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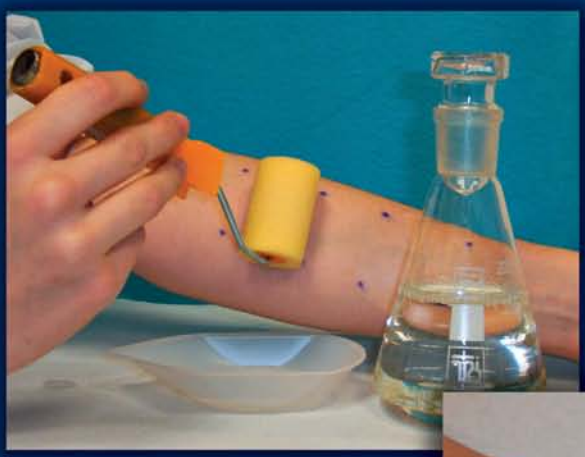
Water and the Stratum Corneum



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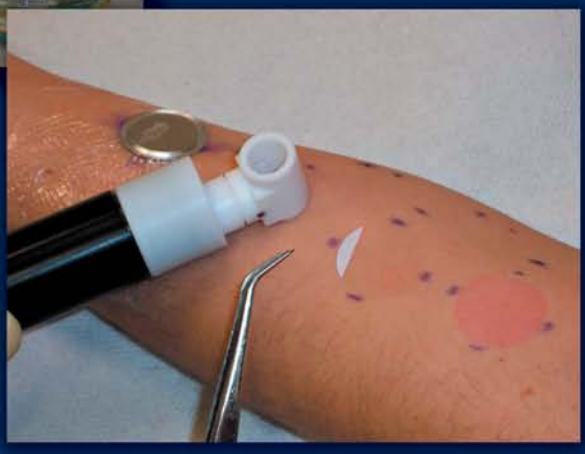

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Series Preface

Our goal in creating the Dermatology: Clinical and Basic Science Series is to present the insights of experts on emerging applied and experimental techniques and theoretical concepts that are, or will be, at the vanguard of dermatology. These books cover new and exciting multidisciplinary areas of cutaneous research, and we want them to be the books every physician will use to become acquainted with new methodologies in skin research. These books can be also given to graduate students and postdoctoral fellows when they are looking for guidance to start a new line of research.

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We are working with these goals in mind. As the books become available, the efforts of the publisher, book editors, and individual authors will contribute to the further development of dermatology research and clinical practice. The extent to which we achieve this goal will be determined by the utility of these books.

Howard I. Maibach, M.D.

Preface

The stratum corneum is the interface between the sometimes harsh environment and the internal milieu of the human body. Our understanding of the stratum corneum has grown over the last two decades from seeing it as an inert surrounding sheet to understanding it as a biological active compartment. The second edition of *Bioengineering of the Skin: Water and the Stratum Corneum* reflects ten years of progress not only of the noninvasive biophysical assessment of skin physiology parameter but also of the perfection of the available devices. Several new authors have added innovative aspects to this edition. The revised chapters reflect the progress in their respective fields. Bioengineering of the skin, or more precisely the biophysical assessment of skin physiology, is moving rapidly from a descriptive approach to a deeper understanding of biophysical and biochemical processes in the stratum corneum (e.g., on stratum corneum barrier function as well on stratum corneum hydration). The current research utilizing bioengineering methods offers reliable and reproducible approaches for product testing in the pharmaceutic and cosmetic industries and in basic research. Functional data can be acquired with low follow-up costs.

We are thankful to all the authors of both new and revised chapters, and we are grateful to Yvonne Herzberg and Erika Dery for assistance in the editorial process. We hope that this new edition of a successful book will enhance communication within the research community and will be useful for scientists starting in the skin bioengineering field.

Joachim W. Fluhr, Peter Elsner, Enzo Berardesca, Howard I. Maibach
Jena, Rome, San Francisco

Biographies

Joachim W. Fluhr, M.D., is senior dermatologist and head of the Skin Physiology Laboratory at the Friedrich-Schiller-University of Jena, Germany. Dr. Fluhr received his medical training at the University of Mainz, Germany, and the University of Strasbourg, France. He earned his M.D. degree in 1992. He served as a resident at the Department of Dermatology at the Municipal Hospital Karlsruhe. He was a visiting researcher at the Department of Dermatology, IRCCS Policlinico S. Matteo, Pavia. Dr. Fluhr did a postdoctoral research fellowship between 2000 and 2002 at the University of California, San Francisco with Peter Elias and Ken Feingold.

Dr. Fluhr is member of the board of the International Society of the Skin (ISBS) and is secretary and cofounder of the International Society of Skin Pharmacology and Physiology (ISP). He has published more than 60 scientific papers and book chapters. His current research interests are the regulatory functions of the stratum corneum pH, models in irritant contact dermatitis, and technical comparison of bioengineering instruments.

Peter Elsner, **M.D.,** studied medicine at Julius-Maximilians-University, Wuerzburg, Germany, from 1974 to 1981, and was trained as a dermatologist and allergologist at the Department of Dermatology, Wuerzburg University, from 1983 to 1987. He received his doctoral degree in 1981 and his lectureship in dermatology in 1987. From 1988 to 1989, he was visiting research dermatologist at the Department of Dermatology, University of California, San Francisco, and from 1991 to 1997, he was consultant and associate professor, Department of Dermatology, University of Zurich, Switzerland. Since 1997, he has been professor and chairman, Department of Dermatology and Allergology, Friedrich-Schiller-University of Jena, Germany. Dr. Elsner has published more than 200 original papers and 20 books. He is a member of more than 30 scientific societies and has served as chairman of the International Society for Bioengineering and the Skin and as a member of the Scientific Committee for Cosmetics and Non-Food Products (SCCNFP) of the European Commission and the European Group on Efficacy Measurement of Cosmetics and Other Topical Products (EEMCO).

Enzo Berardesca, **M.D.,** is director of clinical dermatology at the San Gallicano Dermatological Institute, Rome, Italy. Dr. Berardesca obtained his training at the University of Pavia and received his M.D. degree in 1979. He served as resident and dermatologist in the Department of Dermatology, IRCCS Policlinico S. Matteo, Pavia, from 1982 to 1987, and as assistant researcher in the Department of Dermatology, University of California School of Medicine in San Francisco in 1987. From 1988 to 2001 he was at the Department of Dermatology of the University of Pavia, acting as head of the Dermatoallergology Unit and of the Skin Bioengineering Lab.

Dr. Berardesca was chairman of the International Society for Bioengineering and the Skin from 1990 to 1996. He has organized several international meetings in Europe on skin bioengineering and irritant contact dermatitis. He is a member of the editorial board of *Skin Pharmacology*, *Skin Research and Technology*, *The American Journal of Clinical Dermatology*, and the *Journal of Cutaneous and Ocular Toxicology*. He is a member of the Society for Investigative Dermatology, the European Society for Dermatological Research, and the Italian Group for Research on Contact Dermatitis (GIRDCA), and is vice-chairman of the European Group for Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO) group. His current major research interests are irritant dermatitis, barrier function, and noninvasive techniques to investigate skin physiology, with particular regard to racial differences in skin function, sensitive skin, and efficacy evaluation of topical products. He is the author of 5 books and more than 200 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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Table of Contents

PART III Water Content

Part I

Introduction

1 Water–Lipid Interaction

Bozena B. Michniak and Philip W. Wertz

CONTENTS

I. INTRODUCTION

The interactions of lipids and water are fundamental to all living organisms [1]. Water is ubiquitous and is often taken for granted, but its properties are unique. The water molecule is an electrical dipole, as the hydrogen atoms have a partial positive charge and the oxygen has a partial negative charge. This results in extensive hydrogen bonding among water molecules as well as potentially between water molecules and solutes. Each water molecule is able to hydrogen bond to four neighbors. Water hydrogen bonds with the hydrogens attached to oxygen or nitrogen molecules in the polar head groups of lipids; similarly, oxygens and nitrogens from the polar head groups can hydrogen bond with hydrogens from water.

Nonpolar lipids, defined as lipids that do not form a lipid–water phase, include triglycerides, cholesterol esters, wax esters, and so forth [2]. These lipids can be suspended in water, forming emulsions. In some cases, for example, fatty alcohols, a surface monolayer can be formed. In the case of a fatty alcohol, the hydroxyl group hydrogen bonds with water at the surface, and the aliphatic chain sticks into the atmosphere above the water. These lipids will not be discussed further in this chapter.

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Polar lipids, which are capable of forming lamellar or hexagonal phases in the presence of excess water [2] will be the main topic of this chapter, with an emphasis on lipids found in epidermis [3,4]. This chapter will include some discussion of fatty acids, phospholipids and phospholipid–cholesterol systems, roles of glucosylceramides, and the ceramide–cholesterol–fatty acid mixture of the stratum corneum. Some comments on cholesterol sulfate and synthetic surfactants will be included.

II. LIPID CLASSES FOUND IN THE EPIDERMIS

A. Fatty Acids

Free fatty acids and their salts generally have very low solubility in water but, depending on conditions, are capable of forming a stable monolayer on top of an aqueous subphase [5], micelles [6], bilayered vesicles [7], or multilamellar tubules — often referred to as myelin figures [8]. When a fatty acid salt, or soap, is dispersed in water, a few molecules go into solution as individual molecules. Once a certain concentration of dissolved monomer is reached — the critical micellar concentration — micelles form. Micelles are globular aggregates of fatty acid molecules with the hydrocarbon chains on the inside and the carboxylate groups on the outside. This arrangement maximizes hydrogen bonding between the carboxylate group and water as well as avoids unfavorable interaction between the aliphatic chains and water. Micelles composed of fatty acid salts are, in part, stabilized by charge repulsion among the carboxylate groups. This is also true of some synthetic surface active agents; for example, sodium dodecyl sulfate. Micelles are capable of incorporating nonpolar lipids or hydrocarbons into their interiors. This is how soap solutions remove grease or oils from surfaces.

Free fatty acid–fatty acid salt mixtures are capable of forming bilayered vesicles when the fatty acid and its salt are present in approximately a 1:1 molar ratio. Carboxylic acids in general have a pKa of about 4; however, when the acids are incorporated into bilayers, they behave like much weaker acids, with apparent pKa values of about 7. Regardless of the mixture of fatty acids and fatty acid salts introduced into an aqueous medium, the degree of ionization will be determined by the pH of the system. The pH at which the micelle-to-lamellar phase transition occurs depends somewhat on the chain length of the fatty acid.

B. Phospholipids and Cholesterol

Most biological membranes, including those in the viable epidermis, are composed mainly of phospholipids and sterols, with cholesterol being the dominant sterol in the animal kingdom [9]. The most common phospholipids include sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine. Phosphatidylserine and phosphatidylinositol are generally present in lower proportions. The phosphoglycerides generally contain a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position [10]. Because of the different polar head groups and fatty acid combinations that can be present in phosphoglycerides, there can be many possible molecular species. This diversity of structure probably reflects the numerous

functional roles of membrane lipids. Sphingomyelin, in addition to being a phospholipid, is also a sphingolipid. In epidermis, sphingomyelin is one of the precursors of the ceramides of the stratum corneum. The phospholipids and cholesterol are not randomly distributed throughout the cellular membrane system. Each membranous organelle has its own characteristic lipid composition [11,12]. For example, nuclear membranes tend to be rich in sphingomyelin and cholesterol, whereas mitochondria contain the relatively unusual four-chain phospholipid, cardiolipin. Furthermore, in biological membranes there is generally an asymmetric distribution of lipids between the leaflets of the bilayer [13,14]. For example, in the plasma membrane of viable mammalian cells, phosphatidylserine and phosphatidylethanolamine are preferentially localized in the inner, or cytoplasmic, leaflet. This distribution is actively maintained through the action of lipid translocater proteins. During apoptosis, lipid translocation is disrupted and a scramblase facilitates a loss of plasma membrane lipid asymmetry [14].

When phospholipids with or without cholesterol are dispersed in an aqueous medium, vesicles spontaneously form [15]. Depending on the composition and method of preparation, vesicles prepared from phospholipids with or without cholesterol may be small (<50 nm diameter) unilamellar vesicles, large (ca. 50 to 2000 nm diameter) unilamellar vesicles, multilamellar vesicles, or plurilamellar vesicles. Multilamellar vesicles consist of concentric spheres of bilayers separated by thin layers of water. Pleurilamellar liposomes consist of one or more small unilamellar vesicles inside a large unilamellar vesicle. Various types of phospholipids–cholesterol liposomes have been used extensively to model biological membranes.

C. Glycolipids

Glycolipids consisting of monohexosylceramides, lactosylceramide, gangliosides, and sulfatides are present in minor amounts in essentially all viable mammalian cells. Galactosylceramides, or cerebrosides, are abundant lipids in tissue of the nervous system, and glucosylceramides are present in relatively high levels in the viable portion of the epidermis [3]. The glycolipids serve in a range of specific recognition functions. Liposomes containing glycolipids have been used to study such processes as cell adhesion [16] and signal transduction [17], and they may be useful for targeting liposomes to a specific tissue location [18-20].

The epidermis contains several structurally unusual acylglucosylceramides, the most abundant and most studied of which consists of 30- through 34-carbon ω-hydroxyacids amide-linked to sphingosine with a glucose moiety β-glycosidically attached to the primary hydroxyl group of the base and linoleate ester-linked to the ω-hydroxyl group [21,22]. Acylglucosylceramide, and an acylceramide produced by deglycosylation (see below), appear to be unique to keratinizing epithelia and can be considered differentiation markers [23-25]. It has been proposed that acylglucosylceramide is involved in assembly of the contents of lamellar granules [26]. Specifically, it has been suggested that acylglucosylceramide induces both the flattening and aggregation of lipid vesicles to produce the stacks of lamellae seen within the granules. With synthetic vesicles, acylglucosylceramide has been shown to cause flattening and aggregation. As noted, acylglucosylceramide is found in keratinizing

epithelia, all of which produce lamellar granules, and is not found in other tissues that do not synthesize lamellar granules [25,27]. In a comparison of the epidermal lipids from different orders of vertebrates, it can be shown that mammals, birds, and reptiles produce lamellar granules and contain acylglucosylceramides [28,29]. Amphibians and fish neither produce lamellar granules nor contain acylglucosylceramides.

In addition to the possible role of lamellar granule–associated acylglucosylceramide in the assembly of lamellar granule contents, it has been suggested that about two-thirds of the acylglucosylceramide is associated with the bounding membrane of the granule [30]. This pool of acylglucosylceramide would be introduced to the cell periphery when the bounding membrane of the lamellar granule fuses into the cell plasma membrane. It has been suggested that the glucose and linoleate are removed at this time and that the resulting ω-hydroxyceramide becomes ester-linked to acidic amino acid side chains on the outer surface of the cornified envelope [30,31].

D. Ceramides

Ceramides are present in many tissues as minor membrane components, but they are major components of the intercellular membranes in the stratum corneum [3,32]. The basic building blocks for human ceramides include sphingosines and dihydrosphingosines, phytosphingosines, and 6-hydroxysphingosines as the basic component, in addition to normal fatty acids, α-hydroxyacids, and ω-hydroxyacids. Ceramide production in the plasma membrane through the enzymatic hydrolysis of sphingomyelin has been implicated as the initiating biochemical event leading to apoptosis in a variety of cell types [33-35].

An acylceramide is derived from acylglucosylceramide associated with the lamellar granule contents after extrusion into the intercellular space. For human epidermis, variants of the acylceramide have been reported that contain sphingosine [32], 6-hydroxysphingosine [36], or phytosphingosine [37] as the base component. On the basis of transmission electron microscopic images [38] and x-ray diffraction data [39,40] it appears that acylceramide plays a critical role in organization of the lipid in the intercellular spaces of the stratum corneum [41,42]. Several similar models have been proposed to account for this unique role [43-45]. This is discussed at greater length in the section dealing specifically with stratum corneum lipids.

E. Cholesterol Sulfate

Although cholesterol sulfate is a minor stratum corneum lipid component, it may have an influence on lipid organization [46], and it has been implicated in the regulation of the desquamation process. It has been demonstrated that hydrolysis of cholesterol sulfate is associated with cell shedding both from human skin *in vivo* [47] and from the surface of a mouse skin organ culture model [48]. Conversely, in the hereditary disease recessive X-linked ichthyosis, cholesterol sulfate accumulates to elevated levels in the stratum corneum, and desquamation does not proceed normally [49]. As a result, the skin surface can become scaly. Cholesterol sulfate

has been shown to inhibit serine proteases that may be involved in degradation of desmosomal proteins [50]. This is probably the basis for its role in regulation of desquamation.

F. Cholesteryl Esters

The cholesteryl ester fraction from stratum corneum consists almost entirely of cholesteryl oleate [51]. It has been proposed that oleate from the *sn*-2 position of phosphoglycerides is transferred to cholesterol in the final stages of the keratinization process. Cholesterol esters are not membrane lipids. Cholesteryl oleate is a liquid at skin temperature, and it has been suggested that it phase separates into isolated liquid phase droplets within the stratum corneum. This could provide a mechanism for its exclusion from the lipid lamellae. Because oleic acid is a known permeability enhancer [52], keeping it out of the lipid lamellae would protect barrier function.

III. STRATUM CORNEUM LIPIDS

A. Chemistry

The lipids of the stratum corneum consist mainly of ceramides, cholesterol, and fatty acids, with small proportions of cholesterol sulfate and cholesterol esters [4].

The free fatty acids found in epidermis range from 16 to 28 carbons in length and are predominantly saturated [51]. In the stratum corneum, these fatty acids represent about 10% of the lipid mass, and the 22- and 24-carbon entities are the most abundant. With the exception of a small proportion of cholesterol sulfate, the fatty acids are the only ionizable lipids in either human or porcine stratum corneum. It has been suggested that ionized fatty acids may be important for the formation of lamellar phases in this compartment [30].

Structures of the ceramides identified in human stratum corneum are summarized in Figure 1.1. Every possible acid–base combination is represented, including three linoleate-containing acylceramides, shown at the top.

As noted above, the acylceramides derived from acylglucosylceramides are structurally highly unusual. The ω-hydroxyacids are 30 to 34 carbons in length, which is long enough to completely span a typical bilayer. Furthermore, the ester-linked linoleate on the ω-hydroxyl group makes it possible to insert this portion of the molecule into an adjacent bilayer, thus riveting the two bilayers together at a molecular level. Transmission electron microscopy using ruthenium tetroxide post-fixation [38] and small-angle x-ray diffraction [39,40] both reveal a predominant 13-nm lamellar spacing, which is thought to be a trilamellar lipid structure. In experiments in which lamellae were reconstituted from extracted stratum corneum lipids, the 13-nm periodicity could be reproduced, clearly demonstrating that this repeat unit does not require any protein. However, if acylceramide was removed from the lipid mixture, then essentially only a 5-nm periodicity was observed [39,40]. It has been proposed that acylceramide molecules in the outer lamellae of the trilaminar units insert their linoleate tails into the central lamella, thus riveting the three lamellae together at a molecular level [44,42].

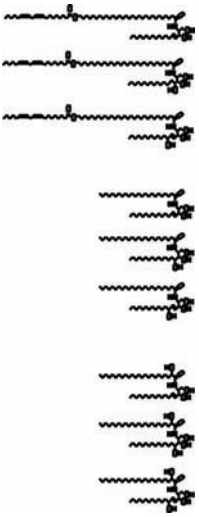

FIGURE 1.1 Representative structures of human epidermal ceramides. The top set of three structures represents the acylceramides containing, from top to bottom, sphingosine, phytosphingosine, and 6-hydroxysphingosine as the long-chain base component. The middle and lower sets of three structures show the ceramides in which the same long-chain bases bear amide-linked normal and α-hydroxyacids, respectively.

All of the ceramides as well as the free fatty acids in stratum corneum are cylindrical in shape, which makes them well suited for the formation of highly ordered membrane domains. This should be advantageous for the barrier function of the skin; however, gel phase or solid membranes could be brittle and incompatible with a pliable skin. The stratum corneum is saturated with cholesterol, and cholesterol crystals have been detected by x-ray diffraction [46]. Cholesterol is capable of making membranes either fluid or stiff, depending on the chemical nature of the membrane components and the relative proportions. It has been suggested that cholesterol in the stratum corneum serves to provide a degree of flexibility to what could otherwise be a rigid and brittle membrane system [30].

B. Phase Behavior

Most biological membranes, including those in the viable epidermis, exist under physiological conditions in a predominantly fluid liquid crystalline phase; however, there may be coexisting membrane rafts in a more ordered state [53]. If cooled, at a certain temperature the liquid crystalline phase can undergo a transition to a more rigid lamellar phase, called a solid or gel phase [2]. The temperature at which this happens is the phase transition temperature and is determined by the chemical properties of the component lipids.

Some membrane lipids, for example, dioleoylphosphatidylethanolamine, are capable of promoting the formation of a nonlamellar inverted hexagonal phase [54]. When incorporated into liposomes, lipids that favor the inverted hexagonal phase tend to be fusogenic, and it is thought that formation of this hexagonal phase is

involved in fusion events in biological membrane systems [55]. This could be significant, for example, in the fusion of the lamellar granule-bounding membrane into the cell plasma membrane. Cubic phases would be transient intermediates in going from a lamellar phase to a hexagonal phase and back again to a lamellar phase [2,56]. Stable cubic phase membranes have been identified in a number of biological systems [56].

Obviously, at the phase transition temperature at which a gel phase would melt into a liquid crystalline phase, the two lamellar phases would coexist. However, it has also been recognized that through lateral segregation of lipids within a membrane, gel phase lipid domains can be formed within an otherwise liquid crystalline membrane [57,58]. Sphingolipid- and cholesterol-rich membrane rafts are thought to be involved in trans-membrane signaling, endocytosis, and exocytosis [59]. Some of these sphingolipid- and cholesterol-rich membrane rafts have proteins called caveolins associated with them [60]. These membrane regions form caveolae (little caves), which appear as small invaginations at the plasma membrane. The caveolae are thought to be involved in the trafficking of cholesterol into and out of cells, among other functions.

The classic view of the phase behavior of the intercellular lamellae of the stratum corneum was put forth by Forslind as the domain mosaic model [61]. In this model, it was proposed that islands of gel phase domains are imbedded in a continuous liquid crystalline domain. Molecules diffusing through these lamellae would do so more rapidly through the more fluid liquid crystalline domains compared with the relatively rigid and impermeable gel phase domains. The regions of least diffusional resistance would be the phase boundaries because these would be the regions with the greatest frequency of chain packing defects. There is now ample evidence from different laboratories, using different physical techniques, that has been interpreted as support for the occurrence of gel phase domains in the stratum corneum [52,62,63]. These gel phase domains could support barrier function, whereas the continuous liquid crystalline domain could provide the flexibility necessary for pliable skin.

In lipid reconstitution experiments, it has been shown that cholesterol sulfate is capable of reducing the proportion of cholesterol in the crystalline state and of causing a shift from a predominantly 13-nm lamellar phase toward an increased proportion of a 4.3-nm lamellar period [46]. These findings have implications for the stratum corneum in recessive X-linked ichthyosis.

More recently, a single gel phase model has been proposed for the stratum corneum lipids [64]. However, the details proposed for this alternative model are not supported by the available x-ray diffraction data [65]. It is possible that a single-phase liquid ordered phase [59,66] could satisfy both the functional needs of the barrier and could fit the physical data. A liquid ordered phase is favored by lipid mixtures rich in both sphingolipids and cholesterol, and the stratum corneum lipid composition meets both of these criteria. A liquid ordered phase has some of the properties of a gel phase, including low permeability, but has some properties more closely resembling a liquid crystalline phase. Although the aliphatic chains are not melted, there is more rapid lateral movement of lipid molecules in this type of lamellar phase.

IV. STRATUM CORNEUM LIPID LIPOSOMES

A. Experimental Applications

The first reported attempt at the preparation of liposomes from stratum corneum lipids was that of Gray and White in 1979 [67]. Because of uncertainty about the composition of stratum corneum lipids, the composition of plasma membranes from granular cells was used as an approximation. As a result, a mixture of ceramides, cholesterol, fatty acids, and granular cells was used. Although vesicles were demonstrated by both freeze-fracture electron microscopy and negative staining, the inclusion of glucosylceramides and the relative proportions of the other lipid components were not representative of the stratum corneum. This was an important step toward preparing stratum corneum lipid liposomes and was one of the first reports of liposomes without phospholipids.

In 1986, liposomes were prepared from porcine epidermal ceramides, cholesterol, palmitic acid, and cholesterol sulfate in the proportions found in the stratum corneum [68]. This was achieved by sonication at 80°C, and electron microscopic methods demonstrated the formation of small unilamellar vesicles. It was shown that vesicles could be formed if either fatty acids or cholesterol sulfate were omitted, but that at least one of these ionizable lipids was required for vesicle formation. These vesicles were subsequently used to investigate roles of acylceramides and acylglucosylceramides. It was demonstrated that acylceramide or acylglucosylceramide could induce aggregation and fusion of the vesicles [69], and the fusion process could also be facilitated by calcium ions [70]. It was further demonstrated that the ability of acylceramide and acylglucosylceramides to induce aggregation did not depend on the nature of the ester-linked fatty acid. The possibility of forming a hexagonal phase was also shown [71].

A number of investigations used liposomes to study the interactions of sodium dodecyl sulfate (SDS) with stratum corneum lipids [72-76]. At relatively low concentrations, SDS readily partitioned into the liposomes until it represented about 20% of the mass of the vesicle. SDS increased the liposomal permeability. At still-higher SDS concentrations, the liposomes were destroyed with the formation of mixed micelles.

B. Commercial Applications

Although there has been a great deal of interest in developing phospholipid-based liposomes for both cosmetic [77,78] and medical applications [79,80], there has been relatively little interest in commercial development of stratum corneum lipid liposomes. Stratum corneum lipid–based liposomes, or liposomes closely resembling stratum corneum lipids in composition, could have several advantages over their phospholipid counterparts, especially for topical use. For one thing, liposomes made of ceramides, cholesterol, and long-chain saturated fatty acids resembling the endogenous lipids should be more biocompatible, thereby minimizing risk of adverse skin reactions. A second potential advantage could be in stability to oxidation. The stratum corneum lipids are highly saturated, which confers long-term stability on exposure to air. Finally, the substitution of stratum corneum lipid

liposomes for phospholipid-based liposomes for topical use could deny surface bacteria a source of phosphorous that could promote their growth.

Among the reasons that there has not been much effort in developing application for ceramide-based liposomes has been a lack of ceramides on an affordable commercial scale. There are now several commercial sources of ceramides [81], and these materials are now being used in a range of skin moisturizer products and at least one hair conditioner, although not yet in liposomal form.

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2 Water–Keratin Interactions

Jean-Luc Lévêque

CONTENTS

I. INTRODUCTION

Keratins are a family of proteins produced by certain epidermal cells in higher vertebrates. Their function is to form an interface between the living medium and the external environment. This function is essential, as it helps to maintain the physical and chemical equilibrium without which life could not exist. As an example, body hair contributes to temperature homeostasis, but it also protects the skin against ultraviolet irradiation. The stratum corneum (SC: the first 10 to 20 μm of the epidermis) controls exchanges between the "inside" and "outside" environments. In the animal kingdom, feathers not only make flight possible but also protect against temperature variations, whereas hooves, nails, and horns are necessary for travel, the search for food, and the maintenance of the species.

These proteins fulfill their role as an interface between the body and the environment thanks to their remarkable physical and chemical properties, which have been the subject of intensive research over the last 50 years. Although all keratins belong to the same family, they show a number of differences in their chemical structure and, above all, in their macromolecular organization. Despite these differences, all keratins contain the amino acid cystine (R-CH2-S-S-CH2-R), responsible for strong covalent bonds not only within a given polypeptide chain but also between chains, which makes these molecules poorly reactive and confers remarkable physical properties.

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Initially, it was the enormous economic value of wool that served as the impetus for research by chemists in large private and university laboratories of the countries concerned (mainly Australia and the United Kingdom), with the aim of modifying this raw material. Later, keratins were used as model proteins in studies by biologists, chemists, biophysicists, and physicists.

As outlined at the beginning of this chapter, keratins have an important interface function, but this can be influenced by environmental conditions that modify their nature and, therefore, their properties. Among these environmental factors, water is one of the most important. Because of their highly intense dipolar nature, water molecules can form bonds with many side-chain endings of keratin. It can also cause internal reorganization of the hydrophobic groups. Water can thereby have a major effect on the physical properties of keratin (Figure 2.1).

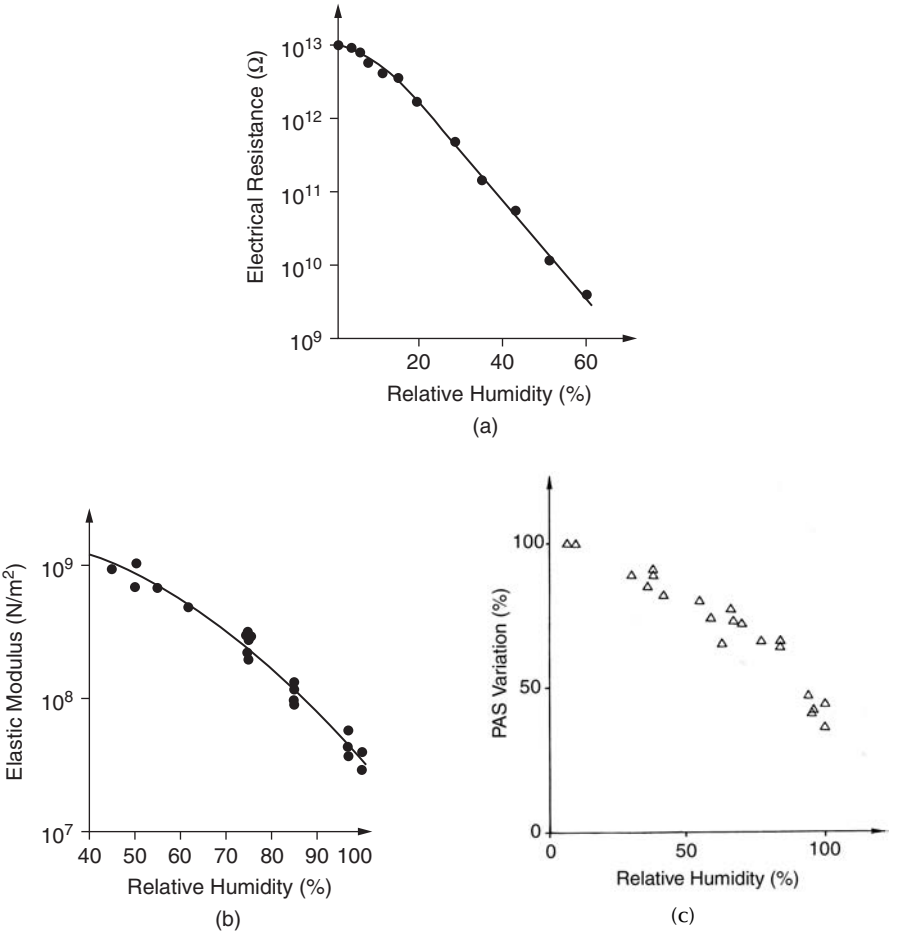

FIGURE 2.1 Influence of the relative humidity on the physical properties of keratin: (a) electrical resistance of natural hair, (b) elastic modulus of SC samples, and (c) photoacoustic properties of SC.

II. SC AND HAIR: SIMILARITIES AND DIFFERENCES

Like the eyelashes and nails (which will not be dealt with here), the SC and hair are part of the large family of keratinized tissues [1]. Both the SC and hair are composed of cells (disk-shaped corneocytes and fusiform cortical cells, respectively) that are connected by an intercellular cement. In hair, the cortical cells are protected by about 10 layers of cuticle cells. These layers play a role in limiting the exchanges between the cortex and the environment. They also limit the water swelling of the structure.

At the microscopic scale, the hair is composed of macrofibrils, which are themselves formed of microfibrils with a diameter of about 80 Å. These microfibrils probably correspond to the tonofilaments in the SC. Protofibrils (or intermediate filaments) with a diameter of about 20 Å have also been described. In hair, these protofibrils contain two α-helices, demonstrated by Pauling in 1951. Questions are now being raised as to the presence of these helices in the SC, which does not produce the X-ray diffraction pattern characteristic of α-keratin. According to Garson et al., the keratin in the SC would be an intermediate between β-keratin and that of feathers [2].

The constituents of these two "tissues" also present similarities and differences. Without going into too much detail, one of the major differences is the fact that lipids represent about 10 to 15% of the mass of the SC but only 2% of hair. The presence of these lipids explains, at least in part, the slow equilibrium kinetics with water.

In SC, lipids form bilayers that, using the transmission electronic microscope, appear to fall under two main types of organization: regular and irregular alternations of clear and dark bands [3]. A chemical model, based on the interdigitation of ceramide molecules, has been proposed to represent this type of organization [4]. X-ray diffraction allows us to measure the reticular distance of these bilayers. They are characterized by two reticular distances at 6.5 and 4.5 nm [2], the second figure being probably the third order of a 13.5-nm reticular distance [5]. It is worth noting that there is no swelling of these bilayers during hydration, as no change in these reticular distances can be detected. This would mean that water molecules are located in the zone between the polar heads of two successive bilayers. Concerning hair, the situation is quite different: Only one diffraction pattern can be distinguished, at 4.2 nm, and the pattern is not always present [6]. From a chemical and physical point of view, there are also important differences.

Using Raman spectroscopy, Gniadecka et al. discovered major differences among SC, hair, and nail in the behavior of the lipids and proteins [7]; a greater fluidity is found in SC. Using differential scanning calorimetry, Golden et al. suggested a correlation between SC lipid fluidity and water content [8]. These studies clearly demonstrate that there is an interaction between lipids and water [9], but a comprehensive model of this interaction still does not exist.

Table 2.1 shows the very large difference in the cystine content of the constituent proteins. The low concentration of cystine in the SC probably explains its low-elasticity module [10]; similarly, the covalent cystine bonds (which are mainly perpendicular to the axis of the hair) probably limit its swelling and, therefore, its

TABLE 2.1
Amino Acid Analyses of Low Sulfur Fractions (residues per 100 residues) According to Fraser

Residue	Hair	Nail	Stratum Corneum
Alanine	6.9	6.5	7.1
Arginine	7.2	6.7	4.5
Aspartic acid	9.3	8.9	9.8
Half cystine	7.6	7.4	2.9
Glutamic acid	16.6	15.2	15.8
Glycine	5.2	6.6	11.6
Histidine	0.7	0.9	1.6
Isoleucine	3.7	3.9	4.6
Leucine	10.2	9.9	9.3
Lysine	3.5	4.5	5.7
Methionine	0.4	0.8	0.5
Phenylalanine	2.0	2.2	3.4
Proline	3.8	4.1	2.9
Serine	9.0	8.7	7.2
Threonine	5.5	5.0	5.4
Tyrosine	2.5	2.8	2.0
Valine	6.1	5.9	5.7

Source: From Fraser, R.B.D., *Keratins*. C. Thomas, New York, 1972.

maximum water uptake. This is illustrated in Figure 2.2, which shows the absorption isotherms for water of the SC and hair. In the zone of moderate humidity, the pattern is similar for the two tissues, probably because the nature of the substrate controls the phenomenon, as we shall see later. Above 80% relative humidity, the open structure of the SC modifies this process. Because of the stratum corneum's strong dilation (the thickness of the SC can increase by more than 100%, though that of the hair is limited to 15%), the structural interaction of the polypeptide chains with the water molecules falls sharply, disappearing above a regain of 30% (see Section VI). Let us first look at the hydration phenomena from an experimental viewpoint and then go into what is known of the different types of water binding to keratin.

III. SORPTION ISOTHERMS OF KERATINS AND OTHER RELATED TECHNIQUES

To investigate the hydration of any substrate, the first step is to determine the sorption isotherm, which reflects the quantity of water that can bind at a given temperature and relative humidity. Such curves are shown for the SC and natural hair in Figure 2.2. As for many other substances, particularly those composed of protein, these curves have a sigmoid aspect and can be divided into three sections.

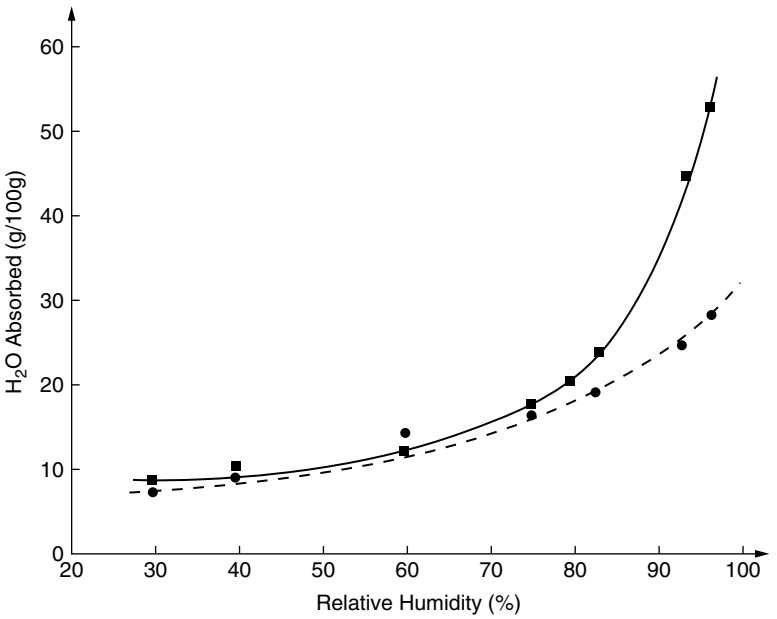

FIGURE 2.2 Sorption isotherms (23°C) for hair (~) and SC (U).

The first section corresponds to water contents between 0 and 9%, at which water uptake is very rapid. This section is the most difficult to study, because it requires deep dehydration of the studied specimen. Beyond about 70% relative humidity, the increase in water content is very rapid — particularly in the case of the SC, which can take up several times its own weight in water, whereas natural hair can only take up about 30% of its dry weight.

As mentioned previously, the two curves diverge widely in this zone of humidity. This type of curve closely reflects the interactions between the chemical groups of a given material and water molecules. The sorption isotherm provides information on the interactions with the various sites in terms of the number of molecules, as well as the condensation of several molecules either on a given site or on a water molecule that has already been bound. The problems relating to the binding energies can only be tackled by means of calorimetric measurements, with or without weight measurements [11].

Finally, spectroscopic methods such as infrared measurements and magnetic resonance spectroscopy deserve a mention, as they can be used to investigate protein–water interactions (see below). X-ray diffraction can also be used to detect the influence of water molecules on the fine structural elements such as microfilaments and protofilaments of the hair or SC [12].

We will now examine the different ways in which water molecules can bind to keratin, using some experimental findings.

IV. TIGHTLY BOUND WATER

When SC or hair specimens have been profoundly dehydrated (moderate heating in a vacuum), water uptake at a relative humidity between 0 and 20% apparently corresponds to the binding of water molecules in a single layer on a number of particular binding sites. Many experimental methods have been used to investigate this phenomenon, including calorimetric measurement [13], infrared absorption [14], mathematical analysis of isotherms for normal or modified samples [15], depolarizing thermocurrents, [16] and electrical conductance [17]. Binding energy with the protein, in the case of human SC, can be seen in Figure 2.4 [18]. The high interaction energy (58 kJ/mol) probably corresponds with a double hydrogen bond with the polar side-groups of two close keratin filaments. This stage of hydration corresponds to the first stage of primary hydration described by Hansen and Yellin [14]. In the case of wool, the drying energy is also high in this region (67 kJ/mol) according to Watt et al. [19], whereas Escoubés found a sorption energy value of 61 kJ/mol for human hair [20].

These values are close to the hydration energy at NH_3+ sites; in contrast, the hydration energy of carboxyl groups (COO–) is only 31 kJ/mol. Watt and Leeder

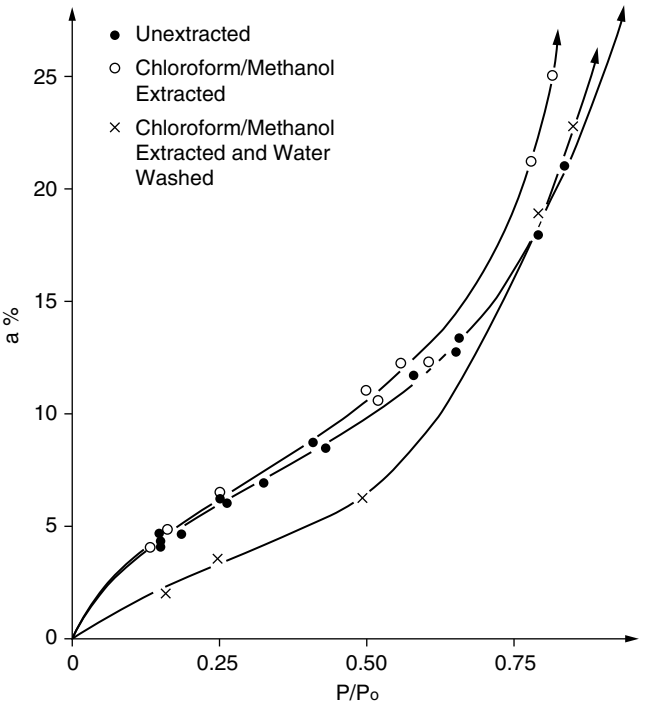

FIGURE 2.3 Sorption isotherms. Water content (a) of SC as expressed by the ratio of the amount of water over the dry weight sample without lipids. ● — ●, unextracted; ○ — ○, chloroform/methanol extracted; and × — ×, chloroform/methanol extracted and water washed.

consider that steric hindrance may explain the better accessibility of COO– groups at low humidity [21]. Thermocurrent studies also indicate preferential binding to carboxyl groups [16]. Precise mathematical analysis of the isotherms in the region of 0 to 20% relative humidity allows one to calculate the number of strongly bound water molecules: BET thermodynamic analysis and Harkins Jura analysis gave values of 5.5 and 7.8%, respectively [12].

V. BOUND WATER

The precise work by Watt and Leeder showed that the percentages of water molecules bound to carboxyl and amino groups accounted for only 46% of the sites occupied at 65% relative humidity; that is, 2.3 and 2.6 molecules at each site on average [21]. At the beginning of the sorption process, there is thus a condensation of water molecules on other hydrophilic sites (e.g., the peptide group or free OH) via hydrogen bonds. This explains the gradual decrease in binding energy illustrated in Figure 2.4. This process allows water molecules to insert between polypeptide chains and leads to a reduction in the interaction between the chains and, thus, facilitates the plasticization of the structure.

The extent to which this process takes place depends on the macromolecular conformation of the chains. In materials containing a large degree of crystalline matter, the quantity of water at equilibrium would tend to be lower than in amorphous substances. As an example, Zeine (70% of α-helices) contains 50% less water than lactoglobulin (10% of α-helices). Contrary to the case of highly bound water, the water molecules bound by hydrogen bonds can mutually exchange protons that migrate within the network of hydrogen bonds. This mechanism explains why electrical conductance and bound-water content are strongly related.

Experiments using differential thermal analysis by Bulgin and Vinson [22] and Walkley [13] gave a figure of about 34% of SC dry weight, which is in agreement with the values obtained by Hansen and Yellin (30 to 35%) [14], who used infrared measurements. These percentages correspond to the normal SC. They are in good accordance with recent results obtained by Gniadecka et al. [7], who conclude that about 20% of the SC total water content is in a free form (tetrahedron water structure). In the case of psoriatic plaques and the stratum disjunctum from patients with senile xerosis, the percentage of bound water appears to be lower [23].

VI. FREE WATER

The experiments cited above show that beyond this threshold value of about 34%, the water present in the horny layer gives rise to a fusion peak at about 0°C, indicating the presence of "freezable" water known as free water. It is noteworthy that this peak does not appear in hair keratin (which never reaches 34% of water content); in other words, hair does not contain free water or freezable water. This is supported by the magnetic resonance spectroscopy experiments by Clifford and Sheard [24] and Lynch and Marsden [25], who found that water molecules are restricted in their movements even at 60°C but do not freeze, whatever the water content. However,

some experimental data have shown that above a water content of 18 to 19%, water molecules have peculiar properties. This is the case of the electrical properties of keratin. Above this water content value, the activation energy of electrical conductance falls from 125 to 58.5 kJ/mol [26]. Similarly, a thermal current peak (a dielectric polarization phenomenon) appears at 130°K, corresponding to a characteristic of the free-water fraction [27].

It thus appears that above a water content value of about 19%, water molecules in hair condense in aggregates that retain the same polarization properties as in the liquid state, but lose the rotational properties of free water, as indicated by magnetic resonance spectroscopy experiments. As a result, the term "free water" is totally inappropriate; "intermediate water" would be more suitable.

As stated above, in normal physiologic conditions, SC water mainly exists in a bound state. When it is totally hydrated, however, Transmission Electron Microscopy studies show that water exist as "pools" or "voids" that can disrupt the structure and even shift lamellar bilayers, creating amorphous intercellular zones [28]. These modifications are, however, reversible [29].

VII. INFLUENCE OF LIPIDS AND HYDROSOLUBLE MATERIALS

Figure 2.3 represents the effect of extracting lipids and water-soluble material (WSM) on the sorption isotherm, and Figure 2.4 shows the changes in enthalpy with water content in the same conditions. The use of statistical thermodynamic methods (BET) has shown that lipid extraction slightly but significantly increases the value of the monolayer (5 to 6%), whereas extraction of WSM reduces it (5 to 3.5%).

In Figure 2.3, it can be seen that water content increases by about 10 to 20%, indicating that some binding sites for water molecules are probably inaccessible when lipids are present. This phenomenon appears to be most marked in the range of high humidities. According to Hey et al. [12], the x-ray diagram for the SC, which gives a 10-Å line (corresponding to the average distance between the keratin chains), shifts by about 1.3 Å when the relative humidity reaches 65%. They logically concluded from this result that, in this relative humidity range, the water is located between the keratin microfibrils.

We have shown that more water penetrates the structure in the high relative humidity range when lipids are removed. This last result, combined with the x-ray results of Hey et al. [12], means that a certain number of lipids are likely to be located inside the corneocytes. This agrees with one of the conclusions reached by Goldsmith and Baden in 1970 [30]: WSM could be an important element in water binding, but the most important phenomenon observed during their extraction was the considerable limitation of the equilibrium kinetics between SC and the atmosphere. Thus, the role of WSM could be to facilitate the passage of water between the lipid bilayers (intercellular spaces) into corneocytes.

There has been far less work done on hair and wool. The amount of lipids in hair has been estimated at about 2% by weight [31], but their influence on hydration has not, to our knowledge, been described. However, permanent waving and bleaching appear to induce greater water uptake in conditions of high humidity.

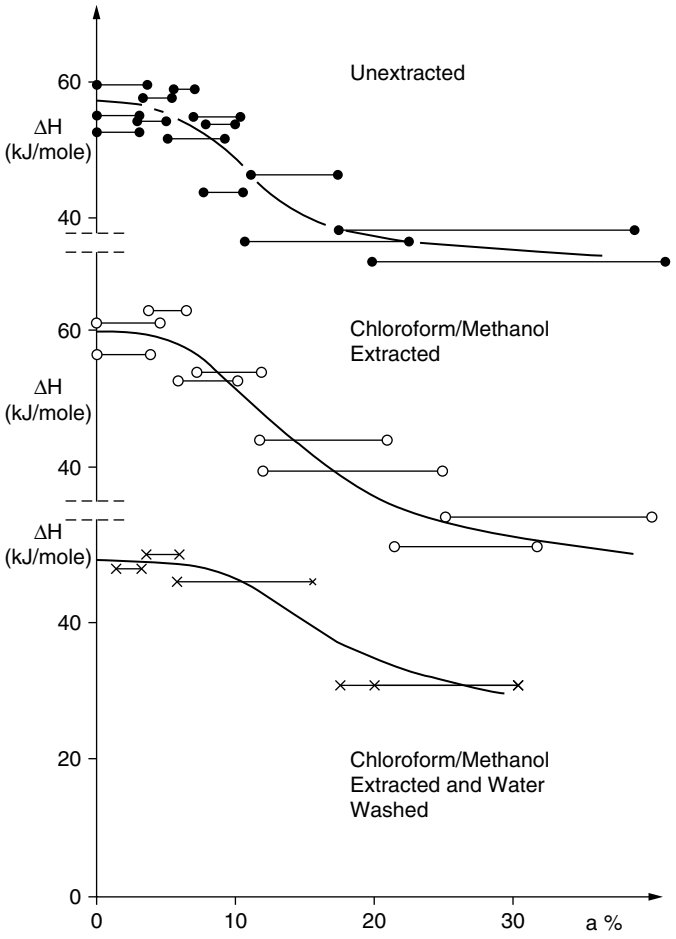

FIGURE 2.4 Enthalpimetric measurements. Water content (a) of SC as expressed by the ratio of the amount of water over the dry weight sample. s — ●, unextracted; chloroform/methanol extracted; and chloroform/methanol extracted and water washed.

VIII. SC AND HAIR SWELLING

There are very few data concerning SC and hair swelling. In regard to SC, a recent study carried out using confocal microscopy concluded that there was 20 to 30% swelling in the lateral dimension versus 3 to 5% swelling in the longitudinal one [32]. Lipid extraction produces a higher lateral swelling, which would be linked, according the authors, to the intercellular lipid structure. It must be noticed that removing polar lipids washes away 12% of water-soluble materials (natural moisturizing factors) that, as mentioned earlier, play an important role in the water–SC interaction. The importance of these natural moisturizing factors can be inferred from the huge swelling percentage of simple corneocytes [33].

Both previous and recent studies on hair demonstrate a 12 to 15% lateral swelling in water versus a 1 to 2% swelling in the longitudinal dimension [34]. This swelling anisotropy in hair and the SC can be explained by the location of the water molecules in the keratin structure. X-ray diffraction shows that there is only very weak interaction between water molecules and the helices (unpublished results).

Water molecules are mainly located in the amorphous part of keratin, between the microfibrils, which are moved away. Removing the hair cuticle by means of a razor blade, for example, results in a much more marked swelling, which demonstrates the role of the cuticle in strongly limiting the water sorption by the hair cortex.

IX. CONCLUSION

Water has considerable influence on the physical properties of both hair and the SC. For example, there is a coefficient of about 100 between the elasticity modules measured in dry and humid conditions [35], whereas electrical conductance is multiplied by a factor of 10,000 between dry and hydrated samples [36]. Major changes are also seen in both optical [37] and thermal properties [38]. The equilibrium between keratin and water is thus essential for keratinized tissues to fulfill their role correctly. Our understanding of these interactions is far from complete. As we saw earlier in this chapter, WSMs play an important role in the hydration of the SC. A major advance was made when WSMs were shown to be filaggrin degradation products [39]. Unfortunately we still do not know exactly how these products (mainly amino acids) influence the degree of hydration, but it may involve a direct effect or the facilitation of water molecule access to the hydrophilic sites on keratin. Similarly, little is known on the role of intercellular lipids, except for their barrier function.

The information presented in this chapter was derived from data obtained *in vitro*. The situation *in vivo* is of course highly different; for example, there is a hydration gradient between the external layer and the internal layers. This gradient is responsible for the continuous flow of water vapor across the SC. In addition, sweat secretion — both continuous and discontinuous — plays an important role in the hydration of the SC.

Finally, surface lipids (essentially sebum) can form an emulsion with water and probably play a role in maintaining surface hydration (even though they do not form a barrier to water vapor). All these problems are difficult to approach *in vivo,* as almost all the methods we have for quantifying hydration are indirect. Fortunately, more specific methods are beginning to emerge: In particular, near-infrared spectroscopy, magnetic resonance imaging, and spectroscopy should prove invaluable in this setting.

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