obviously relates to the fact that the basal cells may be in different phases of the cell division cycle, whereas the stratum spinosum cells are more synchronized in their development. K and Cl covary with mass in the basal region, but the variation is more independent in the stratum spinosum region as expected from cross-section distribution.[13] As expected S covaries to a great extent with the mass distribution. In stratum spinosum and stratum basal more conspicuous variations in relation to mass are seen in the P distribution.

There is an extensive variation in the Fe and Zn distributions in the basal region. The Fe content is close to or below the detection limit in the upper region; the Zn content shows some peaks above the detection limit. Single off-limit values in these trace elements were seen.

Ca appears to stay rather constant within each horizontally scanned band, which is consistent with the fact that the increase in Ca toward the stratum corneum is likely to be related to the physiological and regulatory effects of this ion.

5.10 ELECTRON AND PROTON PROBE DATA FROM PATHOLOGICAL SKIN

5.10.1 Psoriasis

An early XRMA study in which compared skin from healthy, normal persons with uninvolved and involved (a stable plaque) psoriatic skin revealed that Mg, P, and K were increased in the involved skin corresponding to what is recorded in highly proliferative, nonneoplastic cells.[32,33] Previously Burkhart and Burnham had recorded a significant increase in P and Ca content in involved psoriatic skin compared to uninvolved skin from the same patients.[34] Later, Kurtz et al. reported corresponding findings from a PIXE study of psoriatic skin, but found no difference between the Zn content of control skin and uninvolved skin from psoriatic patients.[35] However, in pinpoint lesions they recorded a significant increase of Zn corresponding to neutron activation analysis data given by Molin and Wester (Table IV).[36,37] Some interesting aspects on the elemental distribution were revealed in a study of uninvolved psoriatic skin.[38] Our PIXE data demonstrated that uninvolved psoriatic skin has a mass distribution with the same general features as that of normal skin, although generally at a lower level (absolute mass content). The P and S distributions are not conspicuously different from those of normal skin.

Ca shows a twofold or even higher increase in the stratum granulosum region compared to normal skin, but in contrast to the Ca distribution in normal skin, that of psoriasis follows the mass distribution more closely. In many sections there is an additional Ca peak in the vicinity of the basal cell layer, but the full significance of this is not clear.

The trace element distributions of uninvolved psoriatic skin merit special comments. The main Fe peak appears closer to the mass distribution peak than in normal skin. Also, there are obvious variations in the Fe content in different strata (cell layers), and the lowermost values are consistently at least twice as high as those in normal skin. Our PIXE investigation substantiates the previously reported finding that psoriatic patients lose Fe through the shedding of stratum corneum cells in lesional areas by demonstrating that clinically normal skin of psoriatic patients contains higher than normal amounts of Fe.[36,37]

The Zn content of the uninvolved psoriatic skin is increased in the stratum spinosum especially, except in one single section where the Zn follows suit with Fe distribution. Such variations are likely to occur as a function of the cell cycle position of a particular cell.

5.10.2 Elemental Distributions in Different Strata of Psoriatic Normal-Looking Skin — Horizontal Scans

In comparison to the control skin there are high mean values and prominent variations in the trace elements, notably Fe and Zn, in the upper level of the epidermis. However, the mass distribution pattern essentially follows that of normal control skin with some variations in the upper layer.

In spite of the fact that the spatial resolution does not allow discrimination between the intra- and extracellular compartments, PIXE data nevertheless reveal some crucial points concerning the physiology of normal-appearing psoriatic skin as opposed to the normal skin. The Ca distribution profile, which in a normal skin remains at an almost constant level over the skin cross section, shows a slight increase in the stratum granulosum region in certain specimens. This differs from data of a previous preliminary study based on selected point measurements in different strata of skin sections.[15] However, with the new information obtained from the elemental maps such a variation is likely to occur as an expression of the continuous changes occurring *in vivo*. Continuous changes like these are represented in similar studies using quench frozen specimens by a "snap-shot" depicting momentarily what are actually transient processes.

In order to elucidate the background to these abnormal elemental distributions, further studies including psoriatic plaques will be needed. Recent data from dry skin of atopics also present elemental distributions, which vary conspicuously from those found in normal.[15,39] These facts challenge our experimental imagination to produce answers to what faults in the cellular mechanisms are at hand in these skin disorders.

5.10.3 Metal Allergy

In the Western Hemisphere Ni allergy has very rapidly grown to be a major dermatological problem. The penetration profile of this metal ion through the skin remained largely unknown, in spite of previous studies on Ni penetration through human skin using XRMA, due to the insensitivity of the method.[13,40,41] In a PIXE study of skin samples from individuals tested for Ni allergy, it was demonstrated that the Ni accumulated in the stratum corneum and that only trace levels passed through the skin barrier.[42] These findings suggest that extremely minute amounts of Ni are needed to elicit an allergic reaction in an Ni-sensitized individual. It corresponds to the observation that just a single, very brief contact with a dry nickel-plated object may elicit an allergic reaction.

5.10.4 Irritant Contact Dermatitis and Effects on the Keratinocytes — EMP Data

Irritant contact dermatitis is a public health problem. Skin exposure to irritants (e.g., water, detergents, and solvents) causes damage to the barrier and induces an inflammation, ultimately contact dermatitis.[43] By combining microprobe analysis with other techniques it is possible to correlate physiological changes (e.g., barrier repair processes) with effects on the keratinocytes. In a series

of *in vivo* and *in vitro* experiments[12–14,44–46] we have been able to relate changes in the Na/K ratio and P content of the keratinocytes to changes in proliferation and up-regulation of inflammatory mediators.

5.10.5 Multidimensional Statistical Analysis Using SIMCA

Primary data are not always easy to interpret to give a functional picture of the tissue physiology. Ratios of elements such as Ca/Zn can provide interesting information when one realizes that Zn may be antagonistic to effects elicited by Ca. But comparisons and correlations of data from different strata, individuals, and disorders are still problematic. From a physiological/biological point of view correlations may provide more pertinent information than straightforward statistics which just provide statistical significances. The recent introduction of multidimensional statistical analysis (SIMCA) conspicuously broadens the possibility of meaningful interpretations of primary data. Using SIMCA to study the dry skin of atopic individuals, it turns out that when we look for correlations between strata of the epidermis and elements, the dry atopic skin proves to be very immature compared to the unafflicted skin of normal (control) individuals. A SIMCA scatter plot shows that the stratum basale and spinosum covariate in the atopic skin, but are well separated in the control skin.[47] Such information suggests that the stratum spinosum of the atopic skin is immature. Multidimensional statistical analysis allows us to understand data in physiological terms and will undoubtedly have an impact on the analysis of skin disorder obtained by biochemical and immunological means.

In a recent study of clinically normal skin from patients with psoriasis, a high Fe content of the horny layer was demonstrated, whereas there was no detectable Fe in the horny layer of normal healthy control individuals.[38] Obviously, this finding in psoriasis demonstrates that the entire differentiating epidermis of these patients is involved in the disorder, whether clinically expressed or not.

5.11 SUMMARY AND CONCLUSIONS

In this chapter, as well as in Chapter 8, the feasibility of skin physiology studies using particle probe analysis has been demonstrated. The EMP or XRMA analysis of biological tissues has allowed the study of physiological processes, which cannot be attacked using common physiological techniques, e.g., microelectrode registrations. An example of "impossible physiology" that was subsequently allowed by the XRMA is the study of the physiology of the differentiating epidermis (Figure 5.2).[48] Thus, we were able to show that the cells on the basal lamina separating the fibrous tissue of the dermis from the cellular tissue of the epidermis are the only cells upholding a normal Na/K ratio. The next cellular level has already suffered an increase in the Na and a decrease in K, meaning that these cells either leak ion or that their membrane pumps are deficient. A consequence of this is that only the basal cells with a normal Na/K ratio can go through the mitosis cycle producing a progeny. The biological meaning of this is obviously one of cell division control, resulting in a smooth skin surface.

Further, the EMP has allowed studies of the water profile over the skin cross section[30,31] the physiological changes at irritant reactions[12–14,44–46] and psoriasis.[32] A comprehensive overview of the EMP application is given in two overview papers.[49,50]

The application of x-ray analysis methods to biological problems has proven to be of great, sometimes unsurpassed, value. Recent developments of computer software, statistical programs, etc. have tremendously broadened the possibility of data retrieval and handling. The reason why we do not find more biologically oriented work in the literature is obviously due to an information gap, that is, an educational problem — biologists/medical researchers know too little about x-ray physics, and physicists know too little about biological systems and the effects of biological tissue preparation.

Particle probe analysis and, in particular, proton probe analysis, which is sensitive to trace element levels in tissue sections have been demonstrated to reveal important details about cellular physiology in the differentiating epidermis of normal and pathological skin. Such a physiological approach will serve to complement data from other techniques. A future collective approach of this kind will make it possible to understand how a dry and eczematous skin develops and also what the mechanisms of subsequent healing are.

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6 Role of Calcium Ions in the Regulation of Skin Barrier Homeostasis

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CONTENTS

6.1 INTRODUCTION

Dry skin symptoms are frequently linked to an impaired skin barrier function, as observed in psoriasis, ichtyosis, atopic skin, and contact eczemas.[1] More precisely, this skin barrier function is connected to the chemical and physical condition of the stratum corneum (SC), the uppermost layer of the epidermis. SC gives protection against desiccation and environmental challenge by regulating water flux and retention.[2] The optimal level of hydration maintained in SC is largely dependent on three components, which are constantly regenerated in this particular skin layer, namely (1) intercellular lamellar lipids, as an effective barrier to the passage of water; (2) corneocytes (SC cells), which provide the tortuous diffusion path, created by the SC layers and corneocyte envelopes, that retard water loss, and (3) natural moisturizing factor (NMF), a complex mixture of low-molecular-weight, water-soluble compounds first formed within the corneocytes by degradation of the histidine-rich protein known as filaggrin. Disturbance to the regeneration processes of these components results in dry, flaky skin conditions.[3]

The importance of calcium in the regulation of skin barrier homeostasis is apparent as calcium is involved in the regeneration process of skin barrier components.[4] Hence, the balance of calcium level in skin is closely related to hydration of the skin. Apart from the skin, this ion plays a crucial role in various processes in the body, including the growth, death, differentiation, and function of immune cells. The role of calcium in skin is found to be more complex than previously assumed. The elucidation of calcium regulation mechanism in skin could be useful to understand and solve skin problems.

6.2 MECHANISM OF CALCIUM CELL SIGNALING

In the body, calcium, in the form of the ion Ca^{2+}, is the most abundant metal ion and fifth (after H, O, C, and N) most abundant element in the body, on both an atom and weight basis. Over 90% of body

calcium resides in bones and tooth enamel. The rest, described as mobile Ca^{2+}, is found throughout body fluids and takes part in various processes, including muscle contraction, blood clotting, nerve excitability, intercellular communication, membrane transport of molecules, hormonal responses, exocytosis, cell fusion, adhesion, and growth.[5]

Mobile calcium is a universal messenger for living things, even in simple organisms and plants. The unique combination of its ionic radius and double charge allows Ca^{2+} to be specifically recognized and to yield tighter binding to receptors to the exclusion of other ions, leading to strong, specific binding.[6] The specificity enables cells to form special receptors to assess signals from calcium. For many parts of the body, Ca^{2+} often acts as a second messenger in a manner similar to cAMP. Transient increases in cytosolic Ca^{2+} concentration trigger numerous cellular responses including muscle contraction, release of neurotransmitters, and glycogen breakdown (glycogenolysis), and also act as an important activator of oxidative metabolism (7, pp. 496–498). Ca^{2+} does not need to be synthesized and degraded with each message transmission, so it is an energy-efficient signal for the cell.[8]

In skin, calcium can provide signals for the cells, either extracellular or intracellular (in the cytosol). The extra- and intracellular signaling may be connected to each other, but may also act separately. In cultured keratinocytes, extracellular calcium levels influence growth and differentiation.[9,10] Low extracellular calcium levels (<0.1 m*M*) induce the growth of keratinocytes as a monolayer with a high proliferation rate, rapidly becoming confluent. In this condition keratinocytes never stratify, but possess many of the characteristics of basal cells; the cells synthesize keratin proteins and are connected by occasional gap junctions but not by desmosomes. High extracellular calcium levels (>1 m*M*) induce differentiation of keratinocytes. Keratinocytes rapidly flatten, form desmosomes, and differentiate with stratification. Moreover, cornified envelopes form in cells of the uppermost layers.[9,10]

The response to signaling is also shown in a progressive way. Keratinocytes grown in a low-calcium media proliferate. Increased extracellular Ca^{2+} inhibits proliferation, while it induces differentiation.[11] On the other hand, differentiation of keratinocytes causes a decrease in responsiveness to extracellular calcium, which may facilitate the maintenance of the high level of intracellular calcium required for differentiation.[12]

Intracellular Ca^{2+} increases with raised extracellular Ca^{2+}.[13–15] This implies that increased intracellular Ca^{2+} is the actual signal to trigger keratinocyte differentiation. Intracellular Ca^{2+} signals are assessed through calcium-binding proteins to induce responses. The major calcium-binding protein in skin is calmodulin. Calmodulin regulates target protein by modulating protein–protein interactions in a calcium-dependent way. Calmodulin regulates many enzymes, for example, adenyl and guanyl cyclase, phosphodiesterase, ornithine decarboxylase, calcium-calmodulin-dependent protein kinase, transglutaminase, and phospholipase, which are also found in skin.[8]

Both intracellular release and transmembrane flux contribute to the rise in intracellular Ca^{2+}.[14,15] The rise in keratinocyte intracellular Ca^{2+} in response to raised extracellular Ca^{2+} has two phases: (a) an initial peak, not dependent on extracellular Ca^{2+} and (b) a later phase that requires extracellular Ca^{2+}.[14] An early response of human keratinocytes to increases in extracellular Ca^{2+} is an acute increase in intracellular Ca^{2+}. Stepwise addition of extracellular Ca^{2+} to neonatal human keratinocytes is followed by a progressive increase in intracellular Ca^{2+}, where the initial spike of increased intracellular Ca^{2+} is followed by a prolonged plateau of higher intracellular Ca^{2+}.[16] The response of intracellular Ca^{2+} to increased extracellular Ca^{2+} in keratinocytes is saturated at 2.0 m*M* extracellular Ca^{2+}.[16,17] The response of intracellular Ca^{2+} to increased extracellular Ca^{2+} in keratinocytes resembles the response in parathyroid cells, in that a rapid and transient increase in intracellular Ca^{2+} is followed by a sustained increase in intracellular Ca^{2+} above basal level. This multiphasic response is attributed to an initial release of Ca^{2+} from intracellular stores followed by an increased influx of Ca^{2+} through voltage-independent cation channels. The keratinocyte and parathyroid cell contains a similar cell membrane calcium receptor thought to mediate this response to extracellular Ca^{2+}. This receptor can activate the phospholipase-C pathway, leading to an increase

in the levels of inositol 1,4,5-triphosphate (IP_3) and *sn*-l,2-diacylglycerol (DAG) — both of which are important messengers — as well as stimulating Ca^{2+} influx and chloride currents,[18,19] IP_3 causes release of Ca^{2+} from internal stores, such as endoplasmic reticulum, further increasing intracellular level to precede a number of calcium-stimulated cellular events.[20] DAG forms a quarternary complex with phosphatidylserine, calcium, and protein kinase C to activate the kinase. This will accelerate terminal differentiation (Hennings et al., 1983). The signal transduction mediated through calmodulin induces other proteins, for example, desmocalmin, which is associated with the formation of desmosomes.[21]

Keratinocytes grown in low-calcium medium (0.02 m*M*) maintained intracellular calcium levels adequate for arachidonic acid metabolism and actually showed increased prostaglandin (mainly PGE_2 and PGF_2) production up to 4.5 times compared to cells grown at normal calcium level (1.2 m*M*).[22] If this is true for the *in vivo* condition, a low level of extracellular calcium — for instance, due to a defective skin barrier — may cause an increase in prostaglandin synthesis, leading to hyperproliferative epidermal disorders, such as psoriasis, which are often associated with abnormalities in prostaglandin production.[23]

6.3 REGULATION OF CALCIUM

The regulation of calcium in skin shows an ingenious adaptation of living organisms to the presence of ions. As Ca^{2+} cannot be metabolized like other second-messenger molecules, cells tightly regulate intracellular levels through numerous binding and specialized extrusion proteins.[24] The concentration of calcium in extracellular spaces (generally $\sim$1.5 m*M*) is four orders of magnitude higher than in the cytosol ($\sim$0.1 μM). In excitable cells, for example, muscle cells, the extracellular concentration of calcium must be closely regulated to keep it at its normal level of $\sim$1.5 m*M*, so that it cannot accidentally trigger the muscle contraction, the transmission of nerve impulses, and blood clotting (7, p. 1144). In other cells, including keratinocytes, the extracellular level is maintained in a specific equilibrium with the intracellular concentration.

What is the importance to keep the intracellular calcium level low? A low calcium concentration makes the use of the ion as an intracellular messenger energetically inexpensive. The movement of calcium ions across membranes requires energy, usually supplied by ATP. If the resting level of calcium in the cell were high, a large number of ions would need to be transported into the cytoplasm to raise the concentration by the factor of ten that is ordinarily needed to activate an enzyme; afterward the excess calcium would have to be expelled from the cell. Normally low calcium level means that relatively few ions need to be moved, with a relatively small expenditure of energy, to regulate an enzyme. In contrast, energetic cost of regulation by the other important intracellular messenger, cyclic adenosine monophosphate (cyclic AMP), is high; it must be synthesized and broken down each time it carries a message, and both steps requires a significant investment of energy.[6] Furthermore, low intracellular calcium is a necessary condition for the phosphate-driven metabolism characteristic of higher organisms. The energy-rich fuel for most cellular processes is adenosine triphosphate (ATP). Its breakdown releases inorganic phosphate. If the intracellular concentration of calcium were high, the phosphate and the calcium would combine to form a precipitate of hydroxyapatite crystals — the same stony substance found in bone. Ultimately calcification would doom the cell.[6] This is likely the case with long-term occupational exposure to high levels of dissolved calcium, for example, in miners,[25] agricultural laborers,[26] and oil field workers,[27] which can result in calcinosis cutis, a benign and reversible hardening of the exposed skin.

The large concentration gradient between extracellular spaces and cytosol is maintained by the active transport of Ca^{2+} across the plasma membrane, the endoplasmic reticulum (or the sarcoplasmic reticulum in muscle), and the mitochondrial inner membrane. Generally, plasma membrane and endoplasmic reticulum each contain a Ca^{2+}-ATPase that actively pumps Ca^{2+} out of the cytosol at the expense of ATP hydrolysis (7, pp. 496–498). Mitochondria act as a "buffer" for cytosolic Ca^{2+}:

if cytosolic concentration of calcium rises, the rate of mitochondrial Ca^{2+} influx increases while that of Ca^{2+} efflux remains constant, causing the mitochondrial concentration of calcium to increase, while the cytosolic concentration of calcium decreases to its original level (its set-point). Conversely, a decrease in cytosolic concentration of calcium reduces the mitochondrial influx rate, causing net efflux of calcium from mitochondria and an increase of cytosolic concentration of calcium back to the set-point (7, p. 531).

Besides the already mentioned Ca^{2+}-ATPase, the transport of Ca^{2+} is regulated by a series of calcium pumps, transport systems, and ion channels. The availability of certain regulatory systems is dependent on the activity of the cells. In excitable cells such as cardiac muscle, the influx of Ca^{2+} to cytosol is regulated by voltage- (or potential-) dependent channels, while the efflux (out of cytosol) is regulated by cation exchanger, such as Na^{+}–Ca^{2+} exchanger.[8] Undifferentiated keratinocytes in the basal layer have different sets of Ca^{2+} transport system than differentiated cells in the upper layers. In basal layer, the system consists of 14-pS nonspecific cation channels (NSCC)[28] and does not possess functional voltage-sensitive Ca^{2+} channels.[15] Differentiated keratinocytes are likely to possess at least two and possibly three pathways of Ca^{2+} influx: (a) nicotinic channel (nAChR); (b) voltage-sensitive Ca^{2+} channels (VSCC, which can be blocked by nifedipine or verapamil); and (c) NSCC, which is not activated by nicotine.[29]

The permeability of skin to Ca^{2+} ions has been known from some dermatoses, such as calcinosis cutis[25–27] and perforating verruciform collagenoma.[30] In a shorter term, calcinosis cutis developed after a 24 h (at least) topical application of an electrode paste containing saturated calcium chloride solution, bentonite, and glycerin, used for examination by electroencephalography or electromyography.[31,32] The permeability of human skin to Ca^{2+} ions *in vitro* shows a marked dependence upon anatomic site. In agreement with the data observed for nonelectrolytes, permeation decreased in the following order: foreskin > mammary > scalp > thigh. Mouse and guinea pig skin show comparable permeability to that of human scalp. Ca^{2+} transport from dermis across epidermis is higher than that from epidermis to dermis.[33,34]

A technique was developed to continuously monitor the low level of Ca^{2+} flux across human SC *in vitro*. The study showed that the flux through untreated human SC was sigmoidal. The steady-state flux had an average of 7×10^{-12} mol/cm^2/s. After the SC was pretreated with acetone or sodium lauryl sulfate, the shape of the curve was similar but the Ca^{2+} flux was significantly higher.[35]

6.4 CALCIUM GRADIENT

As mentioned earlier, there is a high calcium gradient between extra- and intracellular domains of keratinocytes, which requires tight regulation. Moreover, a calcium gradient is present within the epidermis, with higher quantities of Ca^{2+} in the upper than in the lower epidermis (as the cell moves from the basal layer to the stratum granulosum).[36] Ca^{2+} concentration increases steadily from the basal region to SC, while this is not the case with other ions.[37] Figure 6.1 illustrates the calcium gradient in human skin in comparison with an actual literature data.[38] Such a gradient is not observed in skin abnormalities related to the formation of abnormal barrier function, such as psoriasis.[39] Studies in mice and rats showed that this gradient exists at the same time as the formation of a maturing skin barrier at the end of gestation. The gradient is then maintained from the newborn throughout the adult life.[40]

It is not yet clear whether the calcium gradient leads to the formation of a mature barrier or the barrier caused the gradient. It may even be both, if the regulation uses a feedback mechanism, as the differentiation will eventually form a barrier leading to the accumulation of calcium ions in the upper epidermis. This high level of calcium will, in turn, guarantee the ongoing process of differentiation toward the formation of corneocytes (horny cells in the SC). The mechanism is thus almost completely autonomous, perpetual, and, if it runs smoothly, requires little correction from the body.

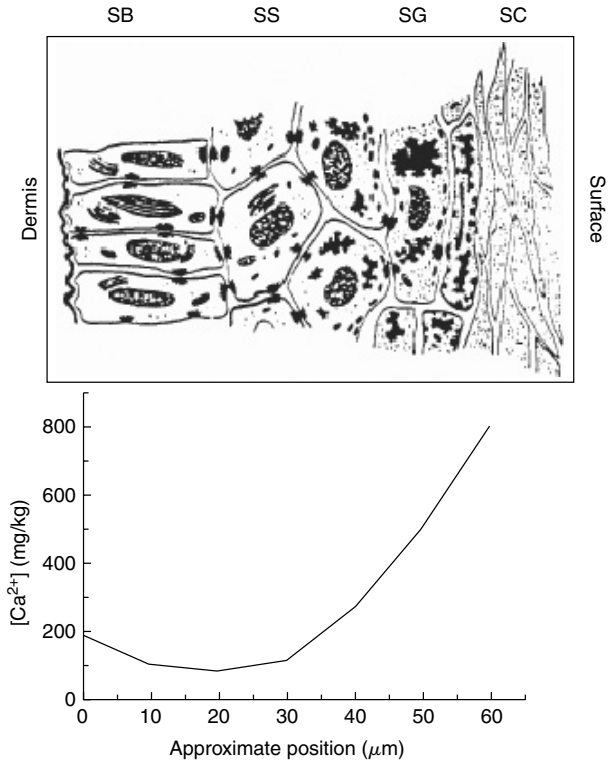

FIGURE 6.1 Illustration of calcium gradient in epidermis based on literature data (proton induced x-ray emission analysis of calcium in sectioned human skin) (Malmqvist et al., 1987). SB, stratum basale/basallayer; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum.

6.5 CALCIUM AND BARRIER REPAIR MECHANISM

Disruption of the barrier with acetone treatment or tape stripping depletes Ca^{2+} from the upper epidermis, resulting in the loss of the Ca^{2+} gradient.[41–43] This is due to accelerated water transit that leads to the increased passive loss of Ca^{2+} into and through the SC.[41,43] One *in vitro* study showed that the permeability of human SC to Ca^{2+} dramatically increased after the SC was pretreated with acetone or sodium lauryl sulfate solution.[35] The decrease in Ca^{2+} levels in the outer epidermis is associated with enhanced lamellar body secretion and lipid synthesis (important components in repair responses).[41,44] However, if Ca^{2+} gradient is preserved by the addition of Ca^{2+} into the media, lamellar body secretion, lipid synthesis, and barrier recovery are inhibited.[44] The inhibition raised by high extracellular concentration of calcium is potentiated by high extracellular K^+.[45] Another study confirmed that barrier recovery is accelerated by the low concentrations of calcium and also potassium during an increased water loss, since water loss may induce a decrease in the concentration of Ca^{2+} in the upper epidermis, which, in turn, may stimulate lamellar body secretion and barrier repair.[46] Furthermore, the inhibition raised by high extracellular concentration of calcium is reversed by nifedipine or verapamil, specific calcium channel blockers.[45] In another study, administration of Ca^{2+} free solutions by sonophoresis resulted in a marked decrease in Ca^{2+} content in the upper epidermis, and subsequently the loss of the Ca^{2+} gradient was accompanied by accelerated lamellar body secretion (a sign of skin barrier repair).[47]

The process of barrier repair in connection with transepidermal water loss and calcium gradient is illustrated in Figure 6.2. Experiment in mice shows that the calcium gradient disappears after acute permeability barrier disruption, and returns after 6 h in parallel with barrier recovery, barrier

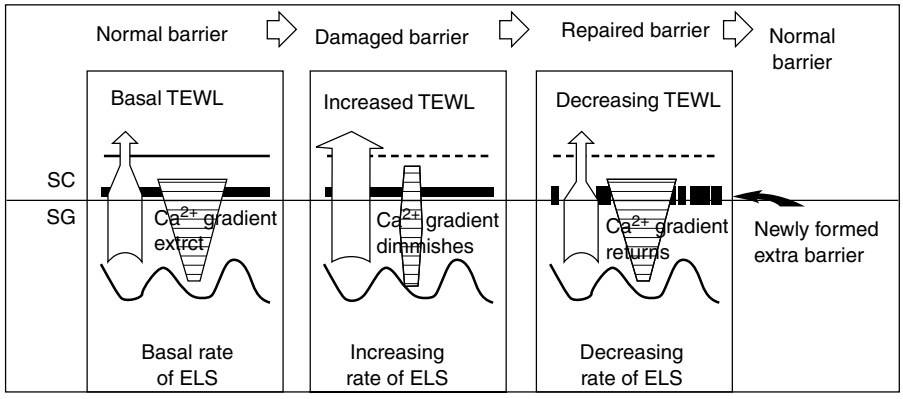

FIGURE 6.2 Illustration of skin barrier repair in epidermis. SC, stratum corneum; SG, stratum granulosum; TEWL, transepidermal water loss; ELS, epidermal lipid synthesis.

function (through restriction of transcutaneous water movement) could regulate the formation of the epidermal calcium gradient.[48]

"It should be noted that the barrier repair in response to the skin barrier disruption is not the same as the normal barrier regeneration process. The response is an emergency step to quickly reduce the transepidermal water loss to its set-point and thereby returning the calcium gradient to its natural condition (41). Once the calcium gradient is normalized, the normal skin barrier regeneration takes place. It is confirmed that addition of high calcium concentration during the barrier disruption process will induce higher influx of calcium into epidermal keratinocytes which delays the emergency skin barrier repair process (new ref. 1). However, during this delay and if the applied calcium concentration is within the right physiological range, the normal skin regeneration process can take place and the normal barrier function is restored without the formation of intermediate emergency barrier. This is indicated in a study on the cultured keratinocytes that extracellular calcium in physiological range of concentration is not a sufficient signal for growth arrest when other growth conditions are optimized (new ref. 2). The restoration of normal barrier function during the application of high concentration of calcium is evident from the effect of bathing in the calcium-rich Dead Sea water to improve skin diseases related to skin barrier impairment (new ref. 3) as well as to enhance skin hydration and reduce inflammation in atopic dry skin (new ref. 4) ."

6.6 CONCLUSION

Calcium ions play an important role in the homeostasis of skin barrier. A change in the barrier will change the calcium ion gradient in skin and lead to disturbance in the skin barrier regeneration process. A severe change might lead to a high degree of calcium signaling, which may induce the activation of various processes, from increased synthesis of skin components or messengers to the inflammatory reactions. All of these are important factors leading to dry skin conditions. The regulation of calcium in skin is therefore necessary to maintain a good skin barrier function and to avoid dry skin symptoms.

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7 Desquamation

Torbjörn Egelrud

CONTENTS

7.1 INTRODUCTION

The stratum corneum is a cellular tissue. Its building blocks, the corneocytes, are highly resistant to physical and chemical trauma. The mechanical strength of an individual corneocyte, emanating from its tightly packed keratin bundles and the cross-linked proteins of the cornified envelope, is outstanding. The mechanical resistance of individual corneocytes is mirrored by the pronounced mechanical strength of the entire stratum corneum, implying a strong cell cohesion within the tissue. The corneocytes and their intercellular cohesive structures are prerequisites for the function of the stratum corneum as the physical–chemical barrier between body interior and exterior, serving as an important part of the barrier as well as a backbone for the intercellular barrier lipids.

The stratum corneum is continuously being formed in the process of terminal keratinocyte differentiation. The rate of stratum corneum renewal is determined by the rate of cell proliferation in the basal layer of the epidermis. The fact that the thickness of the stratum corneum is fairly constant at a given body site implies that a fraction of the most superficial parts of the stratum corneum must be continuously shed at a rate that balances *de novo* production of corneocytes. This process, desquamation, normally occurs invisibly with shedding of individual cells or small aggregates of cells, resulting in the smooth appearance of the skin surface associated with a "normal" skin condition. Disturbances in this process, due to either increased production of corneocytes or a decreased rate of cell shedding, results in the accumulation on the skin surface of only partially detached cells with or without a concomitant thickening of the stratum corneum. The severity of the disturbance may vary from modest to very pronounced, from a barely visible scaling combined with a feeling of roughness and dryness of the skin surface to the accumulation of thick brittle scales such as in psoriasis or in the various forms of ichthyosis.

Thus, it can be concluded that there must be mechanisms within the stratum corneum, which are responsible for a well-regulated desquamation. A closer look at the criteria that must be fulfilled by these mechanisms suggests that they are likely to be of significant complexity. As stated previously, the barrier function of the stratum corneum depends on a strong cohesion between individual

corneocytes. The elimination of cell cohesion, a prerequisite for desquamation, would be deleterious if it took place in the barrier-forming parts of the stratum corneum. Under normal conditions the turnover time of the stratum corneum is two to four weeks. Moreover, corneocytes are "dead" in the sense that they have no protein synthesis, they have no active turnover of cell surface structures, and they are unresponsive to cellular signaling. Thus, chemical reactions leading to structural and functional changes within the stratum corneum may be considered as the final steps of a series of events initiated in viable parts of the epidermis. The process, which occur spontaneously without further input of regulatory signals, but yet in a well-regulated manner, depends on enzymes and other components produced by still living keratinocytes. In other words, at the time when a viable keratinocyte of the stratum granulosum is transformed to a corneocyte of the stratum corneum, the cell and the tissue it becomes part of must be "programed" in a way that allows the cell to be strongly linked to contiguous cells for a certain period of time, after which its cohesion to its neighbors should decrease to an extent, which will eventually allow it to be shed from the skin surface.

It seems reasonable to believe that a better understanding of desquamation and the mechanisms involved would give us possibilities to design better treatments for skin disorders associated with disturbances in stratum corneum turnover, be they common "dry skin problems" or results of more or less handicapping skin diseases. One strategy to understand desquamation would be to first identify mechanisms of cell cohesion in the stratum corneum, the structures involved, and the changes these structures undergo as cell cohesion decreases. The next step would be the identification of chemical reactions taking place, which would immediately give clues as to the nature of enzymes likely to be involved. Another fruitful strategy would be to elucidate the molecular basis and pathophysiology of diseases such as ichthyoses (see Chapter 8). The elucidation of ichthyosis-like conditions induced by certain drugs may also be expected to be productive in this context.[1,2]

The most likely site at that the events that eventually lead to desquamation take place is the stratum corneum intercellular space. As described in other chapters of this book, the chemical composition, organization, and interactions of this part of the stratum corneum are extremely complex. The stratum corneum intercellular space may be considered as a multiphase system consisting of a complex mixture of lipids in which structural proteins, enzymes, and other nonstructural proteins; a range of low molecular weight substances with different degrees of hydrophilicity; and water in low but significant concentrations are dispersed and interact with each other. A full understanding of stratum corneum cell cohesion and desquamation will rely on our understanding of the complex interactions of the many constituents of the intercorneocyte space. Although important steps forward have been taken in recent years, much has still to be learned. It should therefore be stated that our present knowledge about desquamation is quite rudimentary. Some clues have emerged, however, and will be summarized below.

7.2 SKIN DISEASES WITH DESQUAMATION DISTURBANCES

An accumulation of scales on the skin surface may be due to either an increased production of corneocytes, such as in psoriasis, or to a delayed desquamation. It may be predicted that conditions with delayed desquamation, once their pathophysiology on the molecular level is understood, will be highly informative with regard to the understanding of desquamation. Two such conditions are recessive X-linked ichthyosis (RXI) and lamellar ichthyosis.

The elucidation of the molecular genetics RXI has had a major impact on our understanding of stratum corneum turnover. Individuals with RXI lack an enzyme, cholesterol sulfatase,[3,4] which catalyzes the transformation of cholesterol sulfate (CS) to cholesterol and free sulfate. As a result there is an accumulation of CS in the stratum corneum intercellular space. Possible mechanisms by which this change in intercellular lipid composition of the stratum corneum can cause disturbances in desquamation, leading to ichthyosis, will be discussed later.

A group of individuals with severe ichthyosis (recessive autosomal lamellar ichthyosis) has been found to have mutations in the gene for epidermal transglutaminase.[5–7] By means of catalyzing cross-linking of constituent proteins, this enzyme plays a crucial role in the formation of the cornified envelope of the corneocyte. How this type of molecular defect can cause ichthyosis is unknown. It may be expected that further studies on this condition will give important contributions to our understanding of desquamation. Similarly, we can expect that the soon-to-come elucidation of the molecular genetics of inherited lamellar ichthyoses with similar phenotypes, but without transglutaminase mutations,[8] will be informative.

7.3 STRATUM CORNEUM CELL DISSOCIATION INVOLVES PROTEOLYSIS

Experimental evidence that protein structures are involved in stratum corneum cell cohesion was presented by Bisset et al.[9] They induced cell dissociation in pig and human nonpalmo-plantar stratum corneum by means of incubation of the tissue in the presence of the zwitterionic surfactant 6-octadecyldimethyl ammoniohexanoate. Cell dissociation could not be induced when the tissue had been pretreated with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The fact that cell dissociation was found only in the presence of EDTA suggested a role also for calcium in stratum corneum cell cohesion.

Lundström and Egelrud[10] found a unipolar spontaneous cell dissociation in pieces of hypertrophic human plantar stratum corneum incubated in a simple buffer. The cell dissociation occurred only at the surface that had faced outward *in vivo*. The rate of cell dissociation was increased in the presence of EDTA. It was inhibited by inhibitors of serine proteases, but not by inhibitors of other groups of proteases. Since the tissue had not been treated with exogenous proteases before the experiments, it was concluded that the observed cell dissociation was mediated by an endogenous serine protease. This experimental system has been used as an *in vitro* model of desquamation. In addition to information about the enzyme(s) involved in the cell dissociation, it has provided information about the nature of the cohesive structures in the stratum corneum.

There is evidence that protein structures are also responsible for cell cohesion in nonpalmo-plantar stratum corneum. When punch biopsies of normal human gluteal skin were incubated in a buffer containing a mixture of the zwitterionic surfactant *N*,*N*,-dimethyldodecylamine and the anionic surfactant sodium dodecyl sulfate,[11] there was dissociation of cells in the stratum corneum but not in the rest of the epidermis. The cell dissociation took place only in the presence of EDTA and was inhibited by the serine protease inhibitor aprotinin.[12] Suzuki et al.[13,14] presented evidence that spontaneous cell dissociation in nonpalmo-plantar stratum corneum could be inhibited by a combination of inhibitors of trypsin-like and chymotrypsin-like enzymes. Thus, nonpalmo-plantar stratum corneum contains endogenous proteases that mediate cell dissociation.

7.4 DESMOSOMES AND CORNEODESMOSOMES

Desmosomes mediate mechanical contacts between viable epithelial cells such as keratinocytes.[15–18] A desmosome is a round or oval, button-like structure with a diameter of 0.2 to 1 μm. It consists of two symmetrical halves, each one belonging to one of two contiguous cells and consisting of an intracellular, a transmembranal, and an extracellular part. Inside the cell, just below the plasma membrane, is the desmosomal plaque. To this structure are linked intracellular keratin filaments as well as glycoproteins belonging to the cadherin family named desmogleins and desmocollins (for a review of desmosomal cadherins, see Reference 19). These glycoproteins cross the plasma membrane, and their glycosylated parts occupy the extracellular space where they interact with their counterparts from the contiguous cell, thus forming a cohesive structure between the cells. In the electron microscope the desmosomal plaque is visible as an electron dense structure, approximately

15 nm in width, on the inner aspect of the plasma membrane. The extracellular parts of desmosomes between uncornified keratinocytes has a moderately electron dense, plate-like appearance, approximately 30 nm in width, and has a zigzag formed electron dense central line. Desmosomes and keratin filaments form functional units, the desmosome-intermediate filament complexes.[17] These complexes link the keratin filament cytoskeleton of individual cells into a network comprising the whole epithelium.

The corneodesmosomes, that is, desmosomes in the stratum corneum, have a somewhat different appearance in the electron microscope.[20–22] Due to the densely packed and electron dense intracellular keratin filaments, it is not possible to identify the intracellular desmosomal plaque. The extracellular plate-like parts of corneodesmosomes have a homogenous and high electron density with no visible central line. Analyses of total number of desmosomes, measured as percentage of the cell periphery occupied by extracellular parts of desmosomes, showed a difference between the stratum corneum in palms and soles and stratum corneum at other body sites. In nonpalmo-plantar stratum corneum the number of desmosomes in deeper layers was comparable to the number of desmosomes in the stratum granulosum, whereas it was only around 20% of this number in the superficial layers close to the skin surface. This was true, however, only if the whole corneocyte periphery was considered. Whereas there were few desmosomes in the central parts of superficial corneocytes, the number of desmosomes per unit length of cell periphery at the overlapping edges of corneocytes was essentially the same as in deeper layers of the tissue. Thus, extracellular parts of desmosomes in the central parts of corneocytes disappear as the cells move upward in the stratum corneum, whereas desmosomes at the edges remain as long as the cells have not been shed. In palmo-plantar stratum corneum the number of desmosomes per unit length of corneocyte periphery is constant and high throughout the tissue until the cells are shed.[23]

The ultrastructural appearance of corneodesmosomes suggest that they are modified during the transition between viable and cornified epidermal layers. Part of this modification may be due to the incorporation of a recently discovered protein, corneodesmosin.[24–26] This is a 52-kDa protein, which is specifically expressed in keratinizing epithelia. In the stratum granulosum it is found intracellularly in association with lamellar bodies. In the transition zone between the stratum granulosum and the stratum corneum, coinciding with the change in the ultrastructural appearance of the desmosomes, corneodesmosin is translocated to the extracellular parts of desmosomes.[27] Immunoblot analyses have suggested that corneodesmosin is continuously degraded to smaller components in the stratum corneum.[26] It is not yet known to what extent this protein contributes to the cohesive capacity of corneodesmosomes. It has been speculated that corneodesmosin degradation may be part of the regulatory events involved in desquamation.[26]

7.5 DESQUAMATION INVOLVES DEGRADATION OF CORNEODESMOSOMES

Evidence that degradation of corneodesmosomes is a prerequisite for desquamation comes from ultrastructural and immunochemical studies. In the so-called retention ichthyoses, in which it is believed that a delayed desquamation causes the thickening of the stratum corneum and the accumulation of squames, there is an increased number of corneodesmosomes in the superficial layers of the stratum corneum.[28,29] In plantar stratum corneum undergoing spontaneous cell dissociation, electron microscopy of dissociating cells suggested that degradation of the intercellular parts of desmosomes preceded the widening of the intercellular space.[30] Chapman and Walsh[31] showed by means of electron microscopy that desquamation in pig skin was associated with morphological signs of desmosomal degradation.

Immunoblot analyses with antibodies specific for the transmembranal desmosomal glycoprotein desmoglein I (DG I) of plantar stratum corneum undergoing spontaneous cell dissociation[30] showed that although the still cohesive tissue contained only intact DG I, dissociated cells contained no

intact DG I, instead they contained degradation products of this protein. Analyses of surface cells that had been shed from plantar skin *in vivo* gave similar results.[32] In xerotic skin superficial stratum corneum contained more extractable intact DG I than in normal skin,[33] suggesting that delayed desmosomal degradation may contribute to the accumulation of squames. Increased amounts of intact DG I in superficial stratum corneum was found also in a mouse model with experimentally induced scaling.[34] Taken together, these ultrastructural and immunochemical results strongly suggest that corneodesmosomes are responsible for cell cohesion in the stratum corneum and that proteolytic degradation of their extracellular parts is a prerequisite for desquamation.

7.6 ENZYMES INVOLVED IN DESQUAMATION

The best-characterized enzyme so far with a proposed function in desquamation is stratum corneum chymotryptic enzyme (SCCE, also named human kallikrein 7; hK 7).[35] The discovery of SCCE was a result of the search for the enzyme responsible for the degradation of cohesive structures in the *in vitro* model of desquamation in hypertrophic plantar stratum corneum. SCCE has several properties compatible with a role in desquamation also *in vivo*.[36,37] SCCE has been purified from plantar stratum corneum.[38] It has been cloned and expressed in mammalian cells.[39] In reduced form SCCE has a molecular mass of around 28 kDa, it is partially glycosylated, and it has a basic isoelectric point. Although having a neutral to alkaline pH-optimum, it is active also at pH 5.5, that is, it is active at the pH of the stratum corneum.[40] SCCE is produced as an inactive precursor with a propeptide seven amino acid residues long. Removal of the propeptide by means of trypsin treatment of recombinant pro-SCCE yields a proteolytically active enzyme.[39] The mechanisms of SCCE activation *in vivo* remain to be elucidated. The deduced amino acid sequence contains the conserved regions typical of serine proteases, but is otherwise, at most, only around 40% homologous with other known human enzymes. SCCE shows similarities, but also significant differences regarding the activity on peptide substrates and the sensitivity to various protease inhibitors when compared to other chymotryptic enzymes such as bovine chymotrypsin and human cathepsin G.[38] This may be explained, at least partially, by the fact that in SCCE there is an asparagine residue in the bottom of the deduced primary substrate binding pouch, whereas this site is occupied by serine and alanine residues in chymotrypsin and cathepsin G, respectively.[39]

Analyses of mRNA from a large number of various human tissues has shown high expression of SCCE only in the skin.[39] Immunohistochemical studies have shown that SCCE is expressed in high suprabasal keratinocytes in the epidermis. In hair follicles and sebaceous glands it is expressed at a site where there is formation of cornified keratinocytes and hence a need for desquamation-like processes. In the oral cavity SCCE staining is found in the cornified epithelium of the hard palate, but not in the buccal mucosa or at other sites with noncornified epithelium. Thus, these findings suggest that SCCE expression is related to a differentiation process, leading to the formation of a cornified squamous epithelium.[41–44]

Results from enzymologic studies have suggested that SCCE has an extracellular localization in the stratum corneum.[45] This has been corroborated by means of immunoelectron microscopy. With this method SCCE was found intracellularly in association with lamellar bodies in the stratum granulosum. In the transition between the stratum granulosum and the stratum corneum, SCCE is extruded to the extracellular space together with the lamellar bodies. In the stratum corneum specific labeling is found only in the extracellular space, often in association with corneodesmosomes.[46]

Results from *in vitro* experiments, catalytic properties, and tissue localization are all compatible with the role of SCCE in the degradation of intercellular cohesive structures in the stratum corneum as part of the events leading to remodeling of the tissue and eventually to desquamation. Increased expression of SCCE in the epidermis of transgenic mice leads to impaired barrier function with increased transepidermal water loss. The transgenic animals have a thickened epidermis and a marked hyperkeratosis, possibly reflecting compensatory reactions.[47–48] There are also other proteases

present in the stratum corneum, some of which may be involved in desquamation.[13,14,37,38,49–51] Of these proteases, a 33 kDa serine protease named stratum corneum tryptic enzyme (SCTE; human kallikrein 5; hK 5)[35,52] with trypsin-like primary substrate specificity may be of special interest. The tissue distribution of SCTE is similar as for SCCE and it has been postulated that SCTE has a complementary role to that of SCCE in degradation of structures involved in stratum corneum cell cohesion during desquamation.[13,53] In addition SCTE is a candidate for being responsible for the activation of the SCCE precursor. Additional information in this respect will be crucial for the understanding of the role of SCCE and related enzymes in the formation and turnover of the stratum corneum.

7.7 REGULATION OF DESQUAMATION

We are very far from an understanding of how and by which mechanisms desquamation is regulated. If we assume, however, that proteolytic degradation of corneodesmosomes plays a major role in desquamation, a number of possible mechanisms can be postulated on the basis of the present knowledge. These are summarized in Table 7.1.

The activation of enzyme precursors is likely to be of central importance. A significant fraction of the total SCCE present in the stratum corneum is in the form of inactive proenzyme.[53,54] A change in the ratio of precursor to active enzyme may be expected to cause marked changes in the rate of corneodesmosomal degradation. *In vitro* pro-SCCE can be activated by pancreatic trypsin.[39] As mentioned earlier SCTE has been suggested to act as an SCCE activator, but this remains to be elucidated. It is possible that SCCE is just one of a number of enzymes constituting a "proteolytic cascade" in the stratum corneum, in which one enzyme serves as activator of another enzyme.

The stratum corneum is likely to contain a number of inhibitors of the various proteases present. CS may be of special interest. Accumulation of CS in the stratum corneum in RXI may be causative of this disease, in which there is evidence of a delayed degradation of desmosomes.[28] CS has been shown to inhibit pancreatic serine proteases *in vitro*, and application of CS on mouse skin *in vivo* causes a scaling condition.[34] In addition to direct effects on enzymes, CS could cause delayed desquamation by acting as a substrate modifier or by changing the physical–chemical conditions in the stratum corneum extracellular space.

Also, in autosomal recessive ichthyosis there are findings indicative of an impaired desmosome degradation in the stratum corneum.[29] The mechanisms involved have not been elucidated.

As mentioned previously for CS, substrate modifications could be of significant importance as regulating factors in proteolytic degradation of cohesive structures. Walsh and Chapman showed that pretreatment with glycosidases made preparations of stratum corneum more susceptible to cell

TABLE 7.1
Mechanisms which may be involved in regulation of desquamation

Enzyme activation
Activation of SCCE
Enzyme inhibition
Cholesterol sulfate
Antileukoprotease
Other protease inhibitors in the stratum corneum
Substrate modification
Glycosylation
pH? Water? Ions? Lipids?

Note: See text for references.

dissociation induced by exogenous proteases, suggesting that proteins involved in cell cohesion may be protected by carbohydrates against proteolytic degradation.[55]

A number of protein protease inhibitors are present in the stratum corneum. Antileukoprotease has been shown to be an efficient inhibitor of SCCE at physiological concentrations.[56] Extracts of plantar stratum corneum contains covalent complexes between SCCE and α1-antitrypsin (Egelrud, T., unpublished observation). Recent findings in the human genetic disease Netherton's syndrome (NS) have given new insights on the potential role of serine proteases and their inhibitors for epidermal homeostasis. In NS there is severe impairment of skin barrier function. The causing mutations have been found in a gene, Serine Protease Inhibitor Kazal type 5 (SPINK5),[57] encoding a complex protein, which after post-translational modifications gives rise to a number of serine protease inhibitors called LympoEpithelial Kazal-Type related Inhibitor (LEKTI).[58] It has been suggested that the lack of LEKTI, which is highly expressed in the stratum granulosum of normal epidermis,[59] may lead to increased and uncontrolled activity of epidermal serine proteases, which in turn would result in a deteriorated barrier.

There are a vast number of other factors which may be expected to influence the rate of desquamation, for instance, by affecting the rate of proteolytic reactions. pH, water, and ion concentrations, and lipid composition may all be expected to be of importance. Experimental data in this area are very scarce, but some speculations can be made. For instance, the pH dependency of SCCE activity could be of importance. SCCE has optimal activity at pH 7 to 8, but close to half its maximal activity at pH 5.5.[36,37] This implies that rather small variations in either direction of the pH of the extracellular space should have effects on the rate of SCCE-mediated protein degradation. In support of this, the rate of spontaneous cell dissociation observed in plantar stratum corneum *in vitro* showed a marked pH dependency, being highest at neutral to weakly alkaline pH and decreasing at lower pH values.[10]

The effects of chelating agents in *in vitro* models for desquamation suggest that divalent ions such as calcium may play a role in the regulation of desquamation.[12,26,60]

The composition of the stratum corneum intercellular lipids may have profound effects on desquamation. In addition to modifying effects on, for example, proteolytic enzymes and their substrates,[34] lipids may also be directly involved in corneocyte cohesion. The effects of cholesterol sulfate have already been mentioned. In addition to RXI, there are a number of other hereditary diseases with disorders of desquamation associated with disturbances in lipid metabolism. Furthermore, scaling as a result of treatment with lipid-lowering drugs has been observed (for review, see References 1 and 2).

7.8 CONCLUSION

A normal desquamation is of crucial importance for the maintenance of the function of the stratum corneum and for a normal skin appearance. In recent years some basic knowledge about stratum corneum cell cohesion and the role of proteolysis in desquamation has evolved. Much still has to be learned, however. In the near future we may expect to obtain information about further enzymes involved in desquamation, and the ongoing elucidation of hereditary skin diseases will give new clues with regards to regulation of mechanisms involved in desquamation. Similarly, further studies on the physical chemistry and the chemical composition, including identification of hitherto unknown proteins, of the stratum corneum intercellular space may be expected to give important contributions to this central area of skin biology.

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Dry Skin and Hyperkeratotic Conditions

8 Ichthyosis — An Inborn Dryness and Scaliness of the Skin

Anders Vahlquist

CONTENTS

8.1 INTRODUCTION

The term *ichthyosis* (latin for "scaly fish dermatosis") encompasses a wide range of various keratinizing disorders with different etiologies (Figure 8.1), but with the common feature of widespread epidermal hyperkeratosis and a dry and scaly skin (for a recent review see reference 1). Although acquired forms of ichthyosis exist (e.g., in association with malignancy, severe malnutrition, lepra, and treatment with lipid-lowering drugs), only the inherited forms will be discussed here.

Depending on the type of ichthyosis and the variable influence of individual and environmental factors, the severity of the skin symptoms may range from mild xerosis and scaling on the extremities mainly appearing in winter time (as in ichthyosis vulgaris) to massive hyperkeratosis and scaling all over the body (as in lamellar ichthyosis). The more severe forms are frequently associated with keratoderma (thickening of the palms and soles), ectropion (constricted eyelids), anhidrosis (inability to sweat), and patchy alopecia (hairloss). Some patients also experience skin erosions and blisters as in bullous ichthyosis, or epidermolytic hyperkeratosis. Pathogenetically, the latter form of ichtyosis is reminiscent of epidermolysis bullosa simplex and pachonychia congenita, that is, genodermatoses, which are also due to reduced cell-cohesion in epidermis but usually not defined as ichthyosis.

Some patients with ichthyosis also have noncutaneous symptoms, which are due to the same genetic defect as the skin condition albeit expressed in another tissue (CNS, immune system, skeleton, etc.); these diseases are collectively called "ichtyosiforme syndromes."

Over the last decade, many new etiologies for ichthyosis have been elucidated, making it easier to correctly identify various subtypes of the disease and to give proper genetic councelling to the patients and parents. Hopefully, this knowledge will also lead to novel therapies for different forms of ichthyosis, including perhaps somatic gene therapy for the most severely affected patients (for review, see reference 2). Today however the therapy is mainly symptomatic and based on topical emollients, keratolytic agents and, in more severe cases, oral retinoids (vitamin A analogs). Because the patients

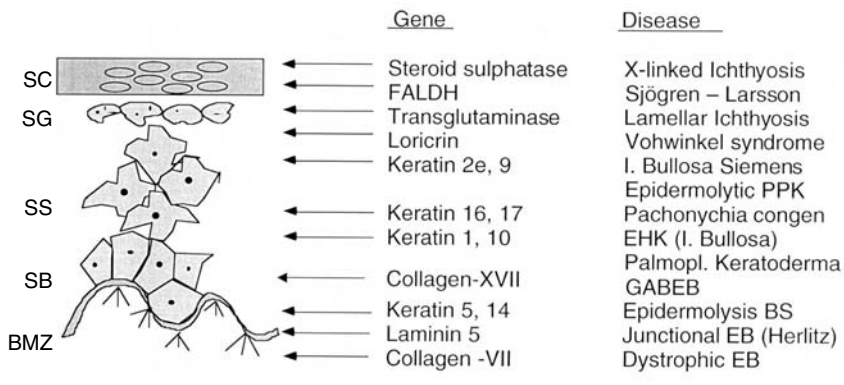

FIGURE 8.1 Examples of gene products which are incriminated in keratinizing disorders in human skin. The top eight of these disorders can be classified as ichthyosis. The lower half of the figure indicates mechano-bullous disorders which are pathogenetically related to some forms of ichthyosis although expressed around the basal membrane thus causing skin fragility and blistering rather than dry scaling. Data compiled from the literature. Abbreviations: SC — stratum corneum, SG — stratum granulosum, SS — stratum spinosum, SB — stratum basale, BMZ — basal membrane zone, FALDH — fatty aldehyde dehydrogenase, PPK — palmo-plantar keratoderma, EHK — epidermoltyic hyperkeratosis, EB — epidermolysis bullosa.

often require life-long treatment with daily applications of emollients all over the body, they are potentially big consumers of such products and usually become experts in their own right on how to treat their skin.

8.2 THE COMMON TYPES OF ICHTHYOSIS

The most common forms of ichthyosis — autosomal dominant ichthyosis vulgaris (IV) and X-linked recessive ichthyosis (XRI) — in many countries occur at frequencies a high as 1/300 and 1/2500, respectively. In fact, the genetic traits for IV and XRI are so frequent that the two diseases sometimes coexist in one and the same family, which may cause confusion as to the inheritance pattern. Although the incidence of IV and XRI is probably similar around the world, climate differences in particular will affect the severity of the disease, and hence its notification by the health care system.

In both types of common ichthyosis, scaling is usually most apparent on the extensor surface of the extremities, but it may also appear on the trunk, especially in XRI (Figure 8.2). Xerosis of the skin is a prominent feature in most patients, but there is no skin inflammation unless ichthyosis is complicated by, for example, atopic eczema (common in IV) or microbial infections.

Clinically, IV and XRI may appear indistinguishable at first sight, explaining why they were not recognized as separate entities until the 1960s when Wells and Kerr did their pioneering work [3]. By and large, XRI starts earlier in life — it may event present at birth[4] — and is usually more severe than IV. When laboratory diagnosing of XRI became possible in the late 1970s, based on the discovery of steroid sulfatase deficiency in these patients,[5] several new features distinguishing XRI from IV emerged[6] (Table 8.1). While histologically the affected skin will show orthohyperkeratosis in both diseases, due to retention of mature corneocytes, the stratum granulosum (SG) is normal in XRI but thin or even missing altogether in IV.[7] On electron microscopy (EM) this defect in IV epidermis will appear as tiny and crumbled keratohyalin granules due to defect processing of profilaggrin.[8,9] The exact genetic mechanism causing IV has not been determined. In fact a recent report claims that there are two forms of IV, one with missing SG and one with normal appearing SG, obviously with different pathoetiologies.[10] This illustrates the difficulty in distinguishing IV and XRI based on histopathology alone. Diagnosing XRI is however facilitated by the fact that on serum electrophoresis the β-lipoprotein fraction of an affected person will show abnormal mobility due to the accumulation of negatively charged lipids.[11]

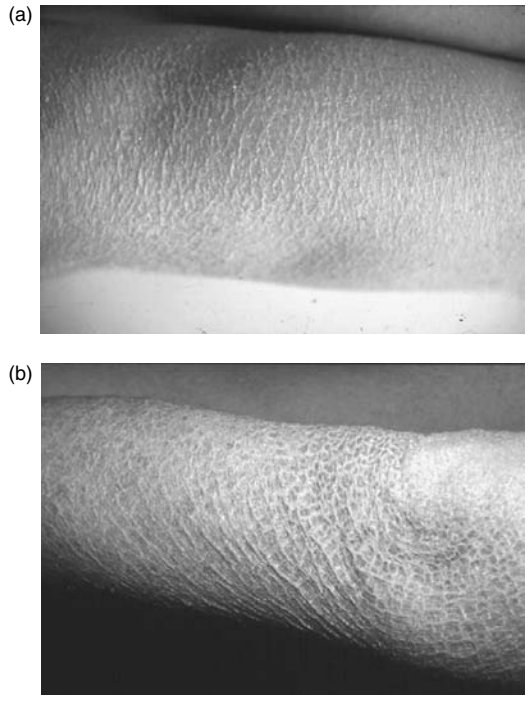

FIGURE 8.2 Extremities showing (a) ichthyosis vulgaris in a 40-year-old woman and (b) X-linked ichthyosis in a 20-year-old man (from the author's files).

TABLE 8.1
Clinical and Biological Features Distinguishing *Ichthyosis vulgaris* (IV) from X-linked Recessive Ichthyosis (XRI)

Features	IV	XRI
Symptoms appearing <6 months	Rare	Frequent
Brownish scales on the trunk	Rare	Frequent
Flexural involvement	Rare	Frequent
Testicular nondescendence	Rare	Frequent
Corneal opacity	Rare	Frequent
Accentuation of palmar creases	Frequent	Rare
Associated atopic eczema	Frequent	Rare
Associated keratosis follicularis	Frequent	Rare
Scanty or absent stratum granulosum	+	−
High CS/low free cholesterol in stratum corneum	−	+
Abnormal mobility of β-lipoprotein	−	+
Deletions or mutations in the STS gene	−	+

8.3 BIOCHEMICAL DIFFERENCES OF THE HORNY LAYER IN IV AND XRI

The lack of filaggrin and keratohyaline in IV horny layer results in a deficiency of the natural moisturizing factor (NMF) — composed of urocanic acid and pyrrolidone carboxylic acid (PCA) — that is, breakdown products of amino acids in profilaggrin.[12] In XRI, on the other hand, there is

TABLE 8.2
Cholesterol Sulfate (CS) and the Skin

Is normally present in stratum granulosum and stratum corneum
Is probably important for the pH gradient in stratum corneum17
Accumulates in stratum corneum of patients with X-linked ichthyosis13
Accumulates in epidermis during chemical carcinogenesis47
Is growth inhibitory to human keratinocytes48
Activates PKC (which phosphorylates TGM1)49
Induces transcription of the TGM gene50
Inhibits certain proteases (SCCE, etc.) in stratum corneum16
Is reduced in epidermis during retinoid therapy51

an accumulation of cholesterol sulfate (CS) and a concurrent decrease in cholesterol in the stratum corneum (SC) due to steroid sulfatase deficiency.[13] Clearly, intercellular lipids are very important for corneocyte cohesion and barrier function. Thus a careful monitoring of transepidermal water loss (TEWL) in XRI patients has shown repeatedly that, despite hyperkeratosis, there is a slight impairment of the epidermal barrier function.[14,15] Interestingly, this impairment can be reproduced in experimental animals via topical application of CS.[15] Recent observations about the multiple effects of CS in epidermis (Table 8.2), makes it possible to speculate about the delayed shedding of corneocytes in XRI. Presumably, hyperkeratosis is not a compensatory mechanism, but reflects a retardation of the desquamation process by CS,[16] possibly via enzyme inhibition.

It is noteworthy that CS, which is a weak organic acid, has been hypothesized to be involved in the formation of a pH gradient over the horny layer. Thus, in an intervention study of patients with ichthyosis[17] we found that the pH gradient, which normally spans from 7 in stratum granulosum to pH 4.5 to 5 on the skin surface, is shifted to more acidic values in XRI patients and to more basic values in IV patients (Figure 8.3). These findings are consistent with the accumulation of CS in XRI, and the lack of urocanic acid and PCA in IV skin. Speculatively, changes in the pH gradient may not just reflect the altered chemical compostion of the horny layer, but might also influence the activity of pH-dependent enzymes operating in the intercorneocyte space.[18] Whether pH variations also influence the transcorneal diffusion of topically applied acids and bases (e.g., salicylic acid, alpha-hydroxy acids, and certain other drugs) remains to be determined.

8.4 TREATMENT OF COMMON ICHTHYOSIS

A primary objective in treating ichthyosis is to remove scales and reduce the dryness of the skin. To accomplish this, several items have to be taken into consideration when prescribing a topical treatment: (1) the age and sex of the patient (children have a thinner skin and a higher skin surface area/body weight ratio than adults thus increasing the risk for systemic toxicity; females of childbearing age should not uncritically be exposed to potentially teratogenic compound), (2) the severity of the disease (thick scales require keratolytic agents, xerosis requires emollients), (3) the extent and location of the skin lesions (whole body application increases the risk for systemic toxicity; face and flexural sites usually need less potent therapy), (4) presence of fissures, erosions, and bacteria in the skin lesions (precludes the use of irritating creams). It is also essential that the health provider evaluates the patient's willingness and ability to apply creams all over the body 1 to 2 times daily for longer periods of time, and has an open attitude to individual preferences regarding cream formulations. It should be kept in mind that cosmetic acceptability of a cream is a *sine qua non* for good compliance and that there are probably as many opinions about "the best cream formulation" as there are patients.

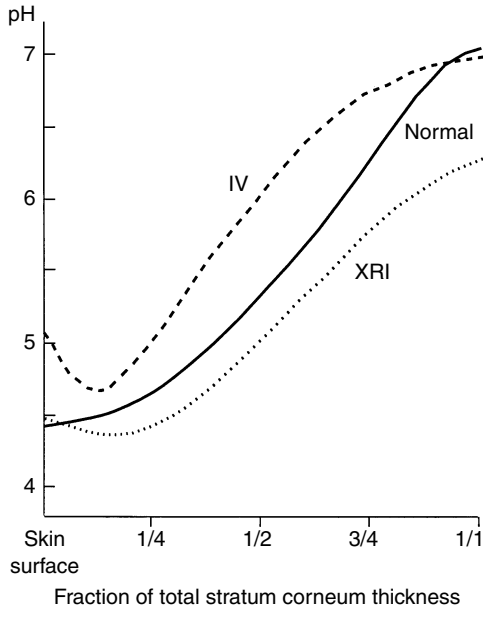

FIGURE 8.3 Schematic representation of the pH gradients over stratum corneum in normal skin, *Ichthyosis vulgaris* (IV), and X-linked Recessive Ichthyosis (XRI) skin, respectively. (Reproduced from Öhman, H. and Vahlquist, A., *J. Invest. Dermatol.*, 111, 674, 1998. With permission.)

In theory, one way of treating IV and XRI would be to substitute the missing components in SC, viz. NMF and cholesterol, respectively. Indeed some success has been reported using cholesterol-containing creams in XRI patients,[19] but on the whole the substitution therapy concept is no viable today. Instead, standard treatment of mild to moderate IV and XRI relies on daily application of emollients containing 2–10% urea,[20] 5–15% lactic acid,[21] or 10–25% propylene glycol or glycerol. Considering that each ichthyosis patient represents a potential mega-user of topical therapy, with a calculated life-time consumption of creams or ointments in the order of 1 t, surprisingly few clinical trials have addressed this problem; in fact, ichthyosis is not even mentioned in the text book on *Evidence Based Dermatology*.[22]

In a recent German study, urea in a new lotion base was found to be highly effective and well tolerated also by children.[23] Using a semiocclusive cream formulation containing lactic acid and propylene glycol (see Section 8.6) we have observed good results in severe cases of IV and XRI (Vahlquist et al., unpubl. observ.). Other treatment options include regular baths (salt and oil), UV-irradiation, and climate therapy in winter. Oral retinoid therapy is rarely indicated for common ichthyosis, except perhaps in the most severe cases of XRI. Topical corticosteroids and vitamin D derivatives (calcipotriol) are usually contraindicated in common ichthyosis because (1) they do not alleviate the disease processes and (2) they are associated with a significant risk for systemic absorption when used extensively on large areas of the body.

8.5 RARER FORMS OF ICHTHYOSIS

The two most severe types of ichthyosis, lamellar ichthyosis (LI) and epidermolytic hyperkeratosis (EHK), are distinct families of diseases with completely different etiologies (see Figure 8.1 and Table 8.3). Nevertheless, LI and EHK have several things in common: they are rare, congenital diseases (prevalence $<1/100{,}000$) with more or less generalized hyperkeratosis and a defective skin barrier, and they usually demands vigorous therapy.

TABLE 8.3
Common and Rare Forms of Ichthyosis

	Ichthyosis vulgaris	X-Linked ichthyosis	Lamellar ichthyosis (nonbullous ichthyosiform erythroderma)	Epidermolytic hyperkeratosis (bullous ichthyosis)
Incidence	1/300	1/3,000 (boys)	1/100,000	1/300,000
Etiology	Defective keratohyaline	Steroid sulfatase deficiency	Transglutaminase 1-plus several deficiency (other causes)	Keratin mutations
Inheritance	Autosomal dominant	Recessive X-linked	Autosomal recessive	Autosomal dominant
Appearance	Early in childhood	Early in childhood	Congenital	Congenital
Symptoms	Retention hyperkeratosis on extremities; better in summer	Brown scales all over the body; associated features; maternal pregnancy abn.	"Collodion baby"; generalized scaling; large, thick scales; ektropion; hypohidrosis	Intense blistering at birth; later verrucous hyperkeratosis, esp. in body folds; keratoderma ±

LI and its closely related variants, nonbullous ichthyosiform congenital erythroderma (CIE) or erythrodermic lamellar ichthyosis (ELI) 1, and congenital ichthyosis with fine/focal scaling (CIFS) or non-LI/non-CIE 4 (Figure 8.4), seem mostly to be caused by abnormalitishing the cornified cell envelope or defective deposition of intercorneocyte lipids. Thus mutations in the gene encoding for keratinocyte transglutaminase type 1 (*TGM1*) cause a recessive disorder characterized by deficient TG-1 activity in the upper epidermis and defective cross-linking of envelope proteins, such as involucrin and loricrin.[24,25] This can be visualized by EM as absent or faint marginals of the corneocytes.[8] We and others have found that about 50% of all cases of LI/ELI are due to *TGM1* mutations usually of the compound heterozygous type.[26] Less readily explained EM findings in LI and ELI include numerous lipid droplets and cholesterol clefts in SC and bizarre accumulations of membrane-like structures in cells from both the granular and the horny layers.[8] These findings point to a multifactorial pathogenesis of LI, CIE, and CIFS, also supported by recent disclosures of several new chromosomal loci and candidate genes for this group of diseases.[27,28] Finding the remaining causes of nonbullous congenital ichthyosis will probably be instrumental in elucidating some of the still unknown mechanisms during normal cornification and also raises the hope for future gene therapy of the most severely affected patients.

8.6 TREATMENT OF LAMELLAR ICHTHYOSIS

The introduction of oral retinoid therapy in the late 1970s was a break-through for many patients with LI, and the pros and cons of this therapy have been discussed at length in the literature (for review, see reference 29). This chapter will focus on external remedies that remain a mainstay of therapy in LI and CIE.

Both emollients and keratolytic agents are commonly prescribed but the treatment traditions differ from one country to another and even from one doctor to another. For example, although urea-containing lipophilic creams are popular in many European countries, mixtures containing propylene glycol or alpha-hydroxy acids (AHA) seem to be the first choice for treating severe ichthyosis in United States. Importantly, the commonly used keratolytic agent, salicylic acid, should be avoided when treating large, eroded skin areas or in small children owing to the risk of systemic toxicity. This

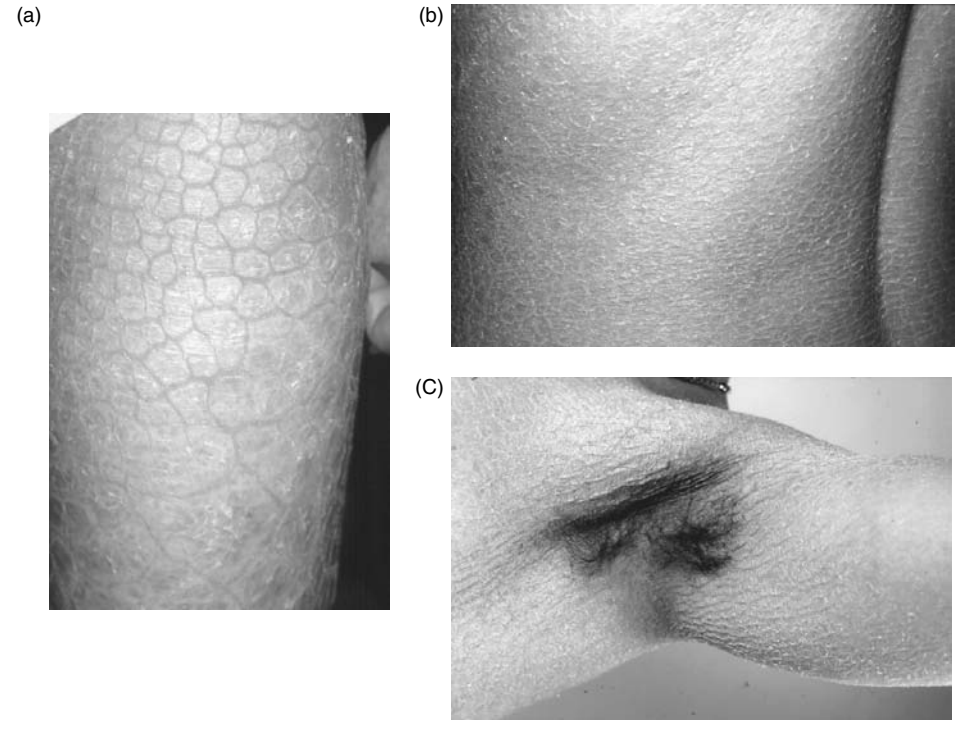

FIGURE 8.4 Examples of clinical variants of lamellar ichthyosis (LI) and nonbullous congenital ichthyosiform erythroderma (NBCIE). (a) Large scales on the thigh of a man with LI due to transglutaminase −1(*TGM1*) mutations, (b) generalized scaling on the trunk of a woman without *TGM1* mutations, and (c) scaling and mild erythroderma in the axillae of a woman also without *TGM1* mutations. (Photos from the author's files.)

is especially important to remember in collodion babies who may otherwise suffer fatal salicylism. By and large, these babies should only be treated with bland, semiocclusive emollients, possibly containing glycerol; even usage of occlusive ointments, such as petrolatum, may increase the risk of bacterial skin infections and septicemia.[30]

In adults, the side effects associated with topical treatment are minimal but the reasons for treatment failures are numerous. Needless-to-say the selection of a cream base (hydrophilic or lipophilic, nonocclusive or semiocclusive) is important not only for the antimicrobial and pharmacologic effects, but also for compliance reasons.

By combining two or more keratolytic agents and moisturizers in the same cream base it is often possible to achieve additative or even synergistic effects without the need of using irritating concentrations of either agent alone. Thus, in a double-blind trial of four different cream mixtures in 20 patients with LI, a mixture of 5% lactic acid and 20% propylene glycol in a semi-occlusive cream for four weeks twice daily was significantly more effective than 20% propylene glycol or 5% urea alone in the same vehicle.[31] However, while hyperkeratosis was virtually abolished in some patients (Figure 8.5) and most patients tolerated continued therapy for many months, the TEWL data actually showed that the skin barrier deteriorated. This pin points an inherent drawback when trying to reduce scaling and hyperkeratosis associated with LI: the excessive accumulation of defective corneocytes in LI probably represents a homeostatically controlled repair mechanism by which epidermis partially compensates for an intrinsic failure of the skin barrier (due to the deficiency of TG-1 or intercellular lipids); therefore an efficient therapeutic removal of corneocytes is likely to precipitate the underlying barrier defect. Admittedly, TEWL is a very sensitive gauge of the barrier function and a modest increase in TEWL may not represent a real problem to the patient. However, also a minor deterioration of the barrier function may increase the transcutaneous penetration of, for example, topically applied drugs and chemicals, a matter of great concern especially in children.

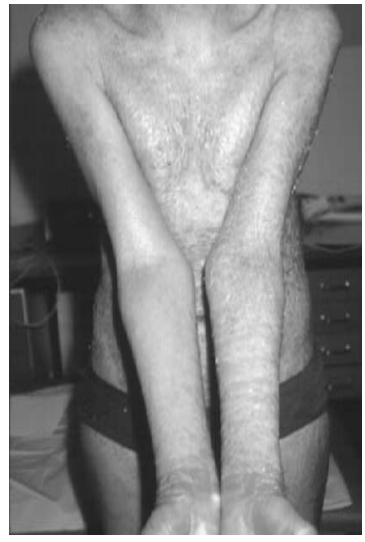

FIGURE 8.5 Patient with lamellar Ichthyosis (due to *TGM1* mutations) who twice daily for 2 mo. received a cream formulation containing lactic acid (5%) and propylene glycol (20%) on the right arm as compared to on the left arm (Reproduced from . . . 31)

Apart from emollients and keratolytic agents, topical applications of more specific drugs, such as retinoids,[32] N-acetylcysteine,[33] liarozole,[34] and calcipotriol[35] have also been tried in LI. Some of these drugs probably act through reducing epidermal hyperproliferation associated with certain forms of LI. Others affect keratinocyte differentiations and hence corneocyte function. Because of the obvious risk of systemic side effects when using these drugs extensively on the skin, and the notorious differences in therapeutic effects seen in different skin regions (strong effects in skin folds, poor effects on palms and soles, etc.), they have not become very popular in the treatment of LI and are usually not available for this indication.

Although treatment of xerosis and scaling is a *sine qua non* in LI therapy, other skin symptoms also need to be taken care of. Thus, ectropion, alopecia, and finger contractions are usually unresponsive to medical treatment, and only occasional patients with anhidrosis will improve the sweating capacity when using potent oral drugs for LI.[36]

8.7 BULLOUS ICHTHYOSIS, A KERATIN DISORDER

EHK and the closely related diseases, ichthyosis bulluosa of Siemens, epidermolytic palmo-plantar hyperkeratosis, and pachonychia congenita, are all due to dominant negative keratin mutations expressed in the suprabasal layers of epidermis.[37,38] Depending on which pair of keratin molecules is affected (K1/10, K2e/9, or K6/16,17), keratinocytes in different parts of suprabasal epidermis will collapse when exposed to mechanical stress. This results in superficial blisters and erosions that easily become infected. Concurrently, other parts of the epidermis may be hyperkeratotic, leading to a mixture of oozing and dry skin lesions with a characteristic malodor.[1] The flexural areas are usually most severely affected, but some patients have a widespread verrucous type of ichthyosis occasionally coupled to palmo-plantar keratoderma (Figure 8.6).

The treatment of EHK is complicated. On the one hand, hyperkeratosis must be reduced to minimize the disfiguring and foul-smelling scales. On the other hand, skin blisters and erosions must be protected from irritation in order to heal. A too potent keratolytic treatment will often aggravate the condition by disrupting the epidermal barrier and increasing the risk for painful and

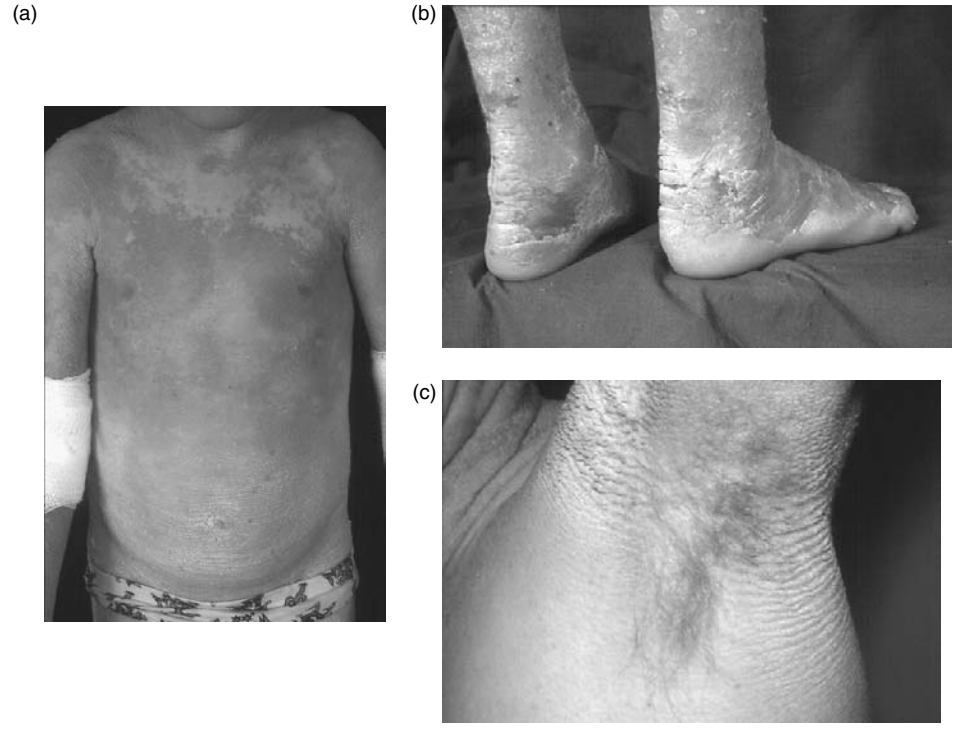

FIGURE 8.6 Epidermolytic hyperkeratosis in a 10-year-old boy showing (a) extensive hyperkeratosis on the trunk, (b) erosions and scaling around the ankle, and (c) verrucous hyperkeratosis in the axilla of a 25-year-old man carrying a spontaneous deletion in one of his K1 alleles (Vahlquist et al., unpubl. observ.).

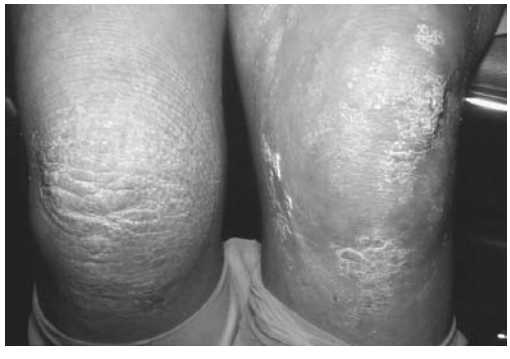

FIGURE 8.7 Successful topical treatment of EHK with a tretinoin-containing cream on the left knee of a woman with inherited K10 mutation (From Virtanen et al, *Acta Derm Venerol* 81, 163–70, 2001. With permission)[40].

easily infected erosions. It is more important than ever in EHK that the treatment of different body areas is individualized.

Retinoids, topical as well as systemic, have been tried in EHK but were often found to be irritating. Nonetheless some patients are improved by oral acitretin,[39] but the dose must be kept low in order to avoid the epidermolytic side effect of the drug. If correctly used, topical tretinoin and tazarotene may also be effective in some patients with EHK (Figure 8.7). Interestingly, the response to retinoid therapy seems to be partially determined by which keratin gene (K1, K2e, or K10) is mutated; patients with K2e and K10 mutations have the best response probably because they tolerate a retinoid-induced down-regulation of K2e expression better than other patients.[40] However the

topical treatment of EHK also relies on the use of bland emollients and a liberal prescription of antiseptics and antibiotics to prevent bacterial infection of the skin.

8.8 ICHTHYOSIFORME SYNDROMES AND OTHER RARE ICHTHYOSIS-LIKE CONDITIONS

Netherton's syndrome (ichthyosis circumflexa and atopy) is due to a deficiency of LEKT1 — a protease inhibitor normally expressed in both epidermis and the thymus.[41] This leads in the skin to exaggerated degradation of corneodesmosomes and a deficient epidermal barrier, which neonatally is reflected in generalized erythroderma and life-threatening hypernatrinemia and septicemia. When the child grows older, ichthyosis and atopy usually become more prominent features.[42] However, the skin barrier remains defective, which must be taken into consideration when prescribing topical treatments.

Darier disease (keratosis follicularis) is an autosomal dominant disorder of keratinization, which usually starts at puberty. Mutations in an endoplasmatic ATPase, which pumps calcium ions across membranes in keratinocytes, have been found to cause the disease.[43]

The patient's skin problems somewhat resemble those in EHK, that is, hyperkeratosis coexists with acantholysis and the epidermis is fragile and easily infected but can be effectively treated with low doses of retinoids.[29]

Among the many types of neuroectodermal syndromes having ichthyosis as presenting symptom, two well-known examples are *Sjögren–Larsson syndrome* and *Refsum disease*, both of which represent inborn errors of the lipid metabolism.[44,45] Although treatment aiming at rectifying the abnormal accumulation of lipid metabolites in skin and nervous tissue improves ichthyosis in some cases of Refsum disease, most patients require additional therapy of the same type as in common ichthyosis. The skin symptoms in Sjögren-Larsson syndrome respond quite well to oral retinoids,[46] but topical treatment with lactic acid/propylene glycol in a semiocclusive cream base (see under lamellar ichthyosis) is also very effective (Gånemo, Jagell, Vahlquist, unpubl. observ.).

8.9 CONCLUSIONS AND PROSPECTS FOR THE FUTURE

Ichthyosis can be a disabling condition requiring laborious treatments several times a day, but may also be a relatively mild disorder, which only occasionally needs application of emollients. From a diagnostic as well as therapeutic point of view, the many different subtypes of ichthyosis represent a problem for the caring physician. For example, a paradoxic combination of barrier failure and massive hyperkeratosis in some types of ichthyosis demands special attention. Choosing the right treatment will be even more important in future when new therapeutic regimes based on more-detailed knowledge about the pathogenesis of ichthyosis will emerge.

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9 Dry Skin in Atopic Dermatitis and Patients on Hemodialysis

Motoji Takahashi and Zenro Ikezawa

CONTENTS

SUMMARY

We investigated the characteristics of the dry skin in patients with atopic dermatitis (AD) and those on hemodialysis (HD) using noninvasive methods. Transepidermal water loss (TEWL), water content, parakeratotic cells, free amino acid and ceramide in stratum corneum (SC), and skin surface pH were examined on lesional and nonlesional skin in the dorsolumbar part of AD patients, HD patients, and healthy normal controls. The water content in SC on lesional and nonlesional skin was markedly lower in the AD patients than in the normal controls. The water content in SC was also lower in HD patients. The level of free amino acids, which represents the natural moisturizing factor (NMF) in SC, was decreased in both patient groups, which corresponded with the decrease of their water content in SC. TEWL was high in AD patients, but that in HD patients was almost similar to that in the controls. The level of the ceramides, which are closely related to the barrier function of SC, was lower in AD patients than in HD patients or in the controls, which was in agreement with the results of TEWL. In the composition of ceramides, the HD patients showed a higher percentage of ceramides 2

and 3 and a lower ratio of 1, 4/5, and 6 in comparison with the controls. No parakeratotic corneocytes were found in the controls or HD patients, while they were found in not only the lesional but also nonlesional skin of AD patients, indicating the presence of mild inflammation even in nonlesional skin. The conversion ratio of ornithine, which is a free amino acid component in SC, to citrulline was lower in AD patients than in HD patients or the controls. This suggested increased epidermal proliferation in AD patients. The skin surface pH value was high in both AD and HD patients, and the latter showed a higher value than the former. Except for pH, the results of all of the measurements in the nonlesional skin of AD patients were found to be intermediate between those of the lesional skin and the normal controls, showing that the lesional skin is physiologically different from the nonlesional skin.

These findings suggested that the decrease in free amino acids (= NMF) and inferior barrier function of SC caused the dry skin of AD patients, but the decrease of NMF mainly caused the dry skin in HD patients.

9.1 INTRODUCTION

Many important allergic aspects of AD have been reported. For example, most patients show high serum IgE levels due to high response of IgE antibody to various allergens such as foods, dust, mites, and fungi. Also, they show an increase of eosinophils and eosinophil-derived mediators such as eosinophilic cationic protein and major basic proteins in blood during exacerbation of AD. Among the nonallergic aspects of AD, the skin is also tends to overreact to irritation and become dry, particularly in winter. Many researchers have analyzed the characteristics of the dryness in skin lesions of AD from the viewpoint of the functions of the SC. Decreased water content in SC,[1,2] enhanced TEWL,[3,4] shortening of the turnover time of SC,[3] reduction of ceramide levels,[5–7] decrease of free amino acid level,[3] and increase of skin surface pH[8] have been reported. However, few investigators have analyzed, compared, and discussed these properties in lesional and nonlesional skin.

On the other hand, although dry skin or skin itchiness has frequently been recognized together with pigmentation in the patients with chronic kidney failure and receiving HD,[9] only a few reports have been made on the characteristics of dry skin (e.g., high pH,[10] decrease of water content in SC, and low TEWL.[11])

Here, we will report the characteristics of dry skin observed in lesional and nonlesional skin of AD patients and patients with chronic kidney failure and undergoing HD from the viewpoints of various functions of SC.

9.2 SUBJECTS AND METHODS

9.2.1 Subjects

The study subjects included 48 patients with AD (27 male and 21 female), 22 patients undergoing HD (7 male and 15 female), and 30 healthy volunteers (15 male and 15 female) who served as controls (Table 9.1).

9.2.2 Skin Regions and Timing for Measurements

Skin measurements were always made on the dorsolumbar region in HD patients and healthy subjects and whenever possible for lesional and nonlesional skin in AD patients. In some cases, meaurements on the nonlesional skin could not be made because a sufficient area could not be obtained.

The targeted skin regions were cleaned with ethanol and distilled water 30 min before the start of measurement. Measurements were made at an ambient temperature of 21 to 23°C and relative humidity of 35 to 50% from December to February when the skin was apt to become dry.

TABLE 9.1
Age Distribution of Subjects

Age distribution (Years)	Atopic dermatitis		Hemodialysis		Normal controls	
	Male	Female	Male	Female	Male	Female
3–9	1	5				
10–19	11	8				1
20–29	7	5			8	11
30–39	6	1			6	3
40–49	1			2	1	
50–59		1		6		
60–69		1	4	4		
70–79	1		3	3		
Total	27	21	7	15	15	15
Mean ± SD	23.3 ± 14.4		64.0 ± 10.1		27.8 ± 4.9	

9.3 MEASUREMENTS

9.3.1 Water Content in Stratum Corneum

Skin surface conductance (μS) was determined with Skicon-100 (IBS Company, Hamamatsu, Japan) by measurement of water content in SC. Nine measurements were repeated at a single point, and the mean was taken after excluding the highest and lowest values.

9.3.2 Transepidermal Water Loss

Transepidermal water loss (g/m^2/h) was determined with Evaporimeter EP-1 (Servo Med Company, Sweden). Measurements were repeated twice, and the mean was calculated.

9.3.3 Skin Surface pH

Distilled water was dropped on the surface of the skin, and pH was determined with a pH meter (Model D-12, Horiba Manufacturing Co., Ltd., Tokyo, Japan).

9.3.4 Parakeratotic Index of Stratum Corneum

The method of Koyama et al.[12] was used to determine the parakeratotic index of SC. A glass plate was attached to the skin with Scotch tape (Sumitomo 3M, Tokyo, Japan) measuring 25 × 19 mm to remove corneocytes. The adherent horny material was stained with hematoxylin–eosin solution for microscopic inspection of nuclei. The results were scored depending on the number of the nucleated cells in the visual field (0 = none, 1 = small, 2 = relatively large, 3 = very large).

9.3.5 Free Amino Acid in Stratum Corneum

The method proposed by Horii et al.[13] was used for the measurement of free amino acid. The SC was stripped with adhesive cellophane tape (Cello-tape, Nichiban Co. Ltd., Tokyo, Japan). The tape was immersed in toluene to remove the SC, which was then washed with toluene several times and dried in a vacuum desiccator. One milligram of dried sample of SC was precisely weighed and homogenized with 0.1% of sulfosalicylic acid. After centrifugation the supernatant was analyzed with a high-speed amino acid analyzer (Model 835, Hitachi, Tokyo, Japan) to determine the level of total

amino acid, ornithine, and citrulline. The conversion ratio of ornithine to citrulline {Cit/(Orn + Cit)} was calculated as an index of amino acid metabolism in the epidermis.

9.3.6 Ceramides in Stratum Corneum

The method of Denda et al.[14] was used to measure ceramides. After SC was stripped with adhesive cellophane tape, it was removed from the tape and washed several times with hexane, followed by drying in a vacuum desiccator. Lipids were extracted from the SC sample in a mixture of chloroform and methanol (2:1). Ceramides were separated with a silica gel column (Bond Elut Sl, Analytichem International, United States) and purified for measurement by gas chromatography (GC-14A, Shimazu Manufacturing Co., Ltd., Japan). The composition of ceramides was obtained by high-performance thin layer chromatography and scanned on a recording photodensitometer (TLC Scanner CS930, Shimazu, Japan).

9.4 RESULTS

9.4.1 Water Content in Stratum Corneum

The water content at both lesional and nonlesional sites was markedly lower in the AD patients than in the normal controls (Figure 9.1). Furthermore, the water content in the lesional skin was lower than that in nonlesional skin, but no significant difference could be recognized between them. The skin surface conductance was also lower in HD patients than in the controls (under one tenth), and the skin of HD patients was dry to the same extent as in AD patients. As the mean age of patients undergoing HD was higher than that in the controls, it is difficult to make a precise comparison without matching the age of the two groups. However, considering the finding that aging did not affect the skin surface conductance on the face,[15] and Kumasaka's[16] report that skin conductance on

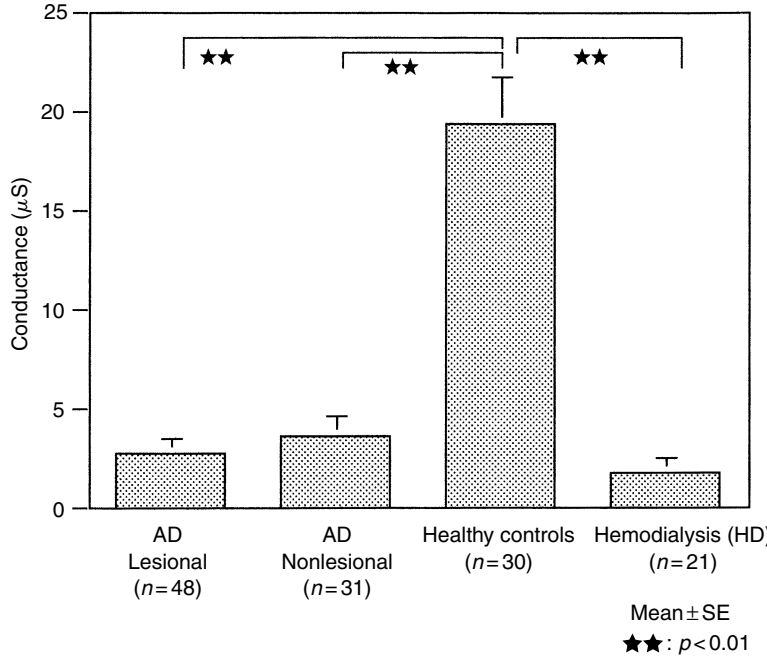

FIGURE 9.1 Water content in the SC of patients with AD and those on HD.

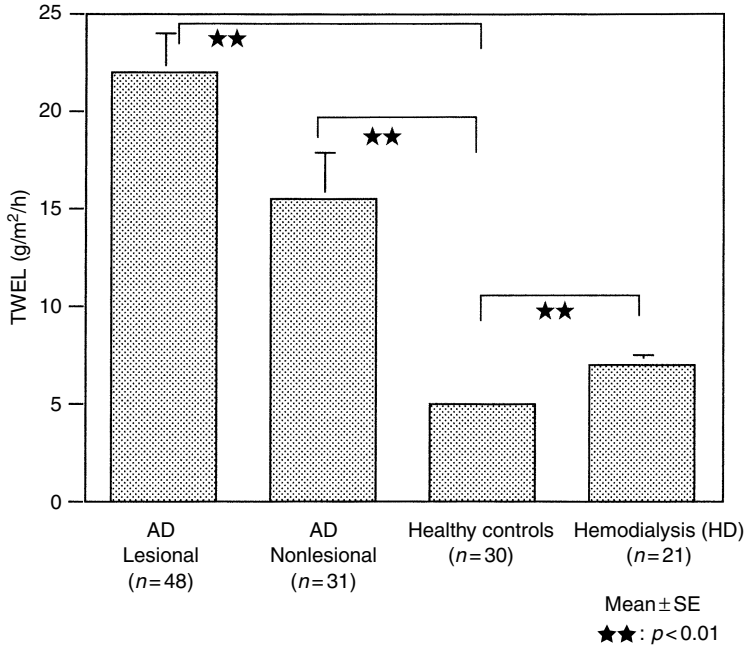

FIGURE 9.2 The TEWL in patients with AD and those on HD.

the crus in the young was about three times as large as that in the elderly, the extent of the dryness in HD patients was larger than the aging effect.

9.4.2 Transepidermal Water Loss

The TEWL in both regions was three to four times higher in AD patients than in the control (Figure 9.2), and the lesional skin gave higher values than the nonlesional skin ($p < 0.05$). On the other hand, the TEWL was only slightly higher in HD patients than in the control (mean value: HD patients, 7.0 g/m^2/h, the control, 5.0 g/m^2/h). The barrier function of SC in HD patients was similar to that in the control.

9.4.3 Skin Surface pH

The AD patients showed a higher pH at lesional and nonlesional sites than the controls ($p < 0.01$). However, the difference between the two sites was not significant. The pH was significantly higher ($p < 0.01$) in HD patients than in the controls (Figure 9.3).

9.4.4 Parakeratotic Cells in Stratum Corneum

Figure 9.4 shows the appearance of typical parakeratotic cells in SC obtained by skin surface biopsy with tape stripping. As Figure 9.5 shows, no nucleated cells were found in either the controls or HD patients, but they were observed frequently on the lesional skin of AD patients. In some AD patients (16 of 31), they were also found on the nonlesional skin.

9.4.5 Free Amino Acid Content in Stratum Corneum

As Figure 9.6 shows, the levels of free amino acids contained in 1 mg of dry SC were in decreasing order of the controls, HD patients, nonlesional skin of AD patients, and lesional skin of AD patients.

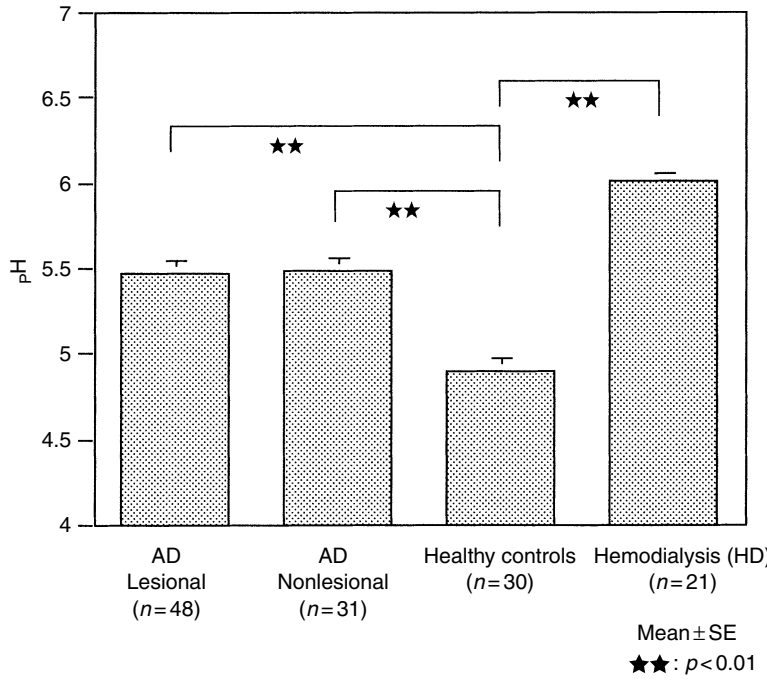

FIGURE 9.3 Skin surface pH in patients with AD and those on HD.

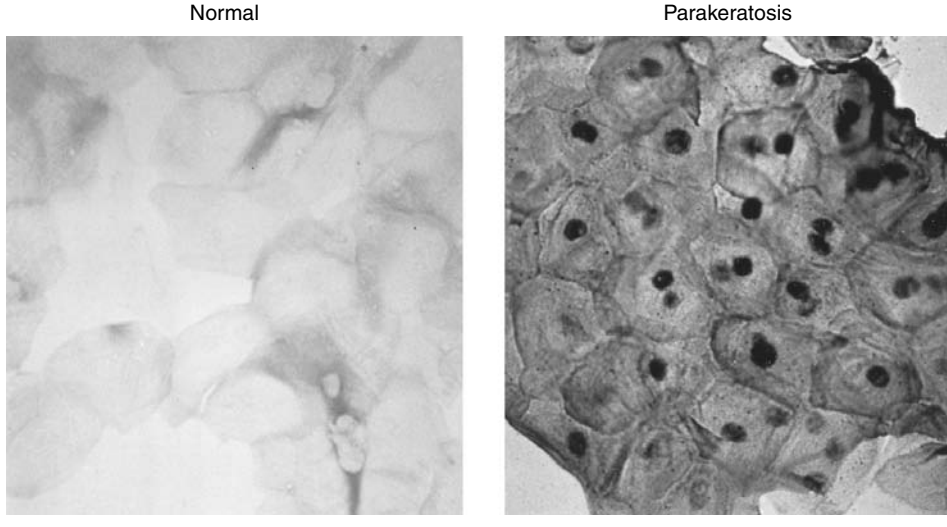

FIGURE 9.4 Parakeratotic cells detected in the SC obtained by tape stripping. Cells were stained with a hematoxylin–eosin solution.

Free amino acids act as a moisture holding factor in SC,[17] and their contents are correlated with skin surface conductance.[13] The amino acid levels in both AD and HD patients who had low skin surface conductance were less than half of the control levels. The conversion ratio of ornithine to citrulline {Cit/(Orn+Cit)} is related to the degree of cornification disorder and is negatively correlated with the epidermal proliferation rate.[12] As Table 9.2 shows, conversion ratios were similar in HD patients and the controls, but showed a significant difference ($p < 0.05$). On the other hand, the conversion

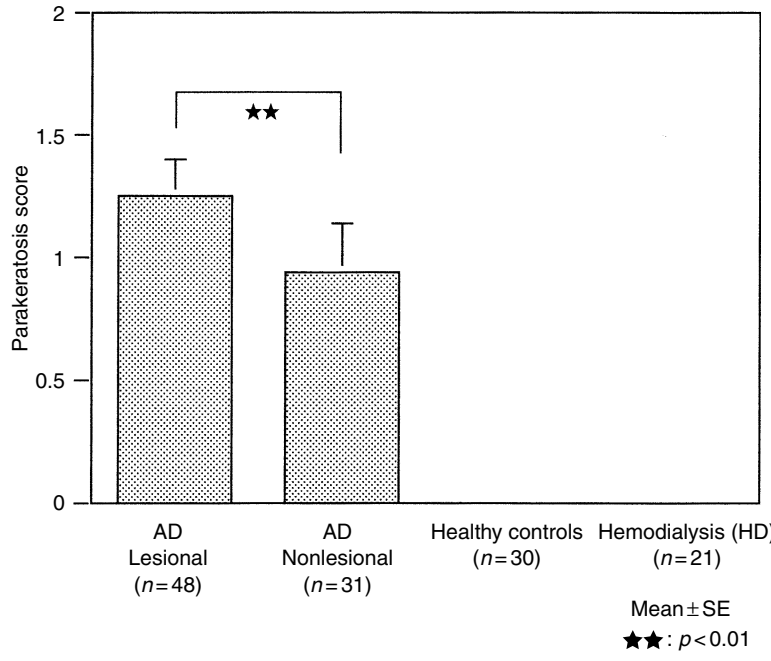

FIGURE 9.5 Parakeratotic cells in the superficial SC of patients with AD and those on HD.

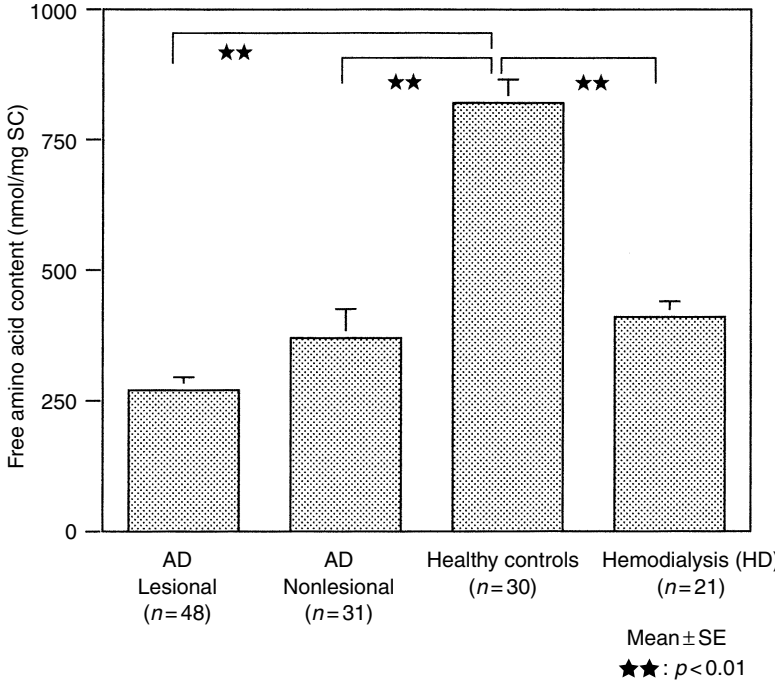

FIGURE 9.6 Free amino acids in the SC of patients with AD and those on HD.

TABLE 9.2
Conversion Ratio of Ornithine to Citrulline

	n	Cit/(Orn+Cit) %
AD lesional	48	69.8 ± 2.8[a]
AD nonlesional	31	75.0 ± 2.6[a]
Hemodialysis	21	88.3 ± 1.3[b]
Healthy controls	30	91.8 ± 1.0
		mean ± S.E.

[a] $p < 0.01$ compared with healthy controls.
[b] $p < 0.05$ compared with healthy controls.

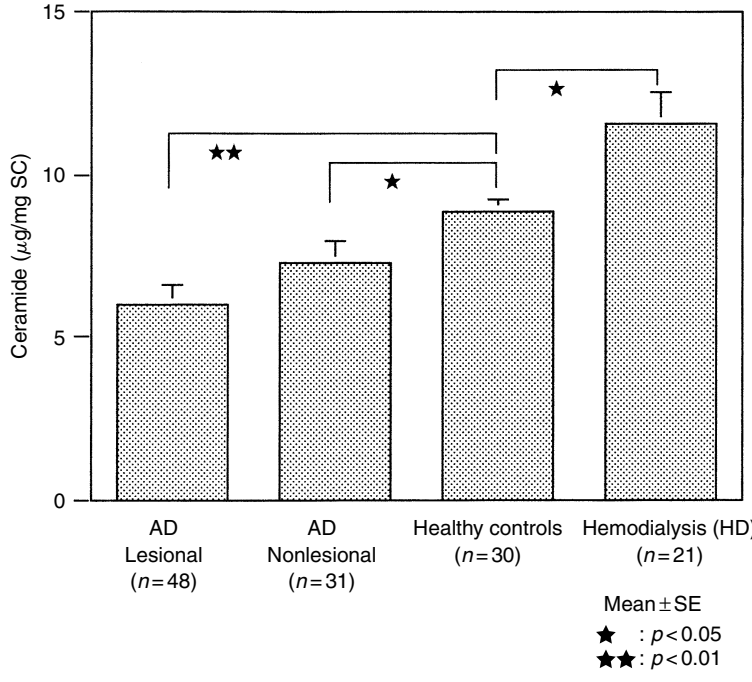

FIGURE 9.7 Ceramide in the SC of patients with AD and those on HD.

ratio of ornithine to citrulline was markedly lower in AD patients, which indicated that the epidermal proliferation was increased in AD patients.

9.4.6 Ceramide Levels in Stratum Corneum

In both lesional and nonlesional sites the ceramide levels were lower in the AD patients than in the controls (Figure 9.7). This result was in agreement with previous reports.[6,7] The lesional skin gave lower levels than the nonlesional skin on average, but no significant difference could be found. However, the levels were higher in HD patients than in the controls ($p < 0.05$). The quantity of intercellular lipids (or ceramide) was closely related to TEWL.[18,19] This study revealed that ceramide levels were high in the control and HD patients whose TEWLs were low, while AD patients with high TEWL showed low ceramide levels.

TABLE 9.3
Characteristics of the stratum corneum in various types of dry skin

Dry skin	Water content in stratum corneum	TEWL	Free amino acids in stratum corneum	Ceramides in stratum corneum	References
Atopic xerosis	Lower	Higher	Lower	Lower	(3),(35)
Senile xerosis	Lower	Normal	Lower	Slightly lower	(20)
Hemodialysis	Lower	Normal – Slightly higher	Lower	Higher	
Seasonal allergic rhinitis	Lower	Normal	Lower	N.D.	(36)
Ichthyosis valgaris	Lower	N.D.	Lower	N.D.	(13)
Induced by tape stripping	Lower	Higher	Lower	Normal	(14)
Induced by surfactant	Lower	Higher	Lower	Normal	(23)

N.D.: not determined

Ceramides are classfied into five species (ceramide 1, 2, 3, 4/5, and 6) according to their polarity. Yamamoto et al.[6] and Imokawa et al.[7] have reported that AD shows a significant decrease in the proportion of ceramide 1, which is a carrier of linoleate and responsible for water-barrier function.

In HD patients, the proportions of ceramides 2 and 3 were high and those of ceramides 1, 4/5, and 6 were low in comparison with the normal controls.

9.4.7 Characteristics of Stratum Corneum in Various Types of Dry Skin

The characteristics of hydration level and barrier function of SC in various types of dry skin were reviewed and summarized in Table 9.3. They are senile xerosis, seasonal allergic rhinitis, ichthyosis valgaris, and experimentally induced dry skin including atopic xerosis and dry skin by hemodialysis. The water content decreased in every type of dry skin and the free amino acids content also decreased corresponding to the decrease of the water content. However, the TEWL or the ceramide levels showed no clear tendency throughout every type of dry skin, especially ceramides showed higher or lower value even though the water content in SC was consistently lower in every type of dry skin.

9.5 DISCUSSION

The characteristics of the dry skin of AD patients have been widely studied. However, those of HD patients have not been studied in detail, although they also show dry skin with itchiness as in AD patients. In this study, we investigated the functions of SC to characterize the dry skin of AD and HD patients by noninvasive methods.

The water content in SC was low both in AD and HD patients, and their skin was obviously dry. However, there was a great difference between them in TEWL. The TEWL was high in AD patients accompanied with extremely inferior barrier function of SC, while HD patients showed a slightly higher TEWL than the controls and their barrier function proved to be in the mostly normal range. The findings obtained in HD patients resembled the symptoms of senile xerosis[20,21] and coincided with those reported by Kamiya et al.[11] The difference in TEWL between AD and HD patients and the

low water content in SC in both groups were explained by the difference in ceramide levels and low free amino acid levels, respectively.

The extent of parakeratosis was determined by the microscopic inspection of nucleated cells in SC. Parakeratotic cells were detected at a high frequency on the lesional skin of AD patients and were also found on the nonlesional skin in some cases, but at a much lower frequency. This indicates the presence of slight inflammation even at the nonlesional site. On the other hand, the absence of nucleated cells in SC indicated that the skin of HD patients as well as normal controls had no inflammation.

Free amino acids in SC are the metabolites of filaggrin originating from keratohyalin granules.[22] Furthermore, histidine is converted into urocanic acid, ornithine to citrulline, and glutamic acid to pyrrolidone carbonic acid.[12] The conversion ratio of ornithine to citrulline [Cit/(Orn+Cit)] and free amino acid content in SC decreased on the scaly inflammatory skin induced by surface active agents or tape stripping.[12,14] In the dry skin, caused by ichthyosis vulgaris[13] or radiation of UV rays,[24] which shows a short turnover time of SC (namely, high proliferation rate in basal cells), the reduced free amino acid levels were recognized.[14,23] The significantly low conversion ratio of amino acids and the observation of some parakeratotic cells even in nonlesional skin suggested that hyperproliferation of keratinocytes induced by slight inflammation caused the decrease in free amino acid levels and the amino acid conversion ratio in AD.

On the other hand, the decrease of free amino acid levels in HD patients could be attributed to the decrease of keratohyalin granules or filaggrin, as found on the crus of the patient with senile xerosis[20,25] or of the elderly,[26] and not to the inflammation nor to the increased epidermal proliferation, because the conversion ratio of amino acid was similar to those in the control and no parakeratotic cells could be observed.

A decrease in ceramide levels and abnormalities in the formation of lamellar bodies and in the extrude process of their components into the corneocytes spaces have been reported in AD patients.[28,29] According to Holleran et al.[30] and Menon et al.,[31] in normal skin ceramides are produced in SC by degradation of glucosylceramides by beta-glucocerebrosidase and by hydrolysis of sphingomyelin by sphingomyelinase. In the epidermis of AD patients an altered metabolic pathway of sphingomyelin was suggested by Murata et al.[32] The activity of sphingomyelin acylase is enhanced, and then large amounts of sphingosylphosphorylcholine and free fatty acids are formed, but the amount of ceramides is decreased. However, no reports on the intercellular lipids in HD patients are available. The ceramide levels were higher than those in the controls. Nevertheless, they had slightly higher TEWL. This might be due to the difference in the composition of ceramides. Though TEWL is correlated with the amount of intercellular lipids, barrier function of the SC also depends on the composition or structure of the intercellular lipids.[33,34] We reported previously[14,23] that on dry skin caused by surface active agents or tape stripping, TEWL was increased with the lack of change in ceramide levels, but the proportion of ceramide 2 was increased while that of ceramide 4/5 decreased. HD patients showed a similar change, and the rise of TEWL was supposed to be caused by the disturbance of the orientation or structure of intercellular lipids. Both AD and HD patients showed a higher skin surface pH than the controls. Ishida et al.[10] have reported that HD patients showed a higher pH on the forehead, forearm, palm, and crus. Anderson[8] also reported that the dry skin in AD or ichthyosis vulgaris showed higher pH. Factors related to skin surface pH are (1) environmental factors, including atmospheric temperature and humidity, and bathing; (2) intracorporeal factors such as menstruation; and (3) factors based on the composition of the skin itself such as SC, sweat, and sebum. However, no established explanations are available yet.

It is quite interesting that the rise in skin surface pH might be related to the drying or itching of the skin in AD or HD patients. The higher pH in HD patients than in AD patients might be partially due to the higher frequency of parhidrosis in the former.

A significant difference was found only in TEWL between the lesional and nonlesional skin of AD patients. However, all the measurements on the nonlesional skin, except for skin surface pH, gave values intermediate between those on the lesional skin and those in the controls.

Therefore, lesional and nonlesional skin might have some differences physiologically as well as clinically.

AD patients showed mild inflammation with induction of the parakeratosis, decreased moisture holding ability, and inferior barrier function, while HD patients showed only reduced moisture holding ability with almost normal skin barrier function but without inflammation. Then, it was concluded that the dry skin of AD patients resulted from the lack of moisture holding factor (free amino acids, NMF) and inferior barrier function of SC, while that in HD patients was mainly attributed to the decrease of the moisture holding factors.

Finally, it is concluded that the hydration state of the skin surface is strongly depend on the content of free amino acids of SC in dry skin, but not on ceramides in lamellar lipids.

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10 Experimentally Induced Dry Skin

Mitsuhiro Denda

CONTENTS

10.1 INTRODUCTION

Dry, scaly skin is characterized by a decrease in the water retention capacity of the stratum corneum (SC),[1] with water content diminished to less than 10%. Barrier function of the SC is usually declined, and transepidermal water loss (TEWL) is increased because of an abnormality on barrier homeostasis.[2] People feel tightness of their skin, and the skin surface becomes rough, scaly, and sensitive. Hyperkeratosis, abnormal scaling, and epidermal hyperplasia are usually observed in the dry skin.[2] Keratinization also shows abnormal features.[2] These phenomena are commonly observed in atopic dermatitis and psoriasis.[3] Dermatitis induced by environmental factors such as exposure to chemicals, low humidity, and UV radiation also shows these features. Thus, many researchers have been investigating the cause and treatment of dry skin, and there is currently great interest in adequate model systems for dry skin studies. In this chapter, I will describe several model systems of dry skin for clinical research of dermatitis associated with skin surface dryness and also mention recent studies to improve the dry skin.

10.2 EXPERIMENTALLY INDUCED DRY SKIN

10.2.1 Dry, Scaly Skin Induced by Barrier Disruption

Barrier disruption is observed in variously induced scaly skin[4] and is known to cause changes in epidermal biochemical processes, including lipid biosynthesis,[5] DNA synthesis,[6] calcium localization,[7] and cytokine production.[8] Up-regulation of specific keratin molecules and adhesion

molecules associated with the inflammatory response is also observed.[9] Because a decline of SC barrier function might be related to many types of skin abnormalities, the role of the SC barrier function has recently become the focus of intense research.

In our daily life, the SC barrier is potentially perturbated by chemicals such as surfactants, detergents, and organic solvents. As a good model of this, Gruneward et al.[10] demonstrated damage of the skin by repetitive washing with surfactant solutions. They treated skin following the repeated use of SLS and N-cocoyl protein condensate sodium as a mild wash substance for one week. In their report, they suggested that repeated washing with even a mild surfactant damaged skin.

Recent studies suggested that intrinsic factors also affect cutaneous barrier homeostasis. Psychological stress delays barrier recovery after artificial barrier disruption.[11] Also, the SC barrier becomes fragile and the recovery rate is delayed with aging.[12] Thus, a dry skin model induced by barrier disruption might be a good model for clinical research.

Previously[13] investigators usually used back or forearm skin for the experiment. It was easier to induce scaly skin on back skin than on forearm skin. In the case of back skin, we stripped SC nine times with adhesive cellophane tape. At that time, the transepidermal water loss (TEWL) value was over 10 mg/cm^2/h and most of the SC was removed. In the case of forearm, to induce dry, scaly skin, stripping for 30 to 50 times was needed. One week after treatment, TEWL was higher than the normal level, skin surface conductance decreased, and SC cell area also decreased (Table 10.1). The skin surface became scaly and flaky. Figure 10.1 shows skin surface pictures of the forearm skin with and without barrier disruption. Abnormal scaling is observed on the surface of skin, which was treated with tape stripping. These phenomena are commonly observed in natural dry skin, such as atopic dermatitis and psoriasis.

Acetone treatment is also used for barrier disruption.[14] Compared to tape stripping, this treatment breaks the SC barrier homogeneously. On the other hand, it takes a longer period of time to break the barrier than by tape stripping.

Treatment with the surfactant is another way to break the barrier, as described earlier.[10] The efficacy depends on each surfactant. Yang et al.[15] suggested that some kinds of anionic surfactant, such as sodium dodecyl sulfate (SDS), affect not only the SC barrier, but also the nucleous layer of the epidermis. Fartasch demonstrated[16] that the topical application of SDS caused cell damage to the nucleated cells of the epidermis and acetone treatment disrupted the lipid structure only in the SC. Thus, if one wants to investigate the effect of the disruption of the SC barrier function, tape stripping or acetone treatment would be better for the study.

The UV radiation also causes decline of barrier function, but this method potentially induces various kinds of responses not only in the epidermis, but also in the dermis.[17]

TABLE 10.1
Change of Skin Surface Condition One Week after Tape Stripping, Measured at 22°C and 55% RH

	TEWL (g/m^{2}h)	Conductance (Mean value/control)	SC cell area (mm^2)
Before treatment	6.3 ± 1.9	1.1 ± 0.3	1047 ± 81
After treatment	8.1 ± 2.5*	0.6 ± 0.3*	956 ± 69***

*$p < 0.05$ and ***$p < 0.001$, significance of difference between normal and scaly skin.

Note: Each value is the mean ± standard deviation from nine subjects. Each value is the mean ± standard deviation from nine subjects.

Source: From Denda M. et al. (1992) *Arch. Dermatol. Res.* 363–367 (with permission).

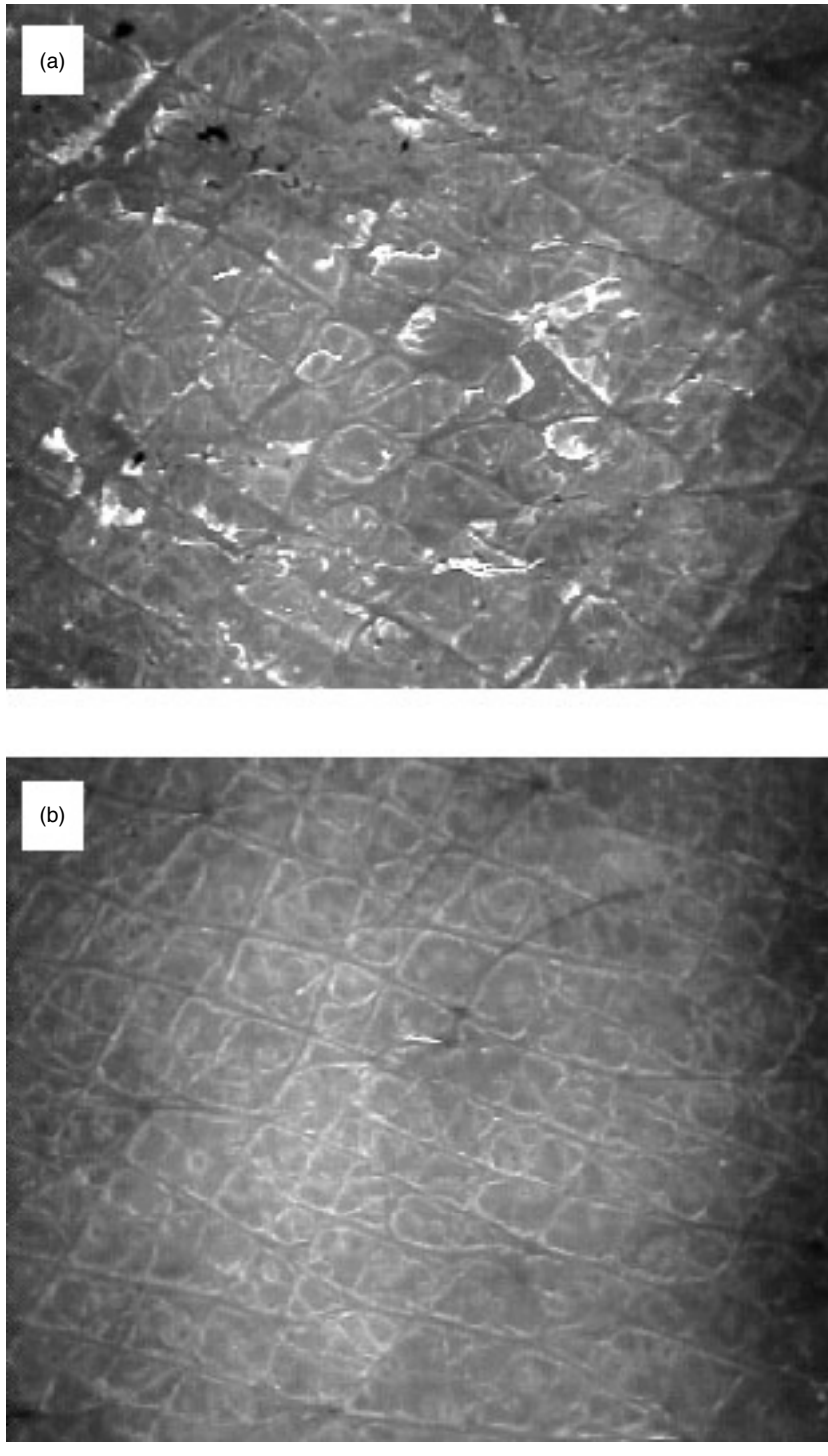

FIGURE 10.1 Dry, scaly skin induced by tape stripping (a) and untreated control (b). Forearm skin was stripped with adhesive cellophane tape 50 times, and 1 week later, skin surface was observed with a microvision system (Hi-Scope, NH-2000, Panasonic, Japan).

The degree of epidermal hyperplasia correlated with the level and duration of barrier disruption.[18] The effects of repeated barrier disruption have been examined using hairless mice. Not only epidermal hyperplasia, but also cutaneous inflammation, was observed in the case of a longer and higher level of repeated barrier disruption by tape stripping and acetone treatment. Since neither the increase in epidermal cytokine production nor the described changes in cutaneous pathology were prevented by occlusion, the hyperplasia in this model should not be attributed to increased water loss, but rather to epidermal injury resulting in the production and release of epidermal cytokines.

Although repeated barrier disruption induces inflammation, epidermal hyperplasia, and abnormal keratinization, there are several histological differences between this model and psoriasis. Gerritsen et al.[19] reported the absence of some characteristic features of psoriasis in the dry skin induced by repeated tape stripping. They also demonstrated that filaggrin expression in the model system was different from that in psoriasis. The mechanism underlying the clonical skin diseases such as psoriasis remains to be investigated.

10.2.2 Dry, Scaly Skin Induced by an Occlusive Surfactant Dressing

Surfactants have also been used to cause artificial dry skin. Many researchers have reported surfactant-induced dry skin.[20] In our daily life, surfactants, that is, detergents, are a potential cause of dermatitis. Thus, the dry skin induced by surfactants has been studied not only as a model system of dry skin, but also for clinical study of skin trouble in our daily life.

The effect of a surfactant on skin depends on the type of surfactant as described earlier. Wilhelm et al. demonstrated the irritation potential of anionic surfactants.[21] They evaluated the effects of sodium salts of n-alkyl sulfates with variable carbon chain length on TEWL and found that a C12 analog gave a maximum response. They suggested that the mechanisms responsible for the hydration of SC are related to the irritation properties of the surfactants. Leveque et al. also suggested[22] that the hyperhydration of SC is consecutive to the inflammation process. They demonstrated that the increase of TEWL was induced by SDS without removal of SC lipids. SDS might influence not only SC barrier function, but also the nucleated layer of epidermis and dermal system associated with inflammation.[23] Recently, no correlation was found between the level of epidermal hyperplasia and TEWL increase on the SDS-irritated skin.[23] Further work would be needed to determine the effects of surfactants on skin.

In our previous study[4] we used human forearm skin or back skin for the study. The forearm skin was treated with a 5% aqueous solution of SDS and an occlusive dressing was applied. After treatment, we washed off the surfactant solution with water and then continuously measured TEWL, skin surface conductance, and SC lipid morphology by ATR-IR for 14 days. The lipid morphology in the SC was altered by the treatment, but recovered to normal within two days. On the other hand, both TEWL and skin surface conductance were abnormal even two weeks after the SDS treatment. Single application of the barrier disruption by tape stripping or acetone treatment did not cause such obvious changes. Thus, the occlusive surfactant dressing affects skin not only on the SC, but also on the nucleated layer of the epidermis and dermis as described earlier. Potentially, this method damaged the skin too much. One should pay careful attention to the concentration of the surfactant solution and period of the occlusive dressing. The damage of skin is different in each person. Application of an occlusive dressing substantially increases the irritant response of the skin to repeated short-term treatments with the surfactant.

During the past decade, several reports have demonstrated the decline of sphingolipid metabolism in atopic dermatitis[24,25] and the cause of dry skin has been shown to be the abnormality of sphingolipid metabolism.[26] However, in experimentally induced dry skin, the total amount of SC ceramide did not change[13,26] but the amino acid content decreased.[13] Recently, Tanaka et al. reported[27] that amino acid content was reduced in the SC in atopic respiratory disease. They suggested that the free amino acid content is the crucial factor of the dry, scaly features of not only experimentally induced

dry skin but also atopic respiratory disease. This is a good example showing that the experimentally induced dry skin model is quite a useful method to investigate dermatitis associated with skin surface dryness.

10.2.3 Dry, Scaly Skin Induced by Dry Environment

Seasonal changes affect the condition of normal skin and may trigger various cutaneous disorders.[28,29] In common dermatitis, a decline in barrier function often parallels the increased severity of clinical symptomatology. All these conditions tend to worsen during the winter season when humidity is low.[30,31] Abundant indirect evidence indicates that decreased humidity precipitates these disorders, whereas, in contrast, increased skin hydration appears to ameliorate these conditions. The mechanisms by which alterations in relative humidity might influence cutaneous function and induce cutaneous pathology are poorly understood.

Recently, low humidity has been shown to stimulate epidermal DNA synthesis and to amplify the hyperproliferative response to barrier disruption.[30,31] SC morphology was also influenced by a dry environment,[32] and abnormal desquamation was observed under low humidity.[33] These results suggest that this model system, that is, dry skin induced by dry environment, is also an important model for clinical research of skin diseases associated with skin surface dryness.

In our study, we used hairless mice.[32,33] Before each experiment, animals were caged separately for at least four days. These cages were maintained in a room kept at a temperature of 22–26°C and at a relative humidity of 40–70%. Animals were kept separately in 7.2-l cages in which the relative humidity (RH) was maintained at either 10% with dry air or 80% with humid air. The temperature was same in all cases (22 to 26°C), and fresh air was circulated 100 times per hour. Animals were kept out of the direct stream of air. The level of NH_3 was always below 1 ppm.

Under a dry condition, epidermal DNA synthesis increased within 12 h.[31] Abnormal scaling and an increase of SC thickness were also observed within 2 to 3 days.[33] When we treated flank skin of the animals, which were kept in a dry condition for 48 h with acetone, obvious epidermal hyperplasia and mast cell degranulation were observed 48 h after the acetone treatment.[30] When the animals were kept in dry condition for more than 1 week, the barrier function was enhanced.[34] However, drastic decrease of environmental humidity induced barrier abonormality,[35] and decrease of water retention capacity of SC.[36] Ashida et al. demonstrated the increase of IL-1α in the epidermis[37] and histamine and mast cell in the dermis[38] under dry environment. Hosoi et al. demonstrated the allergic response enhanced by low environmental humidity.[39] These studies provide evidence that changes in environmental humidity contribute to the seasonal exacerbations and ameliorations of cutaneous disorders such as atopic dermatitis and psoriasis, diseases, which are characterized by a defective barrier, epidermal hyperplasia, and inflammation. Because these responses were prevented by occlusion with plastic membrane, petrolatum, and humectant[30] this dry skin model is a good system to evaluate clinical methods to solve skin problems.

10.3 NEW STRATEGIES TO IMPROVE DRY SKIN

As described earlier, dry, scaly skin looks like skin diseases can be induced by several artificial methods. These are useful model to find out new strategy to improve dry, scaly skin condition. Following are the examples of recent studies about new methods to cure dry, scaly skin.

10.3.1 Protease Inhibitor

Denda et al. previously demonstrated[40] that *trans*-4-aminomethyl cyclohexane carboxylic acid (*t*-AMCHA), an anti-fibrinolytic agent that activates plasminogen, improved the barrier homeostasis and whole skin condition. After barrier disruption, proteolytic activity in the epidermis increased within 1 to 2 h. This increase was inhibited by *t*-AMCHA. Topical application of *t*-AMCHA or

trypsin-like serine protease inhibitors accelerated the barrier recovery. Moreover, topical application of *t*-AMCHA mitigated epidermal hyperplasia induced by repeated barrier disruption. These findings suggested that manipulations that injure the stratum corneum activate the plasminogen/plasmin system and the increase of the extracellular protease activity is detrimental to barrier repair and may induce pathologic changes in the skin. Kitamura et al. also reported[41] the efficacy of this agent to dry skin. The protease balance might be important for the barrier homeostasis and skin pathology.

10.3.2 Nuclear Hormone Receptor Activator

Feingold and his coworkers demonstrated an important role of nuclear hormone receptor on epidermal differentiation and stratum corneum barrier formation. Activation of PPARα Peroxisome proliferator-activated receptor α by farnesol also stimulated the differentiation of epidermal keratinocytes.[42] Cornified envelope formation, involcrin, and transglutaminase protein, and mRNA levels were also increased by the activation of PPARα. Interestingly, the inflammatory response was also inhibited by the treatment.[43] They also showed that topical application of PPARα activators accelerated the barrier recovery after tape stripping or acetone treatment and prevented the epidermal hyperplasia induced by repeated barrier disruption.[42] Regulation of the nuclear hormone receptor would open a new possibility for improvement of the cutaneous barrier.

10.3.3 Histamine Receptor Antagonist

Histamine receptors are related to skin barrier function.[44] Three different types of histamine receptors, H1, H2, and H3 have been reported. First, topical application of histamine H1 and H2 receptor antagonists accelerated the barrier repair. Histamine itself, H2 receptor agonist, and histamine releaser delayed the barrier repair. Histamine H3 receptor antagonist and agonist did not affect the barrier recovery rate. Topical application of the H1 and H2 receptor antagonists prevented the epidermal hyperplasia induced by barrier disruption under low humidity. The mechanism of the relationship between the histamine receptors and the barrier repair process has not been elucidated yet.

10.3.4 Regulation of Receptors of Neurotransmitters in the Keratinocytes

Recently we demonstrated that a variety of receptors of neurotransmitters exist in epidermal keratinocytes and moreover, they are strongly associated with skin permeability barrier condition and epidermal hyperproliferation.[45–47]

Activation of calcium permeable receptors such as purinergic P2X receptor or NMDA receptor delayed the barrier recovery after barrier disruption and enhanced epidermal hyperplasia induced by barrier disruption.[45,47] Topical application of antagonists of these receptors prevented these pathological changes. On the other hand, topical application of agonists of chloride permeable receptors such as GABA(A) receptor or glycine receptor accelerated the barrier repair and prevented the epidermal hyperplasia.[46]

Not only ionotropic receptors but also metabotropic receptors are associated with cutaneous barrier homeostasis. β2-adrenergic receptor antagonist prevented epidermal hyperplasia induced by barrier disruption.[48] In the case of metabotropic receptors, the level of intracellular cAMP in the epidermal keratinocytes is associated with cutaneous barrier homeostasis and epidermal hyperplasia.[49]

Ectoderm-derived keratinocytes and neurons show a similar expression of those receptors. Both of them play a crucial role as the interface of information between body and environment. Both systems are regulated by nonlinear ion dynamics. A physicochemical study of the nonlinear dynamics is necessary for further understanding of both intelligent systems.

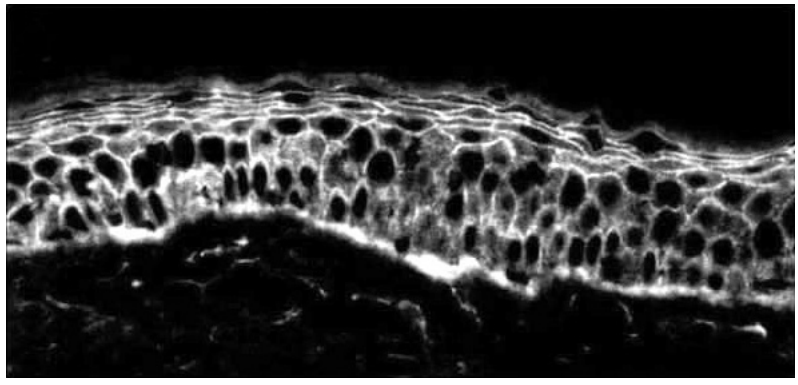

FIGURE 10.2 Pain receptor VR1(TRPV1) is localized in human epidermal keratinocytes.

10.4 THINKING EPIDERMIS: *SENSOR DEVICES IN THE EPIDERMIS*

Epidermis is an interface between body and environment. The basic mechanism of the smart system of the epidermis has not been clarified yet, but epidermal ion dynamics in the epidermis plays an important role as a signaling system.[47,49] As described earlier, a variety of receptors of neurotransmitters are found in the keratinocytes. Recently, the existence and function of vanilloid receptor subtype 1 (VR1, TRPV1) in epidermal keratinocytes has been demonstrated (Figure 10.2).[50,51]

VR1 is a cation channel and originally it was discovered as a polymodal pain receptor in the nerve system.[52] The role of VR1 in the epidermal keratinocytes has not been clarified. It might be associated with sensory perception of the skin. Temperature and osmotic pressure sensitive receptors are also found in the epidermal keratinocytes.[53,54] Epidermal keratinocytes might play a crucial role as a sensor against environmental changes.

Previous reports demonstrated that exposure to a dry environment made skin more sensitive pathologically.[34,37–39] The signaling system of the epidermis against environmental changes has not been clarified yet. The cultured keratinocyte, however, an oscillation of intercellular calcium is induced by the air exposure (see Figure 10.3).

The mechanism of the induction and role of the calcium oscillation have not been clarified. The nonlinear ion dynamics might play an important role in the sensor system of the epidermis.

10.5 CONCLUSION

As described previously, one can induce dry, scaly skin, which shows features very similar to dermatitis such as atopic dermatitis and psoriasis. Use of this experimentally induced dry skin should enable the discovery of a new clinical methodology to cure or care for skin problems. Recently, several excellent *in vitro* skin models have been reported. Although they are also very useful models for the study of cutaneous metabolism, their function and microstructure are still different from those of intact skin. On the other hand, the mechanisms underlying abnormal desquamation, that is, scaling in the dry skin such as atopic dermatitis, are not completely known. Sato et al. reported[55] the inhibition of protease in the SC induced scale without affecting epidermal mitosis. This result seems to be no direct relationship between skin surface appearance and epidermal proliferation. However, decline of SC barrier function induced epidermal hyperplasia, as described earlier.[30] The loss of water content from SC also induced epidermal DNA synthesis.[30] Further mechanistic studies on each of the dry skin features are required.

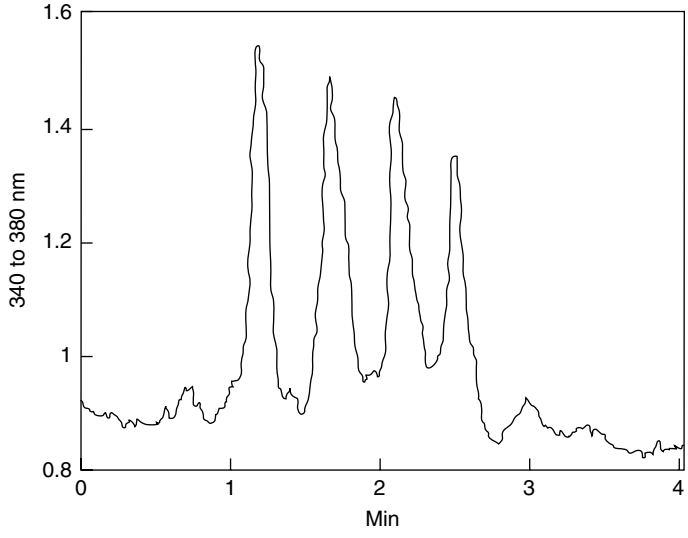

FIGURE 10.3 Oscillation of intercellular calcium in the cultured keratinocyte immediately after air exposure. Vertical axis shows the ratio of emission 340 to 380 nm.

The models described in this section should help clarify the biochemical mechanism of dry skin and lead to improvements in the clinical treatment of various skin problems associated to skin surface dryness.

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11 Dryness in Chronologically and Photo-Aged Skin

Ehrhardt Proksch

CONTENTS

11.1 INTRODUCTION

Elderly people complain about their skin status in high proportion; this proportion may be as high as over 80% at the age of 80. They most often complain about the unsightliness of the skin, skin spots, pruritus, and stinging. Sometimes they complain about what is called "senile pruritus." In most cases "senile pruritus" may result from dry skin conditions. Dry skin is often localized on the lower legs and the outsides of the upper arms; dryness may also occur in the face. Dry skin may develop into eczéma craquelé, especially when stressed with repeated exposure to hot water and detergents. This condition may even develop into a generalized eczema and erythroderma. Erythroderma is much more common in the elderly. The cause of erythroderma is often difficult to identify. The most common cause for erythroderma in the elderly is generalized eczema. Eczema may be related to atopic constitution or to contact dermatitis. Dry skin in the elderly may also be related to psoriasis or irritated (eczemateous) psoriasis. The incidence of psoriasis increases with age. Dry skin may also be related to T-cell lymphoma, which causes localized or generalized mycosis fungoides. The initial stage, often called parapsoriasis, is especially difficult to distinguish from eczema. A special type of mycosis fungoides, Sézary syndrome, shows erythroderma and itching and is sometimes also difficult to distinguish from generalized eczema or psoriasis. Dry skin can be an important finding in renal and hepatic diseases and in hypothyreodism, diseases, which are much more frequent in aging. Very seldomly eczéma craquelé may be an indicator of an internal malignancy.[1]

11.2 SKIN AGING

In developed countries, interest in cutaneous aging is the result of a progressive, dramatic rise over the past century in the absolute number and the proportion of the population who are eldery.[2] Skin aging is due to the conjunction of intrinsic (chronological aging) and extrinsic factors (principally photo-aging, but several other environmental factors, including cigarette smoking, also contribute).

Chronological aging is a generalized process involving every organ and every cell of the body including the skin. The appearance of the skin may be an indicator of the conditions of internal organs. For example, people with senile dry skin commonly also exhibit dry eyes, which may be part of Sjögren's syndrome.[3] Werner's syndrome (WS), a representative progeroid syndrome with chromosomal instability caused by the mutation of RecQ type DNA/RNA helicase, manifests skin changes similar to those observed in systemic sclerosis (SSc). In addition, patients with WS show a variety of the signs and symptoms of normal aging in early stages of life: gray hair, alopecia, muscle atrophy, osteoporosis, cataracts, hypogonadism, diabetes mellitus, hyperlipidemia, atherosclerosis, malignancy, brain atrophy, and senile dementia.[4]

The mechanisms of aging are still only partly known. The process of intrinsic skin aging resembles that seen in most internal organs and is thought to involve decreased proliferative capacity leading to cellular senescense and altered biosynthetic activity of skin derived cells. Senescent cells undergo three phenotypic changes: they irreversibly arrest growth, they acquire resistance to apoptotic death, and they acquire altered differentiated functions. The growth arrest is very likely critical for the role of replicative senescence in tumor suppression, but may be less important for the aging of organs such as the skin. On the other hand, the altered differentiation may be critical for compromising the function and integrity of organs like the skin during aging. Senescent keratinocytes and fibroblasts appear to accumulate with age in human skin. Moreover, senescent cells express genes that have long-range, pleiotropic effects — degradative enzymes, growth factors, and inflammatory cytokines. Thus, relatively few senescent cells might compromise skin function and integrity. Moreover, by altering the tissue microenvironment, senescent cells may also contribute to the rise in cancer that occurs with age.[5] Cellular manifestation of intrinsic aging includes decreased life span of cells, decreased responsiveness of cells to growth signals, which may reflect loss of cellular receptors to growth factors, and increased responsiveness to growth inhibitors. Cells from patients with WS have a striking limitation in their *in vitro* replicative life spans and undergo extensive chromosomal rearrangements.[6] Many syndromes associated with premature aging involve dry skin.

Extrinsic aging, more commonly termed photo-aging, also involves changes in cellular biosynthetic activity, but leads to gross disorganization of the dermal matrix. Environmental factors are atmospheric pollution, wounds, infections, trauma, anoxia, cigarette smoke, and hormonal status. These factors have a role in increasing the rate of accumulation of molecular modifications and have thus been termed "factors of aging." All these factors share as a common feature the capability to directly or indirectly induce one of the steps of the micro-inflammatory cycle. This triggers a process leading to the accumulation of damage in the skin resulting in skin aging. Factors involved are intercellular adhesion molecule-1 (ICAM-1), extracellular matrix, collagenases, myeloperoxidases, and reactive oxygen species.[7] Dermal fibroblasts possess a finite replicative capacity of 50 to 100 doublings, and then cease replicating in response to growth factors. Senescent dermal fibroblasts overexpress metalloproteinase activities that may explain the age-related atrophy of extracellular matrix architecture. Also structural changes in the telomeric region of the genome have been discussed in skin aging.[8]

Skin aging is subject to both endogenous and exogenous factors. Some areas, such as on the trunk, age mainly indogenously. Other areas, especially the face or hands, are also affected by exogenous factors. This has a significant impact on the condition and appearance of the skin. Clinically, chronologically aged skin has a pale appearance and shows fine wrinkling. Solar irradiation causes skin to appear prematurely aged. This photo-aged skin shows deeper wrinkle formation, laxity, and leathery appearance. Both types of aged skin share fragility, impaired wound healing capacities, and

higher vulnerability.[9] Aged skin also shows mottled pigmentation. Histology revealed an overall thinning of the skin including the epidermis, dermis, and subcutaneous fat tissue in chronologically aged skin. In sun-damaged skin there is a thinning of the epidermis, but with a marked stratum corneum hyperplasia. The dermis exhibits thinning, curling, and frequent branching of elastic fibers evolving into an almost complete replacement of disorganized fibers with accompanying amorphous masses. The photo-aging correlates with increased solar elastosis and is inherently different from chronologic aging. Warren et al.[10] found an increase in elastine synthesis and increased degradation of collagen fibers subsequently degrading to what histopathology perceives as solar elastosis. This has also been described as an accumulation of elastotic material. The staining intensity of elastin in protected skin significantly decreased, whereas in sun-exposed skin the intensity gradually increased. The accumulated elastin in facial skin was morphologically abnormal and appeared to occupy the areas of lost collagen. In facial skin the collagen fiber architecture appeared disorganized after the fouth decade. In protected skin, type I and III collagen staining was altered only after the eighth decade, while in sun-exposed skin the relative staining intensity significantly decreased with age.[11] Also, the water content of the skin is greatly influenced by ground substances, which may be responsible for wrinkling and laxity of the skin accompanying cutaneous aging. Therefore, water content in the skin is presumed to be a critical determinant in cutaneous aging. Jung et al. aimed at clarifying the change in water content and the content of glycosaminoglycans (GAG) of rat skin in relation to aging. A significant decrease of water content in aged rat skin, which may be related to the change of GAG with intrinsic aging of the skin, was found.[12]

11.3 BIOPHYSICAL MEASUREMENTS IN AGED DRY SKIN

Obviously, measurements of stratum corneum hydration in aged and aged dry skin are important. In a comprehensive study comparing the skin of the lower leg in elderly versus young subjects with either normal or dry skin, we found a slight, but not significant, increase in stratum corneum hydration in normal aged compared to normal young skin using the well-known Corneometer®. The stratum corneum should have at least 10% water; some authors claim that 20 to 30% is ideal.[13] Hydration was significantly reduced in young and aged dry skin compared to the age-matched controls. The lowest values were obtained in young dry skin (but not significantly lower than aged dry skin).[14] An additional study on the skin of the lower leg found a striking decrease in skin surface hydration in elderly xerotic skin.[15]

Transepidermal water loss (TEWL) in aged and aged dry skin has been examined in several publications. We found decreased TEWL in aged compared to young normal skin. Also, dry skin showed decreased TEWL in young as well as in aged skin. Overall, aged dry skin had the lowest TEWL. We suggest that the described increase in the thickness of the stratum corneum in aged and especially in aged dry skin, together with reduced skin temperature and blood flow, may be the reason for the low TEWL under basal conditions.[14] Wilhelm et al. examined biophysical parameters in skin aging, comparing a group with a mean age of 27 to a group with a mean age of 71 years. They did not find significant differences in sebum, capacity (as a marker of hydration), and pH. However, TEWL was significantly lower in the aged population.[16] This shows an unperturbed barrier function in aged skin under basal conditions. However, after stressing the skin by tape stripping, barrier repair was delayed in aged skin.[17] Barrier repair in aged dry skin has not yet been examined.

11.4 LIGHT AND ELECTRON MICROSCOPY STUDIES IN AGED DRY SKIN

Histologically, elderly xerotic skin showed an atrophic nucleated epidermis and a threefold increase in corneocyte size. This was accompanied by an approximately 50% increase in the number of

stratum corneum cell layers as well as a corresponding decrease in stratum corneum turnover time. Electron microscopy revealed decreased size and number of keratohyalin granules and failure of the granules to aggregate.[15]

11.5 EPIDERMAL DIFFERENTIATION IN DRY AND AGED DRY SKIN

The hallmarks of dry skin (xerosis) are scaliness and loss of elasticity. Decreased hydration and disturbed lipid content of the stratum corneum are also well-known features. The frequency of dry skin increases with age. Previously, we examined whether the known features of dry skin are related to changes in epidermal proliferation and differentiation. In addition, age-related changes in normal and in dry skin were examined. Sixty-two volunteers were divided by clinical grading and biophysical measurements into groups with young/normal, young/dry, aged/normal, and aged/dry skin. Biopsy samples taken from the lower legs (exhibiting the most severe dryness) were examined by two-dimensional gel electrophoresis and by immunohistochemistry for epidermal proliferation, epidermal keratins, and cornified envelope proteins. We found a slight increase in proliferation in both groups with dry skin compared with normal skin of the corresponding age. In aged/normal compared with young/normal skin there was a significant decrease in proliferation. However, epidermal proliferation was the same in aged/dry skin as in young/normal skin. For epidermal differentiation, an age-independent decrease of keratins K1 and K10 and an associated increase in the basal keratins K5 and K14 was detected in dry skin. There was also an age-independent premature expression of the cornified envelope protein involucrin. In contrast, loricrin expression was not influenced by dry skin conditions. Therefore, we concluded that normal epidermal differentiation is necessary for skin homeostasis. Stimulation of aged skin by skincare products should not cause hyperproliferation with disturbed epidermal differentiation. Stimulated skin should be carefully examined by appropriate immumunohistochemical and biophysical methods to detect any alterations in epidermal proliferation, differentiation, and skin barrier function.[14]

11.6 AMINO ACIDS AND FILAGGRIN IN DRY AND AGED DRY SKIN

Dry skin (xerosis) is one of the characteristics of aged skin. Both intrinsically and extrinsically aged skin is prone to dry skin conditions. Dry skin mainly results from changes of the stratum corneum, which, in turn, result from changes in epidermal differentiation. Stratum corneum proteins and lipids are crucially involved in proper water binding, as the elasticity of the stratum corneum needs a certain amount of water. Water content and thickness of the stratum corneum contribute to skin surface morphology.[18] Dry skin may be caused by a decrease in stratum corneum amino acids, which are natural moisturizing factors and which may be derived from filaggrin breakdown.[19] Jacobson et al. examined the effects of aging in xerosis on the amino acid composition of human skin. They found reduced content of free amino acids in old/normal compared to young/normal subjects. Xerosis did not appear to effect the amino acid composition of samples from young or old subjects.[20]

Filaggrin is regarded as a cornified envelope protein[21]; the cornified envelope proteins are responsible for the mechanical resistance of the stratum corneum. Filaggrin breakdown products were supposed to be an important source of the water binding proteins in the stratum corneum.[19] In aged skin, filaggrin is immunohistochemically decreased compared with to levels found in young skin. However, a recent study by Takahashi and Tezuka (2004), showed that the total amount of amino acids in the stratum corneum was larger in aged senile dry skin than in young skin. The expression of filaggrin mRNA in aged skin was, however, similar to that in young skin. This finding suggests that the immunohistochemical decrease in filaggrin in aged skin may be caused by promotion of filaggrin proteolysis in the upper layers of the stratum spinosum.[22] Previously, Tezuka examined

terminal differentiation of aged facial epidermis immunohistochemically. In old age, the epidermis tends to become dry and flaky, especially on the lower legs. However, this is less common in the face, although long-term ultraviolet light irradiation has important effects on the differentiation of facial keratinocytes. The authors found a striking decrease in filaggrin content in the skin of the lower legs but not in the face in aged skin.[23] However, Scott experimentally found alterations in the metabolism of filaggrin in the skin after chemical- and ultraviolet-induced erythema. The time between formation and breakdown of the filaggrin was much reduced in the hyperplastic epidermis resulting from the UV irradiation.[19] This altered filaggrin metabolism may be the cause for dryness in the photo-aged facial skin.[25] We recently found a broadening of the staining zone for filaggrin in atopic dermatitis lesional skin (with reduced hydration). However, the staining intensity was reduced,[24] a quantification of filaggrin content in atopic dermatis (which has not yet been performed) may reveal a decreased amount of the protein in eczema. Overall, the role of amino acids and filaggrin in dry and aged dry skin needs further examination.

11.7 UREA AND GLYCEROL IN AGED DRY SKIN

Wellner et al.[26] described reduced stratum corneum content of water-binding urea in atopic dermatitis and in dry skin. In aged skin either a slightly reduced urea content after the age of 65 in callus skin[27] or a highly significant reduction[28] in the stratum corneum have been reported. These findings may explain the effectiveness of urea-containing emulsion in the treatment of aged and aged dry skin.

Glycerol is effective in enhancing hydration of the stratum corneum in dry skin.[29] Recently it has been shown that the epidermal water/glycerol transporter aquaporin-3 in deficient mice leads to severely impaired stratum corneum hydration.[9] In these mice, stratum corneum glycerol content was reduced threefold. And it was shown that glycerol replacement corrects the defects in these mice.[30] However, it has not yet been published whether changes in the aquaporin-3 transporter occur in human dry skin or in aged dry skin.

11.8 EPIDERMAL LIPIDS IN AGED DRY SKIN

Several works have focused on the role of stratum corneum lipids in aging. Rogers et al. found significantly decreased levels of all major lipid species, in particular ceramides, with increasing age. The relative levels of ceramide-1-linoleate were reduced in aged skin, whereas ceramide-1-oleate levels were increased.[31] To elucidate the mechanisms involved in the decrease of ceramide levels in aged skin, Jin et al. examined both the activities of beta-glucocerebrosidase, which is a major enzyme in ceramide production, and of ceramidase, which is an essential enzyme in ceramide degradation in the stratum corneum of aged skin. The authors found no changes in beta-glucocerebrosidase activity in aged skin; however, there was an age-related upregulation in ceramidase activity. The increase in ceramidase activity may be a cause for the reduced ceramide content in aging.[32] Ghadially et al. examined changes in lipids in aged mice. Although the total lipid content was decreased in the stratum corneum of aged mice, the distribution of ceramides (including ceramide 1), cholesterol, and free amino acid was unchanged. Moreover, a normal composition of esterified, very long-chain fatty acids were present. Finally, stratum corneum lamellar bodies displayed normal structure and dimensions, but were focally decreased in number, with decreased secretion of lamellar body contents.[17] De Paepe et al. described that the decrease in lipid concentration during aging depends on the anatomical site. Therefore, these variables should be controlled in a reproducible and standardized way in order to study the direct relationship between skin condition and barrier lipid composition.[33]

Akimoto quantified stratum corneum lipids in xerosis and asteatotic eczema in aging. On leg skin they found an age-related decline in total lipids. In healthy leg skin, there was age-related decline in the total ceramide. In contrast, in xerosis and in asteatotic eczema suffering significantly reduced water-holding properties, no definite decrease, but rather a slight increase in ceramide quantity, with

the same composition of each individual ceramide as compared to healthy age-matched controls, was found. The authors stated that the observed decrease in the stratum corneum total lipid content may well explain the high incidence of winter dry skin in older people. However, the progression toward asteatotic eczema cannot be accompanied solely by a decrease in ceramide quantity.[34]

Lipid composition and epidermal differentiation in photo-aged human dry skin has not been examined in detail. It has been shown that acute UV-irradiation increases lipid and ceramide content.[35,36] Also, it has been described that the levels of free fatty acids (FFA) and squalene in aged skin were significantly lower. The composition of linoleic acid decreased in the older group by 40%, suggesting age-dependent loss of oxidatively vulnerable polyunsaturated fatty acid. Sunlight exposure for 1.5 h did not change levels of FFA and squalene, or FFA composition. However, squalene hydroperoxide increased by 60-fold, suggesting that hydroperoxide is produced by singlet oxygen.[37]

The UV iradiation has been shown to be involved in membrane lipid peroxidation and this induces matrix metalloproteinase-1-expression.[38] Therefore, the use of antioxidants has been suggested in treating and preventing skin aging.[39] Whether this also works in preventing dry skin in aging is unknown.

11.9 SEBACEOUS LIPIDS IN DRY AND AGED DRY SKIN

The role of sebaceous lipids in dry skin remains controversial. Although some authors denied a function of sebaceous lipids in dry skin,[40] others described that the excretion of sebum onto the skin surface in xerosis is reduced, suggesting that either the activity or the number of functioning sebaceous glands is reduced and that selective changes in the synthesis of specific sebaceous lipids occur in dry skin.[41] Akimoto et al.[34] analyzed sebum-derived lipids present in the stratum corneum and found a significant decline in free fatty acids in xerosis and asteatotic eczema as compared to age-matched healthy controls, and a similar decline in triglycerides in the above three groups when compared to younger controls. While the number of sebaceous glands remains the same during life, sebum levels tend to decrease after menopause in females, whereas no major changes appear until the eighth decade of life in men.[42] This parallels the decline in androgen levels, but this cannot be the sole factor because there is so much overlap in sebaceous gland activity between men and women. Downing et al. further stated that the increased occurrence of dry skin in the elderly has been shown to be unrelated to the sebum secretion rate. This is not unexpected, as children, with even less sebum, rarely have dry skin.[40] Therefore, sebum secretion may be one factor of many in dry skin of the elderly.

11.10 BATHING AND CLEANSING IN DRY AND AGED DRY SKIN

Skin care in aged or photo-aged dry skin to ameliorate the signs and symptoms is important. The value of bathing and cleansing in dry skin has been discussed in several publications. It is certainly important to avoid contact with high concentrations of soaps or detergents and hot water for a prolonged period in dry skin, and especially in aged dry skin. In my opinion the composition of the detergent is more important than the type of detergent or its pH. The value of bath and shower oils also remains controversial. Several commercial products claim the importance of bath oils for the treatment of dry skin, however, in a recent publication the irritant potential of bath and shower oils has been described and it has been found that there are significant differences between several products. Instead of protecting the skin some formulations may induce subclinical injuries and delays in barrier function recovery.[43] Aged dry skin is prone to irritation.[44] Nevertheless, some people with dry skin report an improvement after using bath oil. In my opinion, after using bath oil, an additional treatment with creams or ointments should be performed in dry and aged dry skin.

11.11 THERAPY OF AGED DRY SKIN

The therapy of aged dry skin is not very different from the general treatment of dry skin. In general, lipid enriched ointments (water in oil emulsion) may be used. However, very greasy ointments based on petrolatum may induce perioral dermatitis in the face. Therefore, in the face it is better to use oil in water creams. Also the treatment depends on the environmental conditions. In winter it is generally advisable to use an ointment for dry skin, whereas in summer (where dry skin is not so severe) a cream may be used. In addition, substances with water binding ability may be used. Urea is widely used for the treatment of dry skin. The concentration of urea in creams and ointments is normally between 3 and 10%.[45] In eczema stinging and burning may occur after treatment with urea-containing ointments. This is less pronounced in dry skin only. In addition, several other compounds may be used for the treatment of dry skin. Glycerol shows positive effects in measurements of hydration[46] taken with the commonly used Corneometer® (however determination of skin hydration by biophysical devices serves as a marker for hydration only and may not necessarily reflect the water content important for the stratum corneum). In addition, lactate and potassium salts have been used to improve dry skin.[47]

Also, ceramide containing creams are in use. It has been shown that lipid supplementation in aging containing a mixture of natural ceramides improves the resistance of aged skin against sodium laurylsulfate-induced dermatitis.[48] Zettersten et al. examined barrier recovery in chronologically aged mouse skin after application of different lipid mixtures. They found that a cholesterol-dominant, optimal ratio of lipids containing cream accelerated barrier recovery.[49] Also, a report on the effects of "physiological mixtures" of lipids including ceramides in atopic dermatitis has been published.[50] However, attempts by several companies to introduce ceramide containing creams or ointments were not as successfully as expected. Because the levels of unsaturated fatty acids are lower in aged and photo-aged skin,[37] treatment with linoleic or gamma-linolenic acid may be of value in aged and photo-aged dry skin.[51] For severely dry skin with a tendency toward eczema, corticosteroids of mild to moderate potency may be used with good response.

The UV radiation causes premature skin aging. This photo-aging is characterized by wrinkles, mottled pigmentation, dry and rough skin, and loss of skin tone. Use of topical vitamin A derivatives like tretinoin can improve photo-aged skin mainly by changing epidermal differentiation.[52] However, skin dryness does not improve and even worsens with retinoid therapy, as well known from its systemic and topical use in several diseases in clinical dermatology.

The role of estrogens in preventing skin aging has been discussed in detail. Estrogen administration has been claimed to ameliorate nearly every sign of aging including skin signs. The skin is a target organ for various hormones and sex steroids, which have a profound influence on the aging process. A decrease in sex steroids thus induces a reduction of those skin functions under hormonal control. Keratinocytes, Langerhans' cells, melanocytes, sebaceous glands, collagen content, and the synthesis of hyaluronic acid, for example, are under hormonal influence. A study on the effect of non-contraceptive estrogen use on skin wrinkling, dryness, and atrophy involving 3875 postmenopausal women in the United States with a mean age of 62 years had been performed. Estrogen use was associated with a statistically significant decrease in the likelihood of senile dry skin and wrinkling. The authors stated that the results strongly suggest that estrogen use prevents dry skin and skin wrinkling, thus extending the potential benefits of postmenopausal estrogen therapy to include protection against selected age- and menopause-associated dermatologic conditions.[53] Gynecologists have seen their future in lifestyle medicine, preventing aging in general and also preventing skin aging by prescribing estrogens. However, a rumor of increased breast cancer rates related to hormone replacement therapy has been confirmed in the highly rated Million Women Study.[54] Therefore, the value of estrogen replacement therapy must be discussed. To minimize cancer risk derivatives of estrogen including phyto-estrogen may be used. Also, topically applied estrogen may show fewer side effects. Topical application of estrogens has a positive effect on skin aging parameters.[55] Phytohormones have structural similarity to 17 beta-estradiol, explaining their estrogen-like effects. However, isoflavonoids

exhibit an inferior biological potency to synthetic estrogens. Although a large number of publications have documented the effects of sex hormones on the aging process, it is obvious that hormone replacement should not be administered as an independent treatment for skin aging.[56]

11.12 SUMMARY

Dry skin is very common in elderly people and steadily increases with age. Dryness may be due to chronological aging superimposed with photo-aging. The pathophysiology involves impaired epidermal differentiation and lipid composition. Aged dry skin is prone to irritation, thus cleansing in aged dry skin should be very mild. Lipid-enriched creams or ointments should be used for the treatment of aged dry skin. Urea, glycerol, and other water binding compounds may be included in topical preparations.

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12 Itch Associated with Dryness of the Skin: the Pathophysiology and Influence of Moisturizers

Brett T. Summey Jr. and Gil Yosipovitch

CONTENTS

12.1 INTRODUCTION

Xeroderma (dry skin) is characterized by a scaly, rough, cracked, and fissured surface, and is intimately associated with the somatosensory sensation of itch. Nearly every dermatological condition that manifests with xerotic skin is accompanied by itch. Atopic dermatitis is the hallmark and most prevalent of the conditions manifesting with dry skin and itch. Other clinical conditions involving dry skin and pruritus include common diseases like psoriasis, seasonal xerosis, pruritus of the elderly, asteatotic eczema, certain ichythoses, as well as systemic diseases such as uremia and human immunodeficiency virus infection. Quite interestingly, patients with ichythosis vulgaris, which is associated with significant xerosis, do not complain of itch.[1]

In addition to primary skin processes, itchy xerotic skin can also be induced secondarily. For example, environmental factors such as rapid variations in relative humidity as well as the extremely windy, cold, and dry weather associated with the winter season induce skin dryness and itch. In addition, many commonly used topical products containing soaps, detergents, alcohol, and other irritants as well as hot water alone also lead to dry, itchy skin.

Despite the well-known clinical coupling of dry skin and itch, studies to objectively compare the degree of skin hydration or measurements of the transepidermal water losses with severity of pruritus have provided conflicting results on this close association. This review will discuss the existing data, breakdown the pathophysiology of xerotic itch, and describe the role of moisturizers in alleviating both entities.

12.2 SPECIFIC PRIMARY CONDITIONS WITH BARRIER MALFUNCTION AND ITCH

12.2.1 Atopic Dermatitis

Atopic dermatitis is the most common itchy dermatosis, with well-documented alteration in the stratum corneum function. Numerous studies have revealed an increase in the basal transepidermal water loss (TEWL) in the stratum corneum of patients with this condition. Of note, this increase in TEWL was also described in the clinically unaffected skin of atopics.[2–4] There have been direct correlations shown between the degree of inflammation and severity of barrier impairment in atopic dermatitis. Despite these findings, to date there have been no definitive reports correlating degree of barrier function with itch variability.

12.2.2 Senile Xerosis

Pruritus of the elderly is another common dermatosis associated with itch.[5] One study has shown that elderly patients with generalized pruritus had a higher degree of skin dryness than in age matched control subjects.[6] This study also demonstrated that skin surface conductance, a marker of stratum corneum water content, was decreased in elderly patients with generalized pruritus. The study was also successful in demonstrating an acquired abnormality in keratinization in these patients. This abnormality manifested with increased intracorneal cohesion compared with the controls. Another study showed an increase in histamine release and hypersensitivity in patients with senile pruritus.[7] Paradoxically, oral anti-histamines are not very efficacious in the treatment of senile pruritus.

12.2.3 Dry Skin and Uremic Itch

Dry skin is the most common dermatologic problem among patients requiring hemodialysis.[8] Among this cohort, itch is also present in 60 to 90%.[9] The association between the degree of itch and the degree of skin dryness in hemodialysis patients has been investigated in several studies; however most could not identify any connection. Young et al. were able to demonstrate this connection, however subsequent studies have not.[10] One study found no difference in the capacitance, a measure of skin hydration, of the skin between hemodialysis patients with and without itch.[11] We have studied stratum corneum hydration in hemodialysis patients versus healthy controls and found skin hydration to be significantly lower in the dialysis group. However, we did not show that this finding correlated with itch.[12] In a separate study we also tried to link skin surface pH changes in hemodialysis patients to itch severity, which was unsuccessful.[13] Of note, however, we were able to show skin surface pH levels to be significantly higher in hemodialysis patients at all body sites. A direct correlation has been demonstrated between impairment of barrier function with elevation in skin surface pH.[14,15] Therefore, this finding provided evidence of an abnormal stratum corneum barrier function in dialysis patients. In a recent study we performed on assessing skin barrier integrity in uremics with end stage renal failure on dialysis, we found a significant impairment in barrier integrity (unpublished results). Although no correlation was found between abnormal barrier integrity and itch, several patients with severe itch had significant impairment in their barrier integrity. Future studies will assess possible correlation between this barrier abnormality and severe itch. Kato et al. studied stratum corneum water content using high-frequency conductance measurements with a surface hygrometer.[16] In addition, the study also assessed the resorption (water uptake) and desorption (water loss) before and after dialysis treatments.[17] Despite these sophisticated approaches, Kato et al. were unable to demonstrate a correlation between skin xerosis and itch.[16] Recently, a clinical study did report subjective evidence of a significant connection between intensity of xerosis and intensity of itch. They reported more hemodialysis patients with moderate to severe dry skin had itch than the patients on hemodialysis with mildly dry skin.[18]

12.2.4 Human Immunodeficiency Virus (HIV) Infection and Xerosis

Xerosis with itch is present in more than 20% of patients with HIV infection, and most commonly is localized on the lower extremities.[19] One study revealed the cutaneous neural tissue density within the papillary dermis and epidermis to be significantly decreased in HIV infected patients versus healthy controls.[20] The study also reported significant differences in neuropeptide concentrations within HIV infected individuals. For example, calcitonin gene-related peptide (CGRP) was reduced in the epidermis in both the upper arms and legs, whereas substance P was found to be reduced only in the upper arms of HIV patients with itch. Dry skin and itch can also be induced iatrogenically via commonly prescribed medications for HIV such as indinavir etc. The standard in HIV treatment involves protease inhibitors, such as indinavir, which have been shown to induce dry skin and itch in over 40% of patients.[21]

12.3 PATHOPHYSIOLOGY OF ITCH IN XERODERMA

The pathophysiology of dry skin involves a complex cycle of interactions beginning ultimately with an alteration in the structure of the stratum corneum. This initial defect can lead to subsequent abnormalities in cell proliferation, keratin expression, surface lipid deposition, pH levels, cytokine concentrations, and water metabolism.[22] Any one of the aforementioned abnormalities could serve as the nidus of itch stimulation. A recent study in mice provided new insights into the mechanism of itch induction in dry skin. Miyamoto et al. artificially disrupted the stratum corneum barrier by the use of either tape stripping, 1% sodium lauryl sulfate, 1:1 mixture of acetone:ether, or plain water followed by 1:1 acetone:ether. The study involved applying one of these techniques to the rostral back of mice, and observed if the decrease in barrier function had some correlation with itch induction. Interestingly, only the mice pre-treated with water and followed by acetone:ether were noted to have increased scratching behavior. In addition, this was also the only group with a significant increase in TEWL. This report points to the properties of water and their effects on the stratum corneum as a possible culprit in itch elicitation. Water can remove the intrinsic surface lipids that serve to keep the stratum corneum hydrated and prevent water loss. In addition, water can also cause transient swelling of the stratum corneum followed by drying out of the surface layers. A possible mechanism for itch stimulation may rest in the epidermal C nerve fibers depolarizing in response to these structural changes (i.e., swelling and shrinking) within the stratum corneum.

The role of mast cells and histamine inducing itch remains unclear in dry skin. It has been shown that histamine concentrations increase 48 hours following acetone treatment in a dry environment.[23] A subsequent study demonstrated an increased number of mast cells and histamine levels in the dermis of hairless mice in response to low environmental humidity.[46] The authors did not examine a relationship between scratching behavior with the increase in mast cells and histamine. Miyamoto et al. used the mouse model treated with water followed by 1:1 acetone:ether to see if they could demonstrate an increase in mast cell number or degranulation; however, they found no difference.[24] Furthermore, they performed the same study on mast cell deficient mice and were able to induce a similar scratching behavior, which suggests that mast cells may not play a definite role in the mechanism of itch in dry skin.

12.4 CROSS-TALK BETWEEN STRATUM CORNEUM AND NERVOUS SYSTEM — A POSSIBLE MECHANISM

Nojima et al. recently demonstrated an increase of scratching behavior and scaly skin in rats treated with water followed by 1:1 acetone:ether. In addition, this report also revealed an increase in Fos-like immunoreactivity within the superficial dorsal horn of these rats. This immunoreactivity serves as a marker of the nerves that are directly stimulated by impairment in barrier function.[25] A correlation

between the increase of immunoreactivity in the lamina I spinothalamic tract and duration of scratching was clear. These results are consistent with former reports of neural fibers in lamina I that are histamine specific in cats and humans.[26,27] These neurons have also been associated with patients suffering from chronic itch that responded to iontophoresis of histamine.[28] This study clearly shows that a cross-talk between the stratum corneum and peripheral as well as central nervous systems exists. This cross-talk is further validated by studies demonstrating that *mu*-receptor opioid antagonists, which are well-known to inhibit itch in the central nervous system, are capable of suppressing itch in the mouse model treated with water + 1:1 acetone:ether.[29,47] Bigliardi et al. used confocal microscopy to demonstrate *mu*-receptor presence within the stratum granulosum.[30] This reveals that keratinocytes may use opioid receptors as another method of cross-talk with itch neural fibers.

With the recent exploration into nerve fiber increases in areas of barrier damage, offer another mechanism of itch transmission in dry skin. Takamori et al. showed that the skin of patients with xerosis had a high density of intraepidermal fibers.[31] Furthermore, studies in rats showed not only an increase in nerve fiber density, but also a significant increase in the expression of nerve growth factor (NGF) in the epidermis. This report offers a mediator, NGF, of neural proliferation; and more specifically offers a mechanism of increased C fiber elongation and penetration in the epidermis of barrier impaired skin. It should be noted that these findings contradict results shown in HIV xerosis, where patients have a global reduction in the nerve fiber density. An explanation for these discrepancies might be that different neuromediators are involved, since NGF has not been found elevated in HIV xerosis to date.

Miyamoto et al. have also demonstrated in the dry skin and itch mouse model (water + acetone:ether treated) that the scratching response can be inhibited by the use of atropine, a nonspecific muscarinic acetylcholine receptor (mAChR) antagonist, and 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP), an M_3 mAChR antagonist.[32] They further showed that M_1 and M_2 mAChR antagonist were not able to inhibit the scratch response. This report suggests the role of acetylcholine, and the M3 specific receptor as a potential player in dry-skin-associated pruritus. In addition, skin biopsies in human subjects with atopic dermatitis were found to have increased levels of acetylcholine compared with normal controls, which suggests that abnormal concentrations of neurotransmitters may also be involved in itch secondary to xeroderma.[33]

Serine proteases like stratum corneum chymotryptic enzyme (SCCE) have been shown to induce pathologic changes in the barrier function causing hyperkeratosis and severe itch.[34] A mouse model has been created with hyperexpression of the SCCE gene; the phenotype reveals dry skin with chronic pruritus. Elevations in epidermal SCCE have also been demonstrated in patients with psoriasis and atopic dermatitis.[35] Other mediators, which may be involved in eliciting itch in dry skin, include tryptase (another serine protease), cytokines (i.e., tumor necrosis factor-alpha), and interleukins.[36] One study reported tryptase to be elevated four times the normal range in patients with atopic dermatitis; and further studies revealed a specific tryptase receptor, proteinase-activating receptor 2 (PAR2), to be present on afferent nerves and keratinocytes with increased expression within the epidermis.[37,48] It is possible, in light of these findings, that serine protease elevations secondary to barrier perturbation are responsible for itch fiber activation in dry skin.

Several neural receptors have been isolated recently within the epidermis of humans and mice. Many of these receptors, such as vanilloid receptor 1, are associated with ion dynamics in the peripheral nervous system.[38] Since skin barrier function is also predicated on ion gradient stability,[15,39] these receptors might be inducing transmission of itch related to barrier impairment.

12.5 THE ROLE OF MOISTURIZERS IN ITCH AND DRY SKIN

Moisturizers and emollients have been used for years in patients with dry skin with some relief in pruritus. Moisturizers are one of the gold standards of treatment in atopic dermatitis, the hallmark

of itchy dry skin.[40] Moisturizers' mechanism of action involves retention of water in the stratum corneum and providing an exogenous barrier to prevent transepidermal water loss. However, the antipruritic mechanism of this treatment remains unclear. Moisturizers containing salicylic acids are known to exert anti-pruritic effects with an uncertain mechanism.[41] Moisturizers such as urea, glycerol, and lactic acid promote desquamation and corneodesmolysis. In dry skin, it is likely that abnormal retention of these cohesive proteins coupled with reduced hydration will alter the mechanical properties of the stratum corneum. This alteration may lead to the stimulation of underlying nerve fibers in the epidermis.

Importantly, not all moisturizers provide the same effect in restoration of the barrier function. Certain lipid mixtures or an inadequate concentration of physiologic lipids actually have been demonstrated to inhibit barrier restoration.[42,43] Newer ceramide-dominant emollients have been developed in efforts to restore the intrinsic physiologic lipid concentration of the skin. One type of ceramide-dominant emollient was shown to significantly improve the overall severity of atopic dermatitis and demonstrated correction of transepidermal water losses in these patients.[44] Unfortunately, studies using ceramide-dominant emollients for patients with atopic dermatitis did not use itch improvement as an endpoint. However, these types of moisturizers likely have a role in the improvement of itch associated with dry skin.

In addition to using moisturizers to improve barrier function with hopes of itch reduction subsequent to this improvement, another approach is to formulate topicals containing both moisturizers and anti-pruritics. Some common topical pruritics currently being used with success are pramoxine and polidocanol.[1,45] This approach has been used extensively internationally, but remains underutilized in the United States. Studies using ceramide-dominant emollients compounded with anti-pruritics would be of interest.

12.6 CONCLUSION

The association between primary and secondary causes of dry skin with pruritus is well known. The mechanism of itch transmission as a result of dry skin and barrier impairment is not as clear. However, a large body of evidence suggests that a cross-talk exists between the stratum corneum and nerve fibers in the epidermis. This cross-talk through an array of possible mediators is the likely process of itch transmission to the central nervous system. Future studies focused on this interaction will help shed light on the mechanisms of itch stimulation in dry skin. Currently, the treatment of itch using moisturizers is limited. Newer ceramide-dominant emollients and concomitant moisturizer and anti-pruritic formulations offer promise. Future investigations may offer specific topical targets and greatly improve the efficacy of topical therapy.

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13 Effects of Moisturizer in Psoriasis

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CONTENTS

13.1 INTRODUCTION

Psoriasis is universal in occurrence. It is a disease of the skin characterized by variable clinical features. The cutaneous lesions are usually so distinct that a clinical diagnosis is easy to make. Psoriatic lesions are classified as erythrosquamous, which indicates that both the vasculature and the epidermis are involved.[1]

Psoriasis is a chronic disease with hyperproliferation of the epidermis and inflammatory reactions of the dermis and epidermis. Psoriasis is characterized by an elevated turnover rate of keratinocytes. The duration of the cell cycle is shortened. Inflammation is characterized by the release of cytokines and an expression of CD4+ cells in psoriatic lesions of affected patients. Scaling marks the clinical feature associated with hyperkeratosis, pruritus, inflammation, and stratum corneum dryness.

The onset of psoriasis constitutes a lifelong treat. The different treatment modalities of psoriasis do not offer cure to the patient. Only disease control or suppressive therapy is possible. The available treatments are intended to minimize the development of skin lesions and the associated symptoms.[2] The aim of any treatment should be the decrease or remission of scaling, pruritus, inflammation, burning, and dryness. The classical treatment is of topical nature including dithranol, coal tar, keratolytical agents, and emollients. Photo-chemotherapy with systemic PUVA, bath PUVA and cream PUVA, photo-therapy with classical UVB-light (wavelength: 300 to 320 nm) have shown to be effective. Methotrexate, etretinate, fumaric acid, and recently biologicals (Efalizumab, Alefacept, Etanercept, Infliximab) have shown their efficacy especially in severe cases.[3–9] Topical therapies are adequate in patients with limited plaque psoriasis or less than 20% body surface area involved. The agent of choice depends on the affected anatomical site, patient preference, cost of medication, likelihood of remission, and possible side effects. In some cases a combination therapy with more than one medication may be indicated.[2] The aim of the present chapter is to analyze the actual knowledge on moisturizing agents in the topical treatment of psoriasis. Psoriasis requires a lasting, stabilizing, stage-adjusted topical treatment. A main component of this treatment in a complete therapeutical concept consists in an adjuvant basic therapy with oil baths and emollients. The most important indications of emollients and moisturizing agents are an adjuvant therapy of classical

psoriasis treatment modalities and the supportive treatment in relapse free phases. Very mild forms of psoriasis should be treated with compounds showing low side-effect rats and good cosmetic acceptance.

For topical therapy Greaves et al.[10] scored emollients *lowest*, taking into account:

- Relapse rate
- Side effects
- Cosmetic acceptance
- Efficacy

followed by keratolytical agents, coal tar, dithranol, and corticosteroids. For the adjuvant therapy of mild cases of psoriasis a low risk rate (side effects, cosmetic problems) and no necessity for a strong and rapid efficacy is required. These requirements are met by emollients, moisturizing and keratolytical agents reducing scaling and subjective discomfort and inducing a better hydration of the stratum corneum. Altered structure and function of the skin measured by increased transepidermal water loss (TEWL), dysfunction of bilamellar barrier lipids, impaired skin permeability and skin roughness can be improved, releaving clinical symptoms and decreasing relapses.[11] Therapeutic moisturizers help to maintain hydration and overall integrity of the stratum corneum.[12]

A second indication for keratolytical and some moisturizing agents (e.g., urea) is the penetration enhancement of topically applied antipsoriatic drugs (e.g., salicylic acid in the dithranol and coal tar treatment or corticosteroids). This may result in an economical benefit.[11]

13.2 EFFECTS OF MOISTURIZER AND KERATOLYTICAL AGENTS IN PSORIASIS

13.2.1 Salicylic Acid

Since the beginning of the 20th century salicylic acid is known to exert a keratoplastic effect. Salicylic acid is widely used as a keratolytic agent in the treatment of hyperkeratotic dermatoses, for example, psoriasis.[13] It is mainly used in concentrations of 0.5 to 60% in almost any vehicle. As mechanism of action for topical salicylic acid desolution intercellular corneodesmosomes resulting in corneocyte desquamation, stratum corneum hydration, corneocyte swelling, and subsequently stratum corneum softening have been proposed.[13] Salicylic acid is most beneficial in extremely thick or scaly psoriatic plaques.[2] It is the most effective of the known keratolytics. Several over-the-counter medicated shampoos and scalp solutions aimed for treatment of the scaly scalp contain salicylic acid. Furthermore compounded ointments with salicylic acid are helpful for localized psoriasis.[13] Moncorps reported in 1929 about different penetration properties of salicylic acid from different vehicles.[14] The concentration does not only depend on the concentration within the same vehicle but also on the type of ointment.[14] The resorption rate of salicylic acid on psoriatic lesions is higher with a faster and longer resorption than on the skin of healthy subjects.[15] The resorption rate also depends on the severity of the inflammation.[15] But the liberation of salicylic acid from different formulations does not correlate with the penetration rate into the skin.[16] An additional study of the same group showed a dose-depending percutaneous absorption of salicylic acid *in vivo*.[17] In contrast to the antihyperplastic properties of salicylic acid on pathological hyperproliferation of the epidermis, a promotion of the epidermopoiesis in normal guinea pig skin has been shown with a 1% salicylic acid–acetone–ethanol solution.[18] The mitotic index rose by 17%, epidermis thickness was increased by 40%, and the thickness of the deep epidermis by 19%.[18] Pullmann et al. in contrast did not find a change in the proliferation rate of psoriatic epidermal cells in humans in an autoradiographic study.[19] Roberts et al. however could show a reduction of stratum corneum cell layers after three weeks.[20] The keratolytic effect was visualized by surfometry and scanning

electron microscopy.[21] Huber and Christophers for a 50% salicylic acid solution could show that corneocytes did not change their morphology while the intercellular structure was altered.[22] This treatment resulted in a desquamation of the corneocytes. *In vivo*, with the silver nitrate staining technique, Nook could prove a keratolytic effect for the combination of a water-soluble ointment containing 5% salicylic acid and 10% urea in comparison to 5 and 10% salicylic acid alone in petrolatum.[23] The combination therapy was as effective as 10% salicylic acid, and significantly more effective than a 5% salicylic acid formulation. The keratolytic effect of salicylic acid 6% in an isopropyl solution has been shown with the cantharidin blister method.[24] Going et al. reported the successful treatment of scalp psoriasis with a salicylic acid gel with a negligible systemic absorption of salicylic acid.[25] The negative interaction of dithranol and zinc oxide in pastes could be partly inhibited by the addition of salicylic acid.[26] The addition of salicylic acid to dithranol formulations improves the clinical efficacy of dithranol due to the antioxidant properties of salicylic acid.[27]

Salicylic acid can be helpful as a monotherapy. Witman reported that it is most commonly used in combination with corticosteroids, enhancing their penetration. Concentration of salicylic acid used for this purpose is 2 to 10%.[2] Such combinations require compounding by a pharmacist and carry the risk of imprecise formulations that are potentially unstable, unsafe, or ineffective.[2]

The major risk resulting from topical treatment of psoriasis with salicylic acid is the potential chronic or acute systemic intoxication with the symptoms of burning of oral mucosa, frontal headache, CNS symptoms, pH deviation (metabolic acidosis), tinnitus, nausea, vomiting, and gastric symptoms.[28–30] These symptoms may occur in topical treatment of large body surfaces, especially in children.[31–33] Even lethal cases have been reported.[34,35] Therefore, a concentration higher than 10%, and an application on larger surfaces especially in children are not suitable. Salicylic acid should not be applied to more than 20% of the body surface area.[13] It should be noted that some topical treatments of psoriasis such as calcipotriol are inactivated by salicylic acid.[36]

13.2.2 Urea

The moisturizing effect of urea in dry and scaly skin conditions is widely studied and accepted.[37–40] Urea is known to exert a proteolytical, keratolytical, hydrating, hygroscopical, penetration enhancing, epidermis thinning, and antipruritic effect.[41] An increased water-binding capacity could be shown under a treatment with a w/o-emulsion containing 10% urea.[42] An increased hydration comparing 10% urea to 5% was not detectable in both o/w- and w/o-emulsions.[43]

In vitro and *in vivo* data showed a decreased DNA-synthesis index with a thinning of the epidermis and a reduction of the epidermal cells.[44] The mechanisms of urea on the epidermis result in an epidermal thinning (approx. −20%), reduction of the cells in the DNA-synthesis in basal layers (approx. −45%) and a prolonged generation time of postmitotic epidermal cells.[45] Recent data suggest that lipid biosynthesis may also be increased by topical application of high concentration of urea.[37] An improved drug liberation of steroids from ointments containing urea has been reported.[46] Furthermore, penetration enhancement for glucocorticosteroids by urea is well-studied.[47–53] Such a penetration enhancement leads to a steroid sparing effect and an increased clinical effectivity of steroid ointments containing urea. The maximum of the steroid penetration is within psoriatic lesions. But it remains still unclear whether the penetration enhancement exert a clinical benefit.

Dithranol in combination with urea is widely used in psoriasis to improve the clinical efficacy, to minimize the dithranol concentration, to achieve the desired effect, to shorten the contact, to get a better hydration of the stratum corneum, and to decrease the proliferation rate of the keratinocytes. Gabard and Bieli showed an increased keratolytical effect of salicylic acid by adding 10% urea.[54] Hagemann and Proksch[55] showed in 10 patients with psoriasis under a 2-week treatment with a 10% urea ointment: increased stratum corneum hydration, a small decrease in TEWL, a reduction in epidermal thickness (−29%), and a decreased epidermal proliferation (−51%). The altered expression of involucrin and cytokeratins as marker for epidermal proliferation was partially reversed.[55]

With topically applied 10% urea ointment, Sasaki et al. showed an improvement of stratum corneum water content, hygroscopicity, and TEWL, in psoriasic patients.[56]

Shemer reported a treatment of scalp seborrheic dermatitis and psoriasis with a 40% urea–1% bifonazole ointment, showing a potential benefit of the combination over bifonazole alone.[57] These authors reported that urea reduces the plaque thickness concluding that this treatment regime is safe, well-tolerated, and effective.[58] Own studies showed that salicylic acid and high-dose urea in five different compounded formulations (German Formulatory NRF) have a keratolytic effect on stratum corneum. The used formulations were an ointment with 20% salicylic acid, an oil containing 10% salicylic acid, a gel with 6% salicylic acid, and ethanol and salicylic acid 6% in an isopropyl solution as well as a paste containing 40% urea. Twenty healthy test persons were investigated. Their stratum corneum was stained with silver nitrate and a photographic developer, and a chromameter was used to determine the amount of discoloration produced by the different study products and drug free vehicles. After 24 h semiocclusive application and 24 h later a significant keratolysis in all areas treated with the salicylic acid containing formulations versus drug free vehicles was found. All formulations were effective, with a significant keratolytic activity measured after occlusion. A special notice was made with oil with 10% urea: here the keratolytical effect was first observed 24 h after occlusion. In this formulation the liberation of urea is retarded.[59]

13.2.3 Alpha-Hydroxy Acids

Alpha-hydroxy acids, for example, glycolic acid or lactic acid, are organic acids present in natural sources such as fruits, wine, and milk. They exert specific and unique benefits on the structure and function of the skin.[60] Alpha-hydroxy acids have been proposed as therapeutic options against exfoliative skin conditions such as psoriasis. They penetrate the epidermal layers, provoking an increase in stratum corneum turnover. The precise mechanism by which alpha-hydroxy acids regulate desquamation is not fully understood.[60] Alpha-hydroxy acids appear to increase cohesion of the corneocytes.[61] Kostarelos revealed synergistic effects between alpha-hydroxy acids and betamethasone lotions in the topical treatment of scalp psoriasis.[62] A combination of a 10% (w/w) glycolic acid with 0.1% (w/w) betamethasone was applied twice daily for eight weeks on the scalp. A synergistic effect between alpha-hydroxy acid and betamethasone on scalp psoriasis was apparent. Furthermore, there was no systemic or topical side-effect experienced by the patients and no irritation was observed.[62]

In an own controlled study we treated 12 psoriatic patients with a glycolic acid lotion 15% versus a 0.05% betamethasone valerate cream.[63] TEWL (Evaporimeter EP1, ServoMed, Sweden), Laser Doppler (Perimed-Periflux, Sweden), and skin color (Chroma Meter CR-200, Minolta, Japan) were taken at baseline and on day 5–10–15 on psoriatic lesions. Erythema a^* value was used for monitoring. The results of the study showed that:

1. The TEWL values decreased significantly within 15 days on both sites, particularly for the corticosteroid treated site ($P < .01$ glycolic, $P < .005$ betamethasone). However, no significant differences in TEWL between glycolic acid and betamethasone could be detected.
2. a^* values decreased significantly during the treatment. Significant differences were found between basal and final readings (glycolic $P < .01$, betamethasone $P < .009$). No significant differences were found between glycolic acid and betamethasone.
3. Laser Doppler values decreased significantly during the study (glycolic $P < .001$, betamethasone $P < .0001$); significant differences appeared at days 5, 10, and 15 between the two products with lower values in the corticosteroid treated site ($P < .05$, .01, and .05, respectively). The results showed significant improvement of TEWL, a^* value, and Laser Doppler after treatment with both products. No significant differences appeared in TEWL and erythema between glycolic acid and betamethasone; on the other hand, a significantly decreased Laser Doppler was recorded in the sites treated with betamethasone confirming

the higher effect of corticosteroid compounds in terms of vasoconstriction and reduction of inflammation. The results were confirmed clinically with a reduction of hyperkeratosis and erythema induced by both treatments. The study shows that AHAs are useful not only in the control of hyperkeratosis, but also in the modulation of keratinocyte proliferation, which occurs in the disorders of keratinization such as psoriasis. AHAs can be regarded as an adjuvant therapy in psoriasis.

13.2.4 ω-Fatty Acids and Psoriasis

It has been shown that oral or topical supplements of eicosapentaenoic acid (EPA) and ω-3 derivatives can decrease, not only skin dryness and scaling, but also the severity of inflammatory skin diseases such as psoriasis.[57,64] ω-3 derivatives can be incorporated into cell membranes. They are utilized as a substrate for phospholipase activity. This may lead to an increase of free EPA, which can be used as a substrate for cyclooxygenase and lipooxygenase activities resulting in an increased production of anti-inflammatory leukotrienes LTB5 and PG3.[65] Abnormal serum fatty acid profiles in Darier's disease, ichthyosis vulgaris, psoriasis, and Sjögren–Larsson syndrome have been reported.[66] Hartop et al.[67] monitored TEWL on different psoriatic plaques treated topically with linoleic acid in comparison to clobetasole.

In an own study we tested topical corticosteroids in combination with 5% lanolic acid.[68] An improvement of barrier function could be detected. Formulations containing ω-3 and ω-6 fatty acids may help in the restoration of barrier properties. Higher efficacy of these products may be achieved by combining different classes of stratum corneum lipids.[68] Escobar et al.[64] showed a clinical improvement of scaling and plaque thickness for topical fish oil compared to the base-treated site in a four week treatment.[64]

In a double-blind, placebo-controlled multicenter study with highly purified ω-3-polyunsaturated fatty acids for topical treatment in psoriasis no statistical or clinical differences between the ω-3-polyunsaturated fatty acid and the placebo-treated lesions were found.[69]

13.3 EMOLLIENTS IN PSORIASIS

Emollients are agents designed to make the stratum corneum softer and more pliant by increasing its hydration. They are the most frequently used products in dermatology.[70] They induce a relative occlusive film that limits evaporation of water from the skin and allows the stratum corneum to rehydrate itself. Three mechanisms of emollients on the hydration of the stratum corneum have been proposed. They can exert a direct hydrating effect by liberating water from the formulation itself. Another mechanism is that the occlusive effect of the formulation can influence stratum corneum hydration and finally they are able to bind water evaporating from deeper part of the skin.[71] Regular use of an emollient or moisturizer is important. Several products are available today, for example, moisturizing creams and ointments as well as bath oils. Creams or ointments are preferable to lotions in psoriatic skin. They tend to be thicker, more occlusive, and therefore more effective.[2] Emollients do not work as a monotherapy and should be used in combination with other therapies. It has been reported, that in chronic plaque psoriasis water-in-oil emollients could be used as a steroid-sparing agent,[72] their capacity of hydrating the stratum corneum leads to enhanced delivery of corticosteroids. The replacement of one of the twice daily application of bethametasone dipropionate treatment by a water-in-oil emollient showed the same efficacy than twice per day application of the same glucocorticoids.[72] In an early study it has been shown, that white soft paraffin may inhibit the development of Koebner response in psoriasis.[73] Finlay reported an effective cream therapy adjunct to dithranol for the treatment of chronic plaque psoriasis.[74] Nola reported that the electrical properties of the stratum corneum change after application of an emollient and that there is also an anti-inflammatory activity of these substances.[70] Witman proposed that patients with psoriasis

should be encouraged to take a daily bath in warm water followed by generalized application of moisturizer containing cream or ointment.[2] A second or third application of a moisturizer during the day might be also beneficial.[2] Tanghetti made an observation study that showed that[75] the use of an emollient or a corticosteroid enhanced the efficacy of tazarotene treatment.

Emollients can cause a few side effects, such as irritant dermatitis, allergic contact dermatitis, fragrance allergy or allergy to other constituents, stinging, acne cosmetica, and pigmentary disorders.[70] The patient acceptance of emollients is generally excellent. An additional advantage of these therapies is the fact that they are inexpensive.

13.4 SUMMARY

In summary psoriasis is a chronic disease and requires lifelong treatment. Treatment options include topical agents, photo-chemotherapy, methotrexate, etretinate, fumaric acid, and biologicals. Topical agents beside dithranol are keratolytical agents and emollients. The most important indications of emollients and moisturizing agents are an adjuvant therapy of classical psoriasis treatment modalities next to supportive treatment in relapse free phases. A second indication for keratolytical and some moisturizing agents is the penetration enhancement of topically applied antipsoriatic drugs. Salicylic acid is widely used as a keratolytic agent in the treatment of psoriasis, mainly in concentrations of 0.5 to 60% in almost any vehicle. It is the most effective keratoltic agent. Different investigations showed the positive effects of salicylic acid and reported of benefits in the treatment of psoriasis. Another effect of salicylic acid is the enhanced penetration of corticosteroids in combinations of these treatments. The major problem in topical treatment with salicylic acid is the risk of an acute or systemic chronic intoxication, so some special features have to be attended when salicylic acid is chosen for topical therapy. Urea has a moisturizing effect that is used in the treatment of psoriasis. It exerts different positive effects on psoriatic skin. Also an improved drug liberation of steroids is known. Urea in combination with salicylic acid or dithranol improves the clinical efficacy and the effects of these therapies. Alpha-hydroxy acids are organic acids that exert specific and unique benefits on structure and function of the skin. Synergistic effects of alpha-hydroxy acids and betamethasone have been reported. AHAs can be regarded as an adjuvant therapy in psoriasis. Emollients are the most frequently used products in dermatology and were designed to make the stratum corneum softer. The regular use of an emollient is important in psoriatic therapy although they do not work as a monotherapy and should be used in combination with other therapies. A water-in-oil emollient can show a steroid-sparing effect and the efficacy of tazarotene was enhanced by use of an emollient. Few authors reported the positive effects of emollients in psoriasis. These agents can cause a few side effects, but the patient acceptance is generally excellent.

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Part II

Formulations

Interactions with the Skin

14 Moisturizers as a Medical, Biological, Psychological, Cultural, and Economic Factor

Gregor B.E. Jemec

CONTENTS

Moisturizers are commonly used to treat both healthy and diseased skin, and therefore bridge the gap between medication and consumer good. Dermatologists routinely recommend them. They are used as prevention of irritant dermatitis, to treat minor skin complaints and as adjuvant therapy in combination with topically applied drugs. This suggests that they are useful in a medical/biological sense.

At the same time, large volumes of moisturizing products are being sold over-the-counter not as therapeutics but as consumer goods. The majority of this market is based on the initiative of individuals and without interfering professional advice. This indicates that the products fulfill a perceived need, and that their role may be more extensive than their seemingly simple composition suggests. The nature of this need is unknown as yet, but it may be speculated that not only commercial, but also cultural and psychological factors are necessary.

It may therefore be speculated that the role of the moisturizer exceeds that suggested by medical evidence and most likely includes nonphysical functions such as cultural traditions and psychological aspects.

14.1 MOISTURIZERS AS MEDICINE

14.1.1 What can we Learn from the Placebo Arm in RCTs?

Randomized controlled trials (RCTs) compare active therapy with placebo. Placebo should be as similar to the active therapy in appearance and feel as to be indistinguishable. This means that RCTs involving topical therapy are compared to the cream base, and the cream base most often has an independent function as a moisturizer. The potential effects of the cream base must be made explicit, as they may influence the efficacy of the product tested. By explicating the effect of the placebo arm of

RCTs it is therefore possible to gain information about the efficacy of moisturizers as monotherapy. The effect of the cream base varies. In a stable, dry, and scaly dermatosis such as psoriasis, active treatment with calcipotriol reduces disease severity as assessed by PASI scores by 56%, whereas the use of the cream base reduces it 35%.[1] Dry skin is also a key diagnostic element in atopic dermatitis and moisturizers are therefore extensively used in this disease. Looking at the placebo-arm in RCTs of topical treatment of atopic dermatitis the placebo effect appears to be in the range of 20%.[2–6] The disease is however also more dynamic, and it waxes and wanes more frequently than psoriasis, which may explain the difference seen between the two disseases.

Looking at the placebo arm of RCTs the data therefore suggest that moisturizers have a small but independent biological/medical effect, which may be estimated at 20 to 35% depending on the underlying condition.

14.1.2 Moisturizers as Preventive Measures and Adjunct Therapy

Moisturizers are commonly recommended as preventive measures and adjunct therapy. The preventive use of moisturizers is established in the treatment of atopic dermatitis and hand dermatitis is established on clinical empirical data. Moisturizers appear to be able to revert some of the barrier deficiencies present in, for example, atopic dermatitis, leading to better management of the disease.[7] The use of moisturizers with high lipid content has also been shown to reduce occupational skin problems in wet industries, providing further empirical evidence of efficacy.[8] Experimental data have questioned that skin susceptibility to sodium laureth sulfate irritancy testing may be increased following application of a moisturizer.[9–11] This would suggest that the empirical observations are influenced by a broader range of factors, which may include nonbiological mechanisms such as, for example, adherence to advice given.

Studies have been conducted of the adjuvant role of moisturizers. Using a moisturizer as an adjunct to active medication of the skin with corticosteroids has been shown to reduce the amount of corticosteroids necessary without affecting the efficacy of the treatment.[12] The mechanism underlying this observation is unclear, but it is suggested that the lipid-content of the moisturizer may play a role.

14.2 THE BIOLOGICAL EFFECTS OF MOISTURIZERS

Specific studies of the clinical effects of moisturizers are obviously hampered by the problem of finding a suitable placebo, and the impossibility of conducting a blinded study. Most studies are therefore longitudinal studies involving specific measurements of anatomical and physiological changes before and after the use of moisturizers. These observations are generally based on studies conducted in human volunteers and using biophysical investigative methods. Although these tests have methodological weaknesses compared to RCTs from a therapeutic point of view, they nevertheless give important information regarding the immediate and prolonged effects of moisturizers.

Moisturizers cause changes in skin hydration,[13,14] skin friction,[15] scaling,[16,17] and mechanical properties.[18–20] Following a single application of a moisturizer a series of changes occur, which reflect the composition of the moisturizer.[21,22] Initial changes appear to relate to the water content of the moisturizer and involve increased evaporation from the skin surface, lowered temperature and softening of the skin. With repeated applications over time changes occur, which are thought to be induced by the lipid phase of the moisturizer. These involve increased hydration (as reflected by electrical changes), reduced scaling, and discrete color changes.[23,24]

The use of specific additives such as alpha-hydroxy acids, urea, or glycerol may strengthen some of these changes.[25–28] The effects of additives can be studied in RCTs and lead to recommendation of specific additives for specific purposes, for example, urea to increase hydration.

Although the general effect is not testable in a RCT there is a considerable body of evidence to support biological effects of moisturizers. In particular the lipidization of the skin appears to influence skin physiology favorably. The use of specific lipids reflecting the composition of naturally occurring skin lipids is however not clearly associated with a superior effect, opening the possibility of secondary changes occurring in the skin following moisturizer application.[29]

14.3 THE PSYCHOLOGY OF MOISTURIZERS

It may be speculated that the actual application of a moisturizer satisfies an atavistic psychological need for physical contact, which is reinforced by the immediate physical effects of the moisturizer. It has been shown that the application of a moisturizer increases the tactile sensations in dry skin, both by lowering the threshold for perception and by allowing better point-discrimination.[30] The use of a moisturizer therefore potentially enriches sensory perception.

These physical aspects of perception are further reinforced by the self-touching involved with application of the moisturizers, which may link directly to basic mammalian psychology. Cognitive mechanisms may therefore also play a role. Touch is one of the stronger senses and the basic importance of touch between individuals has been widely explored in other mammalian species. It appears as a crucial element in the rearing of offspring as well as in social contacts between adults of many species. Common experience would suggest its effects are even more profound in humans where touch plays a strong role in both social and sexual bonding and thus affects many aspects of life. These effects span a wide range of human life.[31,32] Affect and attention in children are modulated by touching, as is the heart rate of adults. Touch by others may lower the heart rate, whereas self-touching increases it. It has been suggested that specific peptides may be related to the effects of touching opening the possibility to study the effects of moisturizer application more objectively.[33]

The role of touching the skin in the definition of self is evident in children, and most likely remain an atavistic reflex in adult life. Holding onto oneself for physical and moral support is a natural part of child development. This is further supported by the increased frequency of self-touching in stressed situations and in apparent accordance with the effects of touch on heart rates. When moisturizers are used this may therefore be speculated to have additional unnoticed psychological aspects, which involve both a sense of security and stress coping. The use of moisturizers may therefore be deeply rooted in both individual psychologies as well as in collective memory.

14.4 MOISTURIZERS AS CULTURE

The earliest records of health behavior suggest that historically human beings have always applied lipids to their skin.[34,35] The reasons have sometimes been medicinal, sometimes cosmetic, but most often the distinction has been blurred as biological understanding was low. Traditional medicine reflects cultural traditions.[36] Topical application of supposedly therapeutic substances is often a large element of traditional medicine, which has continued undiminished through time in popular thought. Early Egyptian medicine, for example, contained extensive directions for the treatment of wounds and the application of ointments.[37] Later "plasters" involving, for example, mustard became an accepted form of treatment of internal diseases such as pneumonia or colds in the population. This form of "classical" medicine is perpetuated in what is now termed "alternative" medicine.

The underlying logic of these treatments is not well described, but it may be hypothesized that simple observations of the skin suggests that it is porous. Without optical aids small holes are visible in the skin, and not only does sweat come out of the skin, but, for example, water or paint is absorbed into the skin causing swelling or lasting discoloration. The skin may therefore naturally be perceived as more porous than we know it to be, and in consequence the application of healing or soothing substances may be thought to cure generalized diseases.[38] What we would now define as medicine

were however not the only substances thought to be absorbed through the skin. Humors thus absorbed included more metaphysical or noble substances as well, for example, the oil used by athletes in classical Greece for cleaning their bodies after competitions. The user was thought to absorb some of the strength of the athlete by using the oil. The concept of percutaneous absorption is therefore culturally not restricted to medicinal purposes, but can even encompass more spiritual values. This ongoing cultural aspect is furthermore most likely reinforced by physical changes in the skin and psychological factors associated with the use of moisturizers or creams applied to the skin.

14.5 MOISTURIZERS AS CONSUMER GOODS

Moisturizers form a prominent part of the skin healthcare market, which currently estimated at US$8 billion. It is a growing market.[39,40] The effect on the economy is however not limited to producers of cosmetics or cosmeceuticals. Moisturizers regularly advertised and as such probably give substantial revenue for all media companies. These creams and lotions therefore form a valuable consumer good which is produced and marketed on an industrial scale, and is subject to the same mechanisms as other consumer goods.

Even the word moisturizer is rooted in marketing. It has almost completely replaced the traditional concept of creams or emollients, although one of the important functions of moisturization is emolliency. It is in essence a marketing concept: Unattractive skin is dry and lacklustre; young and attractive skin is moist and supple. Dry skin needs moisture. Moisture is obviously provided by moisturizers. The heavy emphasis on this chain of reasoning has convinced a large number of women that their skin is dry, although it may not be possible to find physical evidence of a sex difference in parameters of dryness.[41] In addition to any biological effects of the moisturizers, their role as consumer goods ensures that they form a part of our commercial environment and the methods used to promote and market moisturizers therefore directly influences human behavior.

14.6 CONCLUSION

There are many reasons to use moisturizers. There is evidence of biological effects, which justify the medical use of moisturizers. In addition, there are however also possible psychological and cultural aspects to the use of these substances, which may provide an underlying drift toward continued use. The application of a moisturizer involves extensive touching either by the self or by another person. Both these forms of touching have psychological implications, which reinforce the use of moisturizers. Historically creams and emollients have been used not only in a medicinal capacity but as cultural elements. Finally, these many aspects are combined in market forces, which strive to meet demand for moisturization and promote expanded use.

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15 New Methodology to Improve Epidermal Barrier Homeostasis

Mitsuhiro Denda

CONTENTS

15.1 SUMMARY

Several new strategies are available to accelerate skin permeability barrier recovery after injury. Here, I will describe our recent work on improving barrier homeostasis with new reagents and new materials, and discuss the implications for clinical dermatology.

15.2 NEUROTRANSMITTER RECEPTORS ON KERATINOCYTES

As I described in another chapter, epidermal keratinocytes carry a series of receptors, which were originally found in the central nervous system as neurotransmitter receptors. These receptors can be categorized in two groups, that is, ionotropic receptors and G-protein-coupled receptors.

Among the former group, receptors that act as calcium ion or chloride ion channels play a crucial role in epidermal permeability barrier homeostasis. Topical application of calcium channel agonists delays the barrier recovery, while antagonists accelerate barrier repair.[1,2,3] Topical application of chloride ion channel agonists accelerates the barrier recovery.[2,4] The results of our studies are summarized in Table 15.1.

The G-protein coupled receptors modulate intracellular cAMP level, which plays a crucial role in epidermal barrier homeostasis.[5] Increase of intracellular cAMP in epidermal keratinocytes by topical application of forskolin delays barrier recovery, while cAMP antagonists accelerate the barrier recovery. Activation of dopamine 2-like receptors (manuscript in preparation), melatonin receptors, or serotonin receptor (type 5-HT1) decreases intracellular cAMP and consequently accelerates the barrier recovery (Figure 15.1), while activation of adrenergic β2 receptors increases intracellular cAMP and delays the barrier repair.[6] Barrier disruption induces an increase of the intracellular cAMP level. Thus, topical application of agonists of receptors that reduce intracellular cAMP accelerates the barrier repair. Our results are summarized in Table.15.1.

Many agonists or antagonists of neurotransmitter receptors are used clinically to treat nervous disorders. Some of them might also be effective medicines for skin problems.

TABLE 15.1
Effects of Neurotransmitter Receptor Agonists and Antagonists on Skin Permeability Barrier Recovery

Ionotropic receptors	Accelerate barrier recovery	Delay barrier recovery
P2X receptor (2)	Antagonist	Agonist
NMDA receptor (9)	Antagonist	Agonist
Cholinergic receptor (Nicotinic) (5)	Antagonist	Agonist
GABA(A) receptor (3)	Agonist	—
Glycine receptor (5)	Agonist	—
G-Protein coupled receptors		
Adrenergic β2 receptor (4)	Antagonist	Agonist
Dopamine 2-like receptor	Agonist	Antagonist
Serotonin receptor	Agonist	—
Meratinine receptor	Agonist	—

The reference number is given in parentheses. – : No effect or not examined.

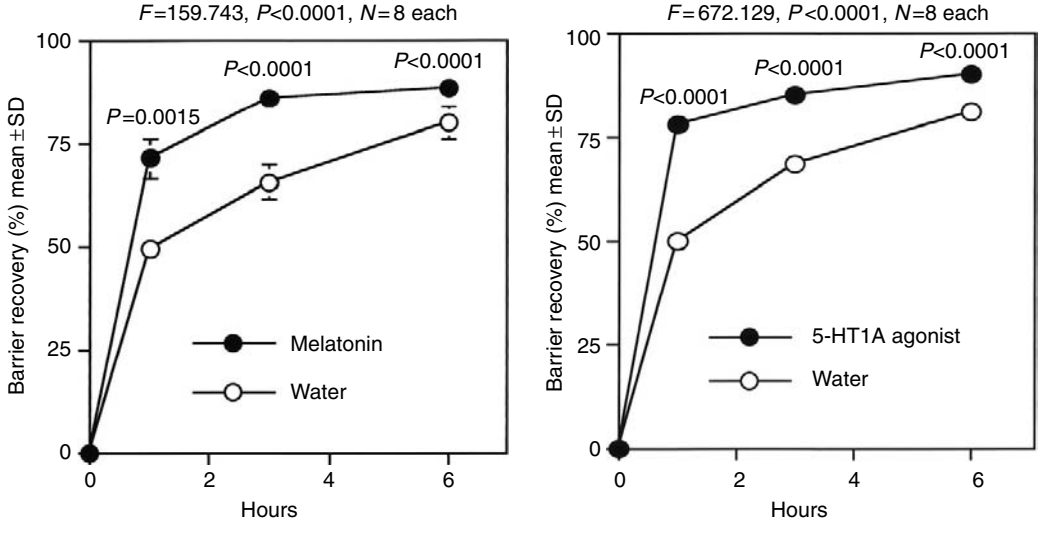

FIGURE 15.1 Topical application of serotonin (5HTA) receptor agonist or melatonin accelerates skin barrier recovery after barrier disruption.

15.3 MATERIALS THAT INDUCE ELECTRIC POTENTIAL ON THE SKIN SURFACE

We previously demonstrated that application of a negative electric potential on the skin surface affects the ion gradient in the epidermis and accelerates lamellar body secretion and skin barrier recovery.[7]

In the field of electrochemistry, several materials are known to induce a stable electric bilayer when attached to another material, without any power supply, so we looked for suitable materials to induce electric potential on the skin surface. Here I will describe our recent work on ionic polymers and barium sulfate as examples, because they are used as ingredients for cosmetic products.

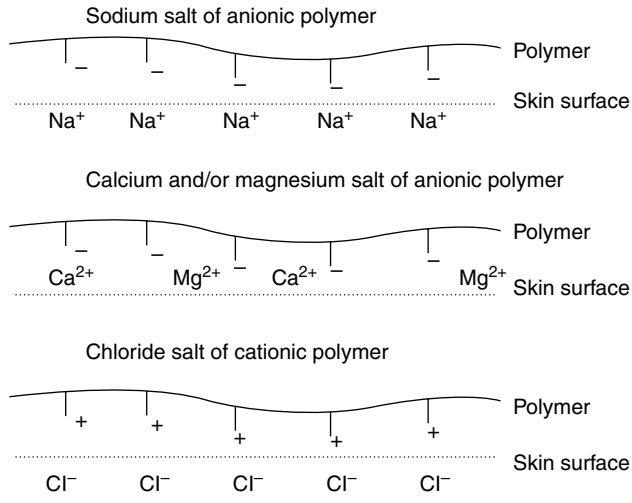

FIGURE 15.2 Schematic illustration of electric bilayers induced by ionic polymers on the surface of the skin. When the counter ion of the anionic polymer is sodium, the skin surface is negatively charged because of the diffusion of sodium ions. Calcium and magnesium ions do not diffuse as easily as sodium ions. Thus, when the counter ion is calcium or magnesium, an electric bilayer does not form. When the counter ion of a cationic polymer is chloride, an oppositely charged electric bilayer is induced, and the skin surface is positively charged.

15.3.1 Ionic Polymers[8]

Topical application of an ionic polymer forms a diffusion electric double layer on the surface of the skin. We evaluated the effects of topical application of ionic polymers on the recovery rate of the skin barrier after injury. Application of a nonionic polymer did not affect the barrier recovery. Application of sodium salts of anionic polymers accelerated the barrier recovery, while that of cationic polymers delayed it. Topical application of a sodium-exchange resin accelerated the barrier recovery, but application of a calcium-exchange resin had no effect, even when the resins had the same structure. Application of a chloride-exchange resin delayed barrier recovery. Thus, topical application of ionic polymers markedly influenced skin barrier homeostasis (Figure 15.2).

15.3.2 Barium Sulfate[9]

Barium sulfate is a stable inorganic material that has been used for contrast media or cosmetic products because of its stability. Since a negative external electric potential accelerates skin barrier repair after barrier disruption, we hypothesized that topical application of barium sulfate would affect the skin barrier recovery rate, depending on the ζ potential.

We demonstrated that barium sulfate particles in aqueous solution have different ζ potentials depending on their surface structure. There was a significant correlation between the barrier recovery rate and the ζ potential of barium sulfate applied topically (Figure 15.3). Barium sulfate with a negative ζ potential significantly accelerated barrier recovery, while barium sulfate with a positive potential did not accelerate, or even delayed, barrier repair (Figure 15.4). Barium sulfate particles with a negative potential had a different appearance from that of barium sulfate particles with a positive potential. The distribution of calcium in the epidermis was also influenced by the polarity of ζ potential. In summary, topical application of barium sulfate with a negative ζ potential prevented epidermal hyperplasia induced by barrier disruption under a dry environment.

These findings suggest a new pharmacological approach toward altering barrier function or epidermal hyperplasia in healthy and diseased skin with inorganic particles.

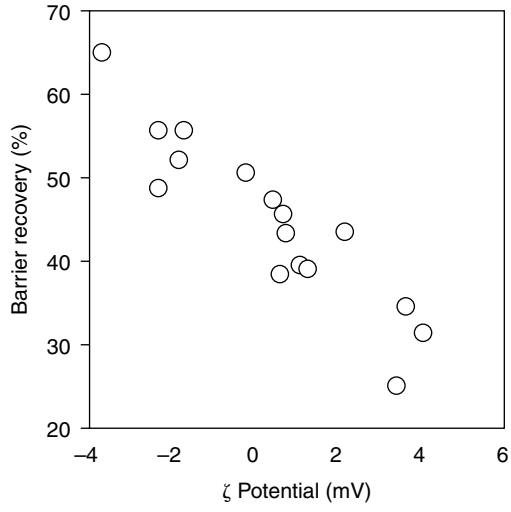

FIGURE 15.3 Correlation between the barrier recovery 2 h after tape stripping and the ζ potential of barium sulfate. There is a significant correlation between the barrier recovery rate and ζ potential.

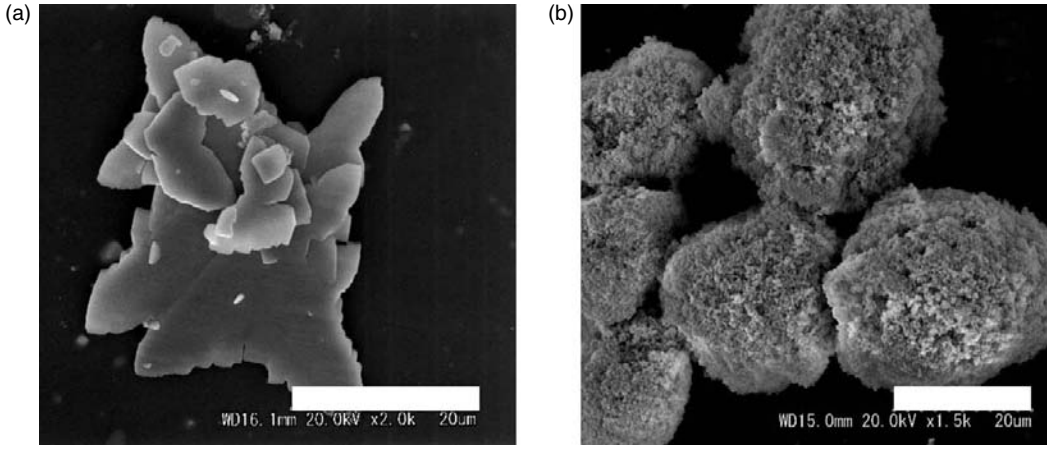

FIGURE 15.4 Scanning electron microscopic observation of two different forms of barium sulfate. In (a) flat board structures are observed (bar = 20 μm). This type has a negative ζ-potential and accelerates skin barrier recovery. (b) shows disordered structure (bar = 20 μm). This type has a positive ζ potential and does not accelerate barrier repair.

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16 Outside and Inside Skin pH

Izabela Buraczewska

CONTENTS

16.1 INTRODUCTION

Nowadays, the importance of pH is considered with respect to many aspects of our lives. Its influence on the health and well-being is often mentioned in a sense of body's internal environment, for example, the impact of pH of food on the digestive system. However, concerns about pH deal not only with the inside of the body, but also with its most external organ, the skin.

The pH-value of the skin surface has been investigated by many researchers since the end of nineteenth century. The acidic nature of the skin surface was first mentioned by Heuss in 1892.[1] In 1928, Schade and Marchionini[2] used the term "acid mantle of the skin" (säuremantel) for the first time. Since this, that phenomenon has become of great interest, and many studies trying to explain its function and the mechanism of formation have been carried out. Nevertheless, many questions remain unexplained.

Considerations about skin pH can be divided into two parts: the outside and inside skin pH. The former applies to the skin surface and the latter to the pH-profile across the epidermis. In the following chapter, the inside and outside skin pH is addressed, as well as its importance and influence on skin barrier function. A short review of methods used to measure the pH is presented as well.

16.2 APPLICABILITY OF THE TERM "SKIN pH"

According to the International Union of Pure and Applied Chemistry (IUPAC), pH is defined as the negative logarithm (base 10) of the activity of hydrogen ions (see Equation [16.1]), and it is

recommended to apply this definition to diluted aqueous solutions ($\leq$0.1 mol/kg).[3]

$$\mathrm{pH} = -\log_{10} a_{\mathrm{H}}, \tag{16.1}$$

where a_{H} is the activity of hydrogen ions.

Considering the definition presented, the accuracy of pH measurements of the skin is questionable. There are two inconsistencies emerging from the definition of pH. First, the skin, especially the epidermis, is not a diluted aqueous solution. Moreover, various residues located on the skin surface may influence the readings, if conducted by devices not designed for existence of many different substances.

The second problem applies to the performance of readings itself. In many cases, when the pH of the skin surface is measured, a small amount of water is applied on the skin before the measurement. Hence, it is not the pH of the skin surface that is measured, but the pH of the aqueous solution on the skin surface.

Both issues are widely discussed by Rieger,[4] who wrote that what is actually measured is "pH of the (extractable) water-soluble constituents of skin." Due to that problem, Rieger proposed to call that measured value not "pH of the skin" but "pH on the skin" or "the apparent pH." That issue is also raised and described by Parra and Paye in guidelines of European Group on Efficacy Measurement of Cosmetics and Other Topical Products (EEMCO).[5]

Despite mentioned considerations, it is widely accepted to use the terms "skin pH" or "pH of the skin" for describing the pH measured on the skin surface by various types of methods. Those expressions are also used in the rest of this chapter, in a meaning of pH-values obtained by any measurement technique. However, it is important to realize, that measured pH of the skin is not the pH in a precise analytical–chemical sense.

16.3 MEASUREMENT METHODS

Methods used to measure skin pH are, from the analytical point of view, of the same type as those for determining the pH values in aqueous solutions. The earliest studies about skin pH were conducted with colorimetric methods, using indicators that change color with pH. This method is complicated, involving collection of indicator solution from the skin. It was simplified by usage of indicator-impregnated strips (foil-colorimetry).[2,5,6]

Potentiometric methods are easier to use and are nowadays the most frequently utilized to measure outside skin pH. They are also used to establish pH in deeper layers of the epidermis, by first exposing them, for example, by tape stripping.[7] The most common potentiometric method is using the hydrogen ion-selective glass electrode with internal reference electrode, which is often called just "glass electrode."[6,8] The electrode is often planar-shaped to make it more easily applied on the skin.[9]

There are also new methods suggested recently, but they are not yet commonly employed.[5] One of them involves ion-selective field effect transistor as a sensor, which requires smaller measurement area compared to the glass electrode.[10] Other possible methods are electron spin resonance imaging and confocal microscopy.[5,11,12] They require treatment of the skin with an indicator substance, which penetrates into the epidermis and allows the pH to be detected in several layers simultaneously.[12]

When measuring skin pH, several issues have to be remembered. First, the interpretation of results has to be done carefully, taking into consideration points described earlier concerning the applicability of the definition of pH on measurements on the skin. Second, one has to realize that there are many substances present on the skin surface like sebum and sweat, as well as material of exogenous origin, for example, cosmetic products, which all can influence the readings.

16.4 FORMATION OF THE pH-GRADIENT

Skin pH is regulated by many substances, shifting pH into lower values by their proton-donating properties. Outside pH is influenced by various substances secreted to the skin surface, like sweat, sebum, and Natural Moisturizing Factor (NMF). Those secretions of eccrine and sebaceous glands contain various acids, like lactic acid, butyric acid, pyrrolidone carboxylic acid (PCA), amino acids, and free fatty acids. Additionally, ingredients of exogenous origin such as metabolites of cutaneous microflora (e.g., free fatty acids) and cosmetic products, can be present.[5,13–15] However, it seems that outside skin pH depends mainly on processes taking place in deeper layers of the epidermis.[13]

The formation of pH-gradient inside the epidermis involves several mechanisms and perhaps not all of them are discovered yet. Currently, it is believed that stratum corneum acidification is regulated by the cooperation of three endogenous mechanisms: histidine-to-urocanic acid pathway, phospholipids-to-free fatty acids pathway, and membrane antiporters.[13] The two former mechanisms consist of formation of acidic substances: urocanic acid and free fatty acids, which have proton-donating properties. In histidine-to-urocanic acid pathway, urocanic acid is formed by the hydrolytic enzyme histadase from histidine, which is obtained from hydrolyzation of filaggrin. This pathway is believed to be the most important mechanism acidifying stratum corneum and also has importance for other metabolic processes in the skin.[13,16] The next mechanism consists of formation of free fatty acids from phospholipids and is mediated by other hydrolytic enzymes — secretory phospholipases.[13,15] The last mechanism involves membrane antiporters (NHE1), which extrude protons in exchange for sodium. It is responsible for acidifying the interface between stratum granulosum and stratum corneum and/or in lower stratum corneum.[13,17]

16.5 OUTSIDE SKIN pH

Outside skin pH has been studied extensively since the first publication describing its acidic properties.[1] Methods used to determine the pH have changed with time, but results are comparable, showing that pH on the surface of healthy, undamaged skin of an adult is slightly acidic, about 5, varying from 4 to 6.[5,14,18–20] It is important to realize that it is impossible to assign only one pH-value to the skin. Variations in outside skin pH appear to depend on many endogenous and exogenous factors such as anatomical site, sex, age, race, circadian rhythm, temperature, humidity, etc. However, studies published so far, often show contradictory results, and it is still not established which factors really have impact on pH and which do not. Few of those factors are described later. More detailed summaries can be found in reviews by Parra and Paye,[5] Fluhr and Elias,[13] and Rippke et al.[14]

Among anatomical sites, intertriginous areas (e.g., axillia) seem to have the highest pH of all body surfaces, having pH shifted toward neutral or even alkaline.[6,13,21,22] Fluhr and Elias[13] proposed that this is caused by decreased urocanic acid formation due to higher humidity of those regions or by sebaceous/eccrine gland distribution. The differences between other anatomical sites are not so clear. For example, measurements conducted by Fluhr et al.[23] on 14 volunteers did not reveal regional differences between abdomen, back, forehead, lower leg, and forearm. In another study, on 574 adults, pH on cheeks was found significantly higher than on forehead.[18]

The difference in skin pH between sexes is also questionable. Few studies show a difference, with men having lower pH than women,[24–27] while others do not.[18,28] It is suggested, that possible pH difference between men and women can be due to sex-hormones, which influence skin barrier function.[13]

Age is an important factor. Outside skin pH changes during the course of life. Infants have neutral or slightly alkaline pH of the skin surface just after birth. It starts to decrease from the first day of life, but it takes a month to obtain pH of about 5.[13,29–34] pH remains almost constant during childhood and adult life and increases in elderly.[13,18,35]

Few studies show that pH of some body areas is influenced also by circadian rhythm.[36–38]

16.6 INSIDE SKIN pH

There is a pH-gradient through the epidermis, changing from acidic values on the skin surface to near-neutral pH of around 7.4 in viable epidermis.[7,11,39] The profile of this gradient from the outside in, has been presented as increasing in a sigmoid way, preceded by an initial slight decrease of pH in the upper layers of stratum corneum.[7,39] Resent research shows a more detailed picture. After the initial increase of pH, there is a dip to acidic values in the interface between stratum corneum and stratum granulosum, but inwards pH increases again, obtaining near-neutral values.[13,40] This profile of the pH-gradient seems to be in accordance with the hypothesis mentioned earlier regarding the formation of low skin pH.

16.7 FUNCTION AND IMPORTANCE OF SKIN pH

Although the acidity of the skin was described long ago,[1,2] its importance and function is still not fully understood. Studies conducted until now reveal the picture of a complex phenomenon, regulated by various mechanisms and fulfilling many different functions.

Since the very beginning, the acidic pH has been linked to skin microflora.[2,41] The acidic pH is supposed to inhibit the growth of pathogenic microorganisms and keep the skin microflora in balance. If the skin pH is elevated, for example, after usage of alkaline soaps, prolonged occlusion, or in skin disorders like atopic dermatitis, the growth of pathogens increases.[42–44]

Recent studies also reveal another important role of skin pH for the barrier function. The pH-gradient is essential for several enzymes located in the epidermis necessary for formation of the skin barrier. Deviation from optimal pH-values can influence their activity, and as a result, abnormal structure and function of stratum corneum may occur.[13,45] One of the pH-dependent enzymes is proteases, responsible for degradation of desmosomes keeping corneocytes together.[39,46–48] Another example is of the enzymes responsible for the formation of lipids necessary for skin barrier formation: ceramides, free fatty acids, and cholesterol.[49] β-glucocerebrosidases and acid-sphingomyelinases are enzymes transforming glucosylceramides into ceramides, and phospholipase A_2 is necessary to obtain free fatty acids.[49–54] The activity of those enzymes is pH-dependent: β-glucocerebrosidases, cholesterol acyltransferase, and one of the acid-sphingomyelinases show higher activity at acidic pH.[55–57] Neutral or alkaline pH is suitable for other sphingomyelinases and phospholipase A_2.[55,58] The importance of pH for activity of mentioned enzymes and therefore for skin barrier, was shown in a recent study on mice. Perturbed skin barrier recovered normally when the skin was exposed to solutions buffered to an acidic pH, while initiation of the recovery was delayed when the damaged skin was exposed to neutral or alkaline pH. This delay in barrier recovery was suggested to be a consequence of a lower activity of β-glucocerebrosidases.[45]

16.8 SKIN DISORDERS AND pH

In some skin disorders, a deviation from "normal" skin pH is observed. The skin of patients with atopic dermatitis has been shown to have elevated outside pH, especially on lesional areas, reaching values even above neutral.[21,43] This can be explained by decreased level of proton-donating substances, for example, urocanic acid and amino acids.[43] Higher pH on the skin surface can facilitate growth of pathogenic micro-organisms such as *Staphylococcus aureus*, which causes problems in patients with atopic dermatitis.[43] Increased pH is also found in children with seborrheic dermatitis.[59]

However, the change of pH occurs not only in the outside skin, but also in a gradient through epidermis as well. For example, in ichtyosis vulgaris, all of the pH-gradient is shifted toward higher values, when in x-linked recessive ichtyosis the effect is opposite.[39] This deviation in pH-gradient has big impact on enzymes located in the epidermis, whose activity is altered.

16.9 MOISTURIZERS, OTHER COSMETIC PRODUCTS, AND SKIN pH

16.9.1 pH of Cosmetic Products

The pH-values of cosmetic products are often stated on the packaging or mentioned in advertisements. Expressions like "pH neutral" or "skin friendly pH" are used, and their role is usually to convince customers about mildness and safety of the product, or its suitability for intended use, for example, low pH of a product for intimate hygiene or sensitive skin.

The majority of cosmetic products, such as creams and lotions — popularly called moisturizers — but also gels, liquid soaps, shampoos, etc., usually do not have extreme pH-values. The first reason for that is the aim to keep their pH similar to the skin pH, in order to avoid irritation. Another cause is to reduce the risk of separation of the product, because extremely acidic or alkaline environment can cause degradation of ingredients. Of course, there are cosmetic products available, with very high or low pH, which can cause irritation, but they are used for special purposes and are not supposed to be in contact with skin for a long time. The examples of such alkaline preparations are those for hair removal or making permanent waves. On the other end of the pH-scale, there are strongly acidic products used for deep skin peeling, for example, based on glycolic acid.

From the literature, little is known about the impact of cosmetic products on skin pH. Skin possesses buffering capacity, which protects it against changes of pH. It has been shown that after application of alkaline preparation, elevated outside skin pH decreases back toward acidic values.[5,14,21] Such change of pH may occur also after application of a cosmetic product. This issue is barely mentioned in case of stay-on products, like moisturizers. Rinse-off cleansing products are investigated more often, in terms of their influence on skin pH and the correlation between their pH and the irritancy potential.

16.9.2 Impact of Stay-On Products on Skin pH

Moisturizers and other similar stay-on products have often pH between 4 and 6. That pH-range is similar to skin surface pH and is often suitable for good physical stability of the cosmetic product. However, there are several moisturizing creams with world-wide acceptance, which have pH of about 7 or even 8, for example, those with stearic acid as the main emulsifier. Also skin protectants based upon zinc oxide often have an alkaline pH.

Stay-on cosmetic products contain ingredients that may affect skin surface pH. Various proton-donating substances are often incorporated into them, serving as pH-adjusters, humectants, or emulsifiers, etc. Sometimes they are the same as those occurring naturally on the skin surface, for example, lactic acid, PCA, amino acids, and free fatty acids. Alkaline substances, for example, sodium hydroxide and triethanolamine (TEA) are often used as well. After application of a cosmetic product on the skin, water and other volatile ingredients evaporate, while other substances stay on the surface and blend with those already present on the skin. As can be concluded from basic chemical knowledge, such application of acidic or alkaline substances may change skin surface pH, depending on the quantity of applied substances, their physicochemical properties, and the buffering capacity of the skin. The question is then, how big that impact is, in which direction pH is changed, and for how long that alteration persists. There is no straightforward answer for those questions, because each cosmetic product can influence skin pH in a different way. The problem of influence of stay-on products on skin pH is very complex and difficult to investigate due to several variables. It has not been studied thoroughly yet, but the growing awareness about skin pH prompts researches to investigate this issue in more detail.

The considerations mentioned earlier also bring up the subsequent questions that wait to be answered, for example, about the influence of moisturizers on pH-gradient inside the epidermis and the activity of enzymes, effect on skin barrier function and skin barrier recovery, or the difference in

impact in case of healthy or diseased skin. One of these issues was investigated in a study, where two moisturizers of two different pHs: one with pH 4.0 and the other with pH 7.5 were applied on skin exposed before to sodium lauryl sulphate (SLS). There was no difference in impact on skin barrier recovery between tested preparations, neither in the early nor in the late stages of the recovery, which suggests that the pH of the studied moisturizers did not have a major impact on the activity of the enzymes responsible for barrier recovery.[60]

16.9.3 Rinse-Off Products and their pH

The effect of various types of rinse-off cleansing products on skin pH has been examined in many studies. There are many types of cleansing products available: liquid soaps, bar soaps, shampoos, cleansing foams, shower oils, etc. Although they differ in their appearance, consistency, foaming properties, or color, they all contain similar ingredients, the most important of them being surfactants, responsible for cleansing properties. There are many types of surfactants available. They exhibit a large variation in the irritancy potential and similarly do cosmetic products containing those surfactants.[61–63]

The ability of cleansing products to change the skin pH in both adults and infants has been investigated. Several studies have shown that usage of alkaline soaps increases the outside skin pH.[64–67] The impact of a long-term usage of an alkaline soap was studied by Korting et al.[64] Outside skin pH of volunteers using the soap repeatedly for few weeks was 0.3 units higher than of volunteers using acidic synthetic detergents. In the same study, short-term effect was studied as well, revealing that skin pH increased directly after washing the skin with both tested products and that increase was significantly higher in case of soap. That elevated skin pH decreased to initial values after about two hours.[64] Another study reported that pH increased 0.45 units when skin was washed with soap of pH 9.5 and slight increase was also found after usage of an acidic product of pH 5.5, as well as after washing the skin only with tap water (0.19 unit).[66] Such results suggest that use of any type of cleansing product may increase skin surface pH, even water. The mechanism behind the impact of cleansing products on outside skin pH is not explained yet. However, it seems that one reason may be that cleansing products remove various substances from the skin surface, among them those responsible for acidification, for example, NMF, lactic acid, free fatty acids, etc.

Similarly as in case of stay-on products, there are several questions waiting to be answered about the impact of pH of rinse-off cleansing products on the skin, its pH, and the skin barrier function. One of the issues investigated was the influence on skin microflora, showing that when skin pH increased after repeated use of an alkaline soap, the count of propionibacteria rose significantly.[64] Moreover, the irritancy properties of cleansing products have often been associated with their pH, but several studies show that there is no direct correlation between those two features.[62,68–70] The reported difference in irritancy potential between cleansers with various pH may depend on the combination of surfactants and their inherent irritating capacity, rather than the pH of the products.[61]

The issue of pH of cosmetic products, their impact on the skin and the consequences of that impact are still not a well-known subject. Understanding of that problem can help not only in the invention of better cosmetic products but also in the avoidance of unnecessary or misleading marketing claims, which often confuse a customer.

16.10 SUMMARY

The knowledge about skin pH has been growing since the last few decades, but there is still much to be discovered. Many issues, for instance, the formation of pH-gradient or influence of various factors like sex or anatomical site on skin pH are still not fully explained. Better understanding of that phenomenon is of great importance for many types of research. In dermatology, it can help in treatment of various skin disorders, especially those connected to altered pH-gradient and impaired

skin barrier function. More information about skin pH is also necessary for research dealing with reconstructed epidermis and percutaneous drug penetration. Moreover, the knowledge about impact of various substances on skin pH would facilitate designing of better cosmetic and pharmaceutical products.

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17 Dry Skin and Use of Proteases

A.V. Rawlings and R. Lad

CONTENTS

17.1 INTRODUCTION

Many researchers consider the stratum corneum (SC) to be a "dead" tissue and most discussions on the SC barrier relate to its permeability characteristics. However, it is largely forgotten that the water lost through the tissue is essential for the functioning of a healthy and biologically very active SC.[1] This imperfect and inbuilt water loss is the key in allowing hydration of the outer layers of the SC in order to maintain its flexibility, but more importantly to provide enough water to allow enzyme reactions that facilitate SC maturation events. One of these events, which is the subject of this chapter, is the aberration of the enzyme-mediated lysis of corneodesmosomes (CD) in the SC (corneodesmolysis) that would normally lead to desquamation of the SC but in this case leads to winter xerosis and the use of topical proteases to treat the condition. The key in precipitating the condition we call "dry skin" or cosmetic xerosis is perturbation of water gradients within the SC.[2] Disruption of the natural moisture barrier leads to reduced proteolysis of key SC structural components called CD (Figure 17.1).[3]

17.2 STRATUM CORNEUM CORNEODESMOSOMES, CORNEODESMOLYSIS, AND DESQUAMATION

The brick and mortar model of the SC was described many years ago but a more complete description of its structure included the "CD," which are modified and specialized desmosomes.[4] These are macromolecular glycoprotein complexes consisting of the cadherin family of transmembrane glycoproteins, desmoglein 1 (Dsg 1), and desmocollin 1 (Dsc 1) together with corneodesmosin (Cdsn). Dsg 1 and Dsc 1 span the corneocyte envelope into the lipid enriched intercellular space between the corneocytes and provide cohesion by binding homeophilically with proteins on adjacent cells. However, within the corneocytes, they are linked to keratin filaments via corneodesmosomal plaque proteins such as plakoglobin, desmoplakins, and plakophilins. Cdsn, after secretion by the lamellar bodies with the intercellular lipids, and certain proteases, becomes associated with the desmosomal proteins just before transformation of desmosomes into CD. Importantly, as these proteins are cross-linked into the complex by the enzyme transglutaminase, their controlled disruption must occur by proteolysis to allow desquamation to proceed.

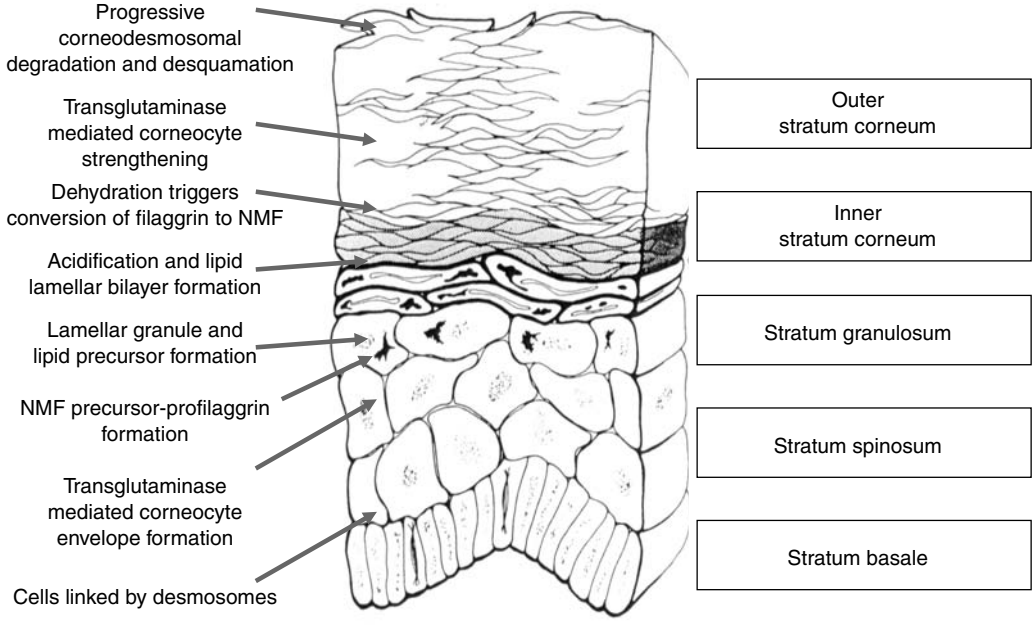

FIGURE 17.1 Typical structure of the epidermis and critical steps in formation of the SC. Modified from Rawlings, A.V., Scott, I.R., Harding, C.R., and Bowser, P.A. *J. Invest. Dermatol.*, 103, 731–740 (1994) and Rawlings, A.V. and Harding, C.R. Moisturization and skin barrier function. 17, 43–48, 2004.

Rawlings et al.[3] (Figure 17.2), first demonstrated the degradation of the CD toward the surface of the SC in humans. This desquamatory process is facilitated by the action of both intracellular and extracellular SC derived enzymes that degrade the corneodesmosomal linkages (see Chapter 7). In summary, several serine (namely stratum corneum chymotryptic enzyme [SCCE] and stratum corneum tryptic enzymes [SCTE[5,6]]), cysteine (stratum corneum thiol protease [SCTP[7]] now known as Cathepsin L-2), and aspartic enzymes (cathepsin E and cathepsin D[8]) are believed to be involved in this process. SC Cathepsin L-like enzyme has also recently been implicated in Cdsn hydrolysis.[9] Only SCTE and not SCCE, however, was capable of degrading isolated Dsg 1 *in vitro*.[10] As these enzymes are members of the kallikrein family of serine proteases they have to be named KLK 5 (SCTE) and KLK 7 (SCCE).[11] KLK 14 is recently reported to be about half of the trypsin activity in the SC.[12] Both KLK 5 and 14 are involved in the activation of pro-SCCE at acidic pH whereas auto-activation of pro-SCTE or via KLK 14 occurs at neutral pH suggesting the presence of a proteolytic cascade of activation of these enzymes in the immature to mature SC.

Cleavage of the corneodesmosomal glycoproteins occurs during desquamation (e.g., Dsg 1). Dsc 1 has been reported to be processed to smaller molecular weight fragments that are still functional (115 to 46/48 kDa fragments).[13] Equally, Cdsn undergoes several proteolytic steps.[14] Cleavage of the N terminal glycine loop domain occurs first at the compactum disjunctum interface (48–46 to 36–30 kDa transition), followed by cleavage of the C terminal glycine loop domain in exfoliated corneocytes (36–30 to 15 kDa transition). The last step appears to be inhibited by calcium resulting in residual intercorneocyte cohesion. Deglycosylases are also involved in corneodesmosome hydrolysis[15] although glycosylation has no effect on Cdsn hydrolysis. Most recently Bernard et al.[16] have also identified an endoglycosidase, heparanase 1, within the SC, thought to play a role in the pre-proteolytic processing of the protecting sugar moieties on corneodesmosomal proteins.

Many of these enzymes have been immunolocalized to the intercorneocyte lipid lamellae. Sondell et al.[17] used antibodies that immuno-react precisely with pro-SCCE to confirm that this enzyme is transported to the SC extracellular space via lamellar bodies. Watkinson et al.[18] demonstrated that

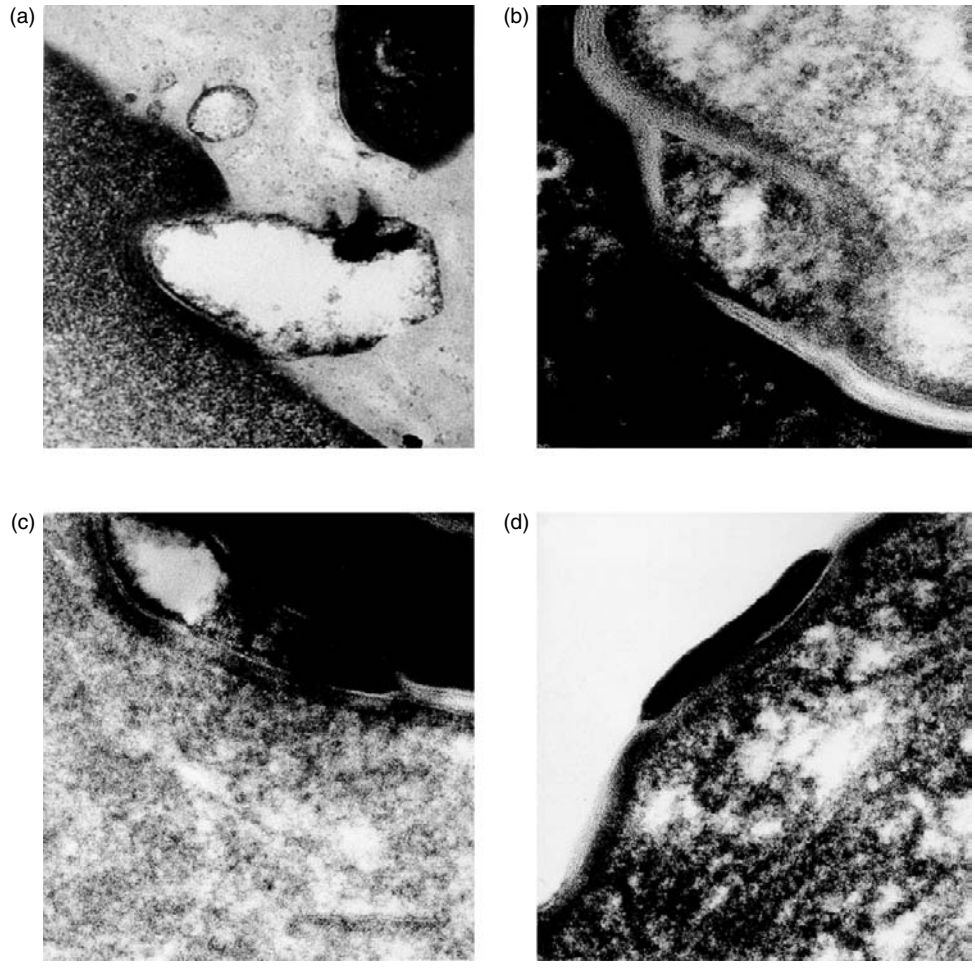

FIGURE 17.2 Electron micrographs of tape strippings of normal skin (grade 1). Degradation of corneodesmosomes (CD) toward the surface of the SC: (a) First strip; CD fully degraded. (b) Second strip; CD partially degraded and encapsulated by lipid lamellae. (c) Third strip; CD partially degraded, vaculation of structure. (d) Third strip, normal CD in contact with lamellar lipids. From Rawlings, A.V., Watkinson, A., Rogers, J., Mayo, A.M., Hope, J., and Scott, I.R. *J. Soc. Cosmet. Chem.*, 45, 203–220, 1994.

the processed enzyme was more associated with the corneodesmosomal plaque. More recently, Igarashi et al.[19] have immunolocalized cathepsin D to the intercellular space, whereas cathepsin E was localized within the corneocytes. Finally, KLK 8 has also been reported to be localized to the intercellular spaces of the SC.[20] Importantly, trypsin activity has also been reported to be present within the corneocytes themselves[21] and may be involved in desquamation. Caspase 14 has also recently been colocalized with CD but its role is desquamation is unknown.[22]

It is obvious that as some of the desquamatory enzymes are found within the lamellar lipids, the physical properties of the SC lipids, together with the water activity in this microenvironment, will influence the activity of these enzymes and ultimately desquamation. The differences in SC water concentration profiles between normal and dry skin influence the enzymic reactions in the SC.[1] Equally, differences in enzyme activity occur on different body sites. SCCE levels, for instance are lower in the axilla compared with forearm skin.[23] Cathepsin D activity is lower on the forehead compared with the forearm,[24] yet SCCE levels are the same. Interestingly, differences in SC turnover

occur on these two body sites. Also SCTE[25] and Cathepsin D levels decrease with age. As a result body site variation and aging need to be considered.

17.3 THE PATHOPHYSIOLOGY OF WINTER-INDUCED DRY SKIN

In dry flaky skin conditions, CD are not degraded efficiently and corneocytes accumulate on the skin's surface layer. Increased levels of CD in soap-induced dry skin were first reported by Rawlings et al.[3] but have been confirmed more recently by Simon et al.[26] Many corneodesmosomal proteins are now also reported to have increased in the surface layers of xerotic skin (e.g., Dsg 1 and Dsc 1; Figure 17.3). There is a close correlation between the levels of Dsg 1 and Dsc 1 in the inner and outer SC in both normal and dry skin. Dsc 1 was reported to be a more sensitive marker of dry skin.[13] More recently Cdsn and plakoglobin were found elevated in dry skin.[26] Interestingly, however, in winter xerosis, the accumulation of the corneodemosomal proteins, Dsg 1 and plakoglobin, correlate with each but Cdsn protein levels do not suggesting that different proteolytic mechanisms occur for the different corneodesmosomal components during desquamation. Simon et al.[26] reported increased immunoreactivity to the carboxy terminal tail of the cytoplasmic portion of Dsg 1. Perhaps the intracellular portions of Dsg 1 are also degraded within the corneocyte (e.g., plakoglobin by the trypsin-like activity or cathepsin E activity reported within the corneocyte matrix). Conversely, Cdsn and Dsc 1 might be degraded by SCCE, SCTE, or cathepsin D in the lamellar matrix.

As reported by Rawlings et al.,[3] the lamellar lipid matrix is also perturbed dramatically in dry skin (Figure 17.4) and reduced levels of ceramides at the surface of the SC. At this time, the full complexity of the different ceramide structure was not known but, more recently, Chopart et al.[27] observed dramatic reductions in the levels of phytosphingosine-containing ceramides in dry skin (approximately 50%), together with a shortening and lengthening of the acyl sphingoid bases sphingosine and 6-hydroxysphingosine, respectively. Van Overloop et al.[28] also clearly demonstrated that the phytosphingosine-containing ceramides were reduced to a greater extent than other

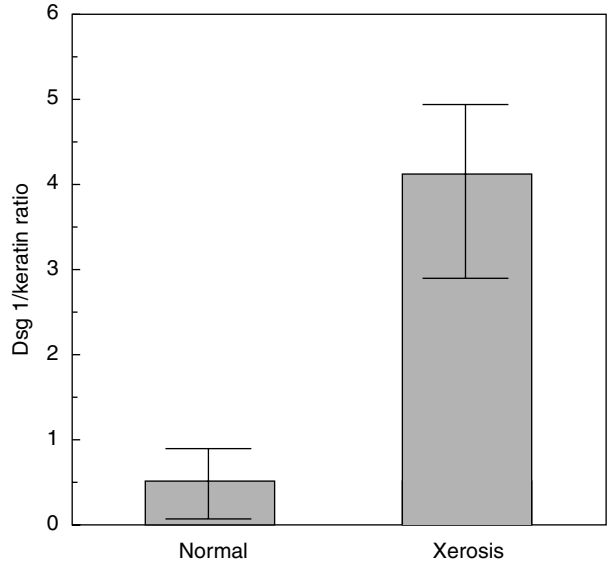

FIGURE 17.3 Histogram showing the increased levels of desmoglein 1 (Dsg 1) in SC of subjects with severe winter xerosis (grade 4) compared with normal SC (grade 1). From Rawlings, A.V., Watkinson, A., Rogers, J., Mayo, A.M., Hope, J., and Scott, I.R. *J. Soc. Cosmet. Chem.*, 45, 203–220 (1994).

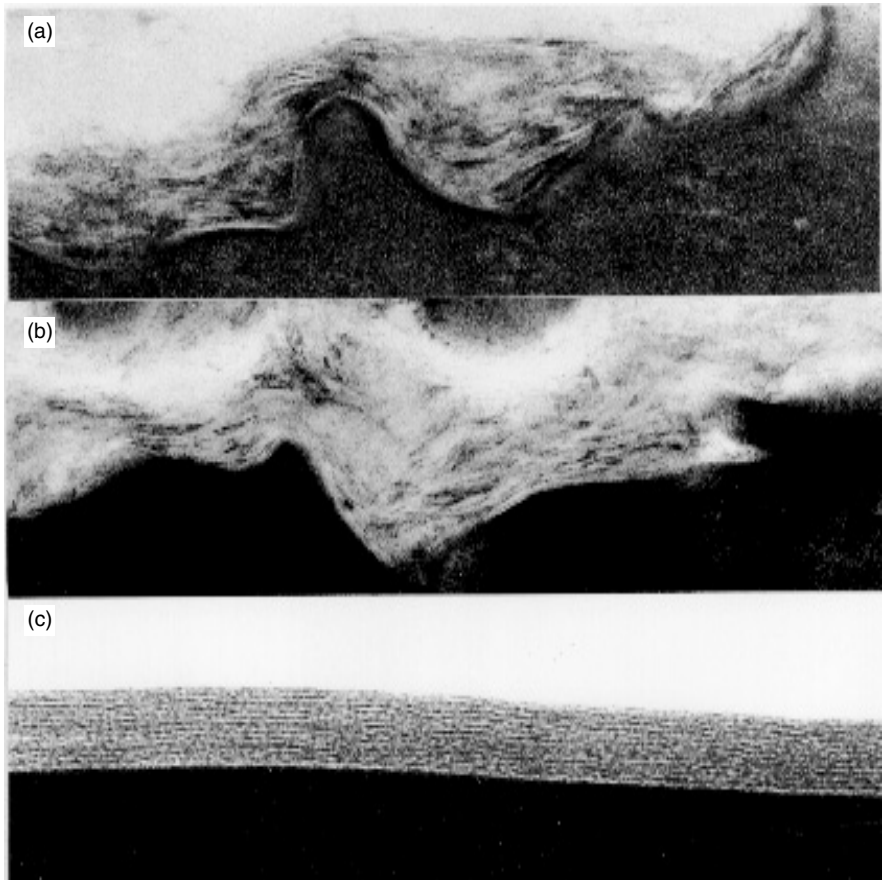

FIGURE 17.4 Organization of SC lipids in tape-strippings of individuals with clinically normal skin. Transmission electron micrographs of tape-strippings. Ultrastructural changes in lipid organization toward the surface of the SC: (a) First strip; absence of bilayers and presence of amorphous lipidic material. (b) Second strip; disruption of lipid lamellae. (c) Third strip; normal lipid lamellae (×200 000). From Rawlings, A.V., Watkinson, A., Rogers, J., Mayo, A.M., Hope, J. and Scott, I.R. *J. Soc. Cosmet. Chem.*, 45, 203–220, 1994.

ceramides, with increasing dryness levels. These changes in lipid composition will, of course, influence the lamellar packing of the lipids. In fact, Schreiner et al.[29] established a reduction of CER EOS and EOH with increased concentrations of sphingosine-containing ceramides (CER NS and CER AS) and crystalline cholesterol in association with a loss of the LPP. However, although the lipid ultrastructure is clearly aberrant in the outer layers of dry skin, more work is needed to ascribe a particular lipid phase. Nevertheless, as the main desquamatory enzymes are found within this lipid matrix, the physical properties of the lamellar lipids will, therefore, influence enzyme activity.

Harding et al.[31] originally reported that SC SCCE levels were reduced in the outer layers of xerotic SC compared with normal skin (Figure 17.5). Reduced activities have been confirmed recently in more extensive studies by Van Overloop et al.[28] who also found that the equally important SC SCTE activities were also reduced.

Several other aberrations in SC biology occur in winter dry skin, which is outside the scope of this chapter, but Figure 17.6 shows a schematic summary of the differences in SC biology in normal (a) and dry (b) skin.[1]

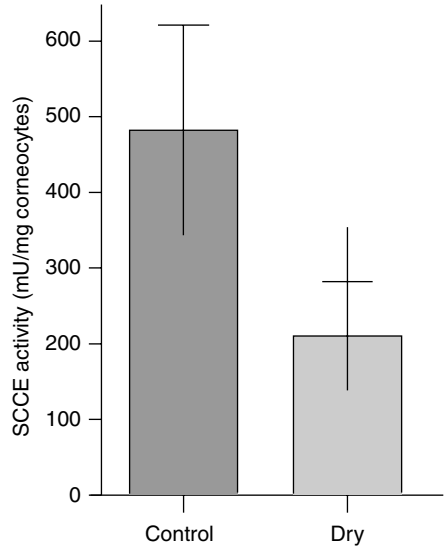

FIGURE 17.5 SCCE activity levels in normal and soap-induced dry skin. From Harding, C.R., Watkinson, A., and Rawlings, A.V. Dry skin, *Int. J. Cosmet. Sci.*, 22, 21–52, 2000.

17.4 THE EFFECT OF TOPICALLY APPLIED PROTEASES

As described earlier, although water (i.e., moisturization) is required to alleviate dry skin, reduced enzymic activity is the cause of the scaling symptoms associated with the condition. These enzymes can be activated for instance with glycerol,[31] hydroxyacids,[25] or urea,[32] but to retain full desquamatory activity topical application of enzymes is required. As all corneodesmosomal proteins persist in the superficial layers of the SC in dry skin, either the full spectrum of skin desquamatory enzymes are required to maximally induce exfoliation or broad specificities are needed in a single enzyme.

Several early studies comparing proteolytic enzymes found no digestion of the SC. Equally, while preparing SC sheets with trypsin the SC sheet remains intact.[34] Other enzymes, however, are capable of degrading the SC for example, use of proteinase K (a nonspecific fungal protease) to prepare the "stratum compactum" cellular layer.[35] However, a variety of bacterial enzymes are capable of degrading the SC. Staphylococci, and in particular *Staphylococcus aureus*, are known to produce several extracellular enzymes including serine, cysteine, and metalloproteases.[36] So far, however, only a clear demonstration of the *in vivo* role of a particular enzyme in the infection process was only demonstrated for epidermolytic toxins that degrade a major desmosomal protein: Dsg 1.[37,38] The epidermolytic toxins (ETA, ETB, ETD) have been identified as a causative agent of staphylococcal scalded skin syndrome.[39] These are only produced by about 5% of strains. These are similar to members of the chymotrypsin-like serine protease family. Serine protease-like proteins (SpI) are also produced by *S. aureus*. Candida also secretes proteases to allow fungal access to the deeper layers of the SC.[40] So some exogenously derived enzymes may degrade the SC or the lack of an effect may just be an access issue. To demonstrate that increased desquamation can occur using exogenous enzymes Bissett et al.[41] using SC cell disaggregation assays in the presence of the zwitterionic surfactant 6-octadecylammoniohexanoate demonstrated that both trypsin and subtilisin can accelerate cell dissociation. Nevertheless, subtilisin enzymes only appear to digest away the superficial layers of the SC when applied topically.[42–44]

Recently the effects of alcalase (another protease from subtilisin family), bovine pancreatic chymotrypsin, and papain (from papaya) have been evaluated on the desquamatory process.[45] Alcalase (or Optimase) is an alkaline serine proteases derived from *Bacillus licheniformis* with

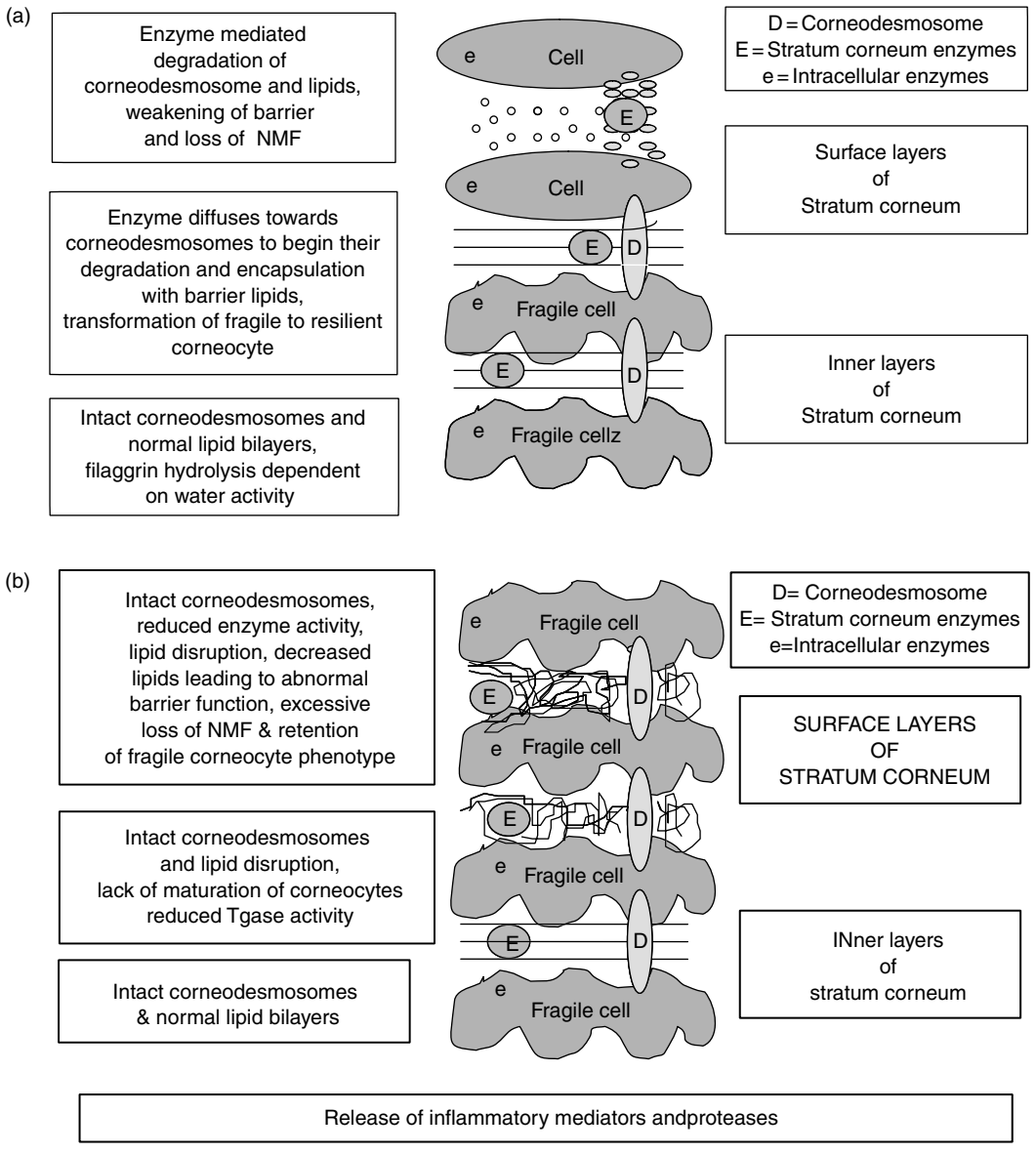

FIGURE 17.6 Summary of SC maturation and corneodesmolysis in normal (a) and dry skin (b).

broad substrate specificity. The effect of topical enzymes has been further evaluated in corneocyte release assays, immunochemical determination of desmocollin 1, and by electron microscopy (EC). First, pig skin obtained from a local abbatoir was cleaned and dermatomed. Skin slices were then placed on the surface of agar (1%) dermal side down and set. Enzymes could then be topically applied to the skin surface and incubated at 80% RH for 24 h at 37°C. Biopsies were then taken from the skin slices and sonicated in buffer. Corneocyte envelopes were prepared and quantified densitometrically in a dot blot assay after staining with Coomassie blue. The effect of topically applied Optimase on desquamation *in vitro* can be seen in Figure 17.7. Almost a doubling of collected corneocytes was determined by this treatment. To confirm the specificity of action of the enzyme on the CD indirect immunofluorescent detection of corneocyte dsc 1 was performed on the prepared corneocyte

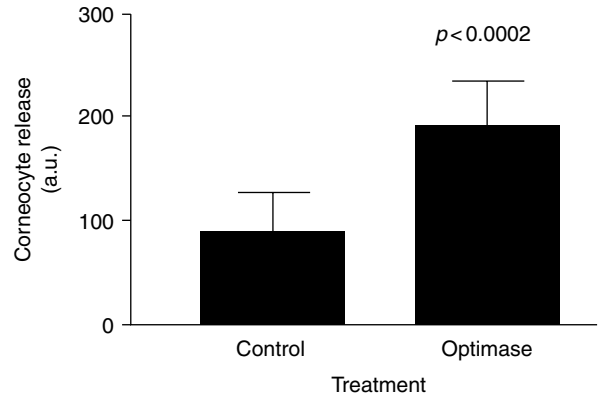

FIGURE 17.7 Effect of topically applied Optimase on desquamation *in vitro*. From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

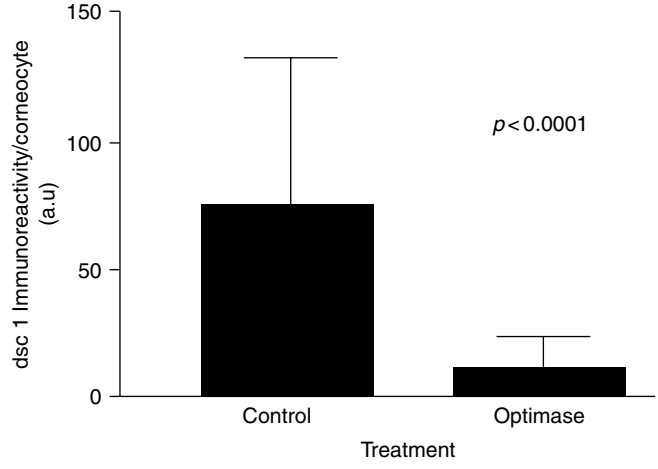

FIGURE 17.8 Effect of topically applied Optimase on corneodesmosome degradation *in vitro*. From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

envelopes using an anti-human dsc 1 antiserum being detected with an anti-rabbit IgG-FITC antibody by fluorescence microscopy and image analysis. The reductions in dsc 1 levels in the pig skin following enzyme treatment *in vitro* can be seen in Figure 17.8. Furthermore, corneodesmosome degradation was followed using electron microscopy. In this example plantar SC was incubated with 100 μg/ml Optimase for 16 h at 37°C, fixed and then treated with osmium tetroxide and uranyl acetate. After sectioning a visual analysis of the CD was conducted by electron microscopy. Intact CD were characterized by the presence of a dense uniform intercellular plaque whereas those in various stages of degradation had a diffuse or dissolving plaque associated with a widening of the intercellular space. Optimase significantly decreased the levels of intact CD as a percentage of the total number in each field (Figure 17.9). These early *in vitro* studies suggested that enzymes may be useful for the treatment of dry skin.

Following a pre-treatment phase of female subjects with soap washing on the legs, baseline visual scaling scores were determined according to the following grades in Table 17.1. In the occlusion studies Hilltop chambers with 0.3 ml of test solutions or control were attached to the skin surface

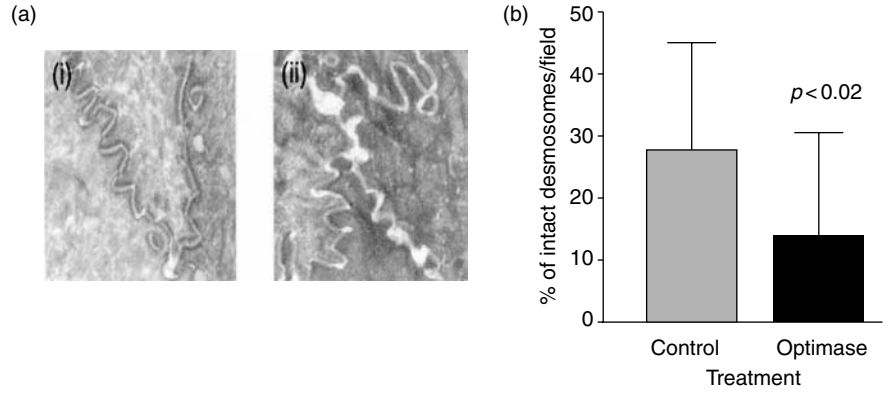

FIGURE 17.9 Effect of aqueous Otimase on corneodesmosome degradation in plantar SC in vitro. (a) Typical electron micrograph images on control and optimase treated plantar stratum corneum. Optimase treated plantar stratum corneum a(ii) shows degraded CD compared control tissue a(i), (b) Effect of aqueous optimise on CD degradation invitro. From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

TABLE 17.1
Clinical grading indices for visual scaling

Value	Visual scaling grade
0	No dryness
0.5	Perceptible dryness & fine white lines
1.0	Fine dry lines, white powdery look and/or some uplifting flakes on less than 30% of the test site
1.5	A more uniform flaking covering more than 30-50% of site
2.0	Uniform marked flaking covering more than 50% of site
2.5	Slight to moderate scaling
3.0	Moderate to severe scaling
3.5	Severe scaling and slight fissuring
4.0	Severe scaling and severe fissuring

with dermatological tape. After treatments for up to 3 h the patches were removed, rinsed with water, and patted dry. Test sites were then visually evaluated for scaling and erythema. The extent of relief of dry skin can be seen in Figure 17.10. Topical application of chymotrypsin (0.5%, 43 GU/ml) alleviated skin scaling by at least a 2 grade change compared with 100% occlusion within 3 h. Heat-inactivated enzymes and vehicle had no effect. GU or glycine unit is the amount of enzyme that at pH 8 and 50°C produces an amount of amino terminal groups from acetylated casein equivalent to μg/ml of glycine. As can be seen in Figure 17.11, increasing exposure time resulted in a greater reduction in visual scaling. Broad specificity bacterial proteases from *B. licheniformis* were also shown to be more effective than topical pancreatic chymotrypsin and papain in clinically alleviating the flaking and scaling.

In dual application studies 0.5 ml of aqueous enzyme or vehicle was followed by 0.3 ml of Vaseline Intensive Care lotion twice per day to the lower legs of female subjects and the relief of winter dry skin was assessed by visual assessment. As can be seen, the effects of Vaseline Intensive Care lotion were marginal for 2 to 3 days only giving a 0.5 unit change in the expert assessment of dry skin whereas the enzyme treatment began to alleviate more effectively with a greater than one grade change in

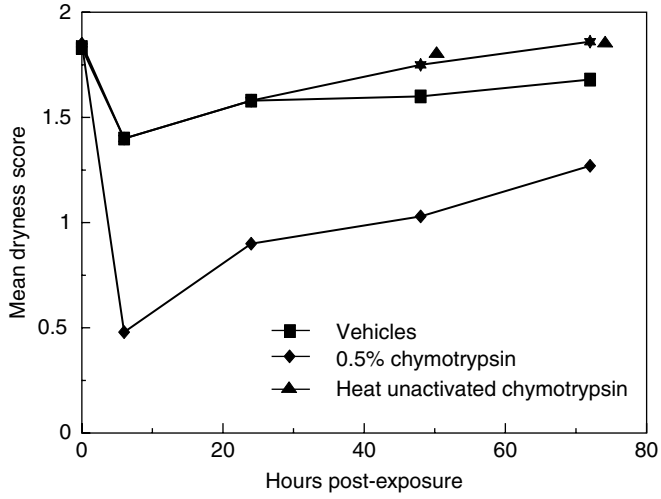

FIGURE 17.10 Effect of bovine pancreatic chymotrypsin on visual scaling after a 3 h occluded application. Vehicle (square), 0.5% chymotrypsin (diamonds), heat inactivated chymotrypsin (triangles). $^*P < 0.05$. From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

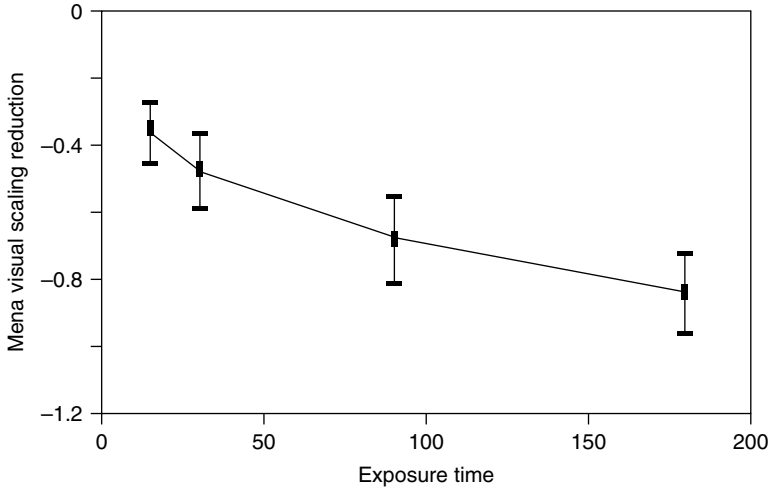

FIGURE 17.11 Effect of protease exposure time on visual scaling. Mean visual scaling reduction is the reduction in visual scaling from baseline after exposure to 0.5% bovine pancreatic chymotrypsin (43 GU/ml). From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

dry skin appearance in some cases (Figure 17.12). Similar effects were observed with either 2.4 or 12 GU/ml with either Alcalase or Optimase. To ascertain if the topical application of the proteases was specifically promoting corneodesmosome degradation, indirect immunofluorescence of dsc 1 was quantified from corneocyte envelopes prepared from tape stripping of human skin after three-days treatment of winter dry skin by Optimase (12 GU/cm^2). As can be seen in Figure 17.13, significant reductions in dsc 1 levels are observed following the treatment. The superiority of Alacalase/Optimase is anticipated due to its broader substrate specificity. These results are similar to the findings of

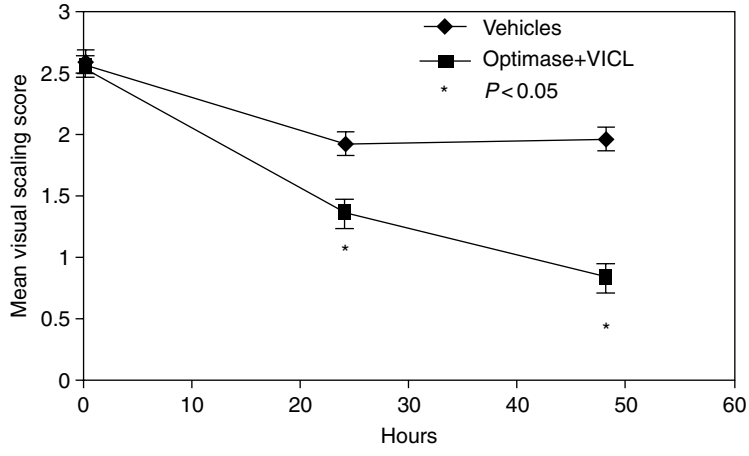

FIGURE 17.12 Reduction in visual scaling achieved using Optimase. Aqueous enzyme was applied followed by Vaseline Intensive Care Lotion. Vehicle (diamonds) and Optimase + VICL (squares). $^*P < 0.05$. From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

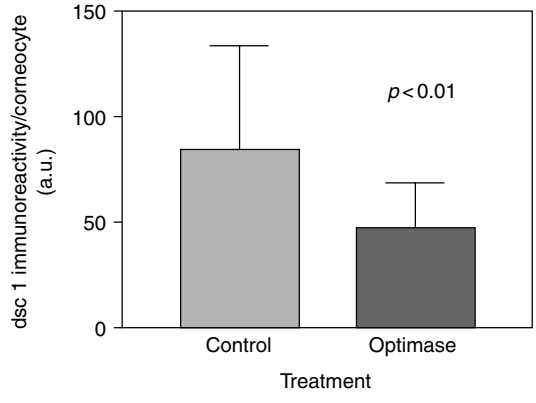

FIGURE 17.13 Effect of topically applied Optimase on desquamation *in vivo*. Soap-induced dry skin was treated with Optimase (12 GU/ml) and moisturizer for three days. Tape strip samples of SC were taken and dsc 1 levels were quantified. From El-Kadi, K., Rawlings, A.V., Feinberg, C., Nunn, C., Battaglia, A., Chandar, P., Richardson, N., Sabin, R., and Pocalyko, D. *Arch. Dermatol. Res.*, 293, 500–507, 2001.

Masunaga et al. who demonstrated that Bioprase from *B. subtilis* in a cleansing preparation improves skin condition over a 14-day period.[46]

Topical application of Cathepsin D-like enzyme from mushroom extract (Actizyme) has also been shown to be beneficial for the treatment of xerosis.[47] In dry skin SC turnover is reduced. This parameter can be measured *in vivo* using the dansyl chloride test. Addition of this enzyme to a formulation increased SC turnover by about 30%. This was of similar order to the effect of hydroxyacids, retinol, and mechanical scrubbing. In contrast to these treatments use of the enzyme did not impair barrier function nor increase stinging scores.

Most recently, Lee et al.[48] investigated the effects of proteases in hand dish washing liquids. Immersing the hands of Korean or Japanese subjects for 15 min in products containing 0.005–0.02%

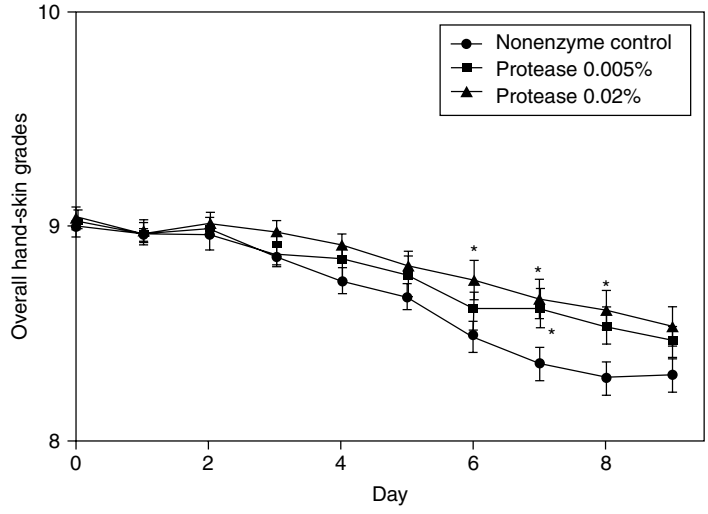

FIGURE 17.14 Time and dose dependent effects of protease-containing dishwash solutions on overall hand-skin condition. $^*p < 0.05$ between nonenzyme control and protease-containing solutions. From Lee, M.Y., Park, K.S., Hayashi, C. et al. *Contact. Dermatitis.*, 46, 75–80, 2002.

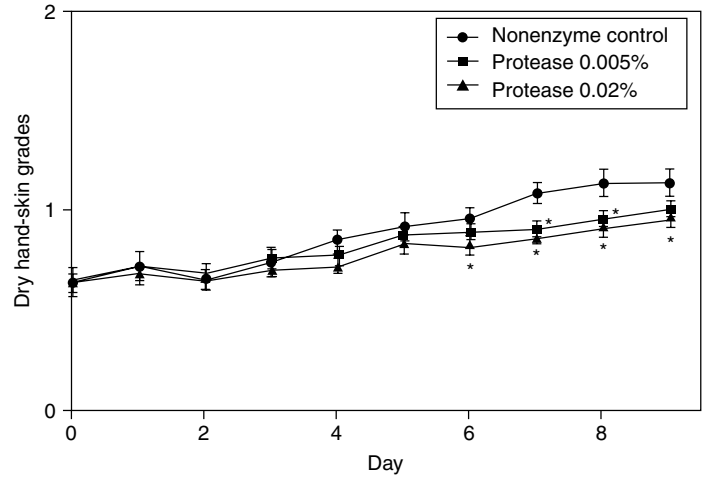

FIGURE 17.15 Time and dose dependent effects of protease-containing dishwash solutions on dryness of hands. $^*p < 0.05$ between nonenzyme control and protease-containing solutions. From Lee, M.Y., Park, K.S., Hayashi, C. et al. *Contact. Dermatitis.*, 46, 75–80, 2002.

protease for 4–9 days the protease containing dish washing liquid was less irritating to skin as judged by less dry skin. As can be seen in Figure 17.14, the enzymes improved skin condition and alleviated skin dryness, as shown in Figure 17.15 (see Tables 17.2 and 17.3 for grading scales). Use of a 0.005% protease did not cause any adverse dermatological effect to atopic subjects compared with nonenzyme control liquids.

In most skin applications stability of the enzymes is a key issue as they are autolytic. This can be achieved by copackaging and use of high concentrations of glycerol to reduce the water activity of the enzyme to stabilize it. On dilution with a moisturizer the enzyme becomes activated.[49] More work is needed to stabilize enzymes in emulsions.

TABLE 17.2
Grading Scale for Overall Hand Condition

Grading scale	Feature
10	Essentially perfect skin
9	Fairly generalized powdery scales primarily on dorsum
8	Patchy to generalized small scales on the dorsum and the interdigital and volar crease area
7	Generalized large lifted scales on dorsum, interdigital web, and palmar digital area
6	Localized to general large lifted scales over the entire dorsum possibly with some shallow fissures

TABLE 17.3
Dry Hand-Skin Grading Scale

Grading scale	Feature
0	No dry skin
1	Slightly lifted or powdery scales in only one area: on dorsum, palmar side, or between fingers
2	Slightly lifted scales covering 50 to 100% of the surface of two or more areas
3	Moderately lifted or powdery scales in only one area
4	Moderately lifted scales covering 50 to 100% of the surface of two or more areas
5	Highly lifted or powdery scales in only one area

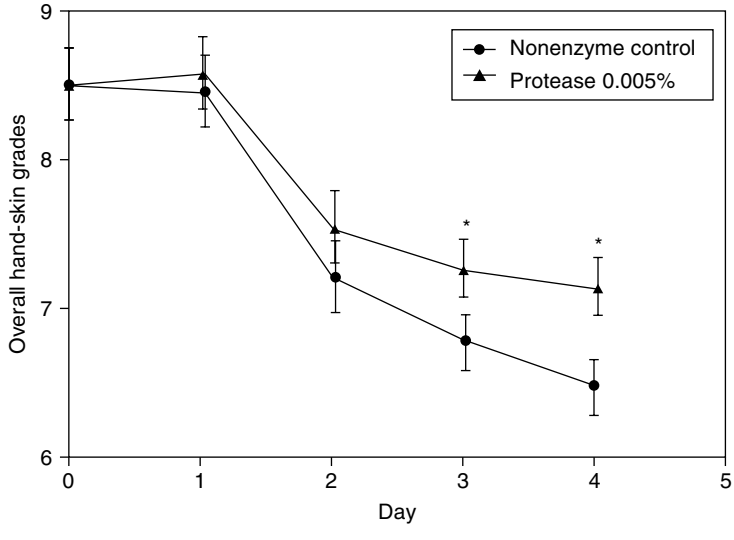

FIGURE 17.16 Time and dose dependent effects of protease-containing dishwash solutions on overall condition of hands. $p < 0.05$ between non enzyme control and protease-containing solutions. From Lee, M.Y., Park, K.S., Hayashi, C. et al. *Contact. Dermatitis.*, 46, 75–80, 2002.

It should be borne in mind, however, that the aberrations in other aspects of SC biology need to be corrected even after using enzymes (see Figure 17.16). Normalization of SC ceramide subtypes and NMF need to be corrected together with transglutaminase activities to allow optimal maturation of immature envelopes that are known to occur in these conditions.[1]

17.5 FINAL COMMENTS AND FUTURE PROSPECTS

The use of proteases in skin care applications has been increasing over the last few years. In particular, proteases provide smoother skin through gentle exfoliation. The proposed mode of action of proteases is to cleave the corneodesmosomal glycoproteins, which in turn make skin look younger and smoother by removing the keratinous dead cell layer. Therefore proteases are used in one or more products of a typical skin care regimen. To name a few product categories, proteases are included in cleansers, masks, exfoliating scrubs, peels, acne creams, and skin moisturizers. Proteases of plant origin, such as papain from papaya and bromelain from pineapple have been the most widely used so far. Recently, proteases from bacterial and fungal origin are coming into greater favor. The reason behind the shift is that the bacterial and fungal protease products are available with a consistent quality and are more economical to produce as they are manufactured by fermentation rather than extracted from plants.

So far none of the skin specific skin proteases have been exploited in skincare products although they have been produced biotechnologically and patented for the treatment of dry skin. As suggested from the *in vivo* studies, use of narrow specificity enzymes would not benefit the treatment of dry skin and in fact all of the skin enzyme specificities will be required for optimal benefit. In this respect not all of the proteases are capable of directly degrading Dsg 1, for instance. Nevertheless, although treatment of dry skin with broad specificity enzymes is highly effective in reducing the skin scaling associated with the condition, correction of the other aberrations of SC biology need to be considered with any formulation.

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18 Effects of Natural Moisturizing Factor and Lactic Acid Isomers on Skin Function

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CONTENTS

18.1 INTRODUCTION

Dry, flaky skin remains one of the most common and vexing of human disorders. Although there is no unambiguous definition of this dermatosis, it is characterized by a rough, scaly, and flaky skin surface that often becomes fissured, particularly during the winter months of the year. The observation that low moisture content is a prime factor precipitating the condition was made by Irwin Blank over 50 years ago,[1] and in many respects these pioneering studies heralded the dawn of moisturization research. Since that time many researchers have investigated the complex process of stratum corneum (SC) maturation in both normal and dry skin and have begun to unravel the biological and physical implications of SC moisturization.

In order to maintain water effectively within the skin the epidermis undergoes a process of maturation or terminal differentiation to produce a thin, metabolically inert, barrier, the SC. This heterogeneous structure has been likened to a brick wall in which the anucleated nonviable cells, termed corneocytes are represented as bricks embedded in a continuous matrix of specialized intercellular lipids (mortar).[2] Each individual corneocyte can be viewed simplistically as a highly insoluble

protein complex, consisting primarily of a keratin macrofibrillar matrix, stabilized through inter- and intra-keratin chain disulfide bonds, and encapsulated within a protein shell called the cornified cell envelope (CE). This latter structure is composed of a number of specialized proteins[3] which are extensively cross-linked through the action of at least two members of the transglutaminase family.[4] Given that elements of the internal keratin matrix are also linked to the interior aspect of the cornified envelope (through both disulfide linkages and again by the action of transglutaminase[5]), each corneocyte can be likened to a single, intricately cross-linked "macro-protein." This extensive protein interaction imparts great strength and insolubility to the corneocyte, an essential feature for the "brick" component of this structure. The overall integrity of the SC itself is achieved primarily through specialized intercellular protein structures called corneodesmosomes[6,7] that effectively rivet the corneocytes together, but which ultimately must be degraded to facilitate desquamation.

The visual appearance of dry skin is now generally accepted to be the consequence of the altered scattering and reflection of light off the rough skin surface resulting from abnormal desquamation. This perturbation to the ultimate step of terminal differentiation emphasizes a critical and often overlooked role of water in the SC, namely, its importance for the activity of a variety of hydrolytic enzymes involved in various aspects of SC maturation and desquamation.[8–11] When the tissue becomes desiccated a loss of overall hydrolytic enzyme activity affects many biochemical processes within the SC. The most widely appreciated symptom of this enzymatic failure is the visible scaling associated with ineffective corneodesmosomal degradation.[12,13] However altered activity of several other enzymes including transglutaminase[14] and lipases[15]can contribute to the formation of dry skin.

Therefore in order to maintain its flexibility, integrity, and critical catabolic activity the SC must remain hydrated, and in healthy skin the tissue contains greater than 10% water.[1,16] In the absence of water the SC is an intrinsically fragile structure, which readily becomes cracked, brittle, and rigid. The maintenance of water balance in the SC is therefore vital to this tissue and is preserved through three major biophysical mechanisms. The first of these is the intercellular lamellar lipids that provide a very effective barrier to the passage of water through the tissue.[17,18] The second mechanism is provided by the proteinaceous corneocytes themselves that also play an important role in contributing to the water barrier.[19] Given that there is only a gradual age-related decline in lipid levels within the SC it is believed that the dramatic increase in corneocyte size plays an important role in keeping water loss (as measured by transepidermal water loss [TEWL]) at a comparable level in young and old skin.[20] The final mechanism is provided by the natural moisturizing factor (NMF), a complex mixture of low molecular weight, water-soluble compound, which is present within the corneocytes.[21] Collectively, the NMF components have the ability to bind water against the desiccating action of the environment and thereby maintain tissue hydration. Historically we have thought of the NMF as an exclusively intracellular component although clearly the consequences of corneodesmosomal lysis and the processing of glycosylated ceramides within the SC invoke the potential presence of intercellular humectants as well.

Usually these three biophysical mechanisms interact precisely to provide a highly efficient barrier against water loss and retain water within the tissue to maintain flexibility and catabolic activity. Nevertheless, this barrier is continually prone to perturbation by both external forces (UV, low RH, cold temperatures, and surfactants), and internal factors (cutaneous disease, psychological stress, and diabetic complications). With decreased performance of the water barrier the increased loss of water from the tissue ultimately leads to the formation of dry skin.

For a proper appreciation of the underlying biochemistry of dry skin we should consider this common condition as a dysfunction of one or more of the vital processes that generate and protect the water-holding capacity of the SC. With this concept in mind, in this chapter we will focus initially on the generation and critical importance of the NMF to SC function. Second, we will consider the effects of topically applied NMF components, and in particular the effects of lactic acid and its isomers, on the alleviation of dry skin symptoms. Finally, we will consider briefly the technologies that can influence NMF generation through stimulation of the synthesis of the NMF-precursor molecules.

TABLE 18.1
The Chemical Composition of NMF

	%
Free amino acids and urocanic acid	40.0
Pyrrolidone carboxylic acid	12.0
Lactate	12.0
Sugars, organic acids, peptides, unidentified materials	8.5
Urea	7.0
Chloride	6.0
Sodium	5.0
Potassium	4.0
Ammonia, uric acid, glucosamine, creatine	1.5
Calcium	1.5
Magnesium	1.5
Phosphate	0.5
Citrate, formate	0.5
Glycerol	ND
Hyaluronic acid	ND

ND. Not determined in this analysis but detected in stratum corneum.

18.2 NATURAL MOISTURIZING FACTOR

18.2.1 The Role of the NMF in the Stratum Corneum

The NMF consists primarily of amino acids or their derivatives such as pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA) together with lactic acid, urea, citrate, and sugars[22] (Table 18.1). These compounds are collectively present at high concentrations within the cell and may represent 20 to 30% of the dry weight of the SC.[23]The importance of the NMF lies in the fact that the constituent chemicals, particularly the PCA and lactic acid salts, are intensely hygroscopic. These salts absorb atmospheric water and dissolve in their own water of hydration, thereby acting as very efficient humectants. In essence, the amount of NMF in the SC determines how much water it can hold for any given relative humidity (RH). In the absence of NMF the SC can only absorb significant amounts of water at 100% humidity, a situation that seldom occurs. It is important to remember that the highly structured intercellular lipid lamellae provide a barrier to reduce the highly water-soluble NMF from leaching out of the surface layers of the skin.[24]

Although the properties of several of the individual components of the NMF have been studied extensively, our understanding of the contribution of individual components and their synergistic behavior to the overall properties of the SC remains relatively poor. Recently, the potent water binding molecule hyaluronic acid has been shown to be naturally present in the SC[25], and the importance of glycerol, present at low concentrations, has been emphasized by the elegant studies of Verkman and coworkers.[26] This group has shown that there is a specific transporter of glycerol in the epidermis[27] and the loss of this protein is associated with major perturbations in SC water retention and mechanical properties.[26] Glycerol is also derived from sebaceous triglyceride breakdown, and again to emphasize the importance of this molecule studies by Fluhr and colleagues have indicated that topically applied glycerol can completely restore the poor quality of SC observed in asebic mice (no sebaceous secretions) to normal.[28] The identification of glycerol and hyaluronic acid in the SC is relatively recent, and in any classical consideration of NMF composition and function these two molecules have been ignored, and moisturization research has focused on four major

intrinsic components: lactate, free amino acids (FAA), PCA, and urea. Fox et al.[29] investigating the humectant capabilities of sodium lactate, demonstrated a 60% increase in water content at 60% RH, whereas, in contrast under the same conditions, glycerol only provided a 38% increase. Laden and Spitzer,[30] after studying the composition of NMF, concluded that since amino acids themselves are relatively nonhygroscopic at skin pH, PCA itself must contribute significantly to the SC water binding capacity. Although it has been demonstrated that sodium lactate is slightly more hygroscopic than sodium PCA at 50% RH,[31,32] both of these salts contribute significantly to the hygroscopicity of the SC. Biologically, this property allows the outermost layers of the SC to maintain liquid water against the desiccating action of the environment.

Traditionally, it was believed that this liquid water plasticized the SC, keeping it resilient by preventing cracking and flaking which might occur due to mechanical stresses. However, under conditions of reduced RH, when water can only provide a transient effect, topically applied lactic acid achieves a long-term plasticization of the SC. Similarly, while developing a skin cream designed to reduce dry and flaky skin, Middleton, (through measuring changes in SC extensibility and water-holding capacity) showed that at around 80% RH sodium lactate and sodium PCA were as effective as other moisturizing agents. Although their benefits were essentially lost on rinsing the SC with water,[33] lactic acid-treated skin retained some residual plasticization benefit. Recent data indicates that lactate plays a critical role in influencing the physical properties of the SC. Lactate and potassium were found to be the only components of the NMF analyzed (although PCA was not analyzed) that correlated significantly with the state of hydration, stiffness and pH in the SC.[34]

Urea, another principle component of the NMF, has also been demonstrated to have similar effects,[35] although no direct comparison with either PCA or lactic acid has been reported.

The general mechanisms by which the NMF components influence SC functionality have been studied extensively. From a physical chemistry perspective the specific ionic interaction between keratin and NMF, accompanied by a decreased mobility of water, leads to a reduction of intermolecular forces between the keratin fibers and increased elastic behavior. Recent studies have emphasized that it is the neutral and basic FAA[36] in particular that are important for helping keratin acquire and maintain its elastic properties. Consistent with these observations Sakai et al.[37] reported that the ratio of acidic amino acids to total amino acids correlated to the resonant frequency a measure of skin stiffness.

These observations clearly emphasize how the NMF is critical for maintaining physical properties of the SC. However, as our understanding of the terminal differentiation and SC maturation process has increased, it has become clear that by maintaining free water in the SC, the NMF also facilitates critical biochemical events. As indicated earlier the coordinated activity of specific proteases and lipases is essential for optimum SC function, and these hydrolytic processes can only function in the presence of water that is effectively maintained by the water-retaining capacity of the NMF. Perhaps the most striking example of this is the regulation of a number of intracellular proteases within the corneocyte that, as we discuss in the next section, are ultimately responsible for the generation of the major elements of the NMF itself.

The generation and maintenance of an acid pH within the SC, the so-called "acid mantle" is critical to the correct functioning of this tissue and there is evidence of a pH gradient within the tissue.[38] Studies from Elias and coworkers point to an essential role of free fatty acids generated through phospholipase activity as being vital for SC acidification,[39,40] whilst Krein and Kermici[41] have recently proposed that UCA plays a vital role in the regulation of SC pH. However, studies on the histidase-deficient mouse (which cannot generate UCA from free histidine), indicate that SC pH in these animals is within the normal range, and this observation rather argues against the importance of UCA.[42] Nevertheless it is likely that other NMF components contribute significantly to the overall maintenance of pH. Collectively the NMF and free fatty acids (derived from phospholipid, ceramide, and sebum breakdown) contribute toward a physiologically important and gradual acidification of the SC toward the skin surface. Although a detailed consideration of the influence of pH on many enzymatic activities within the SC is beyond the scope of this chapter, there is a growing realization

that pH directly regulates barrier formation and homeostasis. Alterations of pH away from its acidic norm of 4.5 to 6.5 is associated with loss of SC integrity and cohesion. This perturbation is due in part to the inappropriate activation and activity of serine proteases involved with desquamation.[43]

18.2.2 The Origin of the Skin's NMF

The precise origin of the lactic acid and urea components of the NMF remains ill defined. They may be derived from the general breakdown of proteins and amino acids (e.g. following arginase activity on arginine). It has also been proposed that urea, like lactate may also be derived in part from sweat.[44] The presence of sugars in the SC represents primarily the activity of the enzyme ∃-D-glucocerebrosidase as it catalyzes the removal of glucose from glucosylceramides to initiate lipid lamellae organization in the deep stratum corneum.[15] In addition the degradation of corneodesmosomes will also release sugars from these glycosylated proteins.[45] Hyaluronic acid, is known to be synthesized in the epidermis by the hyaluron synthase family of enzymes, at least one of which is synthesized by keratinocytes.[46] This glycosaminoglycan may indeed be responsible for the Alcian blue staining reported in the SC by the team led by Voorhees.[47] Finally, staining of isolated corneocytes and CE with a range of fluorescently labeled lectins has revealed the presence of N-acetylglucosamine.[48] The persistence of lectin staining following the harsh isolation procedures required for CE evaluation suggests that these sugars are covalently attached, but they may subsequently be released by ∃-D-glucosaminidase known to be present in the tissue.[48]

Historically a major focus of interest has been the origin of the FAA and their derivatives within the SC, which together represent over 50% of the NMF. Studies conducted by Scott and Harding during the early 1980s[49–52] lead to the conclusion that all of the amino acid components of the NMF were derived specifically from a single, high molecular weight, histidine-rich protein, which represented the major component of the F type keratohyalin granules (KHG).[53] Based upon the ability to these histidine-rich proteins to aggregate keratin fibers *in vitro* into macro-structures reminiscent of the keratin pattern seen in the SC *in vivo*, Dale and coworkers named this class of basic proteins filaggrins,[54] and the phosphorylated precursor protein subsequently became known as profilaggrin.

Although studies by other groups have confirmed that filaggrin is a major source of intercellular FAA,[55] it is probably incorrect to accord it as the status of being the *only* source of these components in the SC. Studies by Jacobsen and team concluded that the FAA composition of human SC could not be accounted for simply by the known amino acid composition of filaggrin.[56] Based on our understanding of the spectrum of catabolic activities intrinsic to the corneocyte we can now consider at least two sources for the FAA present in the stratum corneum: filaggrin and corneodesmosomes. In addition, SC keratins also undergo a small decrease in molecular weight during SC formation and may make a minor contribution (unpublished observations).

Corneodesmosome hydrolysis initiated deep within the SC may lead to the production of an *intercellular* pool of osmotically active solutes. Warner, in studying the disruptive nature of hydration on human stratum corneum ultrastructure observed the presence of water-filled cisternae in the intercellular space and suggested the site of corneodesmosomal degradation as a focal point for production of NMF.[57] The precise contribution of intercellular humectancy to SC function remains to be established and caution must be taken with extrapolation of data from these hyperhydration studies. The dramatic size of some of the cisternae observed in these studies lead Warner to suggest that leaching of NMF from within the corneocyte could contribute to the phenomenon. Nevertheless, the presence of water around corneodesmosome has also been reported in studies where the tissue was not subjected to such extremes of hydration.[58] It is of interest to note that Nguyen[59] has also proposed the presence of intercellular (although in this case filaggrin-derived) humectants following observations of keratinocyte behavior *in vitro*.

Recently, a protein named hornerin has been identified in mouse skin.[60,61] Based on its amino acid sequence and distribution in skin it has been proposed to fulfill a similar role to profilaggrin/filaggrin in murine skin. The conditions under which it is expressed remain to be determined but it is tempting to

speculate that this protein may compensate for the absence of profilaggrin synthesis in the flaky mouse mutant, recently described by Presland.[62] In this mouse model although profilaggrin is synthesized, it is a truncated form that is not proteolytically converted into filaggrin (and hence to FAA) in the SC. Despite this defect the mouse SC recovers from a marked barrier impairment and dry flaky skin after birth to produce a functionally normal SC within three weeks. Based on the importance of profilaggrin synthesis, and the consequences of its failure to be synthesized, it would be anticipated that this gene-deletion would lead to persistent abnormal scaling and dryness. Clearly the mouse can compensate for this deficiency, and although the NMF composition of this mouse model has not been evaluated it is assumed that there is a compensatory degradation of a protein with a profilaggrin-like amino acid composition to derive the appropriate NMF profile of FAA. Alternatively, compensation via increased lipid synthesis and altered cornified envelope composition may occur. There may of course be a far simpler explanation. Normal adult mouse skin has a dramatically reduced filaggrin level compared with its neonatal counterpart, and the need for filaggrin and its derivatives may decline naturally as other mechanisms mature within the skin, and most noticeably the animal grows a coat of fur protecting against moisture loss and UV irradiation.

Interestingly, the human genome project has indicated the presence of a hornerin-like gene close to profilaggrin on chromosome 1q21, but its expression has not been reported or studied in man to date. It remains to be established whether, in otherwise healthy skin, an age-related decline in the ability to synthesize profilaggrin can be compensated for by synthesis of another proteolytically labile protein.

Despite the continued inconsistencies in our understanding of the contribution of nonfilaggrin-derived proteins to intracellular NMF, the synthesis and controlled proteolysis of filaggrin, remains pivotal to our understanding of how the barrier responds to changes in the external environment,[63,64] and how abrupt changes in RH can induce abnormalities in barrier homeostasis.[65]

18.2.3 Synthesis and Degradation of Profilaggrin

Studies conducted in our laboratory indicated that profilaggrin was rapidly dephosphorylated during the transition of the mature granular cell into the corneocyte and then underwent selective proteolysis to form lower molecular weight, highly basic species within the SC.[50]

However, regardless of the putative structural function proposed for this family of proteins within the SC, by Dale in a landmark paper[52] it was clear that keratin-aggregation, was at best, a transient role restricted to the early formation of the SC. Radiolabel pulse chase,[49] immunohistochemical,[66] and biochemical studies[50] confirmed that filaggrin, with the exception of a minor incorporation into the cornified CE,[4,67] and occasional persistence due to altered processing[68] does not persist beyond the deepest two or three layers of the SC (Figure 18.1). First, it becomes extensively deiminated through the activity of the enzyme peptidyl deiminase (PAD),[52] which serves to reduce the affinity of the filaggrin/keratin complex. Second, it is rapidly and completely degraded through small peptides to FAA. Finally, specific constituent amino acids are catabolized further to form specialized components of the NMF.

Foremost among these catabolites are PCA itself (derived primarily by the nonenzymatic cyclization of glutamine[49]), and UCA, a natural UV-absorber[69] formed by the action of the enzyme histidase on histidine[70] (filaggrin catabolism summarized in Figure 18.2).

18.2.4 Control of Filaggrin Hydrolysis

Although the precise nature of the protease system (filaggrinases) catalyzing filaggrin breakdown remains to be identified, they are primarily serine proteases. The actual "trigger" that initializes the proteolysis at a discreet but variable location within the SC is the water activity gradient present across the tissue. The discovery of this mechanism was elucidated following careful observation of changes in filaggrin distribution during SC maturation of fetal and newborn skin.[63] In normal adult

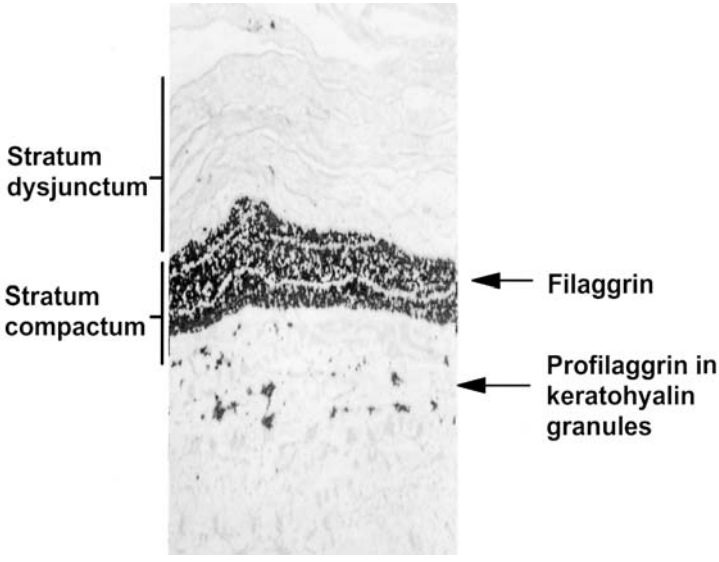

FIGURE 18.1 Distribution of filaggrin in human stratum corneum. Immunoelectron micrograph of human facial skin (9-year-old male). Ultrathin sections were incubated with rabbit-antihuman filaggrin followed by incubation with goat-antirabbit/colloidal gold (5 nm diameter).

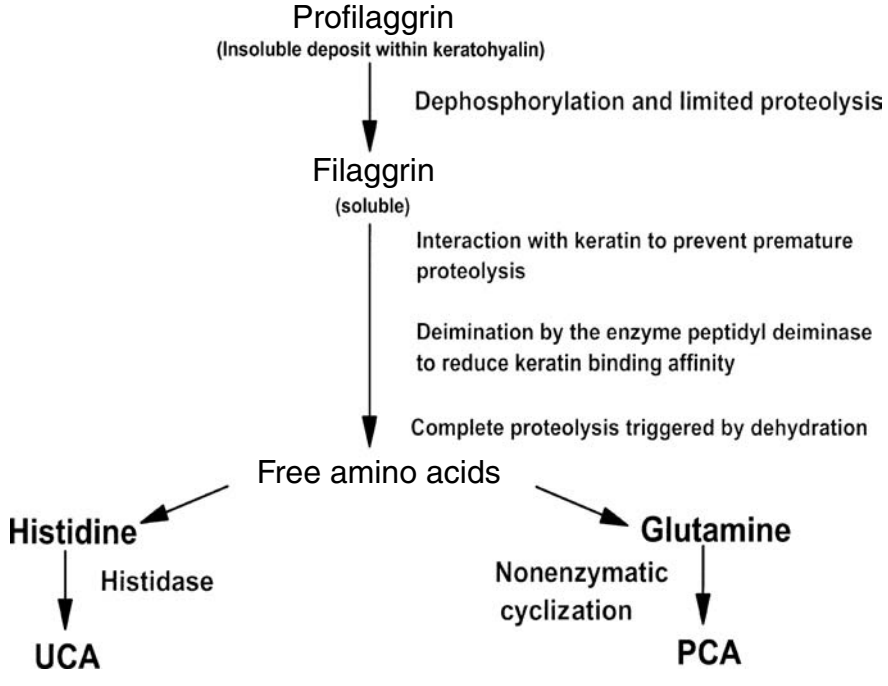

FIGURE 18.2 Schematic representation of profilaggrin catabolism during terminal differentiation.

skin filaggrin is only detected in the innermost layers of the SC (Figure 18.1), whereas in newborn and fetal tissue there is no indication of any proteolytic breakdown of filaggrin in the outer regions. However, within a few hours of birth, the breakdown of filaggrin is initiated in these regions. This triggering could be prevented in a very humid environment, which indicates the possibility that

the water content of the SC is a critical factor. Subsequent studies on filaggrin breakdown in isolated SC revealed that hydrolysis only occurred if the SC was maintained within a certain RH range (70 to 95%). Similarly, if the skin was occluded for a long period[68] filaggrin hydrolysis was blocked, the corneocytes remained filled with the protein, and the NMF level of the SC fell close to zero.

It is now appreciated that the water activity gradient within the SC and the water flux through this tissue at rest and following damage, are intimately involved in several aspects of tissue homeostasis, notably in relation to water barrier repair.[71] However, the observations on the control of filaggrin catabolism, originally made over 25 years ago, represent some of the earliest studies to demonstrate and emphasize the dynamic nature of SC maturation.

At first sight the process by which the skin generates the NMF within the SC seems absurdly complex. However, the logic of Nature's complexity becomes apparent once it is appreciated that the epidermis cannot afford to generate NMF, either within the viable layers or within the newly formed immature corneocyte itself, due to the risk of osmotic damage. It is imperative that the activation of the filaggrin protease systems is delayed until the corneocyte has flattened, strengthened and moved far enough out into the dryer areas of the SC to be able to withstand the osmotic effects of the concentrated NMF pool. The epidermis circumvents the potentially harmful effects of osmotic pressure resulting from the inappropriate premature hydrolysis of filaggrin through two strategies. First, profilaggrin, once synthesized, is precipitated within KHG where it acts as an insoluble and, most importantly, an osmotically inactive repository of the NMF. Second, the interaction between keratin and filaggrin forms a proteolytically resistant complex, which prevents premature proteolysis of the filaggrin (an intrinsically labile protein containing 10 to 15 mol% arginine residues[49]) during the intensely hydrolytic processes, which accompany SC formation.

In summary, these mechanisms are part of an elegant, self-adjusting moisturization process within the SC that allows it to respond to different climatic conditions. This mechanism ensures that it is only as filaggrin containing corneocytes migrate upward from the deepest layers and begin to dry out (and the water activity within the cell decreases) that certain proteases, by a poorly understood mechanism, are activated and the NMF is produced. The point at which this hydrolysis is initiated is independent of the age of the corneocyte[66] and is dictated ultimately by the environmental humidity. When the weather is humid the proteolysis occurs almost at the outer surface. In conditions of extreme low humidity the proteolysis is initiated deep within the tissue so that all but the innermost layers contain the NMF required to prevent desiccation. An appreciation of filaggrin form, function and fate helps to understand the water distribution, altered morphology and swelling properties of isolated SC maintained at differing hydration levels,[72] and offers an explanation for the transient reduced water-holding capacity of the SC of newborn infants.[73]

Immunocytochemical and stereological studies indicate that corneodesmosome hydrolysis is also initiated in approximately the same layer of the SC as where filaggrin is rapidly hydrolyzed. These dramatic changes in protein distribution account for the differential staining properties of the stratum dysjunctum/compactum (unpublished observations) and suggest that the enzymatic activation of the two classes of proteases (one *intra*cellularly based for filaggrin hydrolysis and one *inter*cellularly based for corneodesmosome digestion) are carefully coordinated.

18.2.5 NMF Levels and Dry Skin Conditions

The failure to either make or process (pro)filaggrin is a major problem for the skin and is associated with various dermatological disorders. The symptoms of ichthyosis vulgaris[74] are closely associated with an inability or failure to make profilaggrin. The absence of KHG histologically has been known for many years, and the NMF content of corneum in ichthyosis vulgaris patients is close to zero. Likewise, in psoriatics there is again a paucity of KHG and the associated SC is essentially NMF deficient.[75] Recently, it has been proposed that the very presence of KHG, revered for over a century as the defining characteristic of the granular layer, are in fact a histological artifact.[76] If this is indeed the case then it is likely that the unusual dual acidic and basic characteristics of

the highly phosphorylated profilaggrin are responsible for the putative aggregation artifact leading to the formation of an insoluble precipitation (which we call KHG) during histological processing. The unusual properties of isolated profilaggrin (then known as the histidine-rich protein) were first noted by Ugel in 1970.[77]

It is noteworthy that research continues to hint at additional roles for profilaggrin within the granular cell to corneocyte transition. An inability to dephosphorylate profilaggrin, following the deletion of the serine-protease Matriptase is associated with dramatic impairment of many stratum corneum events, and has lead to the suggestion that profilaggrin dephosphorylation is *the* pivotal event initiating terminal differentiation.[78] Such a critical role is supported by the unusual metabolism of profilaggrin observed in oral epithelium, particularly in the case of the hard palate, where an inability to process profilaggrin leads to an altered keratin pattern and a highly keratinised tissue.[79] Just as the physical properties intrinsic to this protein may represent the driving force for KHG formation (by artifact or by design), then the dramatic changes to these properties once profilaggrin is desphosphorylated (and cleaved) may initiate a cascade of events in the rapidly changing late granular cell. Although it is clear that in all these conditions several aspects of keratinization are impaired, the inability to produce or retain NMF within the SC appears to be a significant factor contributing to the overall manifestation of the skin problem.

Reduced NMF levels are also implicated in the more common dry skin conditions. Subjects with atopic dermatitis have decreased levels of NMF,[80] and FAA levels have been reported to decrease significantly in dry, scaly skin induced experimentally by repetitive tape stripping.[81] Additionally, a significant correlation exists between SC hydration state and the FAA content of elderly individuals with skin xerosis.[82]

Traditionally, components of the NMF are measured following extraction of corneocytes recovered from superficial tape-strippings, or from direct extraction of the skin surface by attaching open-ended chambers to the skin and eluting with small volumes of aqueous buffers or dilute surfactant solutions. By analysing sequential tape strips recovered from the same site profiles of how NMF levels change with depth can be constructed. These profiles indicate that the levels of NMF decline markedly toward the surface of the skin. This is typical of normal skin exposed to routine soap washing where much of the readily soluble NMF is washed out from the superficial SC.[83]

Individual NMF species can be measured by High Performance Liquid Chromatography (PCA and UCA), colorimetric assays (FAA) or by enzymatic assays (lactate and glycerol).

Most recently Puppels and co-workers to determine the concentration of defined NMF component non-invasively *in vivo* in the SC have pioneered the use of confocal Raman microscopy.[84] Figure 18.3 shows depth profiles for the major filaggrin derived components, urea and lactate obtained using this technique. Evidence of leaching from the skin surface is characteristically seen in most profiles and the precipitous drop off in levels of filaggrin derived components deeper in the SC indicates the boundary at which filaggrin hydrolysis is rapidly initiated.

Our own studies have suggested that there is a significant age-related decline in the level of certain NMF components, most noticeably PCA (unpublished studies). The decline in PCA production probably reflects the cumulative effects of actinic damage as it was observed in SC recovered from the back of the hand (photodamaged) of elderly individuals, but not from the inner aspect of the biceps (photoprotected) in the same population. Taken together with electron microscopy studies that report decreased numbers of KHG in senile xerosis,[85] these results suggest that the intrinsically lower NMF levels present in aged skin, compared with young skin, reflect a general reduced synthesis of profilaggrin. In addition, it is likely that in aged skin the loss of NMF becomes more pronounced as elderly individuals also show an age-related decline in water barrier repair.[86]

However, a recent publication from Takashashi and Tezuka has suggested that the content of FAA in the SC is actually *increased* in both senile xerosis and in "normal" aged skin compared to young.[87] Indeed these observations are consistent with earlier observations on the age-related increase in the levels of certain FAA primarily found in filaggrin (serine, glutamic acid, glycine) made by Jacobsen.[56]

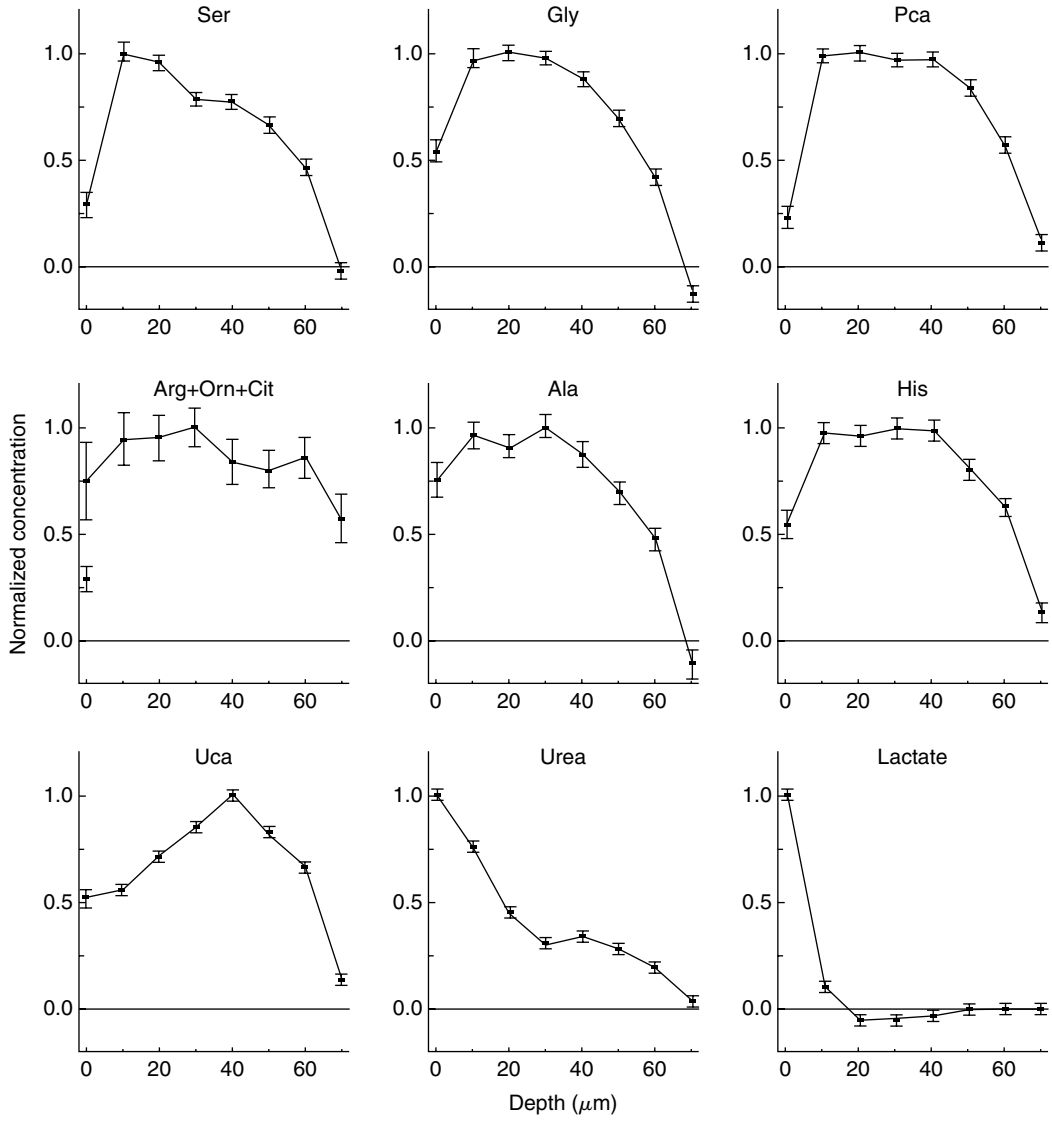

FIGURE 18.3 Semiquantitative *in vivo* concentration profiles of NMF and sweat constituents in the stratum corneum of the thenar as determined by Raman spectroscopy (From Caspers, P.J., Lucassen, G.W., Carter, E.A., Bruining, H.A., and Puppels, G.J. l. *J. Invest. Dermatol.*, 116, 434–442, 2001).

Given that profilaggrin synthesis has been shown clearly to undergo a significant age-related decline,[85,87] on several body-sites (although reportedly not the face[88]) then as discussed earlier, the conclusion must be that other sources of protein are contributing to the overall FAA pool.

Further speculation however is unwarranted at this time as it is likely that choice of body site, the nature of induction of xerosis (natural versus surfactant/solvent induced), differing methods of RT–PCR and filaggrin extraction and quantification protocols may all contribute to the current lack of clarity in our understanding.

Studies in UV and hexadecane damaged skin indicate that the endo- and exo-proteases (filaggrinases) responsible for filaggrin degradation are extremely robust enzymes, effectively degrading all filaggrin present in the SC during and immediately after an acute insult.[66] Nevertheless,

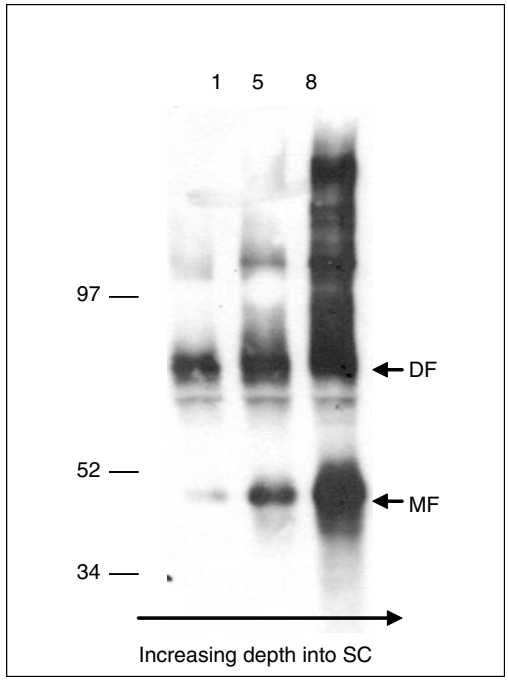

FIGURE 18.4 Persistence of filaggrin-related material in superficial SC. Samples of SC collected by consecutive cyanoacrylate stripping of human forearm skin, extracted, analyzed by PAG electrophoresis, and probed with antifilaggrin antibody following Western blotting (strippings 1, 5, and 8 shown). Mature filaggrin (MF) decreases toward skin surface and is absent from most superficial tape-stripped sample. Higher molecular-weight, deiminated, and protease resistant filaggrin variant (DF) persists into most superficial layers. Molecular weight calibration in kDa indicated on left.

we have recently observed that in aged photodamaged skin there is a minor perturbation in filaggrin processing leading to the persistence of a high molecular weight filaggrin-related material in superficial SC (see Figure 18.4). It appears that in some individuals an imbalance in the activity between the enzyme PAD and general filaggrinase activity may lead to the formation of a form of filaggrin in which complete deimination (through continued PAD activity) renders the protein refractive to filaggrinase activity. Essentially the complete conversion of arginine to citrulline residues on the filaggrin proteins removes trypsin-sensitive protease sites on the normally protease-labile protein. As we have previously shown that it is filaggrin degradation rather than filaggrin deimination that is sensitive to changes in external RH,[68] it is likely that frequent changes in environmental humidity may exacerbate dry skin conditions in part by favoring the formation of this "protease-resistant" filaggrin.

Finally, we recently reported that the allelic polymorphism recognized in the profilaggrin gene may be linked to a predisposition toward dry skin.[89] The profilaggrin gene codes for either a 10, 11, or 12 filaggrin-repeat, and therefore an individual can be 10:10, 10:11, 11:11, 10:12, 11:11, or 12:12. Using a PCR-based approach we have determined individual profilaggrin allelotypes and identified an inverse association between the 12 repeat allele and the frequency of self-perceived dry skin ($n = 89$, $p = 0.0237$). This novel observation could not be explained by a simple reduction in NMF production, and provides further circumstantial evidence for profilaggrin itself (rather than filaggrin or NMF) playing a critical role in epidermal differentiation.

Clearly in dry flaky skin conditions where corneodesmosome degradation is frequently and characteristically perturbed then generation of amino acid-derived intercellular humectancy will also be decreased potentially leading to a further reduction in protease activity.

In summary, the various processes leading from profilaggrin synthesis to conversion to filaggrin and then to NMF are under tight control. However, these controls are perturbed in different ways by a range of factors including UV-light, exposure to surfactants, and, of course, rapid changes in environmental humidity. It is generally accepted that these very different causes can all lead to reduced NMF and contribute to the complex phenomenon known as dry skin.

18.3 THE EFFECT OF TOPICALLY APPLIED NMF

Moisturizing ingredients have been used widely in skin care products for the treatment of dry skin for many years. In fact the use of oils for smoothing skin is reported as early as 2300 B.C., although it was not until the work of Blank in the 1950s[1] that research focused on water-imbibing substances to retain moisture in the SC. This section will discuss briefly the effects of PCA, urea, glycerol, and lactic acid on human SC function *in vivo*.

18.3.1 Pyrrolidone Carboxylic Acid

A considerable amount of work has been performed evaluating the effects of PCA and its salts *in vitro*. However, surprisingly only a limited amount of work has been reported on the influence of PCA topically applied on human skin. In one such study Middleton and Roberts[90] demonstrated that lotions containing PCA were more effective at treating dry skin compared to a placebo lotion.

18.3.2 Urea

Urea is a major component of the NMF, and it has been used in hand creams since the 1940s. This unique physiological substance has proven to be a potent skin humidifier and descaling agent[91] and in high concentrations it has been shown to be an effective treatment for dry skin, being more efficacious than salicylic acid and petroleum jelly.[92] Urea containing moisturizers are also reported to influence barrier properties of the skin, reducing TEWL,[93–96] increasing skin capacitance, and reducing irritant reactions. Corresponding lotions containing glycerol as the humectant had no comparable effect on reducing TEWL. Although the precise mode of action of urea is unknown, the improved barrier function may be related to increased corneocyte size resulting from reduced keratinocyte proliferation. High concentrations of urea have also been reported recently to enhance lipid biosynthesis.[97] Finally, in combination with lactic acid, urea has also been shown to be an effective treatment of ichthyosis[98] and in combination with polidocanol urea is reported to improve juvenile atopic dermatitis.[99]

18.3.3 Lactic Acid

Lactic acid, as well as being a component of the NMF, is also a member of the class of molecules called alpha hydroxy acids (AHAs), which exert specific and unique benefits on skin structure and function. Although originally described for the treatment of dry skin-related disorders, their pleiotropic properties include influencing skin cell renewal and other antiaging benefits, which have become the focus of considerable interest in recent years.

The first recorded use of lactic acid was in 1943 by Stern who used it for the treatment of ichthyosis,[100] and in the early 1970s and 1980s Middleton[101] and Van Scott and Yu[102,103] demonstrated the efficacy of these short chain AHAs in ameliorating dry skin in moisturization efficacy studies.

Other researchers[104–106] have also shown that racemic mixtures of lactic acid ameliorate the common problem of winter xerosis. Typical effects of lactic acid in moisturization efficacy studies

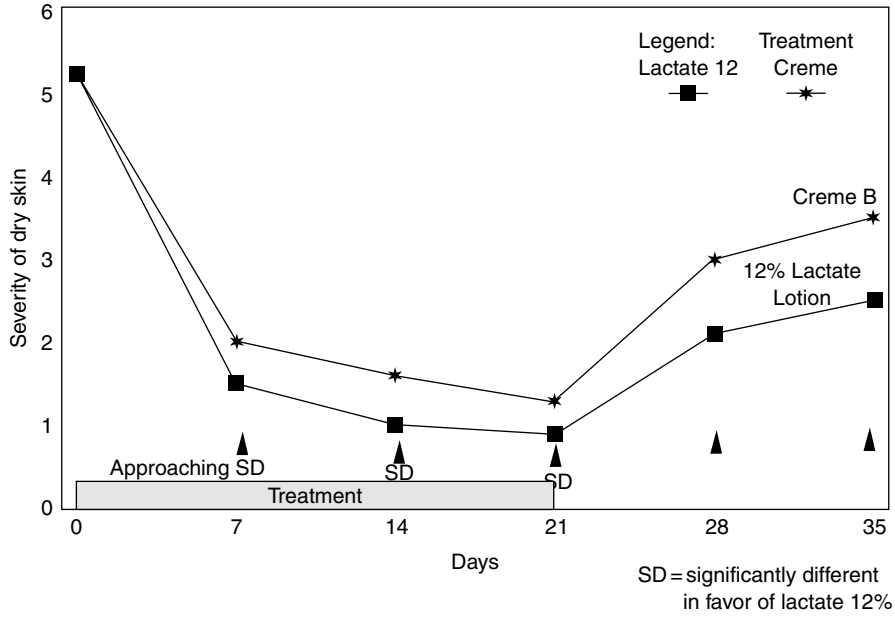

FIGURE 18.5 Improvement in dry skin condition following twice daily applications of a 12% lactic acid formulation (Reprinted by permission of publisher from Wehr, R., Krochmal, L., Bagatell, F., and Ragsdale, W.A. *Cutis*, 23, 205, 1986. Copyright 1999 by Quadrant Healthcom Inc.)

are shown in Figure 18.5. However, as is the case with other humectants, application of lactic acid alone fails to ameliorate the symptoms of dry skin, and coformulation with occlusive agents is required to help retain the humectant bound water within the surface layers of the SC. Typically, we have found that lotions containing barrier lipids (ceramides) and lactic acid provide synergistic relief of dry skin.[107] These results are similar to those found with lotions containing barrier lipids and glycerol[108] and we believe that these lotions then act by increasing enzymatic activity within the SC leading to corneodesmolysis. More recently, the relative efficacy of the different isomers of lactic acid has been studied to help decipher its mode of action in improving SC resilience. *In vitro* lactic acid increased the production of ceramides by keratinocytes, and the l-isomer was found to be more effective than the d-isomer.[109] Similar effects were observed *in vivo* where in a four-week study topically applied lactic acid increased SC ceramide levels and l-lactic acid was seen to be the most active isomer. These changes were associated with improvements in SC barrier performance measured by changes in TEWL following a challenge to skin with sodium lauryl sulfate (Figure 18.6) and by a decrease in the expression of dry skin in the regression phase of a moisturization efficacy study. Significant improvements in these parameters were observed following application of lotions containing l-lactic acid and d,l-lactic acid but not d-lactic acid. In the studies outlined previously a significant increase in the ratio of ceramide 1 linoleate to ceramide 1 oleate may also have contributed to the improvements in SC performance. Ceramide 1 linoleate is of critical importance to the SC, where it functions as an important modulator of lipid phase behavior.[110]

Recently, Berardesca et al.[111] have also reported the ability of a number of AHAs to improve SC barrier and prevent skin irritation (Figure 18.7).

In a pivotal clinical study evaluating the effects of lactic acid on photodamaged skin,[112] an 8% l-lactic acid formula was found to be statistically significantly superior to the vehicle cream in reducing the overall severity of photodamage, mottled hyperpigmentation, sallowness, and skin roughness. Furthermore, the benefit of lactic acid on skin roughness was confirmed instrumentally following laser profilometry of silicone replicas taken from the cheek area. The results indicated that

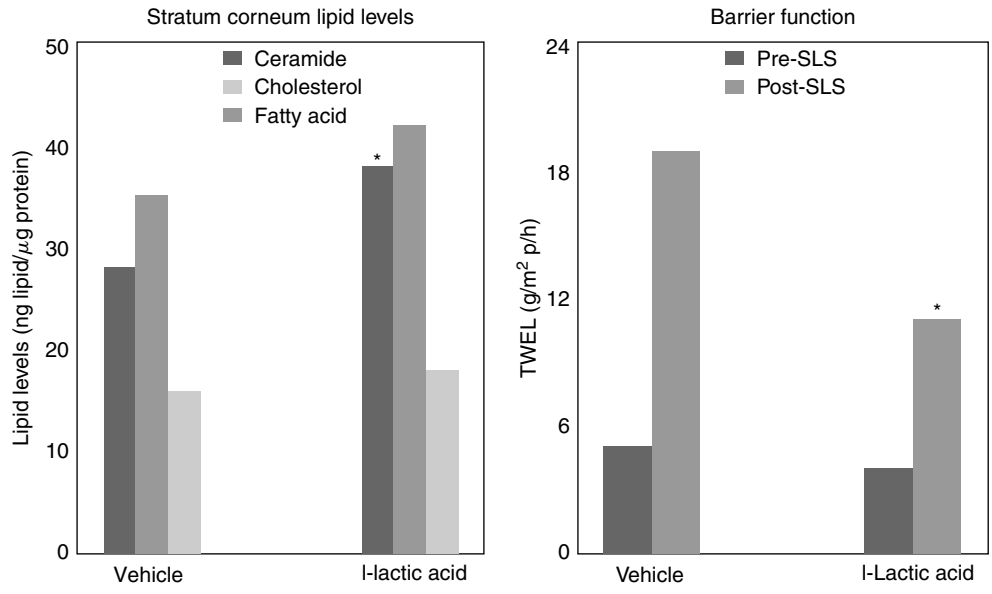

FIGURE 18.6 Effect of lactic acid on SC lipid levels and barrier function following a 1-month topical application of 4% lactic acid in an aqueous vehicle. TEWL evaluated before application of SLS patch and 24 h after removal (*$p < 0.05$). (Reprinted with permission by publisher from Rawlings, A.V., Davies, A., Carlomusto, M., Pillai, S., Zhang, K., Kosturko, R., Verdejo, P., Feinberg, C., Nguyen, L., and Chandar, P. *Arch. Dermatol. Res.*, 288, 383, 1996. Copyright 1999 by Springer-Verlag, New York.)

the l-lactic acid formula substantially reduced the roughness of the skin compared to the vehicle cream regardless of the roughness parameter calculated. Generally, the improvement in skin roughness was of the order of 25 and 10% compared to baseline values for the lactic and vehicle creams, respectively. Although the exact mechanisms that explain these observations are not known, we have shown that lactic acid imparts changes to SC lipids and increases epidermal turnover rates that should lead to the formation of smaller corneocytes. Further studies are in progress to understand more clearly the mode of action of lactic acid in the effective treatment of photodamaged skin.

Most recently, Nakagawya et al.[34] demonstrated that topical application of potassium lactate restored stratum corneum hydration after NMF extraction and exhibited a significantly higher restorative effect than sodium lactate. The authors speculate that this is due to the structure-destructive properties of the potassium ion and may influence hydrogen bonding in the keratin matrix.

18.3.4 Saccharide Isomerates

Mixtures of sugars, saccharide isomerates, have been shown to be effective humectants. These isomerates mimic those found naturally in skin as a result of the hydrolysis of glucosylceramides. In clinical studies Smith[113] has shown that these isomerates reduce the visual appearance of dry skin, increase skin hydration, and reduce stinging to lactic acid.

18.3.5 Glycerol

As glycerol has now been identified in the SC it can be considered as a component of NMF. It is the archetypal moisturizer. It enhances desquamation by acting as a corneodesmolytics, that is, it aids the proteolytic degradation of corneodesmosomes[114] (Figure 18.8.) Equally, however, it also enhances the transglutaminase mediated corneocyte envelope cross linking and ceramide esterifying

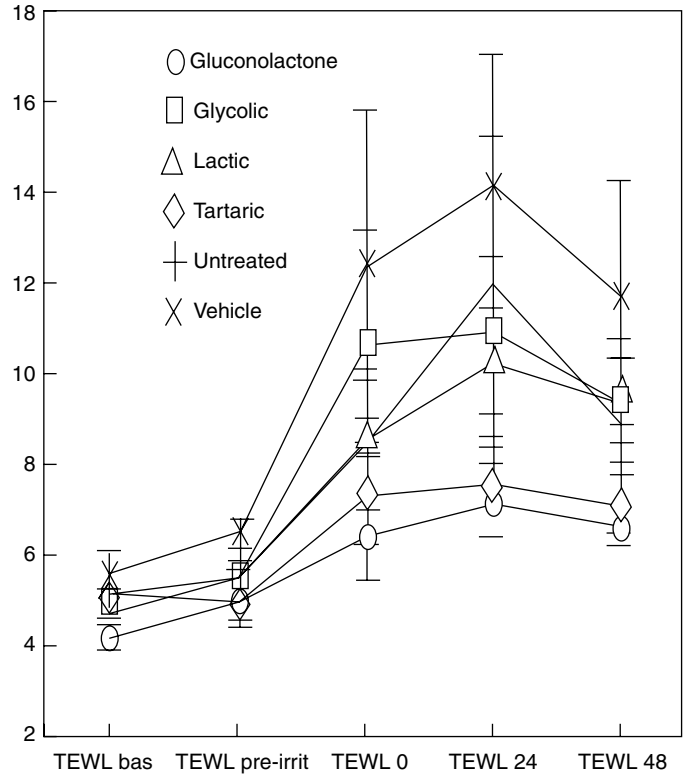

FIGURE 18.7 TEWL after sodium lauryl sulfate SLS challenge (g/m^2/h). Lower barrier damage was detected in alpha hydroxy acid treated sites compared with vehicle and untreated sites (Reprinted with permission of publisher from: Berardesca, E., Distante, F., Vignoli, G.P., Oresajo, C., and Green, B. *Br. J. Dermatol.*, 137, 934–938, 1997. Copyright 2004 by Blackwell Publishing, Oxford, UK.)

events essential for the normal functioning of the stratum corneum.[14] Nevertheless, at conventional levels of use even glycerol's effects of supplementing the NMF moisturizing system needs to be enhanced by combination with other occlusive materials. Petroleum jelly or lipid-based systems are clinically more effective when combined with glycerol,[108] and in fact a synergistic alleviation of dry skin is apparent (Figure 18.9).

18.4 ENHANCING PROFILAGGRIN SYNTHESIS

Given the importance of the profilaggrin/filaggrin family of proteins to skin condition, and the fact that synthesis declines with age and is readily perturbed by UV-irradiation, many researchers have sought to enhance synthesis.[115] A promising approach is through modulation of gene expression particularly through specific members of the nuclear hormone receptor family. Gene expression is regulated by the interplay of specific transcription factors and the nuclear hormone receptors are transcription factors that regulate many important cellular functions. This superfamily of receptors has been divided into five major subgroups depending upon their dimerization and DNA binding properties. The class II subfamily consists of nuclear receptors that form heterodimers with the retinoid X receptor (RXR).[116] Some of the transcription factors that form heterodimers with the RXR's include the retinoic acid receptor (RAR), the peroxisome proliferator receptor (PPAR), the liver X receptor (LXR) and the farnesol X receptor (FXR). Stimulation of these receptors, in particular, regulates keratinocyte proliferation and differentiation.

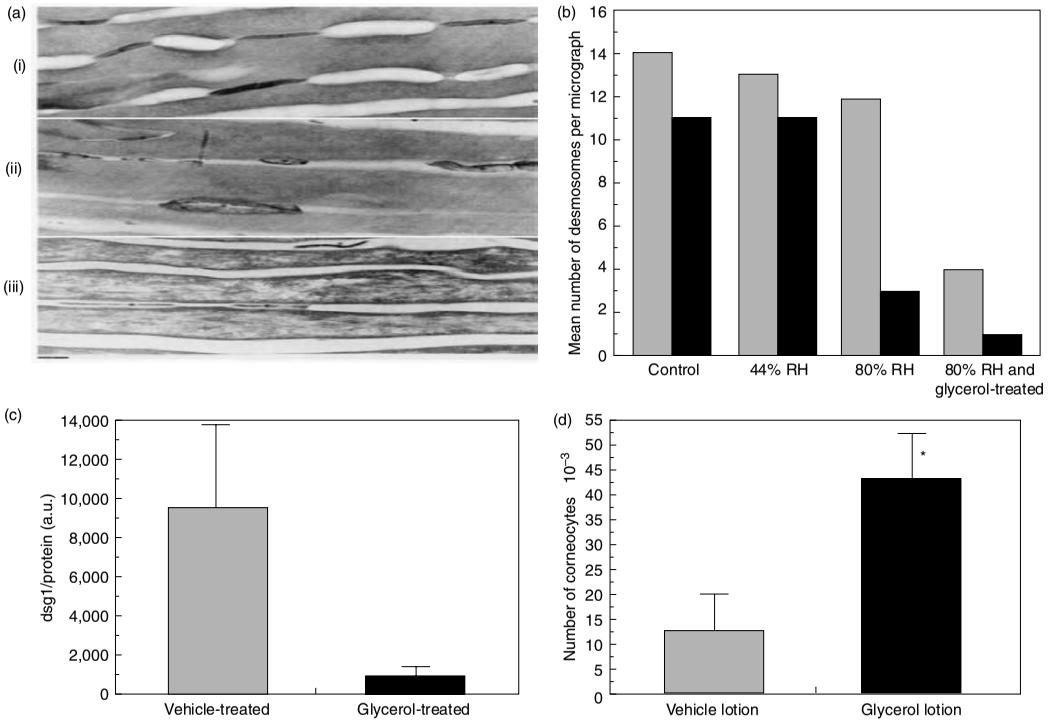

FIGURE 18.8 (a) Osmium tetroxide-fixed stratum corneum. (i) Control tissue no treatment and incubated at 44% RH. Note electron dense corneodesmosomes are fully intact. (ii) Tissue incubated at 80% RH for 7 days. Note the partial degradation of corneodesmosomes. (iii) Tissue incubated at 80% RH following 5% glycerol treatment. Note the paucity of corneodesmosomes and virtually complete degradation of their structures. (b) Comparison of the number of corneodesmosomes in control stratum corneum and stratum corneum incubated at 44% RH, 80% RH, and 80% RH following 5% glycerol treatment. Note the decrease in intact corneodesmosomes in 80% RH-treated samples and the significantly reduced number of intact (black boxes) and total (gray boxes) corneodesmosomes in glycerol-treated tissue incubated at 80% RH. (c) Comparison of the effect of 5% glycerol on desmoglein 1 digestion at 80% RH. Note the dramatic decrease in desmoglein 1 levels in glycerol-treated samples. (d) Comparison of the effect of lotions with and without the addition of 5% glycerol on corneocyte release. (Reprinted with permission of publisher from Rawlings, A.V., Harding, C.R., Watkinson, A., Banks, J., Ackerman, C., and Sabin, R. *Arch. Dermatol. Res.*, 287, 457–464, 1995. Copyright 2004 by Springer, Heidelberg.)

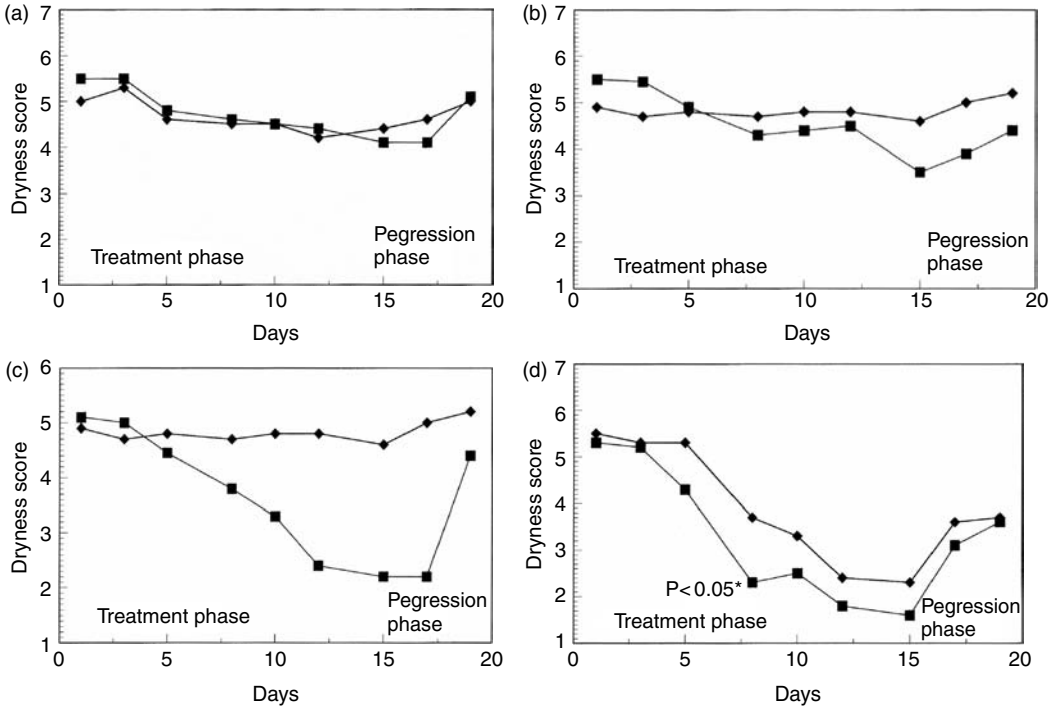

FIGURE 18.9 Moisturization efficacy tests: (a) Comparing the effect of 1% glycerol (square) to a no-treatment control (triangle). (b) Comparing the effect of a lotion containing 1% phospholipids, 2% cholesterol, and 1% stearic acid (square) to a no-treatment control (triangle). (c) Comparing the effect of a lotion containing 1% phospholipids, 2% cholesterol, and 1% stearic acid plus 1% glycerol (square) to a no-treatment control (triangle). (d) Comparing the effect of a lotion containing 1% phospholipid, 2% cholesterol, 1% stearic acid plus 5% glycerol (square) to a lotion containing 1% petrolatum, 2% cholesterol, 1% stearic acid plus 5% glycerol (triangle). (Modified from Summers, R.S., Summers, B., Chandar, P., Feinberg, C., Gursky, R., and Rawlings, A.V. *J. Soc. Cosmet. Chem.* 47, 27–39, 1996.)

18.4.1 Peroxisome Proliferator Activated Receptor

Peroxisome proliferator activated receptors are a recently discovered family of nuclear transcription factors.[117–119] Three PPAR receptor types, PPAR alpha, PPAR beta and gamma/delta, PPAR gamma have been characterized. Like other nuclear receptors PPARs bind to response elements within the promoter region of the DNA of the target gene in the form of homo or heterodimers together with the ubiquitous RXR. On binding ligands, corepressors dissociate from the transcriptional machinery complex and coactivators bind to initiate gene transcription.

PPARs are activated by a wide range of molecules including the fibrate hypolipidemic drugs and a range of saturated and unsaturated dietary fatty acids, eicosanoids and prostanoids.[120,121] Recently, triterpenoids such as ursolic and oleanolic have also been reported to stimulate the alpha receptor[122] and increase filaggrin biosynthesis.

The epidermis has been shown to express the three PPAR variants with PPAR delta being the predominant subtype.[123–125] All PPAR receptors improve epidermal differentiation and increased filaggrin levels *in vitro* and in animal studies.[123,125,126] Watkinson et al.[127] recently extended these observations in a clinical study and reported that topical application of petroselinic acid, a known PPAR alpha agonist, increased epidermal filaggrin levels significantly compared with the vehicle control in a repeat patch study for 21 days (Figure 18.10). Niacinamide or its free acid is also a PPAR agonist and Oblong[128] has reported that niacinamide increases filaggrin biosynthesis by keratinocytes.

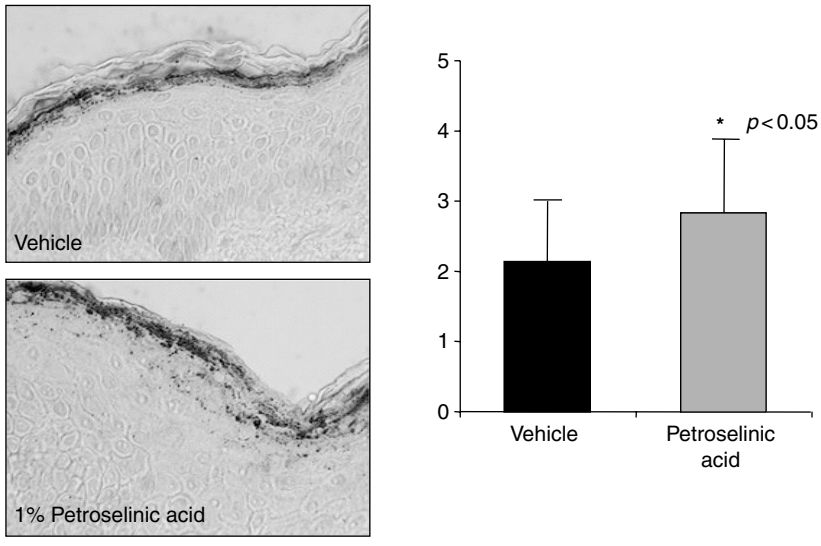

FIGURE 18.10 Increased synthesis of profilaggrin/filaggrin in human axilla skin detected by immunohistochemistry following a 3 week application of a 1% petroselinic acid formulation.

18.4.2 Liver X-Receptor and Farnesol X-Receptor

Two other new nuclear receptors have been shown to increase epidermal differentiation: the LXR and the FXR. Farnesol and juvenile hormone activate the FXR leading to improved epidermal differentiation. Two genes encode for the LXR proteins, LXR alpha and LXR beta, and both are activated by various oxysterols the most potent being 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S) 25-epoxycholesterol and 7-hydroxycholesterol. Cholestenoic acid also acts on this receptor. *In vitro* these agents also increased epidermal filaggrin levels.[129,130]

18.5 FINAL COMMENTS

The NMF is essential for normal functioning of the SC. Working together with the SC lipids this pool of low molecular weight compounds assists in the retention of water within the corneocytes, a capability that is vital for the integrity of this barrier, and its mechanical properties. Hydration of the SC is also essential for the normal functioning of numerous enzymatic processes that are pivotal, not only for desquamation, but also for the generation of the NMF itself. Perturbations to either of these two biophysical mechanisms can lead to xerotic problems. Applications of lotions containing a variety of the constitutive NMF components have been shown to improve SC extensibility properties, desquamation performance, water barrier quality and to alleviate the symptoms of dry and aging skin. However, our understanding remains incomplete, and the location and activity of water within the SC and its effects on the physical and biochemical properties of this unique tissue will continue to be a quest for stratum corneum biologists for many years.

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19 Clinical Evidence for the Use of Urea

Marie Lodén

CONTENTS

19.1 INTRODUCTION

Urea is a physiological substance occurring in human tissues, blood, and urine. The amount in urine is of the order of 2%. The extraction of pure urea from urine was first accomplished by Proust in 1821, and it was first synthesized by Wöhler in 1828.[1] Urea is also a major constituent of the water-soluble fraction of the stratum corneum, as a component of the natural moisturizing factor (NMF).[2] The level of urea in the stratum corenum is significantly reduced in patients with atopic dermatitis.[3]

Folklore is rich in references to the healing properties of urea. The Babylonians of about 800 B.C. are known to have used it. In the beginning of this century, urea was employed in the treatment of infections, particularly infected wounds and ulcers, infection of the ears, infected tooth sockets, infected malignant growths, and of burns.[4,5]

The most well-known dermatologic effects from urea appear to come from its generally accepted property of unfolding proteins, thus solubilizing them and denaturing them.[5–7] Pieces of upper epidermis kept in saturated urea solutions change mechanically and lose their original quaternary structure.[6] Urea can also be used for avulsing dystrophic nails, and a preparation with 40% urea has been shown to be slightly more effective in removing the nail than a formulation with 22%, but it was also more irritating.[8] Urea is also used as a keratoplastic agent (at 40%) to increase the bioavailability of the drug in the treatment of onychomycosis.[9] Concern has been expressed about the use of urea in

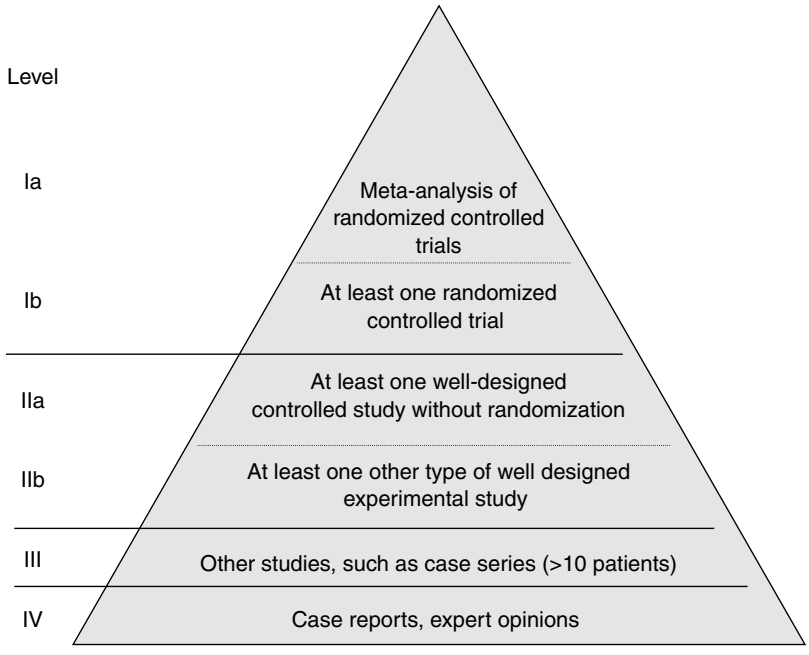

FIGURE 19.1 Types of evidence and their level in the evidence hierarchy.

moisturizers, with reference to the risk of reducing the chemical barrier function of the skin to toxic substances.[6] It has been used for treatment of a variety of dry skin disorders, for example, psoriasis, ichthyosis, atopic dermatitis, hand dermatitis, hyperkeratosis of the feet, seborrheic dermatitis, solar keratosis, perioral dermatitis, and environmentally induced dryness.[1,10] The influence of urea on the skin permeability is considered important, since disease activity in inflammatory dermatoses has been suggested to be driven by abnormalities in the skin barrier function.[11,12]

Different types of evidence can be ranked in term of importance when decisions about clinical interventions are made (Figure 19.1).[13,14] For example, the confidence from randomized controlled trials gives stronger evidence for treatment effects than open studies. Moreover, apparently conflicting results between studies may be compatible when a statistical meta-analysis of the data has been performed. This chapter will give a brief summary of evidence on the treatment effects of common dry skin disorders with urea-formulations. Furthermore, data on the influence of urea on the skin barrier function will be reviewed.

19.2 CHEMISTRY AND BEHAVIOR

Urea (carbamide, carbonyl diamide, CAS no 57-13-6, molecular weight 60.08) is a white, crystalline, and quite inexpensive powder. The substance is hygroscopic, freely soluble in water, slightly soluble in alcohol, and practically insoluble in ether.[15] It is readily incorporated in topical formulations by virtue of its solubility. However, urea in solution hydrolyzes slowly to ammonia and carbon dioxide.[15] In one patent it is claimed that lactic acid retards the decomposition of urea.[16] Unstable preparations may need to be stored in a refrigerator.[15]

Urea attracts water and immersion of psoriatic and ichthyotic scales in 5 *M* urea show that they will absorb 38% of water at 85% relative humidity.[17] The water-holding capacity of ichthyotic scales is increased by 100%, from 9 to 18% by the treatment with a urea cream (10%) for 3 weeks.[18] Addition of equal concentration of sodium chloride claims to give a synergistic effect in regard to

the water-retaining property of human skin than for any of the compounds alone at a comparable concentration.[19]

Urea is easily absorbed into the skin.[3,20] Solutions containing 20% urea have been proposed to reduce experimentally induced itching,[21] but the effect appears too weak to justify the use of more dilute preparations as antipruritics on their own.[4,5,21,22] Urea has also been proposed to influence epidermal proliferation in healthy human skin and in guinea pig ear.[23,24] After short-term contact with a saturated urea solution incorporation of 3H-thymidine in DNA was reduced and a thinning of epidermis was found. After long-term exposure to urea, lasting more than two to six weeks, no further thinning occurred, and there was no tendency for atrophy during this period. [23,24] No changes in the binding forces within stratum corneum have been found after 6 h occlusive exposure of normal skin to 10% urea.[20] Urea does not seem to influence the lipid matrix of the skin, since no influence on the transition temperatures of mouse skin lipids was found by exposure to 12% urea.[25]

19.3 CLINICAL STUDIES ON UREA-CONTAINING MOISTURIZERS

19.3.1 Psoriasis

Different types of evidence exist for the clinical efficacy of 10% urea in the treatment of psoriasis (Table 19.1). Early clinical data from a clinical study on various types of hyperkeratosis showed no superior effects on from 10% urea cream compared to ordinary aqueous cream BP in the treatment of psoriasis.[10] However, five psoriatic patients with chronic therapy-resistant lesions obtained soft and pliable skin after treatment with 10% urea, but no effect on erythema was observed.[17] Psoriatic lesions on the extremities (at least 5 cm in diameter in size) also showed clinical improvement after two weeks of treatment with an ointment containing 10% urea (Basodexan® S ointment) in a placebo-controlled study on ten patients.[26] Higher values of skin capacitance (suggested to reflect skin hydration) were also noted on urea-treated areas. Increased hygroscopicity and water content were also obtained after treatment with 10% urea ointment in patients with psoriasis vulgaris.[27] Moreover, urea treatment reduced epidermal proliferation, measured as an altered expression of involucrin and cytokeratins.[26] Treatment of psoriasis vulgaris with 10% urea-formulations support clinical efficacy at evidence-level Ib (cf. Figure 19.1).

19.3.2 Ichthyosis

The efficacy of urea in the treatment of ichthyosis has been investigated in several clinical studies. Different types of evidence exist for the clinical efficacy and the data support at least evidence-level Ib

TABLE 19.1
Treatment of Psoriasis Vulgaris with Urea-Formulations

Conc. %	*N*	Design	Results	Reference
10	5	Open	Softening effects	17
10	4	Randomized, double-blind, bilateral, versus reference	No difference to aqueous cream BP	10
10	10	Randomized, bilateral	Increased hydration compared to petrolatum	27
10	10	Randomized, bilateral, double blind, placebo	Clinical evaluation and capacitance measurement show superiority to placebo	26

TABLE 19.2
Treatment of Ichthyosis with Urea-Formulations Data

Conc. %	*N*	Design	Results	Reference
10	7	Open, bilateral	Improvement, better than control cream	17
10	17	Open, no control	Improvement	1
10	84	Randomized, double-blind, parallel, versus references	Better than other preparations	29
10	7	Randomized, double-blind, bilateral, versus reference	No difference to aqueous cream BP	10
10	14	Randomized, double-blind, bilateral	Better than placebo	18
10	30	Randomized, double-blind, bilateral, reference cream with 10% urea	Both creams effective, the one with pH 6 preferred to the one with pH 3	31
10	60	Randomised, double blind, bilateral	Better than placebo	30

(Table 19.2). In seven patients with severe ichthyosis a pronounced keratolytic effect was noticed and the skin became soft and pliable after treatment with high concentrations (about 10%).[17] After treatment with 10% urea (Calmuril®) the number of stratum corneum cell layers was reduced in 6 of 11 patients with ichthyosis.[28]

In a double-blind trial on 84 outpatients with ichthyosis vulgaris or X-linked ichthyosis a 10% urea cream (Calmuril) was statistically significantly better in controlling the clinical signs of ichthyosis than three other preparations (salicylic acid ointment, oily cream, and E45 cream, Boots).[29] The patient's assessment did not reveal any statistically significant difference between the groups, which may have been due to inexeperienced individuals. Significant clinical improvement was also noted in 14 patients with ichthyosis after treatment for 3 weeks with 10% urea compared with treatment with the base. None of the patients complained of irritation.[18] Also in 60 children with ichthyosis the improvement was more pronounced in the extremity treated with a 10% urea lotion (Laceran) than in corresponding placebo-treated extremity.[30]

Two preparations containing 10% urea on 30 patients with ichthyosis associated with atopic dermatitis improved skin conditions equally well, but both investigators and patients preferred a cream containing multisterols, phospholipids, and fatty diols (pH about 6) to the other cream (Calmuril) containing betaine and lactic acid (pH about 3).[31]

19.3.3 Atopic Dermatitis

Strong evidence exists for the clinical benefit to use urea in the treatment of atopic dry skin (Table 19.3). A 10% urea cream (Laceran, Beiersdorf, Germany) produced improvement of the xerosis and the pruritus, but somewhat less of the erythema compared to those of a base cream (Essex base cream, Schering-Plough).[32] No results from treatment of the cream base were reported. Two patients felt irritation during treatment with the urea cream and therefore dropped out of the study. A water-in-oil emulsion (Laceran lotion) with 10% urea induced significantly higher skin capacitance (indicative of increased hydration) than the corresponding placebo lotion.[33] Improvement in clinical skin condition could be observed in parallel to the increase in skin hydration.[33]

A 5% urea cream (Canoderm®, ACO Hud AB, Sweden) increased skin hydration (measured as capacitance)[34] and showed similar efficacy as a 4% urea cream also containing 4% sodium chloride as active ingredient (Fenuril®, ACO Hud AB, Sweden) in a double-blind, randomized, and parallel study on 48 atopic patients.[35] The clinical and instrumental assessment showed improvements in both groups during the treatment period.[35] In another study on atopic dry skin, the 4% urea-formulation

TABLE 19.3
Treatment of Atopic Dermatitis with Urea-Formulations

Conc. %	*N*	Design	Results	Reference
5, 10	?	Open, uncontrolled	Urea formulations increase skin hydration	37
10	18	Randomized, double-blind, bilateral	No difference to aqueous cream BP	10
10	20	Single blind, "placebo" controlled	Clinical improvement, decreased TEWL	32
10	38	Randomized, bilateral, double-blind, placebo	Clinical improvement, increased hydration (capacitance)	33
4, 5	48	Randomized, double-blind, parallel, 4% versus 5%	Clinical improvements, no difference between products	35
5	15	Bilateral, blind evaluation, untreated control	Increased hydration (capacitance) reduced susceptibility to SLS	34
4	109	Randomized, double-blind, parallel, versus 2 reference creams	Clinical improvement, urea superior	36

(Fenuril®) was superior to a glycerin-containing cream according to the clinical assessment of dryness.[36]

The level of evidence for the clinical efficacy of 4 to 10% urea in the treatment of dry skin in patients with atopic dermatitis support evidence-level Ib. A formal meta-analysis may give further support for level Ia.

19.3.4 Hand Dermatitis

One of the first clinical studies on urea in a cream was published in 1943.[4] Two hundered and twenty-five hospital personnel were given two jars of cream, one with 3% urea and one without urea, and were requested to use one on each hand. Both the investigators and the patients experienced better results with the urea cream, in that the skin seemed softer, smoother, and even whiter.[4] Patches of slight dermatitis were reported to improve by the application of urea cream.[4]

In a clinical study on cracked, chapped hands, which often occur in winter time and as a result of wet-work, the effect from a 10% urea cream (Calmurid®) was not superior to that of aqueous cream BP.[10] Two preparations containing 10% urea were found to be helpful therapeutic agents in a double-blind, bilateral study.[31] Both investigators and patients expressed preference for the cream containing multisterols, phospholipids, and fatty diols (pH of about six) to the other cream (Calmuril®) containing betaine and lactic acid (pH about three). Some patients noted burning sensations after treatment with the latter cream (Calmuril®).

Different types of evidence exist for the clinical efficacy of 3 to 10% urea in the treatment of dry and chapped hands (Table 19.4). The data support evidence-level II.

19.3.5 Other Conditions

Creams containing 3% urea ($n = 23$) or 10% urea ($n = 24$) were applied to one of the volar forearms on individuals with some evidence of dry skin for three weeks.[38] Both creams improved the skin with respect to dryness characteristics, as evaluated by a dermatologist, measurements of electrical capacitance and conductance, and tape assessments of scaling. Both creams were considered equally effective.[38]

A cream containing 4% urea (Fenuril®, also containing sodium chloride as active humectant) was significantly better than corresponding placebo in reducing dryness and scaling on 26 patients

TABLE 19.4
Treatment of Dry Hands with Urea-Formulations

Conc. %	*N*	Design	Results	Reference
3	250	Bilateral, placebo	Urea cream better	4
10	18	Randomized, double-blind, bilateral	No difference to aqueous cream BP	10
10	30	Randomized, double-blind, bilateral, reference cream with 10% urea	Both creams effective, the one with pH 6 preferred to the one with pH 3	31

with asteatosis.[19] The effect of active treatment was excellent on dryness and scaling, as judged by the doctor as well as by the patients. A 10% urea lotion[22] (Eucerin®) and 10% urea cream[39] (Eucerin®) proved to be more effective than its vehicle in another placebo-controlled bilateral study on 60 and 36 elderly volunteers, respectively, as evaluated by capacitance measurements. No difference between the treatments was noted by the patients, although both lotions reduced skin dryness and itching. Six patients reported erythema from treatment of the urea-containing lotion and two of these also experienced erythema from the vehicle. Two patients reported pruritus from the urea lotion and one from the vehicle. One patient also reported skin exfoliation.

Treatment of xerosis on the plantar surface of the feet for two weeks gave more pronounced improvement in skin roughness, fissures, and dryness by a 40% urea cream (Carmol 40) than from a 12% ammonium lactate lotion (Lac-Hydrin).[40] No change in transepidermal water loss (TEWL) was noted from urea-treatment. Both therapies showed sustained benefit during the next two weeks. Furthermore, a cream containing 10% urea and 4% lactic acid provided faster and better improvement with significantly less xerosis regression in patients with diabetes.[41]

Different types of evidence exist for the clinical efficacy of 4 to 10% urea in the treatment of dry skin (Table 19.5). The clinical data support evidence-level Ib in elderly patients with dry legs. Moreover, hyperkeratosis in feet support the use of urea (10% or more) at level Ib.

19.3.6 Combinations with Corticosteroids

Urea has also been used in combination with steroids. Only fair results were obtained in combination with 1% hydrocortisone in an open study on atopic dermatitis, disseminated neurodermatitis, hand dermatitis, and seborrheic dermatitis; and no improvement in patients with psoriasis, pustular psoriasis, solar keratosis, and perioral dermatitis.[1] However, addition of 10% urea to 0.1% betametasone cream gave superior results than the steroid cream alone in subacute atopic eczema.[43] Moreover, treatment with 10% urea cream containing hydrocortisone has been shown to be clinically better than treatment with other hydrocortisone preparations in a single-blind and bilateral study on 12 patients with atopic eczema.[17] All patients became more soft and smooth in the skin. One patient reported a burning sensation after application of the urea cream to freshly excoriated lesions, but no other side effects were noted.[17] Also 4% urea in combination with 0.5% hydrocortisone was superior to an ordinary 1% hydrocortisone cream in the treatment of dry eczema.[19] A combination of 10% urea moisturizer (Basodexan) and one 1% hydrocortisone preparation with 10% urea was evaluated in an open, uncontrolled, multicenter study on 1905 patients with atopic dermatitis.[44] Over the 12-month observational period, a total of 84% of the patients were exclusively treated with the two trial preparations and only 16% required additional treatment with other corticosteroids. Some patients experienced smarting sensations and itching, but the underlying skin disease may well have accounted for some of these problems.

TABLE 19.5
Treatment of Other Types of Dryness with Urea-Formulations

Diagnosis	*N*	Conc. %	Design	Results	Reference
Dry skin	47	3, 10	Randomized, blind evaluation, untreated control	Less scaling, improved hydration	38
Dry senescent skin	26	4 urea + 4 sodium chloride	Randomized, double-blind, bilateral, placebo controlled	Clinical improvement, urea cream better than placebo	19
Dry senescent skin	36 + 36	10	Randomized, double-blind, bilateral, placebo controlled	Clinical improvement in both groups, higher moisture values in active	39
Dry senescent skin	60	10	Randomized, double blind, bilateral, placebo controlled	Differences in skin capacitance, not clinically	22
Dry senescent skin	23	4	Randomized, bilateral, double-blind	Improved, no difference to reference cream without sodium chloride	42
Hyperkeratosis feet	18	40	Randomized, bilateral, double-blind, versus reference with 12% ammonium lactate	More rapid effect from urea	40
Hyperkeratosis feet	8	10	Randomized, double blind, bilateral	No difference to aqueous cream BP	10
Heperkeratosis feet	40	10	Randomized, bilateral, double-blind	Urea superior to vehicle	41

19.4 EFFECTS OF UREA ON THE SKIN BARRIER FUNCTION

The use of moisturizers with urea has been questioned, with reference to the risk of reducing the chemical barrier function of the skin to toxic substances.[6] Treatment of normal skin with a lipid-rich formulation (urea-free) increases skin susceptibility to the irritant sodium lauryl sulfate (SLS)[45,46] and increases skin reaction to nickel in sensitized individuals.[47] Furthermore, in studies on patients with an impaired barrier function, the composition of the moisturizer determines whether the treatment strengthens or deteriorates the barrier, although one might expect an improvement in association with improvement of the clinical signs of dryness. For example, the elevated TEWL was not normalized in cleaners and kitchen workers[48] by a lipid rich cream and in atopics treated with ammonium lactate,[49] despite clinical improvement. In addition, treatment of xerotic legs in elderly with a lotion with 15% glycolic acid increased TEWL and also the susceptibility to topically applied irritants.[50] Also in patients with lamellar ichthyosis increased TEWL was induced by treatment with 5% lactic acid combined with 20% propylene glycol.[51]

Clinical studies have also addressed the effect of urea as a penetration enhancer and barrier influencing substance. These studies will be reviewed in the following sections.

19.4.1 Normal Skin

Urea has been shown to be an efficient accelerant for the penetration of different drugs.[52–57] Increased levels of hydrocortisone, triamcinolone acetonide,[53] dithranol,[54] and retinoic acid[52] were found in various layers of isolated human skin after 1000 min of exposure time to creams containing 10 to 12% urea. Also the penetration of ketoprofen through isolated rat skin was enhanced by the addition of urea.[56] Furthermore, it has been shown that the time of onset of erythema, induced by hexyl nicotinate, was significantly reduced by simultaneous exposure to an oily cream containing urea.[55]

However, not all studies support the belief that urea is an effective penetration promoter.[25,58–61] For instance, the latency time to induce erythema was not changed by three-weeks treatment with a moisturizer containing 5% urea[62] or by pre-treatment of forearm skin with an aqueous solution of 10% urea.[59] Moreover, urea (10%) had a minimal effect on the penetration of hydrocortisone through excised human and guinea pig skin.[58] Hydrocortisone acetate was even retarded through hairless mouse skin with increasing concentrations of urea (up to 12%).[25]

Measurement of TEWL is another way to study the effect on skin barrier function by various treatments. *In vitro* measurements on piglet stratum corneum suggest that urea markedly decreases TEWL.[61] Studies on humans indicate that treatment for a limited number of days (1 to 2 days) with 5 to 10% urea appears to increase TEWL, whereas longer treatment times (10 to 20 days) decrease TEWL.[32,63,64] *In vivo* TEWL measurements have also been combined with challenge of the skin with an irritant (SLS) to elucidate possible changes in susceptibility to irritation.[63,65] These studies show that the irritant reaction after exposure to SLS was significantly lower after urea-treatment for 20 days than in the untreated skin.[63,65] A decreased susceptibility to SLS was noticed also after three applications of both 5 and 10% urea moisturizers, although this decrease in susceptibility was not preceded by a reduction in TEWL.[63] The improvement in skin barrier function due to the inclusion of urea in the formulation has been confirmed in a placebo-controlled study.[66] A significantly lower TEWL and subsequently lower skin susceptibility to SLS were found in the urea-treated skin compared to the placebo-treated skin.[66] However, no change in skin reactivity to nickel in sensitized individuals was found after three-weeks treatment with 5% urea (Canoderm).[64]

Hence, different types of evidence exist for the barrier-influencing properties of 4 to 10% urea in normal skin (Table 19.6). The data support evidence at level Ib for reduction of TEWL and decreased susceptibility to SLS. No evidence exists for decreased susceptibility to other noxious substances.

19.4.2 Diseased Skin

Topical treatment of psoriasis by 10% urea cream has been found to reduce epidermal DNA synthesis and change epidermal proliferation, without influencing TEWL.[26] However, in ichthyotic skin TEWL was slightly reduced by the application of 10% urea for three weeks.[18] Moreover, in patients with dry atopic skin, a 5% urea cream (Canoderm®) has been found to reduce TEWL on the back of the hands[35] and also to make skin less susceptible against irritation to SLS.[34] In another double-blind study on atopic patients a 4% urea-formulation (Fenuril®) was superior to a 20% glycerin-moisturizer in lowering TEWL.[36] The same 4% urea cream did not reduce TEWL in another study on atopics,[67] probably because of less efficient experimental design with too few patents. After two days of treatment of atopic patients with 10% urea cream (Laceran), TEWL tended to increase, but after seven days of treatment a significant decrease was noted in the atopic patients.[32] Furthermore, the urea treatment increased the level of some extractable lipids from the skin, which was suggested to be due to enhanced skin lipid synthesis, but might also have been derived from applied cream lipids. In dry skin of environmental origin, no influence on TEWL was noted after treatment with a 3% urea cream, whereas TEWL decreased in skin treated with 10% urea cream.[38]

In surfactant-damaged skin a 5% urea cream has been shown to promote barrier recovery.[65,66] The acceleration in barrier recovery was mainly observed as a more rapid decrease in TEWL. Furthermore, a recent placebo-controlled study proved that urea was responsible for the accelerated barrier recovery

TABLE 19.6
Influence of Urea-Treatment on Skin Barrier Function in Normal Skin

Design	*N*	Conc. %	No of days of treatment	Marker of barrier function	Results	Ref
Randomized, untreated control, blind evaluation	28	5, cream	20	Time to erythema from hexyl nicotinate	No difference	62
Bilateral, water control	10	10, solution	One exposure	Time to erythema from benzyl nicotinate	No difference	59
Not reported	20	5, 10, cream	Simultaneous exposure	Time to erythema from hexyl nicotinate	Shorter from 10%	55
Randomized, untreated control, blind evaluation	25	5, cream	20	TEWL, Nickel reactivity	Reduced TEWL, no change in reactivity	64
Randomized, bilateral, double-blind versus placebo	28	5, lotion	14	TEWL, SLS-reactivity	Reduced TEWL and reduced reactivity	66
Randomized, untreated control, blind evaluation	12	10, cream	20	TEWL, SLS-reactivity	Reduced TEWL day 10 and 20. Reduced reactivity day 20	63
Randomized, untreated control, blind evaluation	14	10, gel	20	TEWL, SLS-reactivity	Reduced TEWL day 10 and 20. Reduced reactivity day 20	63
Randomized, untreated control, blind evaluation	13	5, cream	14	TEWL, SLS-reactivity	Reduced TEWL, reduced reactivity	65
Randomized, double-blind	12	5, gel	1,5	TEWL, SLS-reactivity	Reduced TEWL, reduced reactivity	63
Randomized, double-blind	12	10, gel	1,5	TEWL, SLS-reactivity	No change in TEWL, reduced reactivity	63

and that the improved barrier function appeared to be of clinical relevance, since the susceptibility to SLS also was decreased.[66] Twice daily exposure to 15% SLS (except weekends) and the urea cream for 15 days induced a slight but significant barrier damage, measured as TEWL, but urea-treated sites appeared less damaged than the vehicle treated.

Different types of evidence exist for the barrier-influencing properties of 4 to 10% urea in dry skin disorders (Table 19.7). The data support evidence at level Ib for reduction of TEWL in ichthyosis and atopic dry skin and evidence at level II for dryness.

19.5 SIDE EFFECTS

Urea is a normal physiological metabolite and is generally regarded as nontoxic. No report on sensitization has been found, despite its wide use in dermatological preparations. In 1943, Rattner patch tested 500 hospital patients, 66 of whom had skin disease, with a 3% urea cream and found no adverse reaction.[4] Clinical and patient assessments of the use of creams with 10% urea or lower give no evidence of skin irritation with inflammation and barrier damage,[38] although occlusive exposure

TABLE 19.7
Influence of Urea-Treatment on Skin Barrier Function in Dry Skin Disorders

Disorder	Design	*N*	Conc. %	No of days of treatment	Results	Ref
Psoriasis	Randomized, double-blind, bilateral	10	10	14	Small decrease (not significant)	26
Ichthyosis	Randomized, double-blind, bilateral	14	10	21	Reduced	18
Atopics	Single blind, "placebo" controlled	20	10	7	Reduced	32
Atopic	Randomized, parallel, double-blind, 4% urea-reference	48	5	30	Reduced	35
Atopic	Randomized, parallel, three groups, glycerin-controlled	109	4	30	Reduced	36
Atopic	Randomized,	22	4	14	No change	67
Dry skin	Randomized, untreated control, bilateral	23 + 24	3, 10	21	No change from three, but decreased from ten	38
Hyperkeratosis feet	Randomized, double blind, bilateral, versus 12% ammonium lactate	25	40	14	No change in TEWL	40

to 20% urea in petrolatum for 24 h causes significant inflammation (i.e., increase in blood flow and skin thickness) and also increases TEWL.[68]

However, some patients report disagreeable skin sensations from urea treatments, like redness, stinging, and smarting sensations.[17,19,31,32,38,44,69] Application of urea to freshly excoriated areas and to skin lesions can give burning sensations.[70] This is not irritation in the ordinary sense and usually does not cause clinically noticeable damages to the skin, but the disagreeable sensations will hamper compliance, especially in children.[1] Furthermore, it may be difficult to treat sensitive body areas, for example, the face, since stinging and other side effects from topical treatment are mainly perceived in the face.[71,72]

19.6 DISCUSSION

Clinical studies demonstrate beneficial effects of some urea-containing moisturizers in the treatment of a variety of dry skin conditions. However, it may be noticed that the moisturizers show large variability in compositions, which may explain possible conflicting results. Not only the concentration of urea, but also the types of emulsions and stabilizers differ between moisturizers. Vehicle ingredients may be important for the final effect. For example, pH and the formation of ammonia

have to be taken into account in urea-containing products. Moreover, the content of emulsifiers, lipids, chelators, and preservatives may influence the effect.[73]

Evidence from at least one randomized controlled trial (level I) show that 10% urea is effective for the treatment of psorisasis, ichthyosis, and dry feet, and 4 to 10% for the treatment of dry atopic skin and senescent skin. Evidence from another well-designed clinical study (level II) supports the treatment of hand dermatitis with urea. Evidence at level I also exist showing barrier improving effects of urea in both normal and in dry skin disorders (atopic skin, ichthyosis). Furthermore, strong evidence exists (level I) for reduced susceptibility to SLS, but not to other external agents. No evidence has been found for successful treatment of seborrhoic dermatitis, perioral dermatitis, and keratosis pilaris with urea.

Moisturizers are encouraged by dermatologists to be used in normal skin to prevent the appearance of dryness.[74,75] Moisturizer therapy is also considered to offer a steroid-sparing alternative to topical corticosteroids in the treatment of atopic dermatitis.[76] However, treatment of normal skin with an emollient without urea has been shown to actually increase skin reactivity to external agents.[45–47] Furthermore, the elevated TEWL values in dry skin remained abnormal, despite clinical improvement.[48,49] In addition, TEWL and skin susceptibility increased in ichthyosis and xerotic legs by treatment with urea-free moisturizers.[50,51] This implies that moisturizers can be divided into four groups, where several of the discussed urea-containing moisturizers belong to group I (Figure 19.2).

Moisturizers that not only diminish the signs of dryness, but also improve an abnormal barrier function and prevent deterioration of a normal barrier are likely to reduce the prevalence of inflammatory dermatosis.[11,12] Thus, measurement of skin barrier function is suggested to be an intermediate biomarker (surrogate parameter) for eczema, which may be the clinical endpoint (Figure 19.3). Another example of a well-known surrogate parameter is blood pressure for the clinical endpoint stroke. However, the validity of barrier function as surrogate parameter for eczema has yet to be established, although the hypothesis of the mechanistic linkage may facilitate the development of improved moisturizers.

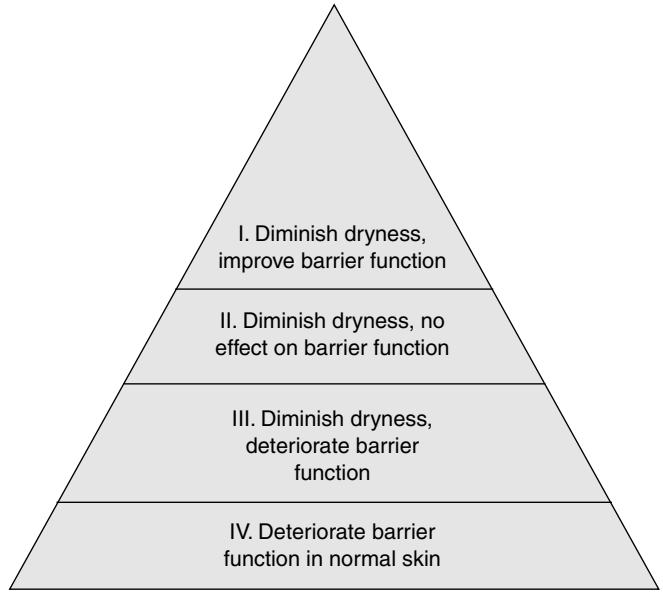

FIGURE 19.2 Moisturizers can be divided into four groups depending on their effect on dryness and skin barrier function.

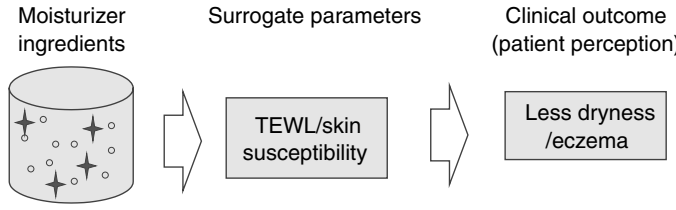

FIGURE 19.3 Measurement of changes in skin barrier function may be a relevant surrogate parameter for the prevalence of eczema.

19.7 CONCLUSION

High level of evidence shows urea-containing moisturizers to be important in the treatment of different dry skin conditions. Since the epidermal abnormality is considered a critical exacerbant of the dermatitis, urea-containing moisturizers may also reduce the prevalence of certain dermatitis by strengthening of the skin barrier function.

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20 Glycerol — Just a Moisturizer? Biological and Biophysical Effects

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CONTENTS

20.1 INTRODUCTION

20.1.1 Glycerol

Gylcerol was discovered in 1779 by the Swedish chemist Scheele and is among the most effective humectant polyols such as sorbitol and mannitol. It is a versatile chemical, and moisturization is due to its high degree of hydroxyl groups, which bind and retain water. Glycerol is found in baby care products and in embalming fluids used by morticians, in glues and explosives; in throat lozenges and in suppositories. Glycerol is a colorless, viscous liquid, and stable under most conditions. Glycerin is nontoxic, easily digested, and is environmentally safe. It has a pleasant taste and odor, which makes it an ideal ingredient in food and cosmetic applications.[1]

Moisturizing agents like glycerol have deeper effects than simply increasing the hydration of the stratum corneum (SC) structural elements. In the last years an increasing number of studies have been accomplished showing new properties of glycerol. Beside the moisturizing benefit attributed to its humectant action, glycerol prevents the SC phase transition. Furthermore, it shows a keratolytic effect by desmosome degradation, influences the protective function of the skin against irritation and penetration of substances through the SC, plasticizes the SC, reduces tissue scattering, stabilizes skin collagen, and accelerates healing processes. Even a virucidal effect of glycerol was reported. The aim of this chapter is to discuss well-known properties of glycerol and to show new aspects in research.

20.1.2 Dry Skin

Skin xerosis is related to changes in SC ceramide levels and a disturbance in their structure, as well as to an abnormalitiy in desmosome processing. The consequence of aberrant desquamation is the retention of corneodesmosomes in the superficial layers of the SC.[2] The intercorneocyte linkages are not broken and the peripheral cell does not detach during desquamation. Large clumps of cells accumulate.[3] The resultant incomplete desquamation leads to the appearance of scaly, xerotic, and eczematous skin. A causative factor in reduced corneodesmosomal degradation is the reduction in proteolytic enzyme activity, which again may be caused by intrinsic or extrinsic factors.[4]

Desquamatory proteases and other enzymes mediate their action in the lipid-rich intercellular space and need free water to be active.[3] Disturbed SC lipid structure results in reduced SC hydration and retention of corneocytes on the skin surface. Subsequently skin xerosis becomes evident due to reduced desmosome degradation.[5] The occurrence of dry skin associated with cold, dry weather may result from an extensive, elevated level of skin lipids in the solid state. Thereby, the material that maintains a higher proportion of lipid in the liquid crystalline state may be an effective moisturizer.[6]

An alteration in the generation of natural moisturizing factor (NMF) also may contribute to dry skin formation. Routine soap washing declines NMF levels at the skin surface due to leaching of NMF from superficial SC.[7] Furthermore, aged skin intrinsically has lower NMF levels than younger skin with decreased number of keratohyalin granules and filaggrin in senile xerosis.[8] Dramatic decrease in the environmental humidity reduces total free amino acid generation (and thus the level of NMF and the capacity of the SC to maintain hydration), and subsequently, induces skin surface dryness in the SC.[9]

Dry skin is further characterized by structural changes in corneocyte envelope (CE) as a result of reduced transglutaminase activity. The enzyme is responsible for the transformation of a soft or fragile envelope into a rigid one. Fragile corneocyte envelopes predominate in dry skin.[9]

Abnormalities in lipid lamellar structure or corneodesmolysis are apparent in scaling disorders like X-linked Ichthyosis, atopic dry skin, or in winter xerosis.[2,10] Susceptibility to dry skin also shows a tendency to increase with age.[3] Exposure to dry environment or extreme shifts in external humidity produces important alterations in underlying skin. Dry environment stimulates epidermal hyperplasia and early markers of inflammation. Shift in external humidity induces a profound defect in

permeability barrier function. The clinical effect of these changes ranges from xerosis to aggravation of pre-existing skin diseases.

20.2 SKIN MOISTURIZATION

20.2.1 Glycerol and Skin Moisturization

Hydration is a key function of the SC. The determinants of SC water content are believed to include the water permeability of the epidermis, the water retaining properties of the SC and the rate of evaporative water loss from the skin surface.[11] The water-retaining capacity of the SC is highly dependent on the phenoptype of the corneocytes, their spatial arrangement, the precise composition and physical packing of extracellular lipids, and the presence of highly hygroscopic compounds between and within the corneocytes.[9]

The SC moisturization is essential for a normal skin physiology. The skin itself preserves water through occlusion (water permeability barrier) and cellular humectancy (NMF). The highly developed lipid lamellae surrounding the corneocytes are a major structural element designed to keep water within the SC.[3] All these lipids are synthesized by the differentiating keratinocytes and form the lipid lamellae during cornification. Lipids help to retain NMF between the corneocytes to allow maximum moisturization of the outer layers of the SC. Effective moisturization helps again to maintain the barrier of the SC. Lipids also influence the activity of certain enzymes within the tissue.[12] Although other lipid species are present in the SC (small amount of phospholipids, glycosylceramides, and cholesterol sulphate), the major lipids are ceramides, cholesterol, and fatty acids. SC lipids are known to be influenced by genetic variation, ageing, dietary influences, seasonal effects, and environmental factors.[12]

The NMF, a mixture of amino acids, derivates of amino acids, and specific salts is a very efficient humectant due to its highly water-soluble and hygroscopic components, which allow absorption of atmospheric humidity and water.[12] Biologically, NMF allows the outermost layers of the SC to remain hydrated despite the desiccating action of the environment. Beside a structural effect due to SC plasticization, NMF also plays a critical role in facilitating key biochemical events.[9] Hydrolytic processes in the SC can only function in an aqueous or semiaqueous environment; an environment effectively maintained by the water-retaining capacity of NMF.[3] NMF is exclusively found in the SC. It is generated by a humidity-regulated proteolytic hydrolysis of filaggrin.[13] This humectant-generating pathway is activated above the stratum disjunctum, as external humidity declines, while the putative aspartate protease, cathepsin B is down-regulated in the lower SC (stratum compactum). Thus, the SC has developed an effective adjustment to environmental conditions in order to be optimally moisturized. This mechanism would not be expected to generate humectants either in the lower SC or under elevated external humidity.

Glycerol is known to diffuse into the SC[14] and retains water in the skin. The water–glycerol mixture than hydrates and plasticizes the skin to prevent dehydration and the resultant physical damage in a stressful environment. Whether glycerol in the viable epidermis can also affect the generation of new SC is not known. Alterations of the course of corneocyte synthesis might result in an SC more resistant to dehydration.[15] Batt and Davis stated, that glycerol acts due to its physical effects on the status of water in the outer layers of the SC.[14] Glycerol may interact with the SC lipid structures or proteins, altering their water-binding and hydrophilic properties.[14] Skin-moisturizing effects depend on the amount of absorbed humectant and their physicochemical properties in SC.[16] It has been reported that the excellent skin moisturization effect of glycerol is due to the high accumulation of glycerol in SC.[16,17] Glycerol forms a persisting deposit/reservoir in the depth of the SC within the lipids without disruption of liquid crystallinity and lamellar structure.[17,18] Ultrastructurally, high-glycerol (25, 40%) caused changes in human skin consisting of intracellular expansion of corneocytes and intercellular expansion between corneocytes (bulking).[18] The expansion was evident throughout the full thickness of the SC. The “bulking” is believed to enhance the resilience of skin exposed to

harsh climatic conditions (enhance barrier characteristics of the SC, which, in turn, leads to a new effective moisturization of the skin). On the other side, undiluted glycerol leads to a dehydration of the skin upon osmotic actions and produced ultrastructurally only minimal or superficial changes in the appearance of the SC.[18]

20.2.2 Glycerol and Skin Hydration

The smallest polyols [ethan-1,2-diol, glycerol and polyethylene glycols (PEGs)] are miscible with water in all proportions, that is, they have an infinite solubility in water. Cohen et al. stated that the higher the solubility of polyols, the higher the ability to absorb water.[19]

Bissett et al. investigated the effect of glycerol formulations on lower legs with dry skin. The effectiveness of glycerol was dose dependent with a maximal benefit at 20 to 40%. An important factor is the total quantity of applied glycerol.[15]

Gloor et al. observed the concentration dependency of the hydrating effect of glycerol. An increase in the dose of glycerol from 5 to 10% in an oil-in-water-emulsion improved the SC hydration and protective effect against the dehydration by tensides.[20]

Fluhr et al. presented similar results.[21] Four different vehicles (water in oil and oil in water emulsion) and two different glycerol concentrations (5 and 10%) were tested. 10% glycerol was more efficient than 5%, independent of the basic formulation. However, the o/w emulsion seemed to be more effective than the w/o formulation.[21]

Okamoto et al. investigated the skin-moisturizing effect of glycerol depending on the absorbed amount in SC and the concentration profile. The skin-moisturizing effect increased linearly with the amount of absorbed humectant in the SC and was dependent on the hygroscopicity of the humectants. A repeated application twice daily for 10 days leads to an accumulation of glycerol in SC.[16]

20.2.3 Glycerol and Hygroscopicity

Humectancy or hygroscopicity is the tendency of a substance to absorb water from the surroundings.[22] Pure glycerol for example, absorbs its own weight in water over 3 days.[11] The connection between *in vitro* humectancy and *in vivo* moisturization is not a simple correlation. Glycerol, which had the lowest humectant activity *in vitro*, from the set glycerol, diglycerol, and triglycerol, was the best eliminating the signs of skin dryness (erythema, SC hydration) in a guinea pig model.[22] The widespread concept has to be challenged that if a material is capable of absorbing water either from the environment or from the skin tissue, then it is a clinically useful moisturizer.

Froebe observed that glycerol behaves as a humectant at high humidity (92% relative humidity), but not at very low humidity (6% relative humidity).[23]

20.2.4 Gylcerol and Evaporation

Changes in transepidermal water loss (TEWL) following glycerol treatment are an instrumental evidence for skin moisturization induced by glycerol.[17] Thereby, single application tests can be predictive of long-term results with humectant-based moisturizers. Electrical measurements of skin conditions correlate well with skin grades.[24] Topical applied water produced only a transient benefit due to the rapid evaporation. Glycerol applied under controlled ambient conditions (relative humidity: not exceeding 65%, room temperature: 20°C) reduced the magnitude of the natural water flux from the skin surface and the rate of evaporation of water from applied aqueous solution.[14,17] TEWL values were significantly and persistently reduced as well as the skin surface profile roughness after treatment with glycerol compared to water treated areas.[14,17] This might be one explanation why seasonal changes caused by low relative humidity can be prevented by glycerol.[25,26]

20.3 PREVENTION OF THE SC PHASE TRANSITION

20.3.1 Skin Barrier Organization — Role of Lipids

Structure of SC and its lipid content affect the permeability barrier function. Visualization studies revealed that the penetration route across the SC resides in the intercellular tortuous pathway between the corneocytes. This implies that SC lipids play a key role in the skin barrier function.[27] Another major controlling element in barrier homeostasis seems to be the epidermal Calcium ion.[28]

In the SC lipids form two crystalline lamellar phases.[27] The mixture of both phases produces the optimal barrier to water loss from SC. The balance between the liquid crystalline and the solid crystal phases is determined by the degree of fatty acid unsaturation, the amount of water, and probably by other yet undiscovered factors. A pure liquid crystal system, produced by an all-unsaturated fatty acid mixture, allows a rapid water loss through the bilayers with a moderate barrier action. The solid system produced with an all-saturated fatty acid mixture causes an extreme water loss due to breaks in the solid crystal phase.[6,23] Studies with mixtures prepared with isolated ceramides revealed that cholesterol and ceramides are very important for the formation of the lamellar phases, and the presence of ceramide 1 is crucial for the formation of the long-periodicity phase.[27] The occurrence of dry skin associated with cold, dry weather for example, may result from an extensive, elevated level of skin lipids in the solid state. Therefore, a material that maintains a higher proportion of lipid in the liquid crystalline state may be an effective moisturizer.[6]

20.3.2 Glycerol and SC Phase Transition

Froebe reported the prevention of SC phase transition *in vitro* by glycerol. Glycerol 10% in a SC lipids mixture inhibited the transition from liquid to solid crystals even when water content was reduced by low humidity (6%). At high humidity, but not at low humidity glycerol acts as a humectant. Therefore, glycerol might act as a skin moisturizer and skin conditioner by inhibiting lipid phase transition from liquid to solid state in dry atmosphere.[23] It is hypothesized that glycerol maintains the fluidity of the lipid membrane through interaction with polar head groups of the lipid bilayers rather than by penetrating the alkyl chains.[23,29] In sum, glycerol seems to enable the skin lipids to preserve its normal structure even when underhydrated.

20.4 KERATOLYTIC EFFECT BY DESMOSOME DEGRADATION

20.4.1 Desmosomal Degradation

Desmosomes are critical structural elements for the cell–cell adhesion complex between adjacent keratinocytes. They are dynamic cell components, whose composition and structure are critical for normal epidermal function, tissue morphogenesis, and differentiation.[30,31] Regulation of desmosomal assembly and disassembly appears to include both internal and external mechanisms.[30] Calcium plays a key role in maintaining desmosomal integrity, while signal transduction between desmosomes and adherent junctions appears important to regulate their assembly and disassembly.[30] Corneodesmosomes are the main cohesive force within the SC.[32] Other components that contribute to the SC cohesion are the van der Waal's forces holding together the lipid lamellae and the corneocyte interdigitation.[32]

The cohesive forces holding the corneocytes together are progressively degraded to allow a regulated cell shedding at the surface of the skin, a process known as desquamation.[3] Thereby, the enzymatic degradation of inter-corneocyte linking structures, or a reduction in intercorneocyte forces, must occur in a carefully controlled manner in order to maintain the integrity, and thus epidermal barrier function.[3] The desquamatory enzymes are believed to be extracellular. The most important

enzymes are the stratum corneum chymotryptic (SCCE) and stratum corneum tryptic enzyme (SCTE) as well as Cathepsin D.[33,34] Their activity is pH dependant. SC lipid phase behavior will influence enzymatic activity. This indicates that the maintenance of the water content of the SC is vital for the normal orderly process of cell loss from the surface of the skin. The SC desquamatory proteases are critically influenced by water activity within the tissue, and desmoglein 1, desmocollin 1, and corneodesmosin degradation are all reduced at low environmental humidity.[35–37] In sum, insufficient SC moisturization and water content leads to defective desquamation.[12,37]

20.4.2 Glycerol and Desmosomal Degredation

Desmosomal degradation has been shown to be a humidity-dependent event. The degradation is significantly reduced at low relative humidity. Rawlings et al. demonstrated that at high (80% relative humidity) but not at low relative humidity (44% relative humidity) glycerol further enhanced desmosomal degradation. This enhanced desmosomal degradation was confirmed by decreases in levels of immunoreactive desmoglein 1, a marker of desmosome integrity. Measurements of the mechanical strength of SC sheets using an extensometer indicated a reduction in the intercellular forces following glycerol treatment. One possible explanation for the effects of high humidity and glycerol on desmosomal structure is that they influence the activity of desquamatory enzymes due to SC water regulating key proteases involved in the protein degradation. Beside the humectant properties of glycerol, the lipid-phase modulating and occlusive properties may also contribute to the improvements in SC desquamatory enzyme activity crucial to desquamatory process.[37] The increase in desmosome digestion following glycerol treatment may be important in subjects, for example, with skin xerosis. The enhanced desquamation seems to be an initial effect to detach nonphysiological scales. In the second step glycerol seems to strengthen the SC integrity.

20.5 PROTECTION AGAINST IRRITATION

20.5.1 Protection against Irritation

Bettinger et al. performed a standardized washing procedure after pretreatment with 10% glycerol-containing o/w emulsion compared to vehicle. The glycerol-containing emulsion inhibited the dehydration of washing in contrast to vehicle.[38] Grunewald et al. investigated different barrier creams including glycerol (10%) applied in an oil-in-water emulsion regarding their efficacy against repetitive washing with Sodium Lauryl Sulfate (SLS). Glycerol-containing o/w emulsion led to a protection against the barrier-damaging and irritating action of SLS. Especially the protection against skin dehydration was remarkable due to the hygroscopic effect of glycerol.[39] Bettinger et al. induced a skin damage by either tape stripping or acetone treatment and applied glycerol (70%) or tap water in an occlusive way.[40] After 5 h they compared the barrier function using biophysical tests. Glycerol associated with occlusion led to a faster reconstitution of the protective skin barrier compared to water. The reactions against DMSO, NaOH, and SLS were significantly diminished in glycerol-treated areas. Glycerol sustains the transepidermal water flow, at least partially, despite an occlusive film and leads to a faster reconstitution of the skin barrier.[40]

Fluhr et al. investigated the influence of glycerol on the recovery of damaged SC barrier function. The skin of the test sites was initially damaged by tape stripping and treated with glycerol (99.8%) (glycerol, glycerol and occlusion, occlusion alone, untreated field) for three days. Glycerol alone and glycerol with occlusion improved barrier function. Occlusion alone did not result in changes in barrier repair and SC hydration, which was controversially discussed in literature earlier.[41–45] Occlusion and glycerol together were capable to enhance moisturizing properties of the system, but not to influence the water flux through deeper layers of the SC and therefore barrier repair induced by glycerol itself. Glycerol, by absorbing water, can stimulate a water flux creating a stimulus for barrier repair. The observed effects were based on the modulation of barrier repair and were not biased by

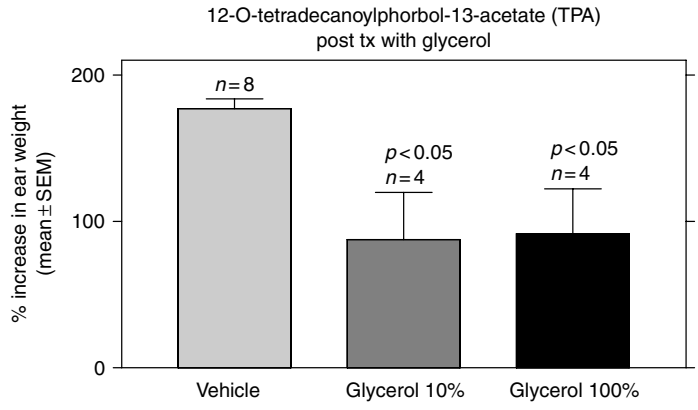

FIGURE 20.1 Both applications (10% aqueous solution and 100%) reduced the TPA-induced ear swelling significantly compared to water (vehicle) by about 50%.

the humectant effect of glycerol.[46] In the second step, an irritation was induced by repetitive washing using 2% SLS solution for 4 days (3 times daily).[46] After that the test areas were also treated with glycerol (25 and 50% glycerol, 33,3% DAC base cream, 41.7 and 16.7% water). The treatment was performed for 3 days, 3 times per day. Even 7 days after the end of the treatment with glycerol an increased SC hydration and a reduced transepidermal water loss was observed. Especially, TEWL has a great importance for repair mechanism of the epidermis after barrier damage.[46] It has been shown that glycerol protects against irritation caused by washing procedure.[38,46,47]

Unpublished data on a TPA (12-O-tetradecanoylphorbol-13-acetate) irritation model[48] could show that glycerol pretreatment prevented ear swelling in a nondose dependent way (Figure 20.1). Irritant contact dermatitis was induced by a single topical application of 10 ml of 0.03% (wt/vol. in acetone) TPA on the inner and outer surfaces of the left ears of male mice. The right ears were treated with vehicle alone (acetone). At 18 h, when TPA-induced inflammation is maximal, ear thickness was measured with a digital caliper (Mitutoyo Corp., Tokyo, Japan). Ear swelling, measured by thickness and weight, was calculated according to the following equation:

$$\text{Ear swelling } (\%) = 100 \times (a - b)/b$$

where a is the thickness or weight of treated left ear and b is the thickness or weight of control right ear. Both applications (10% aqueous solution and 100%) reduced the TPA-induced ear swelling in hairless mice significantly in comparison to water (vehicle) by about 50%.*

20.5.2 Penetration Enhancing Effect of Glycerol

Bettinger et al. described a penetration-enhancing effect of glycerol. A significant increase in hexyl nicotinate penetration on a glycerol-treated site was observed.[40] The explanation for the effect includes the interaction of glycerol with intercellular lipids, the inhibition of the lipid transformation by glycerol, the desmolytic effect of glycerol, and the hydrating effect of glycerol.[40]

20.5.3 Accelerating the Healing Processes

The restorative properties of high-glycerin therapeutic moisturizers are hypothesized to be related to a glycerol reservoir within the SC. This provides a mechanism for enhancing barrier characteristics

*This study was performed in collaboration with A.J. Sagiv, School of Pharmacy, Cell Pharmacology Unit, University of Jerusalem, Israel.

of the SC, which, in turn, leads to a new effective moisturization of the skin. The suggested role of glycerol in normalization of barrier function is essential in the healing of dry skin and in wound healing.[18]

20.5.4 Protection against X-ray and 365 nm Ultraviolet Light

It has been reported that glycerol protects bacterial cells and transforming DNA against both x-rays and 365 nm ultraviolet light. The mechanism whereby glycerol acts is unknown.[49]

20.6 INFLUENCE ON PHYSICAL PROPERTIES OF THE SKIN

20.6.1 Mechanical Properties of the Skin — the Role of the Epidermis

The epidermis plays a role in skin mechanics. Thereby, hydrophilic as well as hydrophobic substances affect mechanical properties of the skin. Changes in skin mechanics can be the result of either a direct influence of a substance on the intercellular matrix, or an epiphenomenona, for example, a physiological shift of water between the tissues aimed to maintain physiological homeostasis.[50] The hydration level of the SC affects its mechanical properties. Increased hydration of the SC influences its extensibility and elasticity.[51–54] Examples from human diseases such as ichthyotic and xerotic disorders indicate that thickening of the SC due to hyperkeratosis and increased corneocyte cohesion is responsible for a marked decrease in the flexibility of the entire SC.[55]

20.6.2 Plasticizing and Smoothing Effect of Glycerol

Batt and Fairhurst investigated the changes in SC, which occurred after application of water, occlusion (4 h), or glycerol. The hyperhydration resulting from complete suppression of TEWL by occlusion induced topographical changes on the skin surface. A general flattening of the skin surface was observed consistent with the swelling of the SC due to hydration of the tissue. Treatment with aqueous glycerol over 4 h induced a significant, long-lasting reduction in surface profile roughness for at least 20 h comparable to those observed after occlusion.[17]

In 1988, Batt et al. again observed the changes in physical properties of the SC following treatment with glycerol or water. The results showed that treatment with water produced a rapid but short-lived response characterized by a reduction in TEWL and in electrical impedance, smoothing of the skin surface profile, and an increase in the coefficient of friction. Application of glycerol-containing solutions (5 and 15%) and products (o/w cream 10% glycerol, o/w lotion 15% glycerol), in contrast, increased and extended the observed effects.[14]

Overgaard et al. investigated the short-term influence of tap water and glycerol on skin mechanics (hysteresis, distensibility, elasticity and resilient distensibility). The substances were applied on the forearms of healthy volunteers in an occlusive way for 10 min. Immediately and 10 min after removal of the test substances measurements were performed. Glycerol created a significant change of hysteresis and distensibility. Water compared to glycerol appeared to have a short-term effect on hysteresis and distensibility, marked by a pronounced increase and a fast return to baseline. Glycerol had a slower increase and a more prolonged effect suggesting that the outermost layers of the skin have been altered more substantially. It might be possible that glycerol attracts water by osmosis from the deeper layer of the epidermis.[50] The rapid onset of changes favors a more direct action but may be due to the ability of each substance to penetrate the SC.[50,56]

Pederson et al. studied the influence of water and glycerol on skin mechanics. Both substances were applied on the forearm and changes in hysteresis and distensibility were quantified.[50] They showed, in contrast to Overgaard et al., that glycerol induced a more rapid onset on the hysteresis (after 3 min) than water. The glycerol effect was detectable until the end of testing (15 min). Distensibility showed a transient increase induced by glycerol, while no changes were seen with water. Altogether, the onset of action for both substances, water and glycerol, was rapid. Therefore the effects were supposed to take place in the outermost layers of the epidermis.[50] The immediate effect of glycerol may be related to the reservoir formation, rather than to a more profound effect to the epidermis.[50,57]

Rigal and Leveque demonstrated in a long-term study a pronounced effect of 10% glycerol (o/w emulsion) regarding mechanical properties of the skin, which persisted up to one week after the treatment (treatment of three weeks).[58]

20.6.3 Reduction of Tissue Scattering

Skin is a highly complex structure consisting of many inhomogenities. Much of the light scattering in biological tissues is due to its variation in polarization, which can be characterized by variations in the index of refraction. Cellular and intercellular components contribute to the scattering properties of the skin.

Vargas et al. applied glycerol to rat and hamster skin and observed an alteration in optical properties.[59] The transmittance increased and a decrease in diffuse reflectance occurred after an application of glycerol on the dermal site of the skin *in vitro*. *In vivo* injection of glycerol allowed a better visualization of structures in the dermis. It was hypothesized that glycerol reduced random scattering primarily by localized dehydration and better index matching with collagen. Glycerol has a refractive index of about 1.47 which is similar to that of collagen.[60] Furthermore, the application of glycerol causes cells in the skin to shrink. A reduction in diameter with no change in refractive index or volume fraction would result in a decrease in scattering contribution from these cells. The complete mechanism that causes reduction in scattering is not fully understood at this time.[59]

20.6.4 Stabilization of Collagen

Native collagen binds glycerol preferentially whereas denatured collagen neither binds nor repels glycerol. The surface interaction of native collagen with glycerol is energetically more favorable than its interaction with water. Glycerol stabilizes the triple-helical structure of solubilized calf skin collagen and may lead to the inhibition of the *in vitro* self-association of monomers into fibrils.[61]

20.7 NEW ASPECTS IN RESEARCH

20.7.1 Aquaporin-3

The aquaporins are a family of small, integral membrane proteins that function as plasma membrane transporters of water and in some cases small polar solutes. There are at least 10 distinct aquaporins in mammals with specific expression patterns in epithelial, endothelial, and other tissues. Studies in aquaporin-null mice indicated a key role for aquaporins in the urinary concentrating mechanism, fluid secretion of glands, brain swelling, skin moisture, hearing and vision, and gastrointestinal absorption.[62]

The mode of action of glycerol both on SC hydration and epidermal barrier function seems to be related to the aquaporin-3 (AQP3) channel. The basal layer of epidermal keratinocytes contains AQP3, a small membrane protein that functions as a facilitated transporter of water and glycerol.[11] Glycerol is transported very slowly into the epidermis and thus its transport rate is sensitive to the intrinsic glycerol permeability of the basal keratinocyte layer.

Mice deficient in AQP3 have threefold reduced SC water content compared to wild-type mice, a reduced skin elasticity, and delayed SC barrier recovery after tape stripping.[63,64] AQP3 null mice express a more than twofold reduced glycerol content in SC and epidermis, without altered serum and dermal glycerol levels. The reduced SC hydration in AQP3 deficient mice could not be corrected by skin occlusion or placement in a humectant atmosphere, indicating that water transport through AQP3 is not a rate-limiting factor on SC hydration. Glycerol applied topically or systemically, but not glycerol analogs, corrected SC hydration defect, reduced skin elasticity and delayed barrier recovery.[11] Analysis of glycerol dynamics indicate an impaired glycerol transport into the epidermis and SC through the relatively glycerol impermeable basal keratinocyte layer resulting in reduced epidermal and SC glycerol content in AQP3-deficient mice.[11] The reduced SC glycerol content has the consequence of a decreased SC hydration due to the water-retaining (humectant) properties of glycerol. Reduced skin elasticity results directly from reduced SC hydration. Another consequence of reduced epidermal cell glycerol content is the delayed restoration of the barrier function after acute barrier disruption.[11]

20.7.2 Sebaceous Glands and Glycerol

Fluhr et al. showed that glycerol regulates SC hydration in sebaceous gland deficient (Asebia) mice.[65] The Asebia mice present normal epidermal barrier function and lamellar membranes despite the presence of sebaceous gland hypoplasia. These mice are characterized by evidence of epidermal hyperplasia, mast cell proliferation, and profound abnormalities in SC hydration (20 to 50% normal). Furthermore, the SC showed a significant depletion, but not an elimination, of nonpolar lipids of presumed sebaceous gland origin. The endogenous glycerol levels were profoundly reduced in SC as well as lipase activity in sebaceous gland structures.[65] The abnormal hydration could be corrected by topical glycerol, while sebaceous lipids, including topically-applied glycerolipids, water alone, and other humectants were ineffective. These results demonstrate the requirement for sebaceous-gland-associated lipases in the generation of the hydrating fraction of glycerol in normal skin. Glycerol generation occurs primarily within the pilosebaceous follicle, rather than at the skin surface.[65] In sum, cutaneous sebaceous glands seem to be an important source for the hydrating fraction of glycerol.

20.7.3 Glycerol and Corneocyte Surface Area

In an unpublished study we could show in an *ex vivo* assay that topical application of glycerol 9.0% compared to NaCl 2.9%, a H_2O control area and an untreated site significantly reduced the corneocyte surface area. The corneocyte surface area was assessed with the VIC method.[66] The corneocytes were collected *in vivo* using a modified detergent scrub technique.[67–69] A metal ring (diameter: 28 mm) was firmly pressed on the ventral forearm. One milliliter of Triton X-200, 0.5% (RADIM, Italy) in 0.075 M phosphate buffered saline (pH 2.5) was pipetted inside the metal ring. The skin surface was gently scrubbed for 60 sec with a Teflon™ stick. The corneocyte containing detergent solution was pipetted into a 1.5 ml Eppendorf-tube and centrifuged with 2800 rpm for 40 sec in order to concentrate the cells. The 20 μl of the cell concentrate were extracted from the bottom of the Eppendorf-tube and transferred onto a microscope slide. The liquid aliquot was dried for 5 min. A videomicroscope picture was taken with VMS 70 A Video Microscope (SCALAR, Japan) with 200× amplification. In order to get a better and standardized contrast, the microscope slice was put on a black evaluation sheet of D-Squame™. An area with well-separated corneocytes was selected and approximately 50 corneocytes on two different sites of the specimen were measured. The images were analyzed using NIH Image™ 1.59, USA on a Macintosh-PC with the same threshold for all pictures. The surface area was calculated in pixels.

Twenty-seven healthy volunteers with a mean age of 42 (range 31 to 56) were included in the study. The mean temperature during the study was 21.7°C (range 24 to 32%) and the relative humidity of 27% (Range 24 to 32%). The three aqueous solutions (Glycerol 9.0%, NaCl 2.9%, H_2O) were

swiped with a cotton wool tip for 2 min on a surface of 2 × 3 cm. The cotton wool tip was soaked with the solutions at the beginning and after 1 min.

Noninvasive measurements were performed for 2 h soaking the surface area. The SC hydration was measured with a capacitance based Corneometer CM 820, visco-elastic parameters [total extensibility (Uf) and elasticity (Ua/Uf)] were assessed using the suction device Cutometer (all instruments: Courage&Khazaka electronics GmbH, Cologne, Germany). The visco-elastic parameters were assessed as surrogate (indirect) measurements of deeper effect on SC hydration[70] while capacitance assessed the more superficial part of the hydration.[71]

The study could show that glycerol induced a shrinking of superficial corneozytes, which is independent from osmotic effects (Figure 20.2). An equimolar NaCl solution had no significant influence on the corneocytes surface area compared to untreated and H_2O treated corneocytes. Only a mild, but significant increase of SC hydration, measured by capacitance was monitored (Figure 20.3).

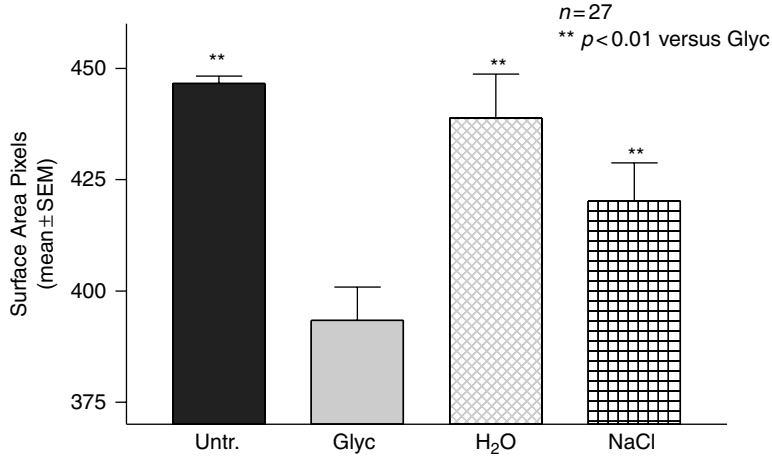

FIGURE 20.2 Glycerol induced a significant shrinking of superficial corneocytes, which is independent from osmotic effects. An equimolar NaCl solution had no significant influence on the corneocytes surface area compared to untreated and H_2O treated corneocytes.

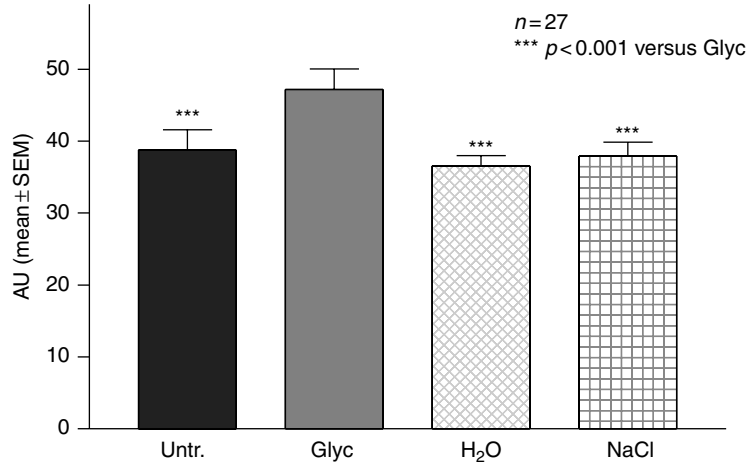

FIGURE 20.3 Only a mild, but significant increase of stratum corneum hydration, measured by capacitance was monitored.

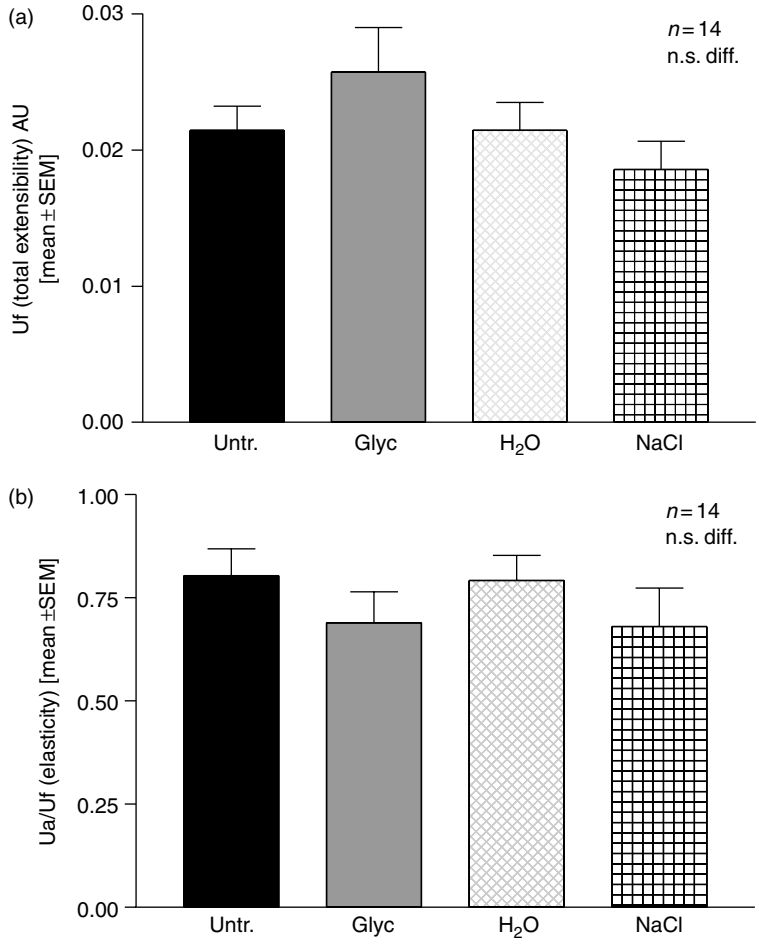

FIGURE 20.4 Deeper effects of glycerol on stratum corneum hydration could be ruled out by assessing the indirect hydration related mechanical parameters of deeper parts of the epidermis, namely the total extensibility (Uf) [Panel a] and the elasticity (Ua/Uf) [Panel b].

However a deeper effect of glycerol in our short-term model could be ruled out by assessing the indirect hydration related mechanical parameters of deeper parts of the epidermis, namely the total extensibility (Uf) (Figure 20.4[a]) and the elasticity (Ua/Uf) (Figure 20.4[b]). Long term application might induce smaller surface corneocytes resulting in a more compact SC. Subsequently this effect might be an explanation of the preventive properties of glycerol-containing formulation in irritant contact dermatitis.[47,72] The mechanisms responsible for this glycerol-specific effect of corneocyte shrinking is yet to be studied.

20.7.4 Glycerol Concentrations and Formulations

The composition of the formulation has been shown to be critical for the delivery of a maximal glycerol benefit.[3] The concentration of glycerol is important. It has been shown that glycerol is an effective moisturizer and skin conditioner when used at levels above 3%.[73] Undiluted glycerin can actually serve to dehydrate skin, based upon osmotic action. Later it was reported that even 1% glycerol has a hydrating effect, at least when applied together with bilayer-forming lipids, phospoholipids, cholesterol, and stearic acid.[5] As described by Fluhr et al. 10% glycerol was more

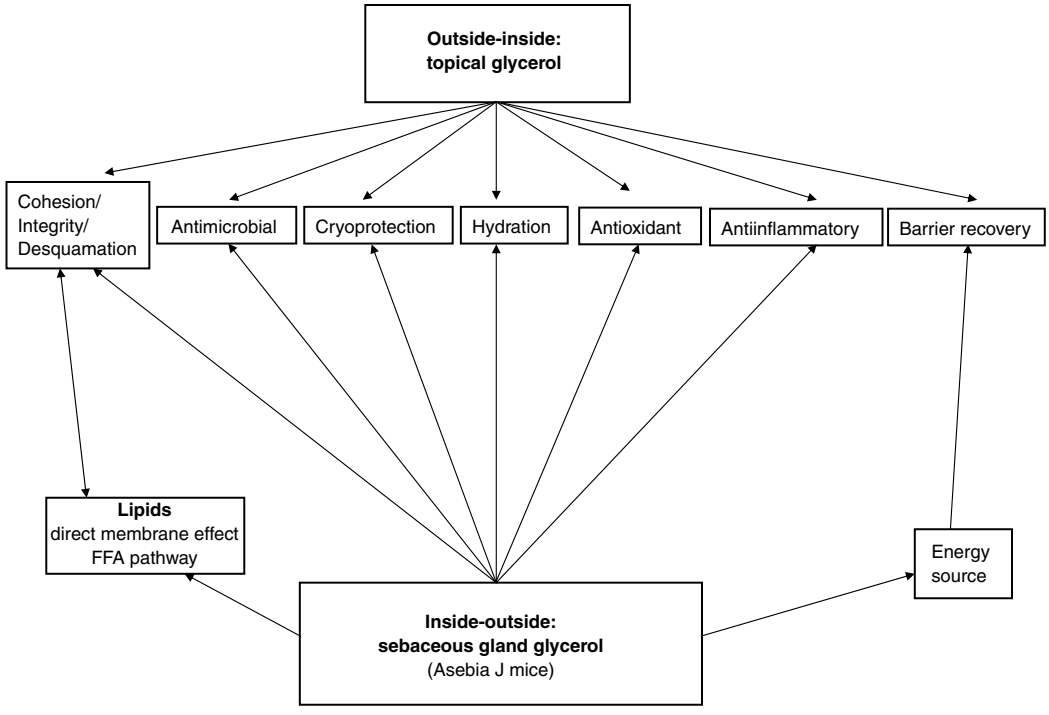

FIGURE 20.5 Current concept on functions of topical glycerol (outside–inside-concept) as well as the functions of endogenous glycerol (inside–outside-concept).

efficient than 5%, independent of the basic formulation used. And the combination of 5% glycerol and 5% urea was more effective regarding the hydrating and protective properties than 10% urea or 10% glycerol. Explanations for the observed effect included the possibility that urea enhances the penetration of glycerol into deeper layers of the SC and thus improves function of glycerol.[21] Observations performed by Bettinger and Fluhr showed that the glycerin effect was more pronounced when used in an o/w emulsion compared to a w/o emulsion.[21,74]

Treatments with glycerol in water reversed the skin dryness induced on the skin of guinea pigs using 2% SLS solution. When dissolving glycerol in medium chain triglycerides (MCT) oil, no moisturizing effect was detected. Without a certain amount of water, glycerol is probably inactive in the MCT oil. The mere presence of an adequate amount of a humectant-moisturizer in a cosmetic product is not a proof of efficiency.[75]

20.7.5 Virucidal Effect

van Baare et al. studied the virucidal action (against herpes simplex virus, poliovirus, and human immunodeficiency virus) of various concentrations of glycerol at different temperatures.[76] Glycerol has a virucidal activity. The virucidal interaction is dependent on its temperature and concentration. Glycerol might influence the enzymatic processes of nucleic acid breakdown.[76]

20.8 SUMMARY

Glycerol is a hygroscopic, nonvolatile, and viscous substance that shows special benefit as a humectant in comparison to liquid and crystalline polyols. Glycerol has been used as an effective moisturizer and humectant in cosmetic products and is recognized as an over-the-counter skin protectant.[73]

Glycerol hydrates the SC.[16,20,21] It is a humectant due to absorption of water from the atmosphere[22,23] and reduces the evaporation rate from the skin surface.[14,17,25,26] It has been shown that glycerol forms a persisting reservoir in the depth of SC (bulking).[16–18] Furthermore, it prevents lipid phase transformation.[23] Improvement of SC desquamatory enzyme activity and desquamation itself is also induced.[37] Glycerol protects against irritation caused by washing procedure,[38,46,47] tape stripping,[40,46] or acetone treatment.[40] The influence on the mechanical properties of the skin includes a plasticizing and smoothing effect of glycerol,[14,17] the reduction of tissue scattering,[14,17,59] and the stabilization of collagen.[61] Even a virucidal effect of glycerol was reported.[76] New research indicates that the mode of action of glycerol both on SC hydration and barrier function is related to the AQP3 channel.[11,62–64] Furthermore, cutaneous sebaceous glands seem to be an important source for the hydration fraction of glycerol.[65] The action of glycerol depends on the concentration[5,20,21,73] and the formulation.[21,74,75]

Glycerol is a key molecule in skin physiology in terms of its primary humectant and biosynthetic functions, and the secondary effects of increased SC hydration.[11] It is not surprising that glycerol is effective in treatment of dry skin conditions due to the fact that dry skin in the broadest sense of the words is associated with aberrant corneodesmolysis,[3] barrier lipid disruption,[3] and alterations in the generation of NMF.[7] Understanding the mechanism of action of glycerol also supports the understanding of diseases associated with dry skin, for example, ichthyosis, atopic dermatitis, winter xerosis, and other. Figure 20.5 summarizes the current concept on functions of topical glycerol (outside–inside-concept) as well as the functions of endogenous glycerol (inside–outside-concept). The latter has yet to be confirmed in *in vivo* studies with human volunteers.

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21 Hyaluronan: Key to Skin Moisture

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CONTENTS

21.1 INTRODUCTION

Skin is the tissue that interfaces with a hostile environment, and that must interpret and respond to signals from the outside world. The brain, derived from neuroectoderm, is actually a glorification of skin, performing the same basic functions as that of skin. The mechanisms that underlie the resilience of skin to the outside world, and the extraordinary ability of the skin to also protect the underlying tissues are just beginning to be understood.

Skin retains a large amount of water, and much of the external traumas to which it is constantly subjected, in addition to the normal process of aging, cause loss of moisture. The key molecule involved in skin moisture is hyaluronan (hyaluronic acid; HA) with its associated water-of-hydration. Understanding the metabolism of HA, its reactions within skin, and the interactions of HA with other skin components, will facilitate the ability to modulate skin moisture in a rational manner, different from the empirical attempts that have been utilized up to now.

Recent progress in the details of the metabolism of HA has also clarified the long appreciated observations that chronic inflammation, and sun damage caused by ultraviolet light cause premature aging of skin. These processes as well as normal aging, all utilize similar mechanisms that cause loss of moisture and changes in HA distribution.

In the past several decades, the constituents of skin have become better characterized. The earliest work on skin was devoted predominantly to the cells that make up the layers of skin: epidermis, dermis, and underlying subcutis. Now it is beginning to be appreciated that the materials that lie between cells, the matrix components, have major instructive roles for cellular activities. This extracellular matrix (ECM) endows skin with its hydration properties. The components of the ECM, though they appear amorphous by light microscopy, form a highly organized structure of glycosaminoglycans (GAGs), proteoglycans, glycoproteins, peptide growth factors, and structural proteins such as collagen and to a lesser extent, elastin. The predominant component of the ECM of skin, however, is HA. It is the primordial and the simplest of the GAGs, and one of the first ECM component to be elaborated in the developing embryo. It is the water-of-hydration of HA that comprises much of the blastocyst, the first recognizable structure in embryonic development.

Attempts to enhance the moisture content of skin, in the most elemental terms, require increasing the level and the length of time HA is present in skin, preserving optimal chain length of this sugar polymer, and inducing expression of the best profile of HA-binding proteins to decorate the molecule.

21.2 HISTORICAL PERSPECTIVE

The term "ground substance" was first used by the German anatomist Henle in 1841,[1] in describing the amorphous-appearing material between cells. It is a mistranslation of "Grundsubstanz," which would be better translated as a "basic," "fundamental," or "primordial" substance. By 1855, sufficient information had accumulated for "Grundsubstanz" to be included in a textbook of human histology.[2] The study of ground substance began in earnest in 1928, with the discovery of a "spreading factor" by Duran–Reynals.[3–7] A testicular extract was shown to stimulate the rapid spread of materials injected subcutaneously, and to function by causing a dissolution of ground substance. Thus, a new field of research was found. The active principle in the extract was later shown to be a hyaluronidase, an enzyme that degrades HA.[8,9]

Ground substance was subsequently renamed "mucopolysaccharides," a term first proposed by Karl Meyer[10] to designate the hexosamine-containing polysaccharides that occur in animal tissues, referring to the sugar polymers alone, as well as when bound to proteins. However, the term "ground substance" persisted for many years afterwards, and could be found in textbooks of Biochemistry, Dermatology, and Pathology as late as the 1970s. It is now established that HA is the predominant "mucopolysaccharide" of skin, and the major component of "ground substance."

Hyaluronan was identified by Karl Meyer[11] in 1938 as a hexuronic acid-containing material that also provided the turgor for the vitreous of the eye. The name hyaluronic acid was proposed from the Greek *hyalos* (glassy, vitreous) and uronic acid. It required 20 years however before the chemical structure of HA was established.[12] It was later found to be a present in virtually every vertebrate tissue, the highest concentrations occurring in the vitreous of the eye, in the synovial fluid in the joint capsule, and in the umbilical cord as Wharton's jelly. However, over 50% of total body HA is present in skin.[13]

The modern era of HA biology began with the realization that HA is a critical regulator of cell behavior, with profound effects on cellular metabolism, and not merely a passive structural component of the ECM. The growth of molecular genetics and progress in the human genome project has facilitated rapid development in the understanding of HA metabolism. The enzymes that synthesize HA, HA synthases (HAS), as well as the enzymes that catalyze the catabolic reaction, the hyaluronidases, are all multigene families of enzymes with distinct patterns of tissue expression. The HA receptors, exist in a myriad of forms, owing their diversity to both variant exon expression as well as to multiple posttranslational modifications. The multiple sites for the control of HA synthesis, deposition, cell- and protein-association, and degradation is a reflection of the complexity of HA metabolism. Their relationships are becoming clarified through the ability to sequence rapidly using the new techniques of molecular genetics. There promises to be an enormous increase in information, and in the understanding of HA biology, as the genes for these enzymes and proteins become sorted out.

21.3 BIOLOGY OF HYALURONAN

21.3.1 Structure

Hyaluronan is a high molecular weight, very anionic polysaccharide. It is a straight chain GAG composed of repeating alternating units of glucuronic acid and *N*-acetylglucosamine, all connected by β-linkages, GlcA$\beta(1 \rightarrow 3)$GlcNAc $\beta(1 \rightarrow 4)$, that can reach 10^7 Da in molecular size. Hyaluronan is the simplest of the GAGs, the only one not covalently linked to a core protein, not synthesized by way of the Golgi pathway, and the only nonsulfated GAG (for reviews, see References 14–17).

The β-linkage is of more than passing interest and not merely a curiosity relevant only to carbohydrate chemists. Glycogen is a polymer of α-linked glucose. Changing to a β-linkage converts the polymer to cellulose. A high molecular weight chain of β-linked *N*-acetylglucosamine is the structure of chitin. Chitin and cellulose are the most abundant sugar polymers on the surface of the earth. Yet such β-linked sugar polymers are not abundant in vertebrate tissues, and the enzymes for their catabolism exist in some suppressed state, for their substrates can survive eons of time.

Glycosaminoglycans and proteoglycans must be distinguished from "mucins," the branch-chained sugars and their associated proteins. These occur more often on cell surfaces, though they also accumulate in the intercellular "ground substance," particularly in association with malignancies. The terms are used carelessly, particularly among pathologists and histologists, and "mucin," "mucinous," "myxomatous," "myxoid," or "acid mucoproteins" unless they have been defined biochemically, may or may not refer to HA-containing materials. This problem has arisen in part because of the ill-defined or unknown nature of histochemical color reactions. A recent example of this ambiguity is the incorrect assumption that the stain Alcian blue has some specificity for HA at pH 3.0 and for the sulfated GAGs at pH 1.5.[18]

Despite the monotony of its composition, without branch points or apparent variations in sugar composition, HA has an extraordinarily high number of functions, as described below. Physicochemical studies indicate that the polymer can take on a vast number of shapes and configurations, dependent on polymer size, pH, salt concentration, and associated cations. Hyaluronan also occurs in a number of physiological states, circulating freely, tissue-associated, and bound to proteins termed hyaladherins.[19,20] The HA can be very tightly associated to hyaladherins through electrostatic interactions. The HA in the ECM of cartilage is an organizer of the matrix, the proteoglycan aggrecan and link proteins decorating the HA in a bottlebrush configuration. The Km (Michaelis constant) of such associations are of such magnitude that HA is not easily dissociated and is not in equilibrium with the HA of the surrounding loose connective tissues.

Hyaluronan also occurs covalently bound to proteins such as inter-alpha trypsin inhibitor, a plasma protein that also functions as a stabilizer of HA-rich structures,[21,22] such as the cumulus mass surrounding the mammalian ovum.[23]

The molecular domain of HA encompasses a large volume of water that expands extracellular space, hydrates tissues, and in the dermis is responsible for skin moisture. It is also a major component in the edema of the inflammatory response. Hyaluronan is capable of expanding its solvent domain over 1000 times its actual polymer volume.[24] Even at low concentrations, solutions of HA have very high viscosity.

By electron microscopy, HA is a linear polymer.[25] It is polydisperse, but usually has a molecular mass of several millions. In solution at physiological pH and salt concentrations, HA is an expanded random coil with an average diameter of 500 nm. Existing models suggest that for high molecular mass HA, super molecular organization consists of networks in which molecules run parallel for hundreds of nanometers, giving rise to flat sheets and tubular structures that separate and then join again into similar aggregates. There is strong evidence that an H_2O bridge between the acetamido and carboxyl groups is involved in the secondary structure. The hydrogen-bonded secondary structure also shows large arrays of contiguous –CH groups, giving a hydrophobic character to parts of the polymer that may be significant in the lateral aggregation or self-association, and for interaction with membranes.[26] This hydrophobic character is perhaps involved in the extrusion of newly synthesized HA chains from the cytoplasmic surface of the plasma membrane where the HAS are located, through the membrane to the exterior of the cell.[27] The unusually stiff tertiary polymeric structure is also stabilized by such hydrophobic interactions.

21.3.2 Function

Hyaluronan, despite the simplicity of its structure, has a surprisingly wide range of functions. In high concentrations, as found in the ECM of the dermis, it regulates water balance, osmotic pressure, functions as an ion exchange resin, and regulates ion flow. It functions as a sieve, to exclude certain molecules, to enhance the extracellular domain of cell surfaces, particularly the luminal surface of endothelial cells.[28] It can also function as a lubricant and as a shock absorber. Hyaluronan can also act as a structural molecule, as in the vitreous of the eye, in joint fluid, and in Wharton's jelly.

Hyaluronan promotes cell motility, regulates cell–cell and cell–matrix adhesion, promotes proliferation, and suppresses differentiation. It participates in such fundamental processes as embryological development and morphogenesis,[29,30] wound healing,[31,32] repair and regeneration, and inflammation.[33–35] Hyaluronan levels increase in response to severe stress, and in tumor progression and invasion.[36,37] Recent studies indicate that HA can also exist intracellularly.[38–40] The intracellular functions of HA are unknown.

Bursts of HA deposition correlate with mitosis.[41–43] Elevated levels promote cell detachment, in preparation for mitosis, as cells leave tissue organization, and enter the transient autonomy required for the mitotic event to occur. Cells must then degrade that HA after mitosis has occurred, to regain adhesiveness, and to reenter the "social contract." The prediction is that HA synthesis occurs as

cells enter mitosis, and that a hyaluronidase activity is activated as cells leave mitosis. The persistent presence of HA also inhibits cell differentiation,[44,45] creating an environment that instead promotes cell proliferation. The elevated levels of antiadhesive surface HA that promotes cell detachment, also permits the embryonic cell to migrate[46] or the tumor cell to move and metastasize.[47,48] The water of hydration also opens up spaces creating a permissive environment for cell movement.

Tissues that contain high molecular weight HA are unusually resistant to invasion and penetration.[49] Blood vessels are unable to penetrate joint synovium, cartilage, and the vitreous of the eye. It is also unusual for tumor metastases to develop in these structures. It may be the large size of the HA polymer that also protects such structures from invasion by parasites. The mechanism by which such high molecular weight structures resist hyaluronidase degradation, and avoid the rapid HA turnover characteristic of the rest of the body is not known. Potent hyaluronidase inhibitors may be involved, a class of molecules about which little is known.

The ECM that surrounds cells also contains variable levels of HA. It is composed predominantly of structural proteins such as collagen and elastin, as well as proteoglycans, and a number of glycoproteins. The HA content is greatest in embryonic ECM, and in tissues undergoing rapid turnover and repair. The basal lamina or basement membrane that separates dermis and epidermis is also considered an ECM structure. The basal lamina contains HA, though the precise structural position is not known. Loss of basement membrane HA in the skin of diabetic patients correlates with skin stiffness.[50]

A number of growth factors are embedded in ECM, concentrated by ECM components where they are protected from degradation. Such factors are presented to cells as mechanisms for growth control and modulators of cell function. Heparan sulfate-containing proteoglycans bind members of the FGF and EGF family,[51] while HA can bind growth factors such as TGF-beta, and also protect them from proteolytic digestion.[52] A complex picture is emerging suggesting that the two classes of GAGs, HA, and heparan sulfate, have opposing functions. An HA-rich environment is required for the maintenance of the undifferentiated, pluripotential state, facilitating motility and proliferation, while the heparan sulfate proteoglycans promote differentiation.

However, the concentration of HA in the ECM can vary widely. Even when the levels are decreased, as in areas of marked fibrosis, HA functions as an organizer of the ECM, as a scaffold about which other macromolecules of the ECM orient themselves. Diameters of collagen fibers can be modulated by levels of HA, the thinner more delicate fibers being favored in regions of high HA concentrations. In fibroblast cultures, the addition of exogenous HA to the medium decreases the diameter of the collagen fibers that accumulate (unpubl. observ.).

The ability of HA to promote cell proliferation is dependent in part on the size of the HA molecule,[53] opposite effects being achieved at high and intermediate sizes. High molecular weight HA is anti angiogenic,[49] while intermediate molecular weight HA moieties are highly angiogenic, stimulating growth of endothelial cells,[48] attracting inflammatory cells, and also inducing expression of inflammatory cytokines.[54–56] Partially degraded HA may have the opposite effect, possibly because it is no longer able to retain and release growth factors such as TGF-β.[52]

The intense staining for HA in psoriatic lesions may in part be due to partially degraded HA, and may be the mechanism for the marked capillary proliferation and inflammation that characterizes these lesions.[53,57–59] Attempts to stimulate HA deposition for purposes of promoting skin hydration must use caution that the HA deposited remain high molecular weight, by preventing free radical-catalyzed chain breaks and by carefully restricting the catabolic reactions of the hyaluronidases.

The most recent development is the realization that HA and associated hyaldherins are intracellular, and have major effects on cellular metabolism. Much of the recent advance comes from the ability to remove the ECM of cultured cells using the highly specific Streptomyces hyaluronidase. Permeabilizing such cells and using confocal microscopy makes it possible to use localization techniques for the identification of intracellular HA and its associated proteins.[38,60] Some of these

intracellular HA complexes appear to be a component of the nuclear matrix in a wide variety of cells.[39,61] They may have importance in regulating the cell cycle and gene transcription.

A vertebrate homologue of the cell cycle control protein CDC37 was recently cloned and found to be an hyaladherin,[62] as was a protein that copurified with the splicing factor SF2.[63] An intracellular form of the HA receptor RHAMM was demonstrated to regulate erk kinase activity. Changes in function of these intracellular, depending on whether or not they have HA molecules attached, confers another layer of complexity dependent on intracellular hyaluronidase enzymes.

The ability of HA to associate with itself, with cell surface receptors, with proteins, or with other GAGs[64] speaks to the versatility of this remarkable molecule. The tight regulation required for HA deposition in association with these multiple and diverse processes depends on net levels of synthesis and degradation.

Hyaluronan is generally produced in the interstitium, in the mesenchymal connective tissue of the body, and is largely a product of fibroblasts. It reaches the blood through the lymphatics. Most of the turnover of HA, approximately 85%, occurs in the lymphatic system. This remaining 15% that reaches the blood stream has a rapid turnover with a $t_{1/2}$ of 3 to 5 min, being rapidly eliminated by receptors in the liver, and also, by unknown mechanisms in the kidney.[65–67] When the hepatic or renal arteries are ligated, there is an immediate rise in the level of circulating HA.[68] Thus, humans synthesize and degrade several grams of HA daily.

During acute stress, such as in shock or with septicemia, there is a rapid rise in circulating HA.[69–72] Such HA may function as a volume expander, as a survival mechanism to prevent circulatory collapse. Some of this rapid rise in HA represents HA recruited from interstitial stores and from lymphatics, and not entirely a reflection of increased synthesis or decreased degradation.[73] However, higher plasma levels of HA do correlate with decreased turnover rates, the $t_{1/2}$ reaching 20 to 45 min in situations of acute stress.

The mean serum and plasma level in healthy young people is 20 to 40 μg/l.[74,75] This value increases with age[76,77] and probably reflects slower clearance, and decreased HA degradative capacity, though this has not been carefully investigated. Hyaluronan also increases in the circulation in liver disease, particularly cirrhosis, and in renal failure reflecting aberrant degradation,[76–80] in rheumatoid arthritis[81] and consistently in some malignancies as a result of increased tumor tissue synthesis.[82]

21.3.3 Embryonic Development

High molecular weight HA participate in many morphogenetic steps during vertebrate development. It is apparent that the polymer is critical for mammalian embryogenesis, as reviewed recently.[83,84]

Hyaluronan is prominent in the very earliest stage of embryogenesis, in maintenance of the undifferentiated state, with its removal required prior to the onset of differentiation.[85] The presence of HA inhibits the process of differentiation, permitting expansion of primordial cell masses. In organotypic cultures of rat keratinocytes, HA suppresses epidermal differentiation,[86] must be removed before the program of differentiation can be initiated.

The developing embryo is rich in HA. The HA creates the spaces permissive for fetal cell migration and proliferation. The HA concentration is high not only in the fetal circulation, but also in amniotic fluid,[87] the fetal tissues, fetal membranes,[88] and in the placenta.[89] The HA levels reach a maximum of 20 μg/ml at approximately 20 weeks of gestation, and then drop until, at 30 weeks gestation, they reach the 1 μg/ml adult-like levels. This corresponds approximately to the time when a "switch" from the scar-free fetal wound healing to the adult-like wound healing with scarring occurs.[90] The factors in the fetal circulation that support such high levels of HA synthesis have been explored and partially characterized,[91] but have not yet been isolated nor fully identified.

The neural crest cells as they pinch off from the neuroectoderm, migrate through the embryonic body in a sea of HA.[46] When these cells reach their particular destination, hyaluronidases remove the HA, and cell migration then ceases. In embryology, as parenchymal glands develop, HA can

be found in the stroma immediately ahead of the arborizing tips, creating the spaces into which the growing glands can grow.[92,93]

The classic studies of Bryan Toole and his laboratory separate embryology into two stages, a model that can be superimposed on the development of virtually all parenchymal organs and vertebrate structures: (1) a primary HA-rich phase in which undifferentiated stem cells involved proliferation and migration, followed by; (2) removal of the HA and the onset of cellular differentiation and morphogenesis.[94]

21.3.4 Hyaluronan Oligomers Have Size-Specific Activities

The extracellular high molecular weight HA polymers are space-filling molecules that hydrate tissues, and are antiangiogenic.[49] These HA polymers are also antiinflammatory and immunosuppressive.[95,96] This derives in part from the space-filling polymers' ability to prevent ligand access to cell surface receptors. The high concentrations of HA in the fetal circulation and amniotic fluid may account for much of the immunosuppression in the developing fetus.

The 20 kDa fragments, products of Hyal-2 cleavage are highly angiogenic,[97,98] and stimulate synthesis of inflammatory cytokines.[99] These HA fragments induce transcription of MMPs (matrix metalloproteases),[100] and stimulate endothelial recognition of injury.[101] Oligomers, in the 6 to 20 kDa size range, induce inflammatory gene expression in dendritic cells.[102–103] Hyaluronan fragments thus are highly angiogenic, inflammatory, and immunostimulatory. Very small HA oligosaccharides also have specific activities. Tetrasaccharides induce expression of heat shock proteins, are antiapoptotic, suppressing cell death.[104]

From these observations, it can be concluded that fragmentation of HA in the course of the catabolic pathway generates products with size-specific and widely differing biological activities, fragments that are involved in essential processes. The working assumption is that HA catabolism is a highly ordered, carefully controlled process, the mechanism for which relies on regulation of the individual enzyme activities.

21.3.5 Wound Healing

Wound healing serves as an example of the orderly regulation that would be required. The ECM in the earliest stages of wound healing is rich in HA. There is also an abundance of inflammatory cells, a necessary component for the normal process of wound repair. In the first stages, high molecular weight HA is deposited, with the ability to bind fibrinogen, one of the first reactions in clot formation.[105,106] The HA also opens up tissue spaces, facilitating polymorphonuclear leukocyte access to the wound site for removing dead tissue, debris, and bacteria. The intermediate-size HA fragments stimulate angiogenesis, followed by fibroblast proliferation. Thus, the processing of the HA molecule by the hyaluronidases are integral to the cascade of events essential to normal wound healing.

In an adult, HA levels rapidly reach a maximum and then drop rapidly,[107–109] reminiscent of the stages in embryology. Decreasing HA levels are followed by increasing amounts of chondroitin sulfate, the appearance of fibroblasts and then deposition of a collagen-rich ECM. In an adult, wound healing often results in scar formation.

In a fetus, however, up to the beginning of the third trimester, wound repair is associated with levels of HA that remain elevated, and the final result is a wound free of scar. Such observations are made in both the experimental fetal rabbit and sheep models, as well as clinically, in term infants following mid-gestational *in utero* surgery. It is on this basis that elevated HA in the wound matrix is invoked as a key to decreased scarring, contractures, and adhesions in adult wound repair. Aspects of wound healing appear to be a strategic retreat to an embryonic situation, followed by a rapid recapitulation of ontogeny.

21.3.6 Malignancy

In malignancy, HA also appears to play a critical role.[47,48,110,111] Levels of HA on the surface of tumor cells correlate with their aggressiveness.[48] In a study of tumor cell-associated HA, the proportion of tumor HA-positive cells, as well as intensity of HA staining are unfavorable prognostic factors in colorectal cancer.[112] However, over-expression of hyaluronidase also correlates with disease progression, as shown in bladder[113,114] and in breast tumor metastases.[115,116] These apparently diverse scenarios may indicate that HA and hyaluronidase are required at different stages in the multistep progression of cancer.

It is well-established that Hyal-1 is a candidate tumor suppressor gene (TSG) product, detected in many tobacco-related lung tumors,[117,118] as well as of the oral cavity and upper airways.[119] This occurs not only at the level of DNA, by homozygous deletion or loss of heterozygosity, but also at the level of RNA. Two splice variants coding for Hyal-1 are transcribed, one variant containing a retained intron that is unable to be translated.[119] The versatility of the cancer cell is such that any mechanism that eliminates an unwanted activity will be used.

However, Hyal-2 can function as an oncogene. Overexpression accelerates tumor formation.[120] It is also a cell surface receptor for some retroviruses, the envelope protein of which mediates oncogenic transformation.[121,122] Paradoxically, Hyal-2 under some conditions functions as a TSG product. Hyal-2 can accelerate apoptosis.[123] Furthermore, an adenovirus-Hyal-2 vector suppresses growth of tumor xenografts in mice.[124] Finally, Hyal-2-over-expressing clones of src-transformed fibroblasts have reduced rates of proliferation (B. Flamion, pers. commun.). Many of these apparent contradictions will become resolved, once the HA catabolic scheme is better understood.

21.4 HYALADHERINS AND RECEPTORS

21.4.1 Hyaladherins

Hyaluronan exists in a number of states in a vertebrate body. Within the ECM, it can be firmly intercalated within proteoglycans and binding proteins in a bottlebrush-like configuration. It can be bound to cells by means of cell surface receptors. Some of the HA exists in a free form circulating in the lymphatic or cardiovascular system. However, even in this relatively free form, there are a number of binding proteins that decorate HA. These are referred to collectively as hyaladherins, a term coined by Bryan Toole.[19] The hyaladherins associate with HA through electrostatic or covalent bonds.[20] It is likely that some of the unique properties attributed to HA are in fact a function of the hyaladherins that are bound to the HA. Growth factors, collagen,[125] and a myriad of other proteins have been identified.

21.4.2 CD44

There are a variety of HA-binding proteins that are broadly distributed, and with wide variations in locations, in the ECM, cell surface-associated, intracellular, both cytoplasmic and nuclear. The same molecule may occur in multiple locations. However, it is those that attach HA to the cell surface that constitute receptors. The most prominent among these is CD44, a transmembrane glycoprotein that occurs in a wide variety of isoforms, products of a single gene with variant exon expression.[126–128] CD44 is coded for by 10 constant exons, plus from 0 to 10 variant exons, all inserted into a single extracellular position near the membrane insertion site.[129] Additional variations in CD44 can occur as a result of posttranslational glycosylation, addition of various GAGs, including chondroitin sulfate and heparan sulfate. CD44 is able to bind a variety of other ligands, some of which have not yet been identified. CD44 has been shown, however, to interact with fibronectin, collagen, and heparin-binding growth factors. CD44 is distributed widely, being found on virtually all cells except red blood cells. It plays a role in cell adhesion, migration, lymphocyte activation and homing, and in cancer metastasis.

The appearance of HA in dermis and epidermis parallels the histolocalization of CD44. The nature of the CD44 variant exons in skin at each location has not been described. The ability of CD44 to bind HA can vary as a function of differential exon expression. It would be of intrinsic interest to establish whether modulation occurs in CD44 variant exon expression with changes in the state of skin hydration. Changes in the profile of CD44 variant exon expression as a result of skin pathologies also await description.

Only one of the many possible examples of the importance of CD44-HA interactions in normal skin physiology is given here. The HA in the matrix surrounding keratinocytes serves as an adhesion substrate for the Langerhans cells with their CD44-rich surfaces, as they migrate through the epidermis.[130,131] In skin pathophysiology, the effect of local and systemic immune disorders on such interactions between Langerhans cells and keratinocytes awaits explication.[132] The ability of HA oligomers of a specific size to stimulate dendritic cells was cited earlier.[102,103]

21.4.3 RHAMM

The other major receptor for HA is receptor for HA-mediated motility (RHAMM),[133,134] discovered and characterized by Eva Turley. This receptor is implicated in cell locomotion, focal adhesion turnover, and contact inhibition. It also is expressed in a number of variant isoforms. The interactions between HA and RHAMM regulate locomotion of cells by a complex network of signal transduction events and interaction with the cytoskeleton of cells. It is also an important regulator of cell growth.[135]

The TGF-β stimulation of fibroblast locomotion utilizes RHAMM. TGF-β is a potent stimulator of motility in a wide variety of cells. In fibroblasts, TGF-β triggers the transcription, synthesis, and membrane expression of not only RHAMM, but also the synthesis and expression of the HA, all of which occurs coincident with the initiation of locomotion.[136]

21.4.4 Strategies and Challenges

Both RHAMM and CD44 may be among the most complex of biological molecules, with locations in an unusually wide variety of cell compartments, and associated with a spectrum of activities involving signal transduction, motility, and cell transformation. The apparent inconsistency of observations between different laboratories regarding the receptors CD44, and RHAMM[137] reflects the subtle ways HA exerts its broad spectrum of biological effects and the myriad of mechanisms for controlling levels of HA expression and deposition. Particularly in the experimental laboratory situation, minor changes in culture conditions, differences in cell passage number, length of time following plating, variations in growth factors contained in lots of serum, or differences in stages of cell confluence have major repercussions in expression of HA, its receptors, or the profile of that decorate the HA molecule.

One of the major challenges and opportunities in Dermatology is to identify the profile of hyaladherens specific for the HA of epidermis and dermis, to characterize these proteins and to understand their function in relation to age-related changes. In an examination of skin as a function of age, the levels of HA did not decrease, as would be expected, but rather the binding of HA to tissue proteins became more tenacious, and the HA became increasingly more difficult to extract.[125,138] Another challenge is to understand how HA as a substrate for degradation by hyaluronidases is affected by associated hyaladherins. It is also reasonable to assume that the secondary structure of the HA polymer is modulated, in part, by the hyaladherins bound to it.

A CD44-deficient mouse has been obtained that has a reasonably normal phenotype,[139] suggesting that other HA receptors may substitute for CD44. These include layillin,[140] endothelium receptor (LYVE-1),[141] andothers that have now been identified using database mining approaches. A convenient tabulation of hyaladherins and HA receptors including database information has recently become available.[83]

21.5 HYALURONAN AND SKIN

21.5.1 General Observations

Hyaluronan occurs in virtually all vertebrate tissues and fluids, but skin is the largest reservoir of body HA, containing more than 50% of the total. Earlier studies on the distribution of HA in skin, using histolocalization techniques, seriously underestimated HA levels. Formalin is an aqueous fixative, and much of the soluble tissue HA is eluted by this procedure. The length of time tissue in the formalin is a variable that may explain the conflicting results that are often encountered. Acidification and addition of alcohol to the fixative causes the HA to become more avidly fixed, so that subsequent aqueous steps are unable to elute HA out of the tissue.[18]

Comparisons have been made of HA localization in skin sections fixed with acid–formalin/ethanol and conventional formalin fixation. Much of the HA, particularly in the epidermis, is eluted during the process of formalin fixation. This suggests that epidermal HA is more loosely associated with cell and tissue structures than is dermal HA. A further incubation of 24 h in aqueous buffer further increases the disparity between the acid–formalin/alcohol and the conventional fixation technique. Once the tissue has been exposed to the acid–formalin/alcohol, the HA association with tissue becomes tenaciously fixed, with little loss of apparent HA observed following additional aqueous incubation, while the formalin-fixed tissues demonstrate progressive loss of HA.

21.5.2 Epidermal Hyaluronan

Until recently, it was assumed that only cells of mesenchymal origin were capable of synthesizing HA, and HA was therefore restricted to the dermal compartment of skin. However, with the advent of the specific techniques for the histolocalization of HA, the biotinylated HA-binding peptide,[142] evidence for HA in the epidermis became apparent.[138,143–146] In addition, techniques for separating dermis and epidermis from each other permitted accurate measurement of HA in each compartment, verifying that epidermis does contain HA.[147]

Hyaluronan is most prominent in the upper spinous and granular layers of the epidermis, where most of it is extracellular. The basal layer has HA, but it is predominantly intracellular, and is not easily leeched out during aqueous fixation. Presumably, basal keratinocyte HA is involved in cell cycling events, while the secreted HA in the upper outer layers of the epidermis are mechanisms for disassociation and eventual sloughing of cells.

Cultures of isolated keratinocytes have facilitated the study of epithelial HA metabolism. Basal keratinocytes synthesize copious quantities of HA. When Ca^{++} of the culture medium is increased, from 0.05 to 1.20 m*M*, these cells begin to differentiate, HA synthesis levels drop,[148] and there is an onset of hyaluronidase activity.[149] This increase in calcium that appears to simulate in culture the natural *in situ* differentiation of basal keratinocytes parallels the increasing calcium gradient observed in the epidermis. There may be intracellular stores of calcium that are released as keratinocytes mature.

Alternatively, the calcium stores may be concentrated by lamellar bodies from the intercellular fluids released during terminal differentiation. The lamellar bodies are thought to be modified lysosomes containing hydrolytic enzymes, and a potential source of the hyaluronidase activity. The lamellar bodies fuse with the plasma membranes of the terminally differentiating keratinocytes, increasing the plasma membrane surface area. Lamellar bodies are also associated with proton pumps that enhance acidity. The lamellar bodies also acidify, and their polar lipids become partially converted to neutral lipids, thereby participating in skin barrier function.

Diffusion of aqueous material through the epidermis is blocked by these lipids synthesized by keratinocytes in the stratum granulosum, the boundary corresponding to the level at which HA-staining ends. This constitutes part of the barrier function of skin. The HA-rich area inferior to this layer may obtain water from the moisture-rich dermis. And the water contained therein cannot penetrate

beyond the lipid-rich stratum granulosum. The HA-bound water in both the dermis and in the vital area of the epidermis is critical for skin hydration. And the stratum granulosum is essential for maintenance of that hydration, not only for the skin, but also for the body in general. Profound dehydration is a serious clinical problem in burn patients with extensive losses of the stratum granulosum.

21.5.3 Dermal Hyaluronan

The HA content of the dermis is far greater than that of the epidermis, and accounts for most of the 50% of total body HA present in skin.[13] The papillary dermis has the more prominent levels of HA than does reticular dermis.[138] The HA of the dermis is in continuity with both the lymphatic and vascular systems, which epidermal HA is not. Exogenous HA is cleared from the dermis and rapidly degraded.[66]

The dermal fibroblast provides the synthetic machinery for dermal HA, and should be the target for pharmacological attempts to enhance skin hydration. The fibroblasts of the body, the most banal of cells from a histologic perspective, is probably the most diverse of all vertebrate cells with the broadest repertoire of biochemical reactions and potential pathways for differentiation. Much of this diversity is site specific. What makes the papillary dermal fibroblast different from other fibroblasts is not known. However these cells have an HA synthetic capacity similar to that of the fibroblasts that line joint synovium, responsible for the HA-rich synovial fluid (R. Stern, unpubl. exp.).

21.5.4 Changes in Hyaluronan with Aging

The HA levels are high in a fetal circulation and fall shortly after birth. After maintaining a steady level for several decades, circulating levels of HA then begin to increase again in old age.[74,77,150] Elevated levels of circulating HA are also found in the syndromes of premature aging, in progeria,[151] and in Werner's Syndrome.[152]

Increased HA levels in the bloodstream decrease immune competence.[153] Various mechanisms have been invoked. An HA coating around circulating lymphocytes may prevent ligand access to lymphocyte surface receptors.[95,96,154,155] The increased HA may represent one of the mechanisms for the immunosuppression in the fetus. The reappearance of high levels of HA in old age may be one of the mechanisms of the deterioration of the immune system in the elderly. The increasing levels of HA with aging may be a reflection of the deterioration of hydrolytic reactions, including the hyaluronidases that maintain the steady state of HA. This is a far more likely mechanism than an increase in HA synthase activity.

The increased HA that is often found in malignancy in the bloodstream[156–159] as well as on the surface of tumor cells[48] may be one of the cancer's techniques for compromising host immune function. It is the probable basis of the failure to rosette in the classic sheep red blood cell rosette test, a former laboratory procedure used to diagnose malignancy.[160,161] The rosetting failure may have been due to the HA coating on the cancer patients' lymphocyte surfaces.

Though dermal HA is responsible for most skin HA, epidermal cells are also able to synthesize HA. The most dramatic histochemical change observed in senescent skin is the marked decrease in epidermal HA.[138] In senile skin, HA is still present in the dermis, while the HA of the epidermis has disappeared entirely. The proportion of total GAG synthesis devoted to HA is greater in epidermis than in dermis, and the reasons for the precipitous fall with aging is unknown. The synthesis of epidermal HA is influenced both by the underlying dermis, as well as by topical treatments, such as with retinoic acids, indicating that epidermal HA is under separate controls from dermal HA.

In contrast with previous *in vitro*[162,163] and *in vivo*[164,165] observations, recent studies document that the total level of HA remains constant in the dermis with aging. The major age-related change is the increasing avidity of HA with tissue structures with the concomitant loss of HA extractability. Such intercalated HA may have diminished ability to take on water of hydration. This decreased volume of water of hydration HA is obviously a loss in skin moisture. An important study for the future would be to define precisely the hyaldherins, the HA-binding proteins, that decorate the HA in senile skin, and to compare that profile with that of young skin, in both the dermal and epidermal compartments. Progressive loss in the size of the HA polymer in skin as a function of age has also been reported.[166,167]

The increased binding of HA with tissue as a function of age parallels the progressive cross-linking of collagen and the steady loss of collagen extractability with age. Each of these phenomena contributes to the apparent dehydration, atrophy, and loss of elasticity that characterizes aged skin.

21.5.5 Skin Pathology Involving Hyaluronan

21.5.5.1 Photo-Aging

Repeated exposure to UV radiation from the sun causes premature aging of skin.[168] UV damage causes initially a mild form of wound healing, and is associated first with elevated dermal HA. As little as five minutes of UV exposure in nude mice causes enhanced deposition of HA (J. Thiele, B. Neudecker, and R. Stern, unpubl. exp.), indicating that UV-induced skin damage is an extremely rapid event. The initial "glow" after sun exposure may be a mild edematous reaction induced by the enhanced HA deposition. But the transient sense of well-being in a long-run extracts a high price, particularly with prolonged exposure. Repeated exposures ultimately simulate a typical wound healing response with deposition of scar-like type I collagen, rather than the usual types I and III collagen mixture that gives skin resilience and pliability. The biochemical changes that distinguish photoaging and chronological aging have not been identified.

The abnormal GAGs of photoaging are those also found in scars, in association with the changes found late in the wound healing response, with diminished HA and increased levels of chondroitin sulfate proteoglycans. There is also an abnormal pattern of distribution.[168] The GAGs appear to be deposited on the elastotic material that comprises "elastosis" and diffusely associated with the actinic damaged collagen fibers. These appear as "smudges" on H&E sections of sun-damaged skin, rather than between the collagen and elastin fibers as would be observed in normal skin.

21.5.5.2 Oxidative Stress

Reactive oxygen species or free radicals are a necessary component of the oxygen combustion that drives the metabolism of living things. Though they are important for generating the life force, they simultaneously are extraordinarily harmful. Organisms thus had to evolve protective mechanisms against oxidative stress. Over the course of evolution, different enzymatic and nonenzymatic antioxidative mechanisms were developed, such as various vitamins, ubiquinone, glutathione, and circulating proteins, for example, hemopexin. Hyaluronan may also be one such mechanism, acting also as a free radical scavenger.[169]

Sunlight (UV light) is an additional generator of harmful oxygen-derived species such as hydroxyl radicals. Such radicals have the ability to oxidize and damage other molecules such as DNA causing cross-linking and chain scission. These hydroxyl radicals may also be destructive for proteins and lipid structures, as well as ECM components such as HA. After a very few minutes of UV exposure, disturbance in HA deposition can be detected. An anomalous situation exists, therefore, that HA can both be protective as a free radical scavenger, and at the same time a target of free radical stress. This paradox may be understood by a hypothetical model in which HA

protects the organism from the free radical stress generated by the oxygen-generated internal combustion, but is itself harmed by the more toxic free radicals generated by the external world, by UV irradiation.

The generation of HA fragments by UV may underlie some of the irritation and inflammation that often accompanies long term or intense sun exposure.[170–173] As discussed above, HA fragments are themselves highly angiogenic and inflammatory, inducing the production of a cascade of inflammatory cytokines. Further complications have occurred in this assembly of metabolic attack and counter-attack reactions that have been compiled in the selective forces of evolution. Unusually high levels of antioxidants are present in skin, such as Vitamins C and E, as well as ubiquinone and glutathione. However, these precious compounds are depleted by exposure to sunlight.[174–176]

To prevent this sun-induced cascade of oxidative injuries, topical preparations containing antioxidants have been developed in the past several decades. Initially, such antioxidants were added as stabilizers to various dermatologic and cosmetic preparations. In particular, lipophilic Vitamin E has been the favorite as a stabilizing agent. However, following oxidation, Vitamin E is degraded into particularly harmful prooxidative metabolites.[177]

In the past several years, increasing concentrations of antioxidants have been used in such skin preparations, in an attempt to create complementary combinations, or to create constant recycling pairs that alternatingly oxidize and reduce each other.[178] Finally, molecules such as HA should be protected by topical antioxidants, to prevent degradation. Topical antioxidants, protecting against free radical damage as well as maintaining HA integrity, may have major effects against natural aging and photo-aging.[179–180]

21.5.5.3 Inflammation

Chronic inflammation causes premature aging of the skin, as observed in patients with atopic dermatitis. The constant inflammatory process leads to decreased function of the skin barrier, accompanied by loss of skin moisture. Presumably, the skin of such patients contains decreased levels of HA. Alternatively, the HA may reflect that found in chronological aging, with a change in the ability to take on water of hydration with enhanced association with tissue structures and loss of extractability. Demonstration of such changes and the precise histolocalization of this decreased HA deposition would be of intrinsic interest, a study that has not been performed yet.

The acute inflammatory process is associated initially with increased HA levels, the result of the cytokines released by the polymorphonuclear leukocytes, the predominant cells of the acute inflammatory process. The erythema, swelling, and warmth of the acute process are followed later by the characteristic dry appearance and the formation of wrinkles. The precise mechanisms are unknown, but may relate to the differences between acute and chronic inflammatory cells and the attendant chemical mediators released by such cells. Alternatively, initiation of a wound healing response, with collagen deposition, may be a mechanism invoked for the premature aged appearance of the skin in chronic inflammation.

21.6 HYALURONAN METABOLISM

21.6.1 Hyaluronan Synthases

A single enzyme is now recognized as being able to synthesize HA, dual-headed transferases that utilize alternately the two UDP-sugar substrates, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine. The HA cytoplasmic product is extruded through the plasma membrane into the extracellular space by means of an ABC transporter system (P. Prehm, pers. commun.) that permits unconstrained polymer growth. Such growth could not occur in the Golgi or on the endoplasmic reticulum where most sugar polymers are synthesized, without destruction of the cell. There are three synthase genes in

the mammalian genome, coding for *HAS-1*, *-2*, and *-3*. They are differentially regulated, with each producing a different size polymer (for review, see[181,182]).

Sequence data of the HAS isoforms suggest that they contain seven membrane-associated regions and a central cytoplasmic domain possessing several consensus sequences that are substrates for phosphorylation by protein kinase C.[181,182] The ABC transporter system proteins required for HA transport through the plasma membrane are encoded at a chromosomal region immediately adjacent to the HA synthase genes (P. Prehm, pers. commun.).

In situ expression of the *HAS-1* and *-2* genes are up-regulated in skin by TGF-β, in both dermis and epidermis, but there are major differences in the kinetics of the TGF-β response between *HAS-1* and *-2*, and between the two compartments, suggesting that the two genes are independently regulated. This also suggests that HA has a different function in dermis and epidermis.

Stimulation of HA synthesis also occurs following phorbol ester (PMA) and PDGF treatment, although a direct effect on HAS has not been demonstrated. Glucocorticoids induce a nearly total inhibiton of HAS mRNA in dermal fibroblasts and osteoblasts.[183] Extracts of dermal fibroblasts indicate that *HAS-2* is the predominant HA synthase therein. This may be the molecular basis of the decreased HA in glucocortcoid-treated skin. However, an additional effect on rates of HA degradation has not been examined.

The parallels among chitin, cellulose, and HA structures, all being β-chains of hexose polymers are reflected in the striking similarity in sequence between the HAS from vertebrates, cellulose synthases from plants, and chitin synthases from fungi. A primordial ancestral gene must have existed from which all of these enzymes evolved that are involved in the biosynthesis of all polymers that contain β-glycoside linkages, an ancient β-polysaccharide synthase.

21.6.2 Hyaluronidases

Hyaluronan is very metabolically active, with a half-life of 3 to 5 min in the circulation,less than one day in skin, and even in an inert a tissue as cartilage, the HA turns over with a half-life of 1 to 3 weeks.[66,67,184] This catabolic activity is primarily the result of hyaluronidases, endoglycolytic enzymes with a specificity in most cases for the β1–4 glycosidic bond.

The hyaluronidase family of enzymes have, until recently, been neglected,[185] in part because of the great difficulty in measuring their activity. They are difficult to purify and characterize, are present at exceedingly low concentrations, have very high specific activities that are unstable in the absence of detergents and protease inhibitors during the purification procedures. Once purified, these enzymes appear to be perfectly stable. New assay procedures have now facilitated their isolation and characterization.[149,186] The human genome project has also promoted explication at the genetic level, and a virtual explosion of information has ensued.

The hyaluronidases fall into three classes[187] based on the analyses of their reaction products: (1) Bacterial hyaluronidases (EC 4.2.99.1) are endo-β-acetyl-hexosaminidases that function as eliminases yielding disaccharides. In marked contrast with their eukaryotic counterparts, they are specific for HA. (2) Endo-β-glucuronidase types of hyaluronidase (EC 3.2.1.36) found in leeches, crustaceans,[188] and some parasites, generate tetra- and hexa-saccharide end products. (3) The mammalian-types of hyaluronidase (EC 3.2.1.35) are also endo-β-acetyl-hexosaminidases, but function as hydrolases, with tetrasaccharides as the predominant end-product. They lack substrate specificity, able to digest chondroitin sulfates (CS), though at a slower rate. In addition, they have transglycosidase activity that generates complex cross-linked chains *in vitro*. This ability has not been documented *in vivo*.

Six hyaluronidase-like sequences are present in the mammalian genome, resulting probably from two duplication events, resulting in three genes, followed by en masse block duplication, generating six hyaluronidase genes. All are transcriptionally active with 0unique tissue distributions. In a human, three genes (*HYAL1, HYAL2*, and *HYAL3*) are found tightly clustered on chromosome

3p21.3, coding for Hyal-1, Hyal-2, and Hyal-3. Another three genes (*HYAL4, PHYAL1* (a pseudogene), and sperm adhesion molecule1 (*SPAM1*)) are clustered similarly on chromosome 7q31.3. They code respectively for Hyal-4, a pseudogene transcribed but not translated in a human, and PH-20, the sperm enzyme.[189,190] The enzymes Hyal-1 and Hyal-2 constitute the major hyaluronidases for HA degradation in somatic tissues, and are the only ones considered here.

Hyal-1, an acid-active lysosomal enzyme, was the first somatic hyaluronidase to be isolated and characterized.[191,192] It is a 57 kDa single polypeptide glycoprotein that also occurs in a processed 45 kDa form, the result of two endoprotease reactions. The resulting two chains are bound by disulfide bonds. This is not a zymogen-active enzyme relationship, since the two isoforms have similar specific activities. Why two forms should occur is unknown. Only the larger form is present in the circulation, while both isoforms occur in urine,[193] in tissue extracts, and in cultured cells. Why an acid-active hyaluronidase should occur in plasma is not clear. Some species do not have detectable enzymatic activity in their circulation,[194] but an inactive 70 kDa precursor form of the enzyme is present in such sera, detectable by Western blot (L. Shifrin, M. Neeman, and R. Stern, unpubl. data). Hyal-1 is able to utilize HA of any size as substrate, and generates predominantly tetrasaccharides.

A human genetic disorder with absent Hyal-1 activity has been identified.[195,196] The syndrome is characterized by short stature, generalized cutaneous swelling, transiently painful soft tissue masses over articular surfaces, and bilateral joint effusions. Histological findings include macrophages from these lesions filled with numerous membrane-bound vacuoles that contained dense flocculent material. Fibroblasts also contained such filled vacuoles, though in lower quantities than tissue macrophages. Plasma HA was 1 to 2 mg/l, 40 times normal, which interestingly, was comparable to plasma HA in mice with deletion of the Hyal-1 gene (A. Csóka, G. Frost, and R. Stern, unpubl. observ.).

Hyal-2[197,198] is also acid-active, anchored to plasma membranes by a GPI (glycosylphosphatidylinositol)-link. Hyal-2 occurs also in a processed soluble form. Again, the difference in function between the two isoforms is not known. Hyal-2 cleaves high molecular weight HA to a limit product of approximately 20 kDa, or about 50 disaccharide units, while Hyal-1 is able to digest the high molecular weight polymer to a limit digestion product consisting predominantly of tetrasaccharides. Hyal-1 and -2 have similar structures, and the difference in their reaction products requires explanation.

The biological properties of HA in aqueous solution is controlled by reversible tertiary structures, as defined by NMR spectroscopy. Evidence suggests a β-pleated sheet-like array stabilized by H- and hydrophobic bonds. Easy transitions between secondary and tertiary structures occur that are convenient mechanisms for switching between functions. The 20 kDa or 50-disaccharide unit is around the size at which such stable tertiary structures are expected to form.[199,200] Polymers greater than 20 kDa provide the preferred substrate for Hyal-2. The enzyme cleaves at a much slower rate once the HA substrate loses tertiary structure. The hyaladherins may also provide additional substrate specificity.[201] The array of hyaladherins that bind to tertiary HA structures may differ from those that bind to HA chains with exclusively secondary structure. The substrate specificity of Hyal-2 may depend on a combination of differences in bound hyaladherins and on secondary versus tertiary structure.

21.6.3 Hyaluronidase Inhibitors

21.6.3.1 Macromolecular Inhibitors

The extraordinarily rapid turnover of HA in tissues suggests that tightly controlled modes exist for modulating steady state levels of HA. The HA of the vertebrate body is of unique importance, and rapid increases are required in situations of extreme stress. Rapid turnover of HA in the normal state

indicates constant synthesis and degradation. Inhibition of degradation would provide a far swifter response to the sudden demand for increased HA levels than increasing the rate of HA synthesis. The ability to provide immediate high HA levels is a survival mechanism for the organism. This might explain the apparent inefficiency of rapid rates of HA turnover that occur in the vertebrate animal under basal conditions. It can be compared to the need to suddenly drive an automobile much faster in the case of an emergency, not by stepping on the accelerator, but by taking a foot off the break.

If inhibition of HA degradation by hyaluronidase occurs, then a class of molecules that have not been explored, the hyaluronidase inhibitors, are very important. It can be postulated that with extreme stress, hyaluronidase inhibitors would be found in the circulation as acute phase proteins, the stress response products synthesized by the liver. These would prevent the ever-present rapid destruction and allow levels of HA to quickly increase.

Circulating hyaluronidase inhibitor activity has been identified in human serum over half a century ago.[202,203] Modifications in levels of inhibitor activity have been observed in the serum of patients with cancer,[204,205] liver disease,[206] and with certain dermatological disorders.[207] This area of biology is unexplored, and though some early attempts were made,[208–210] and even though a review appeared,[211] these hyaluronidase inhibitors have never been isolated nor characterized at a molecular level.

Cultured cells secrete hyaluronidases into the culture media, away from the cells. Such a phenomenon does not occur within tissues. The production of unopposed hyaluronidase activity would cause great havoc in tissues. Simultaneous deposition of hyaluronidases and their inhibitors is a reasonable scenario, one that parallels control of the matrix metalloproteinases by their TIMPs (tissue inhibitors of MMPs).

Inhibitors of mammalian origin, such as the serum inhibitor or heparin, are far more potent than the relatively weak inhibitors of plant origin. Hyaluronidase inhibitors of animal origin would provide a means for enhancing levels of HA in skin, and represent an important research area in attempting to enhance skin moisture.

21.6.3.2 Low Molecular Weight Inhibitors

Classes of lower molecular weight inhibitors of hyaluronidase have been identified, some of which come from folk medicines, from the growing field of ethnopharmacology. Some antiinflammatories as well as some of the ancient beauty aids and practices for freshening of the skin may have some of these compounds as the basis of their mechanism of action.

Those that have been identified in recent times include flavonoids,[212–214] aurothiomalate,[215] hydrangenol,[216] occurring in the leaves of Hydrangea, tannins,[217] derivatives of tranilast,[218] curcumin,[219] an extract of the spice turmeric, glycyrrhizin,[220] found in the roots and rhizomes of licorice (*Glycyrrhiza glabra*), used as an effective antiinflamatory agent in Chinese medicine.

Clinically, heparin used as an anticoagulant, has potent antihyaluronidase activity,[221] as does indomethacin,[222,223] a classic nonsteroidal antiinflammatory agent, and salicylates.[224]

More recently, dextran sulfate[225] and a derivative of Vitamin C, L-ascorbic acid-6-hexadecanoate (A. Botski, et al., pers. commun.), have been shown to be potent inhibitors.

21.6.4 Nonenzymatic Degradation

The HA polymer can be degraded nonenzymatically by a free radical mechanism,[226] particularly in the presence of reducing agents such as thiols, ascorbic acid, ferrous, or cuprous ions. This mechanism of depolymerization requires the participation of molecular oxygen. The use

of chelating agents in pharmaceutical preparations to retard free radical catalyzed scission of HA chains has validity. However, a carefully monitored effect of such agents on HA chain length in human epidermis has not been attempted. Whether such agents can also affect the integrity of dermal HA in protecting them from free radical damage, and whether these agents have any substantial effect on the moisturizing properties of skin HA remain important questions to be answered.

21.6.5 A Scheme for Hyaluronan Metabolism

It is well established that HA is taken up by cells for degradation[227] through the CD44 receptor.[228,229] The high molecular weight extracellular polymer is tethered to the cell surface by the combined efforts of CD44 and the GPI-anchored enzyme Hyal-2. The hyluronan-CD44-Hyal-2 complex is enriched in specialized microdomains. These are invaginations of the plasma membranes composed of cholesterol and gangliosides termed lipid rafts, significant because they also recruit a large number of key signaling molecules. One category of lipid rafts is caveolae, structures rich in the proteins caveolin and flotillin. Hyal-2 interacts with CD44 and with a Na^+-H^+ exchanger termed NHE1 that creates an acidic microenvironment for the acid-active hyaluronidase enzyme.[230] The HA is cleaved to the 20 kDa limit products corresponding to about 50 disaccharide units.

The CD44, a multifuntional transmembrane glycoprotein that is the predominant HA receptor, is expressed in a number of different isoforms. The variant exons of CD44 specifically involved in the interaction with Hyal-2 and NHE1 in the process of HA binding, uptake, and degradation have not been determined. The Hyal-2-generated hyaluronan fragments are internalized, delivered to endosomes, and ultimately to lysosomes, where Hyal-1 degrades the 20 kDa fragments to small disaccharides. Two lysosomal β-exoglycosidases, β-glucuronidase and β-*N*-acetyl-glucosaminidase, participate in this degradation.

Evidence for the latter comes from human I-cell disease. Fibroblasts from patients with I-cell disease, lacking the mannose receptor pathway for lysosomal enzyme uptake, have an apparent HA storage disorder and stain intensely for HA (R. Stern and B. Steinmann, unpubl. observ.). The tetra- and hexa-saccharide products of HA degradation are too small to be detected by the HA-binding peptide staining reaction. This suggests that the β-exoglycosidases participate actively in the degradation of 20 kDa HA fragments all along the catabolic cascade, and not only at the terminal steps.

The specific defect in I-cell disease is the enzyme *N*-acetylglucosamine-1-phosphotransferase, an enzyme essential for the synthesis of the mannose-6-phosphate recognition marker that targets enzymes to lysosomes. Failure of this enzyme causes misrouting of most newly synthesized lysosomal enzymes. Plasma from patients with I-cell disease have normal levels of Hyal-1, but elevated levels of the two β-exoglycosidases,[231] suggesting that Hyal-1 is transported to lysosomes by a pathway different from the mannose-6-phosphate route. Without the β-exoglycosidases, larger sized HA oligosaccharides appear to accumulate in lysosomes. What may be missing is the trimming of these HA fragments to a size sufficiently small to diffuse out of lysosomes into the cytoplasmic compartment. It may be that only monosaccharides are able to diffuse out of lysosomes. Alternatively, HA fragments may leave the lysosome through specific transporters, as other metabolites do, such as amino acids and other sugars. Such putative transporters may have certain size restrictions, explaining why the larger HA fragments in I-cell disease cannot exit lysosomes. Regardless of the mechanism, it appears that oligomers that stain with the HA binding peptide reaction accumulate in I cell disease as a result of backup within the lysosomes, a phenomenon that does not occur in normal fibroblasts.

A scheme for hyaluronan catabolism is presented below (Figure 21.1).

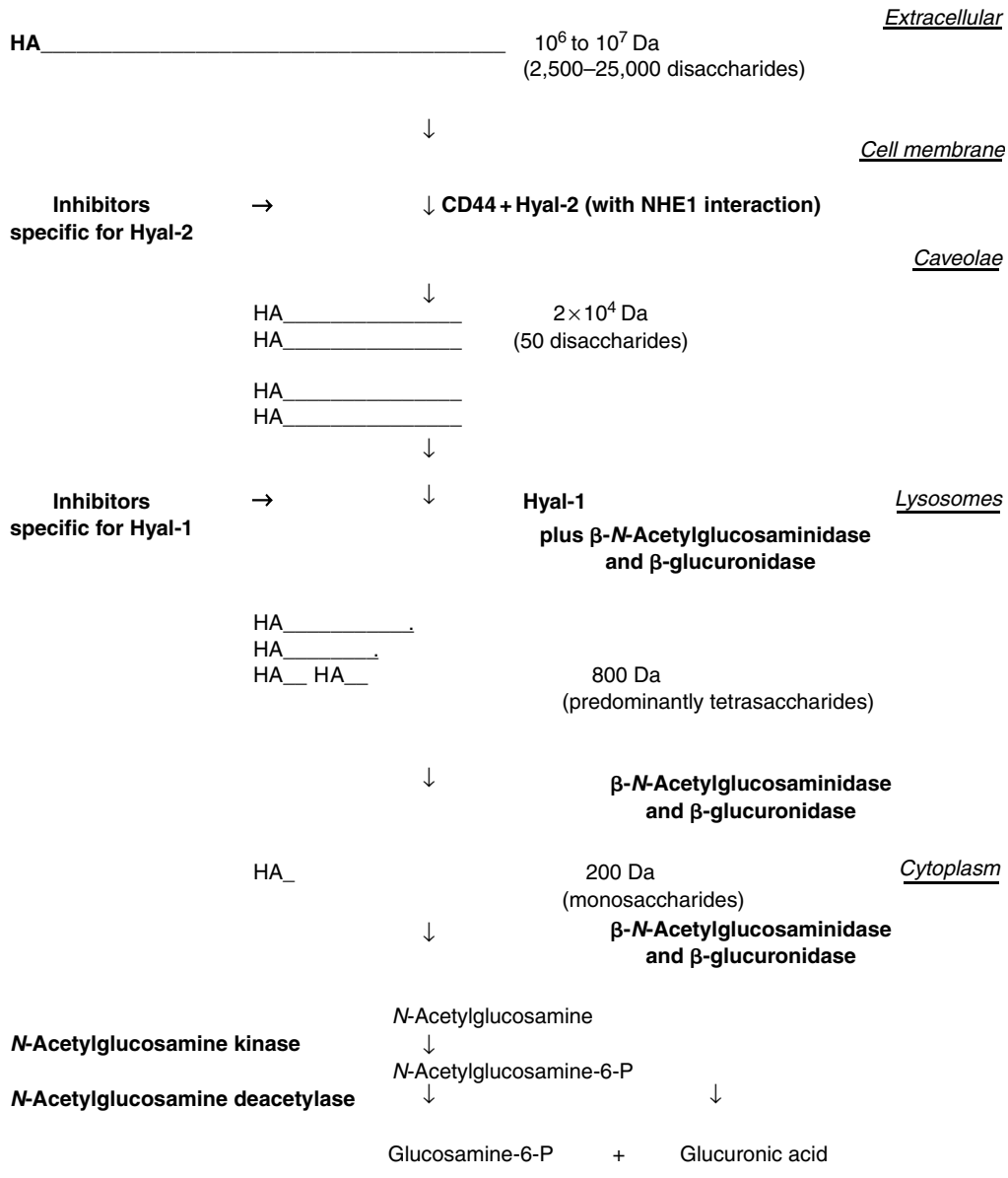

FIGURE 21.1 Scheme for Hyaluronan Catabolism. (Reprinted by the courtesy of the European Journal of Cell Biology, Elsevier Press.)

21.6.6 The Hyluronasome, A New Mini-Organelle

Based on the observations described above, it is possible to invoke a new mini-organelle specific for HA metabolism, termed the hyaluronasome. Parallels between glycogen and HA metabolism are the basis of this formulation. A glycogen mini-organelle occurs in both liver and muscle tissues. The hyaluronasome may resemble the glycogen granule, each involved in the metabolism of large carbohydrate structures, glycogen being a branched chain polymer of α-linked sugars, and HA, a straight chain polymer of β-linked sugars.

> Readily visualized by the electron microscope, glycogen granules appear as bead-like structures localized to specific subcellular locales. Each glycogen granule is a functional unit, not only containing carbohydrate, but also enzymes and other proteins needed for its metabolism. These proteins are not static, but rather associate and dissociate depending on the carbohydrate balance in the tissue. Regulation takes place not only by allosteric regulation of enzymes, but also due to other factors, such as sub-cellular location, granule size, and association with various related proteins.[232]

Such observations may be applicable to the hyaluronasome. A multiprotein membrane-associated complex that contains HA synthetic activity has been described.[42,233] This hyaluronate synthase complex may be a component of the hyaluronasome, containing synthetic as well as catabolic activities, a functional unit that could provide response mechanisms dependant on the metabolic state of the cell.

Suggestive evidence comes from several sources. Cultured cells treated with low concentrations of hyaluronidase increase their levels of HA synthesis.[234–236] Treatment of isolated membrane preparations with low concentrations of hyaluronidase has a similar effect.[234] This is compatible with a feedback mechanism enabling cells to sense levels of HA that have been synthesized. Exogenously added hyaluronidase cleaves newly synthesized HA chains as they are being extruded through the plasma membrane,[27] informing the cell that inadequate amounts of HA have been synthesized. The hyaluronasome, lying just under and partially embedded within the plasma membrane, could rely on a servomechanism using a receptor such as CD44 for relaying such feedback messages. Higher levels of hyaluronidase modulate the profile of expression of CD44 variant exons,[237,238] thus providing the exquisite controls necessary for such regulatory mechanisms.

Levels of HA that cells deposit must respond to various physiological states including growth phase,[239] confluence, inversely related to cell density in both fibroblasts,[240] and keratinocytes,[241] mitosis and cell detachment from the substratum,[242] calcium concentrations,[149,243] anoxia and lactate,[244] viral transformation,[245] and serum stimulation.[91,246] Preliminary immunolocalization data indicate that some of the HAS and hyaluronidases colocalize (A. Spicer et al., pers. commun.). All of this evidence supports, albeit indirect and tentative, the existence of the hyaluronasome structure.

The hyaluronasome, because of its ability to respond to extracellular events as well as to the intracellular metabolic state of the cell may contain HA receptors such as RHAMM and CD44, HA synthase enzymes, the hyaluronidases, hyaluronidase inhibitors,[247,248] the β-exoglycosidases, and HA-binding proteins such as HABP1.[249]

The hyaluronasome can regulate levels of HA deposition with great precision by allosteric regulation of the enzymes contained therein utilizing not only hyaladherins and related proteins, but perhaps by posttranslational modifications such as phosphorylation and sulfation. Levels of specific phosphorylated proteins are utilized in the analysis of signaling transduction pathways. However it was the phosphorylases that degrade glycogen and related proteins of glycogen catabolism that provided the paradigm for protein phosphorylation as a control mechanism.[250] Similar modifications applied to the control of HA catabolism would be in that tradition. However, it must be maintained that the existence of the hyaluronasome is highly speculative. Extensive immunochemical and histolocalization studies are required to establish such a mini-organelle.

21.7 MODULATING HYALURONAN DEPOSITION

21.7.1 The Lactate Effect

Markedly enhanced levels of HA occur in the stroma surrounding malignant tumors. The HA stimulates cell motility and hydrates tisues, creating spaces into which tumor cells can move in the process of invasion and metastatic spread. Lactate is usually the product of anaerobic metabolism. However, cancer cells produce lactate even when oxygen is abundant. The ability of malignant cells to generate lactate, even in the presence of sufficient quantities of oxygen is known as the Warburg effect. We

postulated that an increased HA in peritumor stroma might be a response to the lactate produced by the tumor. In this way, the host is commandeered to participate in its own destruction by the malignancy.

As described above, early in wound healing, there is an increase in HA. This transient increase correlates with hypoxia and the production of lactate that follows the compromised local blood supply. A cause and effect was documented in this laboratory between enhanced levels of HA and lactate production.[251,252] Lactic acid is an alpha-hydroxy acid, the latter being a frequent additive to skin preparations. Enhanced HA deposition and the attendant water-of-hydration may be a common mechanism for the enhanced appearance of skin when such lotions are used.

21.7.2 Alpha-Hydroxy Acids

Fruit compresses have been applied to the face as beauty aids for millennia. The alpha-hydroxy acids contained in fruit extracts, tartaric acid in grapes, citric acid in citrus fruits, malic acid in apples, mandelic acid in almond blossoms and apricots are thought to be active principles for skin rejuvenation. Such alpha-hydroxy acids do stimulate HA production in cultured dermal fibroblasts (unpubl. exp.). The results of such alkaline preparations may depend more on their peeling effects rather than on the ability of alpha-hydroxy acids to stimulate HA deposition.

Lactic acid,[253,254] citric acid,[253,255] and glycolic acid,[253,256–258] in particular, though frequent ingredients in alpha-hydroxy-containing cosmetic preparations, have widely varying HA-stimulating activity in the dermal fibroblast assay. Some of these mildly acidic (pH 3.7 to 4.0) preparations may owe their effectiveness to their traumatic peeling, astringent properties, with constant wounding of the skin. The cosmetic effects of these preparations of alpha-hydroxy acids, including lactic acid, involve increased skin smoothness with the disappearance of lines and fine wrinkles.

Long-term use, however, results in thickening of the skin, in both the epidermal and papillary dermal layers, because of a mild fibrous reaction. This results from a reaction similar to diffuse wound healing, and explains the increased thickness and firmness of both dermis and epidermis. The increased collagen deposition documented in skin after prolonged use is consistent with a wound healing effect.[259] Preparations of alpha-hydroxy acids, as would have been found in the fruit compresses of ancients have yet to find current cosmetic equivalents, though such vehicles are actively being sought.[260]

21.7.3 Vitamin C

The structure of ascorbic acid resembles an alpha-hydroxy acid, which is generally not appreciated. Ascorbic acid is present in most fruits, and may underlie some of the effects attributed to fruit extracts. Vitamin C has pronounced HA-stimulating effects in the fibroblast assay. But its antioxidant activity confounds the effects it may induce. The deposition of HA is stimulated when Vitamin C is added to cultured fibroblasts. The most profound changes occur in the compartmentalization of HA. The preponderance of the enhanced HA becomes cell-layer instead of being secreted into the medium.[240,261] The chemical reactions catalyzed by ascorbic acid that bind HA to cell or matrix components are not known.

As aforementioned, derivatives of Vitamin C and their analogs can function as hyaluronidase inhibitors. In particular L-ascorbic acid-6-hexadecanoate is a potent inhibitor (A. Botski, et al., pers. commun.). Vitamin C itself, D-isoascorbic acid, and dehydroascorbic acid are also inhibitors.[262] Thus, some of the ability of Vitamin C to enhance HA deposition may be attributed to its inhibiton of hyaluronidase.

The ability of ascorbic acid to degrade HA in the presence of divalent cations, particularly iron and copper further complicate the role of Vitamin C in HA biology.

21.7.4 Vitamin A

Hyaluronan hinders the onset of differentiation, as discussed earlier. Retinoic acid retards the differentiation of epidermal keratinocytes, as shown in skin organ cultures, a result of the ability of retinoic acid to stimulate HA deposition.[263–265] Retinoic acid leads to the accumulation of HA in the superficial layers of the epidermis by stimulating HA synthesis specifically in keratinocytes. Some of this accumulation occurs as expanded intercellular HA, which may account for the weakened cohesion of keratinocytes observed both *in vivo* and *in vitro*.

Topical applications of retinoic acid derivatives reduce the visible signs of aging and of photodamage,[266] though there is little correlation between the histologic changes and the clinical appearance of the skin. Initial improvement in fine wrinkling and skin texture correlates with the deposition of HA in the epidermis.

While Vitamin D is considered the "sunshine vitamin," Vitamin A has been accepted as an apparent antidote for the adverse effects of sun exposure, and assumed to prevent and repair cutaneous photodamage.[266] Application of Vitamin A derivatives do reverse some of the sun damage to skin, particularly the roughness, wrinkling, and irregular pigmentation.[267,268] For the over-40 generation, brought up in an era of "suntan chic," appropriate preparations to restore or to prevent further deterioration of skin are critically important.

Impairment of the retinoid signal transduction pathways occurs as a result of prolonged UV exposure. Down regulation of nuclear receptors for Vitamin A occurs,[269] resulting in a functional deficiency of Vitamin A. Application of Vitamin A derivatives would appear to be an obvious treatment modality. Topical application of Vitamin A does increase the HA in the epidermal layer, increasing the thickness of the HA meshwork after prolonged treatment.[270] Vitamin A thus enhances repair, as can be demonstrated in photo-aged hairless mouse model.[271] The decline in GAG, and in particular HA deposition that occurs with UVB irradiation, can be entirely prevented by retinoic acid treatment.

21.7.5 Vitamin E

Radical scavengers such as α-tocopherols prevent oxidative degradation of HA. In tissue culture systems, the addition of Vitamin E to the medium prevents spontaneous degradation of HA,[272] as does superoxide dismutase. In Vitamin-E-deficient animals, there is a decrease in GAGs in tissues, including HA.[273] This could be reversed by the addition of Vitamin E to diets,[274] suggesting that tocopherol supplements can enhance HA in human skin.

21.7.6 Vitamin D

Vitamin D, and in particular, the hormonally active di-hydroxy form, is a regulator of the proliferation and differentiation of skin cells, including not only epidermal kertainocytes, but also dermal fibroblasts and adipocytes. A result of prolonged UV exposure is dermal fibrosis, the excessive deposition of collagen and other ECM components within the dermis. The commandeering of mesenchymal cells to become fibroblasts, and the conversion of adipocytes to fibroblasts are thought to be the underlying mechanism. Pretreatment of skin with Vitamin D prevents the disappearance of adipocytes and the accumulation of fibroblasts. The appearance of HA, the first step in the wound healing response that initiates the cascade that leads to accumulation of the fibrous reaction, can be prevented by such treatment,[275]

21.7.7 Steroids

Topical and systemic treatment with glucocorticoids induces atrophy of skin, bone, as well as a number of other organs, with a concomitant decrease in GAGs, in particular HA. In human skin organ cultures, hydrocortisone has a bimodal effect. At low physiological concentrations, 10^{-9} M,

hydrocortisone maintains active synthesis and turnover of HA in the epidermis, while at high concentrations, 10^{-5} *M*, hydrocortisone reduces epidermal HA content. The effect is achieved through both decreased synthesis as well as decreased rates of degradation.[276] The high concentrations of cortisone also enhance terminal differentiation of keratinocytes and reduces rates of cell proliferation.

Hydrocortisone is also a potent inhibitor of HA synthesis in fibroblasts. *HAS-2* is the predominant synthase of dermal fibroblasts, of the three HA synthase genes. Glucocorticoids induce a rapid and near total suppression of *HAS-2* mRNA levels.The inhibition of HA deposition thus appears to occur at the transcriptional level. Progesterone inhibits HA synthesis in fibroblasts cultured from the human uterine cervix.[277] The steroid effect on HA appears to be system-wide. Hydrocortisone, as well as dexamethasone suppresses the ability of TGF-beta to stimulate HA synthesis through the p38 MAP kinase induced activation of the HAS genes.[278,279] Edema is one of the four cardinal signs of acute inflammation. The ability of glucocorticoids to suppress inflammation occurs in part by their ability to suppress the deposition of HA, the primary mechanism of edematous swelling that occurs during the inflammatory response.

Skin is also an important target organ for estrogens. The estrogenic effect on skin is well characterized, as well as the effect of estrogen withdrawal. A major effect of estrogen is the increased levels of HA deposition and the associated water of hydration. Topical estrogens are also able to enhance HA deposition in skin, as documented in the hairless mouse skin model.[280]

The isoflavones found in soy bean extracts, such as genistein and daidzein, that are phytoestrogens, are also able to enhance HA deposition.[281,282] Their estrogen-like structures may account for their ability to enhance HA deposition.

21.8 DERMATOLOGIC AND COSMETIC PERSPECTIVES

There is a requirement for skin substitutes in a great number of clinical situations. In patients with extensive burns, insufficient skin is available for autologous split-thickness skin grafts. Resurfacing of the burned area can occur with autologous cultured epidermal cell autografts. However, this is dependent on the functioning dermal support, a problem that has given rise to a number of reasonable approaches. Cadaver skin dermis has the problem of possible contamination and potential infection. A synthetic dermis has the requirement for an HA content that will support epithelial migration, angiogenesis, and differentiation. Various methods have been examined for modifying natural HA to provide materials with properties similar to the native polymer. Many derivatives of HA have been formulated.[283–285] Such materials could provide flat dressings that can be seeded with fibroblasts. These artificial dressings could also be seeded with cultured autologous keratinocytes, and with laser-drilled microperforations, the keratinocytes can migrate through the membrane onto the wound bed. Such applications are already in use and result in complete healing with a minimum of scarring.

It is anticipated that in the coming years, a number of HA-derivatives will appear for clinical application in Dermatology that contain cross-linked HA polymers as well as HA-ester derivatives obtained by the conjugation of the carboxylic acid of HA with various drugs in their alcohol forms. The HA polymer, because of its intrinsic biocompatibility, reactivity, and degradability, will have many uses in the rapidly expanding field of tissue engineering and in the tissue substitutes of the future.

The natural moisture of skin is attributed to its HA content. The critical property of HA is its ability to retain water, more than any known synthetic or naturally occurring compound. Even at very low concentrations, aqueous solutions of HA have very high viscosity. The advantage of using HA in cosmetic preparations was recognized very soon after its discovery. Difficulties in preparing large-enough amounts of HA free of contaminating glycoproteins, lipids, and other tissue materials prevented its convenient use in commercial preparations including its use in cosmetics. Even currently, low levels of contamination by DNA in HA preparations are considered the source

of an inflammatory response,[286] Indeed, the proinflammatory activity of the contaminating DNA in HA preparations may be the source of the inflammation attributed to intermediate-sized HA oligomes.

Initially, HA was isolated from rooster combs. This HA was highly purified, and used in ophthalmology as a visco-elastic to replace fluid loss following cataract surgery. The revolution in biotechnology and molecular genetics made it possible more recently to engineer bacteria with augmented HA production, by amplifying the HA synthase genes. This generates a material much lower in molecular weight that has the additional disadvantage of frequent contamination by residual bacterial pyrogens. Such HA, processed from vast fermentation of engineered bacteria has reduced the price of HA drastically, bringing the price into a range that is reasonable for its use in cosmetics. However, this genetically engineered HA of bacterial origin is not of sufficient purity for injectional use.

Many of the cosmetic preparations that contain HA have a concentration of 0.025 to 0.050%, sufficient to give the preparations a very smooth and viscous feel. Such solutions, applied to the skin form hydrated films that hold water for considerable periods and confer the properties of a moisturizer.

Currently, research is underway to modify HA in such a way as to make it more stable and to confer very specific properties. Another direction in such research is to combine it with other materials, such as chondroitin sulfate and modified sugar polymers, to simulate more closely the associations that HA has in its natural state in vertebrate tissues. Since the low molecular size HA fragments are highly angiogenic, defining the optimal size of the HA polymer for cosmetic purposes should be a major goal of such research.

Ethno-pharmaceuticals have long provided Western medicine with a wide variety of drugs. These same sources may provide the cosmetic industry and Dermatology with additional materials. Recent examples are the ginsenosides, major active ingredients of ginseng, which when applied topically, induce expression of the *HAS-2* gene and increase skin content of HA.[287] A myriad of other such agents from folk medicines await identification.

21.9 FUTURE PERSPECTIVES

The biology of HA and its metabolic cycles are in their infancy. The enzymatic steps that constitute extracellular and intracellular HA cycles are beginning to be sorted out. The goals that lie before us are to identify all the reactions involved, and to devise mechanisms for modulating these reactions, with the ultimate goal of enhancing skin appearance and increasing the moisture content of photodamaged and aging skin.

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22 Hydrophilic Pastes

Bernard Gabard and Christian Surber

CONTENTS

22.1 INTRODUCTION

The majority of dermatological textbooks, even some newer ones, describe pastes as semisolid, stiff preparations containing a high proportion of finely powdered material, such as zinc oxide, titanium dioxide, starch, kaolin, and talc, incorporated at relatively high concentration in a suitable vehicle.* These vehicles are by the majority lipophilic or greasy, and the properties of the pastes are globally described as cooling, drying, exudate absorbing, and protecting.[1–7] In a recent publication, a critical review of the evidence available to assert these statements was conducted.[8] It was concluded that "serious doubts must arise from the available explanations and the various formulas of pastes and their absorptive features." Detailed investigations showed that first the powders themselves presented very different absorptive features, and further that two-phase, lipophilic pastes did not absorb moisture independently from the inner phase (powder). On the contrary, three-phase pastes consisting of an hydrophilic two-phase emulsion and a high concentration of powder (inner phase) showed considerable water uptake. It was concluded that not only the "active component(s)" of a paste, that means the powder itself or the mixture of several powders, but also the vehicle used to manufacture the paste is of major importance for the final effect on the skin. Based on these statements, a classification of the pastes was proposed[8] (Figure 22.1).

After a short reminder of the published results, we extend these *in vitro* experiments *in vivo*, and we investigate in a more detailed fashion the interaction of semisolid pastes with the skin. Emphasis was put on hydrophilic pastes, however, whenever necessary and for comparison purposes, results obtained with lipophilic pastes will be shown as well.

22.2 MATERIAL AND METHODS

22.2.1 Test Products

The test products are all commercially available and are shown in Table 22.1.

* Some textbooks (e.g., References 2 and 4) consider a concentration of at least 10% of solid material necessary for the product to be a paste. Others (References 3 and 7) require 20 to 50%.

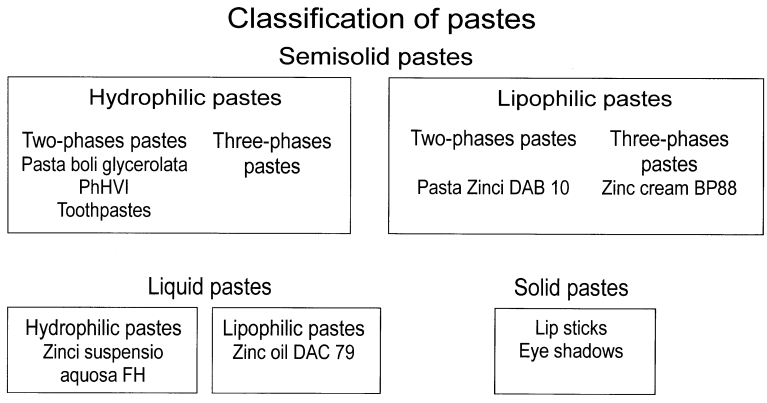

FIGURE 22.1 Classification of pastes. (Modified from Juch, R.D. et al., *Dermatology*, 189, 376, 1994. With permission.)

TABLE 22.1
Composition of the Test Products, as far as Known or Readable from the Packaging Declaration

	Tested pastes and their main components (%)							
	ZnO	**TiO_2**	**Talc**	**Kaolin**	**Starch**	**Water**	**Lipids**	**Glycerol**
Lipophilic								
LP1	46					(?)	(?)	
LP2	17				17	14	46	
Hydrophilic								
HP1	10	10	10	11		29	0	25
HP2	25		25			30 (?)	0	20
HP3		20				53	25	
HP4[x]		16				33	25	
HP5[xx]	25					25 (?)	(?)	25

Note: (?): Approximate or unknown; x: contains also 10% NMP; and xx: contains also 25% $CaCO_3$.

22.2.2 Methods

1. Evaluation of the absorptive features of different powders: these were quantified according to Enslin as previously described.[8,9] Briefly, a thermostated glass cylinder with a porous membrane on one end was filled with water and connected to a graduated capillary tube at the other end. The membrane was covered with the powder. Water absorption through the membrane was measured by the variation in the liquid level in the capillary tube.
2. Evaluation of water absorption properties of pastes *in vitro*: this experiment was conducted as described in Reference 8. Briefly, 10 g of each paste were uniformly distributed on the bottom of a Petri dish, thereby ensuring that the surface of the paste was absolutely plain and unruffled. The preparation was covered with 20 ml of distilled water and left for 30 min at 20°C. The water was removed, and the surface of the product was dried with a soft tissue. The absorptive feature of the paste preparation was calculated from the weight

difference before and after incubation. In a second step, considering that some pastes may be dry after being on the skin for a while, the absorptive features of the same test products were measured in a similar way after pre-drying the preparations at 50°C to weight constancy.

3. Evaluation of the occlusive properties of different pastes *in vitro*: 2 g of test product were carefully spread over the surface of agar-filled Petri dishes. The dishes were weighed and kept at room temperature in a box covered by a protecting cloth (start values). Further weighings were taken at days 1, 2, and 5 thereafter. For control purposes, white petrolatum and a plate without any test product were included in the test. Each experiment was conducted in triplicate.
4. Evaluation of the occlusive properties of different pastes *in vivo*: these were evaluated *in vivo* on tape-stripped skin exactly as described in Reference 10. Briefly, the stratum corneum of the forearm of healthy volunteers was tape stripped until the transepidermal water loss (TEWL) attained values between 40 and 50 g/m^2/h. After a rest period of 1 h, 2.5 mg/cm^2 of the test products were carefully applied on the stripped sites using a gloved finger, and the TEWL was measured at different times until 120 min after application. Percent changes relative to a nontreated control site were calculated over time. Positive control was white petrolatum.
5. Interactions of the pastes with the skin *in vivo*: all *in vivo* measurements were conducted in a climatized room under standardized temperature and humidity conditions (22°C, 45 ± 5% rh). Six healthy volunteers participated in the study. In the first part, after measurement of skin hydration with the NOVA DPM 9003,[11] the pastes were randomly applied at a rate of 10 mg/cm^2 on different areas (2 × 2 cm; including one untreated control area) of the ventral forearms for 5, 30, and 120 min. Thereafter, the pastes were removed with a soft paper tissue and skin hydration was measured at 1, 2, 3, 4, 5, and 15 min. The second part of this study was conducted on the same volunteers following exactly the same procedures, but the skin was preliminary hydrated by an occlusive application of a moisturizer (an O/W lotion containing 5% urea and 10% glycerol) for 1 h. This was intended to mimic a clinical situation where the pastes are applied on wet skin states with the explicit goal of drying the skin.

22.3 RESULTS AND DISCUSSION

The absorptive properties of commonly used powders such as titanium dioxide (TiO_2), zinc oxide (ZnO), kaolin, cornstarch, and methylcellulose were shown to differ considerably when evaluated under standardized conditions (Figure 22.2). The highest water absorption was shown with ZnO and kaolin, followed by cornstarch and TiO_2. Methylcellulose formed a gel with water that prevented the entire soaking of the powder, and thus water absorption remained low.

The paramount role of the vehicle in modulating the absorptive properties of a paste is shown by the results on water absorption *in vitro* (Figure 22.3). First, lipophilic pastes did not absorb water significantly even after previous drying to constant weight. This confirms our former results.[8] Second, hydrophilic pastes absorbed water in significant amounts. Most of them absorbed more water after than before drying to weight constancy. Based on these results, one may distinguish the following categories:

- Pastes showing poor absorptive features in wet state, but strongly absorbing in the dry state (HP1, HP3 to a lesser extent).
- Pastes showing relatively good absorptive features in wet and dry states (HP2).
- Pastes showing poor absorptive features (HP5).
- Pastes showing better water absorption in the wet state than in the dry state (HP4).

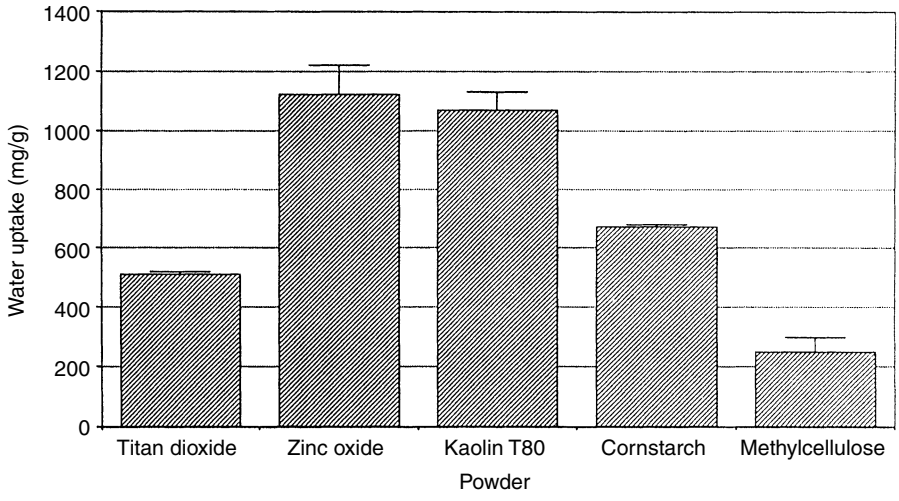

FIGURE 22.2 Absorptive features of commonly used powders determined by the method of Enslin[8]; n = 3, means ± SD. (From Juch, R.D. et al., *Dermatology*, 189, 375, 1994. With permission.)

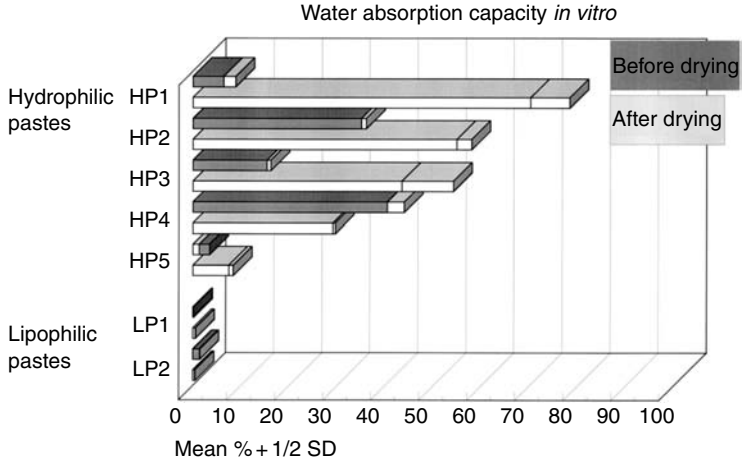

FIGURE 22.3 Water absorption properties of five hydrophilic (HP1 to HP5) and two lipophilic (LP1, LP2) pastes *in vitro* before and after drying to weight constancy; n = 3, means + HSD.

From this, it is obvious that it is not possible to use any hydrophilic paste in any given dermatologic situation. Apart from water (exudate) absorption, which may be a significant (or desirable) component of a paste's action on the skin, occlusion is another factor of importance in situations where skin protection is required.

The results of the *in vitro* occlusion tests are given in Table 22.2. They are, at a first glance, in accordance with what would be expected from the composition of the pastes. The hydrophilic pastes were not or only slightly occlusive; on the contrary, the lipophilic pastes show strong occlusive properties. A closer look reveals that differences were measured among the hydrophilic pastes, at least concerning their capacity to interfere with water loss from the agar plate. The pastes formulated with a small percentage of lipids in the vehicle (HP3, HP4; see Table 22.1) showed a slight occlusive effect. This was confirmed *in vivo* (Figure 22.4). However, compared to the occlusive effect of the lipophilic pastes, the diminution of TEWL seen after application of a hydrophilic paste such as

TABLE 22.2
Occlusion *In Vitro*: Water Loss at Day 5 (g; Means ±SD)

	Water loss (g)	% From untreated control
Controls		
Untreated	12.2 ± 1.1	100.0
White petrolatum	1.6 ± 1.2	13.1
Lipophilic pastes		
LP1	0.05 ± 0.01	0
LP2	0.22 ± 0.02	1.3
Hydrophilic pastes		
HP1	11.6 ± 0.5	95.2
HP2	10.7 ± 0.8	87.8
HP3	9.1 ± 0.4	74.5
HP4	8.8 ± 0.1	72.7
HP5	11.4 ± 0.5	93.8

Note: Means ± SD (n = 3) of water loss at day 5.

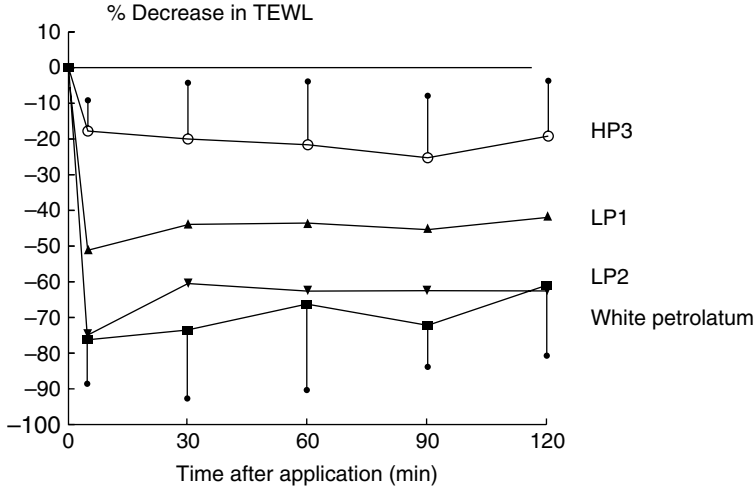

FIGURE 22.4 Occlusive properties of different pastes on stripped skin *in vivo* (percent decrease in transepidermal water loss) of n = 6 healthy volunteers. HP3: hydrophilic paste; LP1 and LP2: lipophilic pastes. For the sake of clarity, means are shown +HSD (HP3) or −HSD (white petrolatum) only.

HP3 is of questionable physiological significance. As expected, in both *in vitro* and *in vivo* models, a strong occlusion was seen after application of white petrolatum. This strong occlusive effect as observed with the lipophilic pastes led to a diminution of the TEWL because of the concomitant increase in the barrier function of the stratum corneum and despite an accumulation of moisture in the horny layer. This justifies the use of such pastes for skin protection, but not for drying the skin.

Summarizing, the results mean that besides using powder with strong absorptive features such as ZnO or, on a second line, TiO_2, pastes purposed to dry the skin should be of the hydrophilic type.

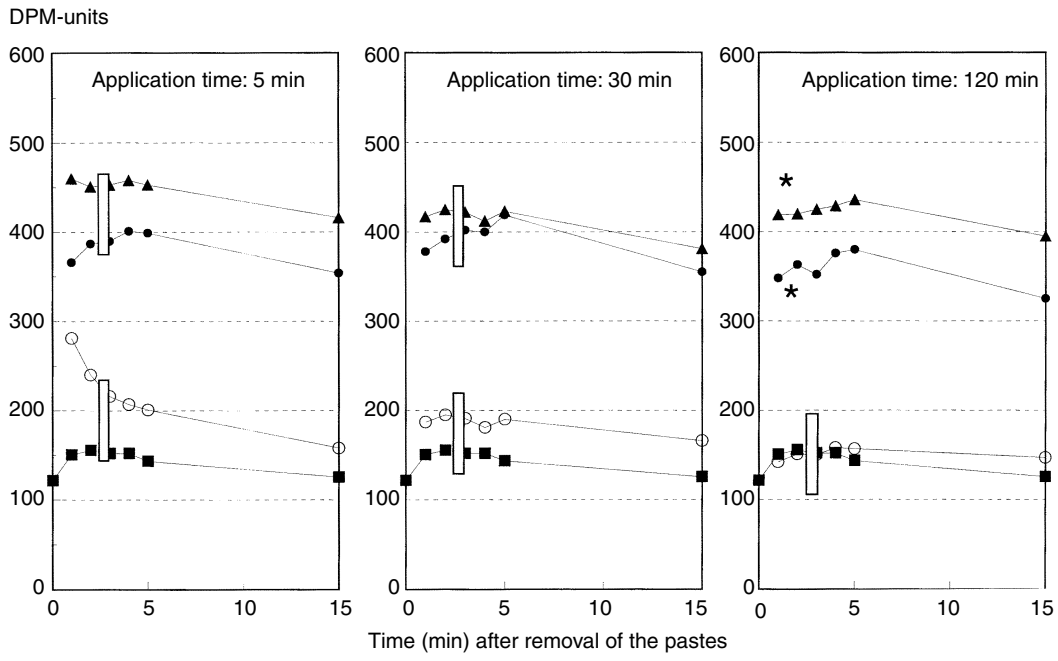

FIGURE 22.5 DPM measurements of skin hydration after application of pastes (10 mg/cm^2) during 5 (left), 30 (middle), or 120 min (right) on normal skin of n = 6 healthy volunteers. The measurements were done 1, 2, 3, 4, 5, and 15 min after removal of the pastes. For the sake of clarity, only means are indicated. Squares: control, normal skin; closed circles: HP1; triangles: HP2; open circles: HP3; ∗: statistically significant differences with the control group and between each other group. M indicates homogenous subsets.

Not only were these shown to significantly absorb water *in vitro*, but they lack a significant occlusive effect which is detrimental in a situation where water evaporation should not be impaired.

The *in vivo* investigations were conducted with three representative hydrophilic pastes: HP1 (poor absorption in the wet state, strong in the dry), HP2 (good absorption in both states), and HP3 (the only paste without addition of a humectant or any other substance; see Table 22.1). The results are shown in Figure 22.5. After a 5-min application of the pastes on normal skin, hydration was significantly higher than in control skin. However, as soon as 2 min after removal of the products, two groups were characterized, enclosing test products not statistically different from each other: HP2 and HP1 on one side and HP3 and control on the other. The same situation was encountered after application of the pastes during 30 min; after 120 min application time, HP2 hydration values were still high and significantly higher compared to HP1. HP3 and control values were no different than before. Thus, after application on normal skin, some pastes significantly and durably enhanced skin moisturization.

As seen in Figure 22.6, application of a moisturizer containing humectants such as urea and glycerol for 1 h under occlusion significantly increased the measured values. There was a gradual decrease in this exaggerated hydration over time, but even after 120 min normal control skin values were not totally recovered. After applying the pastes on this hydrated skin for 5 min, measured values remained high in all groups. However, as soon as 2 min after removing the paste, HP3 values were significantly lower than the other ones and also lower than control values. If the pastes remained on the skin for 30 min, a significant drying effect was measured for HP3 only. For both other pastes, hydration values remained higher than the control ones. After application for 120 min, the situation was even more obvious: HP1 and HP2 did not change the hydration values of the skin, whereas HP3 and control values showed no significant differences. Thus, we were not able to show any drying of the skin surface with hydrophilic pastes containing humectants, even if the skin was preliminary hydrated

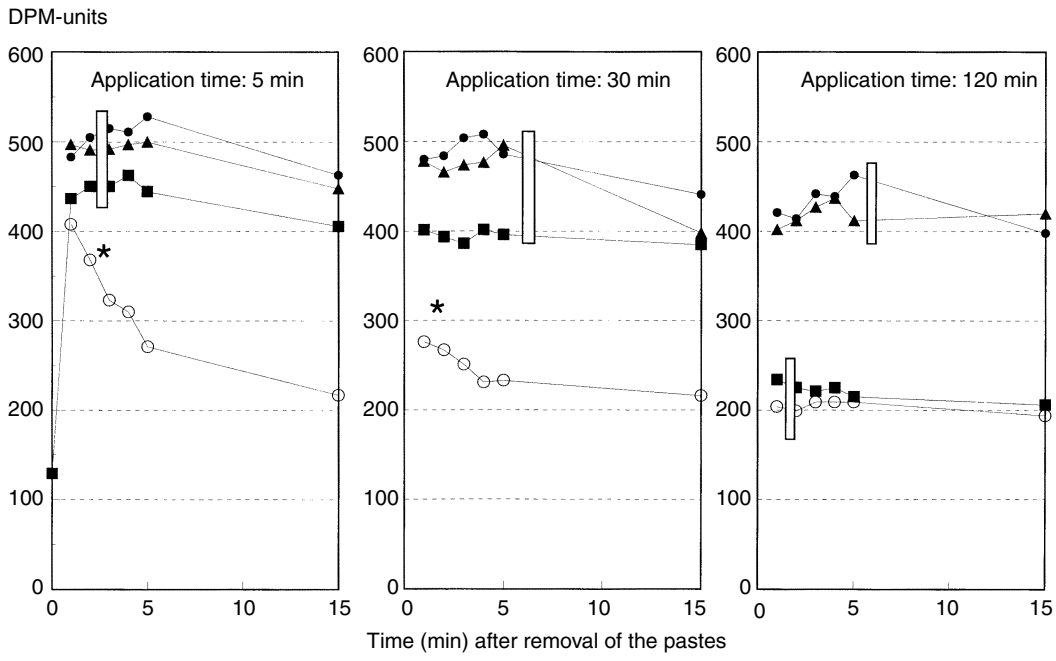

FIGURE 22.6 DPM measurements of skin hydration after application of pastes (10 mg/cm^2) during 5 (left), 30 (middle), or 120 min (right) on previously hydrated skin of n = 6 healthy volunteers. The measurements were done 1, 2, 3, 4, 5, and 15 min after removal of the pastes. For the sake of clarity, only means are indicated. Squares: control, hydrated skin; closed circles: HP1; triangles: HP2; open circles: HP3; ∗: statistically significant differences with the control group. M indicates homogenous subsets.

by occlusive application of a moisturizer. On the other hand, a more simple hydrophilic paste was indeed able to induce a faster dehydration after treatment than measured on a nontreated control zone left open. It is not possible to clearly prove that these results are due to the presence of humectants in the pastes. We consider it likely and see the measured values as the results of a competition between water absorption (which indeed was measured *in vitro*, see Figure 22.3) and water binding in the stratum corneum by the humectants. On the other hand, these differences could also have been due to a different water evaporation from the skin surface. Therefore, we measured the skin surface water loss with an evaporimeter after application of HP2 and HP3 on hydrated skin for 30 min, following the guidelines of the European Society of Contact Dermatitis.[12] The results (Figure 22.7) show that the water loss after HP3 was always higher than after HP2. However, this effect remained for short duration. Therefore, it is likely that the differences in water content of the vehicles themselves might have been at the origin of the differences in water evaporation.

22.4 CONCLUSION

In conclusion, after investigating *in vitro* the water absorption capacities of the main "active" component(s) of pastes, the powder(s), we showed that hydrophilic, but not lipophilic, pastes absorb water to a significant degree. This pointed out a paramount role for the vehicle incorporating the powder. Different categories were noticed, particularly when considering the absorptive capacities after predrying of the pastes. Drying is a phenomenon occurring on the skin possibly after a certain time that thus may contribute to drastic changes of the water-absorbing properties of a paste. Further, hydrophilic pastes showed no or only a slight occlusion, whereas highly occlusive properties were confirmed *in vitro* and *in vivo* for lipophilic pastes.

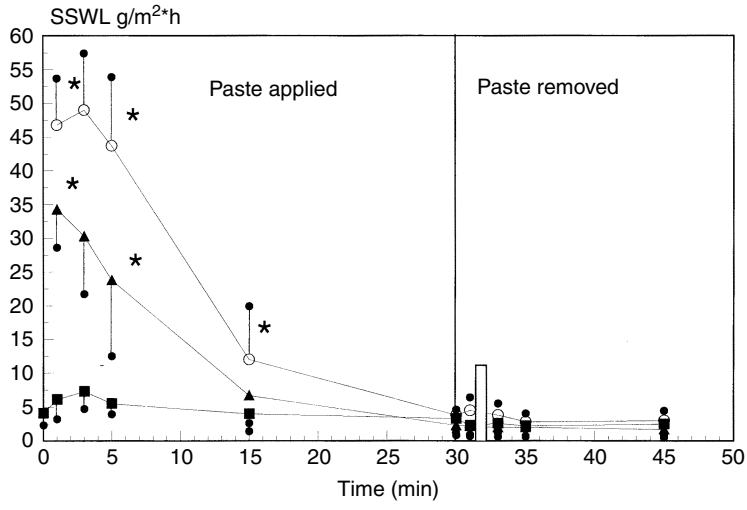

FIGURE 22.7 Measurement of skin surface water loss (Evaporimeter EP-1, Servomed, Stockholm) during application and after removal of two hydrophilic pastes for 30 min on previously hydrated skin of n = 6 healthy volunteers. Means ± HSD for the sake of clarity. Closed squares: control, hydrated skin; triangles: HP2; open circles: HP3; ∗: statistically significant differences with the control group. M indicates homogenous subsets.

In vivo, hydrophilic pastes showed different interactions with the skin. Some pastes clearly hydrated the skin, others could indeed remove water from a preliminary hydrated horny layer. Elements contributing to these properties may be the presence of humectants such as glycerol, contributing to a long-lasting presence of water on the skin in the first case, or the acceleration of skin surface water loss, contributing to an accelerated removal of water from a hydrated horny layer in the second case. However, this represents, in our opinion, at most one of the elements contributing to the measured events and may simply be due to a different water content of the pastes.

We conclude that pastes cannot be pooled in a single group and be generally characterized as "drying" and "exudate binding." Lipophilic pastes did not bind any water at all and were highly occlusive. Thus, they are likely to hydrate the skin through an impairment of the transepidermal water loss. They should be preferably used for skin protection. Hydrophilic pastes, on the other hand, hydrated the skin or maintained an elevated hydration state if they contained humectants. Only an hydrophilic paste without any additional component was able to reduce a hydrated state and led to measurably decreased skin hydration values.

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23 Petrolatum

David S. Morrison

CONTENTS

23.1 INTRODUCTION

"The secret to younger-looking skin!" "The best moisturizer there is!" These statements are often heard when consumers talk about various cosmetic products, but it is surprising that this high praise also refers to the very common and not-so-elegant material known as petroleum jelly, or petrolatum. So, what exactly is this decades-old ingredient that elicits such comments from people?

Petrolatum is a purified material consisting of a complex combination of hydrocarbons with an ointment-like consistency and is derived from petroleum (crude oil). Based on its origin, it would seem that the properties of petrolatum would vary dramatically depending on the type of crude oil used. However, since different types of crude oils have widely differing properties (depending on the source of the oil), only certain waxy crudes are suitable for the manufacture of petrolatum.

Petrolatum has been used as a skin care product since its discovery by Robert A. Chesebrough in 1872.[1] In his patent, Chesebrough stated that this material is useful as a chapped hand treatment. At that time, one of the main benefits of petrolatum was that it did not become rancid (oxidize) as did the commonly used fats of that day. Even today, some skin care product developers use unsaturated oils and other products that need to be preserved against oxidation. Technology has advanced to the point where such preservation is possible, but the desire for highly effective skin care ingredients that are consistent in quality and do not readily oxidize (such as petrolatum) still exists.

Although the refining (and thus purity) of petrolatum has been improved over time, its form remains essentially unchanged from the original. In the European Union (EU), the refining of petroleum substances is important, since different petroleum-derived products are found in different categories under the Dangerous Substances Directive (67/548/EEC) based on their processing history.[2] One of these products is petrolatum, which surprisingly is listed under Category 2 carcinogens. However, *this classification of petrolatum does not apply if the full refining history is known and the raw materials from which the petrolatum is made are not carcinogens*. This exception clause is termed "Nota N" in the Directive

and applies to all types of petrolatums, including petrolatums of the highest purity as well as those that are unrefined. If the materials from which petrolatum is made contain less than 3% DMSO-extract according to test method IP 346, then they are considered noncarcinogenic.[2]

Since Category 2 carcinogens are not allowed in cosmetics according to the EU's Cosmetics Directive (7th amendment), suppliers of petrolatum to the cosmetic industry need to show that their materials are not carcinogenic based on the Dangerous Substances Directive. Of course, when the full refining history of the petrolatum is known and the products from which the petrolatum is made are not carcinogens, then the petrolatum is *not* classified as a carcinogen, and it is allowed for use in cosmetics. In addition, we have found no human or animal data that would indicate that such refined, high-purity petrolatums are carcinogenic. (For a more complete discussion of this issue, see reference 3 and references therein.) Finally, it should be noted that petrolatums commonly used in cosmetic and medicinal applications around the world meet the appropriate pharmacopoeia standards. For example, in the United States, typical petrolatums used for these applications are of *U.S. Pharmacopoeia* quality and pass strict FDA (U.S. Food and Drug Administration) requirements for both direct and indirect food contact (21 CFR 172.880 and 21 CFR 178.3700).

Not long after Chesebrough's discovery, the moisturizing benefits of petrolatum were more widely recognized, primarily in terms of its medical applications.[4] These days, the many benefits of petrolatum (petroleum jelly) are still being touted in the media around the globe, mainly for the treatment of dry, chapped skin.[5–9] In addition, petrolatum has been cited as useful for adding shine to the lips,[5,6] cheeks,[5] and eyelids;[10] moisturizing the feet;[6,11] conditioning eyelashes;[12] and stopping cuts from bleeding.[7] It has applications as a makeup remover[7] and as a facial moisturizer.[13] Presumably because of petrolatum's then newly recognized properties as a skin treatment product, it was included in the 1880 edition of the *U.S. Pharmacopoeia*[4] and is still listed today. In the modern-day *Pharmacopoeia*, there are actually two listings for this material, "Petrolatum" and "White Petrolatum," with the differences being the colors and ignition residues of the two products.

23.2 SKIN MOISTURIZATION BY PETROLATUM ALONE

Petrolatum's skin moisturization properties are clearly due to its occlusivity,[14] and this material is often considered to be the most effective ingredient available for moisturizing dry skin.[15] Petrolatum blocks the evaporation of water from the skin (transepidermal water loss; TEWL), thus keeping the stratum corneum well hydrated.[16] It should be noted that the term "moisturization" is commonly used to describe the action of occlusive agents on skin, even though water is not actually added to the skin.

The lack of oxidation of petrolatum is due to the nature of the hydrocarbon molecules that are present in this substance. During the oil refining process, the hydrocarbon material is hydrogenated (saturated) to create oxidation-resistant molecules throughout, from the liquid oil to the solid waxes. This property enables pure petroleum jelly to be marketed as having the benefit of a long shelf life. The lack of rancidity also gave early cosmetic formulators a new ingredient that could be processed with little regard to possible degradation.

As technology progressed over the years, scientific techniques were developed to quantify the ability of various ingredients to "moisturize" the skin. Cutaneous impedance is one method that has been used to determine how well certain products moisturize the skin, with a decrease in impedance typically indicating an increase in skin hydration. Interestingly, measurements made after application of petrolatum show an initial *increase* in impedance that is due to the resistance of petrolatum, and thus its occlusivity, rather than to dehydration of the skin.[17] Results from a later study by Lodén and Lindberg[18] revealed the drawbacks associated with electrical skin measurements. It was noted that this method should not be solely relied upon to determine skin moisture content; thus, other

methods also should be used to verify (and possibly support) the findings of skin moisturization studies. Recently, skin capacitance values have been compared with skin hydration as determined by ultrasonography (echographic image analysis).[19]

The moisturizing ability of petrolatum also has been determined with a spectroscopic technique for evaluating skin hydration, opto-thermal transient radiometry (OTTER).[20] This study reported that the hydration level of skin *in vivo* increased from 45% (initially) to approximately 80% at 2 h after application of petroleum jelly.

One of the most popular and commonly used methods for determining the effectiveness of skin moisturizers is to grade the xerosis on the lower legs of panelists with both visual and tactile assessments.[21,22] Using this method, Kligman determined that petrolatum was an extremely effective moisturizer.[22]

Another very practical and useful method for evaluating skin moisturizers is by the direct measurement of TEWL on human skin.[23] Not surprisingly, several studies that incorporate this test method have proven that petrolatum is an excellent moisturizer.[24–26] It should be noted that in many studies on TEWL, petrolatum is used as a positive standard when evaluating other cosmetic emollients, since petrolatum is nearly always the most occlusive TEWL barrier material tested. It is often the standard by which other ingredients and formulations are judged.

In a recent study on gelled mineral oils by our laboratory, petrolatum was evaluated in a clinical TEWL study and compared with light mineral oil and a gelled light mineral oil.[27] The study panel consisted of 15 subjects who had extremely dry skin (baseline TEWL $>7.0\ g/m^2/h$). The test materials were applied three times (at 1 h intervals) to the volar surface of the subjects' forearms, with TEWL measurements taken 1 h after each application. The results (Figure 23.1) clearly indicate that the improvement in TEWL as a result of using petrolatum is superior to that using both the light mineral oil and the gelled light mineral oil, with petrolatum reducing TEWL by an average of 33% over 3 h.

Similar studies of moisture transport across a barrier also have been done using *in vitro* methods that determine the movement of water vapor across a film coated with a measured amount of the material to be tested. These are rapid methods for rough determinations of occlusivity and work very well for screening several ingredients at a reasonable cost. Once again, petrolatum's occlusivity has been shown in tests of this nature.[28,29]

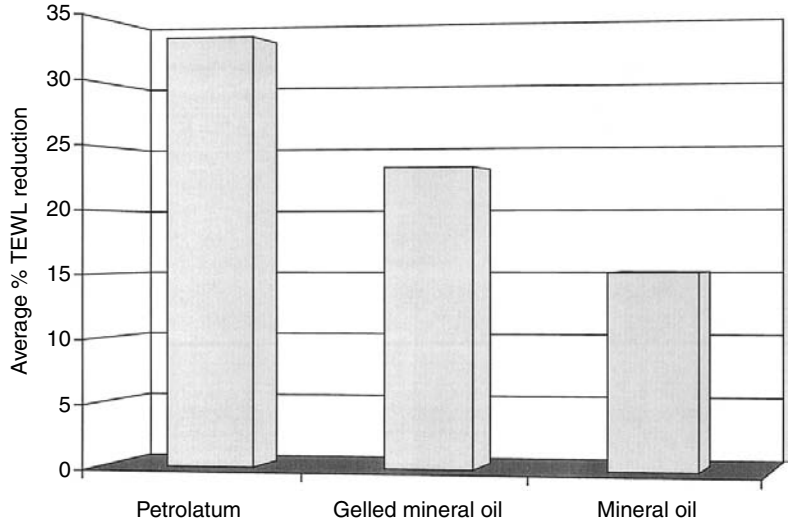

FIGURE 23.1 The average percent reduction in TEWL compared to untreated skin (over 3 h) for some hydrocarbon ingredients.[27]

23.3 SKIN MOISTURIZATION — PETROLATUM IN COSMETIC COMPOSITIONS

The use of petrolatum in cosmetic compositions is evident simply by looking at the ingredient listings on commercially available skin creams and lotions. Although new ingredients that are beneficial to skin are always being discovered and incorporated into skin care products, petrolatum seems to be a material that never falls out of favor.

Most investigations on skin moisturization evaluate petrolatum alone. The moisturizing efficacy of a petrolatum-containing cream was reported by Prall and coworkers in one study and compared with those of two other creams, one containing urea and one containing alpha-hydroxy acids.[30] In both cases, the petrolatum-containing product was found to be comparable to the other formulations as judged by skin dryness.

Numerous patents have been issued over the years for topical cosmetic products that contain petrolatum. Of course, the owners of a patent wish to protect as many ingredients in a formulation as possible, and when hydrocarbons are suitable ingredients, petrolatum is invariably one of the ingredients covered by the patent. In no way does this diminish the importance of petrolatum as an ingredient (whether it is a preferred ingredient or not); rather, it supports the frequent use of petrolatum as a skin care ingredient for cosmetic formulations and recognizes its application in many different types of cosmetic compositions. Examples of recent patents include a diaper rash treatment for patients of all ages that can incorporate petrolatum as the composition's base,[31] an improved ointment base that utilizes a high molecular weight petrolatum fraction for skin moisturization and other applications,[32] and rinsable skin conditioning products that deposit personal care ingredients and conditioning agents onto the skin.[33]

Despite the hydrophobicity of petrolatum, it also has been cited in patents related to skin cleansing products, such as moisturizing bar soaps with petrolatum as a key ingredient,[34–37] liquid personal wash compositions,[38,39] and skin sanitizers.[40] In 2002, a unique skin cleansing technology was reported in which a bar soap was formulated with three common cosmetic ingredients (including petrolatum).[41] It was discovered that this formula inhibits the attachment of bacteria to the skin after washing with the soap. Even when petrolatum was incorporated by itself in the bar soap, fewer bacteria attached to the skin than when the base soap was used (no petrolatum); however, the greatest benefit was obtained when the three ingredients were used together. It is believed that after washing, a barrier remains on the skin that prevents bacterial attachment.

The benefits of petrolatum has been claimed as an ingredient in skin care products designed to reduce wrinkles,[42,43] in products for emolliency, protection, moisturization, and skin conditioning,[44–52] and as a base for dispersing other skin care ingredients.[53,54] Interestingly, it also has been incorporated into cosmetic powders, such as talcum powders, where it acts to condition/moisturize the skin.[55]

23.4 SKIN MOISTURIZATION — PETROLATUM IN DERMATOLOGICAL APPLICATIONS

Even though petrolatum is highly effective at moisturizing dry skin, some critics would charge that this material should not be used on skin and should certainly not be recommended by medical doctors for their patients. It is purported that petrolatum is comedogenic (clogs the pores), as evidenced by its greasy, ointment-like consistency. However, petrolatum is actually noncomedogenic,[56–58] which is unrelated to its physical properties.[59] In fact, some ingredients that have a drier feeling on the skin and are less greasy than petrolatum are actually highly comedogenic when tested neat.[56,57] Nevertheless, these products are likely to be used as ingredients (at low concentrations) in various formulations that are then determined to be noncomedogenic.

Petrolatum continues to be used quite extensively in dermatological applications, primarily for three purposes: (1) as an inert patch testing base, (2) as a vehicle in the dermal application of pharmaceuticals, and (3) as a treatment product itself.

23.4.1 Patch Testing

The fact that petrolatum does not cause irritation[60] explains its frequent use as a suitable vehicle for patch testing of contact allergens[61–63] and irritants. Since this material is unreactive, various substances can be tested with no concern about interference from the substrate. It is a traditional patch testing base for anhydrous materials, especially for fragrances and fragrance ingredients.[64] Advantages include the uniform suspension of powders in petrolatum, the holding of test material in place very well, the lack of evaporation, and good solubility properties. Tests for photoallergy, phototoxicity, irritation, allergic reaction (repeat insult patch test), and diagnostic testing for allergic contact dermatitis are some of the types of studies that frequently use petrolatum as the test vehicle.[65–67] Petrolatum also has been compared with an aqueous system when evaluating a water-soluble allergen.[68]

23.4.2 Drug Delivery

Petrolatum can be used in the delivery of pharmaceuticals to the skin. Although petrolatum itself does not penetrate below the stratum corneum, its ability to solubilize lipophilic materials and suspend hydrophilic solids has made it a commonly used transdermal drug delivery vehicle. Being lipophilic, it is able to incorporate more lipid-soluble pharmaceutical actives than emulsions. Petrolatum's film-forming capability and occlusive nature allow a consistent, steady "flow" of active ingredients to the skin. For example, topical pharmaceutical creams can be applied to the skin, followed by application of petrolatum over the cream. The petrolatum induces hydration of the skin (via reduction in TEWL), and this hydration increases the penetration of the pharmaceutical active into the skin. Additionally, the layer of petrolatum prevents the cream from rubbing off the skin.[69] Liposomal formulations incorporating petrolatum also have been studied as part of topical drug delivery systems.[70]

23.4.3 Treatment Products

Probably the most widely reported information concerning petrolatum's effect on damaged skin was a 1992 article by Ghadially and coworkers.[71] Newspapers across the United States reported the results of this study, which showed that, when applied to skin that had been damaged by acetone, petrolatum accelerated the recovery of the skin's normal barrier properties. It was noted that this is in contrast to materials that are highly impermeable to water vapor (such as polyethylene films) which hinder barrier repair. However, another publication has indicated that the repair of the permeability barrier in human skin is *not* delayed by occlusion.[72]

Although the permeability barrier can be repaired to a presumably greater extent using a complete mixture of physiologic lipids, petrolatum, which remains restricted to the stratum corneum, provides a more rapid barrier repair. The delay in barrier repair when using physiologic lipids has been attributed to the time necessary for lipid uptake and processing by the skin.[73] Recent publications have indicated that petrolatum-based emollients give positive results in the treatment of hand dermatitis,[74,75] and a lipid-containing skin cream was reported to be similar to neat petrolatum when applied to barrier-compromised skin.[76] This benefit from skin care products containing petrolatum is well known within dermatological circles. At the American Academy of Dermatology's Derm Update 2003, one speaker discussed key guidelines for winter skin care. The most important tip was for the patient to moisturize the skin with lotions and creams that contain effective ingredients such as petrolatum.[77]

In addition to the repair of the epidermal barrier, petrolatum has been used in other types of dermatological treatments. Petrolatum can affect the transmission of UV light during phototherapy

to skin, and the transmission of different wavelengths of UV light changes as the thickness of the applied material is varied.[78] Also, it has been reported that a petrolatum-based ointment provides favorable treatment for premature infants: bacterial colonization of the treated skin was decreased, and the frequency of dermatitis was reduced.[79] In another article, petrolatum was shown to inhibit tumorigenesis in skin irradiated with UVB light, and petroleum jelly even reduced tumor yield when applied after irradiation.[80] Other uses include postlaser skin resurfacing treatment;[81] application to burns, cuts, and abrasions;[82] moisturizing and protecting the oral cavity during medical and dental procedures;[83] and use as a wound care ointment.[84,85] In one study, the use of white petrolatum in postsurgery wound care showed an infection rate similar to that in patients who used Bacitracin ointment, thus prompting the authors to refer to petrolatum as "an effective, safe wound care ointment."[85] These authors also estimated the dramatic cost savings that would be realized by switching from antibacterial ointments to white petrolatum.

In 2004, Draelos and coworkers reported the effect of a petrolatum-containing body wash on the treatment of xerotic eczema. In this study, the researchers found that the patients who used a petrolatum-containing body wash in addition to moderate corticosteroid therapy had improved significantly more than the patients who used a more potent topical corticosteroid and a typical synthetic detergent cleansing bar. Thus, the petrolatum's skin treatment benefits were clearly evident, even when it was contained in a wash-off product.[86]

As a dermatological product in sports medicine, petrolatum is frequently used as a lubricant to prevent blisters on feet[87] and other areas where chafing can occur.[88,89] It has been recommended in a variety of sports such as tennis[90] and rowing.[91] During winter sport participation, athletes can prevent nosebleeds by moisturizing the nostrils with petrolatum.[92] In addition, petrolatum is often a primary ingredient in analgesic ointments and balms designed for sore muscle relief.

Not surprisingly, petrolatum has been cited as a major ingredient in patents that describe products for topical skin treatment.[93–95] Some specific applications include ophthalmic ointments,[96] lip care products,[97,98] and aftershave preparations.[99]

23.5 SKIN MOISTURIZATION — PETROLATUM IN PAPER AND RELATED PRODUCTS

During the past several years, the market has seen "new and improved" facial tissue products that are softer than the standard facial tissue or contain lotion to "soothe sore noses" and the surrounding skin. Constant wiping with a facial tissue can irritate the skin, so a product containing emollients is likely to reduce irritation. Petrolatum can be used as an inexpensive yet effective emollient in various types of tissue paper products.[100] In another example, petrolatum was employed as a skin conditioning agent in an antiviral and antibacterial lotion that can be applied to either facial tissue[101,102] or toilet tissue.[101]

Diapers are related products that also benefit similarly from the addition of emollients such as petrolatum. Petrolatum can not only reduce the adherence of bodily waste to the skin but also provide emolliency.[103–107] When applied to the cuffs of diapers, petrolatum also imparts lubricity and minimizes abrasion on the skin.[108] Finally, petrolatum has been formulated into an emulsion that is applied to various cleansing wipes (e.g., baby wipes).[109]

23.6 CONCLUSION

Petrolatum is widely used as a classic skin moisturizer.[110] Its uses range from cosmetic skin care products to dermatological treatments to patch test substrates to tissue paper emollients. As long as people require soft, supple, moisturized skin, petrolatum will be a key ingredient in meeting that requirement.

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24 Phospholipids, Metabolites, and Skin Hydration

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CONTENTS

It is now generally accepted that stratum corneum as the uppermost layer of the skin is a biosensor that regulates metabolic responses of the skin to the changes in the environment.[1] Variations in environmental humidity affect the rate of the permeability barrier synthesis. The chain of events involved in this response includes (1) detection of a change in skin hydration (e.g., due to increased transepidermal water loss), (2) activation of a variety of enzymes including phospholipase D (PLD), and (3) modulation of the rate of the pro-barrier to barrier lipid transformation.[2] In this chain of events, PLD not only controls the rate of lipid transformation, but is also involved in the release of water-soluble metabolites, which function as organic osmolytes[3] and at the same time exert their biological protective activity.[4,5] These metabolites play an essential role in the maintenance of a balanced skin hydration.

Phosphatidylcholines (PCs) are the major precursors of these metabolites. Depending on the saturation of their fatty acid residues, at physiological temperatures these substances are either in fluid-like or in gel-like state. When applied topically, both types of exogenous PCs are taken up by the biosensor stratum corneum. The fluid-state PCs penetrate readily all the way to the epidermis; they have a therapeutic potential and can be used as penetration enhancers. The gel-state PCs penetrate only through stratum corneum; they have no therapeutic potential but can be used as enhancers of penetration through stratum corneum. A recently developed cosmetic product Physiogel AI (Stiefel Laboratories, Inc.) was designed to deliver exogenous supplement of PCs to the pool of endogenous

phospholipid metabolites. It furnishes these metabolites in the form of a matrix of gel-state PC. Clinical studies conducted up to date indicate that Physiogel AI not only satisfies the requirements set up for guaranteeing an efficient barrier repair, but it also ameliorates inflammation and pruritus, and normalizes impaired skin hydration.

24.1 INTRODUCTION TO PHOSPHOLIPIDS AND PHOSPHATIDYLCHOLINES

Phospholipids are components of lecithins, and within this complex mixture[6] they have been applied to the skin since the earliest days of cosmetics. Phosphatidylcholines (PCs) are the most abundant of phospholipids in lecithins and in the majority of biological membranes. PCs in pure form are used in the pharmaceutical and the cosmetic industries. Toxicological aspects of PCs in topical use have been reviewed recently[7]. Two different forms of PC are used by the cosmetic industry today:

1. *Fluid-State PC.* This PC is extracted from soybean, and its molecule contains mainly unsaturated fatty acids. Its transition temperature is approximately 0°C, and it is referred to as the fluid-state PC. The PC molecule has structure-forming properties, and is therefore used as an excipient, both in drugs and in cosmetic preparations. In addition, it is used as an pharmacologically active drug substance in oral, systemic, and topical formulations.
2. *Gel-State PC.* Hydrogenated soybean PC (HPC) contains only saturated fatty acids of which 85% is stearic acid. The transition temperature of HPC is 50–55°C, as compared to 50°C of the SC lipids. It is used in topical formulations as an excipient in drugs and in cosmetics. In contrast to the fluid-state PC, it forms lamellar structures in water, similar to the lamellar structures of the permeation barrier in SC. Two synthetic PCs, distearylphosphatidylcholine and dipalmitoylphosphatidylcholine, with transition temperatures of 55°C and 38°C, respectively, are used as excipients for systemic drug formulations.

24.2 EPIDERMAL DIFFERENTIATION PROCESS OF THE LIPID BILAYER: FROM EPIDERMIS TO STRATUM CORNEUM

Multicellular organisms, including mammals, consist of cells that are organized into different organs. The homeostasis of these organs depends on biological membranes made from phospholipids arranged in bilayers. Within the "friendly" internal milieu of the mammalian organisms, these bilayers, because of their transport, sensory, and enzymatic functions, must be in a fluid state. Mammalian organisms as such can be regarded as compartments separated from its environment by the epidermal permeability barrier located in stratum corneum. However, in contrast to the interior milieu of the multicellular organisms, the biosphere around the mammalian organisms is dry, gaseous, oxidizing, and full of harmful radiation, chemical and biological factors. In order to protect the organisms against this hostile environment, the bilayers of the epidermal permeability barrier are in a gel-state form, are more hydrophobic, and are organized in lamellar sheets. This barrier, which provides efficient protection against most of the harmful external factors, is synthesized in a process of epidermal differentiation in which the polar, fluid-state, and partly hydrophilic pro-barrier lipids are converted to nonpolar, gel-state, and hydrophobic barrier lipids.

24.3 EPIDERMAL BIOCHEMICAL DIFFERENTIATION: PHOSPHOLIPIDS TO METABOLITES

The transformation of the fluid- to gel-state lipids is achieved by the catabolism of the pro-barrier lipids and the subsequent synthesis of the barrier lipids. In stratum basale, up to 60% of the lipids

are fluid-state phospholipids. During the differentiation process, all these phospholipids are completely catabolized, and as a result stratum corneum contains no fluid-state phospholipids whatsoever. Stratum corneum is composed solely of gel-state lipids of which 50% are ceramides. Until recently, only the presence and the right proportion of ceramides to fatty acids and cholesterol were regarded as the critical factors in the maintenance of an intact permeability barrier. Even today, the critical role of phospholipid metabolites in the skin tends to be ignored. In other organs the importance of these organic osmolytes is recognized and they are the subject of intensive research.[8]

24.4 BIOLOGICAL EFFICACY OF PHOSPHOLIPID METABOLITES

Phospholipids and PLD are ubiquitous in the mammalian organism. The matrix of biological membranes of all cells and cell organelles is composed of phospholipids. PLD is activated by nearly all stress factors.[9] This activation results in the release of phospholipid metabolites. They include a range of small water-soluble molecules many of which have essential regulatory roles. The highest concentrations of these metabolites in the skin are in the deepest layer of stratum corneum.[2] It is in this layer in which the epidermal permeability barrier is conceived and in which keratinocytes differentiate to corneocytes. In other words, this is the boundary region that separates and protects the internal milieu of the mammalian organisms from the hostile dry-land terrestrial environment. The phospholipids and their metabolites are summarized in Figure 24.1.

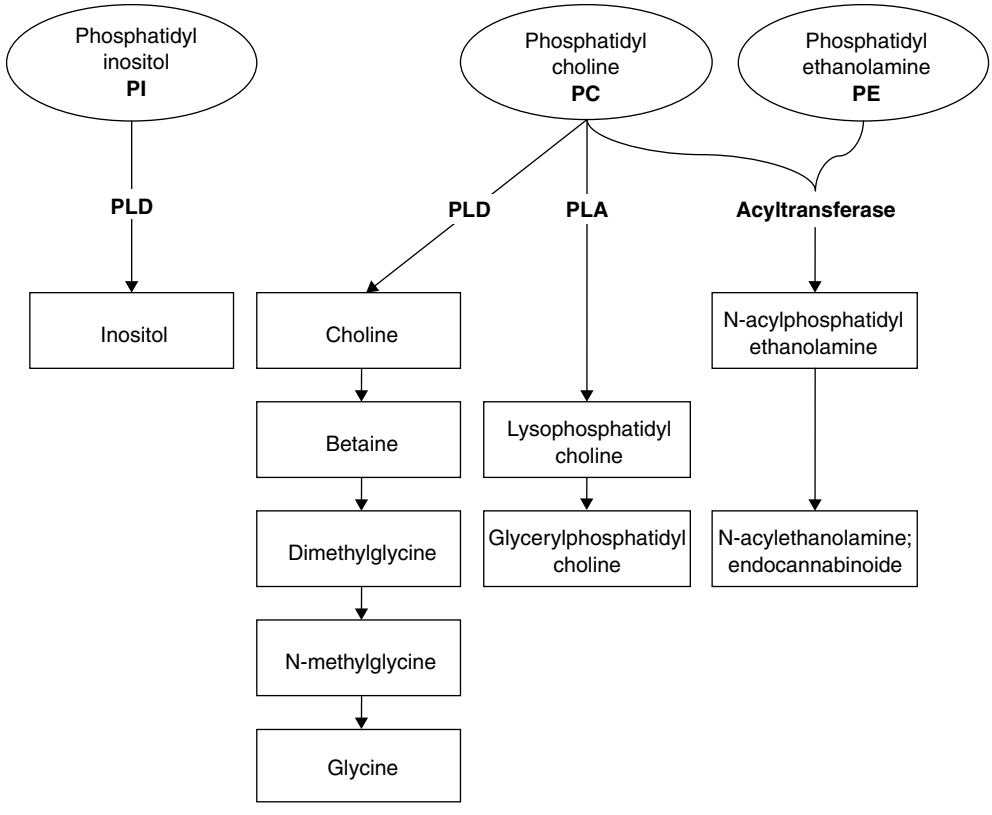

FIGURE 24.1 Phospholipids and their metabolites.

The following biological actions of these metabolites could be of relevance for skin hydration and homeostasis:

1. Organic osmolytes such as inositol, betaine, glycerolphosphatidylcholine, glycine, and n-methylglycine control cell volume by binding water without changing of ion concentrations.[8] Dry skin is less hydrated, less capable of binding water, and volume of corneocytes is reduced. The more reduced is the volume of corneocytes, the easier is the penetration through it and the greater is the susceptibility of skin to chemical irritation.[1]
2. Betaine ameliorates sodium lauryl sulfate-induced skin inflammation.[10]
3. Betaine is used in liver diseases to combat alcoholic cirrhosis. Betaine may also be valuable in the treatment of disorders of the skin. The underlying mechanisms of the action of betaine are not clear, but it has recently been suggested that they may play a role in the maintenance of redox homeostasis.[4]
4. Lysophosphatidylcholine protects mice in the lipopolysacharide-induced septic shock.[11] The fact that the lipopolysacharide increases the concentration of cytokines (the same effect is observed upon the epidermal barrier disruption), and that lysophosphatidylcholine normalizes the cytokines level, provides the theoretical basis for treating disorders of the skin.
5. N-acylphosphatidylethanolamine accumulates in stratum granulosum.[12] It is catabolized by PLD to N-acylethanolamines, a group of trendy substances also named endocannabinoids. It is believed that their biological importance is manifold.[5] N-palmitoylethanolamine is of especial interest in skin research since it is the most abundant endocannabinoid in stratum granulosum.[12] It is seen as a new type of antioxidant,[13] has a cell protective action,[14] and functions as a signaling molecule.[15] As a naturally occurring anti-inflammatory substance[16] it is also comparable to some currently used potent drugs.[17] The synthesis of N-palmitoylethanolamine and other endocannabinoids increases during stress from a variety of causes.[18]

24.5 TOPICAL APPLICATION OF PHOSPHATIDYLCHOLINE

There might be a rationale for the topical application of exogenous barrier lipids in helping the repair of the damaged permeability barrier. Successful filling of the "holes" in the barrier by ceramides may stop transepidermal water loss and may lead to barrier repair. But it is equally possible that ceramides only cause occlusion of the "holes" in the barrier, and that they actually stop the epidermal differentiation that is essential for skin renewal. In addition, ceramides are extremely hydrophobic: their ability to hydrate is so limited that their inclusion in topical formulation in sufficient concentrations is still not possible today. It might therefore make more sense to reach for the pro-barrier lipids and their metabolites: Their topical application would enlarge their endogenous pool, thus enhancing synthesis and renewal of the permeability barrier. The prerequisite of this approach is that the topically applied PCs and their metabolites penetrate stratum corneum and reach the layers where the barrier is assembled. An additional advantage of PCs as compared to ceramides is their superior hydrophilicity. One PC molecule binds 20 molecules of water, and by penetrating stratum corneum it transports this amount of water to deeper layers of the skin.

24.6 PHOSPHATIDYLCHOLINE AS AN ACTIVE DRUG SUBSTANCE AND AS A STRUCTURE-FORMING "INERT" EXCIPIENT

There are two "realities" in the world of the phospholipid science. One reality is that phospholipids are structure-forming molecules. Nature uses these molecules to construct biological membranes

and mixed micelles; humans use them to make emulsions and liposomes. This implies that phospholipids are structurally important but pharmacologically inert. This picture is portrayed in a recent handbook, *Phospholipids*,[19] which does not even mention the use of phospholipids as drugs or dietetic supplements. The second reality is that PCs are molecules that are pharmacologically active and functionally essential. Until recently this idea was not popular. A recent review summarizes actions of the fluid-state PCs and offers a hypothesis in which PCs play a key role in the correction of redox imbalance.[4]

This duality of the phospholipid world has been recently reviewed.[20] The dual action of PCs and other phospholipids is highly relevant to cosmetics and dermatology.

24.7 EFFECTS OF THE TOPICAL APPLICATION OF FLUID-STATE PHOSPHATIDYLCHOLINE

Differential scanning calorimetric analysis shows that there is an interaction between a fluid-state PC and a model stratum corneum lipid mixture in water. This interaction results in a gradual exchange of lipids, fusion of the two systems, and increased fluidity of the model barrier structure. The interaction is complete within 4 h. This very much contrasts with 24 h that the gel-state PC needs to achieve the same result.[21] The results also show that the fluid-state PC penetrates significantly deeper to the rat[22] and human skin[23] than the gel-state PC. These findings were independently confirmed in an experimental study in which the depth of penetration was visualized and in which the fluid-state PC reached the living part of the epidermis.[24]

The following three groups of human studies provide circumstantial evidence that the fluid-state PCs penetrate into the dermis where they may be catabolized to generate biologically active substances:

- Application of fluid-state PC in a liposomal form significantly decreased both the microcirculation in the dermis,[25] and the erythema induced by UVB-light.[26] The latter result could be explained by a series of reactions in which UVB-light induced generation of hydrogen peroxide, activation of PLD,[27] and formation of PC metabolites with anti-inflammatory effects.[16]
- An anti-acne effect was reported by two groups of workers, both using fluid-state PC in different formulations. One group treated acne vulgaris Type 2 for 28 or 56 days. The control was provided by the untreated contra-lateral side of the face. There was, on an average, 64% reduction in the number of commedones and 75% reduction in efflorescence's on the treated side.[28] These findings were confirmed by results of another independent study.[37]
- Application of fluid-state PC also initiates biochemical changes in cell cultures. Fluid-state PC increases cellular lipid fluidity and decreases the rate of proliferation of HaCaT human keratinocytes. No toxicity was observed.[29] Choline, which is a metabolite of PC and the precursor of the organic osmolyte betaine, is actively transported to the keratinocytes. The relevance of this finding is not understood.[30] Acetylcholine is synthesized, secreted, and degraded in human keratinocytes.[31] In addition, PLD, which generates choline from PC, is involved in the differentiation of keratinocytes.[32]

24.8 EFFECTS OF FLUID-STATE PC MATRIX LOADED WITH SUBSTANCES

As outlined above, fluid-state PCs penetrate through stratum corneum to the epidermis. In this process, those molecules that are embedded in the PC matrix get transported along. For long it has been thought that a prerequisite for a successful penetration is the encapsulation of the drug substance into a PC liposome. This assumption has been challenged recently.[33]

The following products, containing a drug substance embedded in a fluid-state PC matrix, have been developed and are now available:

Brand name	Indication	Drug substance
Complex 15	Face crème	Dimethicone
Diclac Schmerzgel	Inflammation, pain	Diclofenac
Dolaut	Joint and muscle pain	Diclofenac
Ecofenac Lipogel	Rheumatoid diseases	Diclofenac
Essaven Gel	Venous microcirculation	Aescin, heparin
Hamemetum Crème	Anti-inflammatory	Hamamelis distillate
Heparin ratiopharm	Sport injuries	Heparin
Pevaryl Gel	Antimycotic	Econazol
Menorest patch	Hormone therapy	Estradiol
Vivelle patch	Hormone therapy	Estradiol

24.9 EFFECTS OF TOPICAL APPLICATION OF GEL-STATE PCs

In another study, fluorescent spectroscopy was used to compare the physicochemical properties of matrices constructed either from the gel-state PC or from the stratum corneum lipids. The transition temperatures were found to be 55°C for the gel-state PCs and 60 to 63°C for the stratum corneum lipids. Further comparison showed that the gel-state PC is more hydrophilic and therefore binds more water. It is also more fluid and more polar.[34]

Differential scanning calorimetry was used for evaluating the interaction between model mixtures of stratum corneum lipids and gel-state PC. The mixing of the systems was complete in about 24 h.[21] The slow interaction of gel-state PC may also apply to its topical application *in vivo*.

These two observations suggest that the uptake of a topically applied gel-state PC matrix alters the biosensor role of the stratum corneum. It results in an elevation of stratum corneum hydration, makes the lipid barrier less rigid and the lipid mixture more polar. This knowledge is relevant for the treatment of dermatitis and dry skin, the conditions in which stratum corneum is less hydrated, less capable of binding water, and abnormally rigid.

24.10 UPTAKE AND TOLERANCE GEL-STATE PCs

A study on human abdominal skin showed that fluorescent dye embedded in a bilayer made from gel-state PC was taken up by the skin,[24] but the dye penetrated only into stratum corneum. The results of this experiment are illustrated in Figure 23.2. Similar results were reported also by others.[22,23,35]

The tolerance of skin of 20 volunteers to the gel-state PCs was studied in comparison to some common emulsifiers. Nine emulsifiers and gel-state PC were tested in the Duhring Chamber using test protocol of Frosch and Kligman.[36] Based on scaling and erythema, gel-state PCs were the only substances that showed no irritation potential.[37] The results are shown in Figure 23.3. Reviewed recently were also the toxicological aspects of the use of gel-state PC in a topical application.[7]

24.11 EFFECTS OF THE TOPICAL APPLICATION OF A GEL-STATE PC MATRIX LOADED WITH SUBSTANCES

The first studies in which fluid-state lipid bilayers were used as a penetration vehicle to promote delivery of a drug substance to the skin were published as early as 1980.[38] In rabbits, triamcinolol

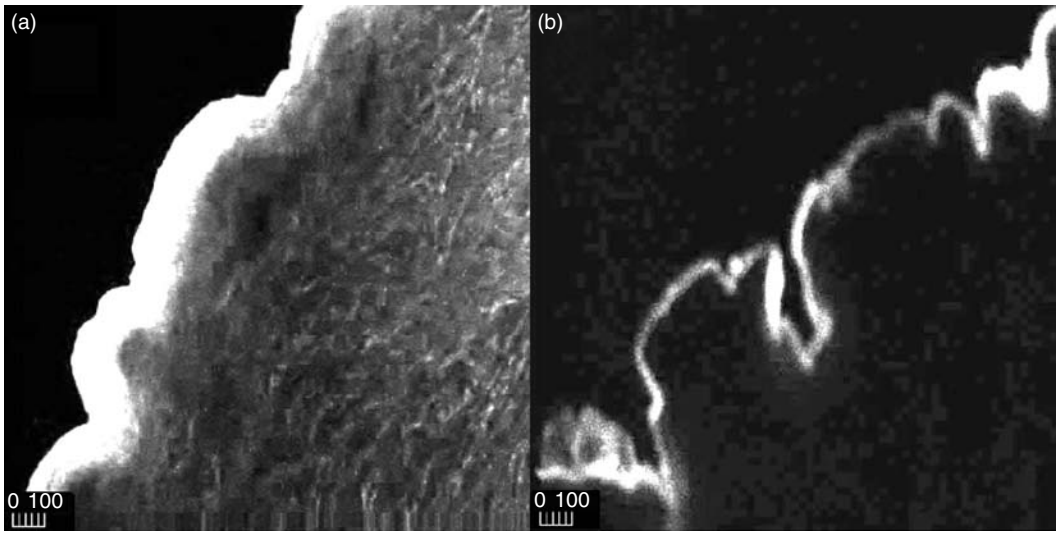

FIGURE 24.2 Uptake of the fluid-state PC (a) and the gel-state HPC (b) by the skin after a 3 h exposure as visualized by the fluorescent dye carboxyfluorescein.[24]

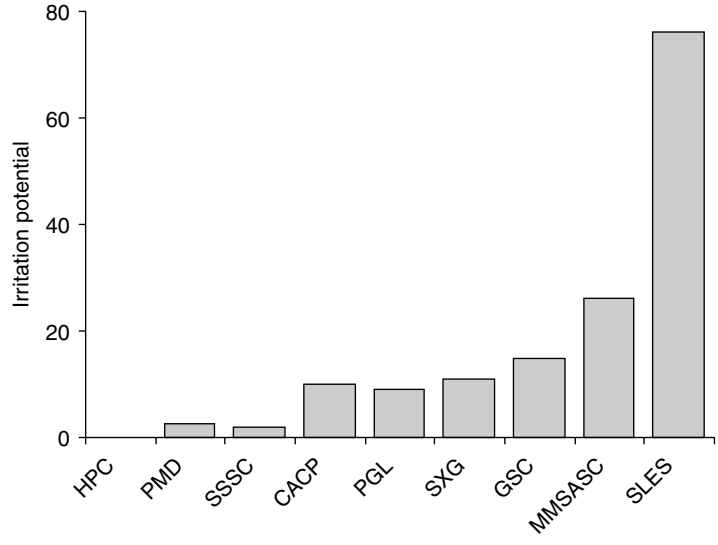

FIGURE 24.3 Emulsifiers and their irritation potential as assessed from the extent of erythema and scaling. HPC, hydrogenated lecithin; PMD, polyglyceryl-3 methylglucose distearate; SSSC, sorbitan stearate and sucrose cocoate; CACP, cetearyl alcohol and cetearyl polyglucose; PGL, polyglycerinlaurate; SXG, saponins-xanthan gum; GSC, glyceryl stearate citrate; MMSASC, macromolecule and stearic acid and sodium chloride; SLES, sodium laureth sulfate (from References 36).

embedded in a fluid-state PC matrix accumulated in the epidermis and dermis, and its urinary excretion was delayed by five days in comparison with the classical gel-state formulation. In another study, the same group of researchers reported that in 24 adult volunteers the local anesthetic effect of tetracain in the gel-state PC matrix lasted for 4 h while no such effect was observed when classical formulations were used.[39]

Based on this research the following drugs were developed and are now commonly available:

Brand name	Indication	Drug substance
Ciproxin	Inflammation; Ear drops	Hydrocortisone, Ciprofloxacin
L-M-X 5	Pain, Anorectal treatment	Lidocain
Repithel	Wound treatment	Povidone iodine
Surpass	Inflammation; Animal treatment	Diclofenac

24.12 EFFECTS OF THE TOPICAL APPLICATION OF GEL-STATE PC MATRIX LOADED WITH PHOSPHOLIPID METABOLITES

As outlined above, gel-state PC matrix penetrates stratum corneum without any damage to the lipid barrier and transports substances into the epidermis. Endogenous phospholipid metabolites are organic osmolytes that possess protective biological functions. Under chronic exogenous provocations the endogenous pool of these metabolites may get exhausted and the exogenous supply might help to replenish and strengthen the endogenous protective mechanism.

In addition, new technology is available, which uses the gel-state PC matrix for stabilizing a formulation containing not only metabolites but also emollients free of surfactants, perfumes, and preservatives. This new technology is used for the preparation of Physiogel AI (Stiefel Laboratories), a new cosmetic product in the market in several European countries. Physiogel AI contains the following metabolites:

- N-palmitoylethanolamine, an endocannabinoid with anti-inflammatory and antioxidant action,[40]
- Betaine, an organic osmolyte, and
- N-methylglycine, an organic osmolyte with a nitric oxide scavenging action.[41]

The results of several open clinical studies, most of them submitted for publication, show that this product:

- protects against light-induced DNA damage using p53 and thymidin dimer as indicators,[42]
- in the treatment of atopical dermatitis is as effective as 1% hydrocortisone,[42]
- ameliorates pruritus in patients undergoing maintenance haemodialysis,[43] and
- relieves pain, itching, and burning in perianale dermatitis.[44]

24.13 DISCUSSION

The permeability barrier located in stratum corneum protects the skin against loss of water at a rate of 1.6 kg per cm^2 per hour. With a body surface of 1.8 $m,^2$ this translates into protection against a potential loss of almost 30 t water per hour.[45] This remarkable and still ill-understood protective action is sometimes overlooked. Even a text book as renowned as the *Molecular biology of the cell* does not mention the existence of this barrier.[46]

When the ancestors of man left the oceans and started to live on the dry land, their most critical acquisition was the capacity to adapt to a continuously changing dry gaseous environment. Humans live not only in hot deserts but also in cold Alaska, in the high altitudes of Tibet and the low altitudes of the Dead Sea. They stay alive in summer and winter. Some humans gratuitously add to the adaptation task of their skin by uselessly washing and perfuming themselves several times a day. This accounts

for the increasing prevalence of atopic dermatitis, indicating a continuous intentional and accidental exogenous damage.[1]

The principle of evolution, to survive by adaptation, is also followed by the skin. Noxious effects on the skin surface and on stratum corneum are translated by signaling systems into messages for the epidermis and dermis. The response is the activation of endogenous protective agents. In the past several years the mechanisms of these protective responses have become better understood. This improved comprehension of a system as complex as the skin should translate into an improved practical treatment of the skin. The first generation of new products is indeed already in the market.

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25 Lanolins

Ian Harris and Udo Hoppe

CONTENTS

25.1 INTRODUCTION

Lanolin has been used by man as a skin emollient for thousands of years.[1] Lanolin (from the Latin *lana* for wool and *oleum* for oil) is another name for wool wax, which is secreted by the sebaceous glands of the sheep (*Ovis aries*) to soften the fleece and protect it against the elements. Lanolin was used by the ancient Greeks (*circa* 700 B.C.), and a method of recovering lanolin from wool washings was described by the Greek physician Dioscorides (60 A.D.) in his *De materia medica*.[2]

Lanolin and its numerous derivatives have been widely used in the pharmaceutical and cosmetic industries for many years as vehicles for active ingredients and for their beneficial effects on skin function.[3] Although purified lanolin is used without incident by millions of people, confusion still exists concerning the possible allergenic potential of lanolin. The extremely low incidence of sensitization of healthy individuals to purified lanolin used in the cosmetic and pharmaceutical industries has been comprehensively reviewed.[4–6]

25.2 PURIFICATION OF LANOLIN (WOOL WAX)

Lanolin is a very complex mixture of esters, diesters, and hydroxy esters of high molecular weight lanolin alcohols and lanolin acids. Being a complex natural product, the method of refinement for lanolin is very important, as this determines the composition, properties, and quality of the purified lanolin.[1,7,8] It is necessary, therefore, to bear in mind that not all refined lanolins are the same. The incredibly complex composition of lanolin also means that it cannot be synthesized.[1]

Wool wax, unlike human sebum, contains no triglycerides and is chemically a wax rather than a fat.[8,9] The wool wax of newborn lambs is thought to consist almost entirely of esters which are very pale in color. These esters are hydrolyzed in the alkaline secretions by bacteria and the environment. The products can undergo further oxidation and degradation. The yield and composition

of the secreted wool wax depend on physiological and environmental factors, such as the age of the sheep, the time of year, the use of pesticides, and the presence of airborne pollutants.[1,7] Therefore, some producers of lanolin use raw material from countries such as New Zealand, which have strict laws on the use of pesticides and which are relatively free from industrial pollution. The crude lanolin is also blended to overcome the problem of variability.

The ancient Greeks (*circa* 700 B.C.) extracted lanolin by boiling the fleece in water. Methods of recovering and refining have been improved to remove dirt, detergents, and other unwanted contaminants.[10] The degradation products of wool wax esters such as oxidized material are undesirable and are the source of color and free acidity. The oxidized material is more polar and more readily emulsifies in the wool washings. As a result the degradation products remain in the aqueous phase and can be removed by centrifuging.[11,12] This process is relatively inefficient, with yields of less than 50%. However, the quality is superior to the older method of acid cracking. The resultant product is refined, and the remaining water and associated detergents are removed to produce anhydrous lanolin.[1]

25.3 COMPOSITION OF LANOLIN

The purity of lanolin and standard tests have been described in the *European Pharmacopoeia* (EP), in *The United States Pharmacopoeia* (USP), and according to other national standards.[13,14] Lanolin is a semisolid with a melting point of approximately 40 ± 6°C and has a molecular weight in the range of 790 to 880 Da. Lanolin is a complex and variable mixture of mainly esters, diesters, hydroxy esters (87.0–93.5%, w/w),[7,8,15] lanolin alcohols (6.0–12.5%, w/w), lanolin acids (<0.5%, w/w), and lanolin hydrocarbons (<1.0%, w/w). The latter are also called "paraffins" and "petrolatum" by the EP and USP, respectively.[13,14,16–18] Approximately 40% of the esters are α-hydroxy esters. Due to the extremely complex nature of lanolin, the true number of different esters present is unknown. Barnett calculated the theoretical number of monoester combinations from random combinations of 69 aliphatic lanolin alcohols, 6 sterols, and 138 saturated lanolin acids to total 10,350.[8] This is most probably an underestimate of the total number of esters, as dibasic acids and dihydric alcohols also occur naturally in lanolin.[19] Further combinations of cyclic mono- and di-esters may be formed by dehydration and from inter- and intra-esterification due to heating during the manufacturing process.[7,8]

The analysis of lanolin has concentrated on the lanolin alcohols (the unsaponifiable fraction of lanolin) and lanolin acids produced by hydrolysis rather than the esters in lanolin itself.[20] Lanolin alcohols belong to three major groups: (1) 69 aliphatic alcohols from C_{12} to C_{36}, (2) sterols (cholesterol and dihydrocholesterol), and (3) trimethyl sterols (lanesterol, dihydrolanesterol, agnosterol, and dihydroagenosterol).[21] The latter have been incorrectly termed triterpenoids. The relative proportion of each group is 22% (w/w) aliphatic alcohols, 35% (w/w) sterols, and 38% (w/w) trimethyl sterols.[8]

The nature of the substance that is responsible for sensitization to lanolin is not clear, but it has a high affinity for the natural free alcohols.[1] Clark has proposed that the incidence of adverse reactions can be virtually eliminated by reducing the level of lanolin alcohols to no more than 3% (w/w).[1] Alternatively, the lanolin alcohols can be intensively purified to eliminate traces of the compounds which may cause sensitization.[22]

The reported number of lanolin acids (C_7 to C_{41}) varies dramatically from 32 to 138.[17,18,21,23–26] The possible explanation for this discrepancy is that different methods were used to produce the alcohols.[8] The lanolin acids comprise four major classes: normal, iso (ω-1-methyl substituted), anteiso (ω-2-methyl substituted), and α- and ω-hydroxy acids. The relative proportions are 12.1% normal acids, 22.1% iso acids, 26.3% anteiso, 27.1% α-hydroxy acids, and 5.1% ω-hydroxy acids.[27,28] Minor constituents include polyhydroxy acids (4.7%) and unsaturated acids (2.1%).[23–25]

25.4 COMPARISON OF LANOLIN TO SEBUM AND STRATUM CORNEUM LIPIDS

Although lanolin and human sebum are both products of sebaceous glands, their compositions are very different.[9] Lanolin contains sterol esters, unlike human sebum, which contains mainly triglycerides, and squalene, which is a precursor of cholesterol.

Cholesterol is a major component of the alcoholic fraction of lanolin.[15] It is also an essential constituent of the lipids of the stratum corneum, which form the epidermal permeability barrier.[29,30] However, the other stratum corneum lipids (ceramides and fatty acids) are different from those of lanolin. Clark and Steel have suggested that the α-, β-, and ω-hydroxy lanolin acids are esterified with diols to form diesters with two long acyl chains, which are similar to those found in ceramides.[16,31,32]

Stratum corneum lipids and lanolin share an important physical characteristic in that they can coexist as solids and liquids at physiological temperatures.[33] A differential scanning calorimetry thermogram of lanolin is similar to that of stratum corneum lipids, showing two broad (heterogenous) phase transitions with midpoint melting temperatures at 21.9 and 38.3°C.[16] The lower temperature peak may represent the transition from a liquid crystal to a gel phase, which has also been described for lanolin alcohols.[34]

25.5 LANOLIN AS A MOISTURIZER

Apart from being a lubricant which reduces friction and roughness, lanolin is an effective moisturizer. That is, when lanolin is applied to dry or inflexible stratum corneum it becomes hydrated and more supple, overcoming the signs and symptoms of dry skin.[35]

Kligman demonstrated that hydrous lanolin, like petrolatum, was able to improve mild to moderate winter xerosis in ten white, young-adult females using a visual scoring system in a double-blind study.[35] A twice daily application to the lower leg produced a successive improvement in the xerosis over a 21 day period. Application of lanolin four times daily was also demonstrated to be superior to twice daily applications. The water in the hydrous lanolin is unlikely to be responsible for the moisturizing as repeated immersion of dry legs in water for 5 min, 6 times a day, for 2 weeks had no effect in relieving xerosis.[35] The beneficial effects of lanolin and petrolatum are not due to the greasy nature of the substances as neither mineral oil, olive oil, nor goose grease had significant moisturizing effects.[35]

Powers and Fox demonstrated that lanolin is semi-occlusive and can reduce transepidermal water loss (TEWL).[36] The application of lanolin and lanolin oil (5.0–6.25 mg/cm^2, equivalent to a film thickness of 54–68 μm) to the inner surface of the forearm reduced the TEWL by 32 and 22%, respectively.[36] This is in comparison with petrolatum which reduced the TEWL by 48%. Spruits reported a 20 to 30% reduction in TEWL using a 50-μm lanolin film.[37] The clinical improvement in xerotic skin is not simply due to a transient reduction in TEWL, because a completely impermeable plastic film applied twice a day had no beneficial effects.

Lanolin appears to penetrate into the stratum corneum, but remains in the more superficial layers.[38,39] Using the tape stripping technique, Clark demonstrated the penetration of anhydrous lanolin (2 mg/cm^2) applied to the flexor aspect of the inner forearm.[40] Almost all of the applied lanolin was recovered and most was removed in the first 15 strippings. Although the bulk of lanolin may remain in the superficial layers, electron-dense lead linoleate and lead oleate topically applied in lanolin were observed by transmission electron microscopy to be localized in intracellular spaces as far down as the stratum granulosum.[40]

Anhydrous lanolin appears to trap some of the water which is moving through the stratum corneum and spontaneously forms emulsions when placed on the skin for only 5 min.[16,31] Cryo-SEM of skin samples following application of lanolin shows vesicles ranging from 0.5 to 300 nm in diameter, which are presumably water droplets that have passed through the skin, subsequently forming a water-in-oil emulsion.[16,31,32]

TABLE 25.1
Effect of Topically Applied Substances on Barrier Recovery

		TEWL (%)	
Treatment	**n**	**45 min**	**4 h**
Vehicle	10	106.9 ± 5.2	69.6 ± 5.5
Stratum corneum lipids (optimal molar ratio)	10	81.7 ± 5.4[a]	44.5 ± 5.3[a]
3% Lanolin	20	81.3 ± 4.1[a]	45.4 ± 3.1[a]
15% Lanolin	10	—	21.7 ± 1.7[a]
2% Petrolatum	10	—	51.7 ± 3.6[a]
10% Petrolatum	9	58.2 ± 10.5[a]	—

[a] Statistically significant relative to vehicle ($p < 0.001$).

Note: TEWL measurements were taken following acetone perturbation of the stratum corneum of hairless mice and following application of substances in a propylene glycol-ethanol vehicle. The initial transepidermal water loss is 100%.

Source: Adapted from Elias, P. et al., *The Lanolin Book*, Beiersdorf AG, Hamburg, 1999. With permission.

Many moisturizers can provide instant relief from xerosis, although the effects are short-lived.[35] Lanolin and petrolatum can be distinguished from other moisturizers in that their effects are long-lived. After application of lanolin or petrolatum for 21 days, the time taken to regress from the improved state back to the original state was 14 and 21 days, respectively.[35] The time taken for lanolin and petrolatum to improve xerotic skin and then, when application is stopped, to regress is approximately equivalent to the turnover time of the stratum corneum. This implies that lanolin and petrolatum may affect not only the anucleated horny layer, but may also change the physiology of the nucleated layers of the epidermis.[35]

In addition to restoring the clinical appearance of xerotic skin, lanolin can also accelerate the restoration of normal barrier function to normal skin that has been acutely perturbed. Elias and colleagues have demonstrated that lanolin accelerated epidermal barrier recovery following perturbation with acetone.[41] Three percent lanolin not only significantly ($p < 0.001$) decreased the TEWL at 45 min, but also after 4 h compared to vehicle-treated sites (Table 25.1). However, the rate of barrier recovery of lanolin-treated sites between 45 min and 4 h was not significantly different compared to vehicle treatment. This indicates that lanolin has an immediate effect on restoring a permeability barrier and does not interfere with the process of lamellar body extrusion and lipid synthesis, which are required for continued recovery. The effect of 3% lanolin on barrier recovery was very similar to that of the optimized ratio of stratum corneum lipids (ceramides, cholesterol, and fatty acids).[42,43]

Other physical methods of assessment of the stratum corneum, such as corneometry and microprofilametry, demonstrate a statistically significant effect of lanolin.[16] The parameters of skin roughness, Rz and Ra, determined by microprofilametry, can be reduced by 2 mg/cm^2 lanolin alcohol or lanolin for at least 8 h after application.[16]

Lanolin and lanolin alcohols are more commonly used in a more complex formulation which provides a cream or lotion of a more acceptable consistency. Figure 25.1 shows that increasing levels of lanolin alcohol in a water-in-oil cream progressively decreased the roughness of xerotic skin in 24 elderly volunteers over a 14 day period, in a dose-dependent manner.[44] Lanolin alcohols also reduced the TEWL of the same volunteers over a 28 day period (Figure 25.2).[44] Petersen demonstrated that the hydration of stratum corneum was higher after application of a cream containing

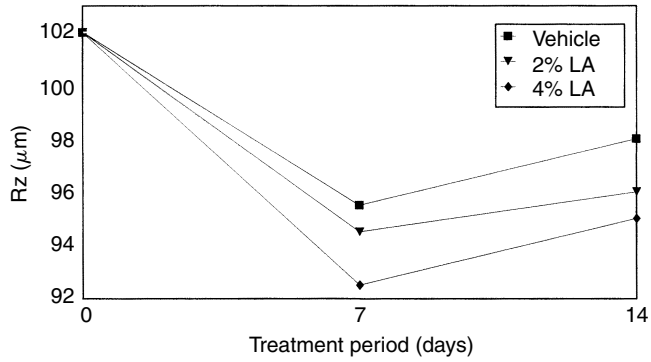

FIGURE 25.1 Lanolin alcohol (Eucerit®, Beiersdorf AG, Hamburg, Germany) reduces skin roughness, as measured by microprofilometry. Lanolin alcohol (2 and 4%, w/w; LA) in a water-in-oil cream containing petrolatum was applied twice daily to the volar aspect of the arm. (Adapted from Sauermann, G. and Schreiner, V., *The Lanolin Book*, Beiersdorf AG, Hamburg, 1999. With permission.)

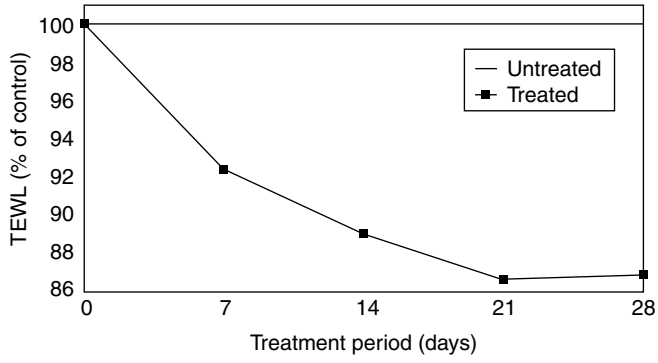

FIGURE 25.2 Lanolin alcohol (Eucerit) reduces TEWL over time. Lanolin alcohol in a water-in-oil cream containing petrolatum (Nivea®, Beiersdorf, AG, Hamburg, Germany) was applied twice daily to dry skin of the lower leg of 24 elderly volunteers relative to the untreated control site. TEWL was measured under controlled conditions 14 h after application of the cream using an evaporimeter. (Adapted from, Sauermann, G. and Schreiner, V., *The Lanolin Book*, Beiersdorf AG, Hamburg, 1999. With permission.)

lanolin (oil-in-water formulation) than after application of petrolatum, using optothermal infrared spectrometry.[45] By measuring electrical conductance, Moss demonstrated that preparations containing lanolin increased the surface hydration.[46] Lanolin can also act as a barrier to the entry of virus particles and irritants into the skin.[44,47]

Lanolin does not have a detrimental effect on epidermal homeostasis of clinically normal skin or healing of wounds. In contrast to oils, such as olive oil and mineral oil, lanolin does not have adverse effects on the homeostasis of normal epidermis.[48] Butcher found that repeated application of olive oil and mineral oil resulted in acanthosis and parakeratosis, which is abnormal proliferation and differentiation.[48]

Chvapil et al. investigated the effects of epidermal growth factor (EGF) in a lanolin vehicle on partial thickness wounds.[49] A beneficial effect of lanolin vehicle was observed with little additional benefit from EGF. Lanolin statistically increased the rate of reepithelialization, the thickness of the dermis, and the number of cells in the dermis. Lanolin is likely to promote wound healing by maintaining a moist environment.[50,51]

25.6 LANOLIN AS AN EMULSIFIER AND A VEHICLE FOR DRUG DELIVERY

Lanolin and lanolin alcohols are excellent emulsifiers, giving oil-in-water emulsions, and can be combined with additional emulsifiers such as cetearyl alcohol to improve stability.[8] The ability of anhydrous lanolin and especially lanolin alcohols to form stable emulsions with up to 300% (w/w) of water distinguishes lanolin from petrolatum in its physical properties.[1] The chemical compositions of lanolin and petrolatum are also very different, as petrolatum is composed completely of hydrocarbons.[16] Lanolin alcohols and petrolatum can be combined to form cholesterized petrolatum, which also has the capacity to form emulsions. For example, a mixture of 65% (w/w) lanolin, 20% (w/w) water, and 15% (w/w) petrolatum can incorporate an equivalent weight of water without changing its consistency.[15,52]

The ability of lanolin alcohols to deliver active substances to the skin is partly due to their surface activity.[15] Lanolin alcohols, which contain a high proportion of cholesterol, have a high surface activity and are able to reduce the interfacial tension of a mineral oil–water system from 52.5 to 5.0 dyn/cm.[8,53] Even at low levels, cholesterol can reduce the interfacial tension of emulsions and dispersed systems.[8,54]

Lanolin has been used for many years as a vehicle for pharmacologically active substances in ophthalmic ointments and topical formulations.[55–60] In addition to being a vehicle for penicillin and other antimicrobial substances, lanolin contains lipids such as 10-methyldodecanoic acid and 12-methyltridecanoic acid, which have antimicrobial activity.[61,62]

25.7 LANOLIN DERIVATIVES

The highly complex nature of lanolin makes it a rich source for fractionation and producing derivatives.[8,16] Lanolin, a semisolid of liquid and solid wax esters, can be further fractionated into lanolin oil and lanolin wax.[63,64] The solid esters of lanolin can be removed using low temperature fractional solvent crystallization. The liquid esters contain a higher concentration of lower molecular weight, branched-chain, hydroxy compounds. Lanolin oil is reported to possess the emollient properties of lanolin with the benefits of being fluid at room temperature and the ability to be spread to form thinner films.[65] It can be solubilized in clear detergent systems to give conditioning properties.[8] The hard lanolin wax esters are used to improve the consistency and to add stability to lip glosses and lipsticks.[8]

The extremely large number of lanolin derivatives has been reviewed by Barnett[8] and Steel.[16] Lanolin derivatives can be formed by acetylation, ethoxylation, propoxylation, alkoxylation, and isobutylation of hydroxy groups, as well as hydroxylation of the double bond in the sterol ester component. Hydrolysis of lanolin can also produce lanolin alcohols and lanolin acids, which like lanolin can be ethoxylated, acetylated, and hydoxylated.

Although cholesterol is essentially insoluble in water, a soluble cholesterol derivative can be formed by reacting it with high levels of ethylene oxide.[66,67] This ethoxylated product has balanced hydrophobic and hydrophilic properties. The increased hydrophilicity makes it useful as an oil-in-water emulsifier or as a stabilizer. Ethoxylated lanolin is used for viscosity regulation, pigment dispersion, and as a solubilizer. Less than 1% ethoxylated cholesterol is effective at reducing the viscosity of anionic lotions to make them easier to pour. Non-ionic systems require more ethoxylated cholesterol for this purpose.[68]

Lanolin has stood the test of time as an emulsifier and skin emollient. Its complex nature has been a rich resource of derivatives formed from fractionation and chemical reactions. Although the composition of lanolin is different from the lipids found on the surface of human skin, lanolin has been demonstrated to be equivalent in its ability to restore barrier function. In addition to the beneficial effects attributable to its physical properties, lanolin may also have a pharmacological effect on the epidermis.

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26 Essential Fatty Acids: Biological Functions and Potential Applications in the Skin

Lesley Elizabeth Rhodes and Amy Storey

CONTENTS

26.1 INTRODUCTION

Burr and Burr reported in 1929 "a new deficiency disease produced by the rigid exclusion of fat from the diet."[1] Rodents fed a fat-free diet showed reduced growth and reproductive failure, accompanied by two prominent changes in the skin, that is, increased scaliness and impaired barrier function.[1,2] Reversal of the features of deficiency by administration of linoleic acid (LA), led to the concept of essential fatty acids (EFA) that cannot be synthesized by the higher animals.[2] Similarities between the clinical features of EFA deficiency and atopic dermatitis led Hansen in 1937 to discover low blood levels of unsaturated fat in atopic children,[3] and he later reported that EFA-deficient infants developed an eczematous rash, which responded to LA supplements.[4] Several studies had previously examined a range of dietary oil supplements in atopic dermatitis,[5–8] with generally reported benefit.

There are now recognized to be two families of EFA, the n-6 and the n-3 fatty acids, derived from LA and α-linolenic acid (ALA), respectively. Although attention previously focused on the effects of EFA deficiency on the skin, interest has shifted to the physiological changes in the skin induced by altered EFA status. The n-6 fatty acids are more abundant and more active in the skin than n-3 fatty acids, but the essential role of the latter in neurological development is established,[9] and there is growing recognition of their function in skin as modulators of inflammation and the immune response. The EFA are now understood to influence skin physiology and pathology via a range of effects including those on skin barrier function, eicosanoid production, membrane fluidity, immune function, cell adhesion, cell signaling, and gene expression.[10]

26.2 CLASSIFICATION AND NOMENCLATURE

Three major families of unsaturated fatty acids are seen in warm-blooded animals, that is, the n-9, monounsaturated fatty acids (e.g. oleic acid, OA), and the n-6 and n-3, both polyunsaturated fatty acids (PUFAs). However, only the n-6 and n-3 families, derived from LA and ALA, respectively, are EFA. These must be obtained from the diet since mammals lack the desaturase enzymes necessary for the insertion of a double bond in the n-6 and n-3 positions of the fatty acid carbon chain. Fatty acid nomenclature is as follows: The first number denotes the number of carbon atoms in the acyl chain and the second refers to the number of unsaturated (double) bonds. This is followed by a symbol n or ω and a number that denotes the number of carbon atoms from the methyl terminal of the molecule to the first double bond. Hence, LA is 18:2(n-6), while the more unsaturated ALA is denoted as 18:3(n-3) (Figure 26.1). These fatty acids must be metabolized to their longer chain derivatives before carrying out many of their activities.

26.3 DIETARY SOURCES

The LA and ALA are mainly obtained from PUFA-rich vegetable oils. LA is found in high concentration in several oils including safflower, corn, and soybean, while ALA is found in linseed, canola, and soya bean.[11] Although most of the n-6 requirement is obtained from dietary LA, small amounts of its longer chain metabolite arachidonic acid (AA) may be ingested from food of animal origin,

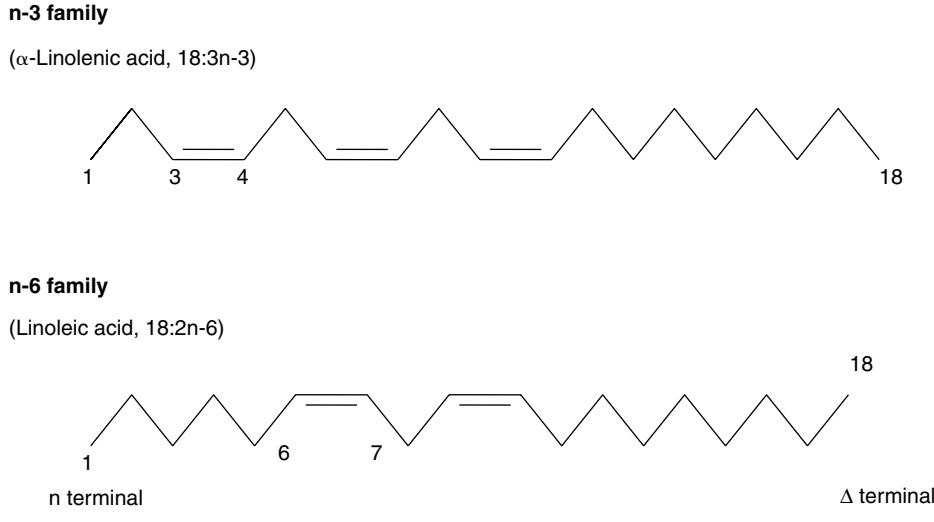

FIGURE 26.1 Chemical structure of α-linolenic (n-3 fatty acid) and linoleic acid (n-6 fatty acid).

particularly meat, liver, and egg yolk. In contrast, the longer chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found mainly in marine animals and oily fish, including mackerel, sardines, herrings, and salmon.

26.4 THE EFA CONTENT OF SKIN

The EFA are found predominantly within the epidermal phospholipids (e.g., phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine), with the pattern of incorporation varying between fatty acids. They are also present in small amounts in cholesterol, and importantly, are found linked with ceramides in the granular layer and stratum corneum, where they play a critical role in barrier function. The most abundant EFA in the skin are LA and AA, comprising approximately 12 and 3.5%, respectively of human epidermal fatty acids.[12] ALA is known to accumulate in the skin of animals, but is found in much lower levels than LA.[13]

26.5 METABOLISM OF EFA

The metabolism of LA and ALA involves an alternating sequence of desaturation and elongation to produce longer chain, more unsaturated fatty acids, including AA (20:4n-6), derived from LA, and EPA (20:5n-3) and DHA (22:6n-3), from ALA (Figure 26.2). All desaturations and elongations take place near the carboxy terminal (Δ) of the chain, allowing the methyl terminal to preserve its relationship to the first double bond. Hence in mammals there is no interconversion between the n-3 and n-6 fatty acids. Fatty acids of the n-3, n-6, and n-9 families compete for the same enzymes, and there is preferential desaturation in the order ALA > LA > OA. Thus the ratio of 20:3n-9 (an OA metabolite) to 20:4n-6 (AA), known as the triene–tetraene ratio, is used to assess for deficiency

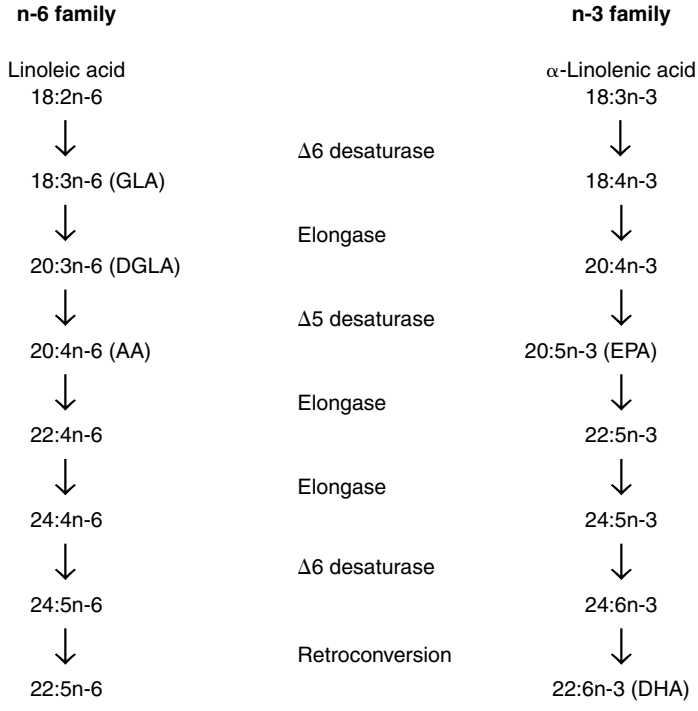

FIGURE 26.2 Metabolic conversion of linoleic acid and α-linolenic acid to longer chain, more unsaturated fatty acids.

of n-6 and n-3 fatty acids, indicated by a ratio of >0.2. The initial step in the metabolism of LA and ALA to the longer chain fatty acids is the rate-limiting Δ6 desaturase, the activity of which is altered by several hormonal and dietary factors. EPA is converted to DHA by two elongation steps to produce 24:5n-3, then a desaturation step by delta-6 desaturase to 24:6n-3, followed by a peroxisomal β-oxidation, retroconversion step to 22:6n-3.[14,15] This pathway is demonstrated for the n-3 PUFAs, although it is also believed to apply for the n-6 fatty acids.

The EFA metabolism is presented in several extensive reviews.[9,16,17] Much of the information concerning EFA physiology and biochemistry has been derived from work in hepatocytes and may be of limited relevance to epidermis since a major role of the liver is to convert dietary lipids into energy stores. Meanwhile, keratinocytes are involved in the fatty acid metabolism required both for normal cellular processes and the specialized role in the permeability barrier. Unlike the liver, the epidermis does not possess the capacity to desaturate at the Δ5 or Δ6 position, and therefore the skin relies on a supply of AA, LA, and ALA from the bloodstream. There is evidence for a distinct fatty acid binding protein in keratinocyte plasma membranes that is involved in EFA uptake into the skin and also recycling of free fatty acids from the stratum corneum.[18] The transport mechanism in epidermis differs from that in hepatocytes since there is preferential uptake of LA over OA, which may function to ensure adequate capture of LA for barrier lipid synthesis.[18]

26.6 FUNCTIONS OF EFA IN THE SKIN

26.6.1 Cutaneous Barrier Function

A major role of the skin is to provide an effective barrier against excessive water loss. Increased transepidermal water loss (TEWL), together with scaling and epidermal hyperplasia, are early features of EFA deficiency. It appears that the hyperplasia is at least partly driven by the barrier defect.[19] While administration of LA restores barrier function and reduces the scaling and epidermal hyperproliferation,[20,21] AA reduces the hyperproliferation, but does not improve barrier function.[22] There is now good evidence that the impaired epidermal barrier is due to the loss of unusual linoleate-containing ceramides (acylglucosylceramides) from the stratum corneum. The structure of the permeability barrier is ascribed to sheets of stacked lipid bilayers (lamellae) surrounding the corneocytes in the stratum corneum. These lamellae are rich in ceramides (sphingolipids), in which LA is the most abundant unsaturated fatty acid.[23] The presence of lamellar bodies, from which the lipid lamellae are derived, is closely associated with the presence of water barrier function.[24,25]

Evidence suggests that LA-linked acylglucosylceramides, present in the upper layer of the viable epidermis, are essential for the formation of lamellar granules and for the subsequent assembly of their contents into lamellae.[26,27] In LA deficiency, the fatty acid is replaced in the ceramide molecule by OA, causing conformational changes that result in inability to form normal lamellae, with associated loss of epidermal barrier.[28] Normally, extrusion of the lamellar granule contents into the stratum corneum is accompanied by the removal of glucose from acylglucosylceramides, resulting in the formation of LA-linked acylceramides, which are abundant in the lipid lamellae of the stratum corneum.[29] The linoleyl moiety of the acylceramide may be further metabolized by lipoxygenase before the barrier function is exhibited,[30] and it is likely that the oxidized metabolite of LA involved is 13-hydroxyoctadecadienoic acid (13-HODE).[17] 13-HODE also reverses epidermal hyperproliferation, possibly by inhibiting protein kinase C activity.[17]

26.6.2 Production of Eicosanoids

The EFA are the precursors of the eicosanoids, namely prostaglandins, leukotrienes, thromboxanes, hydroxy fatty acids, and lipoxins. These important extracellular mediators at low concentrations have critical roles in skin homeostasis. At high concentrations they are involved

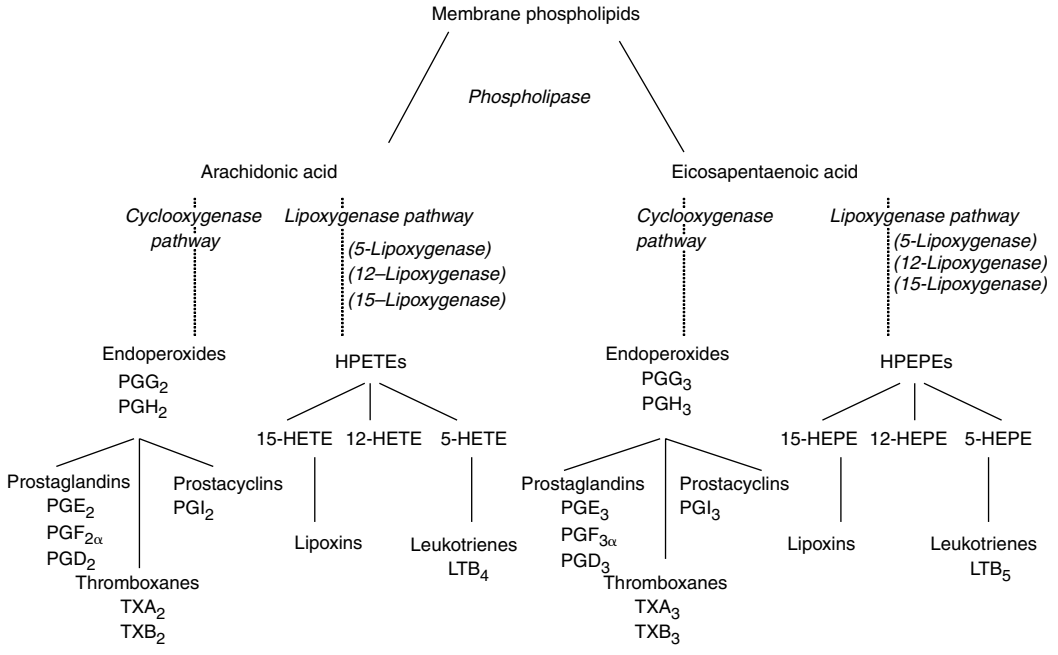

FIGURE 26.3 Metabolism of arachidonic acid and eicosapentaenoic acid via the cyclooxygenase and lipoxygenase pathways.

in modulating the inflammatory process and cell proliferation,[30,31] and also appear involved in epidermal carcinogenesis.[17,31,32] AA is converted into a range of mediators (Figure 26.3), and in many respects, the n-3 metabolites can be regarded as partial agonists of the n-6 eicosanoids, often producing similar, but less potent actions.[16]

The EFA stored in the phospholipids of cell membranes are released by phospholipases, and then undergo oxidative transformation by the cyclooxygenase (COX) pathway to prostanoids and by the lipoxygenase pathway to hydroxy fatty acids and leukotrienes. The metabolism to prostanoids is catalyzed by two isoenzymes of COX, a constitutive (COX-1) and an inducible form (COX-2). The main products of COX metabolism of AA are prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, and PGD_2. In addition, AA is converted via 15-lipoxygenase to 15-hydroxyeicosatetraenoic acid (15-HETE) and lipoxins, by 12-lipoxygenase to 12-HETE, and by the 5-lipoxygenase pathway to leukotriene B_4 (LTB_4), LTC_4, and LTD_4.[17] Oxidation of 5-HETE by activated inflammatory cells produces a further proinflammatory metabolite, the eosinophil chemoattractant 5-oxo-6,8,11,14-eicosatetraenoic acid.[33,34] While many of the oxidative metabolites of AA have potent proinflammatory actions, including leukocyte chemoattraction by LTB_4 and 12-HETE, vasodilatation by PGE_2, platelet aggregation and vasoconstriction by thromboxane A_2 (TXA_2), others such as 15-HETE,[35] PGI_2, and a PGD_2 metabolite, 15-deoxy-PGJ_2,[36] have antiinflammatory potential. A number of AA-derived metabolites, such as PGE_2, 12-HETE, LTB_4, and 5-HETE, also have tumor promoting properties, including enhancing tumor cell survival, stimulating cell proliferation, inhibiting apoptosis, increasing the production of reactive oxygen species (ROS), promoting tumor angiogenesis and tumor cell adhesion [reviewed in 37]. The parent n-6 fatty acid LA can also be metabolized directly by COX and lipoxygenase, to 9-HODE and the antiinflammatory 13-HODE,[38,39] respectively. In addition, the LA-metabolite 13-HETE has been shown to inhibit cell proliferation and enhance apoptosis.[40,41]

The fatty acids of the n-6 series and n-3 series compete with each other for metabolism by COX and lipoxygenase to eicosanoids. While AA is converted to potent inflammatory and tumor promoting mediators, EPA is metabolized to the less active PG of the 3 series, LTB_5 and

15-hydroxyeicosapentaenoic acid (15-HEPE).[16,30,42] EPA has recently been shown to be the preferential substrate for lipoxygenase in comparison with AA.[43] EPA and DHA are more potent than ALA at suppressing AA-derived eicosanoids.[44] Moreover, a rise in dietary γ-linolenic acid (GLA, 18:3n-6) causes increased production of dihommogammalinolenic acid (DGLA, 20:3n-6), which while being a precursor of AA in the liver and other tissues, also competes with AA for metabolism to produce PG of the 1 series, leukotrienes of the 3 series, and 15-hydroxyeicosatrienoic acid (15-HETrE), a potent lipoxygenase inhibitor.[39] Novel oxygenated metabolites generated from EPA and DHA, resolvins of the E series (Resolvin E1 or RvE1) and D series (Resolvin D1 or RvD1), respectively and the conjugated triene, docosatrienes, have recently been isolated from inflammatory exudates and tissues enriched with DHA.[45–47] They are antiinflammatory and neuroprotective,[48,49] and the dihydroxy acid-containing docosatrienes have been recently termed "neuroprotectins."[49]

Dietary fatty acid modification has been found to have a profound influence on epidermal fatty acid composition and subsequent eicosanoid production.[50–52] Human supplementation studies with n-3 fatty acids taken as Maxepa® (Seven Seas, Hull, UK; 18% EPA, 12% DHA) 10 g daily for 3 months, resulted in an increase in epidermal n-3 fatty acids from <2 to $>24\%$ of total fatty acids, with a consequent increase in the n-3 to n-6 ratio.[51] This dietary regime has also been shown to result in an approximately 70% reduction in suction blister fluid PGE_2 levels, in association with a reduced erythemal (sunburn) response to UVB challenge.[52] Reduction of UVB-induced skin PGE_2 levels has subsequently been confirmed in a randomized controlled supplementation study.[53] In an animal model, dietary evening primrose oil (EPO) supplements, which are rich in GLA, caused a significant rise in epidermal DGLA content, accompanied by increased generation of PGE_1 and 15-HETrE.[54,55]

26.6.3 Modulation of Cell Signaling

Membrane fluidity is dependent on lipid content, fluidity generally increasing with level of unsaturation.[56] Dunham et al. demonstrated that in keratinocytes *in vitro* the plasma membrane viscosity ranges over a biologically large factor of two depending on the fatty acid profile of the growth media, with the highest viscosity seen in EFA deficient cells.[57] Since cell-membrane associated proteins are very sensitive to changes in their lipid environment,[58] lipid composition has an important influence on many physiological processes including enzyme and receptor function.

Many extracellular signals act by inducing hydrolysis of cell membrane phospholipids, resulting in the liberation of second messengers, including diacylglycerol (DAG), inositol phosphates, PG, and protein kinase C. Activation of phospholipase C, which can be stimulated by AA,[59] causes degradation of phosphatidyl-inositol 4,5 biphosphate (PIP_2) to inositol triphosphate (IP_3) and DAG. The DAG activates protein kinases, which in turn activate a range of cellular proteins by phosphorylation,[60] while IP_3 acts synergistically to activate cells by increasing ionic calcium. Stimulation of phospholipase A_2 by cell surface signals leads to the release of AA, which is available for eicosanoid synthesis, and free AA may also result from the hydrolysis of DAG. AA and LA can act as second messengers by activating protein kinase C,[61–63] which subsequently activates components of the MAP-kinase signaling cascade, may be an important regulator of intracellular calcium concentration,[64] and regulates epidermal proliferation. On the other hand, EPA and DHA appear to downregulate protein kinase C.[65–67] It is therefore evident that modification of EFA content of membrane phospholipids can potentially influence many vital cell-signaling pathways.

Changes in EFA status affect the activity of several membrane-associated enzymes and proteins.[68–71] Reduced adenyl cyclase activity occurred in EFA-deficient animals,[72] while in animals supplemented with n-6 or n-3 fatty acids, increased adenyl cyclase activity was seen in cardiac membranes.[73,74] However, the opposite effect has been reported in other membranes, possibly reflecting differences in initial fatty acid composition.[75] n-3 PUFAs have been shown to activate membrane Ca-ATPase and inhibit Na, K-ATPase in isolated basolateral membranes from rat duodenal enterocytes[76] and inhibit both Ca-ATPase and Na,K-ATPase activity in synaptosomal membranes isolated from rat cerebral cortex.[77] EFAs have the ability to modify neuronal Ca-ATPase activity

and Na+ channels by directly binding to the channel proteins.[78] It is thought that rather than acting directly on the channel protein, PUFAs alter the phospholipid membrane tension adjacent to ion channel proteins to cause a conformational change.[79]

26.6.4 Modulation of Gene Expression

The n-6 PUFAs have been shown to upregulate COX-2 expression,[80] whereas n-3 PUFAs have been shown to suppress its expression in tumors,[81,82] in association with decreased proliferation of cancer cells and reduced tumor angiogenesis.[83] n-3 PUFAs have also been shown to suppress tumor growth via a COX-independent pathway.[84] DHA, in comparison with LA, has also been shown to decrease the expression of other oncogenes implicated in tumor promotion including ras[85] and bcl-2 expression,[86] resulting in inhibition of mitosis and enhanced apoptosis of cancer cells, respectively.

The n-3 fatty acids, in particular DHA, have been shown recently to inhibit TPA-induced activation of the transcription factor, AP-1.[87] AP-1 transcribes genes involved in cell proliferation, metastasis, and cellular metabolism, and is activated during cellular transformation and tumor promotion. n-3 PUFAs have also been shown to suppress expression of NFκB, a transcription factor responsible for a wide range of inflammatory and immunomodulatory activities, also with tumor promoting activity.[88,89] This inhibition may occur by prevention of phosphorylation of the subunit IκB-α.[90,91] Other transcription factors affected by EFAs include peroxisome proliferator-activated receptor (PPAR) isoforms, and recent research suggests n-3 PUFAs improve insulin sensitivity, possibly via PPARα or PPARγ.[92] All three PPAR isotypes: PPAR-α, PPAR-β/δ, and PPAR-γ, have been identified in keratinocytes. PPAR-β/δ and PPAR-γ have been shown to stimulate keratinocyte differentiation,[93,94] suggesting there might be a role for PPARs in skin disorders involving aberrant differentiation.

26.6.5 Modulation of Immune Function

The EFA of the n-6 and n-3 families influence immune cell proliferation, their activity, and cytokine production.[95,96] Both n-6 and n-3 PUFAs have been shown to inhibit lymphocyte proliferation, the production of interleukin (IL)-2 and natural killer (NK) cell activity *in vitro*. n-3 PUFAs also reduce the production of proinflammatory cytokines, namely interleukin (IL)-1 and tumor necrosis factor (TNF)-α, by peripheral blood monocytes, macrophages, and lymphocytes.[97–99] They inhibit monocyte antigen-presenting functions by reducing surface expression of HLA-DR[100] and downregulate the T-helper 1-type response, involved in chronic inflammatory diseases, possibly by altering CD28 membrane receptor function.[101,102] In human skin cells of both epidermal and dermal origin, that is, keratinocytes and fibroblasts, EPA and DHA independently significantly reduced basal and UVB-induced IL-8 secretion.[103] Interleukin-8 is a powerful chemoattractant for neutrophils, which cause local tissue damage through release of toxic oxygen intermediates, indicating an immunomodulatory role for the n-3 PUFAs in UVB-induced skin effects. In addition, TNF-α-induced IL-8 secretion by keratinocytes was also shown to be reduced by the n-3 PUFAs, implicating a broader role for the n-3 PUFAs in skin inflammation. Other *in vitro* studies have shown that EPA reduces UVB-induced IL-6 secretion by keratinocytes, although inexplicably there was superinduction of UVB-induced TNF-α after EPA.[104] These antiinflammatory properties are not consistently observed in animal and human n-3 PUFA supplementation studies;[53] further studies are indicated in this area.

The n-3 PUFAs are reported to reduce expression of endothelial adhesion molecules VCAM-1, E-selectin, and ICAM-1, therefore influencing leukocyte–endothelial cell interactions and leukocyte migration across the endothelium.[105,106] Oxidized EPA has been shown to be a more potent inhibitor of leukocyte–endothelial interaction, *in vitro* and *in vivo*, than EPA.[107] Since EFAs regulate intercellular adhesion, it has been speculated that the skin changes that are observed in EFA deficiency, may be due, at least in part, to damaged cell adhesion.[108] n-3 PUFAs and GLA supplementation enhance E-cadherin expression in cancer cells and this possibly reduces the invasiveness of these cells.[109]

26.6.6 Modulation of Oxidative Stress

The PUFAs, particularly n-3 PUFAs, are unstable molecules prone to free radical attack and oxidative degeneration. However, it is conceivable that modest degrees of oxidative stress and lipid peroxidation associated with n-3 PUFAs can have beneficial effects. The suppression of cell growth and downregulation of oncogene activity attributable to by n-3 PUFA and GLA may be conveyed via peroxidative mechanisms, since these effects can be prevented by the addition of antioxidants.[110–112] On the other hand, additional vitamin E has been reported to confer further protective effects in n-3 PUFA supplementation studies.[113] The effects of n-3 PUFAs on oxidative status and lipid peroxidation are not consistent between reported studies; variation in baseline levels of fatty acids and antioxidants may contribute to the differences observed. n-3 PUFAs have been shown to upregulate the antioxidant enzymes glutathione transferases and manganese-SOD in mice[114] and decrease SOD in humans.[115] In a human study of UVR-exposed skin, mixed EPA and DHA supplements significantly increased thiobarbituric acid reactive substances.[51] However, on purified EPA, and using a lower UVR dose, no changes were seen in skin malonaldehyde levels and inconsistent changes occurred in skin vitamin E and C content.[116] Observations of increased lipid peroxidation in association with protective effects (i.e., reduced haemolysis, reduced sunburn), have led to the speculation that the unstable n-3 PUFAs could possibly act as a free-radical buffer, protecting more vital structures from attack.[51,117] Further, n-3 PUFAs are also reported as enhancing resistance to free radical attack in association with reduced plasma lipid peroxidation.[118]

26.6.7 Modulation of Apoptosis

It has been recently reported that EFAs are involved in initiating the apoptotic pathway in many cancer cell lines.[119,120] GLA, EPA, and metabolites produced from AA and EPA following UV-irradiation, induced apoptosis in the human promyelocytic leukaemia cell line, HL-60.[121,122] In a melanoma cell line, DHA induced apoptosis and inhibited cell growth.[123] In addition, n-3 PUFAs have been shown to reduce DNA damage and increase apoptosis in the rodent models of dextran sodium sulphate-induced inflammation[124] and azoxymethane-initiated colon tumorigenesis.[125] Since n-3 PUFA-induced apoptosis of tumor development can be inhibited by the addition of antioxidants,[126] a role for ROS in PUFA-mediated cancer cell apoptosis is suggested. PUFA-induced apoptosis of cancer cells is also thought to be modulated by activation of cell cycle regulatory proteins.[127] ROS and lipid peroxides produced may cause mitochondrial dysfunction, resulting in release of cytochrome c and activation of caspase-3.[128]

26.7 EFA IN CLINICAL DERMATOLOGY

26.7.1 Photodermatology and Skin Cancer

Dietary n-3 fatty acid supplements reduce the sunburn response in healthy humans.[51,116,129] In addition, an open study of subjects with the immune-mediated photosensitivity disorder polymorphic light eruption (PLE) found an increased threshold for UVA-provocation of rash after supplementation.[52] Human photoprotection with mixed n-3 PUFAs is accompanied by incorporation of EPA and DHA into epidermal lipids and significantly reduces skin levels of PGE_2.[51,52] Increased lipid peroxidation may be found in skin post-supplementation[51] although this is not a consistent finding between studies using different supplements and methods of assessment.[116] The mechanisms of the photoprotective properties are likely to include reduction in the inflammatory response due to a shift in the balance from the synthesis of n-6 eicosanoids toward the less active n-3 products. There is evidence for a major role of prostaglandins in the sunburn response, and leukotrienes might also be involved through their role in leucocyte chemotaxis.[17,129–131] Modulation of oxidative stress may also play a role in human photoprotection. In a randomized controlled study, EPA supplementation for three months

provided significant protection against the sunburn response, reduced UVR-induced skin p53 by 50%, and protected against *ex vivo* UVR-induced single strand breaks in peripheral blood lymphocytes, while UVR-induced skin cyclobutane pyrimidine dimers were not reduced.[116] This suggests that n-3 PUFA may protect against oxidative but not direct DNA damage.

Studies in hairless mice show a significant suppression of UVR-induced skin cancers during oral n-3 fatty acid supplementation.[132] UVR-induced carcinogenesis is augmented by high levels of PUFA,[133,134] but it is now evident that there is a need to distinguish between long chain n-6 fatty acids, which promote, and n-3 fatty acids, which inhibit, photocarcinogenesis.[132,135] The effects may be analogous to the respective promoting and inhibiting activities of n-6 and n-3 fatty acids in models of colon and breast cancer.[136] There is evidence for an influence of n-3 PUFA and other COX inhibitors on the promotion stage of carcinogenesis, through suppression of PGE_2 and modulation of UVR-induced immune suppression.[137–143] In addition, protective effects may operate through modulation of oxidative stress, lipid peroxidation, apoptosis, cell signaling, and gene regulation (see earlier sections). In humans, a case-control study of males with nonmelanoma skin cancer showed an inverse relationship between skin cancer risk and dietary fish intake.[144] A further case-control study revealed a tendency for a decreased risk of squamous cell skin cancer with increased n-3 fatty acid intake.[145] Hence studies implicate a protective role for n-3 PUFAs in skin cancer, although it is clear that further human intervention studies are necessary.

The COX-2 is over-expressed in skin cancer, as in many other types of cancer,[31,146–148] and promotes cellular hyperproliferation and tumor angiogenesis while suppressing apoptosis.[149] Significantly elevated levels of lipoxygenase products have also been found in skin and other cancers, where they may promote tumor growth. Many inhibitors of COX and lipoxygenase arrest cancer progression and these enzymes are therefore currently being targeted for cancer chemoprevention.[31,45,149–153] Application of COX-2 inhibitors following UVB exposure has been demonstrated to be effective in reducing tumorigenesis in animal models.[154,155] Oral n-3 PUFA ingestion offers a safe[156] alternative to systemic nonsteroidal antiinflammatory drugs for inhibition of COX-2 and lipoxygenase, in addition to having a range of other apparently anticarcinogenic properties, and hence is of potential value in skin cancer chemoprevention.[157]

26.7.2 Atopic Dermatitis

A series of studies over many years have suggested the existence of an abnormal EFA pattern in the tissues of subjects with atopic dermatitis.[3,158,159] The plasma levels of LA and ALA are normal, and hence there is no evidence of dietary deficiency of EFA. However, low plasma levels of LA metabolites, that is, GLA, DGLA, and AA, and also ALA metabolites EPA and DHA, are reported and are interpreted to be consistent with defective functioning of Δ6-desaturase.[158,160] Similar findings have been reported in the peripheral blood monocytes in atopic asthma and allergic rhinitis sufferers,[161] and in umbilical cord blood in infants at risk of atopy, where the biochemical abnormality is proportional to the IgE level.[162] Atopic women are also reported to have low levels of GLA, DGLA, and AA in breast milk.[163] However, a more recent study showed no difference in the plasma PGE_1 and PGE_2 levels between adults with atopic dermatitis and healthy control subjects, making it unlikely that Δ6-desaturase deficiency is the key metabolic defect in atopic dermatitis.[164] Abnormalities of lamellar body ultrastructure,[165] altered sphingomyelin metabolism,[166] and reduced levels of ceramide 1[167] are also observed in patients with atopic dermatitis, raising the possibility that a defect in barrier function may account for the dry skin of eczema.[168]

Several clinical trials of n-6 EFA supplementation in atopic dermatitis were performed in the 1980s, usually with the GLA-rich EPO (Epogam®, Scotia Pharmaceuticals, Guildford, UK).[169–173] Dietary EPO raises the DGLA:AA ratio, resulting in increased generation of antiinflammatory and immunomodulatory eicosanoids. Most of these studies were reported to show some clinical improvement of atopic dermatitis following supplementation, particularly with respect to itching. In 1989, the manufacturer's research institute performed a meta-analysis of published and unpublished EPO

supplementation studies.[174] This was reported to show a significant clinical benefit in both adults and children, and a correlation between clinical improvement and rise in plasma EFA. The controversy regarding this meta-analysis, including the lack of data for perusal in the public domain, has been recently reviewed.[175] Recent double-blind studies of EPO alone, and of combined EPO and fish oil, showed no benefit of either treatment in adult and childhood atopic dermatitis,[176] while EPO in atopic children showed no advantage over sunflower oil.[177] Similarly, a study of EPO in chronic hand dermatitis showed equal improvement in active and control groups,[178] in keeping with a high placebo response rate in dermatitis.

Dietary supplementation studies with n-3 fatty acids alone have generally not been promising in atopic dermatitis. An initial double-blind study reported a subjective improvement on fish oil compared with the control OA, but no objective improvement on physician assessment.[179] A further double-blind study using EPA with saturated fatty acids as the control, showed equal improvement with both supplements and the benefit was attributed to increased clinician guidance,[180] while a multicenter study showed a similar improvement in clinical score in subjects taking fish oil or corn oil.[181] The latter results might possibly reflect a beneficial effect of both EFA-containing oils, but more likely imply a placebo effect, and illustrate the problems posed both in selection of a suitable control and the interpretation of such studies.

A recently reported meta-analysis of EFA in atopic dermatitis identified 19 trials of GLA and 5 trials of fish oil, which matched their inclusion criteria of placebo-controlled trial.[182] It was concluded that supplementation with EFA has no clinically relevant effect in dermatitis. Furthermore, the U.K. Medicines Control Agency recently withdrew the product license for GLA supplementation in atopic dermatitis.

26.7.3 Psoriasis

Disturbances in lipid metabolism occur in the skin in psoriasis. Increased phospholipase A_2 activity is seen in lesional and nonlesional skin, while phospholipase C activity is elevated in lesional skin.[183,184] Increased elongase activity is also observed in psoriatic epidermis. A local increase in AA occurs, and this appears to be preferentially metabolized by the lipoxygenases, resulting in a marked increase in 12-HETE and LTB_4, while there appears to be a relative or absolute reduction in metabolism by the COX pathway.[185] Leukotriene B_4 is a very potent chemoattractant, and topical application causes epidermal hyperproliferation in addition to neutrophil microabscesses.[186] A defective transmembranous cell-signaling system is also suggested by elevation of both IP_3 and DAG in the psoriatic plaque.[187]

Epidemiological studies reveal a very low prevalence of psoriasis in Greenland Eskimos.[188] Eskimos have high tissue levels of the n-3 fatty acids EPA and DHA, attributable to their high consumption of marine lipids.[189] In contrast, psoriatic plaques contain elevated levels of the n-6 fatty acid AA and its metabolites LTB_4 and 12-HETE. Dietary n-3 supplements result in suppression of these levels due to substitution by less active eicosanoids, including LTB_5 that is a less potent neutrophil chemoattractant and stimulator of keratinocyte proliferation than LTB_4.[190,191] These findings have led to several clinical studies of n-3 (EPA + DHA) fatty acid supplementation in psoriasis, with conflicting results. While most of these studies have reported a mild-moderate improvement in the clinical features of psoriasis,[190–193] an eight week study using olive oil as a control showed no benefit.[194] A further multicenter double-blind study showed no advantage of four months treatment with fish oil over control (corn) oil.[195]

It has been suggested that the above therapeutic approach may be too simplistic.[196] There might be a genetic difference in EFA metabolism in Eskimos, since they are also noted to have high DGLA levels accompanied by low AA levels, consistent with a lack of the enzyme $\Delta 5$-desaturase.[196,197] When Eskimos change from their traditional marine diet to a westernized diet, tissue levels of EPA and DHA fall, but the AA level remains low relative to DGLA. Since DGLA is converted to PGE_1, which has antiinflammatory properties, it is conceivable that the low prevalence of psoriasis in this

population might be partly attributable to the higher DGLA levels.[196] Hence, double-blind studies have been performed of combined EPO and n-3 fatty acid supplements (Efamol Marine®, Scotia Pharmaceuticals, Guildford, UK) in psoriasis, but again no improvement was seen.[198,199]

26.7.4 Acne Vulgaris

Low levels of LA are found in the sebum of acne sufferers,[200] and levels appear inversely related to sebum secretion rate.[201] It has been hypothesized that this local EFA deficiency may lead to the follicular hyperkeratosis and occlusion of acne, and that an increased supply of linoleate might possibly ameliorate the condition.[202] In support of this, digital image analysis revealed that topical application of LA over a one month period reduced the size of microcomedones.[203] Decreased levels of LA may also contribute to acne inflammation by failing to inhibit phagocytosis and ROS generation by neutrophils.[204]

26.7.5 Wound Healing

The effects of EFA on wound healing are variable, some studies report no effect, while others report a beneficial[205] or a detrimental[206] influence. In animal experiments, dietary GLA reduced ionizing radiation-induced adverse skin effects when given over the time course of expression of the damage, raising the possibility of increased therapeutic gain for patients undergoing radiotherapy.[207]

26.8 CONJUGATED LINOLEIC ACID

There has also been much interest in the anticarcinogenic properties of conjugated linoleic acid (CLA), derivatives of LA found particularly in cooked meats and processed dairy products.[208,209] The isomeric products differ from LA in the position and configuration of the double bonds, and it is suspected that the *cis* 9, *trans* 11 and *trans* 10, *cis* 12 isomers are the most biologically active. These LA derivatives are incorporated into keratinocyte phospholipids in the same distribution as LA, where they result in decreased AA content and PGE_2 synthesis.[210] Studies of CLA administration in mouse carcinogenesis models give evidence of inhibition of both the initiation and promotion stages of chemically-induced skin cancer.[211,212] Potential mechanisms include modulation of eicosanoid synthesis, signal transduction and oxidative stress, inhibition of cell proliferation, induction of apoptosis, and modulation of immune function and gene expression.[209,210,212,213] CLAs have recently been shown to inhibit cell proliferation in cancer cell cultures,[214,215] induce apoptosis in cancer cells,[216–218] inhibit preadipocyte differentiation[219] and angiogenesis,[220] and reduce production of NO and PGE_2 and NF-κB activity.[221] Conjugated fatty acids derived from n-3 PUFAs also show promising antitumor properties. Conjugated EPA (CEPA) and conjugated DHA (CDHA), induce apoptosis in numerous cancer cell lines.[222] CEPA has been recently shown to be more effective at suppressing tumor growth in DLD-1 human colon tumor cells transplanted into nude mice, than either CLA or EPA supplementation.[223]

26.9 EFA STATUS IN THE MODULATION OF CUTANEOUS RESPONSES

It is clear from the above discussion that alterations in dietary lipid intake can profoundly affect cell membrane composition in human skin, and consequently influence many physiological and pathological processes. While deficiency of LA results in loss of barrier function and hyperproliferation, an altered n-3 and n-6 EFA balance may influence many processes including a wide range of inflammatory and immune responses, and skin carcinogenesis. There is evidence that alterations in eicosanoid production may contribute to many of the activities, while there is also an increasing understanding

of the contribution attributable to EFA modulation of signal transduction, gene expression, lipid peroxidation, and apoptosis.

After many years of controversy, the consensus opinion is now that there is little role for the use of EFA in atopic dermatitis, and this is reflected by removal of the product license in the United Kingdom. The etiology of the common inflammatory skin disorders, eczema and psoriasis, is still poorly understood, and EFA supplementation is addressing just one potential factor. In addition, since AA is metabolized to both pro- and antiinflammatory metabolites, more selective inhibition may be needed. Of more current clinical interest is the use of n-3 PUFA in prevention of carcinogenesis. Effective inhibition of COX and lipoxygenase products by n-3 PUFA gives the potential for powerful anticarcinogenic properties. Additionally, fatty acid supplementation has the advantage of a high safety profile compared with nonsteroidal antiinflammatory drugs, for use as an approach to skin cancer prevention.[157] Further research is needed to explore the manifold mechanisms of action of EFA in skin cells, their optimal balance in the skin, and the effect of their manipulation on skin immune responses and carcinogenesis.

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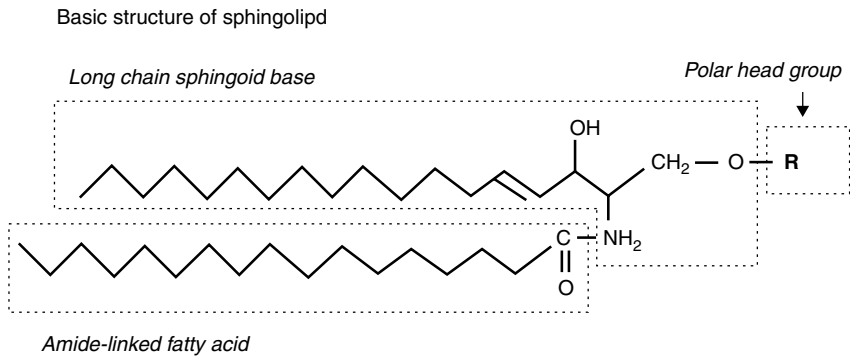
27 Sphingolipids: from Chemistry to Possible Biologic Influence on the Skin

Hisashi Wakita

CONTENTS

27.1 CHEMISTRY OF SPHINGOLIPIDS: OVERVIEW

Over 300 types of sphingolipids are synthesized in various mammalian cell types. Structurally, sphingolipids are composed of a long-chain aliphatic 2-amino-1,3-diol (sphingoid base), an attached amide-linked fatty acyl chain varying in length from 16 to 24 carbon atoms, and a polar head group at the 1-position (Figure 27.1). The diversity of sphingolipids originates from a variety of head groups: ceramide has a hydroxyl at the 1-position, sphingomyelin has phosphorylcholine head groups, and glycosphingolipids contain carbohydrate head groups. Glycosphingolipids are further classified according to the sequence of sugars and the chemical bonds which link them together: cerebrosides have a single glucose or galactose, other neutral lipids such as latotosylceramide and trihexosides have higher order glycose units, and acidic glycosphingolipids contain one or more sialic acid residues (gangliosides) or sulfate monoester groups (sulfatides). For every sphingolipid there is a corresponding lysosphingolipid, which has the identical polar head group at the 1-position, but lacks the amide-linked fatty acyl group at the 2-position. For example, deacylation of ceramide produces sphingosine, a representative of free long-chain bases (FLCBs). Sphingosine can be further converted to highly biologically active metabolites such as sphingosine-1-phosphate via sphingosine kinase-catalyzed phosphorylation at the 1-position,[1] and *N*,*N*-dimethylsphingosine via amino-dimethylation.[2]



R	Sphingolipid
Hydrogen	Ceramide
Phosphorylcholine	Sphingomyelin
Single sugar	Cerebroside
Sugar chains with sialic acid	Ganglioside
Sugar chains with sulfate	Sulfatide

FIGURE 27.1 Structural formulas of sphingolipids.

27.2 CHEMISTRY OF SPHINGOLIPIDS IN THE SKIN

Epidermal sphingolipids play important roles in cell construction, growth, and differentiation of keratinocytes and in cohesion, desquamation, and a permeability barrier formation of the stratum corneum. To better understand these functions, the structure and composition of the epidermal sphingolipids have been elucidated.[3] Sphingolipid composition generally changes dramatically during differentiation, development, and oncogenic transformation. In the epidermis, the amount of sphingolipid such as FLCBs, ceramides, glucosylceramides, and gangliosides is increased with keratinocyte differentiation, especially with transition from the granular layer to the stratum corneum. In contrast, almost complete disappearance of glycerophospholipids is observed during this process.[4] Therefore, the stratum corneum is one of the richest tissues containing sphingolipids, and, in fact, various biologic functions of sphingolipids on the body surface have been revealed.

27.2.1 Free Long-Chain Bases

The major FLCBs in the stratum corneum of human skin include both dihydrosphingosines (sphinganines) and sphingosines (sphingenines) with 18 to 20 carbons, in addition to some phytosphingosines (hydroxysphinganines).[5–7] Moreover, an FLCB with three hydroxyl groups and one double bond (6-hydroxysphingosine) was specifically identified in the stratum corneum of the human skin.[6,7] Although the biological significance of such a variety of FLCB molecular species remains unknown, the relative percentage of each molecular species shows site-related differences in normal skin and differs between normal and pathologic skin conditions.[6] Compared with the stratum corneum of normal lower legs, molar percentages of FLCB having 18 carbons and those with 20 carbons were higher and lower, respectively, in normal plantar epidermis. Psoriatic scales and hyperkeratotic stratum corneum from the clavus and plantar keratoderma contain increased levels

of FLCB with 18 carbons and decreased levels of FLCB with 20 carbons, possibly reflecting the abnormal keratinization in hyperkeratotic skin conditions.

27.2.2 Glycosphingolipids

Although glucosylceramides are the predominant epidermal glycosphingolipids,[8] various classes of glycosphingolipids such as acylglucosylceramides and gangliosides have been identified in epidermis and cultured keratinocytes.

27.2.2.1 Epidermosides

One group of sphingolipids characteristic to epidermis is acylglucosylceramides (AGCs). Hamanaka et al. reported that human epidermal AGCs consisted of *N*-(*O*-linoleoyl)-ω-hydroxy fatty acyl sphingosyl glucose and *N*-(*O*-linoleoyl)-ω-hydroxy fatty acyl phytosphingosyl glucose and named the epidermal AGCs "Epidermosides."[9] The main role of epidermosides in the stratum corneum is thought to be participation in the formation of the epidermal permeability barrier in conjunction with other lipids including (acyl)ceramide, cholesterol, and free fatty acids.

27.2.2.2 Gangliosides

The existence of trace amounts of gangliosides in epidermis had been reported already by Gray and Yardley in 1975.[8] However, they could not identify the individual compounds. Paller et al. demonstrated that the total ganglioside content of the epidermis was about 1 μg of lipid-bound sialic acid per milligram of dry weight and compromised 0.1% of the total epidermal lipids.[10] They determined that GM3 was the predominant ganglioside of the epidermis followed by GM2 and GD3, and polysialylated gangliosides such as GT1b were also presented in trace amounts.

Not only biochemical analysis, but immunohistochemical staining with antibodies against gangliosides has similarly revealed the existence and distribution of gangliosides in epidermis. Nakakuma et al. showed that epidermal keratinocytes reacted with an anti-GM3 monoclonal antibody, but not an anti-GD3 mAb.[11] Expression of GM3, predominantly in the stratum corneum, was reported by Paller et al.[12] In contrast, Hersey et al. detected GD2 in the basal and spinous layers of the epidermis, whereas neither GM3 nor GD3 was detected in normal skin.[13] However, the epidermis adjacent to naevi and primary melanoma strongly expressed GD3.[13]

One of the biologically important gangliosides in epidermis is 9-*O*-acetyl-GD3. The ganglioside was initially thought to be a surface marker for basal cell carcinoma of the skin because of the presence of the ganglioside in basal cell carcinoma, but not in normal epidermis.[10,14] However, 9-*O*-acetyl-GD3 was identified as CDw60 antigen,[15] which is expressed on a subset of lymphocytes such as activated human B lymphocytes[16] and Th2-type CD8+ T (Tc2) cells,[17] and has been implicated in the control of cellular proliferation. CDw60 antigen was also expressed in activated epidermal keratinocytes in psoriasis vulgaris. Moreover, T cell lines obtained from lesional skin of psoriasis vulgaris up-regulated CDw60 expression in cultured normal keratinocytes via IL-13.[18] This finding suggests the role of 9-*O*-acetyl-GD3 in the pathogenesis of psoriasis vulgaris. Furthermore, alterations in the amount and composition of individual gangliosides on neoplastic and activated keratinocytes may lead to novel therapeutic interventions.

27.3 POSSIBLE INFLUENCE OF SPHINGOLIPIDS ON THE SKIN

Few reports have investigated the therapeutic and cosmetic applications of sphingolipids on skin, except for ceramides, which can be used for treatment of dry skin such as atopic dermatitis. However, various *in vitro* studies have demonstrated the biological effects of sphingolipids on differentiation

and proliferation of keratinocytes, which are the predominant cell type in epidermis. Therefore, it is possible in the future that sphingolipids might be utilized as an ingredient in topical medicine or cosmetics. In this chapter, the biological effects of sphingolipids on cultured keratinocytes are mainly discussed.

27.3.1 Free Long-Chain Bases

The discovery that sphingosine inhibited protein kinase C (PKC) activity spurred interest in sphingolipids as modulators of cell function.[19] In epidermis, it was initially speculated that free sphingosine liberated from ceramides in the stratum corneum may provide a feedback mechanism for regulating the differentiation process. Based on this hypothesis, we investigated the direct biologic action of sphingosine on a transformed human keratinocyte cell line and reported the proliferation-promoting effects of sphingosine on the cells.[20] However, the effects of sphingosine on cultured, normal human keratinocytes remain to be elucidated. In contrast, some studies have examined the biological effects of topical application of sphingosine on the skin. Arnold et al. assessed the effects of sphingosine following tape stripping, which is a potential PKC activator, on the level of induction of ornithine decarboxylase (ODC), and found that application of 0.1 *M* sphingosine resulted in a decrease in ODC activity of approximately 50%.[21] Gupta et al. demonstrated that sphingosine inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation, hyperplasia, induction of ODC activity and ODC mRNA, and activation of PKC in mouse skin.[22] Their data are compatible with the hypothesis that PKC is a major mediator of the phorbol ester response and that PKC inhibitors may have therapeutic potential in the treatment of inflammatory skin diseases such as psoriasis.

In epidermis, however, unusually large concentrations of free sphingosines are found in the stratum corneum, where free sphingosines make up about 0.5% of the total lipids.[23] Therefore, it is doubtful that temporary release of a small amount of sphingosine could have any important effect on keratinocytes.[7] In addition, FLCBs in epidermis are "detoxified" by forming a complex with cholesterol sulfate,[24] possibly because of the highly cytotoxic nature of FLCBs. However, at the very surface of the skin, cholesterol sulfate disappears during desquamation and released FLCB might play a physiological role at this site. In fact, Bibel et al. have demonstrated a role for sphingosine in the stratum corneum as a cutaneous antimicrobial barrier.[25–27] Their *in vitro* examination of the antimicrobial activity of stratum corneum phospholipids and sphingolipids showed that only the FLCBs were effective against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Micrococcus luteus*, *Propionibacterium acnes*, *Brevibacterium epidermidis*, *Pseudomonas aeruginosa*, and *Candida albicans*. Of note is that antimicrobial activity was duplicated *in vivo* by topical application and microbial challenge, suggesting the usage of FLCB as a topical applicant for antimicrobial regimen.[26,27]

27.3.2 Glycosphingolipids

Glycosphingolipids in cellular membranes generally play two major functional roles: as mediators of cell–cell and cell–substratum interaction and as modulators of transmembrane signaling.[28] In epidermis, however, the main role of sphingolipids, especially ceramides, has been thought to function as an extracellular barrier in the stratum corneum, the nonliving epidermal layer. Since the barrier function of sphingolipids is discussed in further detail in other sections, we have focused mainly on the biological effects of sphingolipids on the viable cell layers.

27.3.2.1 Epidermosides

In addition to participation in epidermal permeability barrier formation, epidermosides are likely associated with autoregulation of epidermal differentiation. Uchida et al. showed that chemically synthesized analogs of epidermosides enhanced keratin synthesis in cultured human keratinocytes

and induced the morphological changes such as enlargement and flattening, which are compatible with the morphology of differentiated keratinocytes.[29] The results suggest that AGC, which appears as a consequence of terminal differentiation of keratinocytes, supports the differentiation process. Since the amounts of acylceramide, a breakdown product of AGC, are decreased in the stratum corneum of psoriasis vulgaris,[30] which shows an altered pattern of differentiation,[31] AGC might be a potential topical medicine in the future.

27.3.2.2 Gangliosides

Direct pharmacological effects of gangliosides on cultured keratinocytes have been vigorously investigated. Suppressive effects of gangliosides on keratinocyte proliferation were initially reported by Paller et al.[32] They showed that (1) ganglioside GM3, which is the predominant ganglioside of keratinocyte membranes, inhibited the growth of cultured normal human keratinocytes without modulating keratinocyte differentiation; (2) GD3, 9-*O*-acetyl-GD3, and GD1b also inhibited keratinocyte proliferation; and (3) GM1, GD1a, and sialic acid had little effect. They suggest that hematoside (GM3) and "b" pathway gangliosides (GD3, GD1b), generated by the preferential activation of sialyltransferase II versus *N*-acetylgalactosaminyltransferase, may be involved in control of keratinocyte growth but not of differentiation. It was subsequently demonstrated that highly sialylated gangliosides, GT1b and GQ1b could promote keratinocyte differentiation.[33,34] However, the pattern of differentiation induced by GT1b and GQ1b seems to be distinct. Paller et al. showed that ganglioside GT1b induced both early (desmosome formation and keratin 1 expression) and late (involucrin expression and cornified envelope formation) phase differentiation markers,[33] whereas Seishima et al. reported that GQ1b, a tetrasialoganglioside containing two disialosyl residues, induced cornified envelope formation and enhancement of transglutaminase activity, which are characteristic steps in the late phase of terminal differentiation in cultured keratinocytes, while GT1b was much less effective.[34] Both reports suggest that GT1b preferentially promotes the initial phase of keratinocyte differentiation, whereas GQ1b predominantly accelerates the late phase of differentiation. These observations suggest that the mechanisms which mediate the differentiation induced by these gangliosides are different, since GT1b could not cause a shift in intracellular free calcium or alter PKC activity,[33] while GQ1b induces a increase in intracellular free calcium and activates PKC[34] via the activation of phospholipase D.[35] Since the transition from the epidermal spinous to granular cell layer is mainly dependent on PKC activation,[36] it is highly likely that the biologic effects of GQ1b on keratinocyte differentiation are mediated by activation of PKC. In contrast, the differentiation modulating effects of GT1b seem to be mainly dependent on its action on the cell–cell and cell–extracellular matrix interaction of keratinocytes. We recently observed that GT1b enhanced the cell surface expression of E-cadherin, which is the major homotypic cell–cell adhesion molecule on keratinocytes and which plays a crucial role in stratification.[37] In addition, the ganglioside GT1b prevents attachment of keratinocytes to fibronectin,[38] which has the ability to inhibit the suspension-induced differentiation of keratinocytes.[39] Therefore, GT1b might promote differentiation by up-regulating cell–cell interaction and down-regulating cell–extracellular matrix interaction, which accelerate the stratification of keratinocytes.

Gangliosides also modulate the biological functions of cutaneous residential cells other than keratinocytes, which might be pathophysiologically relevant. For example, gangliosides GM2, GM3, and GD1a augmented anti-IgE-induced mediator release from human skin mast cells,[40] suggesting that gangliosides optimize IgE-receptor–ligand interaction and that alterations in cellular gangliosides could thus induce enhanced releasability, as observed in atopics.

27.3.3 Acidic Phospholipid Autacoid

Much attention has recently focused on lyso-formed phospholipids, including both glycerophospholipids such as lysophosphatidic acid (LPA) and lysosphingolipids such as sphingosine-1-phosphate (S-1-P) and sphingosylphosphorylcholine (SPC) (Figure 27.2), since they elicit diverse cellular

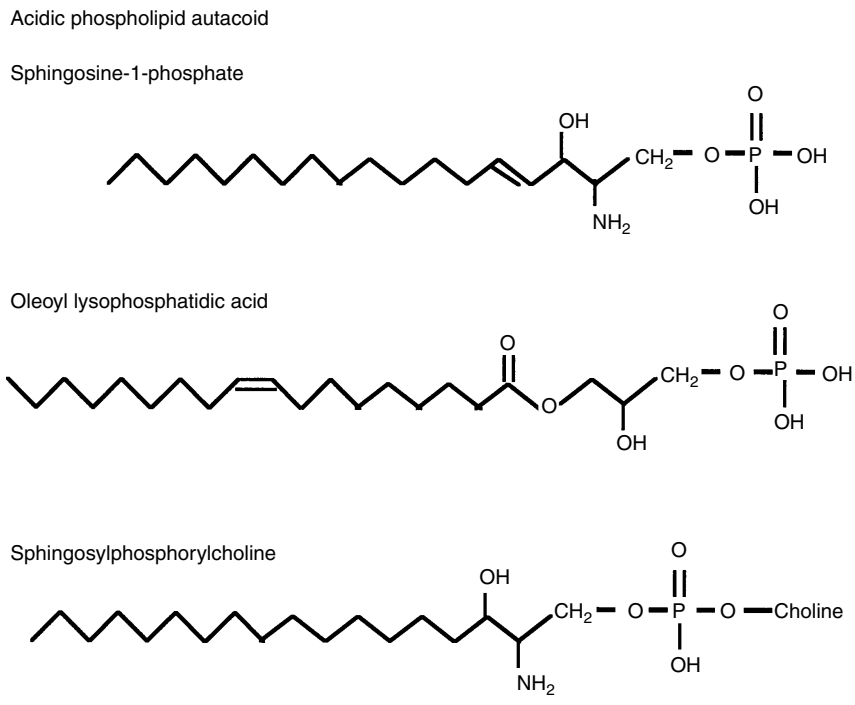


FIGURE 27.2 Structure of acidic phospholipid autacoid.

effects that range from mitogenesis to the prevention of programed cell death via the interaction of specific cell surface receptors.[41–43] Therefore, these active lipids are termed the "acidic phospholipid autacoid (APA) family of lipid mediators."[44,45] Since the main source of APA is platelets and APA is released from activated platelets[46,47] a role for APA lipids in the wound healing process has been speculated. In fact, the topical application of SPC has been reported to accelerate cutaneous wound healing in the diabetic mouse.[48,49] SPC is a potent mitogen of cultured keratinocytes,[49] in addition to dermal fibroblasts.[48] LPA is also mitogenic for keratinocytes via the induction of TGF (transforming growth factor)-α.[50] In addition, LPA induces both the active and latent forms of TGF-β in cultured keratinocytes and increases involucrin synthesis in differentiation-committed keratinocytes.[50] The effects of LPA on TGF-α and TGF-β production by keratinocytes likely have *in vivo* relevance, as concluded from rodent studies involving topical LPA treatment.[50] Although, the biologic effects of S-1-P on keratinocytes remain to be reported, S-1-P promotes the morphogenesis of endothelial cells via induction of cadherin-mediated cell–cell interaction[42] and we thought that S-1-P could enhance the E-cadherin-mediated cell–cell interaction of keratinocytes. Since E-cadherin is crucial for normal epidermal tissue morphogenesis,[51] S-1-P released from platelets might also modulate reepithelization in healing of cutaneous wound.

In conclusion, this chapter discusses the composition of sphingolipids in epidermis and their possible influences on the skin in the context of recent findings regarding the direct action of sphingolipids on cultured keratinocytes. Although it is commonly thought that sphingolipids are rather cytotoxic because of their detergent-like characteristics, we hopefully await their successful application on the skin as therapeutic agents in the treatment of various cutaneous and other human disorders.

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28 Effect of Moisturizers on the Structure of Lipids in the Outer Stratum Corneum of Humans

Keith D. Ertel, Ronald R. Warner, and Ying. L. Boissy

CONTENTS

28.1 INTRODUCTION

Lipids in the stratum corneum (SC) account for only about 15% of its weight, yet they constitute the primary barrier of the skin,[1–5] forming a protective sheath that shields us from desiccation and environmental insults.[6] These barrier lipids exist in the SC intercellular space as highly organized lamellar bilayers that are readily visualized by the marriage of transmission electron microscopy (TEM) with RuO_4 staining.[7,8] The lamellar organization consists of a unique pattern of alternating electron-lucent and electron-dense lamellae forming repeating structures[7–10] that are often referred to as Landmann units.[10] This lamellar structure appears throughout most of the SC thickness,

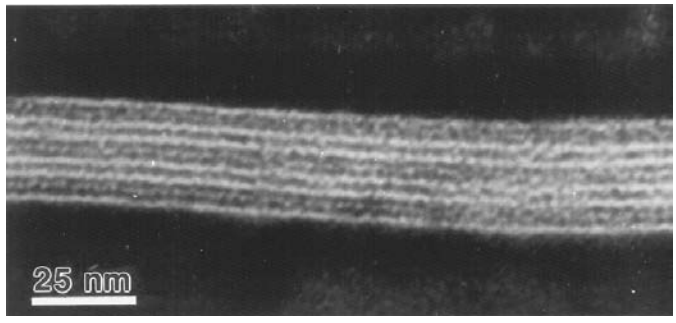

FIGURE 28.1 Normal structure of the lipid lamellae in the intercellular space. Shown are three Landmann units separating the darkly staining corneocytes above and below this intercellular space. Bar = 25 nm.

with variability occurring primarily in the number of Landmann units that bridge the intercellular space.[8,10–12] However, more diverse structures have been described in the outer SC,[11–14] perhaps reflecting environmental impact[12,14] or inherent differences in lipid composition.[15–18]

This chapter focuses on the lipid structure found in the outermost layers of the SC in humans. We present a modified TEM technique to investigate this structure, attempt to systematize and understand the variability in lipid structure observed in the outer SC, and explore the effect of moisturizers on the outer SC at microscopic and macroscopic levels.

28.1.1 Inner Stratum Corneum Lipids

The Landmann-unit structure of intercellular lipid lamellae is illustrated in Figure 28.1. This structure is found throughout nearly all of the normal SC. Swartzendruber et al. proposed a plausible molecular model that accounts for the electron-lucent and electron-dense lamellar structure of the Landmann unit.[10] These Landmann units are dynamic in nature. At least in the inner and middle SC they are altered by age,[19] disease,[8,11,20–22] and hormonal status[23,24]; by experimental solvent treatment[25–27] and topical inhibitor treatment.[28,29] They are known to reform spontaneously following solvent extraction,[5,26] and topical application of certain lipids is also reported to effect lamellar repair and barrier improvement.[29–33]

28.1.2 Outer Stratum Corneum Lipids

In contrast to the more extensive studies of the intercellular lipids of the inner and middle SC, there are few studies of the lipid structure in the outer SC. Evidence suggests that the intercellular lipid composition in the uppermost layers of the SC differs from that found in the lower layers.[34] The outer SC lipids also exhibit structural variability compared to the inner and middle stratum corneum, both with regard to lipid ordering and lateral packing[16,35] and the number of intercellular lamellae, which increases from the usual two or three to in excess of 100 bilayers.[13] For normal skin with little or no visible dryness the outer SC intercellular space is filled with an amorphous lipid material, whereas in soap-treated skin with pronounced visible dryness this space is filled with numerous disorganized lamellae.[14] A separate *in vitro* study using human skin substrate also showed disordered lipid lamellae in the outer SC following soap treatment, less lipid disruption following treatment with a soap/glycerin/oil bar, and normal lamellae following treatment with an isethionate-based bar.[12]

To the extent that lipids are involved in corneocyte cohesion,[36–39] the lipid structure in the outer SC is presumably very important for proper desquamation. However, because the outer SC interfaces with the surrounding environment, its lipids are the most susceptible to structural alterations caused by environmental insult or consumer products that often contain surfactants or solvents.[5,12,30] While

the quantity of SC lipid is apparently not a primary determinant of dryness in normal skin,[40] there may be a functional relationship between the lipid structure of the outer SC and skin dryness.

If consumer products containing soaps or solvents can damage the outer SC lipid structure, then products like moisturizers might also have an impact on this structure. For example, glycerin is reported to increase water binding in the SC and act as a corneodesmolytic,[41] inhibit humidity-induced SC lipid crystalline phase transitions,[42] and speed barrier recovery.[43] Maleated soybean oil inhibits crystalline phase transitions and reduces water loss in model SC lipid systems.[42] And petrolatum, which is often viewed as a gold standard for moisturization, can permeate the upper layers of the SC, affect SC lipid structure, and accelerate barrier repair.[29,44] Conversely, there is evidence that single components of physiologic lipid mixtures and some moisturizers interfere with recovery following experimental barrier disruption.[31,45,46]

Studies employing mixtures of physiological lipids provide important insights into how topical application of these products can impact SC lipids. However, moisturizers sold in the mass market are often quite different from these specialty formulations, being based on more common moisturizing ingredients. Although commercial moisturizers typically improve skin condition, relatively little is known about *how* they effect this improvement. These products appear to provide a continuum of effects ranging from the purely cosmetic, such as temporarily camouflaging visible dry flakes, to more functional effects such as abetting biological repair processes.[47] As noted previously, one mechanism by which the latter might occur is by aiding the digestion of desmosomes that are abnormally retained in the outer SC, thereby enhancing the desquamation process.[41,48] Another mechanism, however, might involve the SC lipids. Moisturizers often contain lipophilic materials, and lipids play a very important role in skin barrier properties,[49,50] so it is reasonable to assume that moisturizers in some way interact with the SC lipids to improve the skin barrier and thereby enhance SC hydration by a mechanism other than simple occlusion.[44,45,49–51]

This chapter investigates alterations in the lipid structure of the outer SC that are induced by moisturizing ingredients and commercial moisturizing products. As a preface to this investigation, we also examine the normal variability in the lipid structure of the outer SC and how it is affected by factors such as age, level of visible dryness, and personal cleanser use.

28.2 TAPE STRIP PROTOCOL

The outer SC was sampled by tape stripping (Scotch Magic Tape 810, 3*M*) using a modification of a previously reported procedure.[14] The tape was applied to the lateral leg surface using gentle pressure and carefully removed after approximately 30 sec. Under stereomicroscope observation, regions of the tape having large clusters of skin flakes were cut out and placed in 0.25% RuO_4 in a 0.1 *M* cacodylate buffer for 1 h at 4°C, rinsed briefly in 0.1 *M* cacodylate buffer, and then dehydrated through a graded acetone series prior to Epon embedding and overnight polymerization at 65°C. Thin sections were cut on an ultramicrotome, counterstained with uranyl acetate and lead citrate, and analyzed in a Philips CM12 at 100 keV. The lipid structure of the SC improves as a function of depth into the SC; by the third tape strip, lipid structure has normalized to the typical Landmann pattern.[14] To focus on the superficial SC only one tape strip was taken, and whenever possible micrographs were obtained only from the outermost 3 to 4 corneocytes, adjacent to the tape. Similarly, to minimize possible artifacts resulting from the mechanical process of tape stripping or from previously uplifted scale, whenever possible micrographs were taken from closely apposed intercellular regions, thus minimizing potential problems of physical trauma or interference from the tape adhesive or applied materials. Since the assessments of lipid structure were qualitative and subjective, tape strip samples were blinded until the analysis completed.

Although this tape-stripping approach is a useful procedure, it does have limitations. For example, there are limitations inherent to RuO_4 staining due to its poor penetration and high reactivity, as discussed previously.[22] These staining limitations are superimposed on the problems of

representative sampling associated with the tape stripping procedure. Only limited areas within a tape strip meet the analysis criteria for TEM inspection, and lipid structure varies even within a single tape strip. Nevertheless, this normal variation in lipid structure is relatively small compared to the large structural changes that are encountered in the outer SC, as will be seen. In our experience, the outer SC lipid structure of an individual's skin is relatively constant over large areas, so that their outer SC lipid structure is quite consistent over an entire leg and similar between legs. The variation that does exist, however, limits the ability to detect small changes in lipid structure. In particular, it is difficult to detect improvements in lipid structure due to the use of moisturizing products when the skin is already in good condition.

Another important limitation is the labor-intensive nature of TEM investigations; the number of samples that can be analyzed in a reasonable time period is small. In the background studies presented here, a minimum of three SC samples were analyzed for each treatment except for mineral oil, where a single sample was analyzed.

28.3 NORMAL LIPID STRUCTURE OF THE OUTER STRATUM CORNEUM

The objective of this study was to observe the lipid structure of the outer SC in a population of people engaged in their usual personal care practices. Accordingly, in this study of normal lipid structure healthy female participants were selected at random without advance knowledge of their usual body skin care practices and without any preconditioning or product use restrictions. The ages of the selected individuals ranged from 22 to 52. Leg dryness was evaluated by an expert grader prior to tape strip sampling.[52]

28.3.1 Young Skin

The lipids of young skin (individuals in their early twenties) with little or no visible dryness typically have a good Landmann unit structure even at the surface of the SC, as shown in Figure 28.2(a). Youthful skin in good condition is invariably associated with closely apposed corneocytes, narrow intercellular spaces, and distinct bilayer structures. In contrast, young individuals with dry skin do not have Landmann units in their outer SC. A variety of intercellular lipid morphologies is observed in different individuals with poor skin grade including fibrous, mesh, and amorphous structures. Usually the intercellular spaces are considerably widened. An example of the latter is shown in Figure 28.2(b), in which the intercellular spaces are filled with an amorphous material having a variety of textures.

28.3.2 Old Skin

Focal domains that are depleted or devoid of lipid bilayers are reported in aged (>80 years) skin.[19] The oldest subject who participated in the present work was considerably younger than this, but we typically did not observe intercellular lipids with a Landmann unit structure in the outer SC in individuals over 40 years of age, regardless of skin condition. It thus appears that loss of SC lipid structure begins much earlier in life than was previously reported, and on this basis we define "old skin" to be skin from a person greater than age 40. An example of lipid structure from an "old" person with good skin condition is shown in Figure 28.3(a). It is common to find lamellae, but these lamellae are seldom present as fully formed Landmann units. Often lamellae are present at the periphery of corneocytes separated by a central band of nonlamellar amorphous/fibrous material as shown in Figure 28.3(a). Other intercellular spaces are simply filled with nonlamellar material (not shown). As with more youthful skin, the corneocytes are nevertheless typically closely apposed. In older individuals with dry skin the intercellular spaces can become spectacularly abnormal. Very widened intercellular spaces are common, usually filled with amorphous material that can contain a great

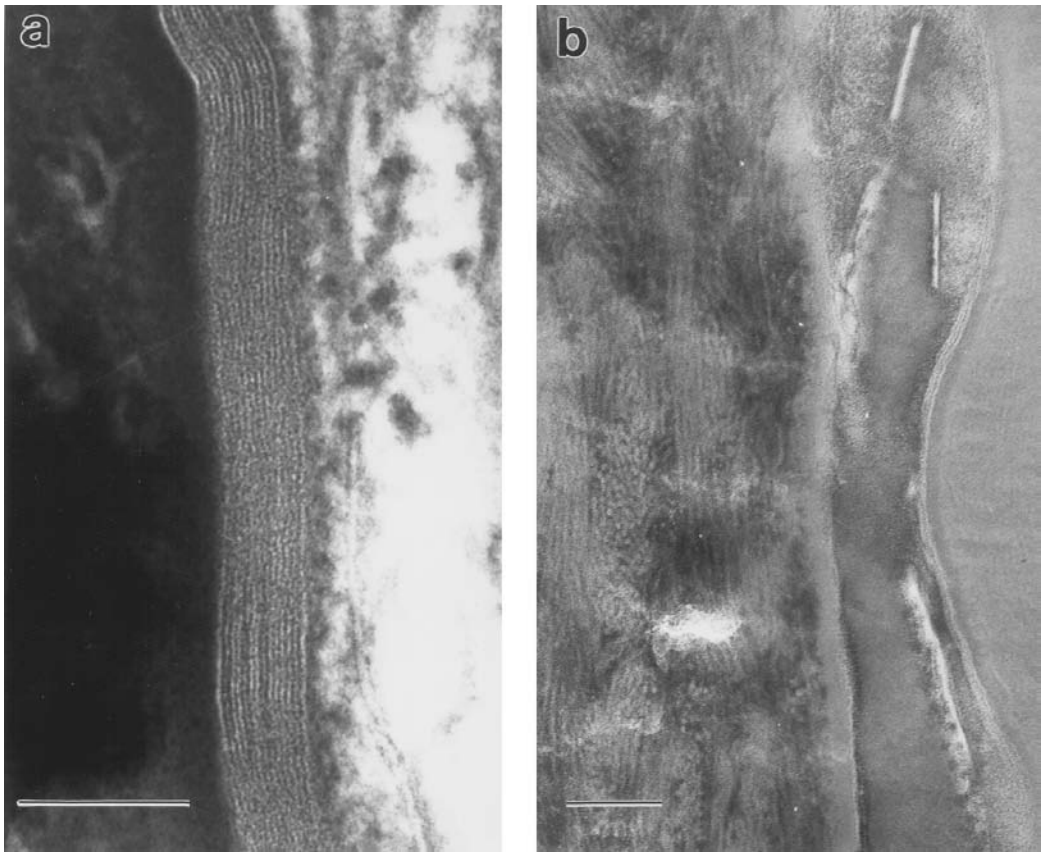

FIGURE 28.2 (a) Landmann units from the outer SC of a person 24-years old, skin grade 0.5. (b) A mixture of amorphous materials with different textures in the intercellular space of the outer SC from a person 28 years old, skin grade 5.0. Bar = 100 nm.

diversity of lipid structures. An example is shown in Figure 28.3(b); the outermost intercellular space appears to consist of a two-phase system, the noncontinuous phase being membrane-bound. Vesicles are apparent. The intercellular spaces are generally widened, many apparently filled with an amorphous material. There is no organized lamellar structure.

28.4 THE EFFECT OF SURFACTANT-BASED CLEANSERS

Surfactants are natural emulsifiers of oils and lipids. This property makes them effective cleansers but also contributes to their ability to impact SC lipids, whether through lipid extraction or lipid compositional changes. Controlled washing of the leg with soap for two weeks results in a worsening of dry skin appearance and produces alterations in the lipid structure of the outer SC. Two distinct altered intercellular structures are observed. In one form, intercellular spaces appear "invaded" by heavily staining globules of a variety of sizes, as shown in Figure 28.4(a). A more frequently observed response to soap use is the formation of profuse disorganized lamellae within widened intercellular spaces, as illustrated by Figure 28.4(b) and as reported previously.[14] In this latter figure, although localized domains of ordered lamellae exist over short dimensions, the lamellae are visualized as single electron-dense and electron-lucent lines with no evidence of the distinct substructure of the Landmann unit.

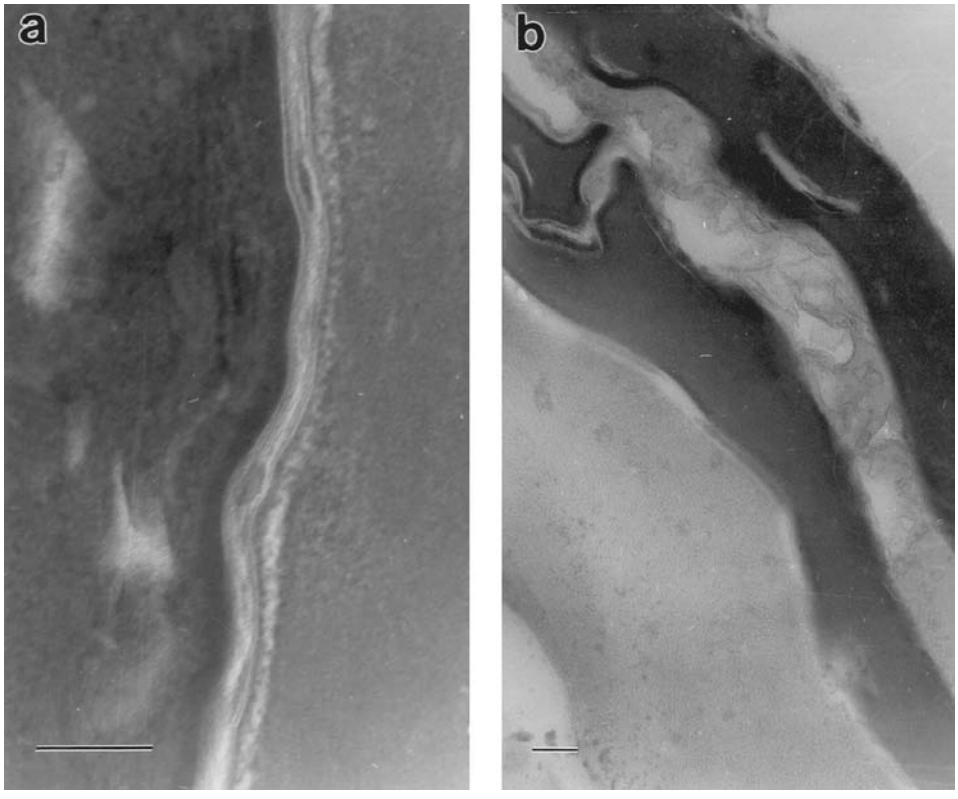

FIGURE 28.3 (a) Lipid structure in the intercellular space from a person 45-years old, skin grade 1.0. The corneocytes are closely apposed and lamellae are frequent, but these lamellae appear somewhat disorganized and do not form Landmann units. The core of the intercellular space is filled with nonlamellar material that is amorphous and fibrous with interspersed granular deposits. (b) Lipid structure from a person 49 years old, skin grade 3.5. The outermost intercellular space contains vesicular structures and membrane-bounded phases. Inner intercellular spaces appear to contain largely amorphous material. Bar = 100 nm.

Synthetic, that is, nonsoap surfactants often exhibit better skin compatibility than soap and are found in a range of personal care products. Cleansers based on these surfactants generally produce less visible irritation and dryness than soap; however, they can still remove significant quantities of lipid from the skin during washing.[53] Effects on SC lipid bilayer structure consistent with those we observed following soap washing were recently reported following controlled washing with cleansers based on "mild" synthetic surfactant systems.[54] Thus, it appears that surfactant-induced changes in the lipid structure of the outer SC are possible with a wide range of cleanser types, not just with soap.

28.5 THE EFFECT OF MOISTURIZERS

The results presented thus far show that the lipid structure of the outer SC varies with age and dry skin condition, and that cleansing products can degrade this lipid structure. We now return to questions raised earlier: do moisturizing ingredients enter the SC, and if so, can they alter the outer SC lipid structure? To address these questions we investigated the effect of neat moisturizing ingredients, reduced-concentration (i.e., "formulated") moisturizing ingredients, and fully formulated commercial products on the lipid structure of the outer SC of the leg following two or three weeks of product use.

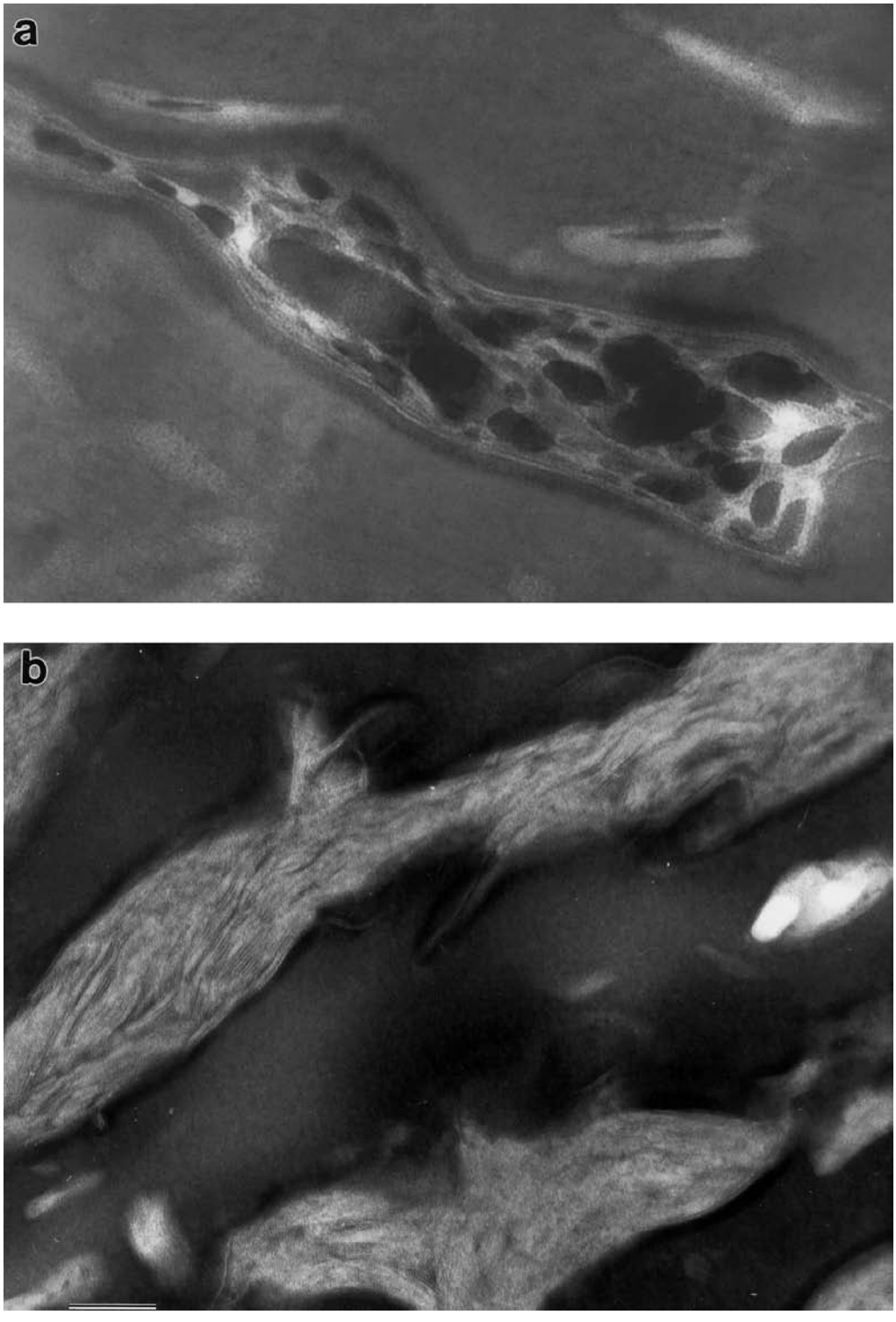

FIGURE 28.4 (a) Soap treatment frequently results in the formation of many darkly staining globular bodies in an amorphous matrix. (b) The signature pattern of soap use is the presence of widened intercellular spaces that are filled with numerous disorganized lamellae without a Landmann pattern. Bar = 100 nm.

For maximum comparative value the focus of this discussion is on results generated in matched studies conducted on a 42-year old male, though similar results were obtained from other subjects. Mineral oil, petrolatum formulated at 10% in an oil-in-water emulsion vehicle containing high levels of humectants, and sucrose esters of fatty acids (SEFA, The Procter & Gamble Company, Cincinnati, OH) formulated at 2 and 10% in the same vehicle, were applied at 3 mg/cm^2 on the lower leg twice a day for two weeks. Neat petrolatum and neat SEFA were applied ad lib twice a day for two weeks. In all cases the final product application was 12 h before tape stripping. All subjects used a syndet-based bar for daily personal cleansing, avoiding direct application of the bar or its lather to the treatment areas.

28.5.1 Mineral Oil

The control, nontreated site is shown in Figure 28.5(a), and the mineral oil-treated site in Figure 28.5(b) (same magnification). In the control skin, the outermost layers contain amorphous material and darkly staining globules. Lamellar structures are found in lower layers but the lamellae do not appear to form Landmann units. Following use of mineral oil the intercellular space is uniformly filled with a smooth-appearing amorphous material, presumably the mineral oil. Intercellular spaces were occasionally focally dilated. There seemed to be little effect of the mineral oil other than as a "spacer" separating corneocytes.

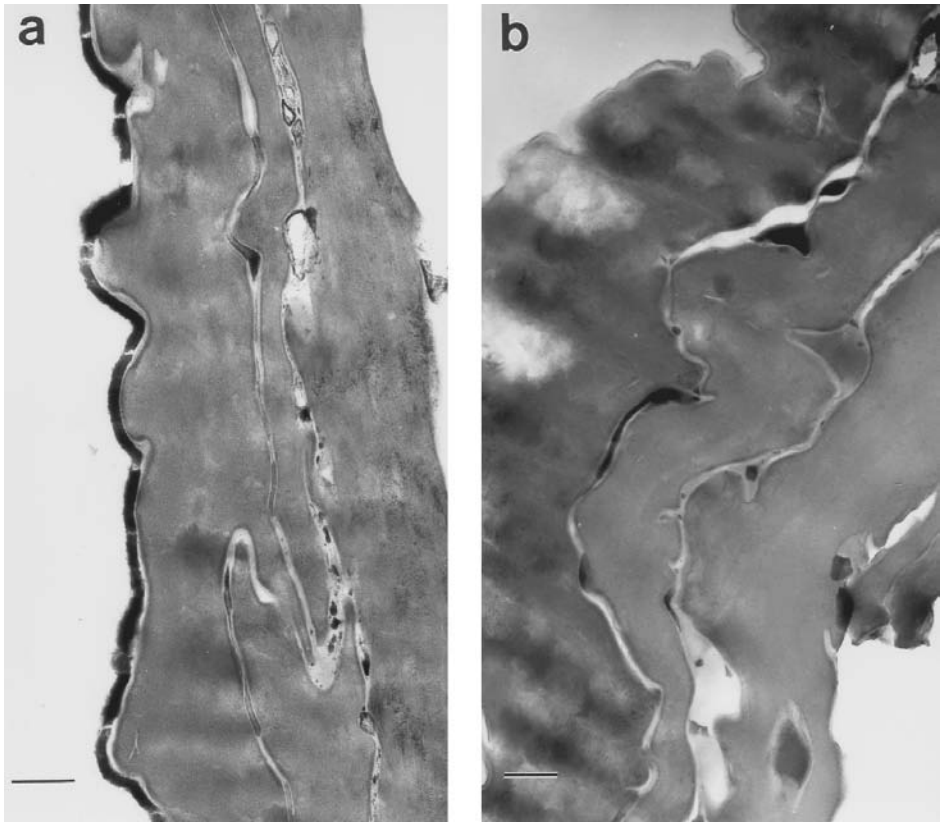

FIGURE 28.5 (a) Control, nontreated site from a 42-year-old male. The outermost (right) layers contain darkly staining globules in an amorphous matrix. Lamellae are present in deeper corneocyte layers, but Landmann units are rare. (b) Treatment with mineral oil results in the formation of large amorphous phases containing some darkly staining material. Bar = 200 nm.

28.5.2 Petrolatum

28.5.2.1 Neat Petrolatum

Petrolatum comprises a complex hydrocarbon mixture that is about 60 to 70% mineral oil, the remainder consisting primarily of paraffin and microcrystalline wax. Despite this composition, the effect of petrolatum on outer SC lipids is distinct from that of mineral oil. As shown in Figure 28.6(a), neat petrolatum forms lamellar-like "streamers" in the intercellular space, as seen previously.[44] The streamers appear to be suspended in a nonstaining or empty intercellular medium,

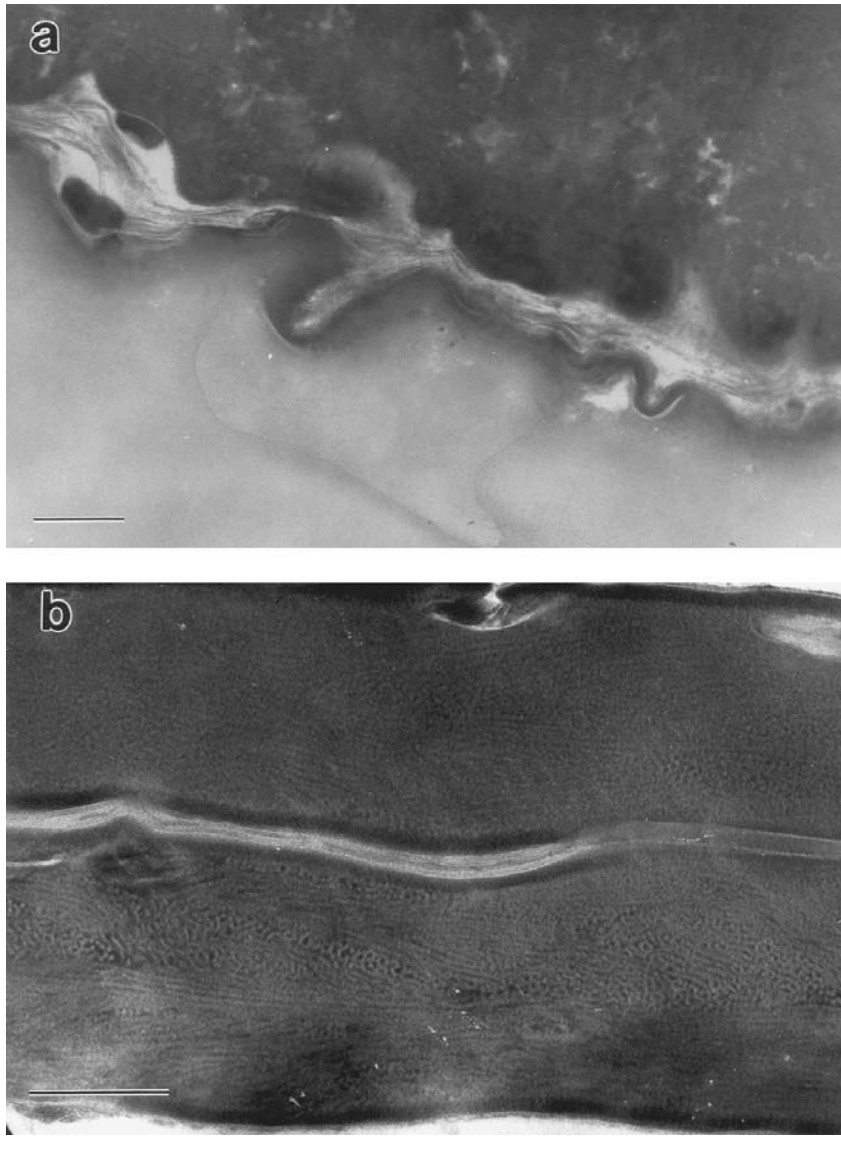

FIGURE 28.6 (a) Neat petrolatum site from a 42-year-old male. Flocculent/fibrous material existing as "streamers" or bands is present within an otherwise empty-appearing intercellular space. (b) Formulated (10%) petrolatum site. Lamellae, occasionally forming Landmann units, are sometimes separated by a thin layer of more darkly staining amorphous material. Bar = 200 nm.

or possibly water. In other areas, petrolatum forms a more continuous amorphous phase, also reported previously.[45] Intercellular structures intermediate between these two appearances are also formed (data not shown).

In other studies, similar streamer and amorphous structures were observed in a young female with dry skin following the above treatment protocol, although the amorphous phase was less prominent. In contrast, the streamer phase was less obvious in older individuals treated with 2 mg/cm^2 twice a day for three weeks.

28.5.2.2 Formulated Petrolatum

The "streamer" phase observed with neat petrolatum (Figure 28.6[a]) was not observed, but amorphous material was common (data not shown). Reasonable lamellae were occasionally encountered, as shown in Figure 28.6(b). Often these lamellae were separated by thin expanses of amorphous material, as shown in the center of Figure 28.6(b). In general, treatment with "formulated" petrolatum resulted in an appearance of the intercellular lipids that was much improved over that of neat petrolatum or mineral oil. The corneocytes were more closely apposed, and Landmann units were more common.

28.5.3 Sucrose Esters of Fatty Acids

28.5.3.1 Neat SEFA

Treatment with SEFA resulted in a very characteristic appearance of the intercellular space, shown in Figure 28.7(a), which we describe as the "SEFA look." The corneocytes are relatively closely apposed, single Landmann units are present at corneocyte margins, and the slightly expanded intervening space is "plugged" with an amorphous material, presumably SEFA. Unlike the other products above, multiple Landmann units are occasionally present, although the multiple units are usually present in short regions within the SEFA "plug," as shown in Figure 28.7(b). Very similar results were obtained in a young female with dry skin.

28.5.3.2 Formulated SEFA

The structure of the lipids in the intercellular space is overwhelmingly the "SEFA look" for both the 2 and 10% concentrations, as shown in Figure 28.8(a). With the 10% concentration, extra Landmann units within the SEFA phase were occasionally seen, as shown in Figure 28.8(b).

In a separate study, 2 mg/cm^2 of 2 or 10% SEFA in a humectant vehicle were applied to the lower leg twice a day for three weeks. The control nontreated site of a 52-year-old female subject, shown in Figure 28.9(a), is characterized by numerous disorganized lamellae characteristic of soap use. Numerous darkly staining globular deposits were also common (data not shown). The humectant vehicle alone resulted in substantial improvement in the outer SC lipid structure of this subject, but Landmann units were not common and many intercellular spaces contained indistinct or amorphous material (data not shown). Notably, the vehicle did not produce the "SEFA look." Following treatment with the 2% SEFA preparation, the "SEFA look" was commonly observed (Figure 28.9[b]), but so too were Landmann units, which is an unusual finding for a person of this age. Following treatment with the 10% SEFA preparation, the "SEFA look" was less common, and Landmann units more common (Figure 28.9[c]).

To conclude this section, previous reports on the beneficial effects of topically applied moisturizing preparations have often focused on optimizing their physiological lipid composition. The results found for the "formulated" SEFA and petrolatum in this work, when viewed relative to the neat materials, suggest that proper formulation of even nonphysiological moisturizing agents will enhance the beneficial effect these materials have on outer SC lipid structure.

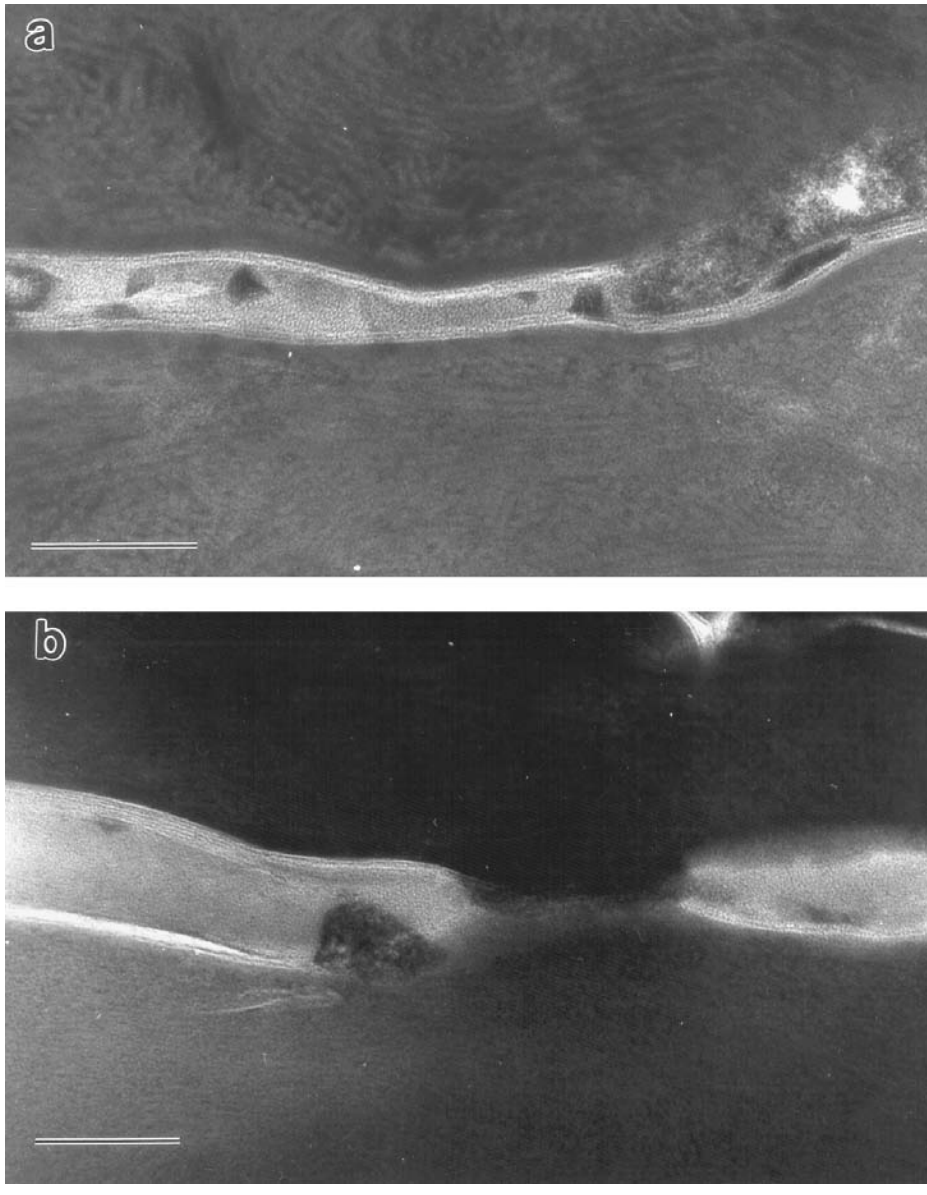

FIGURE 28.7 Neat SEFA site from a 42-year-old male. (a) Corneocytes are closely apposed, with well-formed lamellae at the corneocyte surface. Between the lamellae is a relatively uniform layer of an amorphous material. This pattern is referred to as the "SEFA look." (b) Occasional multiple Landmann units are present in the intercellular space. The length of the double Landmann units is always relatively short. Bar = 100 nm.

28.5.4 Product Comparisons from Clinical Studies

28.5.4.1 Neat Petrolatum versus Neat SEFA versus Glycerin-Based Moisturizing Lotion

Products were applied at 2 mg/cm^2 to the lower leg twice a day for three weeks. Typical results are presented from a 52-year-old female panelist. Petrolatum use resulted in an intercellular space containing diverse intercellular structures including darkly staining globular material, amorphous

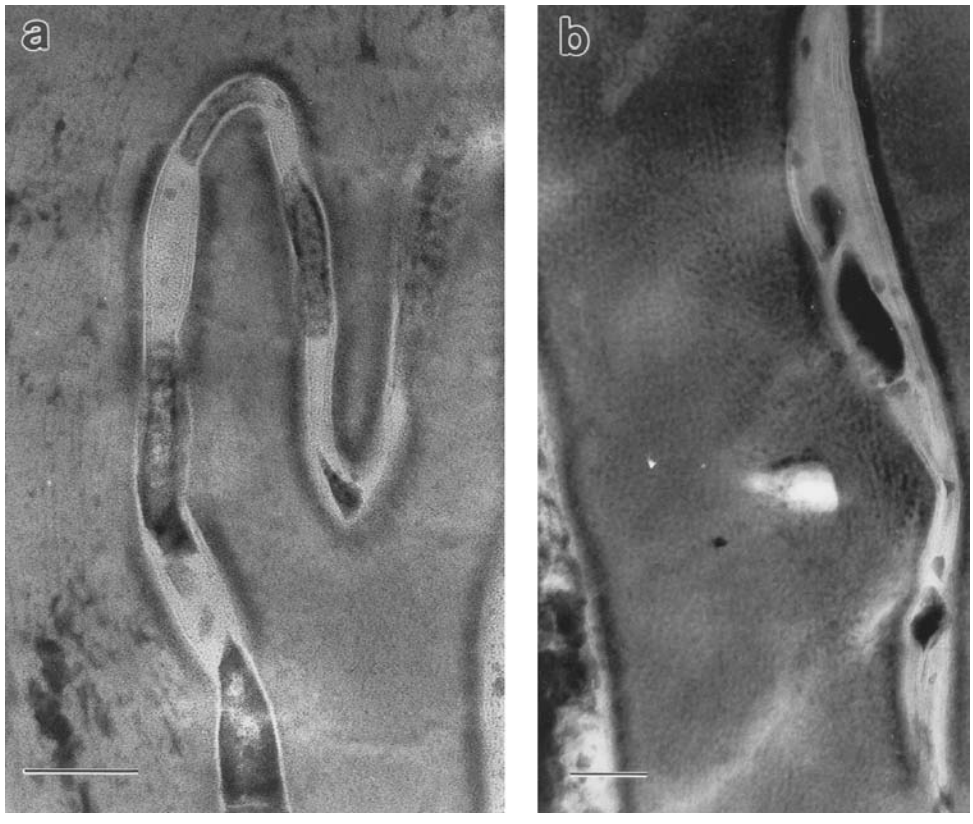

FIGURE 28.8 (a) Formulated (2%) SEFA site from a 42-year-old male. The characteristic "SEFA look." (b) Formulated (10%) SEFA site. In addition to the SEFA look, an extra Landmann unit is present within the amorphous material. This extra Landmann unit is slightly separated from the peripheral lamella, which is common. Bar = 100 nm.

regions, and some lamellae, but few Landmann units, as shown in Figure 28.10(a). SEFA substantially improved the intercellular structures, as shown in Figure 28.10(b), including the occasional formation of multiple Landmann units characteristic of younger skin (insert, Figure 28.10[b]). In striking contrast, a glycerin-based lotion yielded a diverse and unusual lipid structure that included mixtures of amorphous and fibrous material (Figure 28.11[a]), phase-separated amorphous lipids (not shown), and frequent bizarre vesicular structures (Figure 28.11[b]). Landmann units were rarely observed. Other workers have reported that glycerin, under open or occlusive application, speeds transepidermal water loss (TEWL) recovery in SC whose barrier function is compromised by tape stripping or surfactant washing.[43] This seeming disparity with the present work could be a result of the higher doses of glycerin applied or the different treatment forms used. Or the effect of glycerin on SC barrier function, as measured by TEWL, might occur deeper in the SC than in the topmost layers assessed in this work.

The blinded TEM studies rated the test materials' potential to improve lipid ultrastructure as: neat SEFA > neat petrolatum > lotion. However, the blinded expert scoring in this study ranked the test materials' ability to improve dry skin oppositely: lotion > neat petrolatum > neat SEFA. This reversal illustrates two of the roles a moisturizer can play; the former showing the materials' potential to effect functional improvement and biological repair, the latter showing their potential to cosmetically improve dry skin.[47] Importantly, these results demonstrate that the cosmetic and functional aspects of a moisturizer's action on skin do not necessarily contribute to the same extent or need not even act in parallel for a given material or product.

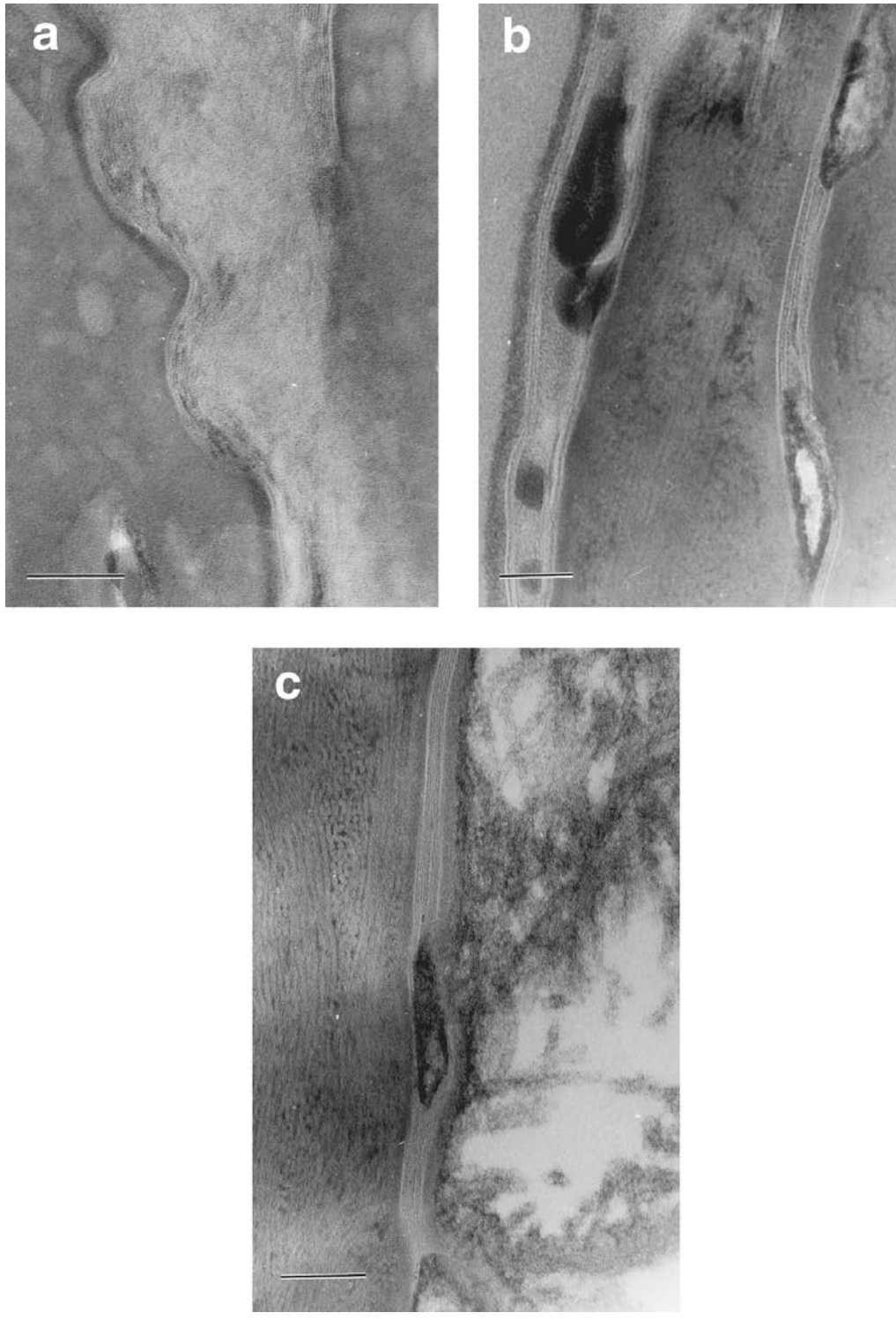

FIGURE 28.9 (a) Control nontreated site from a 52-year-old female. The characteristic lipid structure resulting from soap use (winter xerosis[14]) is evident — compare with Figure 28.4(b). (b) Use of formulated (2%) SEFA results in the SEFA look, as well as Landmann units. (c) With use of formulated (10%) SEFA, Landmann units are commonly observed. Bar = 100 nm.

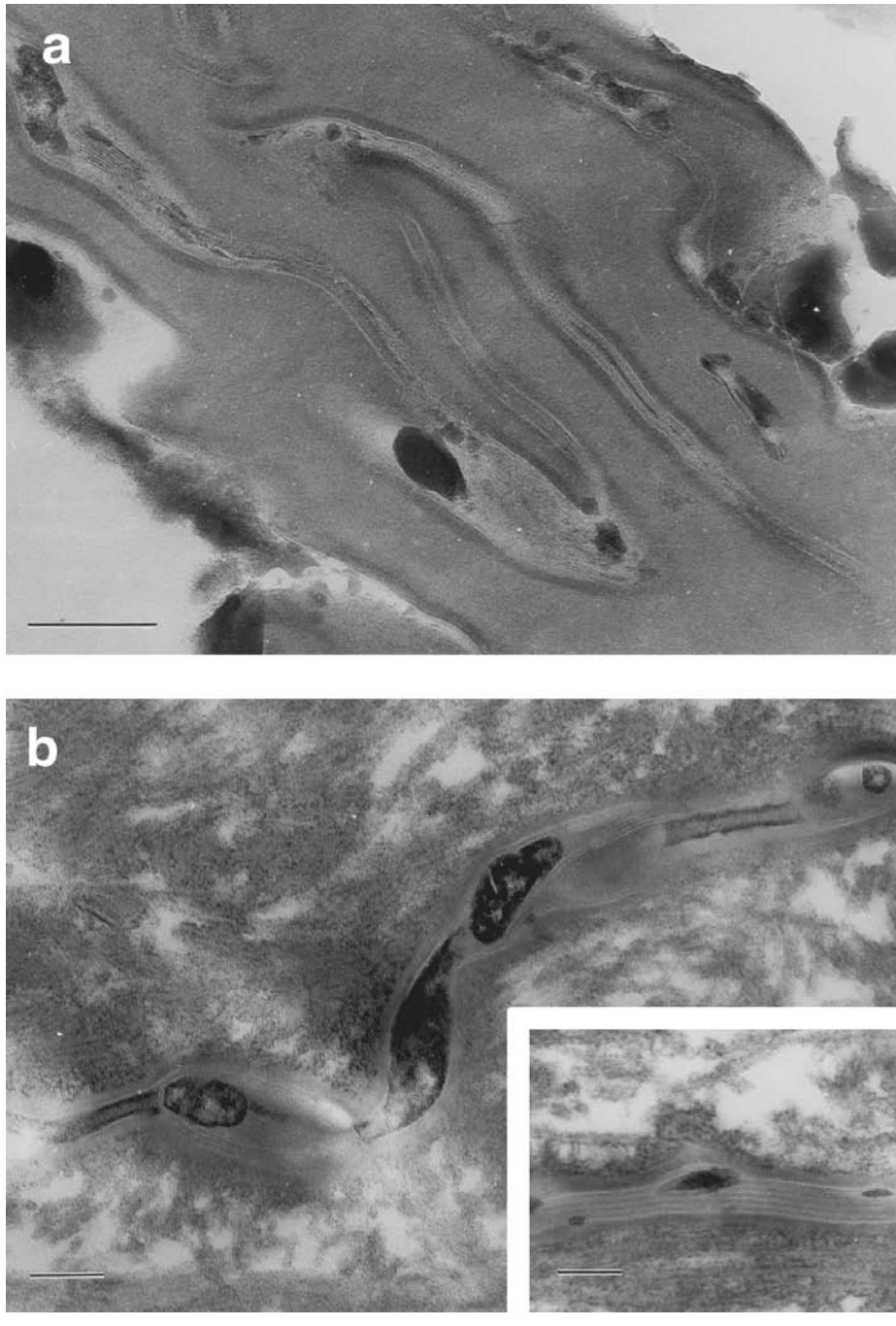

FIGURE 28.10 Neat-petrolatum-treated site from a (different) 52-year-old female. (a) A great variety of intercellular structures are present, but the "streamer" phase typical of petrolatum (Figure 28.6[a]) was not seen. Amorphous regions and expanded intercellular regions containing many darkly staining globular regions are very common, as are lamellae without a Landmann pattern. Landmann units were rare. (b) Neat-SEFA-treated site. The SEFA look is evident. The dark spindle-shaped structures near the center of the micrograph are presumably desmosomes undergoing degradation. In many areas with the SEFA look, multiple, short-length Landmann units are common in the intercellular space, as shown. Normal well-formed Landmann units are relatively common, as shown in the insert. Bar = 100 nm.

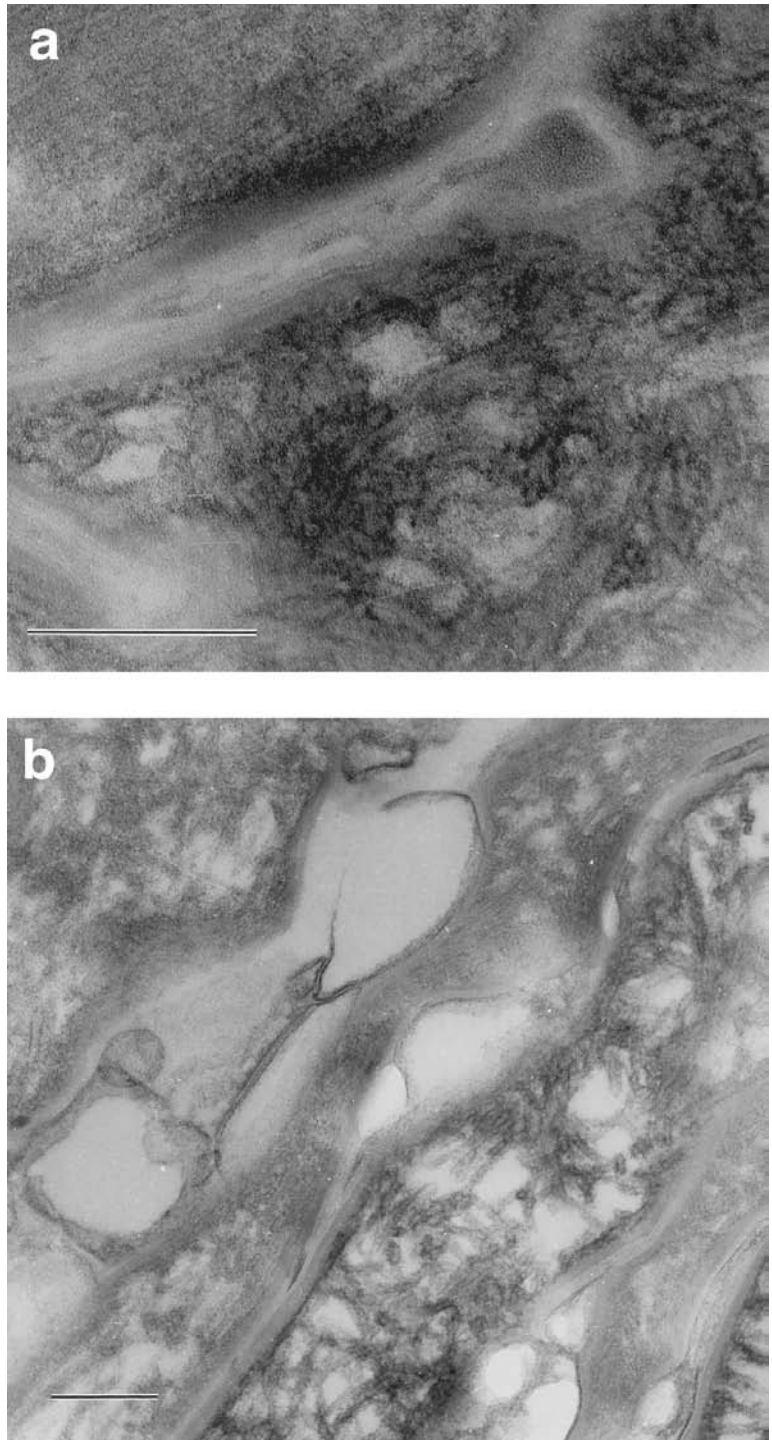

FIGURE 28.11 Site of glycerin-based moisturizing lotion from the 52-year-old female of Figure 28.10. The structure of the intercellular space is unusual. (a) Many areas contain amorphous and fibrous material in the intercellular space. (b) Other areas contain vesicles, membrane-bounded compartments, and a mesh-like material. Bar = 200 nm.

28.5.4.2 Moisturizing Body Wash versus Synthetic Bar + Glycerin-Based Moisturizing Lotion

In a clinical study, a body wash treatment at 10 μl/cm^2 (rinse-off application) and a glycerin-based lotion treatment at 1 μl/cm^2 (leave-on application) were applied to the medial aspect of the legs of female panelists once daily for 25 days. Good repair of the lipids in the intercellular space was routinely obtained with the body wash, which contains 17.5% petrolatum as a skin benefit agent, as illustrated by a particularly dramatic improvement shown in Figure 28.12. The control (water only) treatment site of a 28-year-old panelist, shown in Figure 28.12(a), is characterized by intercellular spaces filled with amorphous material. The effect of the body wash on this subject's outer SC lipids is shown in Figure 28.12(b). The majority of the intercellular space is filled with Landmann units, although amorphous material was occasionally found in some regions. The effect of a syndet bar followed by application of a glycerin-based moisturizing lotion is shown in Figure 28.12(c). This bar/lotion regimen resulted in a clear improvement relative to the control but many intercellular regions are still dilated with amorphous material. Although lamellae are present, Landmann units are relatively rare.

A more typical response produced by these treatments is shown in Figure 28.13, which is from a separate clinical study that used the same treatments applied for only 14 days. The 48-year-old panelist had a moderate amount of skin dryness and the SC at the control site exhibited an intercellular lipid structure similar to that of Figure 28.3(a), that is, a good lipid structure for that age. In this case the body wash resulted in no dramatic change in intercellular lipid structure, shown in Figure 28.13(a), although there was significant improvement in the visual skin grade. The limited improvement in outer SC lipid structure might reflect the shorter treatment period, the decreased dosing compared to the moisturizer study, or possibly the impact of age. Because the aged stratum corneum barrier exhibits a reduced resistance to insult and slower repair than in young skin due to diminished lamellar body secretion,[19] and because the SC turnover rate also typically slows with age, the functional benefits of topical moisturizers might require more time to manifest in "old" skin than in younger skin. Regardless, these results show that while lipid structure was not improved, treatment with the body wash preserved existing lipid structure. In contrast, use of the syndet bar followed by the glycerin-based lotion degraded lipid ultrastructure in the outer SC, as shown in Figure 28.13(b). The intercellular spaces contain amorphous and "fuzzy" material, and prominent disorganized, undulating lamellae. Nevertheless, the visual skin grade was dramatically improved with this latter regimen, again illustrating the distinction between a moisturizers' cosmetic and functional effects.[47]

28.6 CONCLUSIONS

An improved understanding of the structure of the SC barrier is of interest for many reasons such as enhancing percutaneous penetration and, as discussed in this chapter, optimizing topical therapy for the treatment of dry or damaged skin. The results of this TEM work show that the lipid structure of the outer SC is quite variable. Typically, the intercellular spaces in the outer SC are considerably widened and filled with nonlamellar material. These data are consistent with earlier TEM studies[13,14] and with an infrared spectroscopic study that found less structured lipids in the outer SC[16] compared to the middle and inner regions.

Contrary to an earlier report that lipids uniformly have an amorphous structure in the outer SC of normal skin with little or no visible dryness,[14] we instead found considerable variation in this lipid structure among individuals. Intercellular lipids in young skin with little dryness typically had a good Landmann unit structure, even at the surface of the SC. This ideal Landmann unit structure was generally absent in young individuals with dry skin or in individuals over the age of 40 regardless of their dry skin level. In attempting to make sense of this variation, we believe we can generalize and conclude that the outer SC lipid structure is related to an individual's age and dry skin condition.

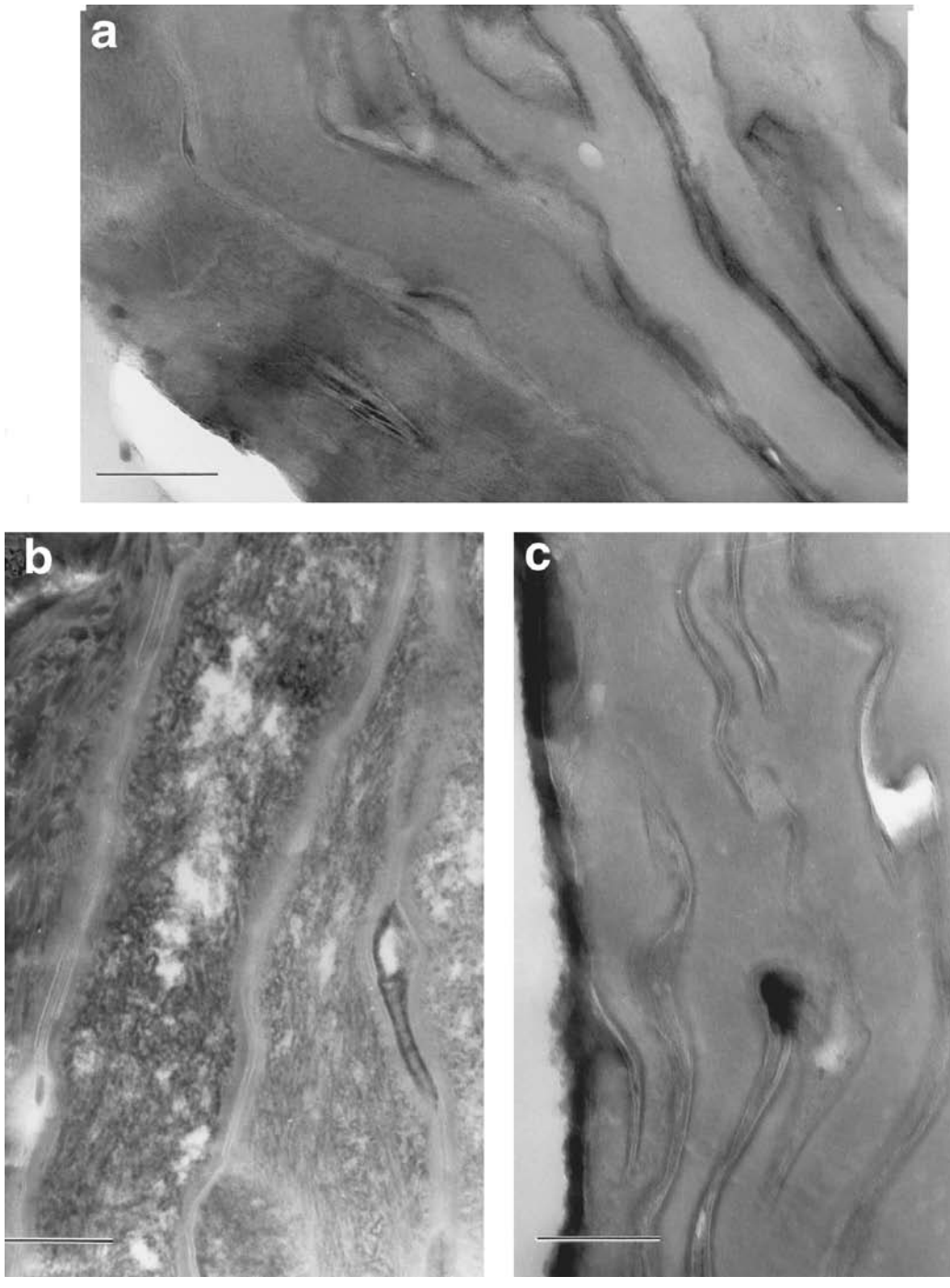

FIGURE 28.12 (a) Control nontreated site from a 28-year-old female. The intercellular spaces are completely filled with amorphous material. Lamellar structures are rare. (b) Site of application of a commercial moisturizing body wash containing petrolatum. Lamellae are common, as are Landmann units. (c) Site of application of a mild synthetic bar followed by a glycerin-based moisturizing lotion. Lamellae are present, but few have the Landmann unit structure. Amorphous material is still common. Bar = 200 nm.

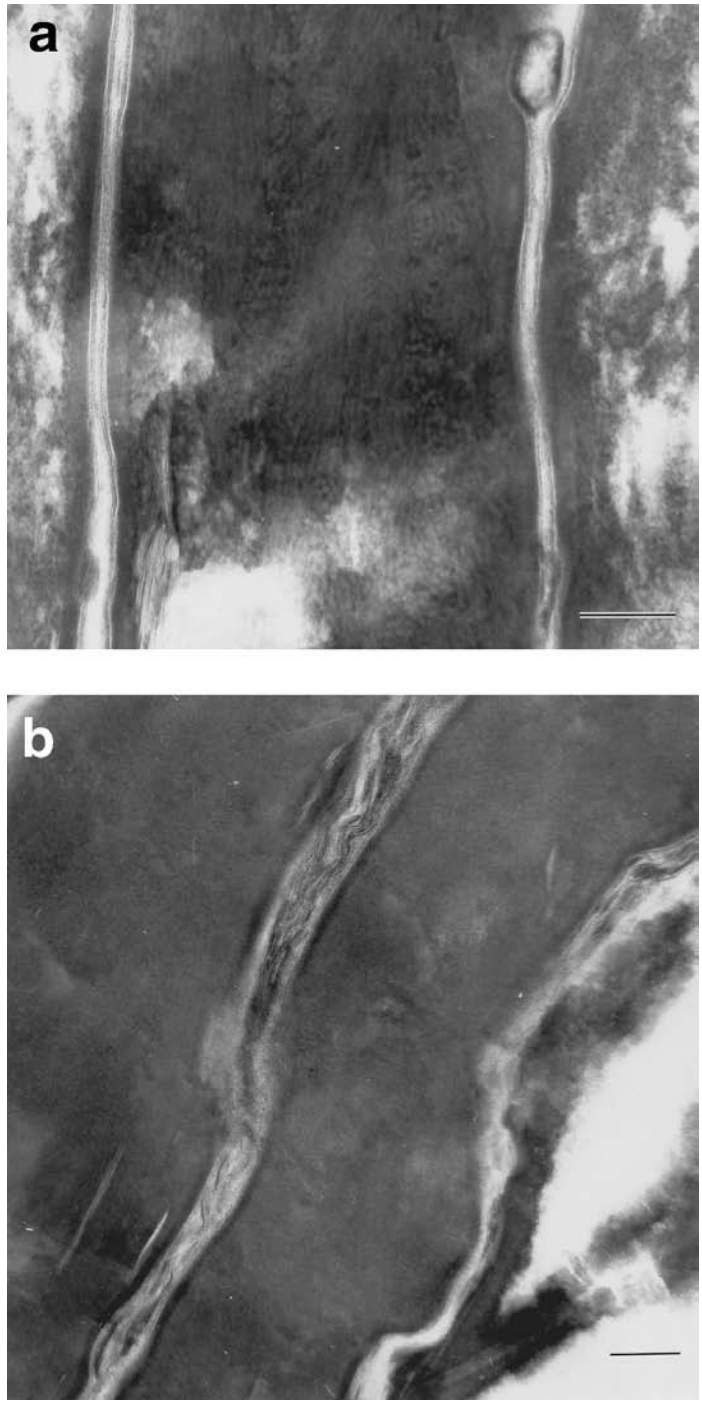

FIGURE 28.13 Tape strips from a 48-year-old female. (a) Site of application of a commercial moisturizing body wash containing petrolatum. The appearance is typical of a person over age 40 with good skin condition. The corneocytes are closely apposed and lamellae are frequent, but the lamellae appear somewhat disorganized and amorphous/fibrous material is present between lamellae. Landmann units are nevertheless easy to find, as shown in the left intercellular space. (b) Site of application of a mild synthetic bar followed by a glycerin-based moisturizing lotion. The intercellular spaces contain amorphous and fibrous material as well as prominent wavy lamellae without a Landmann pattern. Bar = 100 nm.

The outer SC lipid structure of older individuals is altered compared to that found in young skin, the older SC having fewer lipid lamellae and more open intercellular clefts.[19] This paucity of lamellae may be due to a decrease in SC lipid synthesis or lamellar body secretion, resulting in a decreased SC lipid content.[19,51] These results are from observations made on individuals of advanced age, 80 years old. However, we observed changes in the outer SC lipid structure of individuals as young as 40 years old. This is not entirely unexpected since literature data support age-associated changes in SC lipids in relatively young individuals. For example, there is a sharp decrease in SC lipid content by age 45 and a nearly constant SC lipid profile afterwards.[55] Total SC ceramides undergo a sharp drop in concentration around age 40,[40] and levels of individual ceramide species also change with age and female hormonal status.[23,56] A challenge for skin moisturizers is to arrest, and ideally reverse, the age-related decline in the SC lipid barrier that accompanies these changes.

The blinded assessment of changes in SC lipid structure generally corresponded well with the independent, expert assessments of dry skin appearance. However, a number of striking outliers show that lipid structure is not the major determinant of dry skin appearance, which is not surprising given the complexity of SC homeostasis. A good example was the neat petrolatum/SEFA/lotion comparison mentioned earlier, in which neat SEFA improved lipid structure but produced only a marginal reduction in dry skin appearance, whereas the glycerin-based lotion apparently degraded lipid structure but yielded skin with minimal visible flaking. This suggests that another mechanism, such as desmosomal breakdown, is a more important determinant of the skin's dry appearance. However, a healthy, nondry SC may ultimately rely more on the integrity of the lipid barrier than on the state of desmosome degradation in the outer SC layers. Such effects may appear over a longer time frame, for example, during the regression period that is used in some clinical protocols, or require the evaluation of endpoints other than dry appearance.

The discrepancy between visual appearance and lipid structure may be a consequence of commercial products being formulated to achieve visual improvement rather than functional change, that is, improved skin health. This is not unexpected since dry skin is readily observed by consumers and is an important signal of the need to apply moisturizer.[47] However, as this work has shown, a reduction in dry appearance does not necessarily mean that there is an improvement in the functional characteristics of the skin, only that it looks better. Thus, relying on a visual endpoint for evaluating moisturizer efficacy can yield commercially successful products that provide a marginal skin health benefit. An example are reports that some commercial moisturizing lotions may actually impede barrier recovery after experimental barrier perturbation.[45,57] A moisturizer should ideally address not only visual skin problems but also address the underlying biological causes to achieve healthy skin; there is a clear need for evaluation tools and endpoints for skin health beyond visual inspection.

We observed distinctive changes in the outer SC lipid structure with use of different treatments from soap to oil. Some of these lipid structures were sufficiently unique to provide unequivocal identification of product treatment, and to a lesser degree panelist age and skin condition. Based on TEM results, we believe that moisturizing materials enter the intercellular space of the SC and become a part of the SC, as was previously shown for petrolatum.[29,44] The mechanism by which a nonphysiological moisturizing material improves skin barrier lipids is uncertain, and multiple processes are likely involved. Given the chemical nature of the materials studied, we consider it unlikely that any of the treatments participated directly or physically in the formation of Landmann units. Of some note is the observation that petrolatum and SEFA were not as effective in reforming Landmann units when applied neat as when they were applied as reduced concentration or fully formulated products. The moisturizing body wash only contains petrolatum and polymers as moisturizing ingredients, which suggests that the quantity of petrolatum or its delivery form is important for promoting the conditions necessary for SC lipid repair. Likewise, for SEFA applied as a formulated product, the combination of humectants with some degree of occlusion may promote the internal conditions needed for the intrinsic formation of Landmann units. A semi-occluded environment is reported to accelerate TEWL barrier recovery following experimental insult[58,59]; this type

of environment might similarly favor lipid bilayer reformation in the outer SC. However, apparent lack of Landmann unit reformation following treatment with the glycerin-based moisturizer suggests that the choice of nonphysiologic ingredient or the manner in which it is formulated is also important to provide conditions that promote this reformation.[60,61] Beyond ingredient and delivery issues there remains the issue of product aesthetics and convenience; a product will benefit skin only if it is used. Of 651 dermatologist respondents in a recent survey, over 60% believed that less than half their adult female patients apply lotion as recommended.[62] Lack of convenience was cited as a factor contributing to this poor compliance by over 83% of the dermatologist respondents. The development of nontraditional product forms to deliver moisturizing benefits, such as moisturizing cleansers[54,63] and moisturizers intended for use in the shower,[62] can provide increased convenience and could improve moisturizer usage compliance.

The SC is a highly complex system and we do not claim to fully understand the lipid structure of the outer SC or its implications on the basis of this investigation. The conclusions reached are therefore predicated on certain key observations and assumptions. We observed the ideal Landmann unit lipid structure in young individuals with little or no skin dryness, the absence of this structure in individuals with a high level of dryness, and the reappearance of Landmann units with treatment by moisturizing products. We therefore assume that this Landmann unit structure is the ideal lipid structure for the outer SC, as it is throughout its lower regions. In a system undergoing desquamation that may involve lipids,[36–39] this is an important assumption. We further assume that this ideal Landmann unit structure in the outer SC is important to skin health and a parameter by which moisturizers' potential to impact skin health should be judged. Both of these hypotheses warrant further testing.

In summary our microscopy study shows that topical moisturizers enter into the SC and can affect lipid structure. The lipid structure is related to visible skin dryness but is not the primary factor determining the level of dryness. For SEFA and petrolatum, formulated products showed a greater restorative effect on ideal Landmann unit lipid structure than did the neat materials. In our experience most of the moisturizing materials and products that we investigated to date are effective at reducing visible dry skin, but far fewer materials are able to substantially reform Landmann units, particularly in individuals over age 40. Is there hope that moisturizers might restore the ideal Landmann unit lipid structure common in the healthy skin of youth? With ongoing work looking at new moisturizing agents, new delivery systems, and alternative product forms, we believe the promise is there, as shown for older individuals in Figure 28.9 and Figure 28.10.

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29 Vitamins and Skin

Krautheim A. and Gollnick H.P.M.

CONTENTS

29.1 INTRODUCTION

Vitamins are essential nutrients, which must be supplied exogenously. They are organic compounds with indispensable biological activities as coenzymes in a multitude of cellular metabolic processes. Vitamin A, retinoids (vitamin A-derivatives), carotenoids, vitamin D, vitamin E, and vitamin K are fat-soluble, vitamin C and vitamins of the B-complex are water-soluble. This is of importance for gastrointestinal absorption in oral supplementation as well as the transdermal penetration for topical applications.

For dermatological treatment the main focus has been on vitamins A and D. Retinoids have been used systemically and topically for the treatment of acne and a variety of hyperkeratotic disorders including psoriasis, ichthyoses, and lichenoid dermatoses as well as skin cancer.[1] Vitamin D-analogs are of great importance for the topical treatment in psoriasis.

Additionally there has been an increasing interest in the role of vitamins, especially the anti-oxidant vitamins, vitamins C and E, and the carotenoids in protection from ultraviolet (UV)- or chemically-induced oxidative stress as well as in the prevention and treatment of photoaged skin. UVB radiation (280 to 320 nm) is mostly absorbed in the epidermis, can directly damage DNA, and

has as such a strong mutagenic potential.[2] UVA radiation (320 to 400 nm) penetrates deeper, can interact with epidermal keratinocytes and dermal fibroblasts, is not absorbed by DNA, but contributes up to 95% of total UV exposure and is considered the most important source of oxidative stress in human skin.[3,4] It leads to indirect damage of DNA via radicals released on the cell. UV radiation leads to photodamage with subsequent photoaging of the skin characterized by wrinkling, scaling, reduced elasticity, dryness, pigment abnormalities, and eventually to skin cancer.[5,6] The main histological features are accumulation of disorganized elastin-containing fibres and reduced amounts of type I and type III procollagens in the extracellular matrix.[7] On a molecular level UVB exposure induces activation of transcription factors like activator protein-1 (AP-1) and nuclear factor κB (NF-κB) leading to the induction of matrix metalloproteinases, which degrade various collagens and other matrix proteins. Mutations of mitochondrial DNA might also be involved in the process of photoaging.[4]

29.2 ANTIOXIDANT VITAMINS C (ASCORBIC ACID) AND E (TOCOPHEROL)

The human skin is supplied with a sophisticated antioxidant defense system against noxious environmental effects.[8] The induced oxidative stress is associated with the generation of reactive oxygen species (ROS), such as hydroxyl radical, superoxide anion radical, hydrogen peroxide, singlet oxygen, hypochlorite, nitric oxide radical, and peroxynitrite. These are highly reactive and can damage DNA, proteins, carbohydrates, and lipids. Thus ROS can affect a variety of biological processes including inflammation, carcinogenesis, photodamaging, and aging.[9,10] Vitamin C is the most abundant antioxidant in the epidermis, while vitamin E might be the most important one. Vitamin E is the collective name for eight naturally occurring molecules, four tocopherols and four tocotrienols, which qualitatively exhibit the biological activity of α-tocopherol. The main *in vivo* effect of α-tocopherol is to act as a chain-breaking antioxidant during lipid peroxidation. Vitamin C plays an important role in the subsequent regeneration of α-tocopherol in this process (Figure 29.1).[11–16] The unique protection of membrane lipids from peroxidation was also shown *in vitro* in unilamellar liposomes, which contained α-tocopherol in the liposomal membrane and ascorbate trapped inside the vesicles.[17]

In the human epidermis the concentration of α-tocopherol was shown to be 90%, the concentration of ascorbic acid 425% higher than in the dermis. The antioxidant capacity of the epidermis is thus, together with enzymic antioxidants like superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, far greater than that of the dermis.[18]

In human skin equivalents UV irradition with a full solar spectrum led to a linear depletion of vitamin E with increasing amounts of UV light, while vitamin C was only markedly decreased at the highest amount of 16.8 J/cm^2.[19]

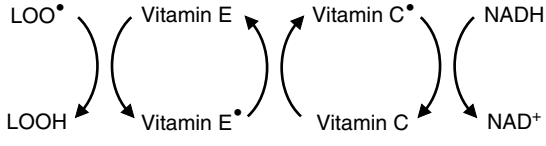

FIGURE 29.1 Pathways of the chain-breaking action of vitamin E in lipid peroxidation and its subsequent regeneration. LOOH lipid hydroperoxide, LOO• lipid peroxyl radical, vitamin C• ascorbate radical (semidehydroascorbate), vitamin E• α-tocopheroxyl radical. The lipid peroxyl radical is reduced to lipid hydroperoxide by tocopherol. The resulting tocopheroxyl radical can be re-reduced by ascorbate. The thus formed ascorbate radical can be reduced to ascorbate by the NADH-dependent semidehydroascorbate reductase.

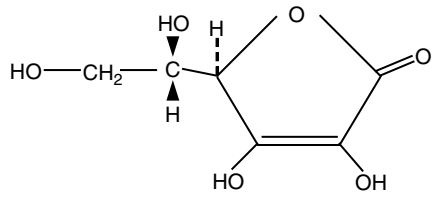

FIGURE 29.2 Chemical structure of vitamin C (ascorbic acid, (*R*)-5-[(*S*)-1,2-dihydroxyethyl]-3,4-dihydroxy-5*H*-furan-2-on).

A study on the photoprotective effect of the topical application of 2% vitamin E and 5% vitamin C in humans showed no effect with the application of each substance alone, but an enhanced photoprotective effect after applying vitamins E and C combined, which was attributed to the regeneration of vitamin E by vitamin C.[20] This enhanced effect has also been shown with the topical application of a combination of 15% ascorbic acid and 1% α-tocopherol to porcine skin.[21] The combined systemic supplementation of vitamins C and E was similarly able to reduce sunburn reactions[22] and to increase the minimal erythema dose (MED), a measure for individual photosensitivity, more than supplementation with either vitamin E or vitamin C alone.[23,24] An oral supplementation with an antioxidative combination of carotenoids (β-carotene and lycopene), vitamin C, vitamin E, selenium, and proanthocyanidins (Seresis®, Pharmaton SA, Lugano, Switzerland) also reduced the development and grade of UVB-induced erythema.[25]

29.2.1 Vitamin C

Vitamin C is the major hydrophilic antioxidant (Figure 29.2). The recommended daily allowance is 75 to 90 mg, increasing to 120 mg during lactation.[26] It acts as an antioxidant by scavenging and quenching free redicals such as superoxide anion radical, hydrogen peroxide, hypochlorite, hydroxyl radical, peroxyl radical, and singlet oxygen.[9] Vitamin C plays a central role in a broad range of biochemical redox reactions and collagen formation. Additionally vitamin C can be regenerated by glutathione as well as NAD(P)H-dependent enzymes and has low toxicity.[5,9,27]

The well-known deficiency syndrome is scurvy. The initial changes are follicular keratosis on the upper arms, back, buttocks, and lower extremities, which are related to a defective collagen synthesis.[28] This is followed by a purpuric follicular rash, swollen, bleeding gums, stomatitis, and epistaxis. Hematomas, especially painful subperiostal, may develop.[29]

A double-blind clinical trial over six months with a 5% vitamin C cream on low-neck and arms as well as a hemiface trial over three months topically with vitamin C containing Cellex-C® (Cellex-C International, Toronto, Canada), both in volunteers with photoaged skin, led to a clinically apparent improvement of photodamaged skin as compared to vehicle. This may have been due to an activation of dermal synthesis of elastic fibers.[30–32] The biochemical involvement of vitamin C in collagen synthesis and collagen synthesis regulation was shown *in vitro* with a specific increase of relative collagen synthesis[33,34] and *in vivo* where topical application of 5% L-ascorbic acid led to an enhancement of steady-state levels of procollagen I and III mRNA and of their posttranslational maturation enzymes.[28] Generally, vitamin C containing topical formulations appear of reasonable use in patients with early photoaging to prevent further photoaging.[35]

In reconstructed human epidermis the presence of vitamin C was required to normalize stratum corneum lipids, which was accompanied by an improvement of skin barrier formation.[36] Interestingly the ascorbic acid concentration in the skin of atopic dermatitis[37] and psoriatic patients[38] measured *in vivo* by microdialysis was significantly lower than in healthy subjects. In psoriasis there was no significant difference in lesional versus nonlesional skin. There has also been demonstrated a decrease of ascorbic acid concentration in skin with increasing age.[39]

Percutaneous absorption of topical vitamin C has already been established for porcine skin increasing to 4- to 40-fold concentrations with repeated application.[40,41] These appeared to be even higher than after oral supplementation.[27] More detailed investigations in porcine skin showed the following results: L-ascorbic acid must be formulated at pH levels less than 3.5 to enter the skin, maximal concentration for optimal percutaneous absorption is 20%, tissue levels are saturated after three daily applications, the half-life of tissue disappearance is about four days. Basically, the penetration of topical vitamin C appears to be critically dependent on formulation characteristics.[42]

One of the main problems of topical application of vitamin C is that it is extremly unstable, so hydrophilic derivatives like sodium ascorbyl phosphate and lipophilic esters with fatty acids were synthesized to improve stability.[43,44] However, an efficient increase in vitamin C levels after topical application of different ascorbic acid derivatives including magnesium ascorbyl phosphate, ascorbyl-6-palmitate, and dehydroascorbic acid to porcine skin could not be shown.[42]

29.2.2 Vitamin E

The α-tocopherol is regarded as the most important lipid-soluble antioxidant in plasma and tissue. The other tocopherols and the tocotrienols are present in much lower concentrations in most tissues (Figure 29.3).[45] Human epidermis contains 87% α-tocopherol, 9% γ-tocopherol, 1% α-tocotrienol, and 3% γ-tocotrienol. The recommended daily allowance is 15 mg, increasing to 19 mg during lactation.[26] Vitamin E preserves the stabiliy of biological membranes and as such of the skin barrier function by protecting against lipid peroxidation via functioning as a chain-breaking antioxidant.[46–49] Additionally it was found to be effective against UV-induced chronic skin damage, tumors, erythema, and sunburn cell formation.[50] *In vitro* α-tocopherol was also shown to scavenge superoxide anion radical, perhydroxyl radical, and hydroxyl radical.[51]

There appear to be skin type associated differences in vitamin E content of skin with higher concentrations in fair-skinned people.[52] Nevertheless the individual epidermal content of vitamin E did not correlate with the MED indicating that it is not a determinant of individual photosensitivity.[53] Also did oral supplementation with vitamin E not affect the clinical improvement of vitiligo lesions.[54]

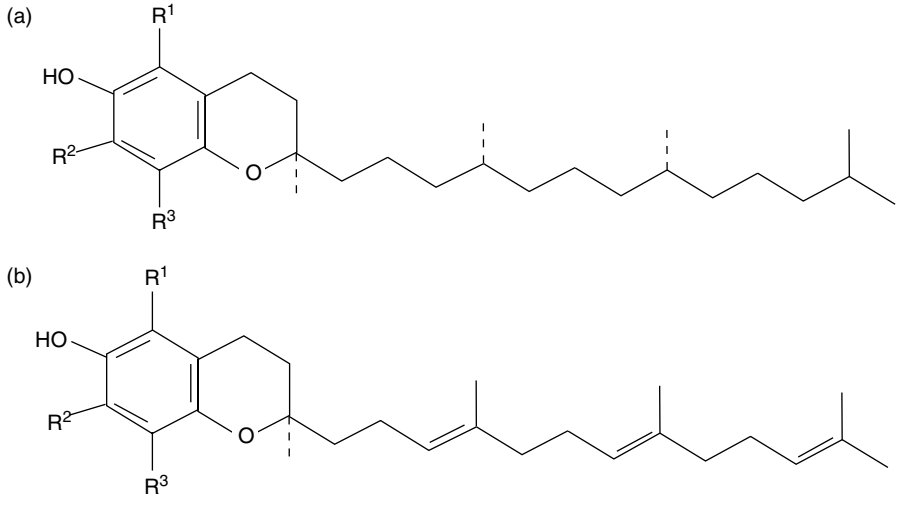

FIGURE 29.3 Chemical structure of vitamin E (term for compounds with a 6-chromanol ring, an isoprenoid side chain and the biological activity of α-tocopherol). (a) Tocopherole: α-tocopherol ($R^1{=}R^2{=}R^3{=}CH_3$), β-tocopherol ($R^1{=}R^3{=}CH_3, R^2{=}H$), γ-tocopherol ($R^1{=}H, R^2{=}R^3{=}CH_3$), δ-tocopherol ($R^1{=}R^2{=}H, R^3{=}CH_3$). (b) Tocotrienole: α-tocotrienol ($R^1{=}R^2{=}R^3{=}CH_3$), β-tocotrienol ($R^1{=}R^3{=}CH_3, R^2{=}H$), γ-tocotrienol ($R^1{=}H, R^2{=}R^3{=}CH_3$), and δ-tocotrienol ($R^1{=}R^2{=}H, R^3{=}CH_3$).

In untreated human stratum corneum there is a concentration gradient of vitamin E with the lowest levels at the surface and the highest levels in the deepest layer.[50,55] This α-tocopherol gradient in the epidermis was confirmed in another study, where the stratum corneum of atopic patients had a twofold higher concentration of α-tocopherol together with a significantly lower lipid peroxide concentration than in healthy volunteers. This less pronounced oxidative stress in stratum corneum of atopic dermatitis patients may result from an increase in cutaneous antioxidant defences due to the chronic inflammation.[56]

Topically applied vitamin E as a 5% solution penetrates rapidly through the skin of mice leading to a general enhancement of skin vitamin E content with the highest concentrations found in the uppermost layers of the stratum corneum and the major portion in the papillary dermis.[45] Due to the observation that vitamin E content of human stratum corneum of the face is much higher than of the upper arm (20:1 for the uppermost layer decreasing gradually with depth) vitamin E was found to be a continuously secreted constituent of human sebum. Thus sebaceous gland secretion appears to be an important pathway for the delivery of vitamin E to the upper layers of human skin.[57] The topical application of a vitamin E containing cream to the face and back of female volunteers led to an increased vitamin E level only in sebum, while a concomitant oral supplementation also increased vitamin E levels in the stratum corneum and plasma.[58]

Vitamin E has a narrow absorption spectrum for UV light with a maximum of about 290 nm. Accordingly acute exposure to UVB but not UVA led to a slight decrease of cutaneous vitamin E in mice.[59] Contrastingly, in another study a concentration dependent depletion of α- and γ-tocopherols in stratum corneum of mice was induced by solar simulated UV radiation.[55] Similarly acute exposure to UVA or UVB led to a depletion of α-tocopherol in stratum corneum of mice.[8] UV irradiation of human skin surface lipids with a solar simulator led to the depletion of vitamin E also. A dose of 4 MED (5.6 J/cm^2) was able to deplete 84.2% of vitamin E.[60] Interestingly α-tocopherol plasma levels decreased significantly more during sun exposure in persons who were previously supplemented with 30 mg β-carotene daily over 10 weeks than in the placebo supplemented control group despite equivalent initial values.[61]

There was a significant increase in epidermal content of α-tocopherol in mice after application of a α-tocopheryl acetate 0.5% cream, thus indicating hydrolysis of α-tocopheryl acetate by endogenous epidermal esterases.[59] When topically applied tocopherol sorbate, α-tocopherol, and α-tocopherol acetate were evaluated in a chronically UVB irradiated mouse model tocopherol sorbate was found to be significantly more protective against skin photoaging than the other two substances. This might be related to a significant reduction in ascorbate radical formation. In an additional assessment of skin wrinkling in these mice tocopherol sorbate was also significantly more protective than the other vitamin E forms with α-tocopherol still being more protective than α-tocopherol acetate.[62] Trolox, a synthetic analog of α-tocopherol might even surpass the antioxidative efficacy of α-tocopherol.[63]

Since vitamin E is very sensitive to oxidation, stable esters have mainly been used for topical formulations and are considered to be safe.[64] As had previously been shown for skin of hairless mice,[65] but not for human skin,[66] the bioconversion of vitamin E acetate by esterases to vitamin E with its known antioxidant effects has recently also been demonstrated in human skin.[48] When supplemented orally α-tocopherol acetate or α-tocopherol succinate get readily hydrolyzed to α-tocopherol in the gut[47] with the natural single stereoisomeric form (RRR-α-tocopherol) appearing to have about twice the systemic availability of the synthetic form (*all-rac*-α-tocopherol).[67]

In a hemiface trial in humans 5% vitamin E reduced rhytides, skin roughness, length of facial lines, and depth of wrinkles more than vehicle.[68] Topical tocopherol acetate was also shown to significantly increase stratum corneum hydration in human volunteers with additionally enhancing the water-binding capacity as compared to vehicle. The optimum concentration for these effects was 5% tocopherol acetate.[69]

In rats and mice the topical application of a 20 to 40% vitamin E ointment suppressed chemical-induced allergic or irritant contact dermatitis comparable to the effects of a 0.5% prednisolone

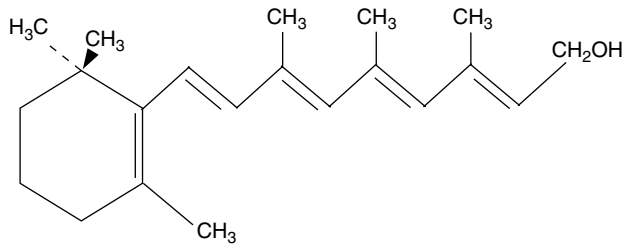

FIGURE 29.4 Chemical structure of vitamin A (*all-trans* retinol, (*all-E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraen-1-ol).

ointment. Furthermore, 20% vitamin E ointment blocked downregulation of skin barrier function induced by contact dermatitis.[70]

29.3 VITAMIN A (RETINOL), RETINOIDS (VITAMIN A DERIVATIVES), AND CAROTENOIDS

The lipophilic vitamin A is a cyclic polyene alcohol (Figure 29.4). The recommended daily allowance is 700 to 900 μg, increasing to 1300 μg during lactation (1 μg of vitamin A equals 12 μg of β-carotene or 24 μg of other provitamin A carotenoids).[26,71]

Vitamin A deficiency is characterized by xerophthalmia, follicular hyperkeratosis (phrynoderma), and generalized xerosis.[29] In hypervitaminosis A the skin becomes dry, rough, pruritic, and scaly and the lips become cracked.

High intake of carotenoids may cause carotenoderma, orange-yellow skin pigmentation on palms, soles and in areas where sebaceous glands predominate.

Vitamin A is mainly taken up either as retinylesters from liver and dairy products or as provitamin A carotenoids from fruits and green vegetables. From the more than 400 known carotenoids about 50% have vitamin A activity, the major one being β-carotene. The vitamin A activity of carotenoids depends in quantity on the conversion rate to vitamin A. Additionally carotenoids have an own antioxidant activity.[72] Retinylesters and carotenoids are converted to *all-trans* retinol, which gets either reconverted to retinylesters, the storage form in the liver and the epidermis,[59] or transported through the circulation in a complex with the retinol binding protein.[7] Apparently it is taken up by skin cells via passive diffusion.[73] In human skin the formation of retinoic acid from β-carotene or the expression of the converting enzyme 15,15′-dioxygenase has not been demonstrated yet.[72]

Vitamin A and retinoids exert their effects on a molecular level through nuclear receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR). These ligand-dependent transcription factors bind retinoids either as homodimers (RAR/RAR, RXR/RXR) or heterodimers (RAR/RXR),[74] which then can induce subsequent target gene expression by binding to the retinoid-responsive elements (RAREs and RXREs) in the promotor region of such genes.[75–77] They also inhibit the expression of genes without retinoid-responsive elements by downregulating the action of other transcription factors such as AP-1 and nuclear factor for interleukin 6 (NF-IL6), probably through mechanisms of competition for commonly required coactivator proteins.[78–80] Retinoid receptors are members of the steroid–thyroid hormone superfamily and exist as α-, β-, and γ-subtypes with differential binding of the different synthesized compounds. The expression of the retinoid receptors is tissue-specific, with RARγ being the predominant type of RAR expressed in human epidermis.[75,78,81,82] Intracellular concentration of retinoids is dependent on cytoplasmic binding by cellular retinoic acid binding proteins (CRABP) I and II,[77] the latter one being the dominant one in the skin.[83]

29.3.1 Vitamin A and Retinoids

Vitamin A absorbs UV light between 300 and 350 nm. After acute exposure to UVA or UVB a dose-dependent decrease of vitamin A was shown in mouse[59] and humans.[84] UV irradiation markedly reduced mRNA and protein of the nuclear retinoid receptors RARγ and RXRα in humans and led to a near loss of retinoic acid induction of the RAR/RXR target genes and the cellular retinoic acid binding protein II thus effectively causing additionally a functional vitamin A deficiency.[85]

Apart from the effective treatment of acne and other dermatological disorders topical application of 0.5% tretinoin (*all-trans* retinoic acid) has long been known to ameliorate changes of photodamaged skin eventually leading to an essentially normal epidermis, to normalize skin pigmentation, to induce angiogenesis and new collagen formation in the papillary dermis, and to eradicate microscopic actinic keratosis.[86,87] These effects have since been confirmed in other studies.[88,89] Thus topical tretinoin has been shown to be an effective treatment of the characteristic changes of photodamaged skin. Topical treatment with retinoic acid is very irritating, but the precursors retinaldehyde and retinol were able to load the epidermis with vitamin A with much better tolerability.[90,91] Subsequently developed topical retinoids also show a much better tolerance profile with at least the same or even better effectiveness.[92]

Embryotoxicity/teratogenicity is the major drawback in the therapeutic use of retinoids. The exposure of the fetus during the first trimester with oral retinoids is known to produce characteristic malformations.[93] There have also been case reports about malformations associated with retinoid embryopathy after the mother had used tretinoin topically during the first trimester of pregnancy.[94–96]

29.3.2 Carotenoids

In skin β-carotene is mainly located in the epidermis[97] and is depleted by eposure to sunlight.[61] The most important action of β-carotene is the quenching of singlet oxygen, a highly destructive reactive oxygen species,[98,99] which might be the reason for the efficacy of β-carotene in the treatment of erythropoietic protoporphyria.[100] A prevention of acute photodamage was shown with reduced erythema formation after exposure to sunlight following oral supplementation of 30 mg/day β-carotene for 10 weeks[61] or 25 mg/day carotenoids, mainly β-carotene, for 12 weeks.[101] Recently an increase in β-carotene plasma levels as well as β-carotene content in cytoplasm of oral mucosa cells was shown with increasing skin type I–IV, suggesting a genetically determined control mechanism.[102] Even though epidemiologic studies suggested a cancer preventive effect of β-carotene the incidence of a first nonmelanoma skin cancer was not altered after oral supplementation of β-carotene 50 mg/day for 12 years[103] or 30 mg/day for 4 to 5 years.[104] However, adverse events were attributed to the β-carotene supplementation of smokers that resulted in an increased risk of lung cancer,[105,106] possibly due to prooxidant abilities of retinol.[107]

Another carotenoid, lycopene, when applied topically at 0.03% in a gel-emulsion followed by UV irradiation reduced erythematous reactions significantly more than vehicle alone in human volunteers. The topical application of 0.5% vitamin E and 1% vitamin C in the same base also reduced erythematous reactions, but not significantly. None of these topical formulations showed a marked difference for hydration or skin barrier function.[108]

29.4 VITAMIN D (CALCIFEROL)

Vitamin D regulates calcium and phosphorus absorption and deposition and serum alkaline phosphatase levels. The recommended daily allowance is 5 μg, increasing to 10 to 15 μg in older age.[109] Vitamin D_3 is synthesized under UVB irradiation in the skin where it is stored and released into the circulation in a complex with the vitamin D binding protein. In liver it is hydroxylated to 25(OH)-cholecalciferol, the hormonal precursor, followed by another hydroxylation step in the

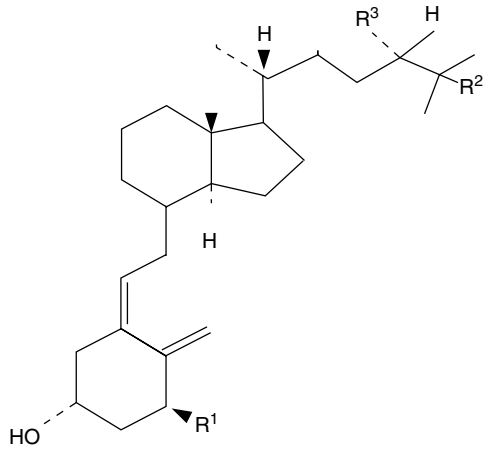

FIGURE 29.5 Chemical structure of vitamin D (calciferol, term for a collection of fat-soluble steroid-like substances that are regulating the calcium and phosphate metabolism). Cholecalciferol ($R^1{=}R^2{=}R^3{=}H$), Calcitriol ($R^1{=}R^2{=}OH, R^3{=}H$).

kidney to 1,25-dihydroxycholecalciferol (calcitriol), the active hormone (Figure 29.5). Deficiency leads to rachitis and tetany in children and osteomalacia in adults.[85]

Topical vitamin D analogs as calcipotriol, and tacalcitol are well established, effective and safe preparations for the treatment of psoriasis vulgaris due to their antiproliferative and prodifferentiating effects on keratinocytes.[110] They can be used either as monotherapy or in combination with other treatment modalities.[111] The main side effect is the increasing risk of hypercalcaemia with increasing amounts of vitamin D analogs applied to the skin.

29.5 VITAMIN B-COMPLEX AND VITAMIN K

There are some well-described deficiency syndromes, the well-established therapeutic use of vitamin K antagonists as oral anticoagulants and the well-known positive effects of pantothenic acid on skin hydration/moisturization and wound healing, which apparently lacks scientific solid base. Apart from that there are not many studies available on the treatment of dermatological disorders with these vitamins, either systemically or topically. Even less is known about transdermal penetration, stability, and formulation dependencies of possible topical applications.

29.5.1 Vitamin B-Complex

29.5.1.1 Thiamin (Vitamin B_1)

The classical deficiency syndrome is beriberi, characterized by anorexia, weakness, constipation, progressive polyneuritis, cardiac insufficiency, and either edema or wasting of muscles.[29] The recommended daily allowance is 1.1 to 1.4 mg.[112]

29.5.1.2 Riboflavin (Vitamin B_2)

Deficiency leads to conjunctivitis, perlèche, sore lips, tongue, and mouth. Additionally seborrhoic dermatitis-like eruptions may occur around the nose, eyes, ears, and the genital area (oro-oculogenital syndrome).[113,29] The recommended daily allowance is 1.1 to 1.6 mg.[112]

Skin wound healing was investigated in a riboflavin-deficient rat model: epithelialization was prolonged, wound contraction slowed and total collagen content reduced by 25%.[114]

29.5.1.3 Nicotinic Acid (Niacin)

The classical deficiency syndrome is pellagra with the triad of dermatitis, diarrhoea, and dementia.[29,115] The recommended daily allowance is 14 to 18 mg.[112]

When a 2% nicotinamide solution was applied twice daily for 4 weeks and compared to vehicle in volunteers with dry skin the ceramide levels in the epidermis increased by 34%, the free fatty acid levels by 67% while the transepidermal waterloss decreased by 27%. This indicates an improvement of a deficient epidermal permeability barrier by an increase in intercellular lipids, especially ceramides.[116] The level of ceramides in the stratum corneum is known to be reduced in atopic dermatitis and aged skin.[117,118]

For mice topical nicotinamide had been shown to have preventive effects against photocarcinogenesis. Oral supplementation of mice with niacin also led to a dose-dependent preventive activity on photocarcinogenesis as well as on photoimmunosuppression concomitantly with its ability to elevate skin NAD levels.[119]

29.5.1.4 Pyridoxine (Vitamin B_6)

A deficiency syndrome is not well-defined in humans. Since pyridoxine deficiency often produces nicotinic acid deficiency, pellagra-like clinical manifestations may occur.[29] The recommended daily allowance is 1.5 to 2 mg.[112]

Apparently the content of the active metabolite pyridoxamine 5′-phosphate is lower in human than in mouse or hamster skin.[120] When inflammatory response was assessed in a pyridoxine or riboflavin deficient rat model, data suggested enhanced inflammation in pyridoxine but not riboflavin deficiency.[121]

There is also a case report of photoallergic reactions to intravenous pyridoxine hydrochloride.[122]

29.5.1.5 Cyanocobalamin (Vitamin B_{12})

Deficiency most often occurs in vegeterians or due to pernicious anemia and is associated with an enlarged red tongue. In dark-skinned patient hyperpigmentation, especially in flexures, may occur.[29] The recommended daily allowance is 2.4 to 2.8 μg.[112]

A left and right comparison of calcipotriol with a vitamin B_{12} cream containing avocado oil in psoriasis patients twice daily over 12 weeks showed a more rapid beneficial effect with calcipotriol, which was significantly better only in treatment week 8. At week 12 there was no significant difference between the two treatment groups, so that this vitamin B_{12} cream containing avocado oil might have potential as a well-tolerated, long-term therapy of psoriasis[123]

In more recent clinical trials the efficacy of a vitamin B_{12} cream was assessed over an eight week treatment period in atopic dermatitis[124] as well as in atopic dermatitis and psoriasis patients[125] in an intra-individual left and right comparison, placebo controlled and double blind. For the atopic dermatitis patients a significant improvement was shown over placebo treatment. In the latter trial there was also a significant improvement, for psoriasis even more marked than for atopic dermatitis. Suggested as possible modes of action were an inhibition of cytokine formation and the scavenging activity of vitamin B_{12} for nitric oxide, which is increased in inflammatory skin diseases.

29.5.1.6 Folic Acid

In defeciency cheilitis, glossitis, and mucosal erosions are common. Grayish brown pigmentation on light exposed skin may also occur.[29] The recommended daily allowance is 400 μg, increasing to 500 μg during lactation and 600 μg during pregnancy.[112]

There has been an association between folate deficiency during the first weeks of pregnancy and neural tube defects, leading to a campaign to encourage folic acid intake. In as how much UV exposure might contribute to folate deficiency is still under discussion.[126–128]

29.5.1.7 Pantothenic Acid

Panthenol is absorbed via passive diffusion after topical or oral application and then enzymatically oxidized to pantothenic acid. This is a component of coenzyme A and acyl carrier protein, and as such of great importance in fatty acid, carbohydrate, and amino acid metabolism. Deficiency leads to uncharacteristic symptoms such as headaches, apathy, gastrointestinal disturbances, palpitations, and paraesthesia typically in the feet, also known as burning feet syndrome. Wound healing is impaired. The recommended daily allowance is 5 to 7 mg.[112]

Topical dexpanthenol, a stable alcoholic analog of pantothenic acid with good skin penetration, acts like a moisturizer, improves stratum corneum hydration, reduces transepidermal water loss, and maintains skin softness and elasticity. In wound-healing activation of fibroblast proliferation has been observed *in vitro* and *in vivo* with dexpanthenol concomitantly with increased elastic and solid tissue regeneration. In skin irritation pretreatment with dexpanthenol resulted in less damage to the stratum corneum barrier, while adjuvant treatment improved the symptoms of dryness, roughness, scaling pruritus, erythema, and erosions considerably. The topical application is well-tolerated with a minimal risk of skin irritation or sensitization.[129–132]

29.5.1.8 Biotin (Vitamin H)

Deficiency can be due to two known genetic disorders of biotin metabolism or be induced by excess intake of avidin, which binds biotin and thus leads to poor absorption. Symptoms are alopecia, eczema around nose and mouth, conjunctivitis, hyperaesthesia, paraesthesia, depression, and muscle pain.[29] The recommended daily allowance is 30 to 35 μg.[112]

29.5.2 Vitamin K

Vitamin K is essential for the synthesis of coagulation factors II, VII, IX, X, and proteins C and S. About half of the daily requirement is synthesized by the gastrointestinal flora. Deficiency leads to an impairment in the coagulation cascade, clinically presenting as purpura, ecchymoses, and hemorrhage anywhere in the body.[29] The recommended daily allowance is 90 μg for females and 120 μg for males.[71]

29.6 CONCLUSION

Vitamin C appears to be a promising ingredient of topical formulations, but larger studies need to be done to establish a stable and skin-penetrating compound and a definite role of vitamin C in skin processes related to oxidative stress. Vitamin E shows promising results in protection from photodamage, but most of the studies have been done with murine skin, so these effects need to be confirmed for human skin. Both vitamins have long been common additives to cosmetic and medical topical formulations due to their antioxidative effects either before or after application. Similarly have both been supplemented systemically for various reasons quite frequently without causing any serious side effects. For topical formulations of vitamin C and vitamin E more studies are on the way from the pharmaceutical as well as the cosmetical industries to get more detailed knowledge about optimal delivery systems, their transdermal penetration characteristics and subsequent effects on human skin. So far there are definite positive results without any signs of adverse effects.

Vitamin A and retinoids have a well-established treatment profile systemically and topically in dermatology, which is limited mainly by embryotoxicity and teratogenicity. When it comes to patient compliance, skin irritation is also a problem. Additonally there have been repeatedly reported positive changes to photodamaged and (photo)aged human skin after topical treatment. Thus less irritating vitamin A derivatives or precursors become increasingly more important ingredients in skin care products for aging skin.

Vitamin D analogs seem to be limited in dermatology to the topical treatment of psoriasis vulgaris, which is not least because of their overall effects on calcium and phosphorus homöostasis.

Apart from pantothenic acid, which has been attributed loads of helpful effects in skin care and occurs quite frequently as dexpanthenol in cosmetic and medical formulations, the other vitamins of the B-complex are so far not known for specific positive results on skin, neither topically nor systemically. Nevertheless, some ideas for applications might eventually lead to new treatment options.

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30 Antimicrobials

Anke Gauger

CONTENTS

30.1 INTRODUCTION

Dry skin and especially eczematous skin is extremely sensible not only for irritants but also for infections due to a disrupted barrier function. Atopic dry skin shows an enhanced transepidermal water loss (TEWL) denoting an impaired water permeability function and a lowered threshold of irritant responsiveness.[1,2] Patients with atopic dermatitis (AD) show a markedly increased rate of colonization or infection with microbial organisms, including *Staphylococcus aureus*. They act in a bidirectional fashion, both as superantigens and as conventional allergens. Increased numbers of *S. aureus* are found in over 90% of atopic eczema skin lesions and even in uninvolved skin leading to exacerbation and maintenance of skin inflammation via different mechanisms: exotoxins, enzymes, superantigens, and others.[3,4] In contrast, only 5% of healthy subjects harbor this organism. The density of *S. aureus* on AD lesions has been shown to correlate with cutaneous inflammation and to contribute to the severity of the disease.[5,6] Not only bacterial, but also viral and fungal superinfections are well-known risk-factors causing acute and severe disease exacerbation. In patients with psoriasis, only 6.7% suffer from skin infections despite the fact that both skin diseases are characterized by defective skin barriers.[7,8] Recently, antimicrobial peptides have been shown to be key elements in the innate immune response system providing the first line of defense in the skin.[9] In comparison to psoriasis, AD skin lesions contain significantly lower levels of important antimicrobial molecules, such as defensins and cathelicidins. In detail, low levels of iNOS, IL-8, HBD-2, and the recently identified HBD-3 transcripts count for the failure of patients with AD to mount an adequate host response against a variety of microbes.[10,11] Thus, while antimicrobial therapy is an important treatment component in the management of AD, neither an increased skin infection rate nor the need

for antimicrobials is noted in patients with psoriasis except the use of antibiotics when streptococcal infections precipitate or exacerbate the disease.

Antiseptics and antibiotics are the two hallmarks of antimicrobial therapy. Although there are several reports about local and systemic antimicrobials for therapy of eczematous skin diseases, there is only little data about prevention.

30.2 COMPOSITION AND FUNCTION OF ANTIMICROBIALS

30.2.1 Antiseptics

Antiseptic agents readily disinfect intact surfaces by decreasing bacterial counts on intact skin. To prevent possible infections, indications for antiseptics include cleansing of intact skin, hand sanitizing, oral rinses, preparation of skin before injections, and surgical preparation and prophylaxis. The goal of surgical preparation of the skin with antiseptics is to remove transient and pathogenic micro-organisms on the skin surface and to reduce the resident flora to a low level.[12] The most commonly used antiseptics are alcohol, chlorhexidine, and iodophors (see Table 30.1).

Ethyl alcohol or isopropyl alcohol in an aqueous solution (between 70 and 92%) is an inexpensive and easily accessible disinfectant, which is rapidly acting and bactericidal as well as germicidal to most viruses, fungi, and other pathogens.[13] However, its residual activity is limited and it should be used in conjunction with a longer-acting disinfectant (i.e., chlorhexidine) for optimal antimicrobial

TABLE 30.1
Antiseptics

Antiseptic	Substance group	Advantage	Disadvantage
Ethyl/isopropyl alcohol	Alcohol	Good antibacteial activity	High irritative potential, low residual capacity
Chlorhexidine	Bis-biguanide	Good antibacterial activity, low sensitizing potential and toxicity	
Povidone-iodine	Povidone-iodine	Good antibacterial activity, low irritative, phototoxic potential and toxicity	Slight allergic potential, restrictive use in patients with thyroid gland disease
Octenidine	Octenidine dihydrochloride	Good antibacterial activity, efficacy against MRSA, low toxicity, good tolerance on mucous membranes	Soluble preparations only
Triclosan	Trichloro-hydroxydiphenylether	Good antibacterial activity, negligible irritative, phototoxic, allergic potential, low toxicity	Slight antibiotic activity, excessive additive in general products
Essential oils	Melaleuca alternifolia (tea tree oil), Farnesol	Good antibacterial activity, antiinflammatory potential	High allergic potential
Gentian violet	Methyl/crystal violet	Good antibacterial activity, low resistance risk, antiinflammatory potential	High toxicity in concentrations >1%, intense color
Polyhexanide	Polyhexamethylene biguanide	Good antibacterial activity, efficacy against MRSA, low toxicity	Solutions and gel preparations only, possible anaphylactic reactions

activity. In addition, even on intact skin alcohol possesses a high irritative and high inflammatory potential.[14]

Chlorhexidine gluconate and *povidone iodine* have emerged as the antiseptics of choice as presurgical prophylaxis. Comparison studies with chlorhexidine, hexachlorophene, and iodophors show chlorhexidine to be the most effective agent. Chlorhexidine is a biguanide that disrupts cytoplasmic membranes and proven to be superior of the two antiseptics in recent studies because of its more persistent activity and broad coverage. It is active against gram-positive bacteria, some gram-negative bacteria, and viruses, and is very safe.[12,13,15] Chlorhexidine's persistence is one of its best attributes. Studies have shown that its germicidal activity persists for more than 6 h after its initial application.[16] However, one disadvantage is the intermediate onset of action. In addition, there are single reports that chlorhexidine can be toxic to the middle ear and irritating to the eyes with direct contact. Therefore, it should be used in these areas with caution, especially when applied together with other antiseptics.[12] Although chlorhexidine is minimally absorbed by the skin and the potential for skin irritation is low relative to other antiseptic agents, contact dermatitis is a frequently reported phenomenon. In addition, there have been fifty case reports of chlorhexidine-related anaphylaxis published worldwide over the past ten years.[17–19] Although resistance rate seems to be negligible, there have been single reports about resistant bacterial strains.[20]

Povidone iodine, the most commonly used iodophor, is a complex of iodine and polyvinylpyrrolidone (povidone). Povidone iodine has a broad spectrum of germicidal activity and is effective against most bacteria (including methicillin-resistant *Staphylococcus aureus* [MRSA]), some bacterial spores, viruses, fungi, and *M. tuberculosis*.[14] Iodophors exert their antibacterial effects through a mechanism of cell wall penetration and oxidation and the release of free iodine.[16] Due to the iodine fraction, iodophors may be able to cause skin irritation, allergy, and toxicity in susceptible persons. Percutaneous absorption and mucous membrane absorption have been documented in former years and, therefore, may be harmful for patients with thyroid disorders, pregnant women, or newborn infants.[21,22] Although povidone iodine shows excellent antibacterial activity with a low resistance rate, its intermediate onset of action is comparable to chlorhexidine.[14]

Octenidine is an antiseptic with proven antimicrobial qualities, which is frequently used as a disinfectant in surgery as well as antiseptic mouthwash with excellent tolerance especially when used on mucous membranes.[23,24] It has even been shown to be effective in eradicating MRSA when used as an octenidine dihydrochloride whole-body wash combined with nasal mupirocin treatment.[25] Due to the low irritant and allergic potential as well a low resistance rate, octenidine seems to be a substance with a promising future.

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenylether) is an antiseptic suitable for formulation in a W/O emulsion with excellent antibacterial activity *in vitro* and *in vivo* against *S. aureus, Klebsiella and Proteus species*.[26] In addition, it has been shown to be effective for eradication of MRSA as well as fungi. Triclosan does not have irritative, phototoxic or allergic, nor mutagenic or teratogenic potential; the toxicity in general is low and so is the sensitizing potential.[27–29] Triclosan and similar additives (e.g., triclocarban) have demonstrated antibacterial and antiinflammatory efficacy when used as an antiseptic wash.[28,30]

Essential Oils have become very popular as naturally occurring antimicrobial and antiseptic agents. Several studies have investigated *Tea Tree Oil (TTO)* (Melaleuca alternifolia) *in vitro* and found antimicrobial properties with susceptibility data on a wide range of bacteria, yeasts, and fungi as well as indirect antiinflammatory responses.[31,32] Koh et al. could even demonstrate that undiluted TTO applied to histamine induced inflammation can reduce mean weal volume.[33] However, the usage of TTO and occurrence of allergic contact dermatitis to TTO have increased simultaneously. There have been several reports about allergic reactions, especially contact dermatitis to TTO even presenting as an extensive erythema multiforme-like reaction as well as immediate systemic hypersensitivity reactions.[34–36] The essential oil contains turpentines (limonen, alpha-pinene, phellandrene) that are potentially allergenic.[37] Other essential oils than TTO (lemongrass, citronella, tuberous blossom, sandalwood, and orange blossom) contain farnesol as the major antimicrobial component.[38] *Farnesol*

has shown a suppressive effect against *S. aureus* with a low irritative and allergic potential.[38,39] *In vitro*, Akiyama et al.[39] found not only an inhibitory effect of Farnesol against *S. aureus* on the horny cells of AE lesions, but also supportive mechanisms of antibiotics suggesting Farnesol as a promising adjuvant agent against *S. aureus* skin infections treated with β-lactam antibiotics.

Gentian violet, a mixture of methyl violet and crystal violet, is a triphenylethane dye with a broad antibacterial activity. Its spectrum of activity covers yeasts and gram-negative as well as gram-positive micro-organisms. *Gentian violet* is active against *P. aeruginosa*, *Proteus*, *Klebsiella*, and *S. aureus* including MRSA, thus covering almost all dermatologically relevant pathogenic bacteria except streptococci. In addition, it has good antifungal activity against both *Candida* and hyphomycetes. The mechanism of action is thought to consist of inhibition of cell wall synthesis and of glutamine precursor synthesis. In addition, *in vitro* antiirritative effects such as significant reductions in dehydration, barrier damage, and irritative hyperemia have been observed.[40] Gentian violet has a low risk of bacterial resistance and contact allergies, but a high risk of toxicity when used in too high concentrations (>1%) in intertriginous areas.[41] Furthermore, due to its intensive color, the unfavorable cosmetical aspect cannot be denied.

30.2.2 Antibiotics

Antibiotics can be either administered topically — as monotherapy or as part of a combination therapy — or systemically.

30.2.2.1 Topical Antibiotics

Topical antibiotics are typically available as ointments and are excellent for use on open wounds. Coupled with the antibacterial action of the antibiotic ingredient, topical antibiotic ointments provide a safe and effective option in wound healing. In addition, topical antibiotics are effective for the localized treatment of primary and secondary pyodermas with minimal systemic side effects.[14] Prophylactic uses include application for traumatic and surgical wounds, burns, intravascular catheters, and eradication of *S. aureus* nasal carriage.[16,42] The advantage of antibiotic therapy in the treatment of eczematous skin will be discussed in the following article considering AD as an example.

Topical antibiotics may be considered to be advantageous over their systemic counterparts because they deliver a higher concentration of medication directly to the desired area and are less frequently implicated in causing bacterial resistance. An ideal topical antimicrobial has a broad spectrum of activity, persistent antibacterial effects, and minimal toxicity or incidence of allergy.

Fusidic acid seems to be the antibiotic drug of choice in inhibition of staphylococci due to efficacy at very low concentrations, regardless of the patient's susceptibility to methicillin or oxacillin.[43] However, due to increased use of topical antibiotics, higher levels of fusidic acid resistance have been noted in areas using larger quantities of topical fusidic acid-containing preparations.[44,45] In contrast to a resistance rate of approximately 10% in the general population, dermatological patients demonstrated nearly 50% resistance rate in a representative study in the United Kingdom.[46] On the other hand, Wilkinson postulates that after 35 years of extended usage of fusidic acid, the level of resistance has been low.[47] Other sources speak of an average prevalence of fusidic acid resistant *S. aureus* between 1 and 3%.[48,49] There is concern that topical use may be driving the selection and dissemination of fusidic acid-resistant (Fus®) *S. aureus* probably leading to a failure of systemic therapy for serious or MRSA infections.[46] Although there was no evidence in a small population study that topical fusidic acid/steroid combination results in an increase in either the prevalence or the population density of Fus® *S. aureus* within a short-term treatment, fusidic acid-containing preparations should nevertheless be used to treat acute skin infections in the short term only.[50]

Mupirocin is a naturally occurring antibiotic available as a cream or ointment, and is unusual in its origin and mechanism of action. It inhibits protein synthesis, actively preventing the incorporation

of isoleucine into protein by binding to isoleucyl transfer-RNA synthetase.[16] Because of this unique mechanism of action, there is no *in vitro* incidence of cross-reactivity with other antimicrobials. Mupirocin is highly effective against aerobic Gram-positive cocci (namely *S. aureus*, *S. epidermidis*, and β-hemolytic streptococci), and some Gram-negative cocci but spares much of the normal flora.[16] Its indications include prophylaxis in ulcers, operative wounds, and burns; treatment of skin infections; and the eradication of nasal carriage of *S. aureus*. In addition, mupirocin has proven useful in the management of secondary pyodermas or superinfection of chronic dermatoses.

Although the incidence of adverse reactions to mupirocin is typically low (occurring in less than 1.5% of patients), several local side effects such as burning, stinging, pain, erythema, and contact dermatitis have been reported. Resistance to mupirocin has been reported but is not common. Some strains of bacteria have a low level of resistance but succumb to high-dose of mupirocin.[14] Due to this fact, it should be handled with extreme care, especially as prophylactic use, in order to prevent further resistance.

Bacitracin is an inexpensive, a low-risk application for causing toxicity, and a readily available topical antibiotic. It is produced by growth of an organism of the *licheniformis* group of *Bacillus subtilis*.[14] Bacitracin is bactericidal for a variety of Gram-positive and Gram-negative organisms. It blocks bacterial cell wall synthesis by inhibiting the regeneration of phospholipid receptors involved in peptidoglycan synthesis. Resistance is uncommon but has been reported in some strains of staphylococci.

Bacitracin is indicated in prophylaxis and treatment of local infections, treatment of secondary pyodermas, as an adjunct in burn treatment, and as prophylaxis in operative wounds. However, it is not indicated in the treatment of chronic ulcers because of the increased risk of allergic reactions. There are several reports of delayed hypersensitivity, acute IgE-mediated allergic reactions, and anaphylactic reactions to bacitracin.[51–53]

Polymyxins are decapeptides isolated from *B. polymyxa* with low cutaneous sensitization potential and negligible systemic absorbance or toxicity.[14,42] The mechanism of action is to disrupt the phospholipid component of the cell membranes through a surfactant-like action, resulting in increased permeability of the bacterial cell.[14,42] Polymyxins are bactericidal against some Gram-negative bacteria, but largely inactive against most Gram-positive bacteria. They may be used for prophylaxis and treatment of superficial wounds as well as for prophylaxis in a surgical wound. They are generally well-tolerated and are most frequently used in combination with other topical antimicrobials for maximum efficacy.[14,42]

30.2.2.2 Systemic Antibiotics

Use of systemic antibiotics should be limited to infectious or pronounced superinfected wounds. Only few situations in dermatologic surgery require prophylactic antibiotics, since in cutaneous surgery postoperative infections are too infrequent and insufficiently severe to justify preventive antibiotics.

On one hand there is prophylaxis of potential endocarditis or infection of prothetic material, which is required in cutaneous surgery according to international recommendations.[54] On the other hand, prophylactic preoperative intraincisional antibiotic treatment may help to prevent postoperative complications in some cases. Hirschmann[55] proposes that in patients with numerous staphylococcal skin infections, oral clindamycin 150 mg every day for 3 months safely reduces further episodes. For recurrent cellulitis, oral penicillin or erythromycin 250 mg twice daily or monthly intramuscular benzathine penicillin decreases subsequent attacks.[55] Huether et al.[56] evaluated surgical wounds at the time of suture removal with or without a single dose of clindamycin as prophylactic antibiotic treatment. Wounds were assessed according to a standardized scheme based on erythema, edema, and the presence of purulent discharge. In addition, bacterial cultures obtained when indicated were also compared. The results of this study support the efficacy of single-dose preoperative intraincisional antibiotic treatment for dermatologic surgery. The authors recommend clindamycin as an inexpensive, safe, convenient, and effective alternative with special emphasis on the relatively high

prevalence of patient-reported penicillin allergies.[56] However, a general advice cannot be given in dermatological surgery and has to be based on specific, internationally accepted guidelines, such as from the German Dermatology Society and the Professional Association of Surgical and Oncological Dermatology.[57]

30.3 EFFECTS OF ANTIMICROBIALS ON THE SKIN BARRIER FUNCTION

30.3.1 Intact Skin

30.3.1.1 Antiseptics

Soaps and detergents have been described as the most damaging of all substances routinely applied to skin. Differences exist between the different surfactants regarding the dehydrating, barrier-damaging and irritating effects, even when concentrations with the same cleansing effect are used.[58,59] Each time the skin is washed, it undergoes profound changes, most of them transient. However, among persons in occupations such as health care in which frequent handwashing is required, long-term changes in the skin can result in chronic damage, irritant contact dermatitis and eczema, and concomitant changes in flora.[60] The normal microflora acts as a barrier against colonization of potentially pathogenic micro-organisms and against overgrowth of already present opportunistic micro-organisms. If the growth of opportunistic micro-organisms is controlled, one speaks of colonization resistance. Administration of antimicrobial agents, therapeutically or as prophylaxis, may cause disturbances in the ecological balance between the host and the normal microflora.[61] A well-balanced bacterial flora prevents establishment of resistant microbial strains and long-term use of topical antimicrobial agents may alter this flora.[62]

Use of antimicrobial soaps is able to reduce rates of superficial cutaneous infections substantially, as demonstrated in multiple studies.[63] Other experimental studies also found a profound reduction in bacteria on the skin with use of antimicrobial soaps, but none assessed rates of infection as an outcome.[60]

For surgical or other high-risk patients showering with antiseptic agents decrease microbial counts on the skin,[64–66] but only in some studies reduced postoperative infection rates could be observed.[67,68] Whole-body washing with a chlorhexidine-containing detergent has been shown to reduce infections among neonates,[69] but concerns about absorption and safety preclude this as a routine practice. Several studies have demonstrated substantial reductions in rates of acquisition of MRSA in surgical patients bathed with a triclosan-containing product.[70] However, abundant use of triclosan in cleaning and hygiene products in general public has evoked an emerging risk factor for antibiotic resistance in the community, since antibiotic potential as well as adaptive resistance to triclosan has already been demonstrated, predominantly in England and Japan.[71–74] Therefore, cleaning or bathing with antimicrobial products is recommended only for persons with increased risk of skin infections or presurgically. Mild, non-antimicrobial soap should suffice for routine cleaning, since bathing or showering relieves the skin sufficiently by mechanical removal of bacteria shed on corneocytes.[60]

In addition, even when prophylactic reduction of the microbial flora is wanted, for example, in handwashing of nurses, with use of antiseptic preparations, no reductions beyond an equilibrium level in counts of hand flora were attained.[75] The numbers of organisms spread from the hands of nurses who washed frequently with an antimicrobial soap actually increased after a period of time; this increase is associated with declining skin health.[76]

In summary, the trend in both the general public and among health-care professionals toward more frequent washing with detergents, soaps, and antimicrobial ingredients has to be evaluated carefully. More washing and scrubbing may rather deteriorate the skin condition and may even increase the risk for harboring and transmitting infectious agents by damaging the skin barrier and disturbing

the microflora.[60] Furthermore, frequent use of antimicrobials in daily public cleaning products may increase the risk of resistent bacterial strains. Antimicrobial cleaning procedures should be limited to a certain group of persons, either with increased risk of infection and its transmission or presurgically. The goal should be to identify skin hygiene practices that provide adequate protection from transmission of infecting agents while minimizing the risk for changing the ecology and health of the skin and increasing resistance in the skin flora.[60]

30.3.1.2 Antibiotics

In intact skin, the use of antibiotics is extremely limited due to their specific antibacterial effect and the possible risk of promoting bacterial resistance.

Recurrent impetigo, furunculosis, or other staphylococcal infections may be a result of pathogenic nasal carriage of *S. aureus*. To reduce postoperative complications, eradication of nasal colonization of *S. aureus* has been extended to colonized health care workers and other susceptible patients.[14] Mupirocin has been found to be the most effective topical antibiotic for the elimination and is effective in reducing subsequent infections. When applied intranasally four times daily for five days, it has been shown to reduce nasal carriage for up to 1 year.[77]

These results were in concordance with a study that examined immunocompetent staphylococcal carriers who experienced recurrent skin infections. The study concluded that an initial five-day course of mupirocin followed by a five-day course of nasal mupirocin every month for 1 year reduced the incidence of nasal colonization and in turn lowered the risk of skin infection.[78]

30.3.2 Atopic Dermatitis

As stated earlier, antiseptic cleaning may be useful in prevention of bacterial infections of the skin, but a prophylactic effect of antiseptics for prevention of eczema has not been shown yet. Due to their mostly irritative and sometimes allergic potential, antiseptics rather deteriorate the skin barrier of intact skin by degreasing the lipid barrier. In addition, they probably enhance susceptibility of dry skin for irritants more than they protect.[17] Late onset hypersensitivity and eczema occur regularly and are well-documented events.

30.3.2.1 Antiseptics

In AD increased *S. aureus* colonization plays a fundamental role; therefore, antistaphylococcal therapy is part of a successful management of the disease. Epidermal lipid deficiencies and barrier dysfunction contribute to enhanced *S. aureus* attachment to the skin and mediate immunological and inflammatory effects including the release of superantigens, additional exotoxins, and exoenzymes, and perhaps bacterial DNA-triggered mechanisms. Therapeutic possibilities include the use of topical antiseptics in cases of microbial-laden atopic eczema, corticosteroids, and specific antibiotic–antiseptic combinations in cases of localized superinfected atopic eczema and systemic antibiotics in cases of generalized superinfected atopic eczema.[48]

Several studies demonstrate the effectiveness of antiseptic therapy in AD; it may be administered either as part of emollients or as wet wrap dressings (see Table 30.1). However, antiseptic therapy is only one part in the successful management of AD by reducing the risk factor *S. aureus*, other therapeutic strategies cannot be neglected.

Triclosan cannot only be applied as an emulsion, but it also has demonstrated antibacterial and antiinflammatory efficacy in eczema therapy when used as an antiseptic wash.[28,30] Likewise, 10% *povidone–iodine* solution as a disinfectant showed excellent antibacterial activity together with improvement of clinical severity.[79] As a 1% solution, *chlorhexidine* digluconate has shown superior effectiveness to triclosan *in vitro*, but may be only suitable for therapeutic use in intertriginous areas or as part of "wet wrap dressings" in the treatment of AD when used as an alcoholic solution.[26,80]

In a comparative study, Stalder et al.[81] found a greater decrease in *S. aureus* colonization in the chlorhexidine group, when compared to *KMn04*, without statistical difference. *In vitro*, the bacterial eradication was even significantly higher in the chlorhexidine group. However, clinical studies concerning bacterial colonization and clinical effectiveness of chlorhexidine containing emollients are still missing.

Although *Octenidine* has an excellent clinical profile in effectiveness and tolerability, octenidine-containing emollients and clinical studies for treatment of superinfected AD lesions are still missing.

Gentian violet has a potent anti-*S. aureus* efficacy *in vitro* and *in vivo*, which was paralleled by reduction of clinical severity of AD.[41]

Gloor et al.[82] discovered an additional antimicrobial activity of a distillate of *Hamamelis* (Aqua *Hamamelidis*) and urea formulated as a topical dermatological preparation that contains both active ingredients. Although mainly used for their antiinflammatory, hydrating, and barrier-stabilizing effects in dermatitis maintenance therapy, the antimicrobial activity of such products is considered a welcome, an added benefit.

30.3.2.2 Antibiotics

Antibiotics can be administered either systemic or topical as monotherapy or part of a corticoid–steroid combination. Antibacterial therapy leads not only to reduction of bacterial colonization, but also in many cases to improvement of AE, even when not actively infected.[83,84]

Topical antibiotic therapy

Topical Antibiotic Monotherapy: Localized impetiginized eczema lesions can be treated successfully with topical fusidic acid or mupirocin, whereas topical application of other antibiotics (neomycin as obsolete aminoglycosid, tetracyclines, or polymyxines) should be avoided.[84] Especially in children with AD, fusidic acid resistance seems to be a particular problem reflecting the chronicity and the extent of the disease (see Section 30.2.2).

Fusidic acid and mupirocin has been proven to be equal in clinical efficacy.[85–87] The risk of allergic contact dermatitis to fusidic acid in patients with AD can be considered very low. In an analysis of multicenter surveillance data in Germany, fusidic acid did not cause any case of sensitization in the subgroup of atopics.[29] Topical neomycin, however, is rarely indicated not only because of inefficacy and high resistance rates, but also because of frequent development of allergic contact dermatitis.[88,89]

S. aureus colonization in the nasal cavity is present in most patients with AD.[90] Correlation of *S. aureus* eradication in the nares and clinical improvement has been shown and is indicated especially in recurrent severely impetiginized eczema.[78,91] For topical treatment of the nasal cavity, mupirocin-ointment has been demonstrated to be effective even in a short-term application of seven days. In localized infections, colonization of chronic AD with *S. aureus* is effectively controlled with mupirocin.[92] In addition, mupirocin cream was found to be similar in efficacy against *S. pyogenes* and *S. aureus* to oral flucloxacillin but significantly more effective than oral erythromycin in mouse surgical models with primary and secondary wounds. The same study found mupirocin cream to be similar in efficacy to cephalexin against *S. pyogenes* and superior to cephalexin against *S. aureus*.[93]

Antibiotic-Steroid Combination Therapy: In the last decades the effectiveness of the combination of topical corticosteroids with an antibiotic has been discussed controversially. Several studies demonstrated a superior effect of an antibiotic–steroid combination versus steroid alone.[94,95] More recently it has been shown in several studies that the advantage of a combination therapy was obvious only when using steroids of low potency whereas no difference in clinical efficacy and bacterial eradication could be observed when using steroids of high potency.[96–98] This phenomenon could be explained by the interaction between superantigen production of *S. aureus* and corticosteroid responsiveness. When T cells are stimulated with superantigens they become insensitive to corticosteroids.[99] Thus, reduction of *S. aureus* superantigen production and augmentation of corticoid sensitivity by

antibiotics leads to an effective combination of antibiotic-topical corticosteroid therapy allowing the usage of low- to medium-potency topical corticosteroids to achieve the same clinical effects as high-potency corticosteroids when used alone.[100] In general, topical antibacterial–corticosteroid combinations can be useful when treating small areas of skin for a limited period of time, but are accompanied by the risk of sensitization and the emerge of resistant strains of bacteria. Systemic antibacterials in combination with topical corticosteroid are more appropriate when large areas are involved.[101]

Systemic antibiotic therapy: Systemic antibiotics are helpful in the treatment of acute exacerbations with diffuse *S. aureus* infection.[48] While erythromycin used to be the most common antibacterial agent in the treatment of generalized impetiginized AE, the newer macrolide antibiotics (azithromycin, clarithromycin, roxithromycin) are more frequently used during the last years. Adachi et al.[102] found that antibiotics with inhibitory effect on protein synthesis can suppress the production of superantigens from *S. aureus*. On the other hand, in *in vitro* studies, superantigen production was not suppressed by antibiotics having an inhibitory effect on either the cell wall or the nucleid acid synthesis. In this study, roxithromycin was the only antibiotic that suppressed replication of DNA coding of superantigens produced by *S. aureus*. These results confirm the use of macrolide antibiotics in the treatment of superinfected AE. However, erythromycin-resistant *S. aureus*-strains have increased worldwide up to 30%.[48,83] Other data source of the National Reference Centre in Germany report a decline of erythromycin resistant strains between 1975 and 1990 (from 21 to 8.5%) but a rapid incline thenceforward (17.5% in 1995). In case of oxacillin-(methicillin)-resistance, *S. aureus* demonstrate a resistance rate of over 80% to erythromycin and over 10% to fusidic acid as a result of parallel resistance.[103] Nevertheless, for macrolide-resistant *S. aureus*, penicillinase-resistant penicillin (dicloxacillin, oxacillin, flucloxacillin, or cloxacillin) and first generation cephalosporins should be used.[100,104] In double-blind placebo-controlled studies, however, treatment with oral flucloxacillin or cefuroxime axetil resulted in a significant reduction of *S. aureus* colonization, but not in a significant improvement of eczema. In addition, recolonization generally seems to appear soon after completion of oral treatment.[91] In case of penicillin- or cephalosporin-allergy, clindamycine or oral fusidic acid are possible alternatives.

An explanation for conflicting results in most studies concerning oral antibiotic therapy could be the fact that the anterior nares as a reservoir of *S. aureus* and the possibility of autotransmission or transmission between patients and their partners often were not considered. Good therapeutic effects were observed when treating the nasal cavity of patient and partners topically in addition to systemic therapy.[48,91]

Silver coated textiles: Recently, a new mode of antibacterial/antimicrobial therapy in AD has been introduced: the use of antibacterial silver coated textiles. In an open-labeled controlled side-to-side comparative trial, silver-coated textiles were able to reduce *S. aureus*-colonization significantly already two days after initiation lasting until the end of the treatment. Even seven days after cessation, *S. aureus*-density remained significantly lower compared to the baseline. Clinical improvement paralleled with reduction of *S. aureus*-colonization.[105]

Silver ions demonstrate two key advantages: they are broad-spectrum antiseptics and are not yet associated with drug resistance. Textiles with antiseptic properties may offer the advantage to enhance the clinical efficacy of topical glucocortisteroids or other antiinflammatory therapy by reducing *S. aureus*-colonization. In addition, an identical clinical efficacy might be achieved by combining textile antistaphylococcal treatment and steroids of less potency and in this way reducing possible side effects of glucocorticosteroids. However, further studies need to be carried out to investigate possible preventive effects and to further characterize potential side effects of the textiles.

30.4 CONCLUSION

Antimicrobial therapy is a milestone in prevention and therapy of infections and certain cutaneous diseases.

Antiseptics are used topically for prevention of bacterial and other infections especially in the setting of healthcare workers. Although they demonstrate excellent antibacterial activity, their usage has to be cautiously evaluated regarding their potential irritative and barrier-disequilibrating effects. When used in therapy of AD, they offer the advantage of a low sensitizing potential and low resistance rate. They can be used in emoillents or as part of an additional "wet wrap dressing" therapy. In diminishing the *S. aureus* density, antiseptics contribute to the stabilization of the skin barrier and to the reduction of eczema severity.

Antibiotics have demonstrated a well-known effect on infected skin. They should be carefully used prophylactically since they might enhance resistance rates of bacteria against common antibiotics. In case of infected or superinfected wounds they do help to recover the skin condition and are part in a successful management of AD.

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31 Moisturizing Cleansers

K.P. Ananthapadmanabhan, K. Subramanyan, and Greg Nole

CONTENTS

31.1 INTRODUCTION

The outermost layer of skin, the stratum corneum (SC), plays an important role in protecting against desiccation and environmental challenges. Optimal hydration of the SC is essential for maintenance and promotion of skin health. Water helps to plasticize SC making it more flexible and resilient to mechanical stress[1] and is also essential for the optimum biological functioning of the SC.[2–4] Various factors including cleansing can cause a loss of hydration of the SC leading to varying degrees of SC dysfunction. Cleansers contain surfactants that interact with the proteins and lipids in the SC, which reduces the water retention capacity and leads to short- and long-term deleterious effects on skin condition. Use of mild surfactants in cleansers provides a significant benefit by reducing the loss of hydration during cleansing and by preserving the integrity of the skin moisture

barrier. In addition to this, cleansers, especially in the liquid form, can incorporate significant amount of emollients/moisturizers that can be delivered and retained on skin during cleansing to provide significant boost in skin hydration, in a lotion like manner. These emollient cleansers, unlike common cleansers, provide significant benefit to the skin such as prevention of dry, tight skin, and in some instances even dryness relief. Moisturizing cleansers when used as part of everyday skin care routine help maintain the SC in a healthy state. In the sections below we examine the importance of moisturization, the science and technology underlying mild and moisturizing cleansers, and methods to evaluate their performance.

31.2 THE IMPORTANCE OF MOISTURIZATION

31.2.1 Hydration of the Stratum Corneum

Extensive research on the biology of the SC has shown that optimal levels of hydration are required for a number of key enzymatic processes leading to the development of a healthy SC.[2–4] SC processes that are influenced by hydration state include desquamation, barrier lipid formation, and natural moisturizing factor (NMF) synthesis. For example, the proteolysis of fillagrin, a critical process to maintaining flexibility and hydration of skin is itself initiated by changes in the water gradient in the SC.[5] It is becoming increasingly clear that normalizing hydration levels in the SC can significantly activate key processes in the living epidermis through an elegant feedback mechanism. Perturbation to the SC barrier leading to altered water flux sets in motion a cascade of events within the underlying epidermis to promote barrier repair and recovery.[6,7] The SC, which in the past was considered nothing but a dead protective tissue, is now recognized to be an enzymatically active biosensor that can regulate activities in the living epidermis.

There is a constant flux of water leaving the skin through the SC. A normal, healthy SC maintains its hydration by controlling the rate of water flux via the lipid barrier and NMF functions. This flux is affected mainly by the structural integrity of the moisture barrier and environmental temperature and humidity. A weakened or damaged barrier will lead to increased water loss from the skin, reducing water content of the SC. It is known that the SC barrier is compromised in several dry skin states such as atopic dermatitis, psoriasis, and winter xerosis.[8] Low humidity leads to an increased rate of water loss from the SC and less water retention in the SC leading to dry, rough, tight skin.

Consumers will alleviate these symptoms by "moisturizing" their skin. "Moisturization" refers to any process that restores the ability of the SC to bind and retain moisture. Typically this is achieved by the use of moisturizing creams and lotions that deliver water to skin along with humectants and emollients, that allow the skin to hold on to the moisture.

31.2.2 Impact of Cleansing on Skin Hydration

Frequent cleansing is known to reduce SC hydration and cause dry, scaly skin.[9,10] It is paradoxical that cleansing, a process that involves saturating skin with water, can actually lead to a net decrease in equilibrium SC hydration. Figure 31.1 shows a schematic of the typical change in skin hydration state during cleansing. There is an initial transient increase in water content of SC during cleansing, but the excess water is quickly lost and water content returns to below baseline values in a few minutes (10 to 15 min).

Although there is a transient increase in skin water content during cleansing, cleansing products can reduce water content of skin:

- In a short term, cleansing reduces water retention ability of SC by removing water soluble NMFs and superficial lipids.[11]
- In a long term, frequent cleansing with harsh surfactants can cause damage to the SC barrier and increase water loss.[12,13]

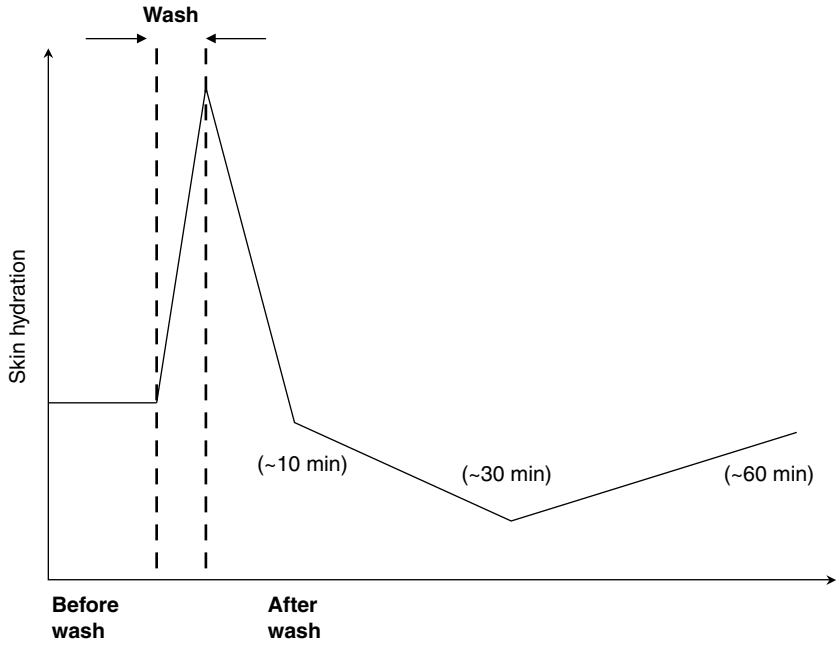

FIGURE 31.1 Schematic of the relative change in skin water content during typical cleansing routine.

The basic function of a cleanser is to promote health and hygiene of skin by removing excess dirt, sebum, and bacteria from skin and promoting exfoliation. However, as explained earlier, cleanser surfactants also interact with SC proteins and lipids, causing damage to the SC barrier, leading to a net loss in SC hydration.

Use of mild surfactant cleansers (as described in Section 31.3) helps mitigate this problem to a large extent. For example, mild cleansing bars, based on synthetic detergents (syndet) are known to be inherently mild and moisturizing to skin as compared to basic soap-based cleansers.[14] A complementary approach to enhance skin hydration after cleansing is to help skin retain some of the moisture it absorbs during cleansing. This can be achieved by depositing emollients, occlusives, and humectants on to the skin that slow down the rate of water loss after a shower and improve SC hydration.

31.2.3 Consumer Perception

In consumer parlance, "moisturization" is a highly desired skin state and expressed in a variety of ways such as soft, smooth, healthy, nourished skin. In the context of moisturizing creams and lotions, it refers largely to the alleviation of the dry skin symptoms and the efficacy is measured by the extent and duration of the relief.

Cleansers induce a perception of tightness, roughness, itch in a short term, resulting from a high rate of dehydration following a wash. Figure 31.2 depicts a typical onset of after-wash tightness on the face immediately after cleansing, as measured by consumer self-perception. Therefore, moisturization from a cleanser mainly connotes an absence of the dehydrating effects of cleansing. This translates to an absence of tightness, roughness, itch immediately after wash and a lack of drying and scaling in the long term. All of this can further translate to a reduced need to apply a moisturizer (especially after showering) in order to maintain a perceivably "moisturized" skin state.

Table 31.1 indicates differences in consumer expectations from a moisturizing lotion versus a moisturizing cleanser.[15] It is interesting to note that for the cleanser, the consumer desire for

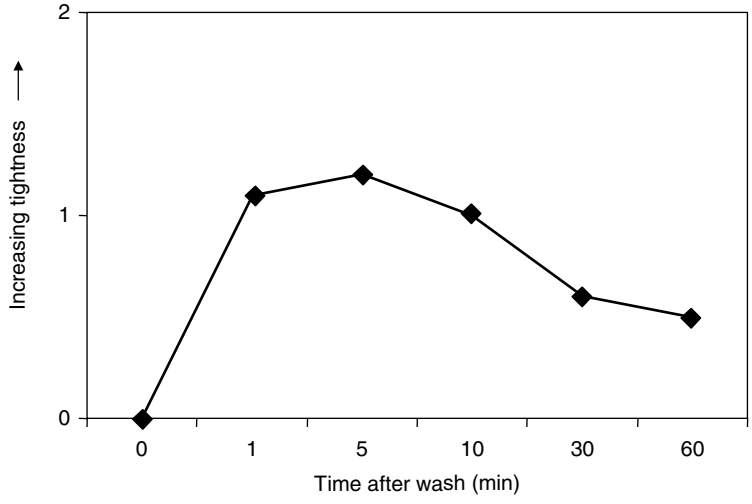

FIGURE 31.2 Profile of the consumer perception of tightness after wash.

TABLE 31.1
Top 5 Consumer Desired Qualities in Lotions and Cleansers

Lotion	Cleanser
Nongreasy feel	Nongreasy feel
Nonsticky feel	Rinses off well
Dry skin relief	Does not dry skin
Softens skin	Smoothes skin
Heals dry skin	Cleans thoroughly

"moisturization" (expressed as "does not dry out skin") is ranked ahead of its primary cleansing function.

As summarized in Table 31.2, consumer perception and manifestation of dryness through cleansing can be described in terms of their technical mechanism. In doing so, routes to ameliorate these negative attributes can be identified, which forms the basis for moisturizing cleanser technologies. This requires an understanding of the complex interaction of surfactants, water, and skin during the cleansing process.

31.3 EFFECT OF SURFACTANTS ON SC

During cleansing, SC is exposed to a relatively high concentration of surfactants (5 to 20%). At these concentrations surfactants have the ability to damage the SC proteins and lipids, and increase the leaching and removal of water-soluble aminoacids, often referred to as skin's natural moisturizing factors (NMFs). The extent of damage will depend upon the nature of the surfactant and the cleansing conditions such as water temperature and hardness.

While it would appear that there is a distinct difference in the mechanisms driving the immediate and longer term consumer perception of cleansing, for the most part it is a matter of degree, related to the increasing interaction of surfactants and skin. For example, superficial dryness seen as an

TABLE 31.2
Short-Term and Long-Term Effects of Cleansing

Symptoms	Technical mechanism	How to measure	Technical solution
Immediate effect of cleansing (short term)			
Tightness and itch	Loss of NMF Protein swelling Differential stress due to rapid dehydration Changes in lipid fluidity	Consumer perception Expert panel Naïve consumer panel Bioinstrumental elasticity	Milder surfactant to remove less NMF and reduce protein swelling Replenish NMF during wash Deposition of emollients and occlusives to moderate rapid water evaporation
Superficial visual dryness	Alteration of the optical properties of the surface cells Loss of surface lipids	Visual expert grading Consumer perception Photography	Milder surfactant to remove less surface lipid and extract less NMF from surface cells Replenish surface lipids Deposit emollients and occlusives
Cumulative effect of cleansing (long term)			
Visual dryness	Aberrant surface desquamation Debonding of cells Loss of flexibility leading to the formation of cracks	Visual expert grading Photography Microscopy	Milder surfactant to extract less NMF from skin and preserve SC lipids Deposition of emollients to hold moisture within skin and enhance surface appearance
Itch	Barrier breakdown leading to an inflammatory response due to diffusion of surfactant into epidermis Debonding of cells and inter-cellular mechanical movement	Consumer Perception Expert panel Naïve consumer panel	Milder surfactants
Erythema/irritation	Barrier breakdown leading to an inflammatory response due to diffusion of surfactant into epidermis Alkaline pH induced protein swelling increasing surfactant irritation potential	Visual expert grading Colorimetry Photography	Milder surfactants pH neutral formulations

alteration in optical properties will, over time, drive deeper and be evident as flaking and cracking. As Table 31.2 shows, the pervasive solution to delivering moisturization from cleansing starts with mild surfactancy. But mild surfactants alone simply reduce the drying effects of cleansing. To achieve active moisturization requires additional technology to counter surfactant effects and enhance skin quality. Therefore, to achieve the goal of a moisturizing cleanser requires both an understanding of how surfactants negatively interact with skin and how moisturizing cleanser technology can minimize that interaction and repair the damage, in both the short and long term.

31.3.1 Immediate (Short-Term) Effects of Surfactants

The SC has about 70% proteins, 15% lipids, and 15% water.[16,17] Most of the water in the SC is present within the corneocyte proteins and is associated with the keratin bundle as well as with the NMFs while the rest of the water is bound within the head-group region of the lipid layer. SC hydration increases markedly during cleansing and the excess water in the corneum evaporates off within 10 to 30 min after the shower. Three aspects govern how the SC hydration changes during and immediately after wash: (1) amount of water that SC absorbs during cleansing, (2) the rate of water evaporation immediately after drying, and (3) the equilibrium SC water content as determined by the humidity and temperature conditions immediately after a wash. All of these changes are influenced by the effects of the cleanser surfactant on skin proteins and lipids.

31.3.1.1 Effects on Proteins

Most of the water absorbed by the SC during cleansing is present within the corneocytes resulting in significant protein swelling. Surfactants increase the swelling further and the extent of surfactant induced swelling is dependent upon the nature of the surfactant. Increased swelling has been shown to be related to irritancy and is useful as a predictor of surfactant irritation potential.[18–20] Figure 31.3 provides a comparison of SC swelling in the presence of surfactant actives in a soap and a syndet bar. Results show that the extent of swelling in the presence of sodium laurate (soap) is significantly higher than that in the presence of sodium cocoyl isethionate (syndet). Other factors such as solution pH and temperature can further affect the swelling. For example, high pH solutions (pH 9+) even without the presence of surfactants have been shown to increase the SC swelling[21] suggesting further evidence for the benefit of pH neutral cleansing.

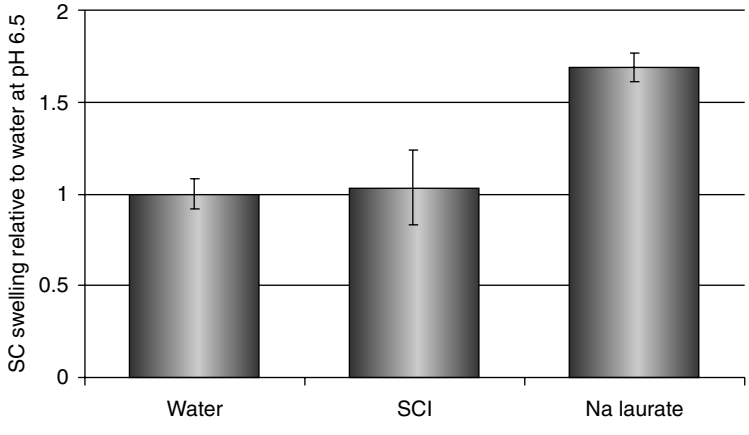

FIGURE 31.3 Swelling of porcine skin SC in sodium cocoyl isethionate (SCI, syndet) and Na laurate (soap) solutions (1%wt). Soap treated SC shows significantly higher swelling than that treated with syndet.

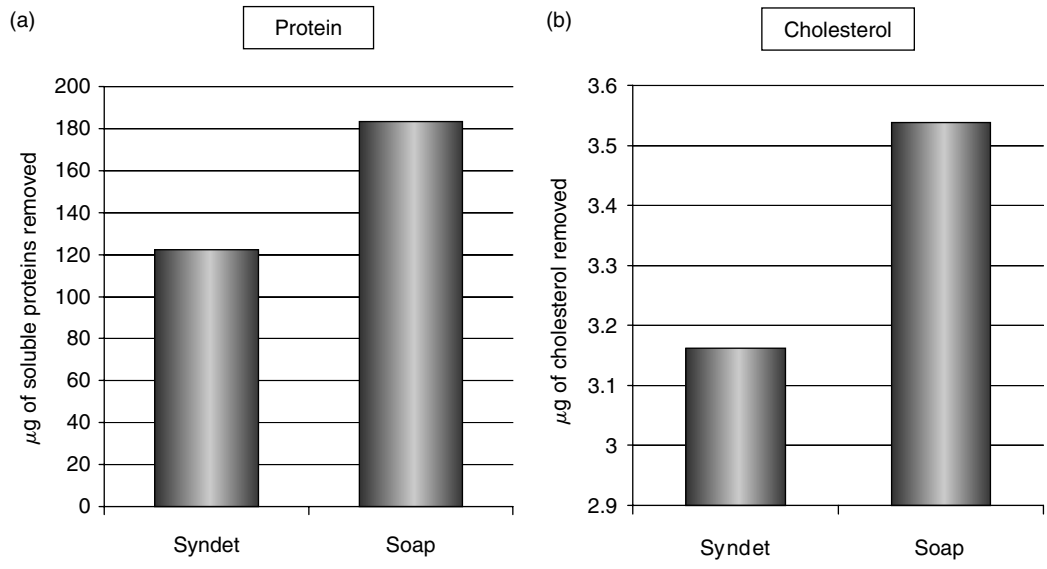

FIGURE 31.4 The amount of water soluble free amino acids (a) and cholesterol (b) removed from porcine skin after a single wash with a syndet bar versus a soap bar. Results show significantly higher removal from the soap washed site.

The excess water taken up by the SC during cleansing is flashed off within minutes after towel drying. How much water is retained in the skin after cleansing is defined by the equilibrium water binding capacity of the corneum, which can be affected by the interactions of the surfactant with the proteins and lipids. Harsh surfactants have been shown to remove NMFs more than by water alone.[22] This may be due to the damage to the corneocyte envelope caused by the harsh surfactant. Surfactant binding to proteins may also reduce the water holding capacity of the proteins. In either case, there is correlation between harshness of the surfactant and the increasing loss of water-soluble proteins. As can be seen in the results of a porcine skin assay (Figure 31.4), the higher loss of water soluble proteins after a single wash with soap versus syndet is consistent with the higher damage potential of the soap.[23]

The interaction of harsh surfactants on SC proteins results in an increase in skin surface water loss (SSWL). This is evident in the results shown in Figure 31.5. Water loss, measured using an evaporimeter immediately after a wash, show that harsher soap induces a higher rate of evaporation than milder syndet. The implications of this high rate of evaporation are examined further.

31.3.1.2 Effects on Lipids

Surfactants are designed to solubilize lipids and therefore, interactions of cleanser surfactants with skin lipids can be expected. Among the three classes of lipids in the corneum, specifically cholesterol, fatty acids, and ceramide, the latter because of its two-tailed and unusually long alkyl chain is not likely to get solubilized by the surfactant micelles. Cholesterol and lower chain length versions of the fatty acids (e.g., C18, C20 fatty acids as opposed to C24 and C28 fatty acids) may get solubilized in the micelle. Note, however, that even without any solubilization of SC lipids by surfactant micelles, simply by surfactant monomer intercalation into the bilayer, stress and damage can be imparted to the lipid bilayer. Insertion of anionic surfactants into the lipid bilayer can induce charge in the bilayer and alter membrane packing and permeability. Results with model liposomes indicate that surfactant insertion into the bilayer is usually the first step toward destabilizing the bilayer, which eventually results in the break-up of the bilayer resulting in mixed micelle formation/solubilization of the liposome.[24,25] In the case of SC, even partial or preferential removal of lipids such as cholesterol

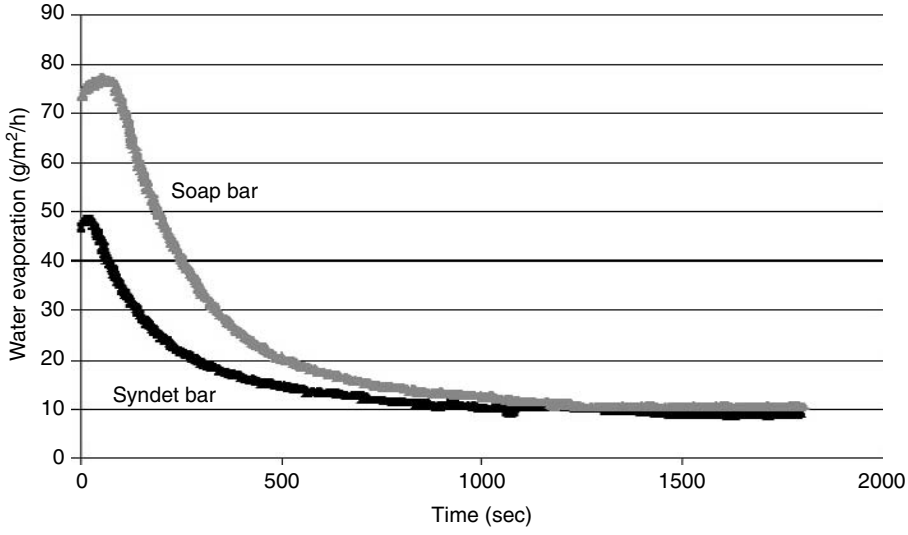

FIGURE 31.5 Water evaporation after a single wash with a soap versus a syndet bar showing initial hyper-hydration due to excessive swelling after wash with a soap bar and a reduced water swelling after a syndet bar wash. The slope of the curves also shows that the rate of evaporation after soap wash is higher, which is consistent with its higher perceived tightness.

can make the bilayer lipid unstable. Results for the removal of cholesterol by soap and the syndet bar are given in Figure 31.4 and show that soap removes more cholesterol than the syndet. While the exact reasons for this difference is not clear at present, it is likely that the high pH of soap allows ionization of the bilayer fatty acids allowing easier cholesterol extraction from the corneum. Yet another factor may be from the increased swelling of soap damaged corneum that allows deeper layers of the SC to be exposed to the cleansing surfactant.

31.3.1.3 Manifestation of the Short-Term Effects on Proteins, Lipids, and NMFs

The above combination of events, specifically, initial hyper-hydration because of excessive swelling and high rate of evaporation to an equilibrium level lower than normal, is hypothesized to be a major contributor to the perception of after-wash tightness. Hypothetical curves of changes to SC hydration immediately after a single wash are given in Figure 31.6 and these are consistent with the *in vivo* SSWL results given in Figure 31.5 as well as those reported in the literature.[19] As water evaporates at a rapid rate from the upper layers, a differential stress is created in the corneum and this is thought to be the origin of the after-wash-tightness. As the evaporation rate reduces to its normal level, the stress is relieved and the tightness disappears. These effects become even more acute under low humidity and low temperature conditions. Low humidity will certainly lower the equilibrium hydration levels in the corneum.

31.3.2 Cumulative (Longer Term) Effects of Repeated Exposure to Surfactants

Continued daily use of cleansers that cause short-term damage can lead to skin dryness, scaling, flaking, erythema, and itch.[26] While detailed molecular mechanisms involved in these effects are not fully understood, based on their current understanding, several possible mechanisms can be hypothesized.

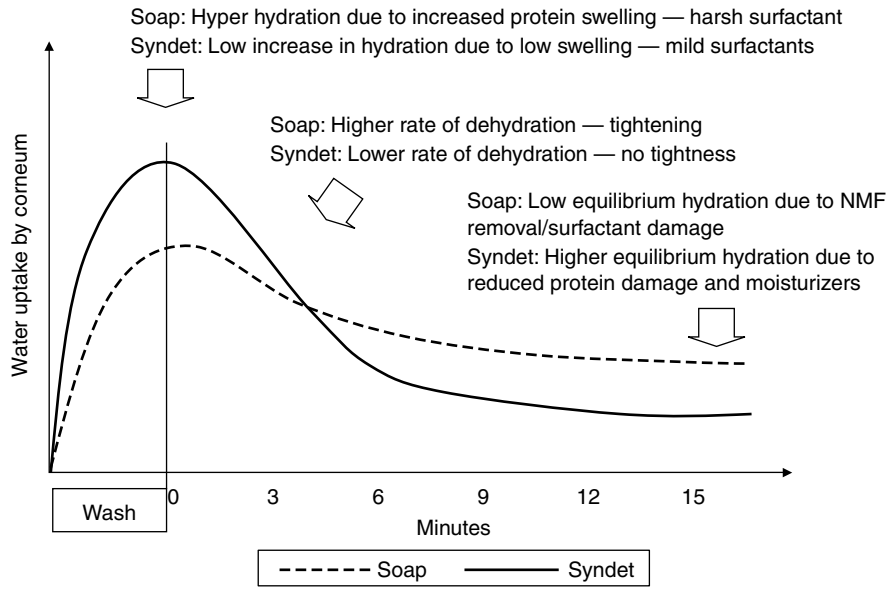

FIGURE 31.6 Hypothetical curves describing changes in SC water level after a wash with a harsh cleanser (soap) versus a mild cleanser (syndet).

31.3.2.1 Dryness, Scaling, and Flaking

Skin dryness is more than just a lack of water in the SC. It is actually a disruption to the biological processes underlying healthy normal skin, that affects both clinical and consumer perception of skin condition.

Consumer perception of dryness has both a visible and a tactile component. Visual effect of dryness is whitening of skin with the development of visible scaling. Dry skin is also physically tighter, more brittle, less soft than moisturized skin. Brittle SC can easily crack leading to chapping and significant barrier damage.

From a materials science perspective, the SC is a laminated composite membrane comprised of two distinct domains, specifically, proteins (corneocyte cells with embedded keratin bundles) and lipid bilayers. Corneocyte cells have covalently attached lipids, which makes them compatible with the surrounding lipid matrix. In addition, corneocytes in different layers are held together by protein "staples" called desmosomes. SC has been designed to exfoliate dead cells in an orderly fashion where the upper layers come off in a layer-by-layer fashion. For this to happen, the desmosomes have to be cleaved by proteolytic enzymes in the SC as the cells approach the outermost layers.

The SC is also designed to maintain certain degree of flexibility and elasticity under normal conditions so that when skin is flexed, it does not crack. Both proteins and lipids contribute to the overall pliability of the corneum. Water and NMFs maintain the flexibility of the corneocytes[27] whereas fluid lipids are thought to maintain the flexibility of the bilayer lipids.

As described earlier, water plays a key role in maintaining a normal SC. Lack of water in the corneum is a primary cause for disrupting several processes in skin. For example, lack of water can lead to visible signs of dryness (whitening), inadequate desquamation, scaling, chapping, and cracking.

Factors that cause excessive swelling followed by reduced water holding capacity of the corneum will allow the corneocytes to swell and shrink repeatedly and this cycling can create stresses leading to de-bonding of the corneocytes from the surrounding lipid matrix. As the situation continues, the effect may propagate down to deeper layers leading to cracking in the SC, a poor barrier, and excessive water loss.

Reduction in the water holding capacity of the corneum can also make the corneocyte proteins brittle and vulnerable to cracking. Keratins in the corneum have a glass transition temperature just below the body temperature[28] and this is sensitive to humidity levels. Glass transition temperature is the point below which the material is brittle. As the humidity/water content of the SC decreases, glass transition temperature increases to values above the body temperature thus making the corneocytes brittle at body temperature.

Presence of water in the SC is essential for the enzymes to cleave the desmosomes and in dry skin inadequate desmosomal degradation can occur leading to accumulation of dry cells. The result is severe dryness with excessive flakiness in the SC.

Similar to water plasticizing the proteins, fluid lipids in the bilayer lipids are implicated in the elasticity of the corneum. Removal of fluid lipids can make the corneum brittle. For example, solvent treatment of the corneum to remove fluid lipids has been shown to make the SC brittle.[29] It has been shown that soap treated corneum behaves somewhat similarly to the solvent treated corneum in the sense that both exhibit a brittle fracture under tension. In contrast, syndet bar treated corneum behaves more like water treated corneum exhibiting a more elastic and pliable structure.

Visible skin dryness has been found to correlate positively with surface hydration, but not necessarily with an increase in transepidermal water loss (TEWL).[30] This suggests that significant barrier breakdown is not a requirement for skin dryness. A continued increase in dryness to values above a certain level may, however, lead to scaling, cracking and chapping, barrier breakdown, and, eventually, to irritation.

31.3.2.2 Erythema and Itch

Erythema (development of redness) and itch are basically inflammatory responses of the skin when irritants penetrate into deeper layers of the SC. In the cleansing context this is usually because of a breakdown of the barrier for reasons indicated earlier leading to penetration of irritant materials. Note, however, that it may not be necessary for the surfactant to penetrate into dermal layers to elicit a response. Communication via production of cytokines in the SC can also elicit a response from the dermis.[26]

Factors that enhance the penetration of surfactants can be expected to increase surfactant-induced irritation. Thus, a swollen corneum will allow increased penetration of the surfactant into deeper layers. The ability of a surfactant to swell the corneum is an indication of its ability to enhance its own penetration into deeper layers and disrupt the cells in the living layer. This may be the scientific basis for the established correlation between the ability of surfactants to swell the corneum and its irritation potential. If the swelling occurs by other mechanisms such as increase in the protein negative charge because of high solution pH,[21] penetration of surfactants can also be expected to be enhanced under these conditions. Thus direct effect of pH 10 by itself on the corneum could contribute to increased surfactant irritation. Changes in lipid layers at pH 10 may also have an impact on irritation in that their increased rigidity may make them more vulnerable to cracking and debonding from the corneocytes and thereby permitting penetration of irritants.

Usually TEWL increases markedly under conditions that result in erythema indicating a barrier breakdown. It is not clear if a breakdown of the barrier itself or the subsequent penetration of irritants into deeper layers is responsible for the erythema. The latter appears to be a more reasonable mechanism.

31.4 MILD AND MOISTURIZING CLEANSER TECHNOLOGIES

It is clear that harsh surfactants have the potential to cause immediate alteration to SC proteins and lipids, and progressively increasing degrees of damage over time that can eventually result in a barrier breakdown. The first step toward mild cleansing is to minimize the damage potential of surfactants

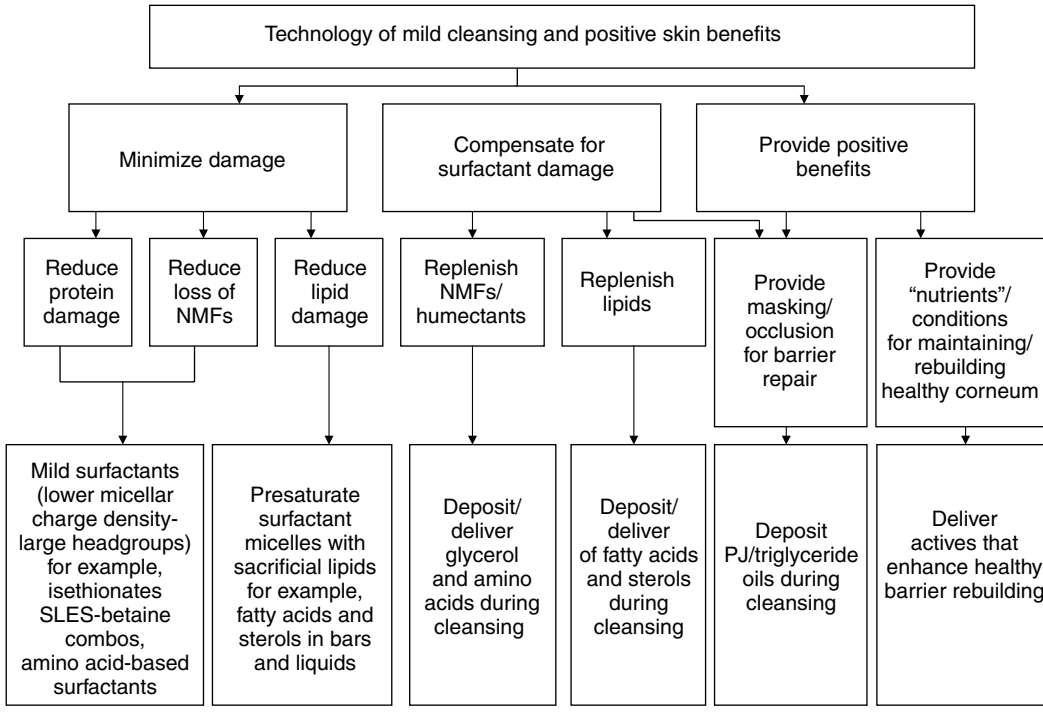

FIGURE 31.7 Currently practiced technology routes to provide mild cleansing with positive moisturization.

to proteins and lipids. The next step is to compensate for the damage and provide positive benefits by incorporating skin benefit agents into the cleanser. Current technological approaches to enhancing the mildness of cleansing systems are depicted in Figure 31.7.

31.4.1 Minimizing Surfactant Protein Damage

As discussed earlier, surfactants that interact strongly with SC proteins leading to their swelling and denaturation have a higher potential to cause erythema, and itching.[18,26] The tendency of surfactants to interact with model proteins has also been correlated with their harshness toward human skin. Thus, higher the tendency of a surfactant to swell SC[18,30] or model proteins such as collagen[31] and keratin,[32] or denature a globular protein such as bovine serum albumin[33] or dissolve a water-insoluble hydrophobic protein such as zein,[34,35] higher is its tendency to irritate human skin. Results of zein solubilization by a number of surfactants is given in Figure 31.8. As can be seen, the tendency of surfactants to interact with proteins follow the order: anionic > amphoteric > nonionic and these are consistent with published results of protein damaging tendencies of various classes of surfactants.

While these empirical correlations are useful as guidelines for formulation work, quantitative correlations between surfactant properties and their protein denaturation tendencies are most useful as a predictive ruler. Based on the hypothesis that protein denaturation is essentially due to massive cooperative binding of surfactants on the protein backbone and the resultant increase in the charge of the protein, surfactant micellar charge was correlated with the zein dissolution tendencies of a variety of surfactants. Results reproduced in Figure 31.9 show that protein denaturation scales with the charge density of surfactant micelles.[36] Results for anionic, zwitterionic, nonionic, and even cationic (absolute charge density without the sign) surfactants are included in the relations given in Figure 31.9. Also included are results for mixtures of surfactants. The strength of the correlation clearly shows that micellar charge can be used as a useful predictor of irritation tendencies of surfactants. This insight allows formulators to develop novel strategies to predict and increase mildness of cleanser

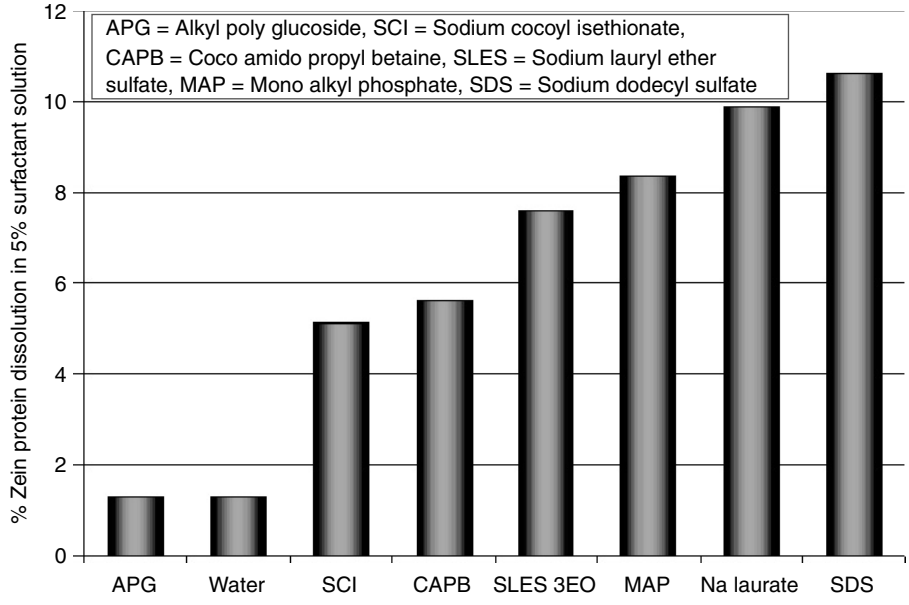

FIGURE 31.8 Protein damage potential of a number of surfactants determined using the zein dissolution test. Higher the zein dissolution, higher is the damage potential of the surfactant.

bases. In general, micelle charge density can be lowered by using surfactants of larger head groups, zwitterionic or nonionic head groups, and synergistic combination of surfactants that allow strong attractive interactions among head groups leading to a reduction in the overall charge density of the micelle.

Blankschtein et al. have concluded that micelle size is a major factor in surfactant induced irritation.[37] As the micelle size increases, penetration of the surfactant into deeper layers decreases and therefore increasing the micelle size is an approach to enhancing mildness. In principle, factors that reduce the micelle charge will increase the micelle size and therefore have the potential to reduce swelling and penetration under cleansing conditions. Note, however, that the inherent tendency of the molecule to cause an irritation response may be related to the charge density of the molecule rather than the micelle size.

Results given in Figure 31.8 shows the Syndet Bar active, sodium cocoyl isethionate, to have significantly less interaction with proteins than soap. This can be attributed to its larger head group area and lower micellar charge density than sodium soaps. Similarly, commonly used surfactant system for liquid cleansers, a combination of sodium lauryl ether sulfate (SLES) and cocoamido propyl betaine (CAPB) is significantly milder than soap as evidenced in Figure 31.9. Again the combination of SLES and CAPB have lower micelle charge density than SLES micelle alone and this can indeed explain its lower irritation potential than that for SLES alone. Synergistic interaction between the anionic and zwitterionic head groups should make this combination mild, especially in the lower pH range where the zwitterionic surfactant may possess a cationic charge because of protonation of the carboxylate group. While syndets are clearly seen as mild (particularly in comparison to soap), Figure 31.10 shows that there is still room for further reducing protein damage from surfactants in both cleansing bars and liquid formulations.

31.4.2 Minimizing Surfactant Lipid Damage

Long-term surfactant damage to the SC lipid extends from the short-term effects resulting in cumulative loss of barrier function and lipid fluidity leading to profound dryness. The results of an assessment

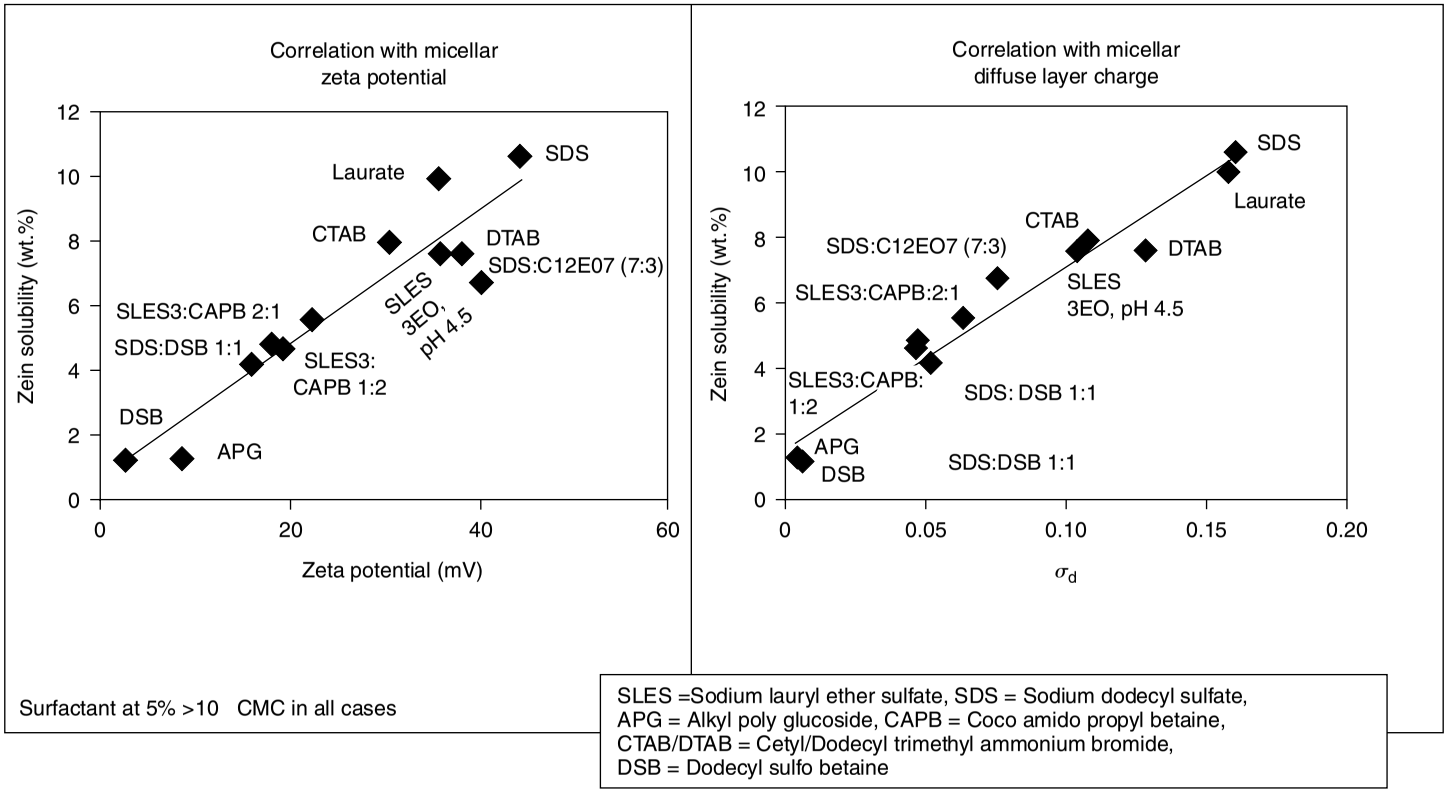

FIGURE 31.9 Correlation of surfactant micellar zeta potential and micelle charge density with zein dissolution showing that protein denaturation potential scales linearly with the micellar charge/potential.

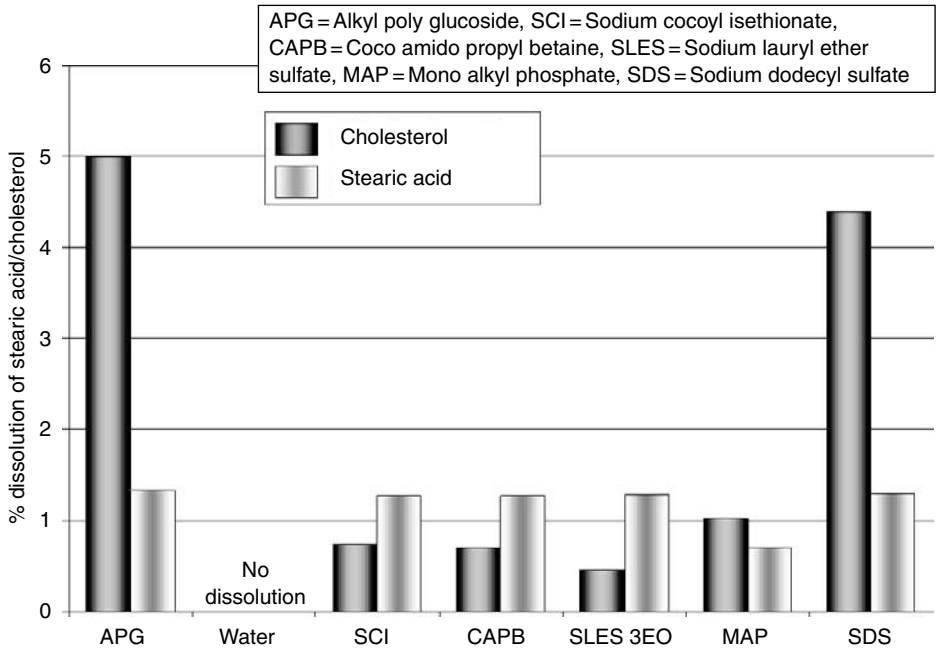

FIGURE 31.10 Lipid damage potential of a number of surfactants determined by the ability of surfactant micelles to solubilize cholesterol and stearic acid.

of lipid damage potential of surfactants as measured by the solubility of stearic acid and cholesterol in 5% surfactant solutions, are given in Figure 31.10. It appears that all the surfactants have some tendency to solubilize cholesterol and fatty acids. Interestingly, alkyl poly glucosides (APG) shows high potential for solubilizing cholesterol in contrast to its relatively low protein swelling tendency. This result shows that mildness toward proteins does not necessarily imply mildness toward lipids, and achieving mildness toward both proteins and lipids simultaneously may require delicate balancing of surfactant properties.

A relatively less understood mechanism, namely the presaturation of surfactant micelles with lipid mimics so that the micelle will have reduced tendency to delipidate the corneum during washing, is an approach to minimize surfactant–lipid interactions. Figure 31.11 shows the clinical benefit of adding high levels of fatty acids to a syndet bar formulation. The hypothesis is that the added fatty acids actually minimize the damage to both proteins and lipids by incorporating into the surfactant micelles, thus making the micelles milder toward both proteins and lipids.[38] Presaturation of the micelles with fatty acids will reduce the tendency of the micelles to solubilize SC lipids or intercalate into the SC bilayer. Also, presence of fatty acids can lower the charge density of the surfactant micelles, thus enhancing their mildness toward proteins.[38]

31.4.3 Compensating for Damage: Enhancing Moisturization

From a technology point of view, the main approach to minimize visible signs of skin dryness and increase skin hydration has been to deposit lipids, emollient oils, and occlusives (such as used in a lotion) under cleansing conditions. The challenges of incorporating high levels of emollients in a stable cleansing formulation and depositing the emollients on skin during the wash process have been largely surmounted by the use of specially structured surfactant formulations with cationic polymers to aid deposition and retention of oils and occlusives on to skin. Typical emollients and

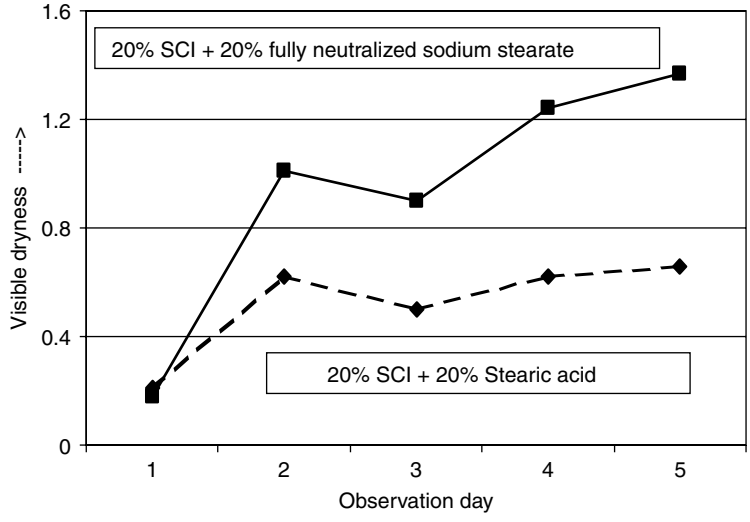

FIGURE 31.11 Change in dryness in a clinical study showing how fatty acid structurants improve the mildness of SCI.

occlusives used in cleansing liquid formulations are vegetable oils (sunflower seed, soyabean) and petroleum jelly. It is a bigger challenge to deliver water-soluble moisturizers such as glycerin and other humectants to skin during washing, and hence hydrophobic emollients are more commonly used in cleansers.

It has been shown that high emollient containing body washes do deposit a significant amount of lipid and emollient material to the skin. A commercial product containing sunflower seed oil triglycerides is found to deposit 10 to 15 μg/cm^2. Figure 31.12 and Figure 31.14 confirm the clinical advantage of such deposition on skin during cleansing. Note that the efficiency of deposition (amount of material transferred to skin versus amount contained in the product) from current technologies is still quite low and is an opportunity for improving performance of these moisturizing body washes. Another opportunity area is to deliver effective water-soluble moisturizers such as glycerin or lactates from a cleanser. These humectant materials are known to increase water holding capacity of the skin when delivered from leave-on products. However, there remains a technical challenge for effectively delivering water-soluble materials from rinse-off systems.

31.5 MEASURING MOISTURIZATION FROM CLEANSERS

31.5.1 Evaluating Moisturization of Skin

Generally when we think about measuring skin moisture we think of lotions where there is both an immediate and sustained positive increase in the hydration state after application. Classic methodologies for evaluating moisturizer efficacy start with dry skin and monitor the improvement benefit of continued product application over days or weeks.[39–41] Even in short-term trials, the lotion effect on skin is typically measured as increase in moisture and the improvement in moisture-related benefits such as smoothness and elasticity.[42]

In contrast, the basis of cleanser testing has historically been about evaluating dryness and irritation potential. Since 1979 when Frosh and Kligman published a seminal paper on the soap-chamber patch test, cleanser moisturization and mildness have been defined as reduced dryness and damage in comparison to soap.[43] As Wolf points out, for decades the desired qualities for soap have

TABLE 31.3
Commonly Used Methods for Quantifying Skin Dryness

Sensory	Consumer perception
	Dry feeling
	Tightness
	Itch
	Tactile roughness
Visible appearance	Expert clinical grading
	Dryness, seen as flaking
	Irritation, seen as erythema
	Instrumental surface measures
	Roughness
	Desquamation
Hydration state	Indirect
	Electrical conductance/capacitance
	Direct
	NIR and Raman spectroscopy
Biophysical/biomechanical	Properties affected by hydration state
	Skin surface water loss
	Elasticity
	Cell proliferation/SC turnover
	Enzymatic activity

been about mildness, gentleness, less irritation, and less drying rather than their primary purpose of cleansing.[44]

As cleansers moved to syndet bars to liquid detergent systems, they became more and more innocuous in the short term and required exaggerated exposure to elicit measurable dryness and damage response. However, as cleansers have begun to move toward active moisturization, methods traditionally associated with lotion-testing can be applied.

Moisturization in skin can be measured in a variety of ways, some of the more common of which are summarized in Table 31.3. It can be measured directly as an increase in hydration in skin or improvement in clinical and sensory symptoms resulting from the improved hydration state of skin. At the most basic level, consumer perceptions can provide a measure of skin feel and appearance but more often are used to quantify the sensory aspects that cannot be measured instrumentally.[45] Expert clinical grading provides a more refined quantitative measure of appearance.[46] The human eye is still the most powerful tool for discriminating subtle changes in appearance.[47] However, bio-instrumentation is required to measure insensible parameters such as the hydration level in skin.[48–50] While methods based on electrical properties of skin are widely used to indirectly measure water content, Near-Infra Red and Raman Spectroscopic techniques are more closely reflective of the actual hydration state.[51,52]

31.5.2 Measuring the Effect of Cleansers on Skin

31.5.2.1 Short-Term Effects

We have seen that in the short term, the changes in skin due to cleansing primarily manifest as changes in sensory perception. Consumer perception methods are the primary means of assessing the transient onset of tightness and itch. Naïve panels can provide comparative data among several cleansers tested but can not provide consistent quantitative measure of performance. Expert panels

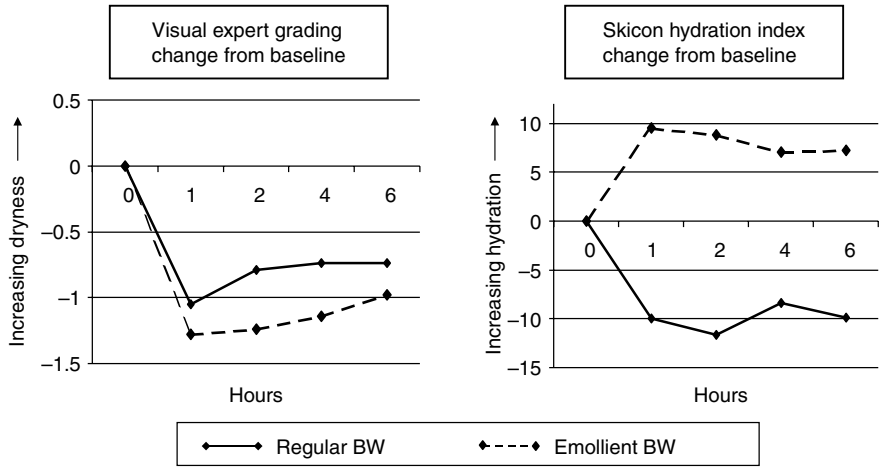

FIGURE 31.12 Comparison of a regular and an emollient liquid BW over a 6 h period following a single wash event.

can provide the quantitative measure, but because sensory attributes are affected by local temperature and humidity, results must still be viewed relative to a known control.

Changes in hydration state can be traced in the short term. Electrical conductance and capacitance of skin can be used to describe the hyperhydration and dehydration cycle of washing. However, it is the equilibrium end-point that defines the final skin state. As cleansers become less drying, we are effectively attempting to measure smaller and smaller changes to final skin state. Yet, as cleansers begin to deliver positive moisturization, these same methods become relevant to describe the benefit. This is particularly important for differentiating actively moisturizing cleanser from ultra-mild cleansers. This is illustrated in Figure 31.12, where visible appearance of dryness and the equilibrium hydration state of skin for 6 h after a single wash are shown. Both regular liquid body wash (BW) and emollient BW show improvement in visible dryness by removal of superficial flakes. However, this is not entirely reflective of the underlying hydration state. Instrumental data, in fact, shows a net loss of moisture for the regular BW as compared to the positive hydration for the moisturizing cleanser.

31.5.2.2 Long-Term Effects

While perceptible and imperceptible changes in hydration can be seen in the short term, real clinical changes to the equilibrium skin state take longer to occur. Small changes in dryness and barrier integrity after washing accumulate over time leading to a breakdown of many physical and biological processes. To model these quickly, a number of exaggerated exposure methods have been developed. Table 31.4 summarizes four widely used methods.[53–58] The first three begin with normal skin and look for the onset of dryness or irritation. Arm and Leg washing use an ordinary, though controlled, wash procedure but increase the frequency of wash events to several per day, in order to more quickly initiate a response. The FCAT procedure increases the response further. It maintains an increased frequency of washing and further exaggerates exposure by leaving lather solution in contact with skin for 90 sec before rinsing. Flex wash increases sensitivity to irritation by using mechanical action to drive product into the antecubital fascia, but in doing so loses sensitivity to dryness. The fourth method, the LCAT, actually begins with mild dry skin to increase response sensitivity and to be capable of measuring active improvement in condition.

Within all of these procedures, the actual measurements continue to focus on mildness and moisturization as defined by the same three aspects used in short-term tests, sensory, visible appearance, and hydration state, with the addition of a measure of barrier integrity using TEWL.

TABLE 31.4
A Comparison of Commonly Used Exaggerated Wash Procedures

Armwash/legwash	Controlled wash of site using gloved hand Bilateral application: two products, paired comparison One to four times washing per day Development of dryness and erythema
FCAT	Controlled wash of sites 15 + 90 sec exposure to lather Two to six sites (up to 3 per arm) Four times washing per day Enhanced development of dryness and erythema
Flexwash	Controlled wash of sites with sponge or pad to antecubital fascia Bilateral application: two products, paired comparison Four times washing per day Enhanced development of irritation/erythema (but loss dryness information)
LCAT	Induce mild dry skin prior to baseline Controlled wash of sites 15 + 90 sec exposure to lather Two to six sites (up to 3 per leg) Four times washing per day Enhanced sensitivity to dryness effects or moisturization improvement benefits

With regular cleansers, a procedure like FCAT (Forearm Controlled Application Test) provides good sensitivity to varying discriminate products based on their drying potential. Looking at soap versus syndet bar, we can compare three clear trends in Figure 31.13: an increase in the visible appearance of dry skin over time, a concomitant decrease in the equilibrium hydration state of the skin, and an increase in the disruption to the moisture barrier evidenced as an increase in TEWL. In all the three measures, the syndet is seen as milder and less drying.

When evaluating moisturizing cleansers, we see a more fundamental change in these trends. The results of an FCAT on an emollient body wash as compared to regular body wash are shown in Figure 31.14. Two distinct features of active moisturization are evident. First, the emollient body wash is showing no negative effect on normal skin appearance. Despite repeated use and exaggerated exposure to the product, the emollient BW provided no significant change in visible appearance of dryness over time as compared to regular BW, which does show increasing dryness. Second, the emollient BW provided a significant increase in skin hydration after five days of repeated use. Taking the moisturization benefit even further, the effect of emollient BW to actually *improve* visible dryness is evident in the results of an LCAT (Leg Controlled Application Test) study (Figure 31.15). In this study design where we begin with mild dry skin, the emollient cleanser can be seen to significantly reduce the visible appearance of dry skin over time. These long-term clinicals demonstrate that positive moisturization seen in the short term (Figure 31.12) is maintained to establish a significantly improved equilibrium hydration state after five days. Thus active moisturization from cleansing is more than a transient effect. This work shows it to provide a sustained improvement in skin condition with repeated use.

31.5.2.3 Advanced Moisturization Measures

The ability of cleansers to positively affect the moisturization of skin can further be measured by evaluating biomechanical properties that are intrinsically linked to hydration state. For example, changes in skin softness are directly related to hydration state, and Figure 31.16 shows how biomechanical

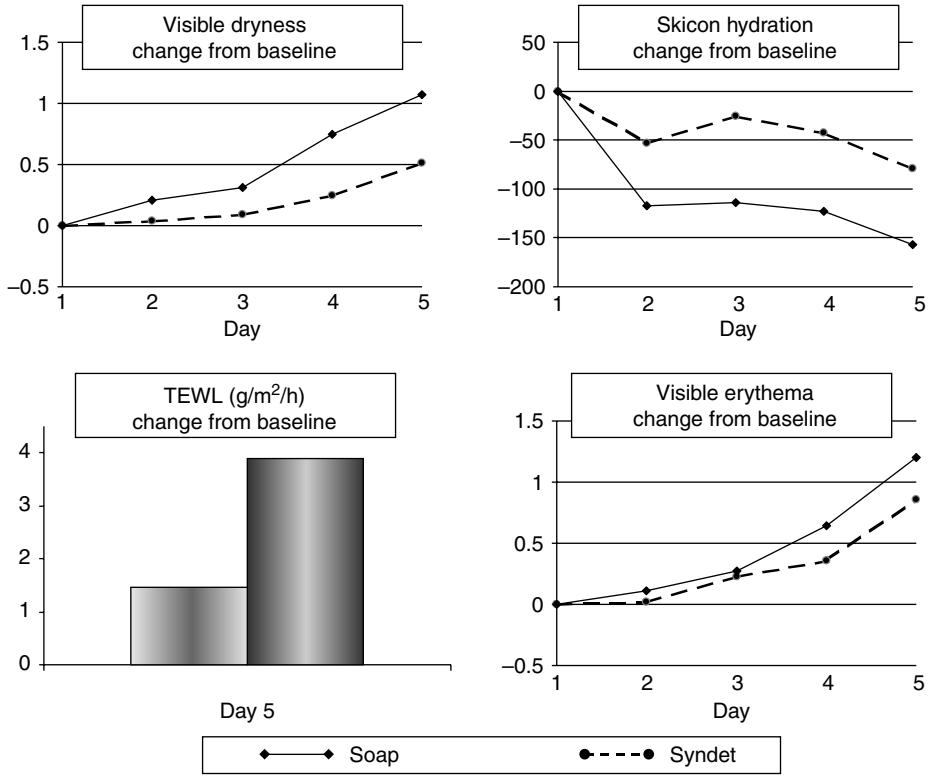

FIGURE 31.13 FCAT study comparing soap and syndet bars shows soap induces higher visible dryness, lower hydration state, greater loss of barrier function, and increased erythema.

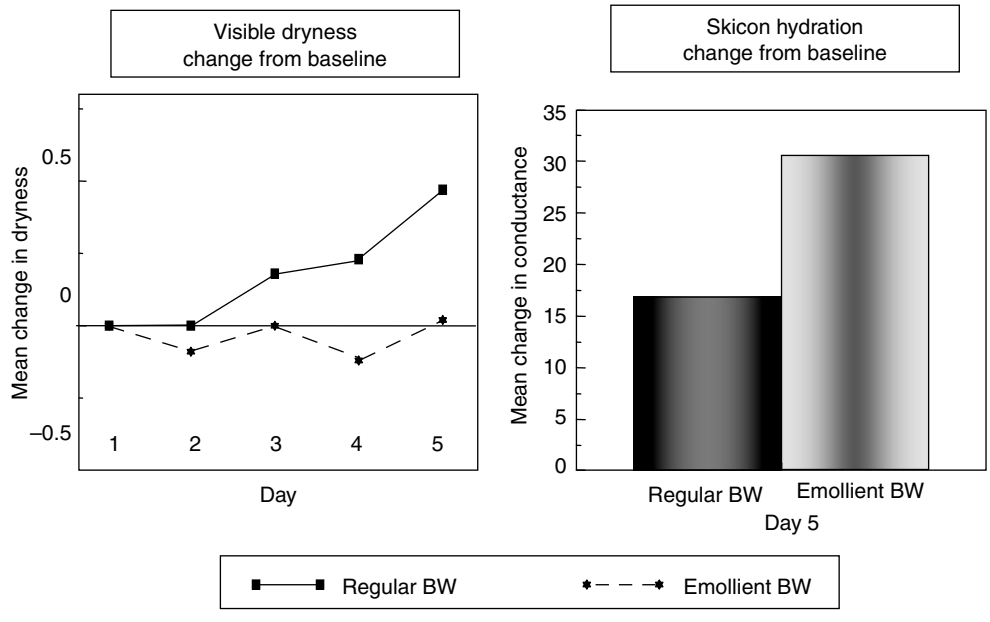

FIGURE 31.14 FCAT study of regular and emollient BW shows that EBW induced no visible dryness and significantly improved the hydration state.

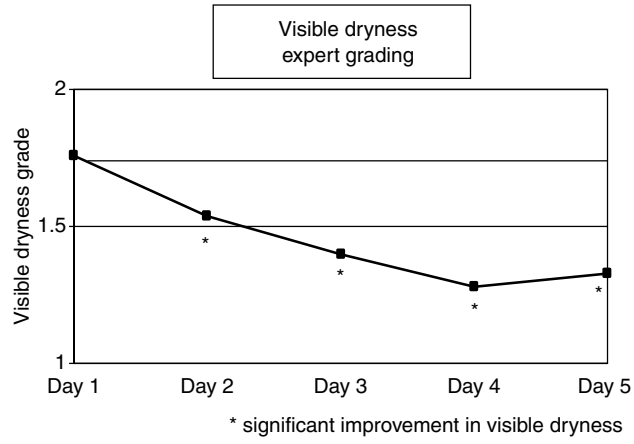

FIGURE 31.15 The LCAT study of emollient BW effect on visible dryness shows a significant improvement in appearance of dryness.

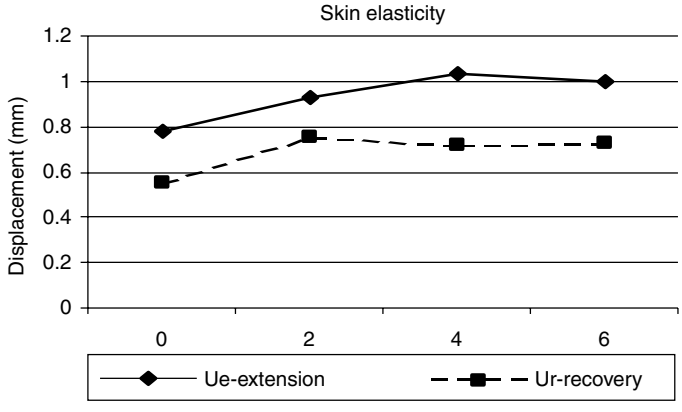

FIGURE 31.16 Effect of emollient BW on elastic properties of skin, as measured using Dermal Torque Meter, over a 6-h period following a single wash event. Ue and Ur refer to the immediate elastic extension and recovery, respectively.

measures can describe changes in elastic properties due to active moisturization from cleansers in the short term.

Recognizing the direct relationship between skin hydration state and regulation of the biological processes of skin, and understanding the significant effect cleansing has on this, it is clear that effective measurement of skin hydration is vital. Electrical conductance and capacitance measurements are indirect measures prone to artifacts. To different degrees, standard instrumentation are influenced by the insulating effect of surface dryness, conductivity of surface films, and the physical contact of probe and skin. More recent methods for the rapid, direct measurement of skin water content are showing excellent correlation with visible dryness.

Near Infra Red (NIR) Spectroscopy provides a noncontact, noninvasive direct measure of SC hydration.[51] It uses IR light, which is absorbed by tissue and the specific wavelengths reflected by the water molecules in that tissue. This technique provides an image of actual water present in skin, which is quantified using image analysis. NIR information can be used to visually show changes in equilibrium water content of SC after washing, which is particularly useful for understanding active moisturization from cleansers. Images shown in Figure 31.17 visibly depict the increase in skin hydration one hour after washing with an emollient BW. These images were taken from a single use

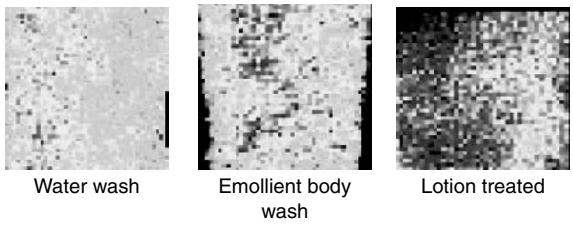

FIGURE 31.17 Near Infrared Spectroscopy images depict the change in skin hydration state one hour after water wash versus a wash with an emollient BW. The increase in dark areas indicate greater hydration after washing with the emollient BW. The change in hydration state for a lotion treated site is included for reference.

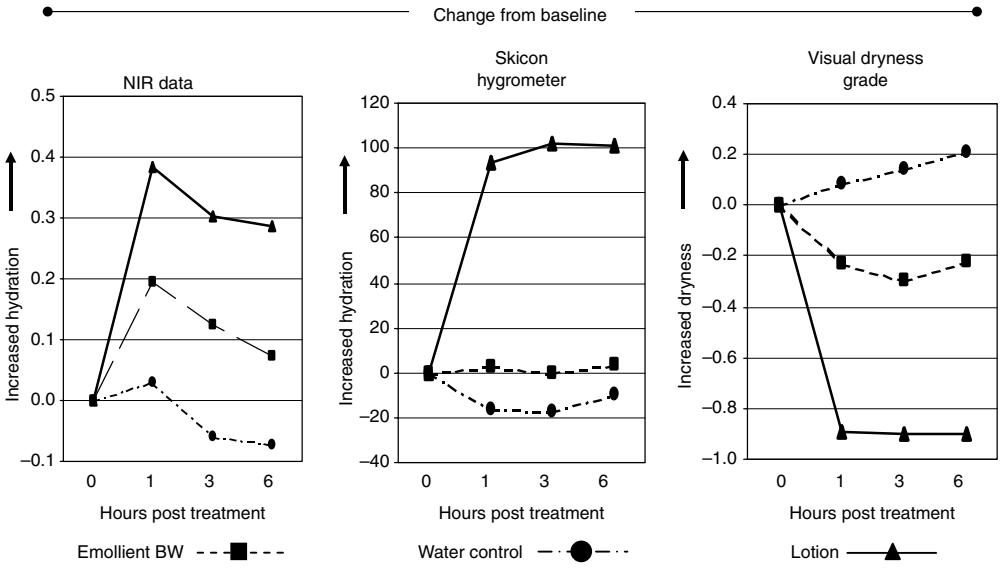

FIGURE 31.18 Near Infrared Spectroscopy analysis provides clear delineation of the hydration profiles of emollient BW relative to water washing or lotion use. NIR shows good correlation with the visible appearance and clearer product differentiation compared to skicon.

trial comparing an emollient BW to water wash control. The quantified results of image analysis are presented in Figure 31.18 and show a clear delineation of the hydration profiles among treatments and good correlation with the visible appearance of dryness. Another emerging technique for direct *in vivo* measurement of skin hydration is Confocal Raman Spectroscopy.[52]

Advanced microscopic techniques such as optical coherence tomography and *in vivo* confocal microscopy have been applied to sensitively evaluate hydration induced changes in the SC. For example, using confocal microscopy, Leeson et al. showed that the morphology of corneocytes at the surface of the skin changes from an irregular, rough arrangement in dry skin to a highly ordered, smooth pattern in moisturized skin.[59]

31.6 CONCLUSION

The cleansing market place has evolved a long way from providing cleansing and hygiene benefit to current technologies that are designed to provide advanced moisturization benefits in the shower. For millennia, cleansing has been synonymous with soap, which is associated with skin dryness. Our understanding is that skin dryness is much more than the superficial removal of moisture from the

SC. Surfactant interaction with lipids and proteins leads to a fundamental breakdown of biological processes that underpin skin health. Mild surfactants have lead to cleansers with significantly reduced drying and damaging potential but only within the last decade have truly moisturizing cleansers begun to emerge.

The technology to clean skin and improve hydration state builds on an understanding of mild surfactancy and adds to it an understanding of skin and moisturization. New understanding of the interaction of surfactants, emollients, and humectants with skin can only lead to cleansers with even broader benefit capabilities. As such, moisturizing cleansers signal a significant reinvention of history's most basic cosmetic product.

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Part III

Skin Measurements

32 Tribological Studies on Skin: Measurement of the Coefficient of Friction

Raja K. Sivamani, Gabriel, W.u., Norm V. Gitis, and Howard I. Maibach

CONTENTS

32.1 INTRODUCTION

Studying the friction of skin supplements other mechanical tests. Friction studies can be conducted with noninvasive methods and give a measure of the skin's health — skin hydration, for example: Naylor[1] showed that moistened skin has an elevated friction response and El-Shimi[2] demonstrated that drier skin has a lowered friction response. Friction provides a quantitative measurement to assess skin.

The friction parameter generally measured is the coefficient of friction. To measure the friction coefficient, a surface is brought into contact with another and moved relative to it. When the two surfaces are brought into contact, the perpendicular force is defined as the normal force (N). The friction force (F) is that force, which opposes relative movement between the two surfaces. From Amonton's law, the coefficient of friction (μ) is defined as the ratio of the friction force to the normal force:

$$\mu = F/N.$$

Modified with permission from *Skin Research and Technology*.

The friction coefficient can be measured in two ways: the static friction coefficient (μ_s) and the dynamic or kinetic friction coefficient (μ_k). The static friction coefficient is defined as the ratio of the force required to *initiate* relative movement and the normal force between the surfaces; the dynamic or kinetic friction coefficient is defined as the ratio of the friction force to the normal force when the two surfaces are moving relative to each other. For simplicity, much of the research has focused on the dynamic friction coefficients wherein the two surfaces move at a relative *constant velocity*. Most of the friction studies on skin have dealt with the dynamic friction coefficient and the subscript k is usually dropped. This overview references the dynamic coefficient of friction unless otherwise noted.

According to Amonton's Law, the dynamic friction coefficient remains unchanged regardless of the probe velocity or applied normal load in making the measurement. Amonton's laws hold true in the case of solids with limited elastic properties. Although Naylor[1] concluded Amonton's Law to be true, later studies by El-Shimi[2] and Comaish and Bottoms,[3] and Koudine et al.[4] have found that skin deviates from Amonton's Law since the friction coefficient increased when the normal load was decreased. El-Shimi[2] and Comaish and Bottoms[3] reasoned that the rise in friction coefficient resulted from the viscoelastic nature of the skin allowing for a nonlinear deformation of the skin with increasing load.

32.1.1 Experimental Designs

Various experimental designs have been devised to measure the friction on skin. They focus on measuring friction by pressing a probe onto the skin with a known normal force, and then detecting the skin's frictional resistance to movement of the probe. The designs fall into two categories:

1. A probe moved across the skin in a linear fashion.
2. A rotating probe in contact with the skin surface.

In the linear designs, the probe movement is accomplished in several ways. Comaish and Bottoms[3] utilized one of the simplest linear designs: they moved the probe across the skin by attaching it to a pan of weights by means of a pulley. Weights are placed in the pan such that the probe slides over the skin at a constant velocity. This allows for the calculation of the dynamic friction coefficient by dividing the total weight in the pan by the normal load on the probe.

More sophisticated linear designs, followed the simple design used by Comaish and Bottoms,[3] but provide motorized unidirectional movement of the probe or the use of a reciprocating motor to move the probe back and forth. In both designs the motorization affords greater control in maintaining the velocity of the probe. Strain gauges measure the friction force as the probe moves along the skin surface. A Biomedical Tribometer, a friction measurement device where the normal load and the probe speed are computer controlled, can also be used.

The second design category measures friction with a rotating wheel pressed onto the surface of the skin with a known normal force. Highley et al.[5] measured the frictional resistance by determining the angular recoil of the instrument as the wheel contacted the skin. They measured this angular recoil by recording the proportion of light that hit a dual element photocell. An electrical signal was then generated in proportion to the frictional resistance. Comaish et al.[6] developed a portable, hand-held device (Newcastle Friction Meter) that relied on a torsion spring to measure the skin's frictional resistance.

An important part of designing a friction measurement apparatus is choosing the probe size, shape, and material. Because friction is an interaction between two surfaces, the probe geometry and material will affect the values calculated for the friction coefficient of the other surface. Also, results will be more accurate when the probe's normal force is maintained at a constant value or continuously monitored; previous methods used to maintain the normal force include spring mechanisms or static weights to weigh down the probe. These parameters are revisited critically later in this article.

Much effort has been spent in understanding how skin friction changes with differing biological conditions and upon the application of various products to the skin surface. These studies have been of interest to various industries that manufacture products meant as skin topical agents because friction measurements can provide clues regarding the effectiveness of their products.

32.1.2 Hydration

Hydration is a complex phenomena influenced by intrinsic (i.e., age, anatomical site) and extrinsic (i.e., ambient humidity, chemical exposure) factors. These factors can affect the mechanical properties of the skin and research has been performed to correlate hydration levels with the skin's friction coefficient.[24] Hydration studies have investigated how increases and decreases in skin hydration correlated with the friction coefficient. In past studies researchers generally induced increases in skin hydration through water exposure. However, decreases in skin hydration were not experimentally induced and dehydration studies were performed between subjects with "normal" skin and subjects that had clinically "dry" skin.[2,12]

32.1.3 Lubricants/Emollients/Moisturizers

Much of the reviewed research has been devoted to ascertaining how the application of certain ingredients influences the skin surface, of interest to the cosmetic/moisturizer and lubricant industries. The studies focused on the effects of talcum powder,[2,3] oils,[2,3,5,14] and skin creams/moisturizers.[7,14,17,24] Hills et al.[15] analyzed how changes in the friction coefficient, following emollient application, differed with temperature.

32.1.4 Probes

Probe geometry and material influence the measured value of the friction coefficient because friction is a probe-skin interaction phenomenon. Few studies have examined probe effects: El-Shimi[2] studied probe roughness and Comaish and Bottoms[3] probe roughness and material.

32.1.5 Normal Load

Friction measurements can offer quantitative insight into changes on the skin surface and the UMT offers technical advances over existing friction measurements. The control of the probe speed and the real-time monitoring of the normal load allow for real-time calculation of the friction coefficient. As seen in Figure 32.1, the control of the load is important because the friction coefficient does not adhere to Amonton's Law. Wolfram[18] theoretically deduced that the friction coefficient would relate to the normal load as follows:

$$\mu \propto N^{-1/3}$$

(where N is the applied normal load to the skin). Sivamani et al.[17] found that the friction coefficient related to the normal load as follows:

$$\mu \propto N^{-0.32}$$

and Koudine et al.[4] found the dependence on the applied normal load to be:

$$\mu \propto N^{-0.28}$$

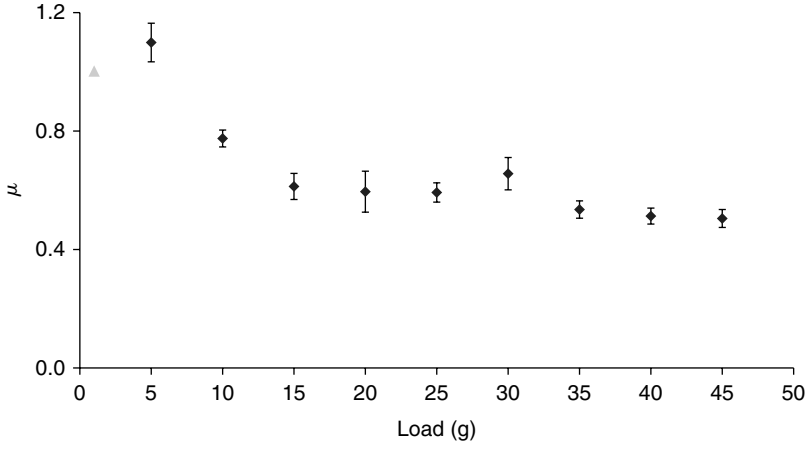

FIGURE 32.1 Friction coefficient versus normal load (50 g Sensor). The friction coefficient increased as the normal load was decreased suggesting that the skin does not follow Amonton's Law. The probe was moved at 5 mm min^{-1} ($n = 4$). Reproduced from Sivamani et al.[24]

32.2 SKIN FRICTION COEFFICIENT VALUES

Friction is an important characteristic of skin because it allows us to execute many of our daily activities. In addition, friction studies offer insight into how skin and the skin surface change across age, gender, race, anatomical site, and chemical applications. This can provide better information about expected skin variations in the population and why certain topical applications are effective. Comparative studies are particularly useful in following how the skin mechanically changes under different conditions.

Previous studies have reported various values for the skin's friction coefficient. Most dynamic friction coefficient measurements most fall in a range of 0.2 to 0.5 (Figure 32.2). Besides natural variations in skin, the wide range in results may be due to differences in probe movement, geometry, controlled monitoring of the normal force, and material chosen to make the friction measurement. In designing the friction measurement apparatuses, the two types of probe movement utilized were rotational and linear. As a result, the linear probe constantly moves over "untested" skin and the rotational probe spins over "tested" skin. This can lead to discrepancies in reported values for the skin friction coefficient. Another important source of variation may be the ability to control the normal force while the probe is testing the skin surface. The skin friction instruments are designed to measure the fictional resistance of the skin and it is assumed that the normal force is constant. During a test the normal force may not stay constant as a result of an uneven skin surface, inaccurate spring, and a nonuniform distribution of static weights placed above the probe head. Therefore, the assumption of a constant normal force may be incorrect and can lead to variation in the calculated friction coefficient. A third source for variation is the choice of the probe material. Because friction is a surface phenomenon between two materials, the choice of the probe influences the numerical value obtained for the friction coefficient.

32.2.1 Hydration

Hydration studies reveal that drier skin had lowered friction while hydrated skin had an increased amount of friction (Table 32.1). However, the skin response is more complex, because very wet skin also has a lowered friction coefficient much like the characteristics of dry skin.[16] Most studies focus on an intermediate zone of hydration where the skin has been moistened without an appreciable "slippery" layer of water on the skin. Results in Table 32.1 show that the increases in friction were

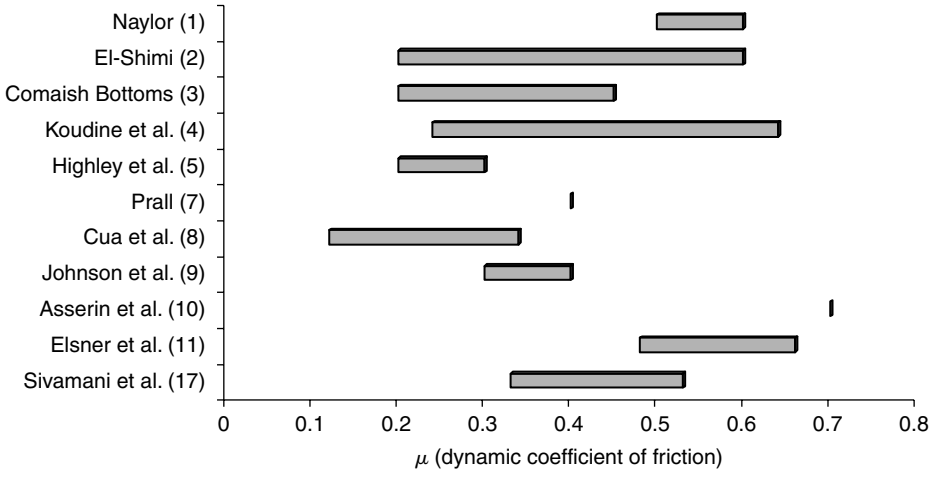

FIGURE 32.2 Outline of the ranges in the dynamic coefficient of friction. These ranges reflect measurement of untreated "normal" skin friction *in vivo*. Reproduced from Sivamani et al.[25]

TABLE 32.1
Comparative Studies of the Changes in Dynamic Friction Coefficient (μ) with Increasing Hydration (Hydration) and Decreasing Hydration (Dryness)

Author	Probe material	% Increase due to hydration $\{(\mu_{moist} - \mu_{normal})/\mu_{normal}\} \times 100$	% Decrease due to dryness $\{(\mu_{normal} - \mu_{dry})/\mu_{normal}\} \times 100$
Naylor[1]	Polyethylene	80	—
El-Shimi[2]	Stainless steel (Rough); stainless steel (smooth)	100–200 (Stainless steel rough) —	28 (Stainless steel rough); 41 (Stainless steel smooth)
Comaish and Bottoms[3]	Wool; teflon	40[a] (Wool); 400[a] (teflon)	—
Highley et al.[5]	Nylon	500	—
Prall[7]	Glass	200	—
Johnson et al.[9]	Glass	100–233	
Lodén et al.[12]	Stainless steel	—	33 (Hand); 41 (Back); 14 (Arm)
Nacht et al.[14]	Teflon	45	—
Sivamani et al.[17]	Stainless steel	55 (*In vitro*)	10 (*In vivo*)

[a]Comaish and Bottoms studied the change in the *static* friction coefficient in their hydration study.

varied and this possibly results from the various probes used. Although the addition of water increases the friction coefficient, this effect lasts for a period of minutes before the skin returns to its "normal" state.[2,5,14,17] The water has an effect of softening the skin and this in turn allows for greater contact area between the probe and the skin. Also, water results in adhesive forces between the water and the probe. Thus, there is more frictional resistance between the skin and the probe and results in a higher friction coefficient.[18] Since the water evaporates in minutes, the skin returns to its "normal" state in the same time frame. For dry skin, the skin becomes less supple and the probe does not achieve as much contact area and this allows the probe to glide more easily over the skin surface. This results in a lowered friction coefficient as seen in the isopropyl study[17] and in prior studies involving subjects

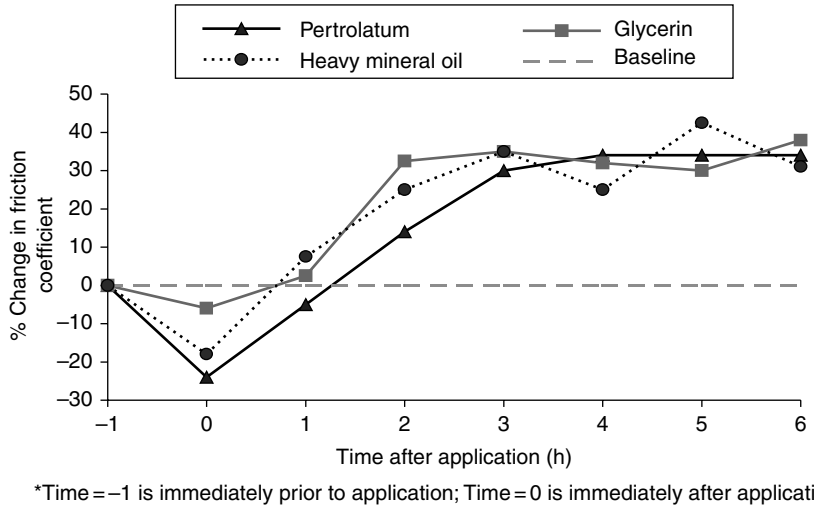

FIGURE 32.3 Effect of lubricant cosmetic ingredient on skin friction coefficient. Amount applied of each material: ~2 mg/cm^2. Reproduced from Nacht et al.[14] (mean of five subjects but *P* value was not published). Time = −1 is immediately prior to application; Time = −0 is immediately after application.

with clinically dry skin.[2,12] The agreement between the experimentally induced dry skin and clinical dry skin is expected.[18]

32.2.2 Lubricants/Emollients/Moisturizers

The studies on lubricants, emollients, and moisturizers are important for cosmetics and products developed to make the skin look and feel healthier. The literature reports that the important qualitative characteristics in skin topical agents are skin smoothness, greasiness, and moisturization.[17,19] Previous research has tried to describe these subjective, qualitative descriptions in a quantitative fashion by correlating them against the friction coefficient. Prall[7] tried to find a quantitative correlation for skin smoothness but was unable to make a direct correlation to the friction coefficient until he added the skin topography and hardness into the analysis. Nacht et al.[14] found a linear correlation between perceived greasiness and the friction coefficient (Figure 32.4).

32.2.2.1 Lubricant Oils

A lowering in the friction coefficient is the initial effect after the application of oils and oil-based lubricants.[2,5,14] Nacht et al.[14] and Highley et al.[5] also showed that after the initial decrease in friction, the oils eventually raised the skin's friction coefficient. The results of the lubricant cosmetic studies by Nacht et al.[14] are shown in Figure 32.3.

32.2.2.2 Emollients and Moisturizers

Prall[6] and Nacht et al.[14] found that the friction coefficient rises with the addition of emollients and creams in a similar fashion to water. However, the effect of the creams lasted for hours while the water effect lasted for about 5 to 20 min.[7,14,17] Sivamani et al.[17] quantified the friction, greasiness, and "stickiness" of the skin following application of creams and treatments (Figure 32.5). Hills et al.[15] also studied emollients, but they examined how different emollients compared against one another and how changes in temperature changed the friction coefficient. At a higher temperature (45°C), most emollients lowered the friction coefficient to a greater degree than at a lower temperature (18°C).

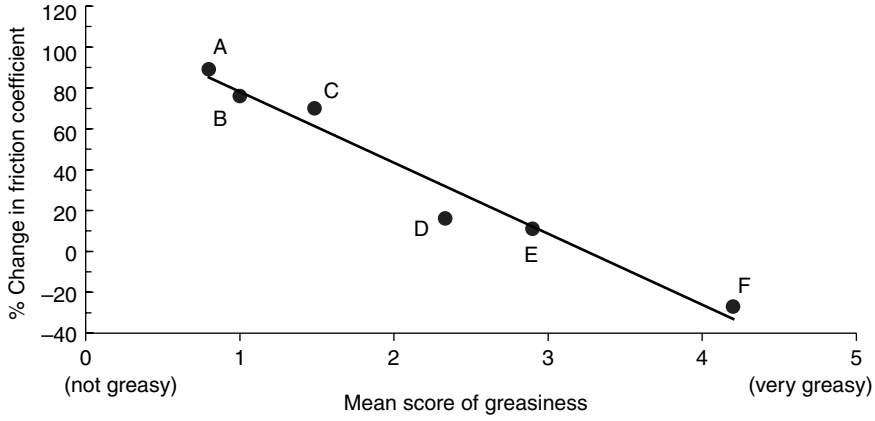

FIGURE 32.4 Correlation between changes in the friction coefficient and the sensory perception of greasiness. A, B, C, D, E, and F represent different creams that were applied to the skin. The reported percent change in the friction coefficient is immediately after application and the greasiness scores were subjective evaluations. Reproduced (from Nacht et al.[14]).

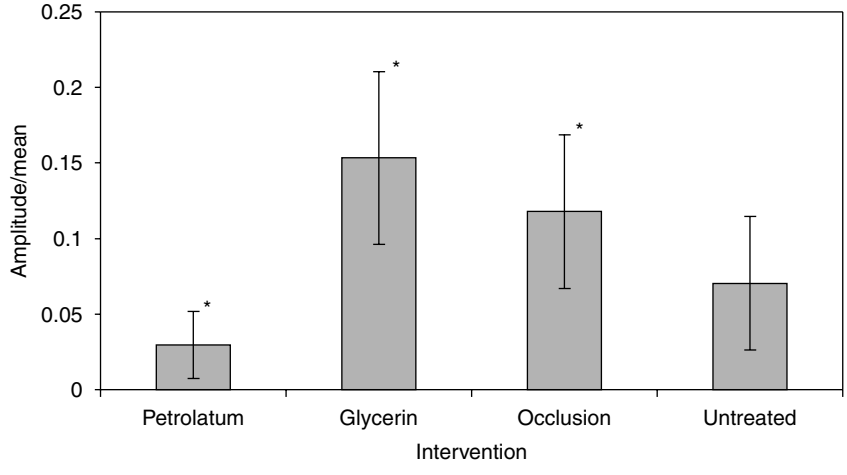

FIGURE 32.5 Amplitude/mean measurements for interventions. The application of glycerin and the PVDC occlusion increased the amplitude/mean of the volar forearm. Also, the addition of glycerin raised the amplitude/mean significantly more than the PVDC occlusion. Petrolatum significantly decreased the amplitude/mean and this is a quantitative evidence of petrolatum's greasiness ($P < .001$). Reproduced from Sivamani et al.[24]

When lubricants/moisturizers are applied to the skin, the skin friction is affected in three general ways.[14,18]

- *A large, immediate increase in the friction coefficient, similar to water application, that follows with a slow decrease in the friction coefficient.* These agents can be interpreted to act by immediate hydration of the skin through some aqueous means to give the immediate increase in friction. In Figure 32.4, creams A, B, and C represent this type of lubricant/moisturizer.
- *An initial decrease in the friction coefficient that is followed by an overall increase in the friction coefficient over time.* These agents are fairly greasy products (Figure 32.3) and this greasiness causes the immediate decrease in the friction coefficient. The eventual rise in

the friction coefficient is probably due to the occlusive effects of these agents.[21] In other words, these products and ingredients act to prevent water loss from the skin, thereby increasing the hydration of the skin. Representations of a few ingredients that elicit this response are in Figure 32.3 and represented as cream F in Figure 32.4.

- *A small, immediate increase in the friction coefficient that then increases slowly with time.* These agents are interpreted to act as a combination of effects seen in the previous two cases. These lubricants/moisturizers have ingredients and agents that serve to both hydrate the skin through some aqueous method, and prevent water loss through some occlusive mechanism. Because of the presence of the these occlusive agents, which tend to be more slippery, the immediate rise in the friction coefficient is lower than in products that fall into the first category listed above. In Figure 32.4, this is seen in creams D and E.

32.2.3 Probes

El-Shimi[2] and Comaish and Bottoms[3] compared probes (Table 32.1) and found that smoother probes gave higher friction coefficient measurements. El-Shimi[2] noted that higher friction coefficient measurements were made with a smoother stainless steel probe as opposed to a roughened stainless steel probe. Comaish and Bottoms[3] found a similar result with two types of nylon probes: a sheet probe and a knitted probe. The sheet probe (the smoother of the two) gave a higher friction coefficient measurement. El-Shimi[2] postulates that the smoother probe forms more contact points with the skin and has a greater skin contact area than the rougher probe, resulting in more resistance from the skin and a larger measurement for the friction coefficient.

32.2.4 Anatomic Region, Age, Gender, and Race

Few studies address the effects of anatomic region, age, gender, or race as they pertain to the friction coefficient. To date, no significant differences have been found with regard to gender[8,22,24] or race.[23,24]

The friction coefficient varies with anatomical site: Cua et al.[8,22] found that friction coefficients varied from 0.12 on the abdomen to 0.34 on the forehead. Elsner et al.[11] measured the vulvar friction coefficient at 0.66, whereas the forearm friction coefficient was 0.48. Sivamani et al.[24] found that the proximal volar forearm had a higher friction coefficient than the distal volar forearm. Manuskiatti et al.[23] studied skin roughness and found significant differences in skin roughness at various anatomical sites. Differences in environmental influences (i.e., sun exposure) and hydration may account for this. Elsner et al.[11] showed that the more-hydrated vulvar skin had a 35% higher friction coefficient than the forearm, and this is in agreement with hydration studies that contend that skin has an increased friction coefficient under increased hydration.

Age-related studies have been contradictory where some authors found no difference[8,22,24] and others found differences.[10,11] Cua et al.[22] showed no differences in friction with respect to age except for friction measurements on the ankle. Elsner et al.[11] also performed age-related tests and found no differences in the vulvar friction coefficient, but observed a higher forearm friction coefficient in younger subjects. They postulate that the skin on parts of the body that become exposed to sunlight can undergo photoaging and thus, forearm skin shows evidence of age-related differences while the light-protected vulvar skin does not.[11] Asserin et al.[10] concluded that younger subjects had a higher forearm friction coefficient than older subjects.

There are few gender-related and racial friction studies; Cua et al.[8,22] and Sivamani et al.[24] found no significant friction differences between the genders. Manuskiatti et al.[23] found no significant racial (black and white skin) differences in skin roughness and scaliness. Sivamani et al.[24] found no differences in volar forearm friction among different ethnicities before and after chemical treatments (Figure 32.7 and Figure 32.8).

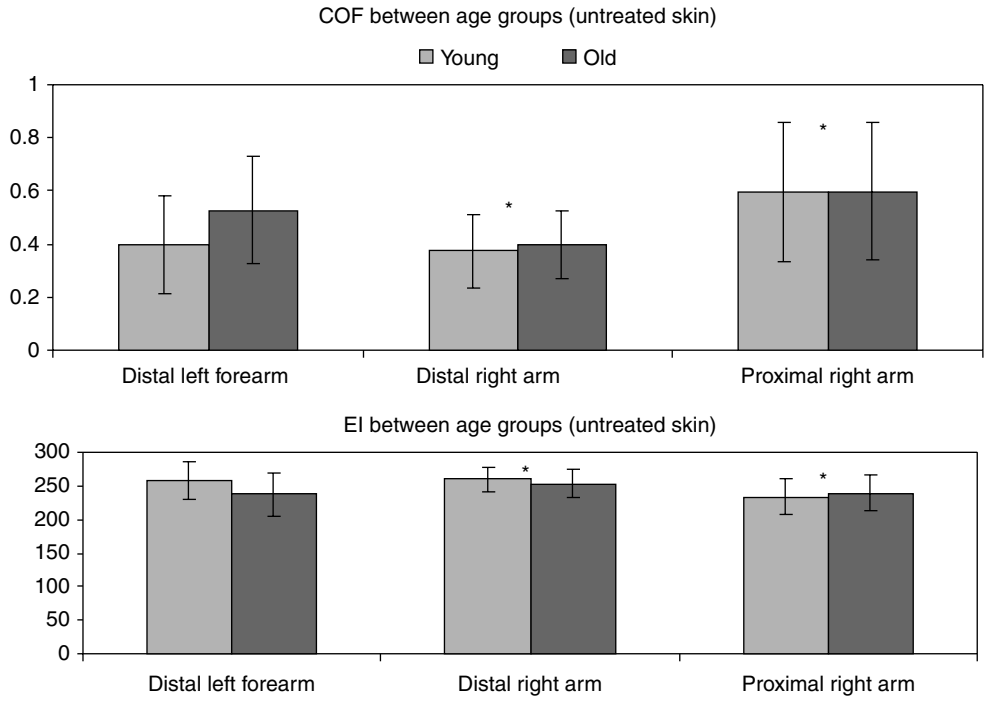

FIGURE 32.6 Age related comparisons of friction and electrical impedance. No significant differences were apparent between old and young skin on the volar forearm. Within each category, the proximal right arm friction and electrical impedance measurements were different from the distal right arm ($P < .001$). Reproduced from Sivamani et al.[24]

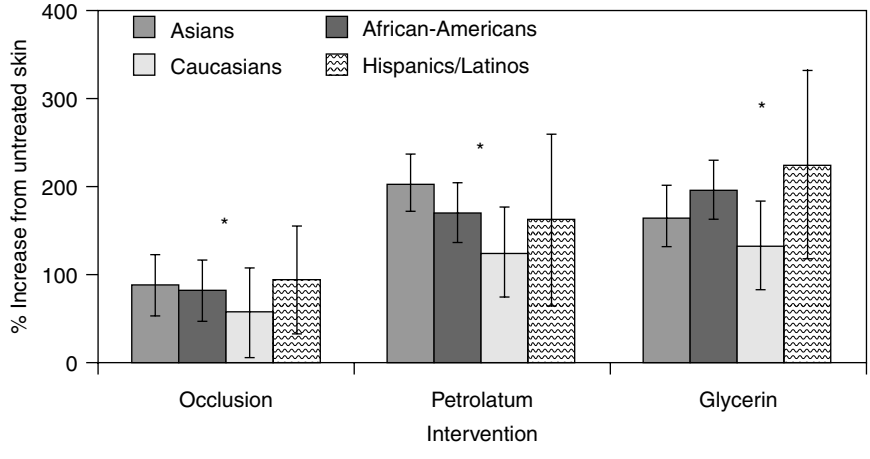

FIGURE 32.7 Coefficient of friction across ethnicity. Data represents increase in friction when compared to untreated skin of the volar forearm. No significant differences were found between the different ethnic groups. Petrolatum and glycerin increased the friction coefficient significantly more than PVDC occlusion ($P < .01$). The increase in the friction coefficient due to petrolatum was not significantly different from the effect of glycerin. Reproduced from Sivamani et al.[24]

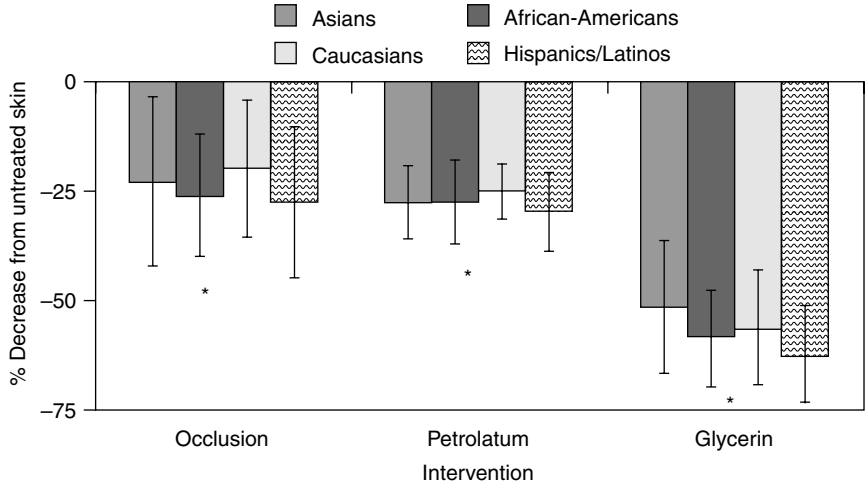

FIGURE 32.8 Change in electrical impedance across ethnicity. Data represents decrease in electrical impedance when compared to untreated skin of the volar forearm. No significant differences were found between the different ethnic groups. Glycerin lowered the electrical impedance significantly more than PVDC occlusion or petrolatum ($P < .01$). The decrease in the electrical impedance due to PVDC occlusion was not significantly different from the effect of petrolatum. Reproduced from Sivamani et al.[24]

32.3 CONCLUSION

Although there have been limited studies dealing with the measurement of the skin friction coefficient, studies demonstrate that differences in skin, because of various factors — such as age and hydration — can be correlated with the friction coefficient. Friction coefficient studies can serve as a quantitative method to investigate how skin differs on various parts of the body and how it differs between different people. It is also a useful method for tracking the changes resulting from environmental treatments — such as sunlight — and when various chemicals are applied to the skin — such as soaps, lubricants, and skin creams. The reviewed studies also indicate that the design of the test apparatus is an extremely important factor, because test design parameters can also have an influence on friction measurements. A better appreciation of the importance of the friction coefficient will become clearer as measurement methods improve and allow for greater accuracy.

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33 Smoothness of the Skin, Complexity, and Instrumental Approach

Jean Luc Lévêque

CONTENTS

33.1 INTRODUCTION

In our everyday life, we are used to handling various objects, heavy or light, solid or fragile, rigid or deformable. In general, we neither break them nor deform them. Moreover, we are able to recognize them just by assessing their shape, weight, texture, and temperature. Yet this sense of touch has not been reproduced to date, even with the most advanced robot technology. While modern technology is able to create or reconstitute a virtual environment, to synthesize sounds and fragrances, designing an artificial hand able to permanently adapt the pressure of its fingers to the nature of the object it grasps is hardly possible in most cases. With regard to the simulation of tactile sensations, some haptic systems that generate forces and vibrations corresponding to the hardness and texture of the object touched have begun to appear, but are far too simple to represent the complexity of contact between a finger and a visco-elastic material. These "force feedback" systems have only recently begun to appear in surgery to allow the surgeon performing surgery to sense tactile sensations in the course of telesurgery experiments.

Touching the skin, however, is essential in clinical practice in order to assess certain skin properties that the other senses cannot detect. In cosmetics, this corresponds to a very common gesture performed every day to evaluate the condition of our skin or to check the effect of a cream or shampoo or when applying perfume. In social life too, it is through touching the skin that we express our feelings to convey sympathy, love, and tenderness.

33.2 MECHANISMS INVOLVED

A light touch of the skin by the finger pad, for example, to assess skin smoothness, brings into play a multitude of physical, chemical, neuro-physiological, and cognitive processes.

At the physical and chemical level, the mechanisms of interaction between two skin surfaces, more or less flat, smooth, and adhesive, govern the sliding movement. According to Wolfram,

adhesive forces probably account for the static friction coefficient (μ_s), particularly when a low applied pressure generates a weak indentation of one surface into the other.[1,2] The nonuniform nature of the skin surface in terms of both relief and chemical properties probably also accounts for the kinetic friction coefficient (μ_k) and the amplitude of frictional fluctuations.[3] As we shall see later, objective measurement of these two parameters only represents one aspect of the skin smoothness concept.

The tactile physiological function is mainly a result of the existence of mechano-receptors or transducers in the skin, which transform mechanical energy into the emission of electrical impulses.[4] Each type of receptor is sensitive to a given frequency band. Paccini receptors can detect very small deformations (a few micrometers) up to a frequency of 300 Hz while Meissner receptors work at a lower frequency (50 Hz). These receptors are perfectly adapted to detect the passage of a fingertip over the skin surface. If we consider a scanning velocity of about 10 cm/s and a density of the microrelief lines of 20 cm, this simple gesture would generate an electric signal of about 200 Hz easily detectable by the Paccini receptors.

In actual fact, tactile sensation over the skin surface is highly dependent on finger pressure. The slow adapting receptors (Ruffini and Merkell) probably determine this pressure, which then generates information relative to skin firmness.

The cognitive process includes the many signals emitted by the skin receptors, which then arrive in the brain. They are processed so as to give rise to a particular sensation and to command verbalization and behavior. Research is under progress in this field but the mechanisms of transformation of electric signals into a sensation are still unknown. Some psycho-physical studies tend to demonstrate that the brain probably selects some of the received information according to the subject's intentions. For example, for assessing the smoothness of a surface, sensitivity is greater the lower the shearing forces.[5]

In fact, the external expression of our sensations and feelings is likely to be affected by the sum of our experiences and the cultural background acquired during our life. Skin smoothness, for example, is experienced from birth but the expression of this sensation is largely governed by our personal history.

As a result of this complexity, skin smoothness is therefore difficult to assess globally in an objective manner. At present, attempts to quantify this cosmetically important quality are only partial, either through a purely physical approach (friction measurement), or by objective cognitive measurements (brain activity) or psycho-physical tests.

This chapter concerns, more particularly, a description of the physical approach to measuring skin smoothness.

33.3 FRICTION MEASUREMENTS

Although there are some recent publications describing sophisticated new experimental approaches,[6] research on friction coefficients actually started some 50 years ago. The basic idea is to measure the force needed to drag a pad along the skin surface. Two types of pad movement over the skin were proposed: either rotation or translation. The probe consists of a plastic, steel, or glass material in the form of a disc or sphere. In spite of the great diversity of experimental procedures and conditions, overall these past studies agree on the following conclusions:[7]

- Amonton's law, stipulating a constant value for the friction coefficient whatever the pressure applied to skin, is not verified for skin.
- Hydration increases the friction coefficient although oily materials generally tend to decrease it (lubrication).
- There are no variations in the friction coefficient according to age, gender, and ethnicity. The effect of age is still under debate.

The main issue is to attempt to provide a better interpretation of the results in terms of skin parameters. The friction coefficient depends on several parameters: microrelief, vertical pressure, skin elastic properties, hydration of the surface, presence (or not) of a greasy film at the interface between skin and the measuring pad, nature of the pad. Several publications describe the influence of all these parameters on the measurement of friction coefficients but results are only qualitative because of the complexity of the phenomenon.

A relatively old publication attempted to link skin texture to the measurement of μ_k. Authors found fairly good correlation between the smoothness of chamois leather, as perceived by experts, and the inverse of μ_k.[8] This *in vitro* result was, however, not confirmed *in vivo* by Prall who found other types of correlations.[9] On the other hand, it has been shown that $1/\mu_k$ correlates very well with the slippery effect of oils. Such results reveal the complexity of the quality of "smoothness": smoothness of skin and smoothness perceived when applying a cosmetic product to the skin correspond to two different sensations.

Figure 33.1 is a plot of the variations in the friction coefficient versus time for two applied loads on the pad during a standard experiment. Such curves allow two coefficients to be defined, one corresponding to the maximum of the curve (static friction coefficient μ_s) and the other corresponding to the asymptotic value (dynamic friction coefficient μ_k). This type of variation is driven by the adhesion forces between the pad and the skin surface. The value of μ_k is lower than μ_s because during displacement of the pad, adhesion bonds have no time to reform after rupturing. Fluctuations during continuous displacement correspond to weak increases in μ (adhesion or "sticking") followed by a decrease in μ (bond rupture or "slipping"). This occurs when the stress induced by the adhesive contact and the shearing stress created by the tangential force are equivalent. These oscillations in the μ_k coefficient are sometime called the "stick-slip" process.

It has been hypothesized that the amplitude of this process is linked to the sensation of skin smoothness.[3] The first argument for such a hypothesis comes from a comparison between the

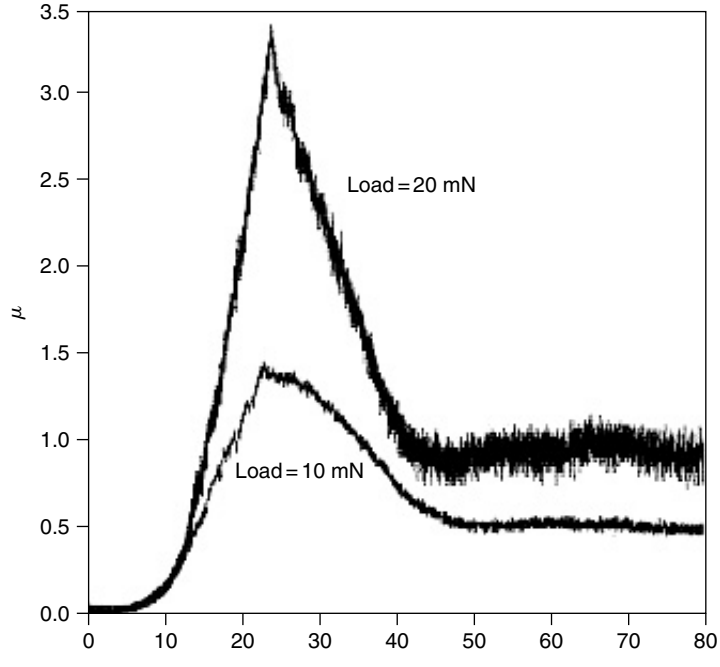

FIGURE 33.1 Variation of the friction coefficient versus time during the recording of an experiment on the forearm. The two curves correspond to two different loads applied on the sliding pad. The maximum of the curves corresponds to the static friction coefficient and the asymptotic value to the kinetic coefficient.[3]

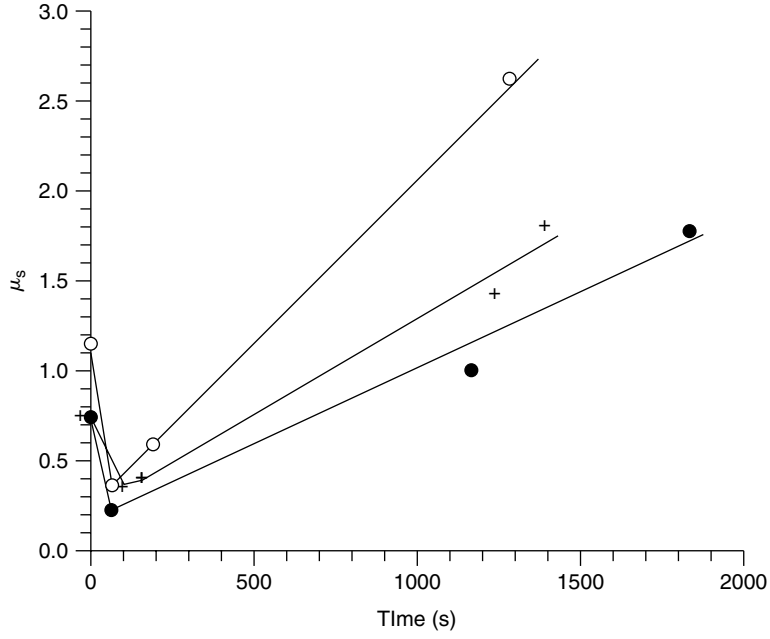

FIGURE 33.2 Variations of the friction coefficient versus time after application of different cosmetic preparations. There is an initial decrease due to lipids followed by a progressive increase due to hydration of the SC.[3]

amplitudes of the stick-slide process of outer and inner forearm skin: this is always higher on the outer part. On the other hand, application of an emulsion, assessed as giving some smoothness to the skin by a panel of experts, decreases this amplitude. This new parameter, called "fluctuations in frictional force amplitude" or (FFFA), could therefore be a good descriptor of skin smoothness.

In fact, such a coefficient also measures nonuniformity of the surface, which is due to the skin microrelief, the presence of squames and hair, and different types and amounts of lipids. These fluctuations are much higher when friction experiments are carried out with a low load on the pad. Measuring FFFA under such conditions could open up new possibilities.

As mentioned earlier, hydration of the skin surface has for a consequence an increase in the dynamic friction coefficient. This increase can be explained by several factors, which are expressed in the formula giving the friction coefficient: $\mu = S(K/E)^{2/3}P^{-1/3}$. Increasing hydration clearly decreases the Young modulus E but also modifies, in an unknown manner, S and K, which are respectively the shear strength of the adhesive contact and the colligative coefficient representing the number of adhesive contacts per unit area. According to Wolfram, water, by dissolving surface protein material, may increase adhesion. When moisturizers are applied to the skin, the increase in μ can be recorded quite rapidly, but follows an initial decrease due to the immediate effect of lipids present in the formulation (Figure 33.2).

33.4 PIEZO-ELECTRIC DETECTION OF THE CONTACT: THE "HAPTIC FINGER"

Dynamic contact between two bodies (e.g., one sliding over the other) generates noise that is characteristic of the hardness of these bodies and of the physical and chemical properties of their interface. Moreover, the velocity of the sliding movement modifies the frequency band of the emitted acoustic waves. The sliding displacement of a finger at the surface of the skin for assessing its smoothness

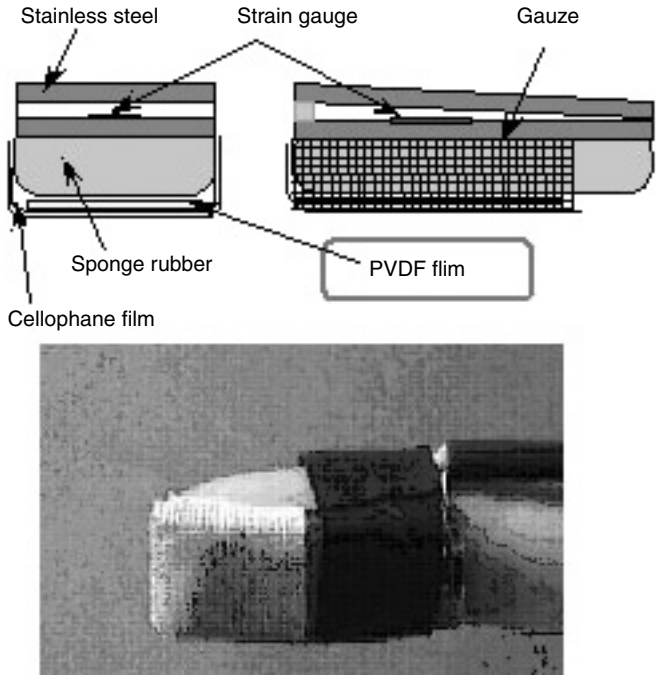

FIGURE 33.3 Structure of the "Haptic Finger."[11]

generates such waves, which can be recorded and analysed. One such promising acoustic method is now under development.[10]

Another way to analyze the waves generated by the sliding movement of the finger over the skin surface is to use a piezo-electric foil, a transducer membrane that generates electrical signals corresponding to the pressure variations of the finger sliding over the skin.

We used a thin foil of poly-vinylidene-fluoride (PVDF) polymer for building a new component, the "Haptic Finger," which, being adjusted at the tip of a finger can be used for touching the skin for assessing its hardness and smoothness, objectively.[11] A strain gauge is mounted in the layered structure of the sensor to control the pressure applied. *In vitro* experiments, carried out on "skin models" made with silicone rubbers of various hardness and roughness, allowed two mathematical parameters to be defined, extrapolated from the electric signal generated in the experiments. The first one is the variance of the signal processed by means of wavelet analysis (WL5-Var), while the second concerns dispersion of the power spectrum density of the signal developed in the frequency domain (FcR). According to our *in vitro* studies, FcR is proportional to the Young modulus (hardness) and WL5-Var is inversely linked to the roughness of the surface under investigation.

An *in vivo* study, carried out on patients with certain skin diseases, allowed us to obtain fairly good correspondence between the measured parameters and the skin assessment given by a clinician. Figure 33.3 represents an example of results obtained *in vivo* from the skin of ten volunteers before and after application of a cosmetic product. With such treatment, only WL5-Var (roughness) was significantly decreased (Figure 33.4).

33.5 COGNITIVE APPROACH

Different types of procedures exist for recording what a person feels during or after application of a cosmetic product, or during or after a given imposed task to evaluate the smoothness of one's own (or another person's) skin. The simplest consists in asking the person to fill in a questionnaire

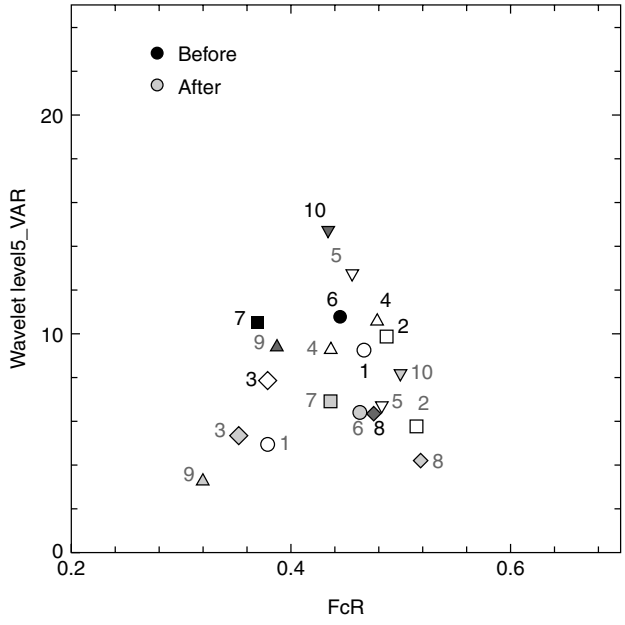

FIGURE 33.4 Measurement of the two parameters generated by the "Haptic Finger," before and 2 h after application of a cosmetic product on ten persons. There is a statistical decrease in "Wavelet Level5_VAR" (Roughness). The FcR parameter (Elastic modulus) is not affected.

where the different attributes of the skin, or of the cosmetic product, are listed with a grading system. There are, for example, standard methods of mood assessment in individuals, such as the "mood adjective check list" (MACL). In certain circumstances, the "mood scale" must be adapted to the population and to the task it has to assess. This approach is more scientifically relevant because the vocabulary chosen corresponds to the most selective and relevant wording, separating out objective from subjective reactions.

For some other tests, psycho-physical methods can be used. For the investigation of skin tactile properties, for example, the stimulus on the skin comes from the application of a series of plastic stimulators presenting two legs separated by different lengths. They are successively applied to the skin of the person who, without seeing the applicator, must decide how many contacts were felt.[12]

As mentioned earlier, any type of stimulation has physiological consequences triggering both personal behavior and verbal expression. To what extent is there a relationship between stimulation and brain activity, on the one hand, and between brain activity and verbalization on the other hand? The first study, which addressed this intriguing yet fundamental question in relation to skin and cosmetic smoothness was conducted in 1999 by Querleux et al.[13] They demonstrated that tactile perception originates in the contra-lateral somatosensory cortex although the subjective part of this perception is marked in the ipsi-lateral sensory region (Figure 33.5). They showed that rough and smooth textures had slightly different localizations in terms of neuronal activity. They also showed that skin-care products amplified sensory perception of the stimulus. This important study is a milestone in this type of research related to sensations generated by the use of a cosmetic product. Indeed, it defines the part played by subjectivity and emotion, an integral part of the cosmetic concept. It also paves the way for a new type of experimental approach in this complex field.

33.6 CONCLUSION

Smoothness of the skin is an important characteristic that cannot be defined and measured simply. Recording the profile of the skin microrelief hardly allows us to predict the perceived feelings

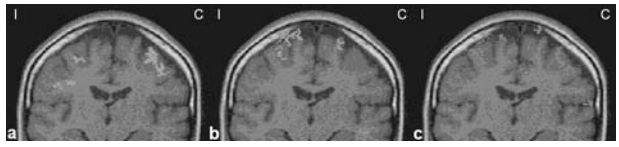

FIGURE 33.5 Activation map superimposed on coronal anatomical MR images in a right-handed subject, in response to passive stimulation. (a) Skin to skin. (b) Perception of a tactile stimulation. (c) Skin to skin with a skin-care product. I: Ipsilateral, C: Contralateral.[13]

we would have by touching it. Besides the microrelief, adhesivity, temperature, and firmness of the surface are all certainly involved in the process. Nowadays, noninvasive methods are able to separately measure each of these parameters, but the human brain works more globally and integrates these physical characteristics of the skin within a more general and cultural background that only some "cognitive" methods can approach. However, measuring the key smoothness-related properties of skin (friction, microrelief, firmness, temperature), in conditions similar to those of everyday life can be of use in designing effective cosmetic or dermatological treatments.

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34 Assessment of Skin Moisturization with Electrical Methods

Enzo Berardesca

CONTENTS

34.1 INTRODUCTION

The importance of water to the proper functioning of the Stratum Corneum (SC) is well-recognized. The reliable quantification of water in the corneum and its interaction with topically applied products is, in fact, essential for understanding skin physiology and developing efficient skin care formulation.

So, it is not surprising that a wide variety of techniques have been developed for measuring SC water content, including electrical measurements (Nova DPM 9003)[1,2]; Skicon 200[2–5]; Corneometer,[2,3] mechanical measurements (Dia-Stron DTM Torque Meter),[6] spectroscopic measurements (Fourier-Transform Infrared Spectroscopy or FTIR)[7]; Nuclear Magnetic Resonance Spectroscopy or NMR,[8] Transient Thermal Transfer or TTT,[8] and direct imaging measurements (Magnetic Resonance Imaging or MRI).[9–11]

Among these different methods, the electrical assessments have become very popular due to their relatively low cost and ease of use.

The use of these techniques has been documented in both normal and diseased skin.[12–18] However, it is also clear that many factors may influence the measurements and can alter the final interpretation of results.[19–21] Furthermore, reproducibility and variability between different instruments and methods can make it difficult to adequately compare data obtained in different laboratories and experiments.

To obtain reliable values, it is necessary to have a standardization of procedures (EEMCO).[21] In this chapter, therefore, attention is focused on the standardization of measurements by determining the variables associated with the method of measuring skin hydration, assessing the extent to which they influence the measured value, and elaborating the techniques by which they may be resolved.

34.2 SOURCES OF ERROR AND ASSOCIATED VARIABLES

The relationship between electrical conductance and water content is not linear, but depends upon the binding state of water molecules to the keratin chains, which is itself described by the water sorption isotherm.[22] It has been customary in the skincare literature to define three types of water according to their strength of binding to the keratin: "tightly-bound" water for water contents from 0 to around 7%, "bound water" between about 7 and 35%, and "free water" beyond. This division can be considered simplistic on the basis of more detailed theory,[23] but is generally helpful. The consequence of variation in water binding strength is that there is no direct proportionality between water content and electrical conductance. Three categories of factors and sources of variation exist in measurements, including instrumental, environmental, and individual-related variables. A detailed account of these influencing variables is given in this section.

34.2.1 Instrument-Related Variables

34.2.1.1 Start-Up and Use

It is very important, independently of the instrument used, that the measurement surface of the probe is absolutely free of dirt particles. Therefore, be aware to clean the measuring head before starting the measurement. In case of cleaning with alcohol it is necessary to dry the measuring surface with a soft tissue, as alcohol and water residues can lead to errors in measurement. The probe should be placed normally to a hair-free skin surface with slight pressure just sufficient to start the measurement process. An inclining position of the probe into the skin or too many hair causes the measuring surface to have an incorrect pressure on the skin.[24] Each of the devices described earlier have a spring in the probe to assure a reproducible pressure. When necessary, shaving or clipping the hair is recommended three days before starting the study.

The instrument should be turned on at least 20 min before measurements are performed. Repeated measurements on the same site can cause occlusion, which results in an increase in the values displayed. Therefore it is recommended to wait for at least 5 sec before repeating a measurement on the same site. It is advisable to measure at least 3 times, once at each of three different but nearby sites, and calculate the median, in order to have more reproducible data.

34.2.1.2 Use of the Probe Protection Covers

The condition of the glass cover of the probe surface has an influence on the measurement results. If this cover has been damaged, it can enable a part of the current flow to reach the skin. This, however, is not dangerous as the current is minor ($>10\ \mu$A) and it will alter the recharge time of the capacitor, and therefore the measured value will increase.

34.2.1.3 Instrumental Variability

The DPM and Skicon readings are usually higher than those of the Corneometer.[2,3,13] Furthermore, it should be remembered that in general different examples of the same instrument type might give variable readings.[5] In general, it appears that the DPM and Skicon are more useful in assessing relatively high water content levels and desorption kinetics, whereas the Corneometer is more

sensitive when assessing relatively dry skin.[4] Furthermore the Skicon is probably measuring SC hydration of more superficial parts of the skin than to the Corneometer. The user should be aware that the electrical-based instruments measure the integer of a field covering a three-dimensional space within the upper parts of the skin. Instead, the DPM readings seem to be less variable than the Corneometer readings.[25] As the Corneometer readings have already been shown to be less variable than the Skicon ones,[13,26] the DPM thus appears as the least variable of the three.[2] Other advantages of a DPM is that it is small, light, portable, and it is not necessary to put it into an electric outlet, thus is very flexible in its use.

34.2.2 Environment-Related Variables

34.2.2.1 Ambient Air Temperature and Humidity

Temperature and relative humidity are known to influence SC water content.[27,28] Under some conditions a linear relationship has been found between "capacitance" and relative humidity.[29] In fact high environmental humidities easily influence the hydration state of the skin surface because the SC is highly hygroscopic. Furthermore chemical changes in environmental humidities have an influence on barrier related factors for example, water holding capacity and thus on SC hydration. Therefore, room humidity should be kept constant. In cases of variation of relative humidity, this variation should be connected in the statistical analysis of variance and, if justified, some suitable normalization procedure such as proposed by Barel and Clarys[29] should be performed. Below 60% relative humidity the influence of the environment is much smaller, however, it is preferable to keep the relative humidity low. Room temperature should be kept constant and at a level to minimize sweat gland activity, hence the range 18 to 20°C is recommended. A steady increase in "capacitance" as room temperature rises above 22°C has been reported,[19] and this may arise from increased perspiration. Seasonal variations due to alterations in temperature and relative humidity occur[28,30–33] particularly in summer the room should be air conditioned. Day-to-day and even diurnal variations are also reported.[34,35]

34.2.3 Individual-Related Variables

34.2.3.1 Anatomical Sites

Regional differences in SC water content have been reported,[13,15,18,25–27,36,37] but when interpreting such results it must be remembered that all these electrical devices give only relative, not absolute, indications of water content expressed in arbitrary units (except by Skicon: μm). The actual relationship between their outputs and true water content is highly complex and also variable. Site-to-site differences in the same anatomical region (e.g., forearm) are reported by some[19] but denied by others.[25] High hydration values appear to exist on the forehead and palms; lower values are observed at the abdomen, thigh, and lower leg.[29] The hydration of contralateral pairs of sites is generally indistinguishable[19] except on the dorsum of the hands. Hence comparative left–right studies of the same selected skin sites are recommended to avoid skin site differences. The influence of body hair should be noted,[24] especially if comparison is made between sites, which are not contralateral.

34.2.3.2 Age and Sex

No significant differences have been reported between males and females when matched by age and tested on the same skin sites.[19,37,38] However, during certain periods of life significant differences may occur. For example, during the initial days of postnatal life, SC hydration is higher than in adults, suggesting that the SC barrier is still in the process of adapting to extrauterine life.[39,40] Based on almost identical values for the parameters of transepidermal water loss (TEWL), SC hydration and pH value, the skin physiology of the child differs very little in SC hydration and barrier function from

that of adults.[41] However, skin aging appears to induce a slow decrease in hydration from around the age of 25 onwards.[19,37,38] In fact, with increasing age, significant decreased levels of all major barrier lipids, changes in cutaneous proteoglycan's size have been described.[42,43] The observation of these dramatic age-related differences suggests that these changes may be involved in the age-related changes in the physical properties of skin.

34.2.3.3 Skin Surface Temperature

It is possible that a skin, which is moist and cool gives exactly the same electrical response to measurements made at a single frequency as a skin, which is dry and warm. To separate and specify potentially confounding influences such as water content, temperature change, and sweat gland activity, it is necessary to use some form of electrical spectroscopic technique, that is, stimulation at three or more different frequencies, or a time-domain approach followed by Fourier transformation.[44–46]

34.2.3.4 Sweating

Thermal sweat gland activity is unlikely if the ambient air temperature is below 20°C, humidity is between 30 and 60%, and the skin temperature is below 30°C, provided that the skin is not exposed to forced convection and no excessive body heat is produced (as a result of physical exercise).[47–49] Subjects in fact, should be relaxed and adapted to the measuring environment for at least 20 min before testing takes place. Therefore, skin should be exposed to the ambient air of the test environment for at least 10 min before taking measurements.[21]

34.3 CONCLUSION

Water plays an important role on the physical properties of the SC, and reliable quantification of water in the corneum and its interaction with topically applied products is essential for understanding skin physiology and developing efficient skin care formulations. Electrical conductance offers a sensitive approach to assessing SC water, even though the exact relationship between instrument readings and actual SC water content is complex and variable, preventing accurate calibration and opening the possibility of misinterpretation.

New methods, more experimental and expensive, are emerging, which can improve the performance of electrical approaches, but for the forseeable future electrical techniques will remain the more basic and widespread methodology, and will remain useful provided their limitations are clearly understood and experimental conditions are designed to overcome the associated difficulties as much as possible.

Finally, it should be remembered that no single type of instrumental test of skin is sufficient; a battery of methods (visual, tactile, instrumental) should always be used to guard against errors of interpretation.

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35 Stratum Corneum Tape Stripping: Relationship with Dry Skin and Moisturizers

Frank Dreher and Howard I. Maibach

CONTENTS

35.1 INTRODUCTION

The outermost skin layer, the *stratum corneum* (SC), consists of corneocytes embedded in lipid layers and represents the main barrier for skin penetration of xenobiotics. Its thickness in healthy adults may vary from 5 to 20 μm, except in the palm and sole where it is much thicker. SC can be removed sequentially by repeated application of appropriate adhesive tapes.[1] This technique, commonly known as "SC tape stripping," is a relatively noninvasive method to investigate the structure, properties, and functions of SC *in vivo*[2] and is the most frequently used for such purposes. Other techniques to remove SC are skin surface biopsy using cyanoacrylate strips and skin scraping.

Since SC was found to be a reservoir for topically applied chemical ingredients,[3,4] its removal by tape stripping has provided useful data on the absorption of products in the skin[5] and may therefore be a particularly powerful method for the evaluation of bioavailability and bioequivalence of topical drug products. The assessment of topical drug bioavailability, which includes the determination of a concentration *versus* time profile of an applied drug in human SC *in vivo* using the tape stripping technique, was described as a dermatopharmacokinetic approach by the U.S. Food and Drug

Administration.[6] The possibility that dermatopharmacokinetic characterization might be an alternative approach to clinical trials in order to assess the bioequivalence of topical drugs, like the use of concentration–time curves for systemically administered drugs, was explored during an AAPS/FDA workshop on "Bioequivalence of Topical Dermatological Dosage Forms — Methods for Evaluating Bioequivalence," in September 1996.[6] However, the draft guidance established during this workshop has still not been finalized nor has been generally accepted; the issue is still under discussion between scientists from industry, academia, and regulatory authorities.

35.2 TAPE STRIPPING TECHNIQUE

35.2.1 Experimental Procedure

The SC tape stripping is carried out by applying an adhesive tape onto the skin surface and further removing it by tearing off. The tape is pressed to a previously delineated skin surface area according to a standardized procedure by applying a constant pressure (e.g., 100 g cm^{-2}), using a weight or spring system over an appropriate time period (e.g., 5 sec). Then the tape is removed with a single continuous motion. The application and removal procedure may be repeated 10 to more than 100 times at the same site. Commonly used tapes for skin tape stripping are stationary tapes (e.g., Scotch® Book Tape no. 810 or 845 from 3M Co., USA), medical tapes (e.g., Transpore® from 3 M Co.) or, for such a purpose specially designed, commercially available tapes (e.g., D-Squame® from CuDerm Inc., USA). Tapes differ in shape, size, composition, and adhesive properties.

Following tape stripping, when performing dermatopharmacokinetic studies, the solute contained in the SC removed by tape strips is extracted and measured using appropriate analytical methods such as HPLC.

35.2.2 Technical Recommendations

Despite the simplicity of tape stripping procedure, numerous artifacts may result in inaccurate conclusions following dermatopharmacokinetic studies. The origin of such artifacts as well as possible ways to avoid them are discussed in the following.

35.2.2.1 Removal of Residual Product

After product application and prior to tape stripping procedure, the product excess has to be removed by wiping with a tissue or cotton swab or by an appropriate skin wash.[6,7] The effectiveness of washing procedure may be validated by simulating skin wash on an inert surface such as the bottom of a glass beaker. Hereby, washing procedure should be modified when the applied product is not completely removed. However, care should be taken to ensure that the washing process (e.g., with an aqueous solution containing a mild detergent) does not bring about subsequent solute extraction or redistribution in the SC. Using such a validated washing procedure, the removal of residual or unabsorbed product is effective after one or at most two tape strips. These tape strips contain superficial product residue, which is not likely to be bioavailable and therefore should not be taken into account when performing dermatopharmacokinetic studies. Improper removal of residual product may consequently have a direct impact on the results obtained in such studies. For instance, inadequate removal of residual topical preparation leads to an overestimation of chemical absorption into the SC.

35.2.2.2 Amount of SC Removed

It is currently recognized, that the amount of SC removed by a single tape strip may be influenced by numerous parameters. Differences in adhesive properties between tape brands[8] as well as between

samples of the same brand may result in different amounts of SC removed per surface unit. Pressure,[9] time course between application and removal,[10] as well as velocity of removal process[9] of the tape further influence SC amount removed. Additionally, SC removal may also depend on intrinsic skin properties related to race, sex, age, skin site, as well as skin condition (e.g., moistened versus dry, sun protected versus photo-aged, healthy versus diseased) or other site-specific inter- and intra-subject differences (e.g., SC thickness). Moreover, it has been recognized that SC amount removed by tape stripping may vary according to the depth. In general, the initial strips remove the largest amount of SC, because they remove the loosely packed squamous cells. Since the cohesiveness between corneocytes increases with depth,[11] decreasing amounts of SC are removed with increasing strips. In addition, product application prior to tape stripping must also be considered as a factor influencing the amount of SC removed by sequential tape stripping.[12] For instance, the vehicle components may alter both adhesive properties of the tape as well as cohesiveness of the corneocytes. Finally, differences in environmental conditions may also affect the amount of SC removed by tape stripping[13] suggesting that tape stripping should be performed under controlled conditions as when measuring transepidermal water loss (TEWL).

A further bias in determining dermatopharmacokinetics may originate from imprecise repetitive application of the tape on the skin being stripped. The use of a thin template to delimit the skin area is a simple way to keep the tape stripping area constant and unchanged during the entire tape stripping procedure.

35.2.2.3 Dissimilar Removal

A major problem in dermatopharmacokinetic characterization is that the removal of corneocytes can be dissimilar. Macroscopic skin furrows, which are commonly present, may lead to such a dissimilar removal of corneocytes yielding cell layers that originate from various depths after SC tape stripping.[14] As a result, a chemical may appear to have penetrated into deeper layers of the SC, whereas in fact the penetration restricted to the top layers. To overcome the problems caused by furrows, it has been suggested to slightly stretch the skin and thereby reduce the furrow depth before carrying out tape stripping. The change in corneocytes cohesiveness after product application may be another cause of dissimilar removal of SC.[2] Dissimilar SC removal might be limited by using less adhesive tapes or applying moderate pressure and shortened tape application time. However, the question whether such adaptations of tape stripping procedure do indeed improve the homogeneity of removed SC layers needs to be further investigated.

35.2.2.4 Time of Tape Stripping Procedure

A further factor influencing dermatopharmacokinetics is the duration of tape stripping procedure. Redistribution of the solute in the SC may occur when tape stripping procedure is performed over a long period of time relative to its lag time for diffusion across SC.[15] Furthermore, lateral spreading of the solute from neighboring, noninvolved skin into the stripped area may happen under certain circumstances.[16]

35.2.3 Estimation of SC Amount Removed by Tape Stripping

As described earlier, the amount of SC removed by tape stripping is highly variable and depends on the way the tape stripping procedure is performed as well as on SC characteristics and conditions.

As a consequence, the amount of SC removed by tape stripping is not proportional to the number of strips removed and has to be determined using accurate and reliable methods.

35.2.3.1 Gravimetric Method

Today, weighing is the preferred method to measure the amount of SC removed on a tape strip.[17] Tapes are weighed before and after stripping and the amount of SC is given by weight difference. High precision balances are needed since a very low amount of SC is removed per square centimeter of tape. However, weighing is time consuming and may be biased by water absorption or desorption during weighing procedure before and after stripping.[17] Furthermore, after topical product application, the weighing of SC is only reliable to some extent since the tape strips may not only contain SC but also applied vehicle and solute.

35.2.3.2 Colorimetric Methods Based on Protein Determination

As an alternative to weighing, a simple colorimetric method based on a commercially available protein assay similar to the Lowry assay was recently proposed.[18] Besides the Lowry assay, total protein content can also be determined using the Bradford assay. Briefly, the total protein assay was carried out by immersing SC containing tapes in a sodium hydroxide solution in order to extract the soluble SC protein fraction (SC is mainly composed of corneocytes filled with keratins) and neutralizing the solution with hydrochloric acid (the protein assay is not compatible with acidic conditions). This method was shown to be accurate and reproducible making it possible to determine even very small amounts of SC adhering to a single tape strip. Furthermore, with the exception of protein containing products and some other compounds interfering with the Lowry or Bradford assay, the uptake of product ingredients into the SC after topical application does not interfere with this colorimetric method. In addition, water content of the SC tape strip has no influence on the colorimetric assay. This method is particularly adapted for hydrophilic solutes, which are chemically stable under alkaline conditions (e.g., hydroxy acids), since they may be easily extracted from the SC adhering to tape strips and can thus be analyzed in parallel. However, this method is less convenient for hydrophobic compounds and obviously for those, which are not stable under the conditions of SC extraction. In such a case, tape strips have to be divided into two parts; one used for SC protein determination and the other for solute analysis.

Another method consists of a spectroscopic measurement of Comassie brilliant blue stained SC protein directly on the tape.[17] In contrast with the colorimetric method described earlier, this method does not require any SC extraction procedure prior to protein assay. However, it has been shown to be variable and not appropriate for quantitative determination since the absorbance of colored SC proteins is negligible as compared to light scattering of the SC material adhering to tape strips.

35.2.3.3 Spectroscopic Methods

Recently, a method based on the evaluation of SC amount adhering on tape strips using UV/VIS-spectroscopy as measured at 430 nm was reported.[19] Unlike colorimetric methods, this technique did not require any previous treatment or staining of SC. SC determination was performed directly on the tape (Tesa Film no. 5529, Beiersdorf, Germany) using a double-beam UV/VIS-spectrophotometer, modified in order to obtain a 1 cm $\times$ 1 cm light beam. The reference beam chamber contained an unused tape. The absorbance at 430 nm originated from light reflection, scattering, and diffraction by corneocyte aggregates on the tape and thus was directly related to SC weight removed by tape stripping. Except products absorbing in the wavelength range of corneocyte absorbance, the uptake of vehicle ingredients and solute into the SC after topical application did not interfere. However, at the present state of knowledge of this method, it cannot be excluded that

differences in SC moisture content as well as factors related to topical product application might have an impact on light reflection, scattering, and diffraction properties of corneocytes aggregates and thus on the spectroscopic measurement. Moreover, as discussed by the authors, the accuracy of this spectroscopic method was particularly sensitive to the occurrence of stacked corneocytes adhering to the tape.

35.3 TAPE STRIPPING IN DERMATOPHARMACOKINETIC STUDIES

As described in the draft guidance set up during an AAPS/FDA workshop, the dermatopharmacokinetic evaluation of a topical drug consists in any measurement of drug content in the skin, which can be determined continuously or at least intermittently for a period of time, and, which may include measurement of SC drug concentration over time in humans.[6] Two topical preparations that produce comparable SC drug concentration versus time curves may be bioequivalent just as two preparations for oral administration are deemed bioequivalent if they produce comparable plasma concentration versus time curves. The successful application of dermatopharmacokinetics thus lies on the assumption that SC concentration versus time curves are closely related to the concentration versus time curves of the drug in nucleated epidermis, hair follicles, sebaceous glands, and dermis, at least for drugs targeting these skin compartments instead of SC.

In order to establish dermatopharmacokinetics according to the FDA draft guidelines, the major part of applied drug absorbed in SC must be removed by applying a sufficient number of sequential tape strips. For that purpose, the draft guidelines recommend a protocol in which at least ten successive tape strips are collected (first and eventually second strip being discarded since they represent unabsorbed drug at skin surface) on a single skin site.[6] The SC absorbed drug is then extracted from combined tapes and analyzed using an appropriate analytical method. FDA draft guidelines recommend expressing the results as the amount of drug per area of adhesive tape. When applying this protocol and ensuring that the majority of drug was removed, the amount of SC recovered by tape stripping would not be relevant. Consequently, no recommendations regarding the important issue of standardizing the amount of SC removed by tape stripping was given. However, such a protocol is only applicable when comparing test to reference product in the same individual assuming that intra-site variability on the test site (e.g., forearm) is negligible. Furthermore, there is large evidence that much more than ten strips should be necessary to remove the entire SC[20] so that the major part of drug contained in the related tissue could be recovered, namely in the case of prolonged product application when steady-state of penetration was reached. In fact, it is not advisable to set up a given number of tape strips for removing the entire SC. It was recently demonstrated that a constant number of strips removed different fractions of SC from one individual to another[21] (and most likely also from site-to-site[22]) even when standardized tape stripping conditions (tape type, pressure, time, removal) were used. For instance, the absolute thickness of intact SC on the ventral forearm may vary from 5 to 20 μm in healthy adults.[21] And, the amount of SC removed by tape stripping after product application may vary considerably according to the preparation applied. Therefore, a given number of tape strips neither removes the same amount of SC, nor the same relative percentage of the total barrier function in different individuals. Consequently, it is not correct to relate the amount of absorbed drug to the tape strip number or to pooled tape strips.

Drug content in SC is not homogeneous but rather forms a gradient through skin.[23] In addition to the tape stripping protocol as outlined by the FDA, dermatopharmacokinetic studies may therefore include the assessment of the drug concentration profile within the SC.[24] For this purpose, the uptake of a drug into the SC is expressed as the amount of drug per mass or volume of SC removed (corresponding to drug concentration in SC) versus SC depth. In order to allow for inter-individual as well as inter-site comparison, SC depth should be normalized with respect to the total SC thickness.[21,25] To normalize data, the cumulative amount of SC removed for each individual is divided by the

corresponding total SC thickness. The total SC thickness may be determined by measuring TEWL in combination with tape stripping assuming a constant SC density of 1 $g\,cm^{-3}$ across SC as well as a uniform SC removal.[26] This measurement may be performed in a skin area adjoining that on which the product is applied.

35.4 TAPE STRIPPING FOR MEASURING SC COHESION

Tape stripping can be further used to investigate intercorneocyte cohesion within the SC by quantifying the amount of SC removed.[11] When using standardized tape stripping conditions, the more SC removed, the smaller the SC cohesion. For instance, tape stripping in combination with a protein assay to accurately quantify SC removal proved to be a sensitive method in detecting keratolytic efficacy of salicylic acid preparations within hours of application.[27] This method may also be useful to measure the influence of moisturizers or other skin care preparations on SC cohesion.

35.5 SUMMARY AND CONCLUSION

The application of tape stripping technique is well-established in dermatopharmacological research and the technique is appreciated as one of the most useful method to remove SC allowing investigation of its structure, properties, and functions. However, despite apparent simplicity, the tape stripping technique entails several technical problems and care has to be taken to avoid misleading conclusions when interpreting data. For instance, results given as a function of tape strip number or pooled tape strips have to be interpreted with care, since the amount of SC removed by tape stripping may be highly variable and may depend on numerous factors related to tape stripping procedure and SC properties. Therefore, SC removal by tape stripping should be determined using accurate and reliable methods. Today, weighing is the most commonly used method for such a purpose. But, due to possible artifacts associated with weighing procedure, other methods such as a colorimetric and a spectroscopic assay were recently proposed to improve the reliability of data obtained on SC amounts removed by tape stripping. However, both methods need further validation before considering that they provide an adequate and more accurate alternative than weighing.

Tape stripping is also used to assess both drug uptake and clearance from the SC making it a particularly powerful method for the evaluation of bioequivalence of topical drug products using a dermatopharmacokinetic approach as described by the FDA. However, implementing the technical recommendations mentioned in this review to avoid problems associated with tape stripping technique and subsequent proper validation are paramount requisites to make tape stripping a viable method for bioequivalence evaluation of topically applied dermatological drug products and use it as a standard technique to pursue the development of safe and effective generic topical products. In addition, recent studies indicate that tape stripping in combination with an accurate method to quantify SC removal is a reliable and sensitive method to measure keratolytic efficacy.

Taken together, sufficient background information exists to begin to permit to the use of stripping method to define the role of individual agents and combinations thereof on so called dry skin syndrome.

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36 XLRS Squamometry Revisited

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CONTENTS

In its most widely appreciated context, the stratum corneum (SC) exhibits an important barrier function extending to protection from ultraviolet light, oxidants, micro-organisms, and toxic xenobiotics. It also protects from loss of water and electrolytes from the body. The SC can also be viewed as a highly specialized structure showing perpetual renewal keeping ideally a steady state in its structure and thickness. It is structurally and biochemically diverse. It possesses a limited form of metabolic activity. It also acts as a unique sophisticated biosensor that signals the underlying epidermis to respond to external stresses.[1,2]

Corneocytes are about 1 μm thick and have a mean area of approximately 1000 μm^2. However, the surface area is dependent upon age, anatomical location, and conditions that influence epidermal proliferation such as chemical irritation and UV irradiation.[3] Corneocyte size increases with age. This is sometimes assumed to be related to the increased transit time within the SC. On most body sites, the SC consists typically of 12 to 16 layers of flattened corneocytes.

Each corneocyte corresponds to a water-insoluble protein complex made primarily of a highly organized keratin microfibrillar matrix. This structure is encapsulated in a protein and lipid-enriched shell. This cornified cell envelope shows differences in maturation among corneocytes. Specific probes allow to distinguish two distinct types of cornified cell envelopes, which have been called "fragile" and "rigid," or "immature" and "mature."[2,4,5]

In some instances, the SC homeostasis is altered. Indeed, the SC representing the interface between the epidermis and the environment is the repository of many biological events that have occurred below it in previous days. It is also affected by diverse and repeated external threats. The genetic background, nutritional status, and the intervention of some physical agents, drugs, cosmetics, toiletries, and other chemical xenobiotics represent other major modulators of the SC structure. Knowledge about the fine SC structure is crucial in many aspects of the dermocosmetic science, particularly when dealing with xerosis and related effects of emollients and squamolytic agents.[6–8]

36.1 XEROSIS ASSESSMENT AND SAMPLING

The formation, composition, structure, and function of human SC have been scrutinized by intense research over the past few decades. Clinical assessments of the SC aspect are based on ordinal grading scales. They often suffer from vague and overlapping definitions.[9] The subjective clinical assessment based on visual and tactile scoring may suffer from variability by inconsistencies from grade to grade and also from poor reproducibility. Variations in the environmental conditions may jeopardize subjective grading systems since hydration swells the outer SC and often camouflages low-grade scaling and dryness.

Investigators were also striving relentless to provide more precise evaluation tools. Bioinstrumentation plays an ever-increasing role reducing grading variation and yielding better distinction between competing drugs and cosmetic formulations.[6,7,8,10–12] One of the ancillary method designed to study the outer SC layers relied on tape stripping. The use of casual sticky tapes often proved to be unreliable for quantitative assessments because there was considerable variation in the adhesion of different batches to the SC.[13] Currently, the standard operating procedures in many laboratories rely on two other sampling methods, namely the cyanoacrylate skin surface stripping (CSSS) and stripping with adhesive-coated discs (SACD).[6,7,14,15]

Groundbreaking work using the SACD method has won international recognition for fundamental research, clinical experimentation, and applied dermocosmetology. The sampling device for SACD is one of the crystal clear pressure-sensitive adhesive-coated discs (D-Squame®, Cuderm Corporation, Dallas, TX; Corneofix®, C+K Electronics, Cologne, Germany; Corneodisc L'Oréal, Paris, France). This specific material provides the required rigidity and adhesion to uniformly sample a fixed area of skin surface. After peeling off the protective seal, the disc is applied to the skin surface using a gauge spring dynamometer to ensure a calibrated pressure usually chosen in the range 100 to 250 g/cm^2. Both the pressure and the time of application of the disc influence the amount of SC removed.[6,7,13,14,16] A short-time (5 sec) SACD procedure removes less corneocytes from the stratum disjunctum than a longer time (1 h) application. This is due to occlusion modifying the SC hydration and cohesion between corneocytes.[9,17–19] A seasonal variation in the amount of harvested SC is possible.[20]

If greasy products have been applied to the skin before sampling, the SACD procedure is impaired giving rise to unreliable data. When such a pitfall is suspected oily product substantivity can be checked before SACD sampling using a Sebumeter SM 810 (C+K Electronics, Cologne, Germany). Prior application of lipid solvents also alters the SC harvesting.[21] In general, however, sampling errors for SACD are expected to be reduced by delipidizing the skin with one application of ether:acetone (1:1). By contrast, repeated applications of ethanol solutions and other solvents significantly alter the physiological desquamation process and reduce the amount of corneocytes collected.[14]

The SACD method is used for different purposes. The most popular goals deal with the evaluation of skin scaliness and internal cohesiveness of the superficial SC. For several years, the challenge of analytical evaluations of SACD allowed exploring various aspects of the structure and biological dynamics of the SC.

36.2 SACD AND SCALINESS

The SC continually renews itself, imperceptibly casting off corneocytes from its stratum disjunctum. The most important adhesive forces holding keratinocytes together come from the desmosomes. During these cells move toward the skin surface, desmosomes are modified into corneosomes (also called corneodesmosomes) in a process following a programed protease-mediated destruction.[8,18,22,23] Desquamation results in the degradation of nonperipheral corneosomes first at the stratum compactum–disjunctum interface and finally the peripheral corneosomes are degraded in the upper layers of the SC. This process leads to loss of corneocytes from the surface of the skin by discrete frictional forces.

Xerosis is the term dermatologists use to describe corneocyte aggregation clinically featuring rough, flaky, and scaly skin. Such a condition, is otherwise known to the laity as dry skin.[7,24,25] In dry flaky skin conditions corneosomes are not degraded efficiently and corneocytes accumulate on the skin surface.[19,26] Thus, failure to degrade corneosomes timely and correctly is the key factor responsible for most flaking conditions. During the xerotic process, corneocytes remain attached to each other with uneven strength until physical forces cause whole rafts of cells to detach partially from the skin. The reduced corneodesmolysis that occurs in xerotic skin disorders is due to reductions in the levels and activities of SC proteases together with elevated levels of corneosomal glycoproteins in the superficial layers of the SC. Additionally, increased levels of fragile corneocytes are associated with reduced transglutaminase activity and corneocyte envelope cross-linking events.[4] The recently introduced silicon image sensing (SIS) technology shows the low water content of the SC in these cases.[27–30]

The SACD can be employed to sample and quantify loose corneocytes and squames from the skin surface. Such a procedure is only valid for conditions where the intercorneocyte bonds are weaker than the SACD adhesion to the SC.[14] In fact, tiny scales found in the common type of facial dry skin, dandruff, and seborrheic dermatitis come off quite easily.[31–42] By contrast, thick compact adherent scales found in ichthyosis and severe winter xerosis of the lower legs[12,31] are not reliably harvested using SACD. This represents a classical pitfall of the method.[14]

When the sampling procedure of SACD is correct, the amount of collected SC can be weighed with confidence.[7,43,44] However, it is time-consuming and often subject to artifacts due to adsorption or desorption of water taking place in the SACD sample.[45] An indirect assessment of the amount in harvested corneocytes was designed by measuring the attenuation of transmitted light using photometry.[46] There may be a bias in such a procedure because a given amount of corneocytes either stacked in clumps or joint in a thin sheet exhibits different optical properties.[7] The same limitation applies to the measure of light reflectance when the sample is placed upon a dark background.[7] The quantitative and qualitative aspects of the scale pattern are probably better appreciated by means of image analysis of the dry SACD samples.[14,47–53] Texture analysis of SACD is another sophisticated method.[54] A series of other innovative methods have also been proposed to provide information about the amount of SC present in SACD.[45,55–58]

Contrasting with the aforementioned assessments, the SC can be studied after staining the harvested SACD. Many dyes are available and their choice depends on the goal of the study. As an example, a mixture of rhodamine B and methylene blue conveniently decorates the corneocytes for microscopic examination. Another quantitative assay is based on the Lowry's reaction of proteins with an alkaline copper tartrate solution and Folin reagent followed by spectrophotometric measurement of the reaction product with maximum absorbance at 750 nm.[59]

A wealth of applications have benefited from staining with a mixture of toluidine blue and basic fuschin in 30% ethanol at pH 3.4. Samples are stained for 1 min and gently dipped into water in order to rinse off the excess of dyes. This step is critical as it is important for not loosing corneocytes from SACD.[7,14] As such, samples are suitable to microscopic examination and reflectance colorimetry. This latter assessment is coined squamometry.[14,31] Basically, the stained SACD samples are placed over a hole cut out of either a glass or plastic slide, which is then placed onto a white reference tile. The color of the specimens is measured by reflectance colorimetry. The most convenient colorimetric parameter is represented by the squamometry index (SQMI) corresponding to the Chroma C^* value, which combines the values of red and blue chromacities following: Chroma $C^* = (a^{*2} + b^{*2})^{1/2}$.

Squamometry is currently used for four main purposes. Accordingly, different names were coined and defined[14]:

- *Squamometry X* refers to the assessment of xerosis and any other scaly condition.
- *Squamometry L* refers to the assessment of the intercorneocyte cohesiveness altered by light, particularly in the ultraviolet range.
- *Squamometry R* refers to the dynamic aspect of the SC renewal.

- *Squamometry S* refers to the assessment of the effect of surfactants on the corneocyte integrity.

36.3 SQUAMOMETRY X

Squamometry X corresponds to the analytical evaluation of xerosis using SACD samplings.[6,7,14,31] A linear correlation exists between the amount of harvested orthokeratotic SC and SQMI. Data are also influenced by the presence of parakeratotic cells and serum deposits as found in spongiotic dermatoses such as atopic dermatitis, irritant or allergic contact dermatitis, seborrheic dermatitis and psoriasis.[32,36]

The efficacy of squamolytic agents, emollients, and other SC biomodifyers is conveniently evaluated using squamometry X.[7,14,39] In general, it is appropriate to take SACD at entry in a given clinical study, as well as after two and four weeks of treatment, which is then stopped. After a two-week post-treatment period corresponding to the regression phase, late SACD samples are collected. SQMI values of the successive samplings show the kinetics of improvement followed by the post-treatment regression.

The efficacy of corticosteroids in inflammatory dermatoses is also conveniently evaluated by the same approach. SQMI values decrease when the effects of inflammation disappear from the SC.[32,35,36]

Squamometry X is particularly gratifying in the assessment of both dandruff severity and efficacy of its treatment.[6,7,14,33,34,37,38,42] Similarly, seborrheic dermatitis can be evaluated noninvasively.[40,41] In these related scalp conditions, some correlation can be established between SQMI and the load in *Malassezia* spp.[33,37,38,40,41,60]

36.4 SQUAMOMETRY L

Ultraviolet irradiation alters the biology of the epidermis. One of the consequences of an acute exposure is altered maturation and desquamation of the SC. The kinetics of the modifications in the SC cohesiveness can be assessed using squamometry L.[14,61] Similarly, the effect of any topical intervention for controlling such a process can be quantified.[62]

36.5 SQUAMOMETRY R

Once the SC stained *in vivo* by an appropriate dye, it is possible to assess the rate of corneocyte renewal in the two following weeks.[63] One of the SC marker is dihydroxyacetone (DHA). The brown color of the SCAD sample can be measured by reflectance colorimetry after placing the DHA–SCAD sample on a white reference tile. After the DHA–SACD colorimetric evaluation, the same samples are stained in a second *ex vivo* step using toluidine blue-basic fuschin dyes to derive SQMI. The latter value serves to assess the amount of collected corneocytes. The squamometry R index is the ratio between the Chroma C^* of DHA–SACD and SQMI. Performing iterative SACD samplings over two weeks shows the kinetics of fading DHA skin color, thus representing an indirect estimation of the desquamation rate.

36.6 SQUAMOMETRY S

The SC is subjected to various environmental chemical threats. Squamometry S was designed to test the effect of surfactants on the SC *in vivo*.[6,7,14,64] Several studies have confirmed the reliability of such a method in predicting irritation by selected xenobiotics[61,65–70] and protection afforded against them by diverse agents.[62,71] As such, squamometry S appears to be a suitable method for evaluating

TABLE 36.1
Ordinal Scales for the Microscopic Assessment of Squamometry S (From Reference 14)

Score	Predominant pattern
Staining intensity	
0	No staining
1	Discrete between corneocytes
2	Spotted on corneocytes
3	Strong in single and clustered corneocytes
4	Strong in most corneocytes
Intercorneocyte loosening	
0	Large and uniform sheet of corneocytes
1	Large clusters, few single corneocytes
2	Small clusters, some single corneocytes
3	Disrupted clusters, many single corneocytes
4	Single/torn corneocytes

the efficacy of barrier substances when minimally damaged conditions are chosen, such as with semi-open applications of the offending product.[71]

Squamometry S is somewhat reminiscent of the *ex vivo* corneosurfametry bioassay although the information is not similar but complementary.[64] Squamometry S entails the application of surfactants or other chemicals onto the skin. Whatever risk exists is small but not zero, all practical steps reducing the risk to the smallest acceptable level must be considered. Hence, the potentially offending agents remain in place for a short period of time only, usually ranging from 15 to 90 min. Such an *in vivo* application can be repeated for instance every second hour when the xenobiotic is weakly aggressive to the skin.[64] At the issue of a single or repeated insult(s), SACD are collected and submitted to SQMI measurements. In addition, microscopic examination is warranted in order to categorize the patterns of corneocyte confluence and intensity of staining.[7,14,34,65,67] Indeed, with increasing aggression, corneocytes may show both increased dye binding and loosening. This is due to the variable combination of protein denaturation, corneosome disruption, and corneocyte lysis. A trained microscopist is endowed to score SACD according to an ordinal scale (Table 36.1) in order to avoid as much as possible inter-observer variations. It is unfortunate that the grading systems differ among research groups.[34,67] Hopefully, image analysis is superior in sensitivity and objectivity to discriminate differences between SACD samples. The most informative values are the percentage of the SACD area occupied by corneocytes and the distribution in size of the corneocyte aggregates relative to that of a single cell. The staining intensity relies on the evaluation of grey levels on a 1 to 8 scale. Such a method is sensitive enough to detect subclinical irritation well before any increase in erythema and transepidermal water loss can be detected.

36.7 CONCLUSION

The SC is not a dead tissue of little importance. SACD is becoming a time-honored method for studying this dynamic and heterogeneous structure. There is strong circumstantial evidence indicating that squamometry test in its XLRS variants affords valuable information. It is a simple, highly reproducible, and sensitive method. In many instances changes induced by physiological variations and environmental threats, and improvement brought by cosmetics and other SC biomodifyers are finely demonstrated by this method. Further experience with squamometry will probably continue to define the relative strength and weakness of the method and perhaps design new variants of it.

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37 Methods for Testing Stratum Corneum Barrier Properties

Ludger Kolbe and Soeren Jaspers

CONTENTS

37.1 INTRODUCTION

Moisturizers are marketed as products that combat the signs of dry skin by delivering moisture to the skin. They are appreciated by the consumer for making the skin smooth and pliable. Since the discovery of Blank[1] that water has a plasticizing effect on the stratum corneum (SC), the main goal of formulators was to develop moisturizers that deliver more moisture. However, enhancement of hydration, softness, and smoothness are short-lived effects, lasting only for hours. Therefore, moisturizers need to be reapplied frequently. In short-term *in vivo* studies the improvement of skin mechanics or plasticity[2] and hydration parameters[3] have been analyzed following a single application of a moisturizer.

The traditional view of moisturizers is that they increase the moisture content of the skin by forming an occlusive, hydrophobic film on the surface of the SC, thereby trapping moisture in the underlying tissue. However, restoration of barrier function after disruption can be accelerated by the application of lipids, like free fatty acids, cholesterol, and nonphysiological lipids like the complex hydrocarbon mixture petrolatum.[4] Barrier repair can be measured within an hour after barrier disruption and leads to normalization of barrier function within hours to days, depending on the initial insult.

Consumers use moisturizers frequently, especially women use them often on twice daily basis for years or even decades. Therefore it is appropriate to ask for the consequences of this life-long treatment on the barrier function of the skin. Fortunately we do not see adverse reactions after

long-term use of moisturizers. Individuals identifying themselves as having sensitive skin will change their cosmetics as soon as they perceive itching, tingling, or other adverse effects. However there are positive consequences of moisturizers on barrier properties, evident only after long-term use, that is, twice daily application for several weeks. Such an improvement of barrier function occurs even in normal skin,[5] but skin conditions with impaired barrier function, like winter xerosis, represent an excellent model for studying barrier improvement by moisturizers. Moisturizers are well known for relieving the symptoms of dry, scaly, and itchy skin. The efficacy of moisturizers for the treatment of dry skin of the lower leg is usually assessed by the regression method,[6] estimating the time course for reduction of scaliness and reappearance after treatment. However, this method gives little information on barrier properties. Experience has taught us that products that are equivalent in removing scales may have different effects on the integrity of the horny layer.

The SC is not only a barrier preventing excessive diffusional water loss, it is also a mechanical and permeability barrier reducing the absorption of substances from the environment. All these barrier properties can be tested in order to gain a complete picture of the state of the horny layer.

37.2 TECHNIQUES

Over the years a panel of methods has been published for testing different properties of the SC barrier (Table 37.1). The combination of tests, employing the application of chemical probes to the skin and the measurement of skin responses with new bioengineering read-out systems allows to measure changes in barrier properties with high sensitivity and reliability. A subjective selection of skin barrier tests will be discussed further in this chapter.

37.2.1 Transepidermal Water Loss

Measurement of the transepidermal water loss (TEWL) is the standard method to determine SC barrier status. A disturbed skin barrier is characterized by high TEWL.[7] The measurement of the TEWL is based on the diffusion principle in an open chamber. The density gradient is measured

TABLE 37.1
Selection of Tests for the Investigation of Skin Barrier Function

Tests involving chemical probes
- Ammonium hydroxide blister test[30]
- Chloroform:methanol burning test[30]
- Dimethyl sulfoxide (DMSO) test[21]
- Erythema after sodium lauryl sulfate provocation assay[26]
- Lactic acid sting test[30]
- Nicotinate test[30]
- Repetitive irritation test[17]
- Sodium hydroxide erosion assay[11]

Bioengineering methods to measure skin responses to chemical probes
- Chromametry[30]
- Evaporimetry[30]
- Hydration measurements with various electrical devices[30]
- Laser Doppler imaging[30]
- Transepidermal water loss (TEWL)

indirectly by two pairs of sensors (temperature and relative humidity) inside a hollow cylinder and is analyzed by a microprocessor, integrated in the handle.

The TEWL measurement has been a valuable tool in barrier repair experiments where repair kinetics were followed by TEWL. However, there are some problems with this method when the efficacy of moisturizers is to be evaluated. Some ingredients of moisturizers like petrolatum are shown to be occlusive.[8] Therefore TEWL readings might reflect the occlusive effect of the cream, rather than an improved, less permeable SC. Measurements performed shortly after application of the moisturizer may represent surface water loss of residual emulsion water instead of TEWL. The TEWL measurements must be performed in an environmental chamber with constant temperature and humidity after an appropriate rest period in order to minimize the contribution of sweat gland activity.[9]

Recently, there was some significant improvement in the engineering of TEWL — Probes (Tewameter TM 300, Courage & Kazaka, Cologne, Germany). Preheating of the probe and usage of new integrated electronic sensors resulted in a significantly reduced measurement period and a considerably more stable signal. As a result of the new assembly the handle is efficiently thermally insulated against the electronics. This new engineering, linked with an accurate and skillful operation and used under constant ambient conditions leads to very significant results.

Newly, so called close chamber instruments become available. The AquaFlux device (Biox Systems Ltd, London, UK), for example, is sealed with a condenser that is maintained at a temperature of −13°C using a Peltier cooler.[10] This leads to significantly higher TEWL values compared to conventional open systems. The possible benefits and fields of application of these instruments remain to be examined in the near future.

37.2.2 Sodium Hydroxide Erosion Assay

This assay is a recently published modification of the alkali resistance test.[11] Sodium hydroxide (NaOH) is strongly keratolytic and rapidly introduces structural defects in the horny layer. Burckhardt introduced the alkali resistance test in 1947.[12] His goal was to develop a procedure that would enable the identification of individuals at increased risk of chemical injury to skin. In some European centers the alkali resistance test subsequently became an accepted screening procedure, in the United States the test never caught on. Researchers after Burckhardt largely found the alkali resistance test to be unreliable and irreproducible.[13,14] Attempts to improve the method failed[15] and therefore the alkali resistance test fell into disuse. However, NaOH has been used as an irritant in other tests.[16,17]

Burckhardt's original test involved application of a drop of 0.5 *N* NaOH under each of three glass blocks for periods of 10 min. The end-point was the time required for ten small vesicles and erosions to develop.

In an attempt to strengthen the reliability and sensitivity of Burckhardts method the procedure was changed substantially and became the erosion assay: Fifteen microliters of 1.0 *M* NaOH were applied to the test site and covered immediately by a circular plastic disc 1.3 cm in diameter to achieve uniform distribution. After one minute the solution was wiped off with a facial tissue; the test site was then gently rubbed with a cotton swab soaked in a solution of blue food coloring. Erosions stain deep blue. If no erosions developed after the first minute fresh solution was reapplied in a series of one minute intervals. Precautions have been introduced in order to avoid severe alkali necrosis, an occasional adverse effect of the original alkali resistance test, by recommending a less ambiguous end-point for the sodium hydroxide erosion assay. The appearance of the first erosion(s) was found to be both safer and more reliable than counting multiple erosions as Burckhardt did. The time in minutes required for the development of the first erosion is recorded as the erosion time. The usual size of an erosion is about 0.5 mm. Exposed sites were allowed to heal spontaneously without treatment or bandaging.

37.2.3 Dimethyl Sulphoxide (DMSO) Whealing Test

The use of Dimethyl sulphoxide (DMSO) in dermatology has a long history.[18,19] In concentrations up to 50% DMSO is used for topical treatment of amyloidosis,[20] but in concentrations of 90% and higher it is known to induce whealing and a flare reaction. The procedure described here is a modification of that described by Frosch et al.[21] for the identification of individuals with sensitive skin. The DMSO reaction can be assessed clinically or measured with the laser Doppler flowmeter.[22] The recent development of laser Doppler imagers[28] make it possible to measure the DMSO induced increase in blood flow with unparalleled accuracy. To elicit the reaction 15 μl of 90%, 95%, and 100% DMSO were applied to three adjacent sites and immediately covered with a circular plastic disc, 1.3 cm in diameter, spreading the solution evenly over the skin. The discs were removed after 15 min and the surface was wiped with tissue paper. Readings were made 5 min later (approximately the peak response) clinically according to the following scale: 0 = no reaction, 1 = discrete, follicular wheals, 2 = flat wheals, with partial convergence, 3 = confluent solid wheal.

The DMSO induced increased blood flow was measured using the moorLDI laser Doppler imager (Moor Instruments Ltd, Devon, UK). The instrument scans a low power laser beam in a raster pattern over the skin. Moving blood in the microvasculature causes a Doppler shift, which is processed to build up a color-coded image of cutaneous blood flow. The mean and standard deviation of the blood perfusion units in a region of interest was calculated.

37.2.4 Sodium Lauryl Sulfate (SLS) Irritation

The SLS is frequently used to induce experimental irritant dermatitis. The reaction is characterized by erythema, increased TEWL, and scaliness. Susceptibility to SLS irritation can be used as an assay for the prevention of irritant reaction by moisturizers.[23–25]

A vast number of different protocols for the induction of SLS irritancy can be found in the literature.[26] Concentrations spanning several order of magnitude have been used for single or multiple applications with exposure times ranging from minutes to days. In order to show the efficacy of moisturizers to prevent SLS irritation low concentrations should be used. With higher concentrations the individual differences between subjects tend to increase. The prevention of irritant dermatitis from a strong insult with high concentrations of SLS is probably beyond the capacity of bland moisturizers and more the domain of barrier creams.

37.3 DATA

37.3.1 Xerotic Leg Skin

37.3.1.1 Erosion Assay

On normal skin NaOH erosions first develop at hair follicles and orifices of sweat gland ducts, usually after 4 to 5 min. They are small and circular in appearance. However, erosions on xerotic leg skin look different, they often develop along cracks in the SC, revealing the weak spots of xerotic skin. Erosion times are significantly reduced, on severely dry skin erosions develop within the first minute.

In a small test group of only five subjects erosion times of the dry skin of the calves were determined before and after four weeks of treatment with either a water-in-oil cream or a 12% lactic acid lotion (Figure 37.1). The assay was performed on four adjacent spots per leg. Spot-to-spot variation was on average less than one minute. In the majority of subjects erosions formed within two minutes. Erosion times on the legs treated with the water-in-oil cream were strongly increased, whereas the opposite legs, treated with the lactic acid lotion showed only slight improvement. This is not unexpected since alpha-hydroxy acids are known for their keratolytic activity which will weaken the

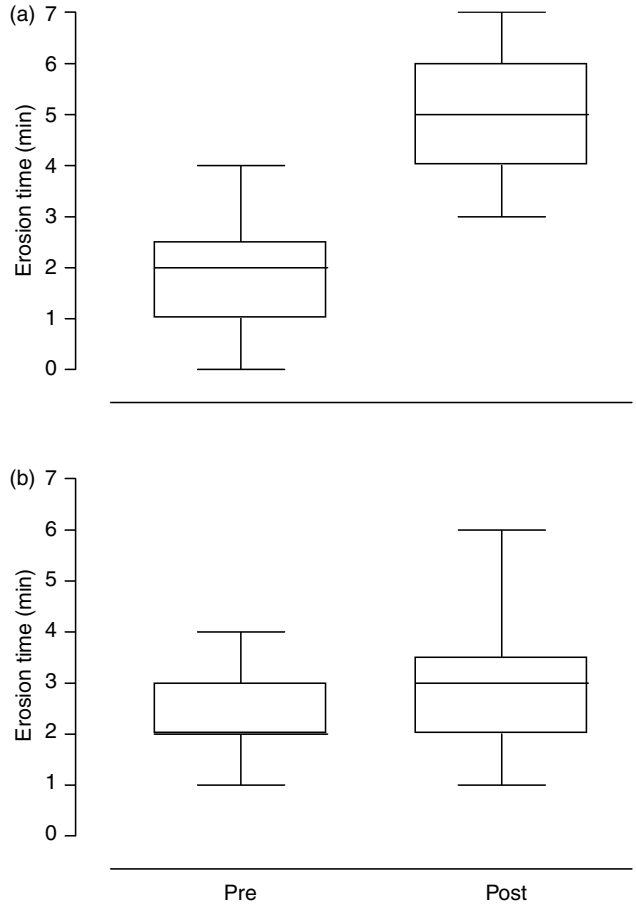

FIGURE 37.1 Effect of moisturizer treatment on sodium hydroxide erosion time of xerotic leg skin. (a) Marked improvement of alkali resistance after treatment with a water-in-oil emulsion for four weeks ($p < .0001$). (b) Slight but significant improvement after treatment with a lotion containing 12% lactic acid ($p < .01$). Erosion assays were performed before and after treatment on four adjacent spots per leg. Data shown as box plots. Statistical analysis was performed with the paired t-test.

horny layer. The improvement by moisturizers is probably not due to a protective moisturizer film in the outer layers of the SC, since there was no significant increase of erosion times after only a few applications. Erosion times gradually increase over the treatment period.

37.3.1.2 DMSO Reaction

The DMSO readily penetrates the SC of dry leg skin and induces an inflammatory response, characterized by a strong whealing reaction and increased cutaneous blood flow. Determination of blood perfusion units with a laser Doppler imager showed a dose dependent response (Figure 37.2). However, after six weeks of treatment with a water-in-oil emulsion the reaction was markedly reduced, only 1 out of 14 subjects responded slightly to 90% DMSO. Laser Doppler imaging is a reliable and sensitive method and the obtained images can be stored in the computer for further investigation (Figure 37.3). Nevertheless, a trained examiner will also get good results with clinical grading.

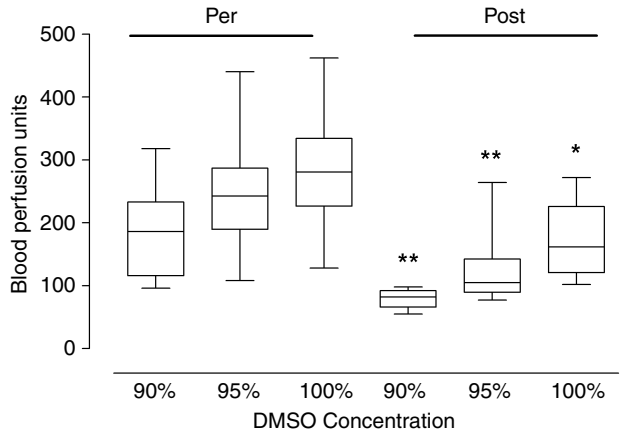

FIGURE 37.2 DMSO response of xerotic leg skin. (a) Dose dependent increase of DMSO induced cutaneous blood flow before on untreated skin. (b) After twice daily application of a water-in-oil emulsion for six weeks the response was markedly reduced. DMSO induced blood flow was measured with a laser Doppler imager. Data expressed as blood perfusion units. Statistical significance was determined using the paired *t*-test ($^{*}p > .05$, $^{**}p < .01$).

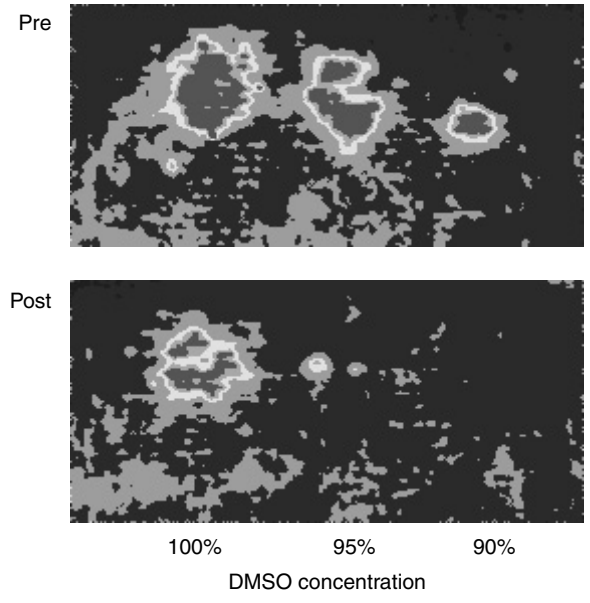

FIGURE 37.3 The laser Doppler imager generates a pseudo-colored image of blood flow. In this gray scale image the various shader of grey represent regions with increased blood flow. The dark background represents the baseline blood flow. At baseline DMSO evoked a dose dependent increase in cutaneous blood flow. After 6 weeks of treatment with a water-in-oil emulsion the DMSO response was markedly reduced.

37.3.2 Normal Volar Forearm Skin

At first glance it might seem a bizarre idea to improve a normal skin barrier. Can there be a better barrier than the normal barrier? However, we know there is considerable individual variation. Marie Lodén[5] demonstrated that indeed some moisturizers improve barrier properties of normal skin. Sensitive skin is a multidimensional phenomenon but at least in part a weak SC barrier contributes

to the problem.[27,28] Moisturizers formulated for individuals with sensitive skin accordingly should be aimed at improving the barrier function.

37.3.2.1 SLS Irritation

Susceptibility to SLS irritancy was analyzed before and after treatment with a water-in-oil emulsion for four weeks. Patches with SLS ranging from 0.025 to 0.1% were applied to the volar forearm of nine subjects for 24 h. At 48 h SLS induced erythema was measured with the Minolta Chromameter and the laser Doppler imager from Moor Instruments (Figure 37.4). The laser Doppler imager revealed a significant lower vascular response to SLS at all concentrations after treatment. There was also a decrease of background blood flow, measured at adjacent unchallenged skin. This probably reflects a subclinical response of the supplying and draining blood vessels in larger area surrounding the exposure sites, which was also reduced by the moisturizer. No significant differences were found

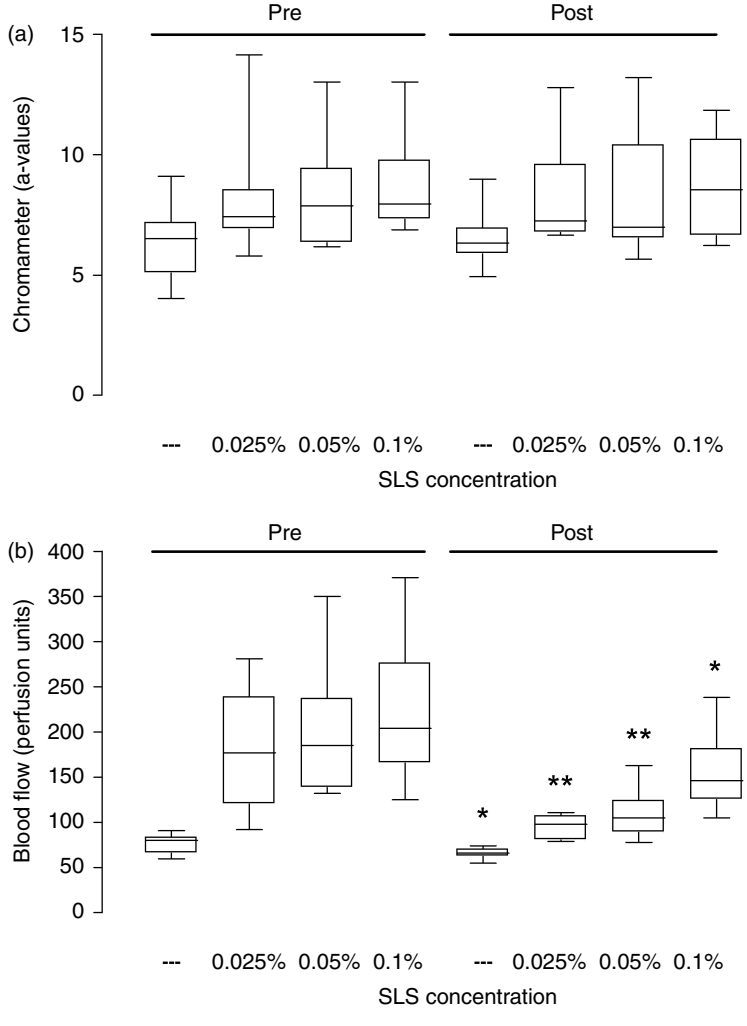

FIGURE 37.4 SLS irritation and moisturizer treatment. (a) No significant differences in skin color (a-values) were detectable with the Chromameter. (b) The same sites as in (a) scanned with the laser Doppler imager showed marked reduction of erythema. Volar forearms were treated with a water-in-oil emulsion twice daily for four weeks. Statistical significance was determined using the paired *t*-test ($^*p > .05$, $^{**}p < .01$).

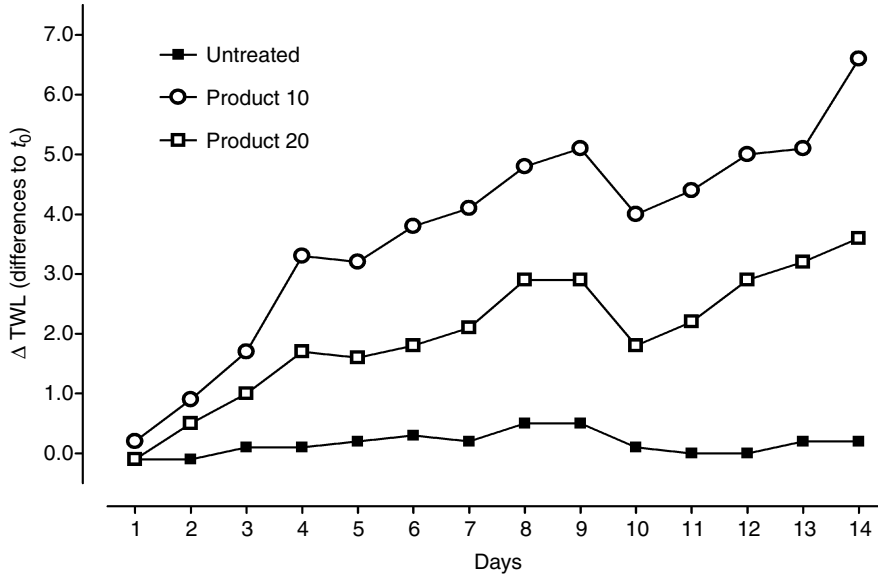

FIGURE 37.5 TEWL after harsh washing. Washing of forearm skin leads to barrier damage characterized by increased TEWL. The improved TEWL probes allow to discriminate between products with different content of surfactants. Barrier damage was more pronounced on areas treated with higher concentration of surfactants. Repair is fast, stop of treatment for one day (day 4 and 9) leads to reduced TEWL readings the next day.

with the Chromameter but DMSO test and erosion assay demonstrated a significant improvement of barrier function (data not shown).

37.3.2.2 TEWL

In this study a washing test on the inner forearm was performed over a period of 14 days. Two products (10 and 20) containing different amounts of surfactants were used for this test (Figure 37.5). TEWL was determined daily using the improved TEWL probe (Tewameter TM 300). It gradually increased over time showing a stronger increase with higher content of surfactant (product 20). The decrease in TEWL between day 4 and 5 and day 9 and 10 is a result of a stop of treatment between these measurements, respectively. The trend of the untreated area is considerably stable, also the run of the curves and the discrimination of the treated sites are very clear and meaningful.

37.4 CONCLUSION

The TEWL measurement is an indispensable method to evaluate barrier function. Particularly the new improved TEWL probes will bring us more reliable data than ever before. But not always is improvement of barrier properties reflected by reduced TEWL. Especially on normal skin where TEWL is already low the slight additional reduction after moisturizer treatment might not reach statistical significance. Furthermore reduction of TEWL might reflect the occlusivity of the product rather than a less permeable horny layer. Hence, additional information must be obtained with other methods. Product occlusivity might interfere with other techniques as well, but by using a set of different methods the problem can be minimized. One can argue that it makes no difference whether a moisturizer is protective because of an improved SC or because the product forms an occlusive layer. However, these are two different mechanisms, which should be investigated separately. The evaluation of protective product layers is a valid approach for the evaluation of barrier creams,

in short-term studies,[29,30] but in long-term studies with moisturizers improvement of the structural integrity of the horny layer itself should be evaluated.

The DMSO, NaOH, and SLS are useful chemical probes because of the different properties of the substances and the different mechanisms by which they induce irritation. Due to its amphiphilic nature DMSO rapidly penetrates the SC and induces whealing and erythema. The aqueous NaOH solution dissolves the keratin layers, thereby introducing structural defects in the horny layer. The mechanism by which an aqueous solution of SLS induces irritation is still unknown, but SLS penetrates the barrier like DMSO, although much slower. Combined, tests with these and other chemical probes provide valuable information on the barrier properties of the SC.

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Part IV

Skin Reactions

38 Sensitive Skin

Heather L. Daughters, Ai-Lean Chew, and Howard I. Maibach

CONTENTS

38.1 INTRODUCTION: DEFINITION OF SENSITIVE SKIN

Facial moisturizers frequently produce burning, stinging, itching, and suberythematous irritant dermatitis. Rates occasionally proximate 20%. The subjective symptoms are generally described by consumers as "sensitive skin."

The term "sensitive skin" has become ubiquitous in the world of cosmetology in recent years, yet no formal definition exists. Consumers use the term to describe a variety of adverse skin reactions to cosmetics and other topical products, as well as skin reactions triggered by environmental (e.g., temperature, wind, pollution), lifestyle (e.g., stress, emotion, diet), and hormonal factors (e.g., menstrual cycle).[1] This all-encompassing interpretation includes the spectrum of dry skin; oily or acne-prone skin; tendency to flushing; and nonspecific sensations of burning, stinging, and itching. Dermatologists and cosmetic chemists use the term to express both a situation of facial skin hyper-reactivity on contact with a variety of topical agents, as well as the occult dermatoses resulting

from or flaring up with topical applications and other exogenous factors.[1,2] Controversy exists over whether the term is reserved purely for visible hyper-reactive responses or for susceptibility to chemically induced stinging and other such sensations.[1] Contrary to consumer perception, few conventional medical assessments of sensitive skin take hormonal or lifestyle factors into account. Thus, sensitive skin remains an imprecise phenomenon. In broad terms, sensitive skin is largely agreed to be a lay term used by individuals who consider themselves more intolerant of topical preparations and environmental conditions than the general population. The onus on the medical practitioner, then, is not merely to label a patient as having sensitive skin, but to diagnose the underlying condition causing his or her symptoms.

Acne-prone skin is often classified under the sensitive skin category by cosmetic consumers. However, as many dermatologists consider it to be a separate clinical entity, acne-prone skin and acneiform eruptions will not be discussed here.

38.2 EPIDEMIOLOGY

Sensitive skin is largely believed to be a widespread phenomenon. Epidemiological studies have shown that the incidence of self-reported skin sensitivity is 51.4% in women and 38.2% in men. Studies also show that 10% of women and 5.8% of men consider themselves to have very sensitive skin.[3] However, consumer-perceived cutaneous reactions are usually scientifically unconfirmed; self-assessment is not an accurate parameter. Furthermore, estimates of the prevalence or incidence of sensitive skin are problematic as the term "sensitive skin" lacks a consistent definition.

The North American Contact Dermatitis Group has published data on a multicenter study of cosmetic reactions from 1977 to 1983 conducted by dermatologists with a special interest in contact dermatitis.[4,5] They identified patients with cosmetic dermatitis as 5.4% of 13,216 patients with contact dermatitis. Other studies have shown that cosmetic-induced subjective skin discomfort occurred more commonly in individuals with sensitive skin (53%) than in those individuals who did not consider themselves as having sensitive skin (17%).[3] The most frequent cause of cosmetic dermatitis was identified as allergic contact dermatitis, although this may have been a misrepresentation of the population due to the special interests of the dermatologists involved. Irritant dermatitis was thought to be under-reported, as it is a diagnosis of exclusion. We feel that although this study serves to alert physicians and consumers to suspect cosmetic reactions, it does not in any way represent the true prevalence of the problem. Individuals with sensitive skin may not have been identified as many experience sensory reactions with no visible inflammation. Frequently, consumers who experience a reaction to a cosmetic product will merely discontinue use of the suspected item, rather than consult a physician. While this action is certainly adequate in treating the symptoms at hand, it hinders both our ability to quantify the percentage of adverse reactions caused by cosmetics, as well as identification of the ingredients that cause these reactions.

In the previous study, 79% of the patients with cosmetic dermatitis were female and 85% were Caucasian. Correspondingly, in a series of skin reactivity studies, Frosch and Kligman concluded that the typical "hyper-reactor" had the following characteristics: white, fair skin, high susceptibility to sunburn and poor ability to tan (Fitzpatrick phototype I or II), and blond or red hair.[6] These features were most prominent in people of Celtic lineage. Accordingly, dark skin is commonly thought to resist chemical injury better, presumably because erythema is less discernible.[7] However, in light of recent studies, ethnic skin has been found to play a complex role in sensitive skin — this has been reviewed by Berardesca and Maibach.[7] Since ethnicity has been shown to play a role in an individuals skin sensitivity, one might also think that atopy would also contribute to the sensitivity of an individual's skin. Studies have shown that the incidence of atopy is higher in women with sensitive skin (49%) compared to those without sensitive skin (27%); however atopic diathesis does not appear to be a predictive factor for sensitive skin because the incidence of self-perceived sensitive skin was almost equal for atopics (49%) and nonatopics (51%).[3]

38.3 SYMPTOMATOLOGY

Although the differential diagnosis of sensitive skin encompasses a range of possible skin diseases, the types of complaints reported are very similar. Burning, itching, stinging, or a tight feeling (due to associated dry skin) are frequently reported symptoms.[2,8] These symptoms vary in intensity from mild to severe and may be intermittent or continuous throughout the day. Onset or exacerbation of symptoms correlates to application of the offending topical product(s). Clinical signs are usually minimal — even though erythema and edema may be evident, these inflammatory changes are often transient and have no long-term clinical sequelae. The lack of objective signs and overall similarity in clinical symptoms poses a challenge to the clinician's diagnostic acumen.

The face is the most common site for cosmetic reactions, particularly in the eyelid area.[4,5,9] Facial skin is highly permeable, due to a thinner stratum corneum and a greater density of appendages (e.g., sweat glands, hair follicles). Moreover, facial skin contains an elaborate network of sensory nerves. The frequency of cosmetic application is also increased at this body site. Although mild inflammatory changes are often masked on the face, in the event that eruptions do occur, they are readily noticed by the consumer.

38.4 CLASSIFICATION AND ETIOLOGY

Skin reaction to moisturizers and other cosmetic products have a varied differential diagnosis, challenging the clinician's ability to pinpoint the underlying cause. Indeed, sensitive skin is often regarded as a complex multifactorial syndrome, rather than a single entity. Maibach and Engasser coined the term "cosmetic intolerance syndrome" to describe this heterogeneous syndrome, whereby certain susceptible individuals cannot tolerate a wide range of cosmetic products.[9,10]

Fisher coined the term "status cosmeticus," a condition in which every cosmetic product applied to the face produces itching, burning, or stinging, rendering the sufferer incapable of using any cosmetic product.[11] The patient with status cosmeticus typically has a clinically unremarkable presentation. They may have a mild malar erythema with slight edema of the eyelids. Sometimes this is accompanied by a follicular eruption. The mild clinical picture usually contrasts vividly with the patients' bitter complaints of burning or stinging sensations. The history usually includes "sensitivity" to innumerable cosmetics, while patch test and "use" test results using various implicated products will be negative. Status cosmeticus may be considered to be at the extreme end of the spectrum of sensitive skin. In practice, the term "status cosmeticus" is only applied to a patient who has undergone a thorough workup and other diagnoses have been excluded.

When dealing with patients with sensitive skin, the following differential diagnoses have to be considered (see Table 38.1), thus permitting a rational approach.

38.4.1 Exogenous Causes

38.4.1.1 Subjective Irritation

Subjective or sensory irritation is defined as chemically induced burning, stinging, or itching sensations without detectable visible or microscopic changes.[2,9] This reaction commonly appears within an hour of application in certain susceptible individuals (known as "stingers") and is usually transient, lasting minutes. Ingredients that cause this reaction may not generally be considered objective irritants and will not cause abnormal responses in nonsusceptible persons (nonstingers). This nonspecific reaction is probably grossly under-reported, due to its transient nature. Furthermore, in specialized skin sites, such as the face and scalp, subtle inflammatory changes are often masked.

Subjective irritation is believed to be the most common cause of sensitive skin and cosmetic reactions.[2] Propylene glycol, butylene glycol, and hydroxy acids are examples of subjective irritants present in modern-day cosmetics.[2,12] Alcohol is also capable of causing subjective irritation, but is

TABLE 38.1
Etiology of Sensitive Skin: Differential Diagnosis[9]

Exogenous	
Subjective irritation	Common; acute onset; burning, stinging, itching within minutes of application
Objective irritation	Common; morphologically difficult to differentiate from ACD, diagnosis by exclusion
Suberythematous irritation	Burning, stinging, itching. Squamometry may show protein abnormality
Allergic contact dermatitis	Uncommon; diagnostic patch test essential
Photoallergic contact dermatitis	Uncommon; diagnosis by photopatch testing
Contact urticaria	Query patient about burning, stinging, itching. Diagnosis by immediate-type testing for wheal-and-flare reaction
Endogenous	
Seborrheic dermatitis	Rosacea Psoriasis
Atopic dermatitis	Common diagnoses, but small percentage have atypical morphology Æ difficult diagnosis
Dysmorphophobia	Rare; diagnosis of exclusion

not commonly known to cause objective irritation, whereas SLS, a strong objective irritant, does not usually cause stinging.[2] This suggests that subjective irritation is not just a mild form of objective irritation.

The precise mechanism of subjective irritation has yet to be determined. Insights concerning the mechanism of subjective irritation may be gleaned from the fact that local anesthetics block the response,[2] and stingers respond more vigorously to vasodilators.[13] The response of sensitive skin individuals was explored more in a study conducted by Issachar et al. This study examined the permeability of a vasodilator, methyl nicotinate, in sensitive and nonsensitive skin individuals specifically looking at its permeability. The study was designed to assess whether the barrier function of the skin is altered in sensitive skin. The study looked at 20 women divided into reactor and nonreactor groups based on their response to 10% aqueous solution of lactic acid. The vasodilatation of methyl nicotinate was measured by a laser Doppler perfusion imager (LDPI) every 5 min for 1 h after the methyl nicotinate was applied. This study revealed a significant difference between the reactor and nonreactor group. Reactors showed a significant increased intensity of response to the methyl nicotinate. This suggests that the correlation between increased penetration of methyl nicotinate and the skin response to lactic acid may be due to the increased penetration of water-soluble chemicals in individuals with sensitive skin.[14] Recent studies utilizing quantitative sensory testing methods, such as the thermal sensory analyzer (TSA), on antiinflammatory agents have provided insight into their action of cutaneous sensation.[15,16] Such studies with sensory irritants and their inhibitors may provide similar insight into the pathophysiology of subjective irritation.[2]

Before confirming a diagnosis of subjective irritation, patients must be patch tested and open tested to exclude allergic contact dermatitis and contact urticaria, respectively. Subclinical contact urticaria, in particular, mimics sensory irritation.

38.4.1.2 Objective Irritation and Nonerythematous Irritation

Objective irritation is defined as nonimmunologically mediated, localized inflammation of the skin, usually resulting from contact with a substance that chemically damages the skin.[2,9] The exact mechanism is unknown, and it is likely that both endogenous and exogenous factors are involved. *In vivo* predictive testing in animals (e.g., modified Draize test, repeated application patch tests,

guinea pig immersion test) and humans (e.g., cumulative irritation assay, chamber scarification test) can detect moderate to strong irritants, allowing manufacturers to eliminate these potential hazards prior to marketing a cosmetic.[17] Mild irritants, however, are more difficult to identify and are sometimes missed. Although irritation normally causes an erythematous reaction, dermatologists may have difficulty identifying low-grade inflammatory changes in the face. Careful examination of the facial area, aided by slight magnification, may be useful in unmasking the diagnosis. Patch testing to rule out allergic contact dermatitis is obligatory. Photoirritation or phototoxicity should also be considered. Some cosmetic products which cause irritant contact dermatitis are soaps and detergents, deodorants and antiperspirants, eye makeup, shampoo, permanent hair-waving products, and moisturizers.[9] It should be noted that many moisturizers contain surfactants and emulsifiers that are cumulative irritants, that is, mild irritants that produce inflammation only after repeated application.[9] This fact is frequently overlooked as moisturizers are commonly used in the treatment and prevention of irritant dermatitis.

Nonerythematous or suberythematous irritation is defined as a state in which the clinical observer sees no abnormality; the patient knows that there is something wrong and may describe it as burning, stinging, or itching. Charbonnier et al. have shown that objective alterations are present and are readily demonstrable by the technique of squamometry.[18] The latter utilizes protein staining and microscopic examination of stratum corneum tape strippings. The greater the protein abnormality, the greater the irritation. This is more discriminating than visual examination and current bioengineering technology.

Management of these patients is difficult as almost any chemical can be an irritant, depending on a host of factors, such as the concentration of the chemical, the mode of exposure, other chemicals in the formulation, and other environmental and constitutional factors.

38.4.1.3 Allergic Contact Dermatitis

Allergic contact dermatitis is dermatitis caused by prior exposure to an allergen leading to specific cell-mediated sensitization. It is classified as delayed-type hypersensitivity, as inflammation develops after a relatively long time interval following the exposure. Clinically, it manifests as a pruritic erythematous eruption, with papules and vesicles at the site of exposure. This is the simplest cause of sensitive skin, in terms of diagnosis and management. Patch testing is the diagnostic gold standard; all the patient's cosmetics and skin care items should be included in the patch testing procedure — it is not sufficient to patch test with the routine series.[9] As the most common cosmetic allergens are fragrances and preservatives, patch testing with the fragrance and preservatives series is imperative.[4,5] Lanolin, a naturally occurring wax emollient, is an uncommon albeit important cause of cosmetic allergy. In 2000, a study published suggested that sensitive skin was actually a subclinical expression of individuals contact allergy to nickel sulfate.[19] Note that many allergenic cosmetics are mild irritants under occlusion — this is a potential cause of false-positive patch test results.

Management of cosmetic allergy is relatively straightforward since the advent of cosmetic ingredient labeling. "Hypoallergenic" formulations of cosmetic products seem to be in vogue at present. Fragrance-free formulations are also available for the fragrance allergic — these are useful, as fragrance allergies are complex and difficult to isolate.

38.4.1.4 Contact Urticaria Syndrome

Most people think that the most common part of sensitive skin is sensory irritation. Contact urticaria syndrome (CUS) comprises a presumably smaller part and is a heterogeneous group of inflammatory reactions characterized by burning, tingling, itching, and a wheal-and-flare response that usually appear within minutes after contact with the eliciting substance.[20] These reactions are transient, disappearing within 24 h, with the majority fading within a few hours. In its more severe forms, generalized

urticaria and extracutaneous manifestations, such as respiratory or gastrointestinal symptoms and even anaphylaxis, may be experienced.[20]

Three mechanisms are implicated in CUS: immunologic (ICU), nonimmunologic (NICU), or uncertain mechanism.[20] ICU is a type I hypersensitivity reaction that is IgE mediated and is associated with atopy. NICU is the more common variety of CUS. NICU due to cosmetics is most commonly caused by fragrances (e.g., cinnamic aldehyde) and preservatives (e.g., benzoic acid and sorbic acid).[2] Parabens have been documented by passive transfer to cause ICU.[21]

Muizzuddin et al. recently studied contact urticaria in an attempt to define sensitive skin objectively.[22] Skin responsiveness was assessed using balsam of Peru, which induces NICU. They found that individuals with self-assessed sensitive skin were more susceptible to NICU. This group was also more susceptible to stinging induced by lactic acid and stratum corneum barrier removal using tape stripping.

Diagnosis of CUS involves a high index of suspicion and appropriate open testing for "immediate" onset lesions. These are easily missed on the face, and careful observation is required.

38.4.1.5 Photosensitivity Reactions

Photosensitivity reactions are adverse cutaneous responses to the synergistic actions of a chemical agent and ultraviolet light.[23] Photosensitivity reactions may be broadly categorized into phototoxic reactions and photoallergic reactions. Phototoxic reactions may be experienced by any individual under appropriate conditions (i.e., appropriate wavelength of ultraviolet radiation and sufficient concentration of phototoxic chemical), while photoallergic reactions are delayed-type immunologic reactions requiring a period of sensitization.[23,24] Photopatch testing is an invaluable diagnostic tool for photoallergic contact dermatitis. This is a modification of the basic patch test procedure — patch test sites of the suspected substance(s) are applied in duplicate; one site is irradiated with ultraviolet light, and the results are compared to the nonirradiated site. A stronger reaction in the irradiated site suggests photoallergy.

Cosmetic products that are photoallergenic include fragrances, such as musk ambrette and 6-methylcoumarin, and sunscreens (e.g., para-aminobenzoic acid and its derivatives, benzophenones, dibenzoylmethanes).[17] Oil of bergamot, previously a popular ingredient in fragrances, has now been eliminated from most perfumes due to its phototoxic properties.[24]

38.4.2 Endogenous Causes

These include atypical or subtle manifestations of dermatologic conditions such as seborrheic dermatitis, rosacea, psoriasis, atopic dermatitis, and ichthyosis. Classic manifestations of such diseases are diagnosed with relative ease. However, diagnostic difficulty arises in the presence of atypical morphology, lesions masked by topical therapy (e.g., corticosteroids), or exacerbations due to other topical agents (e.g., skin care products).[2,10]

A thorough clinical review sometimes directs the clinician to the correct diagnosis. In other cases, time is required for other stigmata to surface. Appropriate diagnostic testing and a prolonged cosmetic elimination program should be implemented in the first instance, but if all else fails, therapeutic trials may be indicated. Topical corticosteroids may sometimes prove useful to break a cycle of cosmetic intolerance syndrome.[10]

Another factor to consider is that patients with endogenous skin disease are frequently more susceptible to cosmetic reactions. One reason is that patients with preexisting skin disease may have skin barrier dysfunction, with consequent increased permeability. Skin hyper-reactivity in atopic patients, particularly, has been gathering interest in recent years. Epidemiologic associations between atopic dermatitis and irritant dermatitis are now supported by skin bioengineering data.[25]

Certain substances have been reported to affect eczematous skin, but not normal skin. One example is parabens, a popular preservative that may sensitize eczematous skin, but rarely causes

reactions in normal skin.[17] Fisher has termed this phenomenon the "paraben paradox."[11] Likewise, lanolin, a popular emollient, is an important sensitizer when applied to eczematous eruptions, particularly stasis dermatitis, but rarely affects individuals with normal skin.[17]

38.4.3 Dermatologic Nondisease

Cotterill used the term "dermatologic nondisease" to describe a group of patients who presented with significant skin symptomatology, but no significant objective skin pathology on examination.[26,27] In his experience, the majority of patients were females and middle-aged people. Burning, itching, or discomfort were the most frequent complaints, and these were most often experienced in the face, scalp, and perineum. Other features that may be present include a preoccupation with imagined excessive facial hair, imagined excessive hair loss, and orodynia. Cotterill found that these patients were commonly depressed, sometimes with suicidal ideation, and often suffered from dysmorphophobia or a disturbed psychological body image. Management of these patients is a delicate matter, as they often react badly to referral to a psychiatrist. These patients also largely fail to respond to any topical or oral therapy, and a placebo response is never seen. When associated with depression, systemic antidepressant treatment may be attempted, but is generally ineffective.

38.5 DIAGNOSTIC TESTS FOR SENSITIVE SKIN

Measurement of differences in skin reactivity or "sensitivity" among individuals plays an important role in the workplace, as well as in the manufacture of safe topical therapeutics and cosmetics. Outlined in the following sections are objective and subjective methods of quantifying the reactivity of human skin to chemicals, which are potential irritants.[6] The experimental basis of such testing is to quantify the differences among individuals to chemicals that produce characteristic responses using a standard reproducible procedure. Individuals classified as hyper-reactors (sensitive skin) and hyporeactors can then be identified.

38.5.1 Objective Methods

1. Ammonium hydroxide blistering time[6] — This test measures the permeability of the stratum corneum barrier, the rationale being that the time taken to raise a blister is a function of the number of cell layers in the horny layer. An aqueous dilution of concentrated ammonium hydroxide is placed in a small plastic well, which is subsequently covered with a glass slip. Careful observation using a magnifying lens is then carried out until a tense blister forms in the well. Tiny vesicles initially appear, and formation of a full blister usually takes a few minutes; the time taken for the full blister to form is known as minimal blistering time (MBT). Lower values of MBT correspond to skin that is more reactive.
2. Dimethyl sulfoxide (DMSO) test[6] — This test measures the diffusional resistance of the horny layer. Equal quantities of three different concentrations of DMSO are applied to three plastic wells for 5 min. DMSO provokes wheeling in human skin. The wheals are scored 10 min after removal of the test fluid using a scale. Individuals with high reactivity are those susceptible to wheeling with the lowest concentration of DMSO.
3. Sodium lauryl sulfate (SLS) test[6] — The SLS attacks the horny layer, making it more penetrable to chemicals and also causing inflammation. Thus, it measures both the horny layer barrier and tissue reactivity to toxic substances. Aluminum chambers are filled with 0.1 ml of 1 and 2.5% aqueous solutions of SLS. The chambers are then applied to the ventral forearm for 24 h. The reactions are scored 3 h after removal of the chambers on a scale. Those reacting strongly to the lower concentration of SLS are deemed more reactive or sensitive.

38.5.2 Subjective Methods

Subjective responses are nerve-mediated sensory responses such as burning, stinging, itching, or pain that may be experienced in varying intensities, but do not induce visible changes that can be perceived by an outside observer. Semiquantitative methods of assessment have been devised to measure such responses.

1. Chloroform–methanol pain threshold[6] — A 0.1 ml solution of equal parts chloroform and methanol (CM) is placed in a plastic well. This mixture rapidly induces sharp pain. As soon as the subject perceives this pain, the fluid is removed and the elapsed time is recorded. Highly sensitive individuals experience pain induced by CM more rapidly than less sensitive individuals.
2. Lactic acid sting test[6] — This test, devised by Frosch and Kligman in 1977,[28] utilizes the subjective irritant, lactic acid, and remains the most popular test for subjective irritation. Lactic acid, as well as a number of other substances, will induce a sharp stinging sensation without overt inflammation in a number of susceptible individuals, known as stingers. Stinging potential of a substance is not strictly related to its objective irritancy. The subject is placed in a hot, humid environmental chamber until profuse sweating is achieved. Then a 5% solution of lactic acid is rubbed over the nasolabial folds and cheeks with a cotton-tipped applicator. The stinging sensation is scored on a scale at 10 sec, 2.5 min, and 5 min.

The methods for assessing reactivity previously outlined are simple, convenient, inexpensive, and noninvasive or minimally invasive.[6] However, cutaneous reactivity depends on many factors. None of the previous methods give a full picture of the characteristics of sensitive skin, only susceptibility of skin to irritants. Subtle manifestations of endogenous cutaneous conditions must still be clinically excluded. Exclusion of allergic contact dermatitis must still be performed by patch testing, exclusion of contact urticaria by open tests or PUT/ROAT, and exclusion of photoallergy by photopatch testing.

One experiment conducted by Giacomoni et al. used the lactic acid sting test on two different cohorts divided into a sensitive and nonsensitive skin group. Volunteers were asked to grade the intensity of their irritation as nil, mild, moderate, or severe (scored as 0, 1, 2, or 3). For more than 80% of the nonsensitive skin volunteers, the score of intensity was a 0 or 1. In the sensitive skin group, 75% of volunteers scored their intensity as a 2 or above 2.[30]

38.6 PATHOPHYSIOLOGY

As the definition of sensitive skin is controversial and its etiology is thought to be heterogeneous, it follows that the pathophysiology of sensitive skin is thus far incompletely defined and probably embraces an array of mechanisms. Clinical manifestations such as contact dermatitis in a hyper-reactor probably have the same mechanisms as in a "normal" person with the same dermatoses. However, some general features causing enhanced reactivity have been identified in individuals with sensitive skin. Increased susceptibility to irritation from exogenous substances may be due to inherent structural features of the skin, for instance, hyper-reactors may have a thinner stratum corneum with a reduced corneocyte area,[31] thus allowing a higher transcutaneous penetration of water-soluble chemicals.[32] A heightened neurosensory input in subjects with sensitive skin, corresponding to an augmented response to cutaneous stimulation, may also lower the threshold to irritant stimuli.[33] Release of a different makeup of inflammatory mediators, which may alter the inflammatory response, has also been implicated in individuals with sensitive skin.[34]

Evidence to support the idea that sensitive skin has a thinner stratum corneum is an experiment using stripping and TEWL. Stripping of the stratum corneum was done by the application of sticky tape and its removal. Sticky tape was applied and removed, taking the upper layers of the stratum corneum. Each time this was done, the TEWL was measured. The average number of tape

strippings necessary for doubling the TEWL was about 10 for sensitive skin while it took about 20 for nonsensitive skin.[30]

One study examining the inflammatory mediators revealed that compared with normal skin, prostaglandin E_2 was increased approximately 3.8-fold ($p < .0002$) in sensitive skin compared to normal skin. Leukotriene B_4 and interleukin-1* showed no differences between normal and sensitive skin individuals.[35] Progress has been made in establishing the pathophysiology of sensitive skin; however, a great deal of work remains to be done in this field.

38.7 SKIN BIOENGINEERING AND SENSITIVE SKIN

Studies on sensitive skin are often performed using subjective self-assessment — this naturally yields results of variable reliability. The current trend is striving toward identification of more objective biophysiological measures of skin sensitivity. Today, skin bioengineering studies are employed to investigate the correlation of various biophysiological parameters with skin reactivity, thereby also conveying some insight into its mechanisms.[29] The advantages of bioengineering instruments are that they are quantitative, noninvasive, and detect subtle changes that would otherwise be undetectable to the naked eye.[36] Examples of bioengineering techniques used to evaluate the pathophysiology of skin reactivity include transepidermal water loss (TEWL), skin conductance, resistance, impedance, blood flow velocity, and skin pH (Table 38.2).[17] Descriptions of these methods may be found in the textbooks of Berardesca et al.[37] and Elsner et al.[38]

Determination of basal biophysiological parameters may identify subjects with sensitive skin. Earlier studies have shown that increased skin susceptibility has been correlated with an increased basal TEWL,[39–41] skin surface pH,[42] and fair skin complexion (measured by chromametric L* values),[43] whereas no relationship was shown for basal skin thickness, skin blood flow, sebum excretion, and skin hydration.[29] However, a recent study by Seidenari et al. utilizing multiple bioengineering techniques showed significant correlations only for capacitance and colorimetric a* values.[44]

Individuals with sensitive skin often have associated dry skin. In a recent study of subjects with sensitive hands, no difference in skin hydration was seen macroscopically between normal subjects and sensitive hand subjects (who had self-perceived dry skin). However, measurement with the corneometer confirmed reduced skin surface moisture in the group with sensitive hands, and D-squame analysis showed greater loss of cohesiveness between corneocytes harvested from the

TABLE 38.2
Bioengineering Methods and Biophysiological Parameters in Sensitive Skin Research[30]

Method	Biophysiological parameter
Evaporimetry	Transepidermal water loss (TEWL)
Colorimetry/chromametry (CIE system)	L*: skin reflectance
	a*: red/green axis
	b*: blue/yellow axis
Laser doppler velocimetry	Skin blood flow
Ultrasound	Skin thickness (edema formation)
pH meter	Skin pH
Corneometer (electric capacitance)	Stratum corneum hydration
Sebumeter	Sebum excretion

sensitive hands group.[45] In this study, no correlation was found between sensitive hands and TEWL or skin redness.

The results of the latter two studies described contradict earlier data. Up to this point, an elevated TEWL had been the most widely accepted biophysiological parameter associated with sensitive skin, due to impairment of the skin barrier function or composition. That further studies are imperative to create a consistent and objective operational definition for "sensitive skin" is affirmed by these conflicting results.

38.8 MANAGEMENT OPTIONS

The first step in management is to identify the causative ingredient(s), as well as the causative mechanism, if possible. As sensitive skin is often multifactorial, the approach to the patient should cover the range of differential diagnoses. Thus, starting with a complete history and examination, clues derived from clinical suspicion should aid in devising a plan for diagnostic testing (Table 38.3).

A thorough history for burning, stinging, and itching identifies sensory irritation and possible CUS. The history should include careful questioning of all topical products applied, as well as the time of onset in relation to exposure. Personal and family history of atopy should be actively sought, as should other skin conditions such as psoriasis and rosacea. A meticulous physical examination may identify other stigmata of atopic dermatitis, psoriasis, or other skin disease.

Patch testing (and photopatch testing) should document the few cases due to allergic contact dermatitis. Apart from the routine battery, testing should be performed with the fragrance and preservative series, as well as any cosmetic or skin care product that the patient uses. Immediate-type testing should be performed if indicated by the history. If systemic symptoms were present, perform only in the presence of emergency resuscitative facilities.

If an ingredient is identified as the causative agent, then avoidance of the ingredient is advised. If, however, no substance is identified or the patient reacts to a wide range of substances, then a prolonged cosmetic elimination program may be considered.[9,10] The patient will be barred from using most cosmetic products for a period of 6 to 12 months. For the duration of the cosmetic elimination program, no soaps or detergents or moisturizers are allowed. Glycerin and rose water may function as a substitute for commercial moisturizers. Lip and eye cosmetics may be used freely if no problems are identified in these areas. Face powder may also be used. After the allotted time,

TABLE 38.3
Management of Sensitive Skin[9]

1. Clinical review: history and physical examination
2. Examine every cosmetic and skin care product
3. Patch test and photopatch test — to rule out contact and photocontact allergy
   Immediate-type testing — to rule out contact urticaria
4. Avoid causative ingredient, if identified by testing.
5. Treat any endogenous inflammatory disease.
6. Cosmetic elimination program — 6 to 12 months.
   Avoid all cosmetics apart from:
   - Lip cosmetics
   - Eye cosmetics
   - Face powder
   - Glycerin and rose water

   After 6 to 12 months, gradually reintroduce one product every one to two weeks
7. Be alert to depression and other neuropsychiatric conditions

gradual reintroduction of one cosmetic product will be instituted periodically, for instance, one product every 2 weeks, so that in the end, a simple skin care regime is devised for the patient.

One study conducted by Hawkins et al. looked at the benefits of mild cleansing, moisturizing, and sun protection as a way to improve skin health and quality. In three groups, those with normal skin, sensitive skin, or dermatologist-assessed highly sensitive skin (mostly rosacea with atopic background in some cases) this skin care regiment showed significant improvements in skin health and quality assessed by expert assessments, instrumental evaluations, and subjective self-assessment.[46] Proper skin care may help some individuals with sensitive skin; however, the lack of a control group in this study makes the results vulnerable to interpretation. In another noncontrolled study, it was concluded that adequately formulated cosmetics (sterile preservative, emulsifier, and perfume-free) may reduce both irritation and sensitive skin. It is also felt that this type of cosmetic could also clinically improve dryness, erythema, and stinging.[47]

Another study conducted looking at menopausal women on or without HRT therapy examined the response if the individuals stratum corneum to variations in environmental humidity, either in air or in response to an emollient. Data showed that the baseline stratum corneum hydration is decreased by a low dew point; however, both HRT and emollients improves the functional properties of menopausal women's skin and can counteract some of the deleterious effects of cold and dry weather.[48]

38.8.1 Hypoallergenicity

Because up to 40% of the population claim to possess sensitive skin,[49] numerous cosmetic and skin care items have been formulated to be "hypoallergenic" or literally "reduced allergy." These are products designed for an individual with sensitive skin. Marketing claims for hypoallergenicity are based on objective tests performed on these products, such as the guinea pig maximization test, repeat insult patch test, cumulative irritancy test, chamber scarification test, photopatch test, and facial sting test (lactic acid test), as well as postmarketing surveillance programs.[50] However, no formal criteria exist for evaluation of hypoallergenic products. Draelos and Rietschel demonstrated the ambiguity of the term "hypoallergenicity" in a recent study.[50] Although 75% of dermatologists believed that the concept was relevant to their clinical practice, dermatologist's perceptions of the hypoallergenicity claim was varied. Most believed that hypoallergenicity embodied skin irritation (72.6%) and contact allergy (87.9%), while opinions were divided over subjective irritation (59.8%), contact urticaria (46.4%), photomediated responses (31.5%), and acne (23.4%). A similar ambiguity exists among cosmetic manufacturers: some hypoallergenic products are low in certain allergens, others are low in certain irritants; some are preservative free; some are fragrance free — the diversity is potentially endless. This is an issue that clearly needs to be addressed.

38.8.2 Antiirritants

Goldenberg and Safrin suggested that the sensory effects of topical irritants may be neutralized by "antiirritants."[51] They proposed three possible mechanisms of action of antiirritants: complexing of the irritant, blocking the reactive sites in the skin, and preventing physical contact with the skin. The main antiirritant cosmetic chemicals are imidazole, hydroxy, and carboxyl compounds. Studies of the safety and efficacy of these antiirritants in cosmetics are ongoing.[11]

38.9 SUMMARY

Sensitive skin is not a single entity, but a heterogeneous syndrome, puzzling both consumer and clinician alike. The definition remains obscure, and so it follows that prevalence and pathophysiology are as yet undetermined. Innovative skin bioengineering techniques have opened up new avenues for

sensitive skin research. Such studies are still in their infancy and continue to be published — this will undoubtedly shed new light on the topic.

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39 Stinging and Irritating Substances: Their Identification and Assessment

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CONTENTS

39.1 INTRODUCTION

This chapter explores the assessment of the irritant potential of cosmetic ingredients and products and details a number of methodologies available, chiefly in human volunteers. The practicalities associated with conducting studies using human volunteers are also examined. To try to increase

the practical utility of the material, rather than present a generic review, focus is on the general strategy and methods employed in our own laboratory. It should be made clear at the beginning that all assessments should be made on a case by case basis; the methods are not to be slavishly applied to the safety assessment of all new moisturizers.

There are two main aspects associated with skin irritation, the physical manifestation resulting from damage or perturbation to the skin barrier, for example, erythema and dryness and that which cannot be seen, but is sensory in nature, for example, stinging and itching. Generally, skin irritation is a transient response and once the irritant stimulus has been removed the skin repairs very swiftly and normal condition restored within a few days. Human volunteers are generally used in skin irritation studies on formulated products, but a sound safety assessment must be performed to ensure that there is sufficient knowledge and assurance on the ingredients for the key toxicological endpoints of concern. Testing in humans allows both the physical and sensory aspects of skin irritation to be examined simultaneously in the species of interest; a significant advantage over animal testing.

39.2 REGULATORY REQUIREMENTS FOR TOXICOLOGICAL TESTING

The majority of moisturizers are manufactured with a view to providing cosmetic benefits, chiefly to maintain the skin in good condition and sometimes also to perfume it. The primary objective of cosmetic product safety legislation is to safeguard consumer safety. In the European Economic Community in the 1970s it was evident that there were considerable differences in the requirements amongst the Member States. In 1976, Council Directive 76/768/EEC,[1] on the approximation of the laws of the Member States relating to cosmetic products, introduced harmonized cosmetic safety legislation into the European Community. Article 2 states that "a cosmetic product put on the market within the Community must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use." Article 7a states that "the manufacturer shall take into consideration the general toxicological profile of the ingredient, their chemical structure and their level of exposure."

The directive does not provide any detail about the types of methodologies that may be used to assess the toxicological profile of the ingredient. In this respect, the Commission's Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) guidance notes,[2] provide some assistance on the testing and safety evaluation of cosmetic ingredients. The 7th amendment to the Directive (2003/15/EC),[3] introduced an animal testing ban on finished products and prototypes from September 2004 and on cosmetic ingredients no later than March 2009. The ban covers animal testing for the majority of toxicological endpoints, including skin irritation.

Irrespective of the intended use of a chemical, be it cosmetic or otherwise, European legislation[4] governing the safe use of chemicals requires a "base set" of toxicity data for any chemical.[5] This directive is designed to guarantee adequate protection for humans and the environment against the potential risks of chemical substances. For the endpoint of skin irritation, traditionally the rabbit[6] has been used to determine whether a material should be classified as R38 Irritant. To date, there are no validated *in vitro* methods for the assessment of skin irritation, but for the purposes of making a judgement on the irritant potential of cosmetic ingredients and formulations there are a number of non-animal methods that may be used as part of a tiered testing strategy.

39.3 SOURCES OF SKIN IRRITATION DATA

Before any practical work is carried out, all possible sources of information on skin irritation should be tapped, including manufacturers data on raw materials. However, this is often no more than basic Draize data from a regulatory rabbit study and is unlikely to be of great utility for safety assessment. In addition, the safety assessor must be aware that the skin irritation potential of a formulation is not

a direct summation of the irritation potential of the ingredients. Other sources of information on the skin irritation potential of individual chemicals include the following.

39.3.1 In *Silico* Models

Only a handful of (Quantitative) Structure Activity Relationship ((Q)SAR) models have been developed in recent years to predict the skin irritant potential of chemicals. The most extensive work appears to be that by Enslein[7], other workers have focused on smaller datasets comprising specific chemical classes such as esters, alcohols, or acids. The real predictivity of all these tools is not fully characterized. Further discussion/details have been published elsewhere.[8,9]

DEREK (deductive estimation of risk from existing knowledge) for Windows contains 25 chemical structural alerts associated with skin irritation.

Hazard Expert contains a number of alerts for skin irritation.

TOPKAT comprises 13 QSAR models and data from which these models are derived (rabbit skin irritation). Compounds and data were collected from national and international journals as well as U.S. government sources and total some 1258 compounds. The chemicals are grouped into five class specific models, heteroaromatic and multiple benzenes, alicylics, single benzenes, and two classes of acrylics. Each model applies to a specific class of chemicals and is further subdivided into two or three groups based on severity.

BgVV The BgVV database has been used to develop specific SAR models for predicting skin irritation and corrosion. These models have been incorporated into a decision support system (DSS). The DSS is mainly a rule based approach, the rules being developed are not only based on substructural features but additionally incorporate specific physicochemical properties such as Log P, molecular weight, and aqueous solubility. The rules have been developed and validated on a total of 1508 compounds of which 199 are classified as hazardous. The DSS is designed to predict EU risk phrases.

39.3.2 *In Vitro* Methodologies

There is currently very little information in the literature regarding the mechanisms of skin irritation. Work performed in-house has investigated the genomic and proteomic profiles of known skin irritants (SLS, benzalkonium chloride, and phenol) applied to EpiDerm™ (3D human skin model supplied by MatTek).[10] These investigations have indicated that signaling pathways are upregulated at an early stage upon treatment with an irritant and thus genes within these pathways could be utilized as potential (early) biomarkers. Different irritants upregulated different genes within an individual pathway. Proteomic analysis of the different irritants have indicated that they appear to operate via different mechanisms of action as there were a very limited number of proteins, which were changed with all the irritants.[11,12] These technologies provide a method of obtaining a global view of the changes, which occur within a given cell or tissue making them very useful for investigating mechanisms of action, which could be combined with the use of 3D human skin models and human volunteer studies.

Currently there are a number of 3D human skin models commercially available, for example, EpiDerm™ (MatTek Corporation, USA), EPISKIN™ (EPISKIN SNC, France), and SkinEthic™ (SkinEthic Laboratories, France). These models have been validated (or are undergoing validation) for the identification of potential skin corrosivity of test materials (EU Annex V method B40 and OECD guideline 431).[13] All the manufacturers provide suggested protocols for the identification of skin irritants, but at present there are no *validated* protocols or prediction models for skin irritation. However, an ECVAM skin irritation validation trial, including the EpiDerm™ and EPISKIN™ models, has recently started (completion due end 2005). The protocols being assessed use a short exposure period to test material (15 min), followed by a model-dependent recovery incubation in the absence of test material. An in-house protocol using EpiDerm™ (similar to that being used in the validation trial) was previously identified and the in-house performance assessed by comparison

with the results of human 4 h occluded patch tests. Irritant materials could be identified, but there was over prediction of borderline materials. Although these assays are primarily being examined for hazard identification, they have the potential for looking at potency as dose-responses can be examined. These assays currently rely on cytotoxicity (MTT assay) as the major end-point (although SkinEthic™ recommend morphology and cytokine measurement as well). In future, skin model assays will need to benefit from the identification of more meaningful endpoints (such as may be identified by studies of skin irritation mechanisms described earlier) to enhance their sensitivity. Further development of the skin models to include all relevant cells is also required; the current models consist of a keratinized epidermis only, although MatTek are piloting a so-called full-thickness model with a dermal-like layer containing fibroblasts.

39.4 HUMAN VOLUNTEER TESTS

Prior to embarking on a skin irritation study in the human volunteer, a full safety assessment must be conducted by a suitably qualified and experienced individual on all ingredients and formulations to be tested.[14] This is of paramount importance to safeguard the health of the participating volunteers through avoidance of adverse health effects. The study must also receive the approval from an Ethical Review Committee and the study must be conducted in accordance with the Declaration of Helsinki (1964) and subsequent revisions.[15]

As part of the safety assessment the assessor must consider the chemical structure and all the available information regarding the toxicological profile of the material. The decision to proceed with human testing should only be made if the information available provides sufficient confidence that the volunteer will not be exposed to undue risk. Key toxicological endpoints of concern for moisturizing products include skin corrosivity, percutaneous absorption, genotoxicity, skin sensitization, skin irritation, systemic toxicity, reproductive toxicity, and phototoxicity.

At the very least, to support limited topical exposure, for example, single application patch test in the human, genotoxicity data are required (*and a favourable judgement on the sensitization and corrosive potential of the material*). The following types of study may be performed using human volunteers.

39.4.1 Acute Skin Irritation

Many of the methodologies involve the assessment of visible signs of irritation, for example, erythema and dryness following a short topical exposure to a material. The assessment may be subjective, made by a trained skin assessor, or quantitative using one of the many bioengineering tools available. For example, damage to the stratum corneum may be assessed using a transepidermal water loss (TEWL) meter and skin dryness by measuring electrical capacitance. However, dermatopathological signs may be useful prequels to visible irritation. A number of cytokines (IL-1$\propto$, IL-1RA) and chemokines (IL-8) are upregulated early in skin inflammation. *Cytokine profiling* may therefore be used to gain useful information on the potential of a material to cause skin irritation. Punch biopsy and blister formation techniques may be used to sample skin for inflammatory mediators; however, both are invasive, with the procedures themselves inducing cytokine production. Tape stripping an area of skin previously exposed to test material and extracting cytokines from the tape has also shown upregulation of inflammatory mediators and is a simple, noninvasive procedure.[16]

Metabonomics (the quantitative measurement of time-related responses to stimuli within the body)[17,18] may prove to be of some use to assess the potential of a material to elicit an irritant response following topical application. The concept being that certain stimuli change the metabolite profile in intermediate biochemical pathways. Analysis of body fluids such as urine, saliva, plasma, biopsy material, etc. produces a "fingerprint" of biochemical changes characteristic of the nature or site of a toxic (or other) effect.

The *4 h occluded patch test* was developed in an effort to reduce animal testing and to obtain more relevant acute skin irritation data. The test is designed to evaluate the potential irritancy of undiluted materials such as detergents, as required by the laws governing labelling of such materials. It is based on the four hour rabbit patch test defined by Annex V of the EEC Dangerous Preparations Directive (DPD ref. 88/397/EEC).[19–21] In April 1997, the OECD issued a draft guideline based on this methodology, but it is yet to be accepted.

A small area of the outer surface of the upper arm is exposed to test and control materials under occlusive cover. The test and control materials are applied to round chambers and taped to the arm with surgical tape. A standard irritant (20% sodium dodecyl sulphate [SDS]) is used as the positive control. Each panellist has up to four patches applied, each patch being applied for an increased duration. Due to the potential irritancy of the test materials a cautious approach is used. The first patch is applied for one hour, the second, third, and fourth patches for two, three, and four hours, respectively. Each patch is applied to a different site on consecutive weeks. This approach allows any unexpected or unacceptable reactions to be limited to a minimum.

Any panellist that develops a positive reaction (defined by an in-house scoring system) to any material, is not retreated with that material at the following exposure(s). All other materials are tested for up to 4 h. If the number of skin reactions to the test material is similar or greater than the control material, then this would indicate that the material should be classified as R38-Irritant.

A *covered patch test (*2×23 *h)* may be conducted to generate information on the skin irritant potential of the test material (as a single ingredient or in formulation) against a control material(s), which when appropriate might be a marketed formulation whose acceptability in the market place is well established. Patch testing, the diagnostic version of which dates back to the late 19th century, is a scientific procedure now widely used by dermatologists to identify materials causing allergic contact dermatitis in a patient. The method can also be used in a predictive capacity to investigate the potential of a product or ingredient to cause irritant contact dermatitis. Although there are a number of variations on a general theme, one typical approach uses a small area of the outer surface of the upper arm, which is exposed to test and control materials under occlusive cover. 50 μl of the test or control materials are applied onto chambers mounted on tape and placed on the upper outer arm. A standard irritant, for example, 0.3% sodium dodecyl sulphate is used as the positive control and water as the negative control. For comparative purposes, a product with a similar formulation and with a satisfactory market history is normally included in the test. Twenty-three hours after application the patches are removed. If no significant irritation is apparent, identical and freshly prepared patches are then reapplied to the sites, removed 23 h later and assessed again one hour later for irritation. A further assessment is taken 24 h after this assessment to monitor recovery.

Patch testing is an artificial procedure, which ensures that the product is in close and constant contact with the skin for approximately 48 h and simulates a worse case exposure scenario. Using the information from the patch test on the new product's irritation potential, a risk assessment for the product can be made; this will take into consideration its intended use at realistic levels and times of exposure. Additional tests that more closely simulate the intended use of the product may be required to confirm the risk assessment.

39.4.2 Cumulative Skin Irritation

Simulated use tests

These are conducted to gain information on the cumulative irritancy of a product. This type of test is designed to mirror the intended use of the product, but exposure may also be exaggerated, to provide a greater margin of safety in the risk assessment on the product and also to provide information on problems that may be encountered should the product be misused. Some methods are designed to simulate the normal use of products, with controlled exposure. The skin irritation is monitored and comparisons made between the test and control product in the same panellist. The controls are

usually similar products that are already marketed and have an acceptable market history. A risk assessment can then be made, which can be extrapolated to support the market place.

The *elbow test* involves applying products to the inside of the elbow up to six times per day for three weeks. This is a sensitive area of skin, and easily defined, which is important since this is a self-application test. This test is useful for products such as body lotions, etc., and also as a preliminary to a face test, where the skin is more sensitive. Each panellist serves as their own control; the test material is applied to one elbow and the control material applied to the other. The panel is balanced according to sex, hand dominance, and initial skin grades of reaction. One half of the panel has the dominant hand allocated to the test material, and the other half has their dominant hand allocated to the control material. The levels of irritation elicited by the test and control treatments are compared. Subjective comments are also taken into consideration. At intervals throughout the treatment period, each site is assessed for visible signs of irritation, for example, erythema and dryness.

The *volar forearm* is a fairly sensitive area of the body as it does not receive a great deal of exposure to the sun. Up to three different products can be tested on each arm and are applied up to six times per day for three consecutive weeks, with the sites assessed at regular intervals by trained skin assessors.

In use tests represent a very valuable tool in the assessment armory. Many personal care products are designed for frequent skin contact, often with very sensitive areas of skin (e.g., the face or underarm). Materials such as face creams and deodorants, etc. must therefore be evaluated for their irritation potential to ensure that they are safe for normal use. A use or exaggerated use test provides data on which a safety assessment can be made. A test material is compared with a control, usually a material of similar formulation that is already marketed, and has an acceptable market history. The frequency of exposure may be exaggerated to maximize the sensitivity of the test. For the duration of the test, panellists are provided with test and control materials and a treatment card to record the daily use of each material. The materials issued at the start of the test are weighed before and after the test, so that the amount of material used can be calculated.

Full-face test. A panel of 60 healthy adults is recruited. 20 panellists are provided with the test material, 20 with control material, and 20 act as untreated controls. The 60 panellists are balanced according to sex, hand dominance, and initial skin grades of reaction. Panellists are asked to apply the materials to their forehead, cheeks, nose, chin, and neck after washing at least twice a day for 21 days. The levels of irritation in each of the three panels are compared to assess the irritancy of the test material relative to the control and the untreated groups. Subjective comments are also taken into consideration. At intervals throughout the study, the face is assessed for the standard parameters, primarily for erythema and dryness at six sites (forehead, right cheek, left cheek, nose, chin, and neck). All relevant panellists comments are recorded and considered in the final evaluation.

Half-face test. A panel of 24 healthy adults is recruited. The panel is balanced according to sex, hand dominance, and initial skin grades of reaction. Twelve panellists apply the test material to the right side of the face and 12 apply control to the right side of the face. The test material is applied to one half of the face and neck and the control material is applied to the opposite side of the face and neck at least twice a day for 21 days. The levels of irritation elicited by the test and control treatments are compared to assess the irritancy of the test material relative to the standard. Subjective comments are also taken into consideration. At intervals throughout the study, the face is assessed for the standard parameters, primarily for erythema and dryness at ten sites (left and right sides of the forehead, cheek, nose, chin, and neck). All relevant panellists comments are recorded and considered in the final evaluation.

39.4.3 Sensory Irritation

Cosmetic and detergent-based materials such as face creams, shower gels, deodorants, etc. are specifically designed for frequent skin contact, even to very sensitive areas of the body. An individual may experience a wide variety of skin reactions to a topically applied material. These reactions are not always visible, for example, erythema or dryness and may be subjective reactions such as stinging,

burning, or itching. Visible signs of skin irritation may not always parallel the subjective effects of a material. It is therefore important that the sensory effects of new or modified materials can be assessed to ensure that they are acceptable for use in the market place.

Stinging is a problem that occurs primarily (but not uniquely) on the face, particularly on the nasolabial folds. The extreme sensitivity of this region is a reflection of its microanatomy, including a more permeable horny layer, a high density of sweat glands and hair follicles, and an elaborate network of sensory nerves. The sensory perception test is designed to detect materials, which cause adverse sensory effects (primarily stinging) within minutes of application, and is similar to that described by Frosch and Kligman.[21] The test involves applying small quantities of material to the nasolabial folds and surrounding area. This site is chosen partly due to its sensitivity, but also because any responses obtained almost invariably remain mild and transient, with little or no visual effects. This latter point is of obvious importance when conducting tests on the face.

Individuals are classified as "stingers," "nonstingers," and "inconsistent," according to whether they are consistently sensitive, consistently insensitive, or inconsistently responsive to the application of 10% aqueous lactic acid to the nasolabial fold. A panel of 24 healthy adults are recruited for each test, consisting of approximately eight panellists from each sting category, the males and females being grouped within the categories. The side allocated for application of the test material is then alternated for each panellist, so that half the panel has the test material applied to one side of the nose and the remaining half on the opposite side. Test and control materials are applied once to the nasolabial folds and surrounding area with cotton wool buds (one material to each side of the face). Panellists are asked to wash off the materials with damp tissues 8 min later. At intervals during the 8 min panellists are asked whether they experience stinging or other sensory effects by means of a questionnaire. The visible condition of the application sites is also recorded, along with any spontaneous comments. Panellists are also followed up 24 h later, to check on any effects after the treatment period. The levels of irritation caused by test and control treatments are compared to assess the irritancy of the test material relative to the standard. Subjective comments are also taken into consideration. Stinging reactions are classified on a scale of 0 (none) to 3 (severe). The total of the scores obtained over the test period then provides an indication to whether the material causes stinging in that individual. Subjective effects other than stinging (e.g., itching), along with visual assessments (erythema) are compared.

39.5 PRACTICALITIES ASSOCIATED WITH HUMAN VOLUNTEER TESTING

39.5.1 Study Design

One of the practicalities which must be considered early in designing a study, is the most appropriate size of the panel. Obviously this will impact on the cost of the study, the statistical power of the study, the number of trained staff needed to conduct the study and the time it will take to execute and report the study.

39.5.2 Description of Study/Informed Consent

It is important when recruiting volunteers onto a study that they are fully informed, both verbally and in writing, about what is required of them. They must be provided with information about the product being tested and the methodology being used, and of any possible risks associated with taking part in the study. It is also important that they understand that they may not fulfil the inclusion/exclusion criteria and are ineligible to take part in the study. It must also be made clear to the individual that they are free to withdraw from the study or that they may be withdrawn from the study if they do not adhere to the required schedule/study requirements. Volunteers must confirm their agreement to take part in the study by giving written consent and responding to any questions posed, which may affect panel selection.

39.5.3 Panel Selection

Under the Declaration of Helsinki, when conducting safety testing, one must always consider the health and welfare of the volunteers and ensure the method is designed such that the volunteers health will not be compromised during a study. Part of ensuring that this occurs is at the volunteer selection phase, when it is good practice to complete an initial review of their general health prior to their being accepted onto a study. This information can be gained by asking the volunteers a number of questions, for example, their age, current skin condition, history of skin disease, and details of any medication that they may be taking. Not all medication will necessarily exclude the individual from participating in a study, but it is generally accepted that has taking medication that has an anti-inflammatory effect or may affect the immune system, should not be included in the panel, as the medication could suppress the inflammatory response, thereby producing false negative results. In addition, women that are knowingly pregnant or mothers that are still nursing are also generally excluded, as are individuals wish a known history of allergic or irritant reactivity to similar product types that are already in the market place.

It is also good practice to ensure that volunteers only participate in one study at a time and if they have recently completed another skin irritation study, the skin must be checked to ensure it has returned to normal condition before they are selected for another study (a gap of 1 month is recommended between studies).

39.6 ASSESSMENT OF SKIN IRRITATION

39.6.1 Visual Assessment

Core to all these methodologies to assess skin irritation, is the assessment of visible effects by experienced skin assessors. Erythema and dryness are often the primary parameters for assessment, but other effects, which may be present and can be assessed as individual assessment parameters include wrinkling, glazing, oedema, and vesicles. The grading scheme that is used in-house is shown in Table 39.1. Experienced skin assessors are able to assess these reactions in a highly reproducible fashion[22] and are able to identify very subtle changes in skin reactivity, beyond that which a consumer may see or consider as being of any relevance.

In order to ensure that the visual assessment captures all irritant responses, it may be necessary to record more than one assessment for any given skin site. This is particularly true for use tests, where topical application is likely to cover a large area of skin. The area of application may need to be divided into several discrete sites, which are assessed separately. For example, the axilla may be split into three sites; the “peak” (generally identified as the mounded area in the centre of the axilla, where the majority of hair growth occurs), the “around” (skin around the peak which usually receives some treatment) and the “creases” (creases that are found crossing through the axilla). Treatment may be discontinued due to a reaction (e.g. well-developed erythema) in any of the three sites.

39.6.2 Bioengineering Equipment

There is a number of bioengineering tools used to complement visual assessment, for example, a transepidermal water loss (TEWL) meter can be used to detect early changes in the integrity of the stratum corneum, prior to the mainfestation of readily visible signs of irritation. The readout from bioengineering equipment is quantitative, and the use of these tools is easily transferable between laboratories, whereas visual assessment is subjective and requires an experienced skin assessor to produce accurate, reproducible data. Bioengineering methods commonly used to assess skin irritation include the following.

TABLE 39.1 Description of Grades Used in Skin Condition Assessment

Grade	Erythema (R)	Dryness (D)	Edema (E)	Vesicles (V)	Wrinkling (W)	Glazing (G)
No reactions:						
0	Nothing visible					
1	A marginal reaction that is detectable, but is not sufficient to be classed as "slight"					
Slight reactions						
2	Perceptible erythema	Perceptible dryness	Perceptible swelling	One or two small vesicles	Perceptible surface wrinkling	Perceptible shiny surface
3	A higher grade reaction than another "slight" reaction, which is not sufficient to be classed as "distinct"					
Distinct reactions (obvious to the eye)						
4	Distinct erythema	Distinct dryness	Distinct swelling	Several small vesicles	Distinct surface wrinkling	Distinct shiny surface
5	A higher grade reaction than another "distinct" reaction, which is not sufficient to be classed as "well developed"					
Well-developed reactions (very obvious to the eye)						
6	Well-developed erythema; may extend beyond site	Well-developed dryness with possible flaking	Well-developed swelling; may extend beyond site	Vesicles covering approximately 50% of site	Well-developed defined wrinkling	Well-developed shiny surface with possible cracking
7	A higher grade reaction than another "well-developed" reaction, which is not sufficient to be classed as "strong"					
Strong reactions (outstanding)						
8	Strong, deep erythema; may extend beyond site	Strong dryness with flaking and possible cracking	Strong "blister like" swelling; may extend beyond site	Vesicles covering most or the entire site	Strong deep wrinkling	Strong refractive surface with possible cracking
9	**and above Open ended scale as necessary**					

Each grade of reaction is for the *whole* of the site, **OR** the subsequent grade on *part* of the site, for example, R1 = perceptible erythema on the whole site, or distinct erythema on part of the site. Reactions of 6 and above are considered too great for further treatment.

39.6.2.1 Evaporimeter

The measurement of TEWL is used to evaluate the barrier function of the stratum corneum. TEWL may be assessed using an open chamber system, in which the water vapor pressure gradient produced above the skin surface is measured. This method allows continuous TEWL measurement, as it does not occlude the skin, making it more suitable for the assessment of patch test reactions.[23] Alternatively, a closed chamber system may be used, which estimates TEWL from the gradual increase in relative humidity inside the closed chamber caused by evaporation of water from the skin surface under the chamber.[24] Probably the biggest limitations of most TEWL meters is the need for a temperature and humidity controlled environment and the necessity for the individual to acclimatize for 10 to 15 min prior to taking readings. It should be noted however, that substantial progress is being made to resolve such practical issues associated with the use of this type of equipment.

39.6.2.2 Colorimeter/Erythema Meter

Colorimeters tend to work by measuring the average colour of the skin over a site. Skin contact is necessary for such measurements, however this can cause variation in readings, as the pressure placed on the skin can alter the skin colour, as can the intensity of light at the site being measured. Another factor that should be taken into consideration, is that the colorimeter produces an average color measurement based on the area surrounding the probe, and this could be misleading if the area to be assessed is smaller or composed of different colors. A more sophisticated instrument is the imaging colorimeter that requires no contact with the skin. The combination of imaging and colorimetry provides a more accurate and reproducible measurement.[25]

39.6.2.3 Capacitance

Equipment is available that is based on the capacitance measurement of a dielectric medium. Any change in the dielectric constant due to skin surface hydration verification alters the capacitance of the measuring capacitor.

39.6.2.4 Laser Doppler Perfusion Imaging

This technique involves the creation of a two-dimensional image of skin perfusion. It operates by emitting laser light on to the skin tissue, which upon partial absorption and diffuse scattering, is then reflected with doppler shifted frequencies from blood cells and with unshifted frequencies from stationary tissue. It is a popular method, as it is easy to use and non-invasive, however subjects must refrain from smoking for 4 h prior to measurements and no caffeine intake is permitted 1 h prior to measurements.[26]

Although such tools aid assessment, rarely will they provide data beyond that which can be obtained with thorough visual assessment of the sites at appropriate time points during a study (e.g., no single bioengineering tool is currently more sensitive than visual assessment techniques when conducted by experienced skin assessors, using sensitive scoring schemes).

39.6.3 Interpretation of Results/Evaluation

The results from each study are considered on a case by case basis and the subsequent analysis and interpretation will be dependent on the type of study and the data collected. Generally, data comparison is made between test and control substances. Standard analysis for the majority of skin irritation studies includes a breakdown of the range of assessment grades elicited by each substance tested, a summary of subjective comments and some form of statistical analysis.

39.6.4 Considerations for Statistical Analysis

This must be decided before the study commences, to avoid biasing the interpretation of the results, and to ensure that a suitable number of volunteers are included in the study to provide a sufficiently powerful test.

A common statistical comparison, is between the test material(s) and the control material(s), to detect any differences beyond those that would occur as a consequence of random probability. In general, the smaller the size of the panel, the lower power the test will have, i.e. it will be less likely to identify genuine differences should they exist. Whether this is an issue hinges on the size of difference that the investigator would like to detect, with the optimum panel size determined by the anticipated variability of the results, which may not be known. A pragmatic approach should be taken toward panel size selection, with a sufficient number to allow some meaningful analysis, but that is not unwieldy in terms of running the study or that is prohibitively costly.

39.7 OTHER FACTORS THAT CAN IMPACT ON SKIN IRRITATION

There are numerous factors that can impact on the irritation seen in any given study and it may be necessary to consider these when interpreting results. It is well documented that, particularly in terms of visible skin irritation, reactivity varies according to the skin site, for example, the forearm is known to be more sensitive than the back. This difference is also seen in terms of sensory effects. The influence of the vagus and trigeminal nerves is evident when testing products on the face, as it is much more sensitive than the upper arm or back. Differences in skin physiology between anatomical sites are also known to contribute to differences in skin sensitivity and consequently reactivity. This is perhaps best illustrated in the axilla, which unique, in that it is a partially occluded site, populated with a number of different types of gland (sebaceous, eccrine, and apocrine). Consequently, the irritation profile at this site is often quite complex, with sensory effects, erythema, dryness, and on occasion folliculitis.

It is well documented that seasonal variation produces changes in skin reactivity. Stronger visible reactions are produced during the colder winter months, often increasing the number of pronounced reactions within a panel quite significantly.[27] In contrast, during the summer, skin that is exposed to sunlight is often more robust and therefore generally less reactive to chemical insult. The effects of seasonal variation on reactivity can be interpreted meaningfully by simply including a positive control such as 0.3% Sodium dodecyl sulphate (SDS) to gauge the level of sensitivity. It is also of use to include a marketed control, with a known history of use in the market place, which can be used to benchmark new products.

Age effects are thought to exist, with skin sensitivity becoming less pronounced from 30 years of age. The density of epidermal nerve fibers is also thought to decrease with age.[28]

39.8 IRRITATION TESTING STRATEGY

General strategies for the assessment of skin irritation potential have been available for many years.[29–31] They are rarely absolute, both in the sense that they neither insist on very specific protocols, nor do they generally try to identify the skin effects of a product in isolation. Typically, the approach that is used is to compare a prototype formulation with appropriate benchmarks, which might be a defined dose of a standard irritant such as SDS, or a safely marketed product of the same type (and which for example can be used in a similar manner in an exaggerated use test). Currently, information gained from *in vitro* methods and *in silico* models are sufficient to allow limited exposure in man, that is, a single topical application to a small area of skin, from which a judgement can be made on the irritant potential of the material for use in the internal risk assessment on the material in

formulation. The real problem lies in the fact that unless the material has been proven to be an irritant by a validated QSAR models, or corrosive by a validated *in vitro* method, only *in vivo* methods are accepted by regulatory authorities for the purposes of hazard identification. However, at the moment, there are no validated *in vitro* methodologies to assess skin irritation, although this may change subject to the results of the ECVAM validation trial (due end 2005). There is also a paucity in knowledge surrounding the mechanisms by which materials cause skin irritation. Investment in the following areas may therefore be helpful in developing robust nonanimal alternatives to assess skin irritation:

- Improve the utility of QSARs to accurately predict the skin irritant potential of materials.
- Conduct research into the mechanisms involved in skin irritation across a range of different chemical classes both *in vitro* and in man, using proteomics, genomics, and metabonomics.
- Develop new, robust biomarkers to assess irritation, compare *in vitro* profiles with those *in vivo*.
- Further develop skin models to include all relevant cell types.

39.9 RISK ASSESSMENT

Information on each of these endpoints is used in the risk assessment of the finished product:

$$\text{Risk} = \text{Hazard} \times \text{Exposure},$$

where hazard stands for the inherent property of a material, exposure stands for the consumer contact with hazard and, risk stands for the probability of adverse event in contact with hazard.

In practice, safety assessment of a product potential skin irritative effect considers various factors:

1. Hazard data on the individual ingredients.
2. Dose response data on the individual ingredients.
3. Any historical data on the general formulation type.
4. *In vitro* irritation data on the formulation.
5. Human skin test data on the formulation compared to market benchmarks.
6. Consumer test data (e.g., feedback from efficacy/preference studies).
7. Ongoing monitoring of marketplace feedback.

Normally, sufficient data will be available for point 1, but generally will not be of much value for the safety assessment, particularly since data from point 2 are rarely available. However, since the skin irritating effects of a formulation are a complex function of the ingredients,[32] the most valuable information is derived from the other five points. Of most note are data from point 5, particularly exaggerated exposure studies where a test product is applied to a defined skin site many times a day and is compared to a marketed product known to have an extensive history of safe use. Valuable information often comes also from more extended consumer use tests, perhaps undertaken in a number of locations to allow for differences in use habits. Ultimately it is not possible to do predictive studies (clinical or *in vitro*) which will predict the market place perfectly, particularly low level complaint rates — which means that point 7, monitoring feedback from the market, is always of importance.

REFERENCES

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40 Sensitizing Substances

A.E. Goossens

CONTENTS

40.1 INTRODUCTION

The most important sensitizing culprits in cosmetics, including skin care products, are perfume components, preservative agents, and also certainly excipients and emulsifiers.[1,2] Perfume components have been attracting more attention recently since reactions to them seem to be increasing over the years, and the literature indicates that routine patch testing of markers in addition to the fragrance mix is required to detect fragrance allergy. With preservatives, important shifts in allergenicity have occurred over the years, and their spectrum varies considerably from country to country. With regard to excipients and emulsifiers, many reports have recently appeared in the literature on both moisturizing preparations — also those intended for "sensitive skin" — and lip-care products. Among the other potential cosmetic sensitizers are antioxidants, natural ingredients, such as herbal extracts and vitamins, and also sunscreens, which are nowadays often being added to skin-care products and are responsible mainly for photoallergic contact dermatitis.

40.2 THE NATURE OF COSMETIC ALLERGENS IN MOISTURIZERS

40.2.1 Fragrance Ingredients

Fragrance ingredients are, in general, the most frequent culprits in cosmetic allergies.[2–7] Katsarar et al.,[8] who investigated the results of patch testing over a 12-year period, found an increasing trend in sensitivity to fragrance compounds, which reflects the effectiveness of the advertising of perfumed products. Sensitization is most often induced by highly perfumed products, such as toilet waters, after-shave lotions, and deodorants,[9] but fragrance-containing skin-care products may also cause contact allergic reactions.[1]

The literature confirms that the fragrance mix remains the best screening agent for contact allergy to perfumes because it can detect some 70 to 80% of all perfume allergies.[10,11] However,

the literature also insists on the need to test with additional perfume allergens. Indeed, testing with additional markers, for example, the individual components hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyral®), farnesol, and citral, as well as with complex natural mixtures[12–16] increases the sensitivity of testing. In the near future, a new fragrance mix (II) will be introduced into the standard series.[17] Because of the increasing importance of fragrance allergy and to ensure that sensitized consumers are adequately informed, 26 fragrance components will have to be labeled as cosmetic ingredients on the packaging (Annex 3 of the Cosmetic Directive[18]). Positive patch-test reactions are frequently associated with a fragrance allergy and often indicate the presence of common or cross-reacting ingredients in natural products, the occurrence of cross-reactions between simple fragrance chemicals, or a concomitant sensitivity.

40.2.2 Preservatives

Preservatives are important allergens in skin-care products alongside cleansers and makeup.[1,2] However, within this class, important shifts have occurred over the years.[2,19] The methyl (chloro)isothiazolinone mixture was commonly used in the 1980s and was then a frequent cause of contact allergies. This frequency has declined considerably in recent years.[4] Since then, formaldehyde and its releasers, particularly methyldibromo glutaronitrile — as used in a mixture with phenoxyethanol, better known as Euxyl K400 — did gain importance in this regard,[1,2,4,19–22] although the frequency of positive reactions observed seems to be influenced by the patch-test concentration.[21,22] Both the methyl(chloro)isothiazolinone and methyldibromo glutaronitrile mixtures are now recommended to be used only in rinse-off products, and they still can be found in some of the leave on products in the market.

The spectrum of the allergenic preservatives also varies from country to country. For example, in contrast to continental Europe where reactions to the methyl(chloro)-isothiazolinone mixture and more recently methyldibromo glutaronitrile have been the most frequent,[2,4,5,19,23] formaldehyde and its releasers have always been much more important, particularly as concerns quaternium-15[2] in the United Kingdom, although its incidence seems to have slightly declined recently.[24] Parabens are rare causes of cosmetic dermatitis. When a paraben allergy does occur, the sensitization source is most often a topical pharmaceutical product, although its presence in other products can be sensitizing as well.[1,25] This is often the case also for other ingredients, such as chlorphenesin,[26] which cross-reacts with mephenesin, which is used in pharmaceuticals. Another recently introduced preservative is iodopropynyl butylcarbamate, which was first reported as a cosmetic allergen by Pazaglia and Tosti in 1999[27] and for which the test concentration seems critical.[28] Its presence in cosmetics is being discussed, not because of its potentially allergenic properties but because of its iodine content (Ian White, personal communication).

40.2.3 Excipients, Emulsifiers, and Humectants

Many excipients, emulsifiers, and humectants are common ingredients in topical pharmaceutical products, which are likely to induce sensitization, and cosmetic products. The classical examples are wool alcohols, fatty alcohols (e.g., cetyl alcohol), and propylene glycol.[1,2] Emulsifiers, in particular, have long been regarded as irritants, but their sensitization capacities should not be overlooked. It is imperative, of course, that patch testing be properly performed to avoid irritancy and that the relevance of the positive reactions be determined. A large number of emulsifiers, emollients, excipients, and humectants have been reported to be contact allergens in moisturizers,[1] including preparations to treat dry lips[29,30] for which pigmented contact cheilitis has also been described.[31]

Table 40.1 lists the emulsifiers, excipients, and humectants that have been reported to be contact allergens in moisturizers over the last five years. For the allergens previously identified, see de Groot.[1]

Some of these substances, because of their low irritancy potential and "skin-mildness," are often incorporated in skin-care products "recommended by dermatologists," "for use on intolerant skin"

TABLE 40.1
Emulsifiers, Excipients, and Humectants Reported as Contact Allergens in Skin and Lip Moisturizers from 1999 to 2004 (NonExhaustive List)

Substances	Literature references
Butylene glycol	1, 32–35
Castor oil and derivatives:	1, 36
glyceryl ricinoleate	1, 37
propylene glycol ricinoleate	38
ricinoleic acid	31
Ceramide (hydrophilized)	39
Cetearyl isononanoate	40
Di-isostearyl maleate	41, 42
Ethylhexylglycerin	43
Glycerin	44
Glycerylisostearate	45
Glycerylmonoisostearate monomyristate	46
Hexyldecanoic acid (isopalmitate)	47
Isopalmityl diglyceryl sebacate	48–51
Laureth-9	52
Maleated soybean oil	53
Methoxy PEG-17 dodecylglycol polymer	54, 55
Methoxy PEG-22 dodecylglycol polymer	55
Pentaerythritol rosinate	50, 56
Pentylene glycol	57
Polyquaternium-7	52
Sodium dihydroxycetyl phosphate	58
Triglycerides: caprylic/capric and synthetic triglycerides	59, 60
VP/eicosene copolymer (already reported previously as allergen in sunscreen products)	61–63

or "for sensitive skin" that have become very popular in recent years. A low irritant potential, however, does not preclude the occurrence of allergic contact dermatitis. Examples of this are butylene glycol[33] and pentylene glycol,[57] that is, aliphatic alcohols with similar uses (solvent, humectant, and antibacterial) to those of propylene glycol, which is considered to be more irritant and allergenic, ethylhexylglycerin (syn.: octoxyglycerin), a skin conditioning agent,[43] and methoxy PEG-17 and PEG-22/dodecyl glycol copolymers, which are alkoxylated alcohols and synthetic polymers used as emulsion stabilizers and suspending and viscosity-increasing agents, and also as skin-conditioning agents.[54,55] Alkyl glucosides, which are condensation products of fatty alcohols with glucose such as coco and lauryl glucosides[64] are often used as mild surfactants and cleansing agents and also as emulsifiers, particularly decyl- and cetearyl-glucoside, and may be hidden allergens in sunscreens.[65]

40.2.4 Antioxidants

Antioxidants form only a minor group of cosmetic allergens. Examples are propyl gallate, octyl gallate,[66] which may cross-react with other gallates and are also used as food additives, and t-butyl hydroquinone, a well-known allergen in the United Kingdom but not in continental Europe.[2]

Some antioxidants are used more specifically in sunscreen products and also in moisturizing products to prevent aging but are rare causes of allergic contact dermatitis in such preparations, for example, tocopherol (vitamin E) acetate, retinol palmitate,[67] and ascorbic acid (vitamin C).[68]

40.2.5 Natural Ingredients

In addition to the vitamins already mentioned as antioxidants, panthenol, a vitamin B derivative[69] and its derivative panthotenyl ether[70] may exceptionally cause contact allergy due to their presence in moisturizers.

Plant extracts and herbal remedies have become very popular in recent years. For example, resveratrol (a phenolic phytoalexin produced naturally in red grape skin and in leaf epidermis of various plants) was recently reported as an allergen in a moisturizer.[57] Protein-derived ingredients, in particular, are often used in skin-care products, especially in those for treating dry skin in atopic subjects (often children). Contact dermatitis (sometimes located mainly on the eyelids[71]) may develop occasionally from oat or *Avena* extract,[72] hydrolyzed wheat protein,[73] and soybean extract.[74] Not only contact dermatitis but also contact urticarial[75] reactions to protein-derived products, sometimes severe,[76] have occurred. Although such reactions seem to be rare and may sometimes be irritant in nature, especially when patch testing atopic subjects,[77] their use has given rise to controversy since subjects (also children) may become sensitized through topical preparations and develop food allergies afterwards, or vice versa.

Other natural ingredients identified as allergens in moisturizers are placenta,[70] chitin (a cellulose-like biopolymer and important structural element of the integuments of arthropods, particularly crustaceans, mollusks, unicellular micro-organisms, seaweed, and fungi), and chitosan (deacetylated chitin).[78,79]

40.2.6 Sunscreens

Because of the media attention being given to the carcinogenic and accelerated skin-aging effects of sunlight, sunscreens are being used increasingly not only in sunscreen products but also in other cosmetics including moisturizers, in which they may be responsible for photocontact and contact allergic reactions.[80] Some sunscreen agents such as benzophenone-3, which may also cause contact urticaria[81] and even anaphylaxis,[82,83] and dibenzoylmethane derivatives have been recognized in the past as being important allergens.[1,2,84] The 4-methylbenzylidene camphor, cinnamates, and phenylbenzimidazole sulfonic acid are only occasional, sometimes even rare, causes of cosmetic reactions. The first cases of reactions to the newer sunscreens have recently appeared: photoallergic contact dermatitis from octyl triazone[85] and octocrylene.[86] In our experience,[2,4,5] the contribution of sunscreens to cosmetic allergy is relatively small despite the increase in their use. The low rate of reported allergic reactions observed, however, may well be because a contact allergy or a photoallergy to sunscreen products is often not recognized, since a differential diagnosis with a primary sun intolerance is not always obvious. Furthermore, the patch-test concentrations generally used might be too low, in part because of the risk of irritancy.

40.3 IDENTIFYING SENSITIZING SUBSTANCES IN MOISTURIZERS

Taking the history of the patient and noting the clinical symptoms and localization of the lesions are critical. Allergen identification for a patient with a possible contact allergy to cosmetics is performed by means of patch testing with the standard series, specific cosmetic-test series, the product itself, and all of its ingredients. We can only find the allergens we look for. For skin tests with cosmetic products

the patients supply themselves, there are several guidelines.[87] Not only patch and photo-patch tests but also semi-open tests (in case of possible irritants), usage tests, or repeated open application tests (ROAT) may need to be performed to obtain a correct diagnosis. Once an allergen has been identified, it is the dermatologist's task to provide specific advice about the products that can be used safely since subjects sensitive to specific ingredients must avoid those products that contain them. Although cosmetics are labeled, providing the allergic patient with a list of cosmetics that can be used is, in our experience, the most practical and effective tactic.[88]

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41 Regulatory Aspects on Safety

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CONTENTS

41.1 INTRODUCTION

Skin moisturizers are used by a majority of the population in different degrees. Skin moisturizers, in addition to their beneficial effect on the skin, must be devoid of any deleterious effect on human health. They must not cause damage to human health under normal or reasonably foreseeable conditions of use.

In most cases, skin moisturizers are intended to keep the skin in a good condition, and are therefore considered as cosmetic/hygienic products and must comply with the legislation for these products. The manufacturer is in principle responsible for the safety of each product. However, in addition, the legislation in many countries may restrict the use of certain ingredients, and demand specific documentation of ingredients and the final product.

Certain skin moisturizers, however, may be considered as medicinal products and are then covered by special documentation and registration requirements not discussed in this chapter.

The main part of the safety assessment of finished products could be based, in principle, on data from the different ingredients used. The many thousands of different products on the market are all derived from a smaller number of ingredients. The toxicological profiles are also adequately studied

on separate substances. To avoid costly duplication of studies and unjustifiable use of animals, toxicity testing on the different ingredients and particularly those of most concern is preferable. For example, the documentation, evaluation, and listing of coloring agents, preservatives and UV-filters within the European Union (EU) and the Cosmetic Ingredient Review within the United States are important.

41.2 DIFFERENT TYPES OF INGREDIENTS

As reviewed in this book, skin moisturizers contain a wide variety of ingredients. A typical basic formula for a moisturizer includes water, polyol, lipid, surfactant, special moisturizer, preservative, and perfume.[1] Some of the ingredients are included to have beneficial effects on the skin, others are needed to get a suitable composition. So far, some types of ingredients have been of most toxicological concern. Preservatives and perfumes are two types of ingredients, which need special toxicological attention. Colors and UV-filters are two other types of ingredients for which special requirements have been made. Within the EU compounds used as preservatives, UV-filter and colors require an evaluation by authorities prior to their use in cosmetic products. The documentation and safety are evaluated by a scientific committee before they are permitted and placed on the lists of the Cosmetics Directive.[2] General and specific requirements that the manufacturer should provide are specified in the "*Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation*" 5th revision.[3]

Within EU the use of a large number of different substances are prohibited or restricted, annex II and III in the Cosmetics Directive. The list of prohibited substances includes for example, some pharmacological active substances (e.g., glucocorticoids, oestrogens), and other substances known to be harmful to the consumers (e.g., certain salicydanilides). Prohibition of substances classified as carcinogenic, mutagenic, or reprotoxic category 1 and 2 have been introduced recently. Substances for which the use are resticted includes for example, boric compounds, fatty acid, dialkanolmides, monoalkanolamines, trialkanolamines, polyacrylamides. An increasing number of substances are covered by the prohibition or restrictions due to evaluations of the responsible scientific committee within the European Commission. The former Scientific Committee on Cosmetics, and Cosmetics and Non-Food Products has recently been replaced by the new Scientific Committee on Consumer Products. The work done by the committee can be followed via the website,[4] where their opinions are made publicly available.

Within the fragrance industry a self-regulatory work is increasing the safety of the substances used if the IFRA Code of Practice is followed.[5] Recently, most of the IFRA guidelines on fragrance ingredients have been included in the legislation within EU.

For other ingredients, for which no regulation is specified from the authorities, the manufacturer has the full responsibility. The manufacturer is responsible to use ingredients and a final composition of the skin moisturizer so that there is no risk of harmful effects for the consumer.

41.3 TOXICOLOGICAL REQUIREMENTS ON INGREDIENTS

To assure that an ingredient does not pose a risk for the human health, all possible toxicological endpoints must be considered. That includes possible acute and chronic effects both locally and systemically. The exact information and studies needed depends on the compound and its properties. As a guidance to the toxicological properties to be considered, *The SCCNFPs Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation* could be used.[3] The general toxicological endpoints are listed in Table 41.1. The first part of the list (points 1 to 6) constitutes items necessary for all compounds. Depending on the properties and outcome of this first part, additional points (points 7 to 9) might be necessary. Considerable skin absorption is an example of

TABLE 41.1
General Toxicological Requirements

1. Acute toxicity
2. Irritation and corrositivity
3. Skin sensitization
4. Dermal/percutaneous absorption
5. Repeated dose toxicity
6. Mutagenicity/genotoxicity
7. Carcinogenicity
8. Reproductive toxicity
9. Toxicokinetic studies
10. Photo-induced toxicity
11. Human data

circumstances where these additional points are necessary. The last two points, 10 and 11, may also be necessary or helpful in certain cases.

It is important to remember that as our knowledge about the effects of different substances on the human body increases, ingredients already in use might need further testing and re-evaluation.

41.3.1 Acute Toxicity

Acute toxicity is necessary to evaluate the amounts that do or, in many cases, do not affect the living organism at a single exposure. It can be necessary for assessment of accidental exposure. However, mostly it is helpful to choose the levels in subsequent toxicological examinations. For new substances it should be performed only for the need in other legal requirements, for example, due to chemical legislation and worker protection. No exact figures are required, ranges or intervals might be enough. Both oral and dermal route might be adequate, but in most cases oral route is used. Acute toxicity is of minor importance for most ingredients in skin moisturizers, but can be important for additives with special effects.

41.3.2 Irritation and Corrositivity

Possible irritative effects of the substance on the skin must be assessed. Preliminary knowledge can in this case be derived from experiences of other substances with similar structure. If any hesitation exists, studies must be performed.

41.3.3 Skin Sensitization

Predictive tests on the potential of the compound to cause skin sensitization are essential. The introduction of new potent sensitizers must be avoided. So far only animal tests are sufficiently reliable to predict a low sensitizing potential, although alternatives can exclude potent sensitizers.

41.3.4 Dermal/Percutaneous Absorption

As skin moisturizers are applied to the minor or major outer part of the human body the skin absorption of ingredients is important to know to make it possible to estimate the systemic exposure. If skin absorption studies are lacking, 100% absorption could be assumed in the safety evaluation.

41.3.5 Repeated Dose Toxicity

To substantiate the safety of the substance for the population exposed, a subchronic study must be performed. The study should be designed to obtain a no-adverse-effect level. A 90- or 28-day study in rats is usually used.

41.3.6 Mutagenicity/Genotoxicity

To exclude substances with mutagenic/genotoxic potential, as a minimum, a combination of two suitable *in vitro* tests is necessary.

41.3.7 Carcinogenicity

Depending on the outcome of previous testing on mutagenicity/genotoxicity and systemic exposure, complete carcinogenicity testing might be necessary to exclude these risks.

41.3.8 Reproductive Toxicity

The risk of different reproduction disorders must be evaluated if there is a considerable systemic exposure, as moisturizers are extensively used within the population.

41.3.9 Toxicokinetic Studies

If there is a systemic exposure of the substance it is important to examine the disposition, metabolism, and excretion of the substance to make it possible to extrapolate *in vitro* and *in vivo* data to man.

41.3.10 Photo-Induced Toxicity

Most of the toxicological studies mentioned previously are needed also for other uses of the substance. However, different types of investigations involving light sources also are, in many cases, a special requirement for use in cosmetic products. This is specifically required when the cosmetic product is expected or intended to be used on sunlight-exposed skin.

41.3.11 Human Data

For many substances used in cosmetics, humans might have been exposed earlier intentionally or unintentionally. All data from these experiences are useful in the safety evaluation both for substances and later for the products.

41.4 SOURCES OF TOXICOLOGICAL INFORMATION

First, a request about all available toxicological information should be made to the raw material supplier. In addition, data from usual toxicological sources, databases, and literature is supplementary. As toxicological studies substitianting safety of a substance are prepared within companies and not usually published, different attempts have been made to make them more available. In the United States the Cosmetic Ingredient Review reports on the safety on different ingredients.[6] Within EU, reports from the Scientific Committee on Consumer Products (former Scientific Committee Cosmetics and Scientific Committee on Cosmetics and Non-Food Products) are available for some ingredients.[4] If suffient toxicological information is not to be found, studies must be conducted.

41.5 METHODS

For the toxicity studies needed, internationally accepted methods as those reported within EU[7] or in accordance with the OECD Guidelines for testing of chemicals are recommended. Tests for assessing photomutagenicity, photoirritationcy, photosensitization, and skin absorption have not been included so far in abovementioned guidelines. Guidance to the types of tests could also be found in the document from SCCNFP.[3]

New studies have to be conducted according to Good Laboratory Practice (GLP). However, old studies should not automatically be invalidated if they do not comply with guidelines or GLP requirements. Due to ethical reasons other testing procedures based on scientifically justified models and procedures can be accepted.

Alternative to methods using animals has developed during the past years and can be followed through the European Commission, which report on the progress of this development in Reference 8.

Recently the testing of final products on animals has been prohibited within the EU,[9] and will cover also ingredients as alternative methods will be developed and accepted.

41.6 SAFETY EVALUATION OF PRODUCTS

In general the safety evaluation of the finished product can be obtained by ascertaining the toxicity profile of the different ingredients. But it is important to evaluate each different toxicological end point and if the documentation is adequate for the assessment.

Important factors to consider in calculating the exposure are, for example, concentration of ingredients in the product, quantity, frequency and area of skin contact, and nature of consumers.

For some of the effects the concentration in the products is most important, for example, the local tolerance on the skin and eye. For some of the other effects it is necessary to estimate the presumed use by a normal or perhaps an eager user and the total amounts are more adequate. Guidance to relevant exposure estimation can be found in part 6 of SCCNFP. "Safety evaluation of finished products" in the notes of guidance.[3] The European cosmetics industry has, for example, estimated the exposure levels to be 0.8 g/day of face cream, 1–2 g/day of general cream and 8–16 g/day of body lotion for a female user.[10] It is also important to predict the use of the special product and the expectations from the single user. Groups of users with especially sensitive skin are important to take into account.

It is also necessary to look at the product as a composition. Possible interactions and potentiations of effects between the different ingredients locally or systemically must be considered. The possibility of different penetration due to the composition and the possible effect on toxicity must be evaluated. For skin moisturizers a lot of experience is gathered for previous compositions and products on the market. For local effects such experience may be reliable, but one has to pay special attention to systemic toxicity, which is very difficult to discover during use by consumers.

Within EU, documentation for each specific product should be readily available at the manufacturer or importer within the community.[2] This so called Product information shall include:

a. The qualitative and quantitative composition of the product.
b. The physico-chemical and microbiological specifications of the raw materials and the finished product and the purity and microbiological control criteria of the cosmetic product.
c. The method of manufacture.
d. Assessment of the safety for human health of the finished product.
e. Name and address of the qualified person responsible for the safety assessment.
f. Existing data on undesirable effects on the human health resulting from use of the cosmetic product.
g. Proof of the effect claimed for the product, where justified be the nature of the effect or product.
h. Data on any animal testing performed by the manufacturer.

Recently, information on ingredients and undesirable effects, points a and f, should be made available by the manufacturer/importer within the EU after request from the public.

41.7 ADDITIONAL TOXICOLOGICAL ASPECTS

Apart from the strictly regulated types of ingredients, colors, preservatives, and UV-filters, the main part can be used under the manufacturer responsibility. From the past we can get important toxicological aspects that ought to be noticed. In some cases toxicological problems have been discovered among constituents such as emulsifiers and emollients.

41.7.1 Contaminants

In the manufacturing process of ingredients a lot of different chemicals are used. The residue levels of these starting materials must be controlled, not only to have a high quality raw material, but also in relation to their toxicological profile. In the past, the residue level of dioxane used in the manufacturing process for ethoxylated substances was considered as a health hazard, as dioxane was shown to be carcinogenic in mice. Recently, maximal allowed residual concentrations of acrylamide, used in the manufacturing of polyacrylamides, have been established within the EU. It is important also to state that the specification of the ingredient during the toxicological testing and evaluation to make the results relevant for the ingredient when used in a cosmetic product.

41.7.2 Formation of New Substances

Under certain circumstances, formation of new substances can be seen during manufacturing, storage or use. Formation of different nitrosamines was found in products with dialkanolamines together with some nitrosating agents. 2-Bromo-2-nitropropane-1,3-diol and 5-bromo-5-nitro-1,3-dioxane are two, but not the only, examples of such substances. This formation of nitrosamines is important to avoid or minimize, as many different nitrosamines are shown to be carcinogenic is animals.

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