Acta Derm Venereol (Stockh) 1993; 73: 12–17

Freeze-fracture Electron Microscopic and Osmotic Water Permeability Studies of Epidermal Lipid Liposomes Derived from Stratum Corneum Lipids of Porcine Epidermis

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Freeze-fracture electron microscopic studies revealed that the liposomal membrane morphology was intact before and after osmotic treatment. This finding suggested that water leakage from the liposomes was not due to fusion of two or more lipid vesicles, but rather to the osmotic salt effect. A stop-flow spectrophotometric study revealed that epidermal lipid liposomes derived from stratum corneum lipids of porcine skin underwent increases of the absorbances with decreases of volume of the vesicles. The initial rate at which the changes in optical density occurs is a measure of the water permeability through the liposomes. The reciprocal of the changes in the absorbance at the equilibrium at different salt osmotic shocks showed a linear dependence on the reciprocal of the osmotic pressure gradient, indicating that epidermal lipid liposomes are an ideal osmometer. The present investigation reports that lignoceric acid is a potent water barrier. Present findings suggest that the initial rate of water penetration decreased in the liposomes made from 30–45% (wt% ratio) of cholesterol and ceramides. Oleic acid as drug penetration enhancer facilitated the water diffusion of the stratum corneum lipid liposomes by a fluidizing effect on the liposomal membranes. Furthermore, ceramides are important in the water barrier properties of the skin. The permeability of water depends upon the amount (wt%) and the type of lipid of the membrane.* Key words: Stop-flow spectrophotometry; Barrier function of lignoceric acid and cholesterol; Oleic acid and linoleic acid as lipid additives (drug penetration enhancers).***

(Accepted August 18, 1992.)

Acta Derm Venereol (Stockh) 1993; 73: 12–17.

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Over the past decades, several investigations have been carried out on the crucial role of the extracellular lipid lamellae of stratum corneum of mammalian epidermis in providing a physiological barrier against transdermal water loss (1–3). However, certain biophysical properties of multiple intercellular lipid lamellae of stratum corneum have not yet been clearly understood. It has been reported that fully differentiated cells in the stratum corneum of mammalian epidermis do not contain phospholipids (4–6). The extracellular lipid lamellae of stratum corneum were predominantly made up of ceramides, cholesterol, free fatty acids and small amounts of cholesteryl sulfate and cholesteryl esters (4–6). The elevated transdermal water loss in people with dry skin and other pathological conditions (3, 8–10) has focussed an interest in understanding the role of the constituent lipids in maintaining the epidermal barrier. Since the intercellular lipid lamellae of stratum corneum provide the major barrier to percutaneous penetration (11–13), it is important to explore the role of various lipids which are one of the components of the stratum corneum lipid lamellar sheet of bilayer liposomes of the mammalian skin. These extracellular lipid membrane sheets of stratum corneum are unique in their lipid composition (5–6, 11). Recently, it has been reported that lamellar lipid membranes could be prepared from liposomes (consisting of stratum corneum lipids) using cellulose ester membrane filters (11). The water flux through the membrane was measured (11). This study, however, did not report osmotic water permeability studies using liposomes derived from various lipids whose composition is similar to the chemical identity of stratum corneum lipids and

Table I. *Composition of lipid mixtures used for making liposome preparations (wt%)*

Cer, ceramides; *CH*, cholesterol; *FFA*, Free fatty acids were lignoceric acid, oleic acid, palmitic acid, linoleic acid, carnauva-palmitic acids; *CS*, cholesteryl sulfate

Mixture[1]	Ceramides	Cholesterol	FFA	CS	DPPC	Egg PC	DCP
A	55	25	15	5	–	–	–
B	50	30	15	5	–	–	–
C	45	35	15	5	–	–	–
D	40	27.5	27.5	5	–	–	–
E	35	40	20	5	–	–	–
F	30	45	20	5	–	–	–
G	–	25	15	5	55	–	–
H	–	20	12	5	63	–	–
I	–	15	20	5	60	–	–
J	–	40	20	5	35	–	–
K	–	32	–	–	–	64	4
L	–	–	–	–	–	96	4

[1] Lipid mixtures were 50% by weight of lipid in the swelling solution and the final concentration of the lipid in the liposomes was 1.8–2.0 mg per ml.

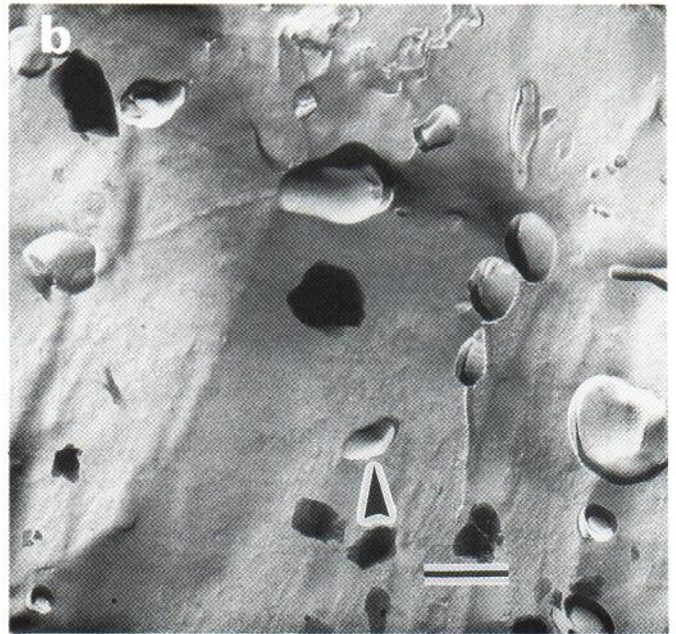

Fig. 1. Freeze-fracture electron micrographs of epidermal lipid liposomes formed by extruding stratum corneum lipids (consisting of ceramides/cholesterol/carnauva-palmitic acids/cs) following the method as described in the text. *a*) epidermal lipid liposomes, control untreated; *b*) the same liposomes treated with osmotic shock of 100 mM NaCl salt solution (final osmolarity) containing 20 mM borate buffer, pH 7.0. The direction of shadowing is indicated by the arrowhead. Bar-200 nm.

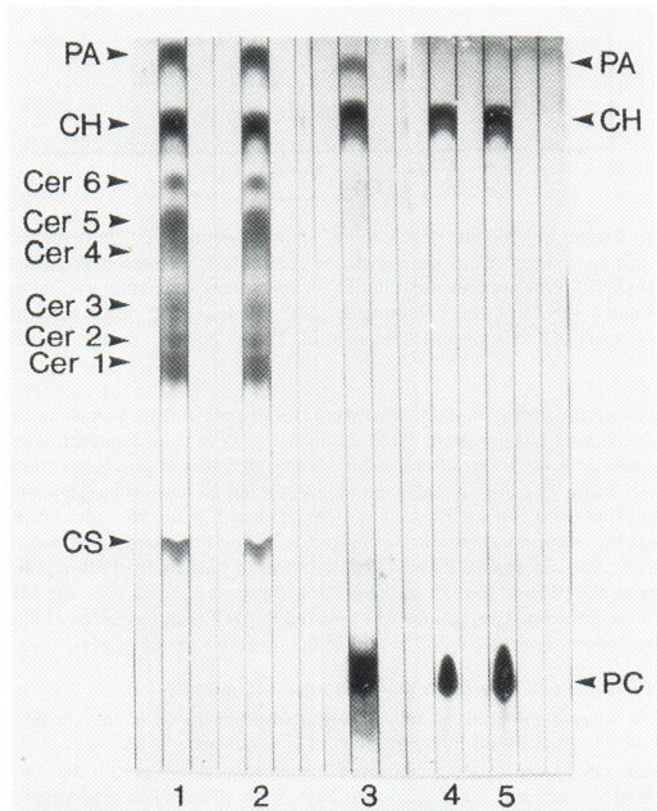

Fig. 2. Thin-layer chromatograms of the starting lipids and the respective liposomal lipids. *Lane 1*, starting lipids of stratum corneum of pig epidermis; *Lane 2*, the respective liposomal lipids derived from stratum corneum; *Lane 3*, the liposomal phospholipids derived from dipalmityol phosphatidylcholine (DPPC) / cholesterol (CH) / palmitic acid (PA) / CS; *Lane 4*, egg lecithin or phosphatidylcholine (PC) starting phospholipids; *Lane 5*, the liposomal phospholipids derived from egg lecithin (PC) / cholesterol / dicetyl phosphate (DCP).

the barrier efficiency of constituent lipids of the liposomes derived from stratum corneum lipids.

In the present study, we investigated the interactions amongst the various lipid components of the stratum corneum in relation to the water barrier function of the mammalian skin. We have, therefore, prepared liposomes from only those lipids whose presence in the stratum corneum of the porcine skin has been established and used these liposomes as model membranes to investigate osmotic water permeability of epidermal lipid liposomes derived from stratum corneum lipids by adding or deleting particular lipid molecules, thereby elucidating the role of these molecules in maintaining the barrier function and in modulating the water permeability of these lipid bilayer liposomes. In almost all of the work reported in the literature (12–16), permeability has been measured through whole skin or through full thickness epidermis in the presence of drug penetration enhancers like ethanol (14), oleic acid (15), and azone (16). This study reports the osmotic water permeability studies of stratum corneum lipid liposomes derived from ceramides/oleic acid/cholesterol/cs and also from ceramides/linoleic acid/cholesterol/cs to give a better understanding about the fluidizing effect of these fatty acids in increasing water permeability of stratum corneum lipid liposomes.

MATERIAL AND METHODS

Epidermal ceramides and other lipids and phospholipids

The epidermal ceramides were isolated and measured by preparative thin-layer chromatography from total lipid extracts of lyophilised stratum corneum preparations of full thickness of pig epidermis as described previously (17). 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

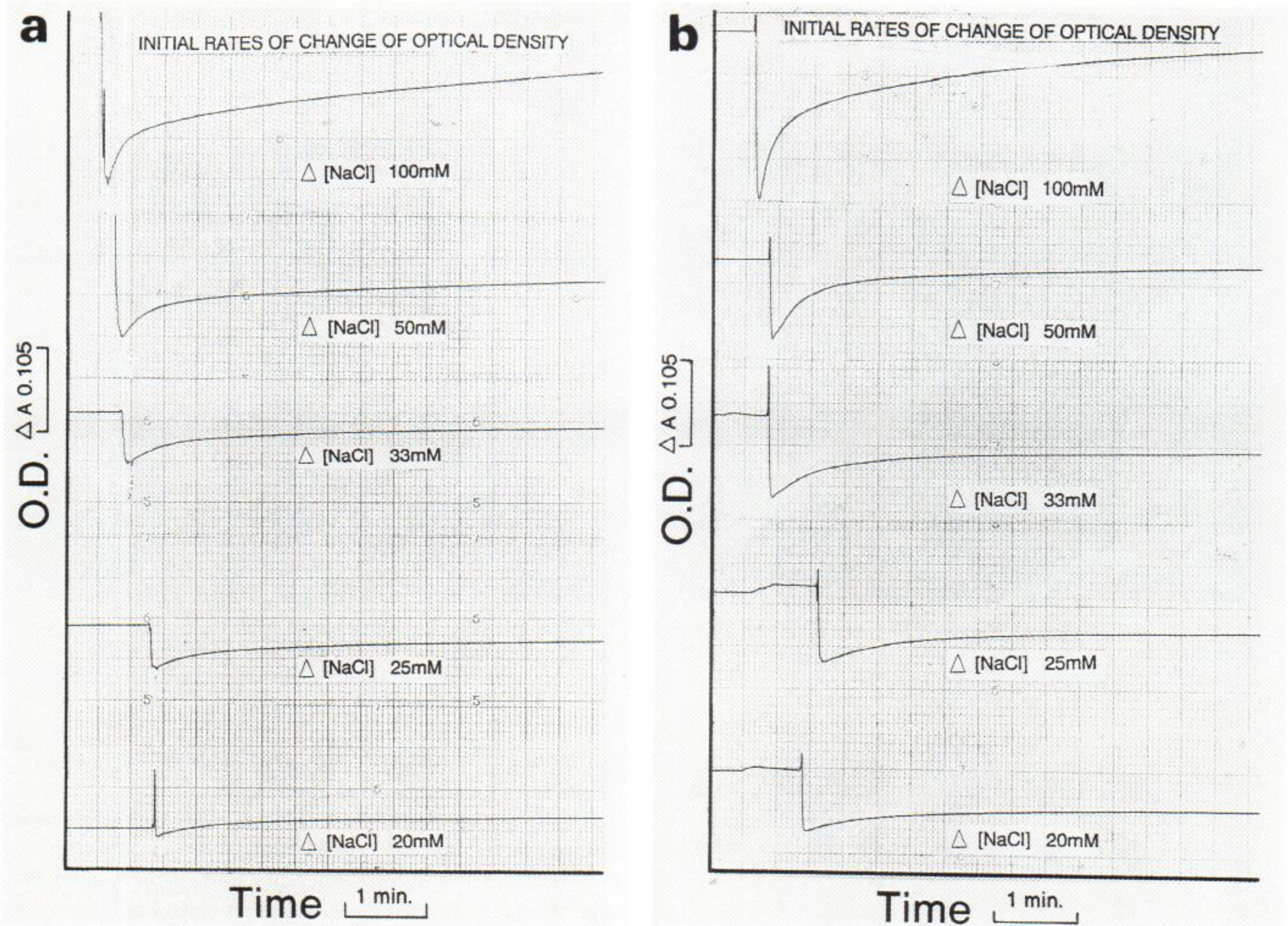

Fig. 3. Typical stop-flow spectrophotometric recordings (traces) for the initial changes in liposome permeability upon exposure to a hyperosmolar NaCl solution in the stopped-flow apparatus. *a*) stratum corneum lipid liposomes which were derived from ceramides/cholesterol/carnauva-palmitic acids/cs; and *b*) the same liposomes which were derived from ceramides/cholesterol/oleic acid/cs. These liposomes were prepared from mixture A (as mentioned in the text) and were treated with different salt osmotic shocks of NaCl solution at pH 7.0 and at 25°C. Each vertical (Optical density at 500 nm in Y axis) small division corresponds to the optical density of 0.0105 and each horizontal (Time 1 inch = 1 min in X axis) small division corresponds to 250 milliseconds.

combining carnauva wax fatty acids (18), 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 stratum corneum (19). Palmitic acid, linoleic acid, oleic acid, lignoceric acid, dicetyl phosphate, cholesterol, dipalmityol phosphatidylcholine and egg lecithin were purchased from Sigma Chemical Co.

Preparation of liposomes

Unilamellar lipid vesicles (liposomes) were prepared from 2 mg of a lipid mixture in chloroform/methanol (2:1, v/v) as shown in Table I, and epidermal lipid liposomes were prepared by the extrusion procedure as described previously (6). Phospholipids liposomes were prepared from egg lecithin and also from dipalmityol phosphatidylcholine from a lipid mixture as mentioned in Table I, both by extrusion procedure (6) and also by following the method of Bittman & Blau (20–21). Briefly, the lipid mixtures were dried under a stream of nitrogen and under vacuum at room temperature. The dried lipids were then swelled or hydrated in a borate buffer (boric acid -sodium hydroxide-potassium chloride, 20 mM), pH 8.5 at 70–80°C , which is above the gel to liquid crystal transition temperature of the stratum corneum lipids (Tc = 60°C) (6). The hydrated lipids were frozen in liquid nitrogen and thawed in a water bath at 70–80°C several times. Large hydrated chunks of lipids were then extruded in a stainless steel device (Lipex Biomembranes, Vancouver, BC, Canada) equipped with a thermobarrel attached to a circulating water bath. Extrusion was performed at 70–80°C through a nucleopore polycarbonate filter (Nuclepore Corporation, Pleasanton, CA), 25 mm in diameter. The dispersions were extruded five times through filters of pore sizes 5 μm and 1 μm. The dispersions were then extruded 10 times through a 400 nm filter and 200 nm filter. The final pH was 8.5 and the amount of lipid in suspension was 1.8–2 mg per ml. The liposome suspensions were dialysed against 20 mM borate buffer at particular pH to equilibrate the pH and the salt concentration. In some experiments, the pH of the liposome suspensions was lowered to pH 7.0 and even to pH 6.0 by dialysis against 20 mM borate buffer having respective pHs.

Analyses of starting lipids and the liposomal lipids

The amount of lipids in the liposome suspensions or in the starting lipids were analysed by quatitative TLC as described previously (18). Aliquots of the aqueous liposome suspensions were spotted and applied to 6 mm side lanes scribed in 20 × 20 cm silica gel plates (Alltech Associates, Inc, Deerfield, IL) on an analytical plate with the starting lipid mixture used in the liposome preparation. For resolution of nonpolar and polar lipids, the chromatograms were developed successively with chloroform/methanol/water (40:10:1, twice to 10 cm), then chloroform/ethanol/acetic acid (190:12:1, to 20 cm), and finally with hexane/ether/acetic acid (70:30:1, to 20 cm). The plates were air-dried, sprayed with 50% sulfuric acid, charred at 250°C and scanned in a Shimadzu densitometer, and the amount of lipid in the liposomal suspension or in the starting lipid material was quantitated (18). The

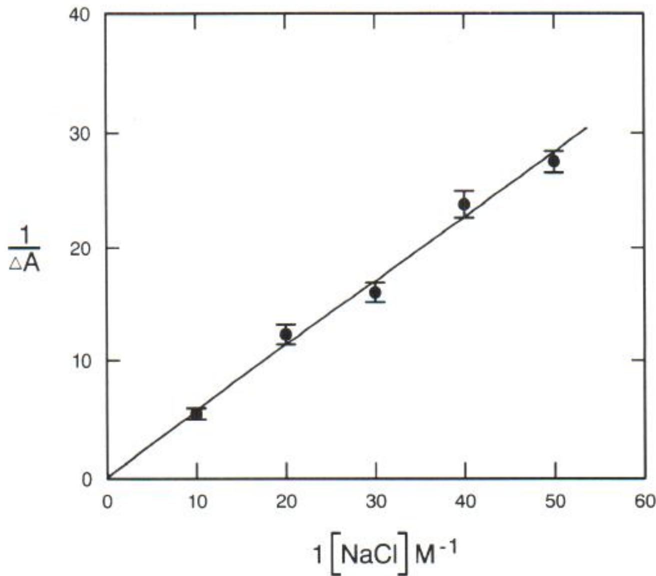

Fig. 4. Absolute change in optical density of stratum corneum lipid liposomes derived from ceramides/cholesterol/carnauva-palmitic acids/cs (mixture A) at equilibrium at various salt concentrations. It shows the relationship at equilibrium between the reciprocals of the absorbance changes at 500 nm and the reciprocals of the changes in the NaCl salt concentrations after osmotic shrinking. Each experimental point represents the average of the data from three individual experiments. The bars represent the standard deviations.

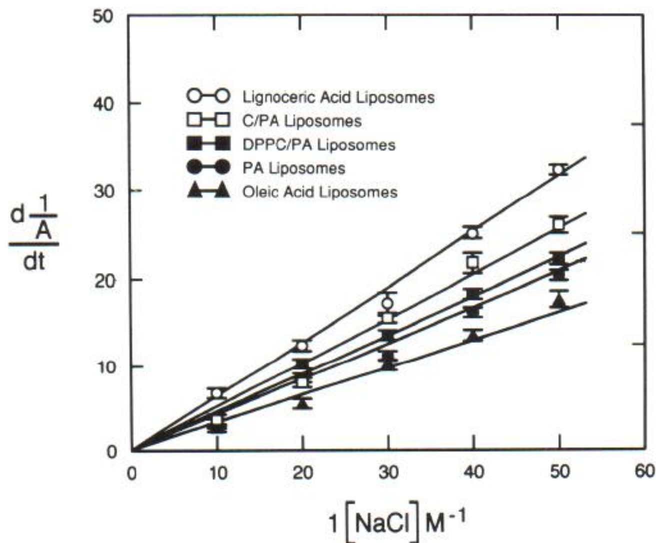

Fig. 5. Plots of initial shrinking rates vs the reciprocal of the osmotic difference of NaCl for the liposomes (mixture E) which were derived from different fatty acid lipid mixtures. (○——○) Lignoceric acid liposomes derived from ceramides/cholesterol/lignoceric acid/cs; (□——□) C/PA liposomes derived from ceramides/cholesterol/carnauva-palmitic acid/cs; (■——■) DPPC/PA liposomes derived from dipalmityol phosphatidylcholine/cholesterol/palmitic acid/cs; (●——●) PA liposomes derived from ceramides/cholesterol/palmitic acid/cs; (▲——▲) oleic acid liposomes derived from ceramides/cholesterol/oleic acid/cs. The absorbances were measured at 500 nm and at pH 7.0. Each experimental point represents the average of the data from three independent experiments and the bars represent the standard deviations.

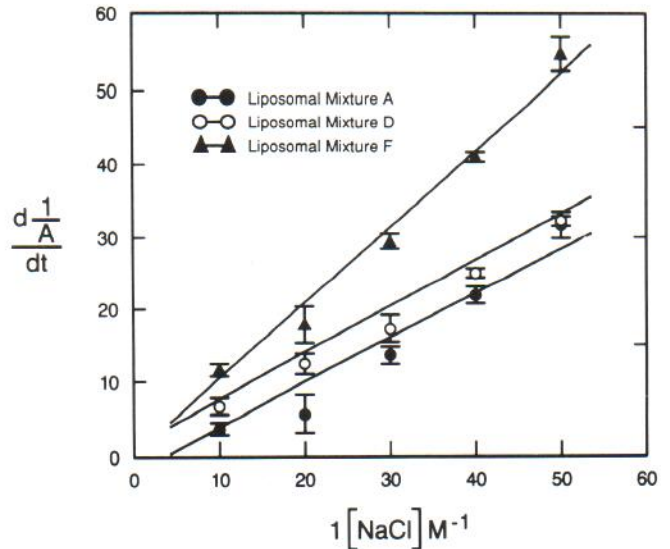

Fig. 6. Plots of initial rates of permeability behaviors in stratum corneum lipid liposomes derived from different wt% of lignoceric acid in the lipid mixture as mentioned in Table I. The absorbances were measured 500 nm and at pH 7.0. Values represent the mean + SEM of three independent experiments.

phosphorous content of the phospholipids was estimated colorimetrically (21).

Freeze-fracture electron microscopy of epidermal lipid liposomes

Drops of liposome suspensions were sandwiched between copper specimen holder plates and quick-frozen in liquid propane at -190°C with a propen jet (cryojet, Balzers AG, Lichtenstein). Freezefracturing was performed following standard procedure in a Balzers BAF 400 D apparatus. The fractured samples were coated with platinum and a carbon replica was made. Replicas were floated on distilled water for 1 h, cleaned with bleaching solutions (6), thoroughly washed with distilled water and picked up on copper grids. The clean replicas were examined in a Hitachi H-600 transmission electron microscope operating at 75 kV.

Liposome osmometry and measurement of water permeability by stopflow spectrophotometry

The osmotic property of the liposomes was investigated by monitoring the shrinkage of the liposomes in a series of hypertonic NaCl solution. Permeability of water through the liposomes was measured by osmotic shock experiments as described by Bittman & Blau (20). Briefly, equal volumes of the liposome suspensions and buffered salt solution of sodium chloride were mixed in the cuvette of a stop-flow apparatus and the resulting osmotic shrinkage was followed in a spectrophotometer, a modified HPLC spectrophotometer detector. The initial change in transmittance or optical density resulting from the scattering was monitored at 500 nm for a period of 30 min -1 h at room temperature (25°C). All permeability experiments were carried out at 25°C at different pHs (i. e, 6.0, 7.0, 8.5.). The initial rate of shrinking was determined by converting the change in transmittance per minute to dA/dt (13, 20).

RESULTS

Morphology of epidermal lipid liposomes studied by freeze-fracture electron microscopy

Fig. 1 shows freeze-fracture electron micrographs of lipid vesicles obtained by extruding epidermal lipid liposomes through polycarbonate filters. The liposomes were predominantly unilamellar and were uniform in size distribution. Fig. 1b shows

Table II. *Effect of pH and cholesterol on water permeability through epidermal lipid liposomes derived from ceramides / lignoceric acid / cholesterol / cs at 100 mM NaCl osmotic shock, 25°C*

CER, ceramides; *CH*, cholesterol; *FFA*, free fatty acids; *CS*, cholesteryl sulfate. The absorbances were measured at 500 nm and at 25°C.

Mixture[1]	Composition of lipids (wt%)				Initial O.D (dA / dt.min^{-1})*		pH 6.0
	CER	CH	FRA	CS	pH 8.5	pH 7.0	
A	55	25	15	5	0.23+0.05	0.28+0.09	0.20+0.08
B	50	30	15	5	0.29+0.06	0.21+0.03	0.19+0.07
C	45	35	15	5	0.27+0.09	0.18+0.03	0.16+0.08
D	40	27.5	27.5	5	0.19+0.09	0.16+0.02	0.15+0.07
E	35	40	20	5	0.17+0.05	0.16+0.9	0.14+0.08
F	30	35	20	5	0.15+0.02	0.12+0.05	0.10+0.08

[1]Lipid mixtures were 50% by weight of the lipids in the swelling solution and the final concentration of lipids in the liposomes was 1.8–2 mg per ml.
*SEM ± (the average values and error limits) were obtained from three different independent experiments.

that the morphology of the liposomes was stable, even with osmotic shock treatment of 100 mM NaCl salt solution (final osmolarity) for 30 min to 1 h.

Stop-flow spectrophotometric studies of osmotic water permeability of the liposomal membranes

Thin layer chromatographic studies revealed no change of lipid composition of liposomes derived from the respective starting lipids (Fig. 2). The initial rate at which the change in optical density occurs is a measure of the water permeation through the liposomal membrane. Typical spectrophotometric recordings (traces) of stop-flow kinetics of water permeability as an index of initial changes of volume in liposomal permeability upon exposure to various concentrations of hyperosmolar NaCl salt solution are shown in Fig. 3. The reciprocal of the changes in the absorbances at the equilibrium at different salt concentrations showed a linear dependence on the reciprocal of the osmotic pressure gradient and indicates that liposomes prepared from stratum corneum have been shown to act as an ideal osmometer (Fig. 4). Figs. 5–6 show the experimental results for the initial rates of osmotic shrinking of liposomes prepared from ceramides/oleic acid/cholesterol/cs and different liposomal preparations derived from stratum corneum lipids consisting of lignoceric acid, palmitic acid, oleic acid, and carnauva/palmitic acid respectively for each particular liposomal preparation and also for phospholipid liposomes composed of DPPC/cholesterol/palmitic acid/DCP or CS. The initial rates of shrinking of these liposomes when exposed to hyperosmolar NaCl solutions show a linear dependence on the osmotic pressure gradient (Figs. 5–6). The marked effect of lignoceric acid on decreasing the initial rate of the volume change is discussed below. The rates correspond to permeation of water. Fig. 6 shows that stratum corneum lipid liposomes derived from different compositions (wt%) of lignoceric acid underwent a marked effect on decreasing the initial rate of volume changes as an index of water permeability with increasing concentration (wt%) of lignoceric acid (C24: 0) of the epidermal lipid liposomes. The results so far indicate little effect of pH ranging from 6.0–8.5 on the initial rate of water permeability of liposomes derived from stratum corneum lipids as shown in Table II, although some effect on the time necessary to achieve equilibrium and the degree of change in optical density at equilibrium was observed at pH 7.0 or above and at pH 6.0. Table II shows that cholesterol lowers the initial rate of water penetration, and the rate decreases as the ceramides to cholesterol ratio (wt%) is varied (Table II), although the primary role of lignoceric acid in the barrier function of skin might be emphasized. Fig. 7 shows the histogram of relative water permeability as a change in optical density/min with respect to the types of lipid liposomes derived from different saturated and unsaturated free fatty acids which are present as unusal lipid components of mammalian skin.

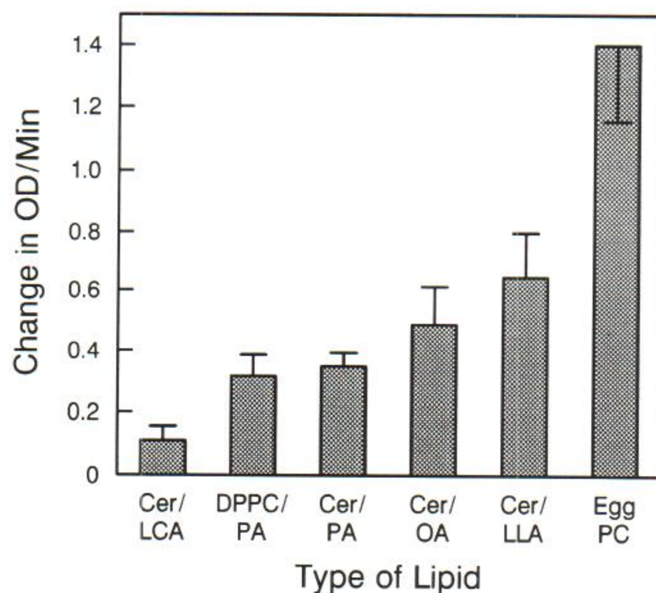

Fig. 7. Plots of changes in initial optical density vs. type of the lipid in the liposomes to show the barrier efficiency of various free fatty acids of the epidermal lipid liposomes and the water permeability behaviors of different fatty acids. Values were represented as means + SEM of three different independent experiments, and lipid composition (wt%) was determined by quantitative TLC as mentioned in the text. *Cer*, ceramides: *LCA*, lignoceric acid; *DPPC*, dipalmityol phosphatidylcholine; *PA*, palmitic acid; *OA*, oleic acid; *LLA*, linoleic acid; *Egg PC*, egg phosphatidylcholine or egg lecithin.

DISCUSSION

In the present investigation, we have studied the liposomal

morphology after osmotic treatment. Freeze-fracture studies revealed that the morphology of liposomes made from lipid mixtures consisting of ceramides, cholesterol, free fatty acids and cholesteryl sulfate was intact before and after the osmotic treatment. This finding suggests that water leakage from the liposomes might be due not to the fusion of two or more vesicles, but rather to the osmotic salt effect.

Next, the water permeability through the epidermal lipid liposomes was studied. Our findings suggest that lignoceric acid is one of the major free fatty acids of the stratum corneum and also a potent water barrier. These findings are in agreement with previous works (5, 19). The permeability of water depends on the amount (wt%) and the type of the lipid of the membrane. The stratum corneum is the outermost layer of skin, which acts as the main barrier for the diffusion of water through the skin (22–25). Cholesterol is one of the lipids of stratum corneum that plays an important role in the water barrier (5, 22–25). The present finding is also in agreement with previous works. The cholesterol in the liposomes drastically affects the fluidity (13). Present findings suggest that the initial rate of water penetration decreases in the liposomal membrane made with 35–40% (wt% ratio) of cholesterol and ceramides. The water barrier property of the skin, which is an important physiological function, prevents the loss of water from the body during dry weather (10). This property is maintained in vivo by linoleic acid ester of acyl ceramides of the stratum corneum (22–25), but it is interesting to note that such unsaturated fatty acids, when applied externally, induce increased permeability of the skin, even though the exact mechanism of inducting water permeability is not yet clear. The present finding indicates that water permeability induced by linoleic acid and oleic acid through the liposomes might be caused by their fluidizing effect, as it has also been observed by others (12, 15, 20).

The water permeability data in this study indicates that this simple liposomal membrane is a useful model system for human skin permeability that could be used for understanding the role of individual components in maintaining the epidermal barrier. Finally, it indicates that water permeability depends on the amount (wt%) and type of the lipid of the membrane and that lignoceric acid is a potent water barrier.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the central electron microscopy facility at the University of Iowa for their assistance and provision of facilities. This study project was supported by grants from the United States Public Health Science (AR 32374).

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