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The essential role of lipid bilayers in the determination of stratum corneum permeability

Anthony J.I. Ward and Charles du Reau

Department of Chemistry, Clarkson University, Potsdam, NY 13699-5810 (U.S.A.)

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Summary

Water permeability of reconstituted stratum corneum using various lyotropic liquid crystal mixtures of simple lipids is found to be similar to that of intact stratum corneum. Optical microscopy showed that the integrity of the bilayer arrangement of the lipid molecules is maintained in the reconstituted material. The formation of the lipid lamellae found in the SC is discussed in terms of stability at the water-amphiphile interface. The relative invariance of the water flux values with the type of lipid mixture chosen is interpreted, therefore, as indicating the prime importance of the bilayer arrangement in determining the barrier function.

Introduction

The permeability properties of the skin have long been topics of interest for areas of investigation in pharmacy, dermatology and cosmetics. In recent times interest has focused on the usefulness of the skin as a potential route for drug administration. Here, two factors that are considered favoring this form of drug delivery are the accessibility of this organ and a potentially better control of the rate and amount of drug released to the body.

The permeability of skin to drugs and water has been thoroughly investigated (Blank, 1952, 1964; Blank and Scheuplein, 1964; Scheuplein, 1965; Blank et al., 1984) with the early indications taken to favor the transcellular route as being the dominant pathway. However, more recent investigations (Elias and Friend, 1975; Nemanic and Elias, 1980; Smith et al., 1982) have shown the essential role of intercellular lipids in percutaneous absorption. It is now generally accepted that the barrier to diffusion is primarily located in the uppermost layer of the epidermis called the stratum corneum (SC). Much progress has been made in understanding the ultrastructure of this tissue and the importance has been demonstrated (Elias and Friend, 1975; Elias et al., 1977, 1979) of the intercellular medium of the SC which contains several types of lipids. The essential role of the intercellular lipids in the barrier properties of the skin has been demonstrated (Imokawa and

Correspondence: A.J.I. Ward, Dept of Chemistry, Clarkson University, Potsdam, NY 13699-5810, U.S.A.

Hattori, 1985) by removal of the lipid component by solvent extraction leading to rapid transepidermal water loss.

Transmission electron microscopy of freeze fractured specimens (Elias and Friend, 1975; Elias et al., 1977) indicated a lamellar or bilayer arrangement for the intercellular lipids. Later, low-angle X-ray diffraction studies (Friberg and Osborne, 1985, 1987) of the epidermal lipids were consistent with the typical geometrical arrangement of a lyotropic lamellar liquid crystal. An important point found in these studies was that this structure was only obtained when the free fatty acids were saponified to an extent consistent with a pH of approximately 5.0 which is typical for in vivo human skin (Rothman, 1954). The observation of a lamellar arrangement of the SC lipids has more recently been confirmed (White et al., 1988) also from low-angle X-ray diffraction studies. However, although no similar bilayer arrangement of the extracted lipids was found, in agreement with the earlier observations, an intercellular protein was postulated to be responsible for the lamellar SC lipid arrangement.

The subject of the associated structures formed by surfactants and lipids has been one which has shown itself amenable to description in basic physical chemical terms. Processes such as amphiphilic association with, in particular, bilayer formation, and the factors determining stability have been discussed in detail (see, e.g., Israelachvili et al., 1976). Recent advances (Helm et al., 1989) in the understanding of bilayer adhesion and fusion is of direct relevance to processes such as the release of the lipid contents from lamellar bodies in the stratum granulosum. It is these extruded contents which have been supposed (Elias and Friend, 1975) to fuse together to form the intercellular lipid medium of the SC.

An important question relating to the observed bilayer structure of the lipids and its influence on the barrier function is that of the relative importances of the molecular arrangement compared to the details of the molecular structures. This paper addresses this question making use of an experimental approach to prepare stratum corneum reconstituted with different bilayer forming lipid mixtures.

Materials and Methods

Materials

The nonionic surfactant *n*-dodecyl pentaoxyethylene glycol ether, $C_{12}EO_5$, was from Nikko Ltd (Japan) and was 100% pure in the alkyl chain and > 98% homogeneous in the polyoxyethylene chain as determined by gas chromatography. Sodium dodecyl sulphate was obtained from B.D.H. Ltd (Specially Pure) and was recrystallized three times before use. Oleic acid from Sigma Ltd was used without further purification. *n*-Dodecanoic acid-d_{25} was obtained from MDS Isotopes and was used without further purification.

Optical microscopy

The textures of the various lamellar phases and the RSC material were observed using a polarized light microscope (Zeiss).

Scanning electron microscopy

RSC discs were fixed in glutaraldehyde 3%. Dehydration was carried out in an ethanol series and the fixed samples were cemented directly to aluminum specimen blocks with silver conducting paint and then coated with a thin layer of gold paint. Images were taken using an International Scientific Instruments model SMS-2 scanning electron microscope.

Nuclear magnetic resonance spectroscopy

^{2}H N.M.R. spectra were obtained with a Fourier Transform N.M.R. spectrometer (IBM NR/250) operating at a resonance frequency of 38.08 MHz for deuterium. Samples were mixed and transferred to standard N.M.R. tubes (10 mm o.d.) and remixed and centrifuged to remove air bubbles. A spectral width of 100 kHz was typically used and 30000 transients averaged. All measurements were made at 30 °C with samples containing 0.5–1.0% (w/w) of the deuterated probe.

Low-angle X-ray diffraction

Samples were sealed in thin-walled glass capillaries (0.7 mm o.d.) and mounted on a thermostated cell holder which was held at 31 °C.

Characteristic lamellar d-spacings were obtained using a low-angle camera (Richard Seiffert) with position sensitive detector (Tennelec PSD-1000) and Nickel filtered Copper radiation ($\lambda = 0.157$ nm).

Preparation of reconstituted stratum corneum (RSC) films

Pieces of human stratum corneum were scraped from the calf skin of volunteers and the corneocytes were isolated after lipid extraction using chloroform:methanol (2 : 1). 5–10 mg of cells and a proportional amount of liquid crystal were suspended in ether and added slowly to water in a petri dish. The area of the suspension was limited by placing a rubber O-ring (1 cm diameter) on the water surface. After evaporation of ether, the petri dish was stored overnight at 0 °C for the hydration of the newly formed RSC film. This was then carefully separated from the rubber O-ring and the cohesion of the film was tested by suspending it in water and vortexing for 1 min (Smith et al., 1982).

Permeability studies

A two compartment glass diffusion cell (Blank et al., 1984) was used for permeability studies. The RSC disc was held between two Teflon discs and clamped between the two compartments. 1 ml of a salt solution of K_2CO_3 (1.4 M) was put in either side of the cell. As a result, the RSC film was only in contact with the vapors, which had a relative humidity of 93%. The diffusion cell was placed in a thermostated bath at 31 °C during 3 days to equilibrate for the hydration of the RSC film.

Once the equilibrium was attained, the donor compartment was spiked with 10 μl of tritiated water (HTO) of known activity (1 mCi/g). Then, 10 μl samples of the receptor compartment were taken at regular intervals and the concentration of HTO was measured using standard scintillation counting techniques (Packard TRI-CARB 3000C Liquid Scintillation System).

It has been demonstrated (Blank and Scheuplein, 1964) that water transport across the stratum corneum is diffusion-controlled. Fick's first law of diffusion, therefore, can be integrated under steady-state conditions. However, the stratum corneum does not act as a totally passive membrane but shows a selectivity for different diffusing molecules which can be expressed by a partition coefficient. As a result, the permeability coefficient, K_p, and flux, J, of tritiated water may be evaluated from:

$$J = K_p(C_1 - C_0) = \frac{KD}{d}(C_1 - C_0)$$

where K is the water-membrane partition coefficient, D is the water diffusion coefficient and $C_1 - C_0$ is the HTO concentration gradient across the membrane and d is the thickness of the membrane.

Results and Discussion

Reconstituted stratum corneum films (RSC) were successfully made with lyotropic liquid crystals based on simple surfactants replacing the normal lipid fraction. Samples were produced using lamellar phases formed by either nonionic or anionic surfactants (Table 1).

These phases were chosen because of their ability to incorporate water over a wide range of compositions (Figs 1 and 2). System B was prepared as a model for the stratum corneum lipids where the nonionic surfactant was used to replace all of the lipid components except for the fatty acid. The rationale behind the use of $C_{12}EO_5$ was that the primary interaction between the polyoxyethylene headgroup with the water is through hydration interactions. In this respect, this is similar to the strong hydration interactions of the ceramides and sterols found naturally in

TABLE 1

Model lipid mixtures

System	Composition (% w/w)
A	$C_{12}EO_5$ (68): Water (32)
B	$C_{12}EO_5$ (55)/Oleate (5.5):Oleic Acid (7.5)/ Water (32)
C	SDS (45)/*n*-Decanol (23)/Water (32)

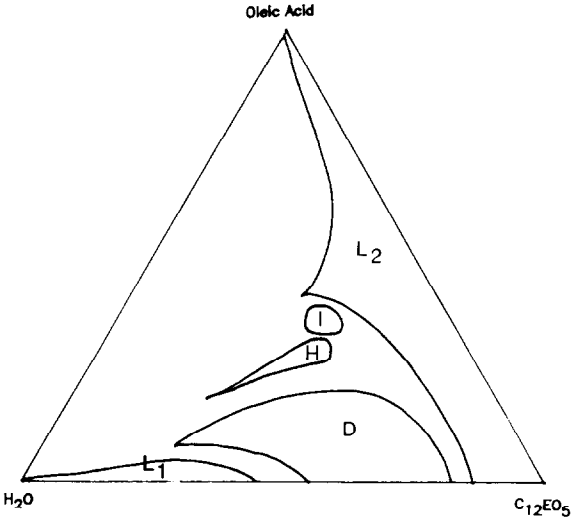

Fig. 1. Ternary phase diagram of $C_{12}EO_5$/oleic acid/water at 298 K. L_1, isotropic liquid; L_2, isotropic liquid; D, lamellar; H, hexagonal; I, cubic.

the SC. In this context the choice of a surfactant-based system to model the epidermal lipids must be founded on a structural analysis of the major features of the natural lipids. The phase behavior of epidermal lipids is known to be typical of a lamellar phase from electron microscopy results (Elias and Friend, 1975), and low angle X-ray diffraction (Friberg and Osborne, 1985; White et al., 1988).

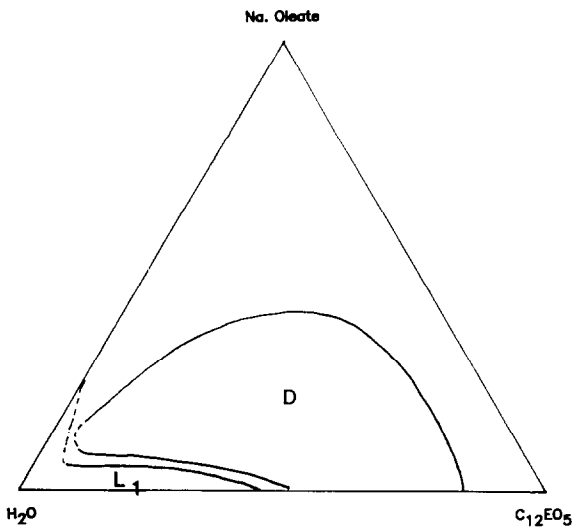

Fig. 2. Ternary phase diagram of $C_{12}EO_5$/sodium oleate/water at 298 K. L_1, isotropic liquid; D, lamellar.

The first important result of characterization of epidermal lipids (Elias et al., 1979; Lampe et al., 1983) is the shift in lipid composition from the basal layer to the cornified layer. It was noted that as the relative amount of phospholipids was significantly reduced, the sphingolipids and neutral lipids became predominant in the SC. In other words, the formation of the diffusion barrier was characterized by the replacement of ionic lipids by nonionic lipids.

Another important finding was the importance of sphingolipids (Gray and White, 1979; Landmann, 1984; Wertz et al., 1986) for the formation of lamellar structures. Finally, the essential role of sphingolipids in the retention of water by the stratum corneum was recently demonstrated in an elegant paper (Grubauer et al., 1989). From these experiments, it can be inferred that sphingolipids play a key role in the phase stability of these intercellular lipid lamellae and that the lamellar arrangement of lipids is essential to preserve the barrier function of the stratum corneum.

Ceramides are complex lipids composed of a long chain amino alcohol base and a long chain fatty acid associated by an amide linkage. The epidermal ceramides typically comprise a base called sphingenine (containing 18 carbons and one double bond) and a saturated fatty acid (containing mostly 22–24 carbons). Very little is known about the spatial structure of these complex molecules. However, Pasher (1976) obtained some useful information by means of crystallography, infrared spectroscopy, thin layer chromatographic behavior and monolayer studies. Some molecular arrangements were proposed and the role of hydrogen bonding was discussed in terms of membrane stability and permeability. These structural observations prove once more that sphingolipid molecules can contribute to bilayer stability of stratum corneum lipids.

The evidence, therefore, supports the idea that the lamellar bilayer structure formed by the lipid components of the SC and which provide its barrier function rely on lipids which are nonionic and have strong hydrogen bonding interaction with the aqueous region. The choice of a simple

nonionic surfactant to model the nonionic components of the SC lipids is, therefore, reasonable. The remainder is present as partially neutralized fatty acid with (note: oleic acid is the most abundant unsaturated constituent at approximately 6% (w/w) of the total SC lipid fraction (Lampe et al., 1983)) with oleic acid being chosen as the model. Even though substances such as oleic acid and SDS are recognized as important perturbents of skin structure, it should be noted that ^{2}H NMR results (Ward and Tallon, 1990) show an increase in dynamic order of the bilayers, i.e., increased order parameters when such solubilizates are incorporated. This is not unreasonable since unless the bilayers can spread at a sufficient rate, incorporation of a solubilizate intercalated between the bilayer forming molecules must necessarily increase the number of all trans conformations of the C–C segments. It may be noted here that barrier integrity is lost in diseases such as psoriasis where increased lipid crystallinity is found. Thus, to some extent, an increase in crystalline nature of lipid bilayers forming chains would be expected. These observations, at first sight, are in contradiction to the generally accepted wisdom in the pharmaceutical literature that percutaneous enhancing agents cause a disordering of endogenous intercellular lipids. This traditional view may have to be reconsidered in view of recent observations (Ward et al., 1989) of identifiable separate fractions of solubilizates in some bilayer systems. Thus, it appears that a certain fraction of a solubilizate such as oleic acid can form a liquid layer located at the center of the bilayer. Such a scenario has previously been suggested (Ward et al., 1988) for simple alkanes solubilized in lamellar phases of $C_{12}EO_5$, and has also been proposed (White, 1981; White and King, 1983; Jacobs and White, 1984) for analogous systems of phospholipids containing solubilized alkane using detailed X-ray and SANS analyses. The degree of neutralization of the fatty acid (41% molar) was chosen to represent a pH in the range 4.5–5.5 again similar to that found for human skin (Rothman, 1954). This system was found coincidentally to accommodate water over a range of contents (25–80% w/w) also similar to that normally found in the skin.

Information about the structure and dynamics of such lamellar phases can be derived using nuclear magnetic resonance (NMR) spectroscopy and low-angle X-ray diffraction. The characteristic spacing of the lamellar structure – the d-spacing – increases linearly with water content and the degree of solvent penetration into the bilayers calculated (Ward and Tallon, 1990) using the expression:

$$P(\%) = \frac{(d_{calc} - d_{exp})}{(d_{calc} - d_0)} \times 100$$

where d_{calc} is the d-spacing calculated assuming no penetration, i.e., $d_{calc} = d_0(1 + \phi)$, d_{exp} is the experimentally observed value and ϕ is the volume fraction of water in the sample. A value of 13% penetration is obtained for system B which compares to 33% found for the fatty acid free system A. This indicates a well-defined bilayer/water interface relative to the system A where the interface has been shown (Ward et al., 1988) to be highly flexible. Confirmation of an ordered bilayer/water interface is also given by consideration of the order profile derived from a deuterated probe molecule, *n*-dodecanoic acid-d_{25}. The deuterium spectrum consists of 12 overlapping powder patterns (Fig. 3) with quadrupolar splittings in the range 2–25 kHz. The order profile of the probe chains (Fig. 4) reflects that of the host bilayer showing an almost linearly monotonically decreasing value along the chain from the headgroup towards the terminal methyl group. The difference between this profile and that previously observed for chains of a nonionic surfactant in the lamellar phase (Ward et al., 1988) is that for system B the order parameter for the α-position is approx. 15% larger than that of the β-position. This is characteristic of bilayer systems with ordered bilayer/water interfaces (Boden et al., 1976; Charvolin, 1976; Seelig, 1977) and is primarily the result of hydrogen-bonding interactions between the solvent and the carboxylic headgroups and electrostatic interactions with the carboxylate groups. A similar profile of order/disorder has also been observed in other models of the SC lipids (Ward et al., 1989).

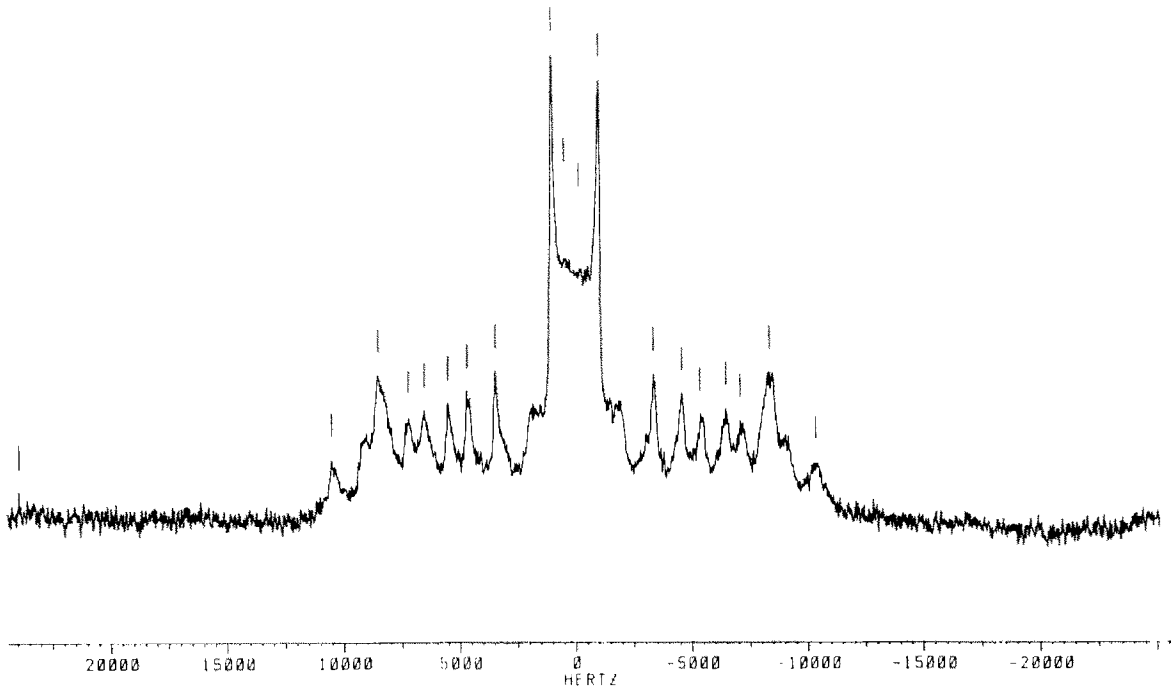

Fig. 3. ^{2}H NMR spectrum of *n*-dodecanoic-d_{25} acid solubilized in lamellar phase of $C_{12}EO_5$/oleic acid/sodium oleate/water (55 : 7.5 : 5.5 : 32 weight percent) at 298 K.

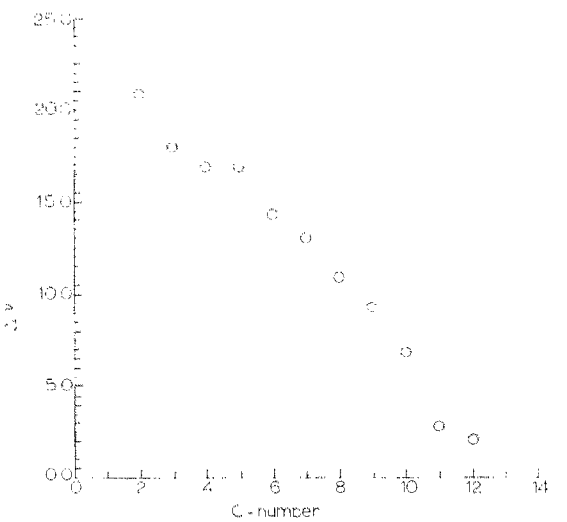

Fig. 4. Quadrupolar splittings, $\Delta\nu$, of *n*-dodecanoic-d_{25} acid in $C_{12}EO_5$/oleic acid/sodium oleate/water (55 : 7.5 : 5.5 : 32 weight percent) at 298 K.

The morphology of the RSC films was examined by polarized light and scanning electron microscopy (SEM). Classical textures associated with lyotropic liquid crystals were observed (Fig. 5) confirming the integrity of the lamellar structure in the reaggregated material. In the case of the SEM observations, the morphology was found (Fig. 6) to resemble that of intact SC. However, although the cells are seen to form layers, their stacking was not as regular as that found in the normal intact SC.

Some consequences of the less regular arrangement of the cells in the reaggregated material in the permeability properties may be expected. The fluxes (Table 2) of water permeation measured for RSC systems made with the lamellar phases A, B, and C are comparable to each other with values in the range 2–4 mg cm^{-2} h^{-1}. It was decided to normalize fluxes to a thickness

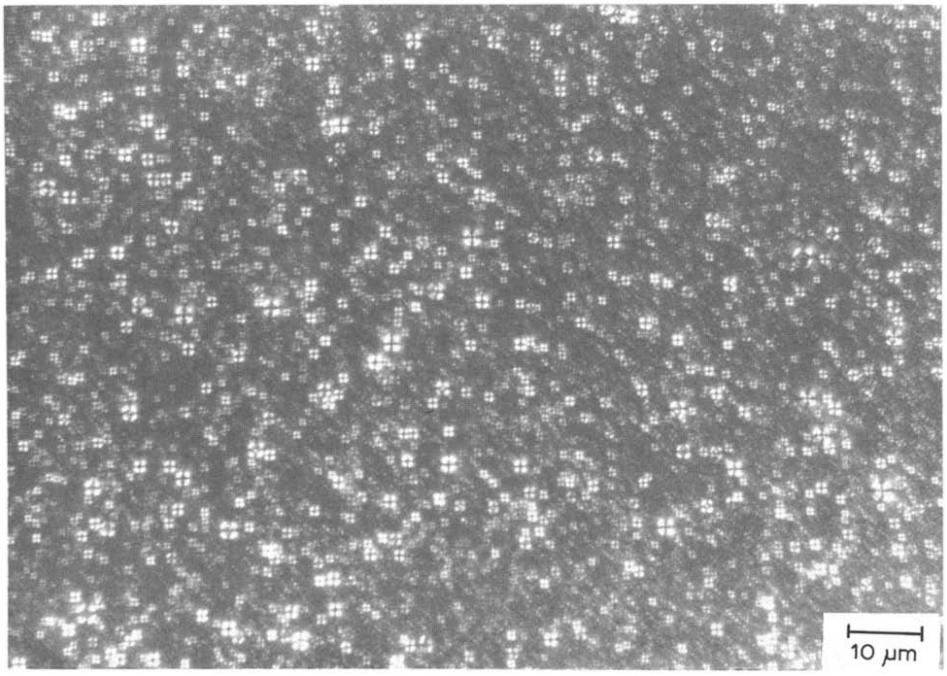

Fig. 5. Polarized light micrograph of RSC film (× 100).

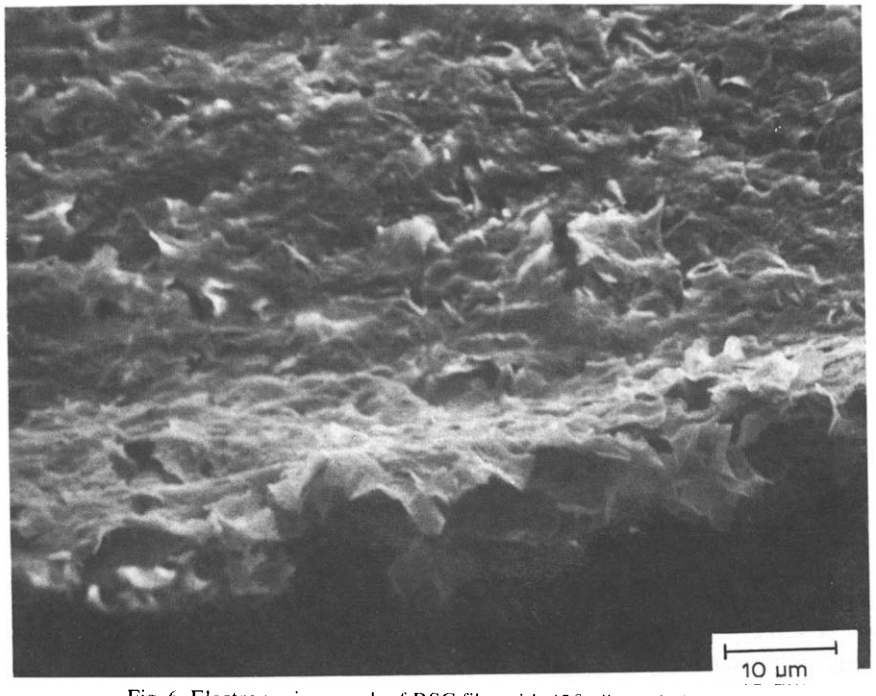

Fig. 6. Electron micrograph of RSC film with 45 ° tilt angle (× 150).

TABLE 2

Water transport through RSC systems

System	Flux (mg cm^{-2} h^{-1}) [a] (mean ± S.D.)	Permeability coefficient (K_p) (cm h^{-1})
Intact SC (Blank et al., 1984)	0.9 ± 0.2 [b]	0.7 ± 0.11
RSC (Kock et al., 1988)	2.0 ± 0.2	
RSC (Friberg et al., 1990)	2.8 ± 0.2	4.1 ± 0.6
RSC System A	2.0 ± 0.5	3.0 ± 0.3
RSC System B	2.4 ± 0.5	3.5 ± 0.5
RSC System C	3.2 ± 0.5	6.6 ± 0.5
RSC (Kock et al., 1988) lipid extracted	7.9 ± 0.2	
RSC lipid extract	4.8 ± 0.5	9.3 ± 0.6

[a] Normalized to 15 μm thickness.

[b] Standard deviations derived from measurements on 14–15 samples.

of 15 μm since it is not always possible to prepare samples experimentally with the same thickness each time. This value of 15 μm was chosen by reference to that found (Blank et al., 1984; Fig. 5) for SC exposed to a relative humidity of 93%. The assumption made in this procedure is that the partition coefficient, K, is independent of the diffusion layer thickness at a given relative humidity. These values are slightly higher than that previously found (Blank et al., 1984) (Table 2) of approx. 1 mg cm^{-2} h^{-1} for intact stratum corneum, but are in good agreement with values obtained for RSC samples made using either the extracted SC lipids (Kock et al., 1988) or a model lipid mixture (Friberg et al., 1990). Similarly, higher flux values (5–6 mg cm^{-2} h^{-1}) were found for cells reaggregated without addition of lipids which were in agreement with the results of a former study (Kock et al., 1988). The higher flux values in these cases give a good reflection of the loss of the barrier function of the SC arising from lipid depletion.

The measured permeability coefficients, K_p (Table 1), fell in the range 3.0×10^{-3} to 7×10^{-3} cm h^{-1} for the three systems studied. In this respect, they are similar to RSC samples made using a model lipid mixture closely simulating the natural lipids (Friberg et al., 1990), the native SC lipids themselves (Smith et al., 1982), and slightly higher than those measured for intact SC (Blank et al., 1984).

These data show that the RSC material has water permeability characteristics similar to that of the intact stratum corneum. The good agreement between samples produced by reaggregation with the different lamellar liquid crystalline systems and that produced using the extracted SC lipids (Kock et al., 1988) is important. Since optical microscopy showed that the integrity of the lamellar bilayer structure was maintained in the RSC, the lack of variation in the flux values indicates that the detailed nature of the bilayer forming molecules is not the main determining factor. It seems that the ability of the lipid fraction to form a bilayer structure is a major determinant of SC permeability to water. Cellular membranes, e.g., erythrocytes, leucocytes, and phospholipid bilayers (Scheuplein and Blank, 1971) exhibit K_p values in the range 0.02–0.2 cm h^{-1}, i.e., one to three orders of magnitude larger than the RSC values quoted here (Table 1) and intact human stratum corneum (Scheuplein and Blank, 1971). Considerations of the detailed pathway through each kind of membrane as expressed in terms of a tortuosity are outside the scope of the present article. It may be argued, however, that the lower permeability of the SC compared to cellular membranes results from differences in effective diffusional pathlengths within a given membrane thickness. If this were the case, a larger difference than the factor of 2–3 observed in the diffusion coefficient of water through the membranes would be expected (PL 2×10^{-10} cm^2 s^{-1}, SC 5×10^{-10} cm^2 s^{-1}). Some difference in the tortuosity of pathways in a system comprising PL bilayers to those formed by polar lipids may be expected. In the former case, the bilayer/water interface is relatively planar and smooth; whereas, for polar lipid systems it is well-known that large fluctuations occur in the lamellae surfaces parallel to the bilayer direction. This would lead to an effectively less well-defined set of bilayer/water interfaces. Further, the dispersion of cells within the bilayer matrix of the SC would provide an obstruction effect further lowering the apparent permeability of the system.

Thus, in terms of developing a system to model SC behavior, systems using simple single-chained surfactants, e.g., systems A, B and C of this work provide as good a representation as those using more complex lipid mixtures (e.g., Friberg et al., 1990) which are more closely akin to the long double-chained unsaturated lipid molecules found in vivo. A further finding of this work, in agreement with an earlier observation (Kock et al., 1988), is that even after the extraction procedure there is sufficient adhesion between the cells for reaggregation to occur without addition of exogenous lipids although the barrier function is substantially reduced. This may be the result of an unextracted lipid fraction tightly bound to the cell surfaces and further investigations of the nature of this interfacial lipid are presently being carried out. It should again be emphasized that there are differences in the degree of interactions in the lipid bilayers considered here and phospholipid systems. The strength of interaction between headgroups, because of their electrostatic nature, is much stronger than in polar lipids in the case of phospholipid bilayers. This produces a much less flexible bilayer system which is manifested in several ways, e.g., the ability to solubilize alkanes is small in PL systems ($< 5\%$) whereas polar surfactant bilayers can accommodate large quantities (Moucharafieh et al., 1979) (up to 55% w/w in the case of hexadecane). Similarly, the strength of interaction with the aqueous component is stronger in the PL case being an ion-dipole as opposed to dipole-dipole interaction.

Acknowledgements

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