Development of a model of the lipid constituent phase of the stratum corneum: II. Preparation of artificial membranes from synthetic lipids and assessment of permeability properties using *in vitro* diffusion experiments

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Synopsis

This paper describes the development of model membranes from liposomes using commercially available synthetic lipids. The composition of the lipid mixture used to prepare the liposomes was based on earlier studies to optimize the strength of interlipid interactions within the bilayers. Model membranes were prepared by extruding liposomes through a membrane filter below the phase transition temperature of the lipid mixture. The resulting membrane filter, with the deposited lipids, was then dried and treated with calcium chloride to allow transformation of the liposomal bilayers into extended sheet structures. The treated membranes were dried and mounted on diffusion cells. A variety of markers was then used to test the permeability characteristics of the model membrane. The results suggest that the membrane prepared using synthetic lipids is a suitable model for permeation characteristics of skin.

INTRODUCTION

In a previous paper (1), the optimization of the composition of liposomes prepared using commercially available semi-synthetic lipids suggested that the interlipid interactions of this mixture were similar to that obtained using lipids extracted from porcine stratum corneum. It was therefore of interest to determine if model membranes of the stratum corneum lipid compartment can be developed by the use of the optimized synthetic lipid mixture. The preparation of model membranes with synthetic or semi-synthetic lipids would also obviate excessive reliance on animal skin studies if they are shown to possess permeability characteristics similar to skin.

The quest to circumvent problems associated with the use of human or animal skin to answer basic questions has prompted an extensive search for a reliable *in vitro* model. Attempts to adapt synthetic polymeric membranes such as cellulose acetate membranes

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(2,3) or dimethylpolysiloxane membranes (4–7) revealed that the nature of the materials comprising such membranes limits their use as a skin model system. A few studies described the formation of membranes by using the corneocytes and lipid extracts of porcine stratum corneum (8,9). Although reaggregation of stratum corneum components resulted in viable membranes, such an approach would not obviate the use of animal skin. Abraham and Downing (10) reported the *in vitro* formation of membranes by use of a mixture of epidermal ceramides, cholesterol, cholesterol sulfate, and fatty acids. Although the authors demonstrated that water transport across their model membrane was similar to that across the stratum corneum, the system suffers the constraint of requiring epidermal ceramides that involve difficult and time-consuming extraction and isolation procedures. Further, no permeation studies were reported with permeants other than water. Indeed, most of the studies on model membranes prepared from lipid mixtures have been solely tested by water transport characteristics (8–11). This study reports the preparation of model membranes from a mixture of synthetic lipids and investigates the permeability characteristics of a variety of markers with a range of physicochemical properties across this model membrane. The model membranes prepared possessed sufficient cohesive properties so as to be easily handled when employed in diffusion studies wherein the membrane was in intimate contact with solutions in the receiver compartment.

MATERIALS AND METHODS

MATERIALS

Palmitic acid, stearic acid, ceramides type III from bovine brain, cholesterol, cholesterol sulfate, N-palmitoyl-DL-dihydrogalactocerebroside, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES free acid), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). The lipids were of the highest purity available and were used as received. Carnauba fatty acid was a gift from Dr. D. T. Downing, Marshall Dermatology Research Laboratories, University of Iowa. The chain lengths of fatty acid components of this mixture have been reported to be in the range C_{24}-C_{30} (12). The radioactive marker, [^{3}H]-cortisol (specific activity 134 mCi/mg) was obtained from ICN Radiochemicals (Irvine, CA). [^{14}C]-sucrose (specific activity 204 μCi/mg), [^{14}C]-estradiol (specific activity 204 μCi/mg), and [^{3}H]-progesterone (specific activity 151 mCi/mg) were purchased from Amersham (Arlington Heights, IL). All other chemicals were reagent grade, and all solvents used were of HPLC grade. Water was double-distilled and deionized using a Milli-Q ion-exchange system.

METHODS

Preparation of liposomes

Liposomes were prepared by the reverse phase evaporation method by using a ceramide:cholesterol:fatty acid:cholesterol sulfate mixture at a weight ratio of 37.7:28.3:17.0:17.0. This ratio was found to exhibit the strongest interlipid interaction determined by monolayer studies (1). Briefly, the lipid mixture contained in a one-liter

round-bottomed flask was dissolved in a chloroform-methanol solvent mixture (2:1 by volume). HEPES buffer (0.05 M), pH 7.5, was then added to the organic solution. The ratio of the organic solution to the buffer solution was 2:1 (v/v). The mixture, which was opalescent, became clear after 18 minutes sonication (Branson sonicator, E-module, Shelton, CT). The organic solvents were then removed by a rotary evaporator at 55°C for palmitic acid systems and 70–72°C for the stearic or carnauba acid systems. After solvent removal was complete, the volume of suspension was adjusted with buffer to obtain a suspension containing 30 mg/ml lipid. The liposomes were then annealed for 30 min at the appropriate elevated temperature. The liposomal suspension was then filtered through a 12-μm pore size Nucleopore™ (Pleasanton, CA) filter to remove any particulate lipids. The filtered suspension was then used immediately to prepare the model membranes.

Preparation of membranes

Membranes without BSA treatment. One ml of the filtered 30 mg/ml liposomal suspension was diluted threefold with 2 ml of the buffer and extruded through a 2-μ pore size Nucleopore filter for non-BSA treated membranes and through a 0.45-μm pore size Nylaflo filter (Gelman Sciences, Ann Arbor, MI) for BSA-treated membranes in an extruder (Lipex Biomembranes, Vancouver, BC, Canada). Extrusion was carried out by immersing the entire extruder assembly in a water bath maintained at 40°C, a temperature well below the phase-transition temperature of the lipid mixture. The nitrogen pressure used was approximately 40 psi. At the end of the extrusion process, typically an hour, the extruder assembly was dismantled and the lipid-covered nylon filter was carefully retrieved and dried in an oven at 60°C. The dried membrane was then weighed to ascertain the amount of liposomal lipid retained on the filter.

For studies of water vapor transmission rates and electron microscopy, the dried membrane was treated successively with 2 ml of 3 mM, 5 mM, and 8 mM $CaCl_2$ solutions in the buffer. Each treatment was separated by 2-hr intervals. The treated membrane was then dried *in vacuo* for at least 24 hr before use.

For diffusion experiments, the lipid filter following extrusion and drying was mounted on a Franz diffusion cell (Crown Glass Co., Somerville, NJ) with a nominal diameter of 2 cm and a receiver capacity of approximately 13 ml. The membrane was placed, lipid side up, on a 0.02-mm thick silastic (Dow Chemical, Midland, MI) cut to O-ring shape, with the inner and outer diameters matching those of the diffusion cell. This was done to ensure a proper seal between the donor cap and the receiver compartment. The donor cap was then placed carefully on the cell and clamped tightly with adjustable clamps. The receiver compartment was left empty and maintained at 37°C. Three ml of 10 mM $CaCl_2$ solution in pH 7.5 HEPES buffer were then added to the donor and the system was allowed to dry over a period of five days before testing with marker solutions.

BSA-treated membranes. The lipid-covered nylon filter, following extrusion and drying, was placed carefully on a glass slide with the lipid side up. Approximately 3 ml of BSA in buffer was then gently added drop-wise to the filter and soaked for 10 min to allow the BSA to spread over the membrane. The filter was then heated at 80°C in an oven for one hour to denature the BSA. This treatment cycle was repeated twice to ensure complete coverage of the filter surface. After the BSA treatment protocol, the surface of the filter membrane exhibited a glossy appearance, indicating the presence of the

denatured protein. The membrane was then mounted on a Franz diffusion cell and treated with 3 ml of 10 mM calcium chloride solution and dried over a period of 5 days before use in diffusion experiments.

Characterization of the model membranes

Electron microscopic studies. Scanning electron microscopy was carried out to determine if the membrane surface was uniformly covered with lipid. Model membranes were mounted on an aluminum holder and sputter coated (Sputter Coater, Polaron Instruments, Model ES 100) to a 15-mm thickness with gold-platinum alloy prior to scanning (International Scientific Instruments, Model DS 130). Transmission electron microscopy was employed to demonstrate the existence of bilayer structures within the lipid matrix of the model membrane. The specimen preparation involved 30 min hydration of the membrane with buffer; 1 hr fixation in 2% glutaraldehyde and 3 hr post-fixation with 1% ruthenium tetroxide (both from Polysciences Inc., Warrington, PA); and serial dehydration with ethanol and final embedment in Spurr's resin (also from Polysciences Inc.). The blocks were then sectioned with a diamond knife and examined by a transmission electron microscope (Phillips Electronic Instruments, Model EM-400) operated at 60 KV.

Resistance to water transport: Water vapor transmission experiments across model membranes. Model membranes were sized to 15-mm diameter and mounted on top of a 50-ml glass vial containing saturated $MgCO_3$ solution yielding ~65% relative humidity at 30°C. The vial was then sealed and incubated in a closed chamber at 32°C. Water evaporation through the model membrane was evaluated by periodic determination of the weight loss from the vial over a 24-hr period (13). The weight loss from an aluminum foil-covered vial served as the negative control, and that from an untreated filter served as the positive control. The cumulative weight losses for the three cases were plotted as a function of time. The flux of water across the model membrane was determined after equilibrium was established.

Diffusion experiments: Permeability of markers across model membranes. At the end of the 5-day period the membrane was dry and ready for use. The receiver compartment was filled with buffer and maintained at 37°C. Care was exercised to ensure the absence of air bubbles between the underside of the membrane and the receiver solution. The receiver solution was constantly stirred with a small Teflon-covered magnet. The set-up was allowed to stand for 30 min to ensure that no leaks were present. Each diffusion cell was calibrated with distilled water to determine receiver capacity. One ml of an aqueous solution of the marker containing sufficient cold drug was then added to the donor. The concentrations of cold marker in the solutions were: 0.1% for sucrose, and saturated solutions for cortisol, estradiol, and progesterone. A minimum of six membranes was used for each marker, and all experiments were carried out under non-occluded conditions. At predetermined time periods, approximately 0.3–0.4 ml of the receiver solution was withdrawn gently using a 1-ml syringe with Teflon tubing attached to the needle via the receiver spout. Before withdrawal of the sample, the solution in the spout was mixed thoroughly with the rest of the solution without the generation of air bubbles or the creation of back-pressure effects. The samples were collected in pre-weighed scintillation vials to accurately determine the weights of solution withdrawn. Fresh buffer, equivalent to that of the withdrawn solution, was then added back to the receiver compartment to maintain constant volume. The samples were then mixed with 10 ml

of Ecolite scintillation cocktail and assayed for radioactivity with a Beckman LS 9000 counter.

RESULTS

ELECTRON MICROSCOPY

Scanning electron microscopy revealed that the model membrane surface was completely covered with a lipid film, although the surface was fairly uneven. Typical micrographs are shown in Figures 1a and 1b. Few lipid crystals could be observed. Figures 2a and 2b show typical transmission electron micrographs of cross-sections of the model membranes. Distinctive bilayer patterns are evident at higher magnifications (Figure 2b). The apparent defects seen as empty spaces extending across the sections at low magnifications (Figure 2a) were found to be artifacts caused by the non-uniform expansion of the bilayers during the fixation process.

WATER VAPOR TRANSMISSION STUDIES

Figure 3 shows a plot of the cumulative weight loss as a function of time for the model membrane system and for an untreated filter. The water flux across the untreated filter
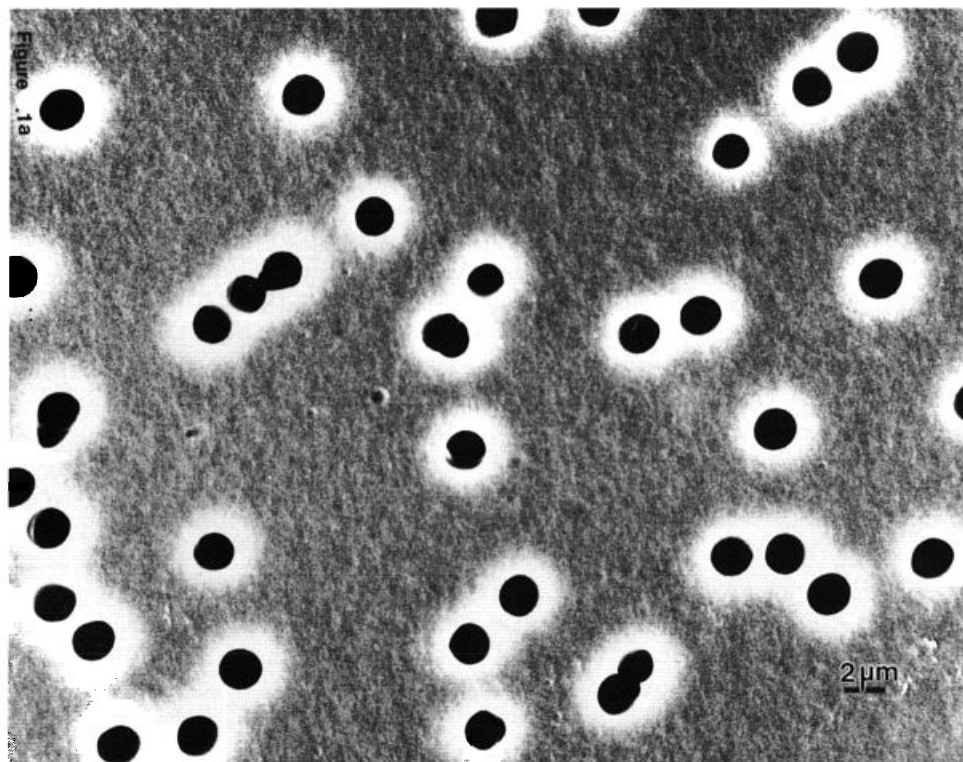

Figure 1a. Scanning electron micrograph of 2-µ pore size Nucleopore filter surface.
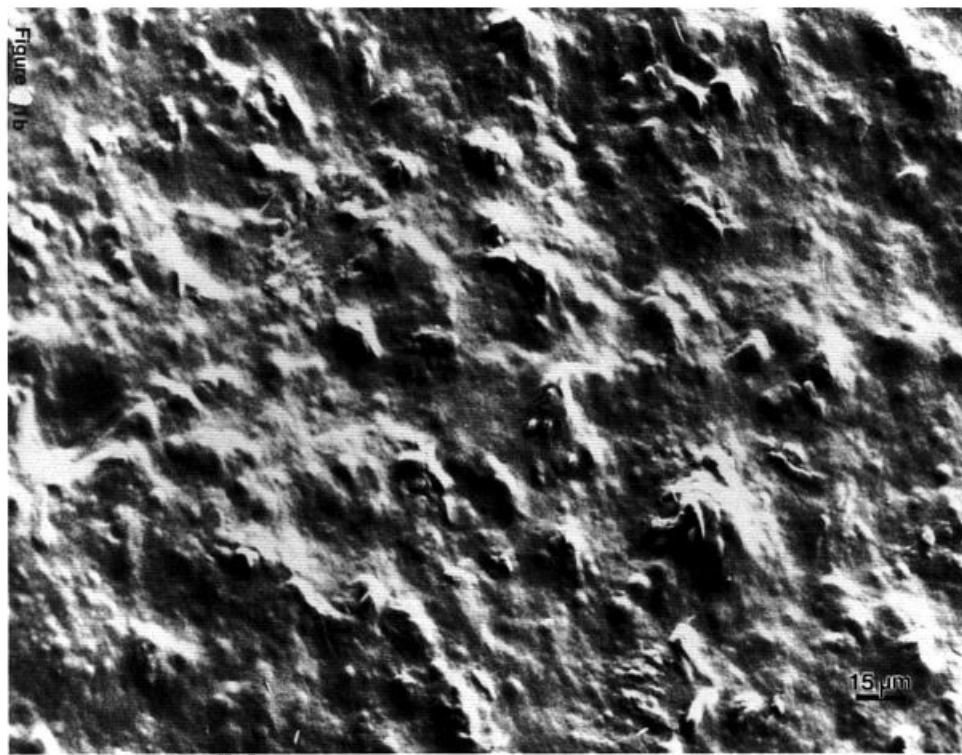
Figure 1b. Scanning electron micrograph of palmitic acid membrane surface.

was 5.76 mg/cm²h, whereas for the model membrane, this value was about two orders of magnitude lower, i.e., 5.20×10^{-2} mg/cm²h.

DIFFUSION EXPERIMENTS

Table I lists the compositions of the lipid mixtures used in the preparation of the model membranes. Figure 4 shows typical permeation profiles across the model membranes for a few of the markers tested. It is observed that plateauing of the profiles occurs earlier for the more hydrophobic markers such as progesterone or estradiol as compared to the more polar cortisol or sucrose. Indeed, the permeation profiles for cortisol, and sucrose were linear over a 24-hr time period. Table II lists the permeability coefficients of the drug markers across the model membrane. The permeability coefficients were calculated by using the linear portion of the permeation profiles after appropriate corrections for changes in donor phase concentration with time were carried out according to the treatment of Flynn *et al.* (14). Table II also lists the corresponding permeability values across human skin (15), along with the octanol-water partition coefficients of the markers (16). Figure 5 shows the correlation between the permeability coefficients across model membranes and octanol-water partition coefficients. Figure 6 shows a plot of the permeability coefficients across the model membranes against those reported across human skin for the various markers.

DISCUSSION

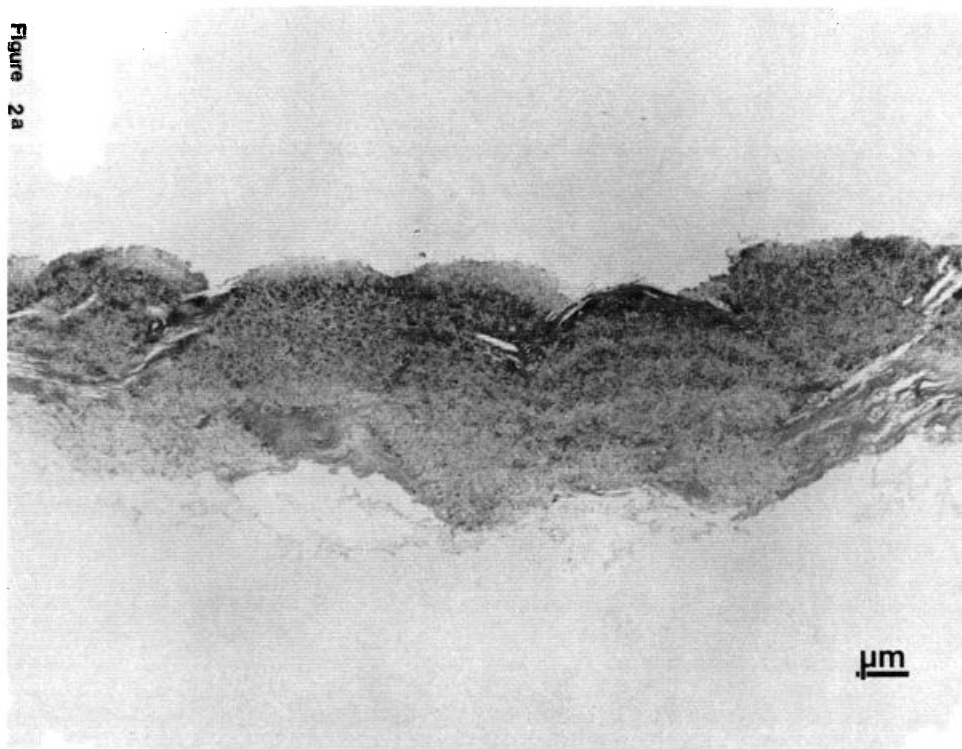Scanning electron microscopy revealed complete coverage of the filter surface by the

Figure 2a. TEM of cross-section of lipid matrix of palmitic acid membrane.

liposomal lipids. It was also evident from transmission micrographs that these lipids were organized as bilayer structures. The water vapor transmission studies showed that the lipid matrix on the surface of the filter provided significant resistance to water transport. The high reproducibility of the water flux across the model membranes ruled out the possibility of major random defects in the model membranes. The water flux across the 60 μm membrane was 0.052 mg/cm^{2}h and agrees well, after accounting for thickness effects, with a value of 0.4 mg/cm^{2}h for *in vivo* water flux across human skin with a stratum corneum thickness of roughly 10 μm (10). The electron micrographs as well as the water vapor transmission rates were obtained using model membranes that were not treated with BSA.

Preliminary diffusion studies with non-BSA-treated model membranes indicated that although the permeability values of hydrophobic markers across these membranes could be ranked according to their hydrophobicities, hydrophilic markers such as sucrose exhibited permeation rates that were excessive and highly reproducible (Table II). Thus, it appeared that although the lipid matrix in the model membrane exhibited barrier properties to markers that were hydrophobic, hydrophilic compounds such as sucrose permeated easily, perhaps across another pathway in the membrane. It is possible that a polar pathway exists in the bilayer matrix, as evidenced by the extremely high reproducibility of both water vapor transmission rates and permeation of sucrose, and not the result of random defects in the membrane. The extensive investigations into the structure of stratum corneum by Elias *et al.* (17–20), as well as those by Downing *et al.* (21–23), have revealed that the cohesive property of the stratum corneum is attributable
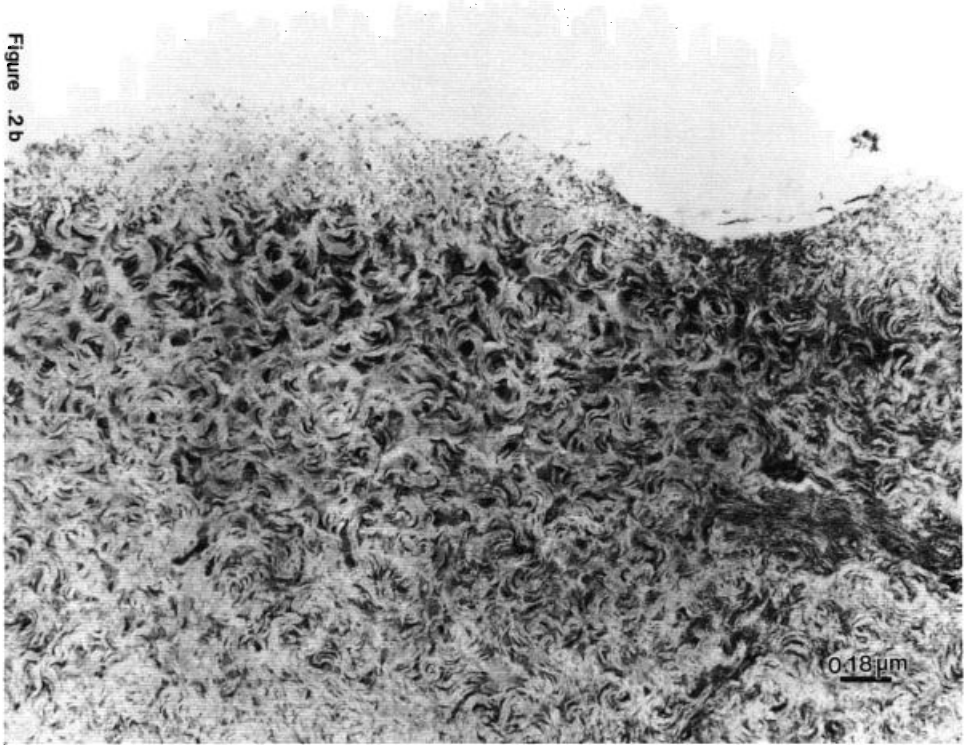
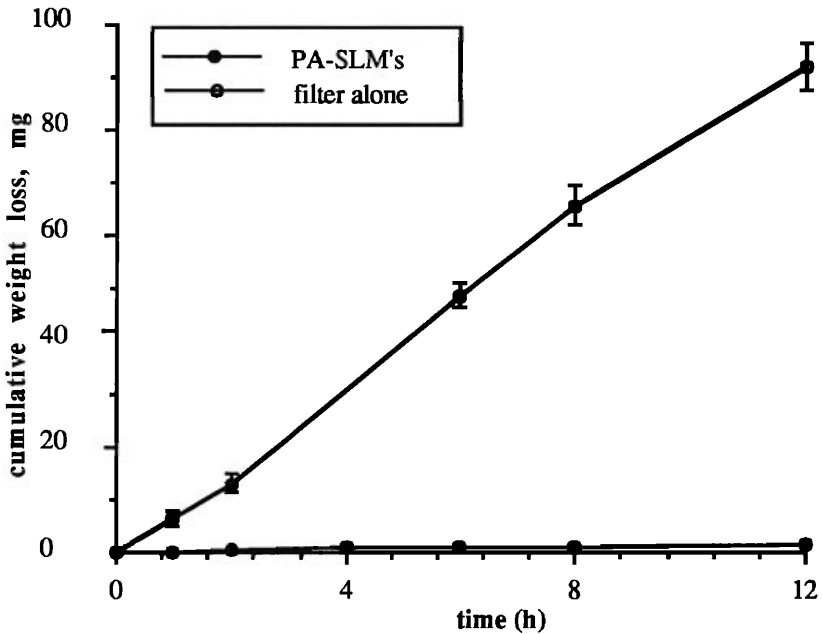**Figure 2b.** TEM of bilayer structures of palmitic acid membrane at high magnification.

Figure 3. Water loss through PLM membranes as compared to untreated filter.

Table I
Lipid Components of Different Synthetic Lipid Membranes

| | Composition (weight percent) | | | | |
Membrane type	Cerebrosides	Ceramides	Cholesterol	Cholesteryl sulfate	Fatty acid
Palmitic (standard)	—	38	28	17	17
Stearic	—	38	28	17	17
Carnauba	—	38	28	17	17
Cerebroside/carnauba	5	40	25	18	12

Each membrane is referred to by its fatty acid component. All quantities are expressed as weight percent.

to covalent linkages between corneocytes and their lipid envelopes. These lipid envelopes, in turn, are interdigitated with the lipid bilayers in the stratum corneum, giving rise to a remarkably cohesive unit. Indeed, the increase in partitioning of hydrophobic compounds into delipidized stratum corneum reported by Surber *et al.* (24) has been explained in terms of regions within the stratum corneum that are exposed upon delipidization and that were inaccessible to the drugs in intact stratum corneum. The implication of strong lipid–corneocyte interactions within the stratum corneum also
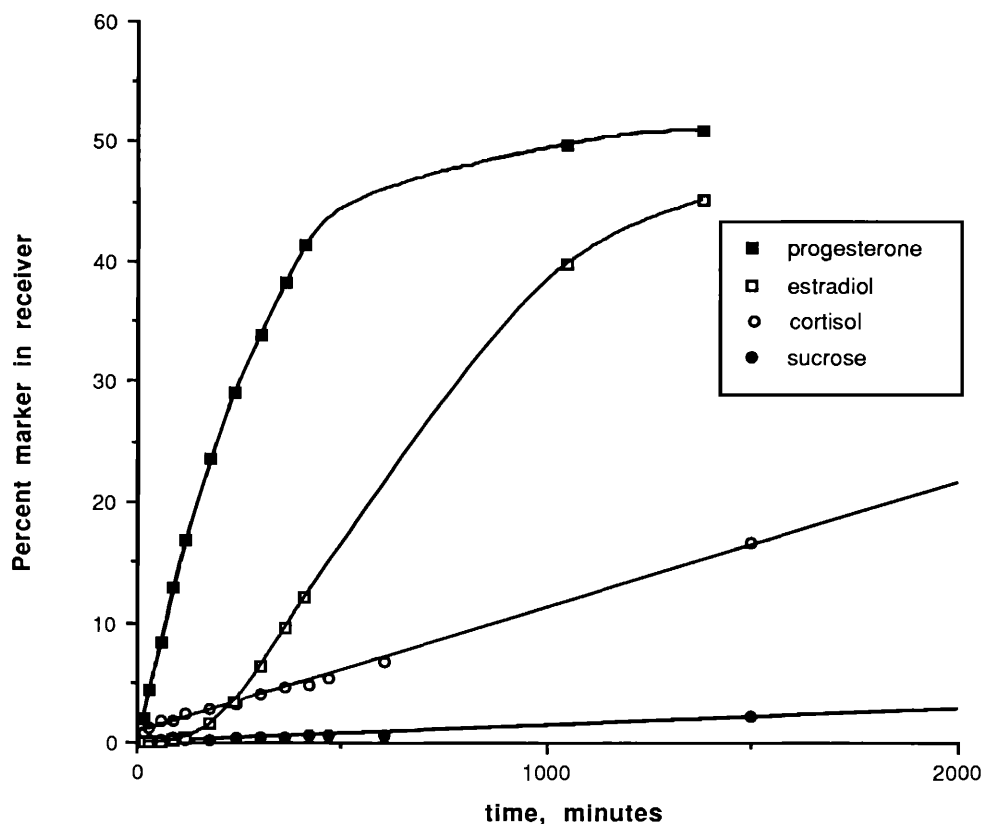

Figure 4. Typical permeation profiles of various markers across artificial model membranes.

Table II

Permeability Coefficients of Various Markers Across BSA-Treated Artificial Membranes

Marker	Molecular weight (daltons)	Partition coefficient (octanol/water)	Permeability coefficient (cm/h)	
			BSA-treated artificial membranes (n = 6)	Human skin (from ref 15)
Sucrose	342	≈0	0.000224 ± 0.000063	≈0
Cortisol	363	40	0.00255 ± 0.00067	0.0003
Estradiol	272	490	0.00536 ± 0.00273	0.03
Progesterone	315	5890	0.0266 ± 0.0064	0.15

Values shown are average ± SD.

suggests that in order to obtain a model membrane that mimics skin permeability characteristics, a lipid matrix alone is not sufficient. In an attempt to simulate corneocyte–lipid bilayer interactions, heat-treated BSA was included in the model membrane to allow (i) the generation of hydrophobic protein microglobules or microspheres within areas of the membrane that may have previously constituted the polar pathways for markers such as sucrose and (ii) the creation of anchor sites similar to corneocytes in
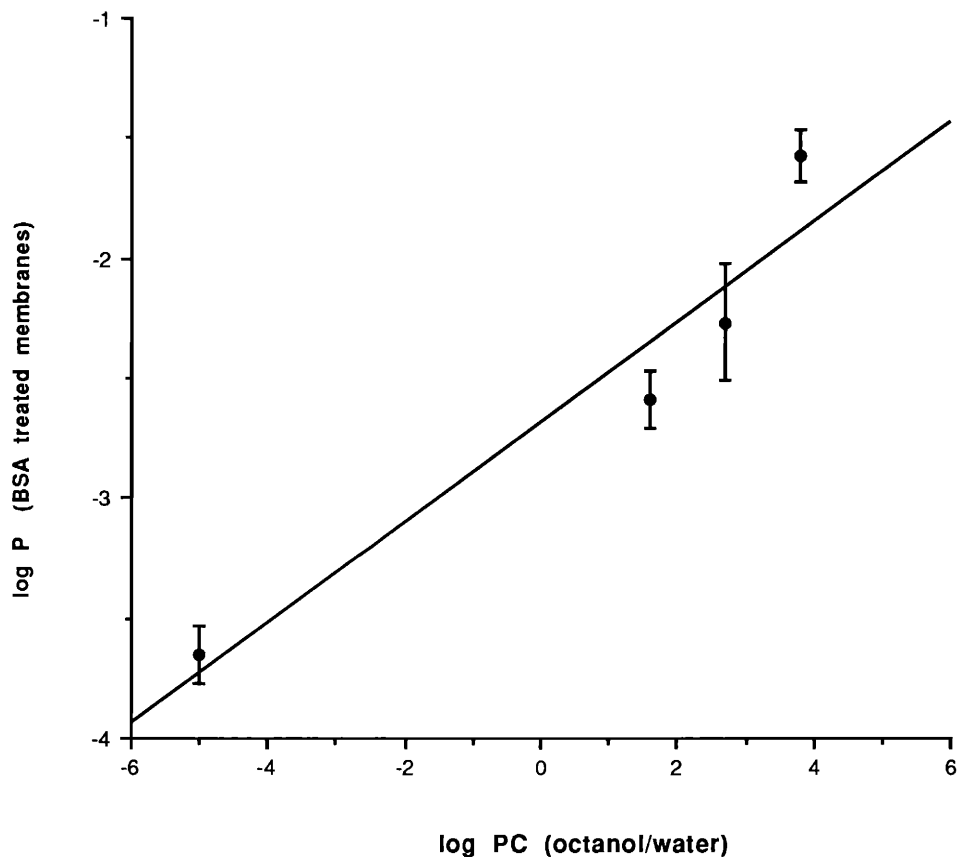

Figure 5. Correlation between permeability coefficients of markers across BSA membranes and octanol-water partition coefficients.
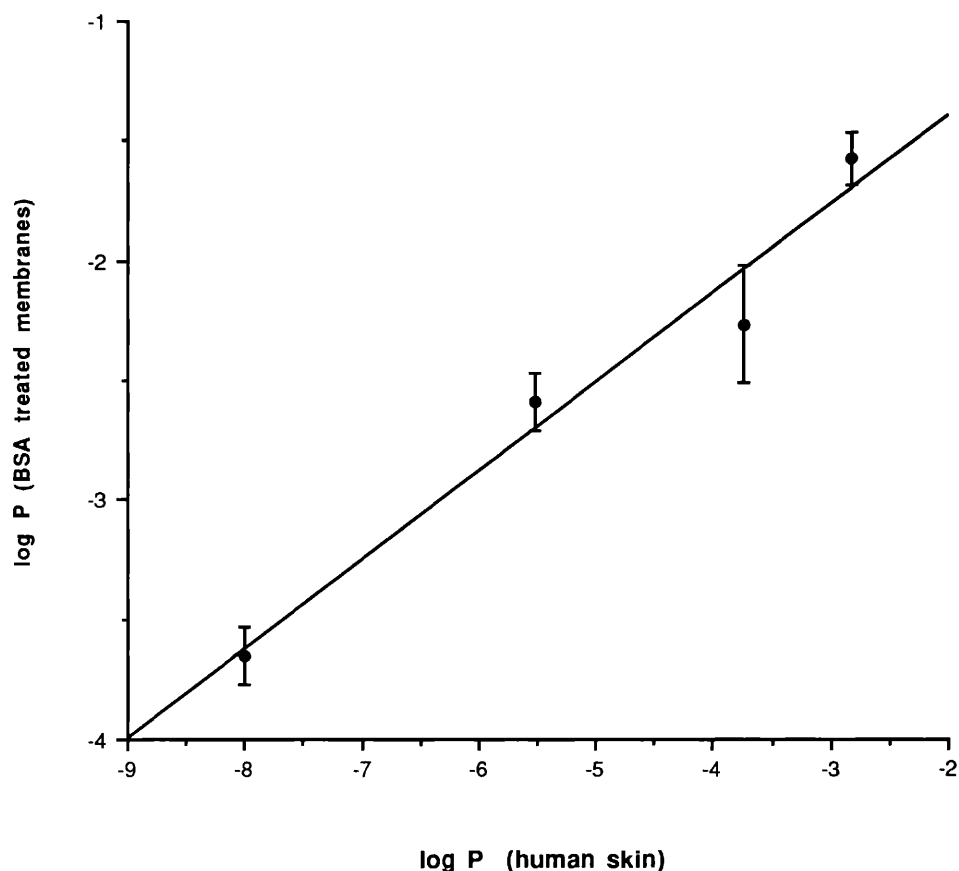
Figure 6. Correlation between permeabilities of markers across BSA membranes and across human skin.

stratum corneum with which the lipid bilayer matrix may interact and result in a more cohesive membrane. The BSA-treated membrane, after calcium chloride treatment, exhibited markedly higher resistance to the permeation of sucrose compared to non-treated membranes. It was also confirmed that heat treatment at 80°C of blank filters alone or of lipid membranes without BSA treatment did not lead to reduction of sucrose permeation across these membranes. It was also observed that both BSA and calcium chloride treatment protocols were essential to the generation of model membranes with high barrier properties to hydrophilic drug markers. Treatment of lipid-laden filters with either BSA only or calcium chloride alone did not suffice. This suggests that the model membrane obtained after the combined treatment protocols probably incorporates interactions between the protein and lipid bilayers via calcium-mediated linkages.

It would be necessary to carry out extensive structural investigations by electron microscopy and other spectroscopic methods to define the interactions at a molecular level. It is clear, however, that the model membranes prepared in this fashion possess excellent potential to screen a variety of compounds for permeation characteristics across human or animal skin. The excellent correlations of permeability values of several markers across the BSA-model membranes with octanol-water partition coefficients (Fig 5, $r^2 = 0.92$) and with available data on their permeability across human skin (Fig 6, $r^2 = 0.97$)

suggest that it is possible to prepare membranes from simple synthetic lipids that are capable of modeling skin permeation characteristics.

ACKNOWLEDGMENTS

REFERENCES

(1) D. Kittayanond, C. Ramachandran, and N. Weiner, Development of a model of the lipid constituent phase of the stratum corneum: I. Preparation and characterization of "skin lipid" liposomes using synthetic lipids. *J. Soc. Cosmet. Chem.*, **43**, 149–160.

(2) C. M. Gary-Bobo, R. Dipolo, and A. K. Solomon, The role of hydrogen bonding in non electrolyte diffusion through dense artificial membranes, *Gen. Physiol.*, **54**, 369–382 (1969).

(3) R. Dipolo, R. I. Sha'afi, and A. K. Solomon, The transport parameters in a porous cellulose acetate membrane, *J. Gen. Physiol.*, **55**, 63–76 (1970).

(4) G. L. Flynn and R. W. Smith, Membrane diffusion. III. Influence of solvent composition and permeant solubility on membrane transport, *J. Pharm. Sci.*, **61**, 61–66 (1972).

(5) F. Bottari, G. DiColo, E. Nannipieri, M. F. Saeton, and M. F. Safarini, Release of drugs from ointment bases. II. In vitro release of benzocaine from suspension type aqueous gels, *J. Pharm. Sci.*, **66**, 926–931 (1977).

(6) C. R. Behl, E. E. Linn, G. L. Flynn, C. L. Pierson, W. I. Higuchi, and N. F. H. Ho, Permeation of skin and sechar by antiseptics. I: Baseline studies with phenol, *J. Pharm. Sci.*, **72**, 391–396 (1983).

(7) S. Tanaka, Y. Takashima, H. Murayama, and S. Tsuchiya, Studies on drug release from ointment. IV. Release of hydrocortisone butyrate propionate from topical dosage form to silicone rubber, *Int. J. Pharm.*, **27**, 20–38 (1985).

(8) W. P. Smith, M. S. Christensen, S. Nacht, and E. H. Gans, Effect of lipids on the reaggregation and permeability of human stratum corneum, *J. Invest. Dermatol.*, **78**, 7–11 (1982).

(9) W. R. Koch, J. L. Burns, and D. L. Bissett, Preparation and characterization of a reconstituted stratum corneum film as a model membrane for skin transport, *Arch. Dermatol. Res.*, **280**, 2152–256 (1988).

(10) W. Abraham and D. T. Downing, Preparation of model membranes for skin permeability studies using stratum corneum lipids, *J. Invest. Dermatol.*, **93**, 809–813 (1989).

(11) S. E. Friberg and I. Kayali, Water evaporation rates from a model of stratum corneum lipids, *J. Pharm. Sci.*, **78**, 639–643 (1989).

(12) P. W. Hertz and D. T. Downing, Ceramides of pig epidermis: Structure determination, *J. Lipid Res.*, **24**, 759–765 (1983).

(13) G. E. Burch and T. Windsor, Diffusion of water through dead plantar, palmar and dorsal human skin and through toe nails, *Arch. Dermatol.*, **53**, 39–41 (1944).

(14) G. L. Flynn, S. H. Yalkowsky, and T. J. Roseman, Mass transport phenomena and model: Theoretical concepts, *J. Pharm. Sci.*, **63**, 479–509 (1974).

(15) R. J. Scheuplein and I. H. Blank, Permeability of skin, *Physiol. Rev.*, **51**, 702–747 (1981).

(16) C. Hansch, Ed. *Comprehensive Medicinal Chemistry: The Rational Design, Mechanistic Study and Therapeutic Applications of Chemical Compounds*, Vol. 6, Cumulative Drug Subject Index; 2nd Drug Compendium, Pergamon Press, New York (1990).

(17) P. M. Elias, Epidermal lipids: Barrier function and desquamation, *J. Invest. Dermatol.*, **80**, 445–495 (1983).

(18) P. M. Elias, Epidermal lipids, membranes, and keratinization, *Int. J. Dermatol*, **20**, 1–19 (1981).

(19) G. Grubauer, K. R. Feingold, R. M. Harris, and P. M. Elias, Lipid content and lipid type as determinants of the epidermal permeability barrier, *J. Lipid Res.*, **30**, 89–96 (1989).

(20) P. M. Elias, E. R. Cooper, A. Korc, and B. E. Brown, Percutaneous transport in relation to stratum corneum structure and lipid composition, *J. Invest. Dermatol.*, **76**, 297–301 (1981).

(21) W. Abraham and D. T. Downing, Interaction between corneocytes and stratum corneum lipid liposomes in vitro, *Biochim. Biophys. Acta,* **1021,** 119–125 (1990).

(22) P. W. Wertz, D. C. Swartzendruber, D. J. Kitko, K. C. Madison, and D. T. Downing, The role of the corneocyte lipid envelopes in cohesion of the stratum corneum, *J. Invest. Dermatol.,* **93,** 169–172 (1989).

(23) D. C. Swartzendruber, P. W. Wertz, D. J. Kitko, K. C. Madison, and D. T. Downing, Molecular models of the intercellular lipid lamellae in mammalian stratum corneum, *J. Invest. Dermatol.,* **92,** 251–257 (1989).

(24) C. Surber, P. Wilhelm-Klaus, M. Hori, H. I. Maibach, and R. H. Guy, Optimization of topical therapy: Partitioning of drugs into the stratum corneum, *Pharm. Res.,* **7,** 1320–1324 (1990).