Preparation of Model Membranes for Skin Permeability Studies Using Stratum Corneum Lipids

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Liposomes were prepared from stratum corneum lipids consisting of epidermal ceramides (55% by weight), cholesterol (25%), free fatty acids (15%), and cholesteryl sulfate (5%). Multiple lamellae were formed by air-drying the liposomal suspensions on hydrophilic filter disks, and water permeabilities through these filter-supported lamellae were measured using a diffusion cell. Ultrastructure of the lipid lamellae was characterized by scanning and thin-section electron microscopy. Water flux data and ultrastructure of the model membranes are discussed in relation to stratum corneum, the horny layer of the epidermis that constitutes the epidermal barrier. J Invest Dermatol 93:809-813, 1989

Permeability Studies

Preparation of Model Membranes for Skin

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Abbriviations:
EDTA: ethylene diamine tetraacetic acid
SC: stratum corneum
SEM: scanning electron micrograph
TEM: thin-section electron micrograph
TMA: trimethylamine

Stratum corneum (SC), the dead layer of the epidermis, represents a highly specialized tissue providing a protective barrier against transepidermal water loss and penetration of other solutes from the environment. SC consists of cornified cells embedded in a matrix of lipid lamellae [1]. These multiple extracellular lipid membranes are believed to constitute the epidermal water barrier [2,3].

In recent years, there has been an increased interest in percutaneous penetration due to development of topical drug products and the increasing presence of potent pharmacologically active environmental pollutants that can penetrate skin. Several reviews have appeared in recent years on epidermal barrier and percutaneous penetration [4-7]. Also, the elevated transepidermal water loss in people with dry skin and other pathologic conditions [8] has evoked interest in understanding the role of constituent lipids in maintaining the epidermal barrier.

The study of transport properties using artificial lipid membranes and other model systems that are sufficiently simple and bear resemblance to the biologic membranes is a crucial step in understanding complex biologic membranes. Some model systems have used multiphasic composites made up of alternating hydrophobic and hydrophilic polymeric layers [9]. Guy and Fleming initiated a different approach where a membrane filter saturated with lipids was used as a model membrane [10].

In the present study, we have prepared and characterized the structure of a model lamellar lipid membrane prepared from liposomes consisting of SC lipids. The lipid lamellae were supported on cellulose-ester membrane filters. The water permeability through these membranes was measured. This novel approach of using lipid lamellar sheets supported by a membrane filter was made possible by knowledge of the structures of lipids that make up the intercellular lamellae of the SC [11,12] and our recent demonstration that SC lipids can form bilayer structures in vitro [13-15].

MATERIALS AND METHODS

Lipids
Epidermal ceramides were isolated by preparative thin layer chromatography from total lipid extracts of pig epidermis, as described previously [11]. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically. A mixture of free fatty acids was prepared by combining carnauba wax fatty acids, which consisted of C20:0 (9%), C22:0 (10%), C24:0 (28%), C26:0 (14%), C28:0 (21%), C30:0 (8%), and small amounts of other chain lengths [16], with palmitic acid (C16:0) to a ratio of 8:2 by weight, forming a mixture close to the distribution of free fatty acids in SC [17].

Preparation of Liposomes
Appropriate volumes of solutions of individual lipids in chloroform:methanol (2:1, by volume) were combined to produce a mixture containing 55% by weight ceramides, 25% cholesterol, 15% free fatty acids, and 5% cholesteryl sulfate as a close approximation to the composition of SC lipids [17] without the small proportions of cholesteryl esters and other minor constituents. The lipid mixtures were dried under a stream of nitrogen and then under vacuum at room temperature. Aqueous dispersions of the lipid mixtures were prepared by suspension in a buffer containing 5 mM trimethylamine (TMA), 0.5 mM EDTA (disodium salt), and 0.02% sodium azide to a final concentration of 2.5 mg lipid per ml. The suspensions were sonicated in a Branson bath sonicator (Branson 12) at 80°C until the suspensions became clear, usually about 2 to 5 min. The preparations were then annealed at 80°C for 15 min. The final pH of the suspensions was adjusted to 7.0 by dialysis for 2 h, at room temperature. Dialyzing buffer contained 5 mM TMA, 0.5 mM EDTA, and 0.02% NaN3 and was reduced to pH 7.0 with HCl. Dialysis was performed using SPECTRA/POR dialysis tubing (M.W. cutoff 3500), and the pH of the dialyzing medium was periodically readjusted to 7 with very dilute HCl.

Preparation of Membrane Filters
Small disks of 1.5 cm diameter were cut from Millipore cellulose acetate/cellulose nitrate GS-type filters of 0.22 μm pore size. The filters were placed on a heating block at 40°C, wetted with distilled water, and the liposome suspension was applied on the filters dropwise, using a Hamilton syringe, to cover a circular area of 0.7 to 0.8 cm diameter. The suspensions were applied in 10-μl increments with 2 to 3 min of

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were highly carrying a lipid filter. of dry nitrogen flowing over the and ing volume suspension. The suspension dried to a smooth lipid compartments of after damage diffusion Permeability Measurements A diffusion cell (Fig 1) was de­ membrant: filters to mems. by this procedure were more uniform with better barrier properties than those formed by applying the entire amount of any given suspension. The suspension dried to a smooth lipid film containing 150–500 μg of lipid per cm², formed from 30–100 μl of the suspension.

Permeability Measurements A diffusion cell (Fig 1) was designed and constructed from Plexiglass. The cell consisted of two compartments of 0.5 cm diameter. The membrane filters were held between two neoprene gaskets mounted between the two compartments. Two hydrophobic gaskets cut out from Millipore GV-type filters (0.22 μm pore size), with a 0.6 cm diameter hole, were used to hold the lipid-containing filters before mounting them in the diffusion cell. The hydrophobic rim prevented seepage of water between the neoprene gaskets and the lipid-carrying filter. The hydrophobic gaskets also provided physical support and prevented damage to the lipid film.

The upper well was filled with water to a depth of about 3 mm and was then sealed with a screw. Water permeating through the membrane filters into the lower compartment was swept by a stream of dry nitrogen flowing over the lower surface of the membrane filter. The effluent gas from the lower compartment was analyzed for its water content by an electrolytic water analyzer (MEECO model W, MEECO, Inc., Watlington, PA). The water analyzer was set up as described by Spruit and Matten [18]. The water diffusing through the membrane was detected as an increase in the water content of the carrier gas (as ppm water vapor by volume) for a carrier gas flow of 100 ml/min (6L/h). This was converted to ppm water by weight as: \( W = P(18/22.4) \), where \( W \) is ppm water by weight, \( P \) is the increase in water concentration in the effluent gas (ppm by volume), and 18 gm/22.4 L is the conversion factor from volume to weight assuming ideal behavior for water vapor. Water flux is calculated as \( F = \frac{(W/A)}{t} \), where \( F \) is the water flux (in g/cm²/L), \( A \) is the area of the membrane (in cm²), for a carrier gas flow rate of 6L/h. This is converted to flux per unit time as \( J = F \times 10^5 \), where \( J \) is the water flux in mg/cm²/h.

**Electron Microscopy** After the permeability measurements, the membrane filters were examined by scanning and transmission electron microscopy. Small pieces of membrane carrying a lipid film were coated with Pd-Au in a sputter coater (Emiscope SC-500) and analyzed in a Hitachi 570 scanning electron microscope operating at 5 kV. For transmission electron microscopy, small pieces of membrane were fixed in 0.2% RuO₄ in cacodylate buffer for 30 min at room temperature, dehydrated in graded acetones, embedded in Spurr’s resin, and sectioned. Silver-gold sections were stained in uranyl acetate and lead citrate and analyzed in a Hitachi H-7000 transmission electron microscope operating at 75 kV.

**RESULTS** Table 1 shows the permeation of water across membranes carrying varying amounts of SC lipids. These measurements were done with disks mounted with the lipid side in contact with water. Permeability measurements were made over periods of 10–15 h, allowing permeability to reach steady state. After a slow initial decrease, the values remained constant after about 4 h. Measurements were done on two batches of lipid membranes formed from different liposome preparations and were in good agreement with each other. Disks containing 150 μg or less of lipid per cm² were highly permeable. The permeability dropped substantially on increasing the amount of lipid per disk to 200 μg/cm². Permeability values were comparable to in vivo measurements when the amount of lipid per disk was in the range of 250–300 μg/cm². The disks were highly impermeable to water when the amount of lipid was 350 μg/cm² or higher. Table II shows the permeability data obtained from disks containing varying amounts of lipid when the measurement was done with the lipid side away from water. These high values suggest damage to the lipid lamellae. The cellulose ester membrane filters sagged upon standing in contact with water for a few hours in the diffusion cell. This could stretch or shrink the lipid film, causing macroscopic damage in addition to disrupting the continuity of the first few lamellae in contact with the surface of the disk. The permeability values increased during the first several minutes of the measurement before reaching steady state value, indicating slow damage to the lipid film as the hydrophilic filter was wetted upon standing in contact with water. Thus the permeability data obtained with the disks with their lipid side contact with water are taken as representative of the actual permeability through these lipid lamellae.

**Figure 2B–D** show the scanning electron micrograph (SEM) of a blank membrane filter. Fig 2B–D show the SEM of membrane filters containing varying amounts of lipid. At lower amounts (150 μg or less of lipid per cm²), incompletely covered pores are seen on the surface, as shown in Fig 2B. Figure 2C shows the surface of the membrane filter containing 250 μg of lipid per cm², where the pores are completely covered with lipid film and the permeability is comparable to that of in vivo measurement (cf Table I). Figure 2D shows smooth continuous lipid film and is representative of the

**Table 1. Water Flux Through Model Membranes Measured with the Lipid Side in Contact with Water (Means of 3–4 Measurements)**

<table>
<thead>
<tr>
<th>Amount of lipid (μg/cm²)</th>
<th>Flux (mg/cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>42 ± 0.49</td>
</tr>
<tr>
<td>200</td>
<td>2.14 ± 0.40</td>
</tr>
<tr>
<td>250</td>
<td>0.48 ± 0.14</td>
</tr>
<tr>
<td>300</td>
<td>0.37 ± 0.12</td>
</tr>
<tr>
<td>350</td>
<td>0.26 ± 0.10</td>
</tr>
<tr>
<td>500</td>
<td>0.21 ± 0.10</td>
</tr>
<tr>
<td>Human forearm</td>
<td>0.37 ± 0.49</td>
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</tbody>
</table>
Table II. Water Flux Through Model Membranes Measured with the Lipid Side Away from Water (Means of 2–3 Measurements)

<table>
<thead>
<tr>
<th>Amount of lipid (µg/cm²)</th>
<th>Flux (mg/cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>2.65 ± 3.71</td>
</tr>
<tr>
<td>300</td>
<td>3.36 ± 0.72</td>
</tr>
<tr>
<td>350</td>
<td>2.41 ± 1.13</td>
</tr>
</tbody>
</table>

disks containing 350 µg or more of lipid per cm². Figure 3A, B show thin-section electron micrographs (TEM) of lipid-bearing disks (250 µg/cm²). Figure 3A shows the thickness of the lipid film (0.7 µm) relative to the thickness of the membrane filter (220 µm). It should be noted that only a part of the filter thickness is shown in Fig 3A. Figure 3B shows little penetration of the liposomes into the upper regions of the pores. Uniform multiple lamellae formed from the liposomes are seen in the upper regions of the pores and on the outside.

DISCUSSION

The permeability results in Table I show a correlation between the amount of lipid used and the barrier efficacy of the membrane. The filter disks were highly permeable when the amount of lipid was 150 µg per cm² or less. SEM of these filters showed surfaces with incompletely covered pores. With the filters carrying 250 µg per cm², which had permeability comparable to the in vivo transdermal water loss, the filter pores appeared to be more completely covered with lipids. The small unilamellar vesicles that were prepared by sonication ranged in size from 20 to 200 nm, as examined by freeze-fracture electron microscopy. The liposome suspensions were applied on wetted disks, and the liposomes did not infiltrate deep into the pores despite the suspensions being continuous with water channels along the filter pores. Some of the smaller liposomes infiltrated into the upper regions of the pores and fused to form lamellae along the cellulose-ester matrix upon drying. Larger liposomes near the 200-nm size range apparently formed the bridge

**Figure 2.** Scanning electron micrographs of filters containing varying amounts of lipid. A: Blank cellulose-ester filter; B: 150 µg/cm²; C: 250 µg/cm²; D: 350 µg/cm². Bar: 1 µm.

**Figure 3.** Thin-section electron micrographs of filters containing 250 µg of lipid per cm². A: Bar: 2 µm; B: Bar: 100 nm.
across filter pores, over which subsequent lamellar layers were formed by fusion of the smaller vesicles as the suspension dried.

Transformation of SC lipid liposomes to lamellar sheets upon drying can be visualized by considering the physical process of drying. As the liposome suspension dries, it becomes more concentrated; as the remaining water leaves the vesicles, they flatten and fuse with each other [19]. The resulting lamellar sheet would be thermodynamically more stable than the flattened vesicles. The sparse amount of salts in the buffer (0.5 mM EDTA and 0.02% NaNO₃) and a volatile base (TMA) facilitated this uniform transformation of liposomes to lamellar sheets by keeping the ionic strength low during the final stages of drying, thus preventing the lipids from being salted out of the suspension. Salts trapped between lamellae during drying would disrupt the formation of a more uniform lamellae by forming channels for the passage of water.

The filter membrane used in this study's SC is much closer than other membranes used in modelling of percutaneous permeability so far. While it is similar to some of the earlier models in having a hydrophobic and a hydrophilic medium [9], the hydrophobic matrix that constitutes the barrier is made up of lipids that resemble those of SC in their composition and morphology. However, the multiple lipid lamellae seen in Fig 3B do not show the same ultrastructural patterns that are seen in electron micrographs of the SC intercellular lamellae [20]. This is due to a random distribution of the constituent lipids in the bilayers made in vitro. It is not clear at this point how much of a role, if any, the asymmetric orientation of lipids in the intercellular lamellae in SC plays in regulating the barrier properties.

The lipid mixture used in this study has a high transition temperature (75 °C [13]). The free fatty acids and the acyl chains of the epidermal ceramides are longer (16-28 carbons) than the predominantly 16- and 18-carbon acyl chains of the phospholipids that are found in most biomembranes. Also, the ceramides, which are the dominant species in the stratum corneum (45-55%), have been suggested to form lateral hydrogen bonding in bilayers [21]. Thus the ceramides, cholesterol, and free fatty acids that make up more than 90% of the lipid used in this study could form extended lateral hydrogen bonding that can condense the bilayers and render them highly impermeable. It should be of interest to investigate the interaction between ceramides and cholesterol. Another factor contributing to the barrier property of the SC lipid lamellae could be the high level of internal interdigitation that is thought to occur in the bilayers formed from these highly heterogeneous lipid chains [22].

The diffusion of water through the hydrocarbon interior and the transport across the headgroup region have been proposed as two possible rate-limiting steps in the transport of water across lipid bilayers [23]. The transport of water through the rigid hydrocarbon interior and across a highly condensed headgroup region of the stratum corneum lipids is thus highly inhibited. It is of interest to compare the total thickness of the lipid layers formed in vitro in the present study with the total thickness of the intercellular lamellae found in intact SC. If we take the SC to consist of 15 horny cell layers, with an average intercellular space 40 to 50 nm thick, then the total thickness of the intercellular lamellae in intact SC ranges from 600 to 750 nm. This is based on numerous thin-section electron micrographs obtained in this laboratory from human, mouse, and pig SC [20] and is far below the thickness estimated by Plewig et al [24]. The total thickness of in vitro lamellae from Fig 3A,B is also 600-700 nm. Thus the total thickness and the barrier properties of the multiple lamellae made in vitro are comparable to the intercellular lamellae of intact SC. However, the amount of lipid required in the model system (250 µg/cm²) is twice the amount of lipid found in intact SC (125 µg/cm²). This may be due to the highly porous cellulose-ester filter (75% porosity with an average pore size of 0.22 µm) that was used as the hydrophilic support medium in this study, whereas the intercellular lamellae in intact SC have a molecularly smooth support medium, the horny cell lipid envelope. The monolayer of lipids that makes up the lipid envelope has been suggested to be interdigitated with the contiguous bilayer of the intercellular lamellae [22].

In a recent study, Firestone and Guy [25] used a membrane filter saturated with lipid for permeability experiments. However, these authors did not characterize the final form of lipid arrangement in the filter and did not report any water permeability measurements. Also, the amount of lipid used was an order of magnitude higher than that used in the present study.

The intercellular lipid lamellae of SC are now widely recognized as the barrier to percutaneous penetration. The present model membrane formed from the same lipids that make up the intercellular lamellae is a relevant model for epidermal barrier. The water-permeability data from the present study indicate that this simple membrane is a useful model system for human skin permeability that could be used for understanding the role of individual lipid components in maintaining the epidermal barrier. This model could also be useful for preliminary studies of percutaneous penetration of active ingredients from different topical formulations.

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REFERENCES