Formation and Characterization of Liposomes from Lipid/Proteic Material Extracted from Pig Stratum Corneum

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ABSTRACT: The formation and physicochemical properties of liposomes prepared from a mixture of lipids and proteins extracted from pig stratum corneum have been investigated. The extraction of this material was carried out with chloroform/methanol mixtures. The sonication of these mixtures at 80°C in water that contained piperazine-1,4-*bis*(2-ethanesulfonic acid) led to the formation of bilayered structures (vesicle size of about 150 nm), which were stable to aggregation for more than 24 h. The interaction of these liposome suspensions (proteoliposomes) at a subsolubilizing level with surfactants indicates that the nonionic surfactant Triton X-100 had the largest capacity for altering liposome permeability, whereas the amphoteric surfactant dodecyl betaine exhibited the smallest. The anionic surfactant sodium dodecyl sulfate showed an intermediate activity relative to that shown by the other surfactants tested. Despite the fact that the proteoliposomes showed negligible permeability in the absence of surfactants, compared with that of phosphatidylcholine liposomes, addition of identical amounts of surfactants resulted after 45 min in similar permeability effects for both. However, the proteoliposomes appeared to be more resistant to the action of surfactants in the initial interaction period.
JAOCS 73, 443–448 (1996).

KEY WORDS: Liposomes, permeability alterations, pig stratum corneum.

The stratum corneum (SC), the outermost layer of mammalian epidermis, consists of flat cells (corneocytes) that are separated by an intercellular matrix. The corneocytes are filled mainly with keratin filaments lying parallel to the corneocyte and embedded in an interfilamentous protein material. Their membrane is doubled inside the cell by a protein envelope. The intercellular matrix is composed mainly of lipids, organized into bilayers that have been postulated both to account for the permeability properties of SC and possibly to ensure the cohesiveness between corneocytes (1,2). The analytical composition of lipids and proteins forming the SC has been investigated extensively (3–6).

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To find out whether SC lipids could form bilayers, Wertz and co-workers (7–9) prepared liposomes from lipid mixtures that approximated the composition of SC lipids at physiological pH. These authors also investigated the interaction of these bilayer structures with the anionic surfactant sodium dodecyl sulfate (SDS) to study its deleterious effect of this surfactant on human skin (10). Furthermore, Blume *et al.* (11) reported that mixing phosphatidylcholine (PC) liposomes with lipid model mixtures for SC lipids could be one mechanism that contributes to the enhancement of permeability of the skin to lipid vesicles. However, it would be interesting to know whether lipid and protein mixtures directly extracted from pig SC could form liposomes.

In recent papers, we have studied the subsolubilizing and solubilizing interactions of different surfactants with simplified membrane models, such as PC liposomes, to establish a criterion for the evaluation of surfactant activity in these structures (12–14). In the present work, we seek to extend these investigations to determine the capacity of the lipid/protein mixtures extracted from the SC to form bilayers, and to investigate their physicochemical properties. The study of the subsolubilizing interactions between these bilayers and some representative surfactants, such as the nonionic Triton X-100, the anionic sodium dodecylsulfate, and the amphoteric *N*-dodecyl-*N,N*-dimethylbetaine (D-Bet), could provide new information about the effect of these surfactants on the permeability of these bilayer structures, taking into account the different physicochemical characteristics of these amphiphilic compounds.

MATERIALS AND METHODS

PC was purified from egg lecithin (Merck, Darmstadt, Germany) according to the method of Singleton *et al.* (15) and shown to be pure by thin-layer chromatography (TLC). SDS was purchased from Merck and further purified by a column-chromatographic method (16). The nonionic surfactant Triton X-100, octylphenol polyethoxylated with 10 units of ethylene oxide and an active matter content of 100%, was purchased from Rohm and Haas (Lyon, France). The amphoteric surfactant D-Bet was specially prepared by Albright and Wilson, Ltd. (Warley, West Midlands, United Kingdom); the active matter

was 30% in aqueous solution, and the free amino content was 0.20%. Piperazine-1,4-*bis*(2-ethanesulfonic acid) (PIPES buffer) obtained from Merck was prepared as 20 mM PIPES buffer adjusted to pH 7.20 with NaOH and contained 110 mM Na_2SO_4. The starting material, 5(6)-carboxyfluorescein (CF), was obtained from Eastman Kodak (Rochester, NY) and further purified by a column-chromatographic method (17).

The standards used for lipid analyses, ceramide type III (Cer), cholesteryl palmitate (Chol-pal), triglycerides (Trig), and cholesterol (Chol), were supplied by Sigma Chemical Co. (St. Louis, MO), and palmitic acid (PA)(reagent grade) was purchased from Merck. The cholesteryl sulfate (Chol-sulph) was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically. The lipids of the highest purity grade available were stored in chloroform/methanol 2.1 under nitrogen at –20°C until use.

Isolation of SC and extraction of lipid and proteic material. Young pigs, weighing 20–30 kg, were killed and brought to the laboratory intact. Most of the hair was removed from the skin surface with animal clippers followed by an electric shaver. Sections of fresh pig skin were placed in water at 70°C for 4–5 min, and the epidermis was scraped off in sheets. The epidermal sheets thus obtained were placed in 100 mL of 0.5% trypsin (Type III; Sigma Chemical Co.) in phosphate-buffered isotonic saline (PBS) at pH 7.4 and kept at 4°C overnight. The SC pieces were then collected on a coarse sieve, rinsed with distilled water, and suspended in a large volume of distilled water. The pieces were then individually transferred to a round flask, to which 100 mL of fresh trypsin/PBS solution was added, and the flask was rotated by means of a rotary evaporator at 100 rpm to provide gentle agitation at room temperature. After 2 h the tissue pieces were again collected on a sieve, washed with distilled water, blotted dry with filter paper, and then kept at –20°C until use (18,19).

The sheets of SC were individually extracted for 2 h with each of three mixtures of chloroform/methanol (2:1, 1:1, and 1:2, vol/vol) at room temperature. The SC was finally extracted with methanol overnight to remove traces of polar lipids (20,21). This extraction also removed some low-molecular weight proteins present in the SC. The different extracts were combined, concentrated to dryness, and weighed and redissolved in chloroform/methanol prior to analysis.

Analysis of the extracted lipids and proteins. The lipid extract obtained was first qualitatively analyzed by TLC. For nonpolar lipids, the chromatograms were developed with 100 mL of hexane/diethyl ether/acetic acid (80:20:1). For polar lipids, the chromatograms also were developed with 100 mL of chloroform/methanol/water (65:25:4). After drying, the chromatograms were sprayed with 50% sulfuric acid and heated to 220°C to char the lipids.

The quantitative analysis of the lipid extract was obtained by TLC coupled to an automated flame-ionization detection (FID) system (Iatroscan MK-5; Iatron Laboratory, Inc., Tokyo, Japan) (22). The lipid extract was directly spotted onto silica gel-coated Chromarods (type S-III) in 0.5, 1, and 1.5 µL with a SES 3202/IS-02 semiautomatic sample spotter with a precision 2-µL syringe (Iatron Laboratory, Inc.). The rods (in sets of 10 mounted semipermanently on stainless steel-racks) were developed with the following mixtures: (i) 70 mL of chloroform/methanol/ water (57:12:0.6) for a distance of 2.5 cm (twice); (ii) 70 mL of hexane/diethyl ether/formic acid (50:20:0.3) to 8 cm; (iii) 70 mL of hexane/benzene (35:35) to 10 cm. A total scan was performed to quantitate all lipid components. The same procedure was applied to different standard solutions to obtain calibration curves for the quantitation of each compound.

The low-molecular weight proteins, removed with methanol overnight after trypsin treatment, as well as some SC sheets, were hydrolyzed with 6 N HCl for 24 h at 110°C to obtain their amino acid compositions. Determination of the amino acid composition of these two hydrolyzed samples was carried out with an Automated Amino Acid Analyzer (Biotronik LC 5001, Hucoa Erlöss, Frankfurt, Germany).

Liposomes preparation–stratum corneum liposomes. The material extracted from the SC was dried under a stream of nitrogen at room temperature. The dried material was then hydrated in PIPES buffer (20 mM PIPES adjusted to pH 7.20 with NaOH containing 110 mM Na_2SO_4) supplemented with 10 mM CF. The final amount of extracted material in suspension was 1 mg/mL as determined by quantitative TLC/FID analysis. The hydration was carried out by sonication (Labsonic 1510; B. Braun, Melsungen, Germany) at 70 W for approximately 15 min (until the suspensions became clear) and at 70°C (7,21).

PC liposomes. Unilamellar liposomes of a defined size (about 100 nm) and a final PC concentration of 1 mg/mL were prepared by extrusion of large unilamellar vesicles that were previously obtained by reverse-phase evaporation (12).

Determination of phase transition temperature. Analyses of proton magnetic resonance (^{1}H NMR) were carried out at temperatures ranging between 25 and 90°C to determine the phase transition temperature of the lipid/protein mixture extracted from pig SC. The ^{1}H NMR spectra were recorded on a Varian Unit of 300 MHz (Palo Alto, CA). The preparation of the dispersed samples was carried out by sonicating 1 mg of extracted material in 1 mL of deuterated water (99.99% D) for 15 min at 80°C. The NMR spectra were measured at intervals of 5°C. The line widths of the CH_2 band at 1.3 ppm were measured, and 1024 scans were accumulated each time. The different line widths were plotted vs. the temperature, and the inflexion point of the curve was taken as a phase transition temperature.

Particle size distribution and internal volume of liposomes. The mean vesicle size distribution (nm) and polydispersity index of liposome suspensions were determined by dynamic light-scattering measurements in a Photon correlator spectrometer (Malvern Autosizer 4700c PS/MV; Malvern, England). The particle size distribution was established by particle number measurement. The sample was adjusted to the appropriate concentration range with PIPES buffer (20 mM PIPES containing 110 mM Na_2SO_4 and adjusted to pH 7.20), and the measurements were taken at 37°C at a reading angle of 90°.

The internal volume of liposomes is defined in our case as the volume enclosed by a given amount of extracted material and expressed as μL/mg. The determination of this parameter was carried out by measuring the concentration of CF encapsulated in the interior of vesicles with an RF-540 Shimadzu spectrofluorophotometer equipped with a thermoregulated cell compartment (Kyoto, Japan). To this end, liposomes containing CF were freed of unencapsulated fluorescent dye by passage through Sephadex G-50 medium resin (Pharmacia, Uppsala, Sweden). After separation, the concentration of encapsulated CF was determined spectrofluorophotometrically after the destruction of liposomes by addition of Triton X-100 (23).

Electron microscopy—freeze fracture. Liposome suspensions were placed on thin copper specimen carrier plates and frozen in liquid propane at −190°C. Freeze-fracturing was carried out in a Balzers 301 apparatus (Balzers AG, Balzers, Lichtenstein), and the specimen was shadowed with platinum and coated with carbon. The replicas were then coated with a support film of Parlodion, applied in amyl acetate, and air-dried before the copper carriers were dissolved by floating in an acid mixture (orthophosphoric/sulfuric/glacial acetic, 1:1:1). The replicas were then washed in distilled water, cleaned in Clorox bleach for 2–3 h, and rinsed several times in distilled water before being picked up on Formvar-coated grids. The Parlodion support film was dissolved by standing in methanol for 30 min. The cleaned replicas were examined in a Hitachi H-600 AB transmission electron microscope (Hitachi, Mito, Japan) operating at 75 kV.

Negative staining. Carbon-coated copper/palladium grids G-400 mesh, 0.5% Taab with 0.5% E 950 collodium films in *n*-amyl acetate were employed. A drop of the vesicular solution was placed on the grid and, after 1 min, sucked down to a thin film with filter paper. Negative staining with a drop of a 1% solution of uranyl acetate was performed. After 1 min this drop was removed with filter paper, and the resulting stained film was dried in a dust-free place. Samples were also examined in a Hitachi H-600 AB transmission electron microscope operating at 75 kV.

Permeability alterations of bilayers. Liposomes containing concentrated CF in the interior of the vesicles hardly fluoresce, but fluorescence strongly increases when CF is released from the interior of vesicles to the bulk aqueous phase. Therefore, the permeability changes of liposomes caused by surfactants were determined quantitatively by monitoring the increase in fluorescence intensity of the liposome preparations due to liberated CF in a RF-540 Shimadzu spectrofluorophotometer (Shimadzu Co.) at an excitation wavelength of 495 nm and emission at 515.4 nm. To this end, vesicles containing CF were freed of unencapsulated fluorescent dye by passage through Sephadex G-50 medium resin (Pharmacia) by column chromatography (23).

The general procedure to assess the effect of surfactants on the release of liposomal content consists of treating aliquots of liposomes (3 mL) with a small amount of buffered solution that contained the surfactants. Thereafter, the proportion of the CF released was calculated by means of the following equation (14):

$$\%\text{CF release} = (I_T - I_0)/(I_\infty - I_0)\ 100 \qquad [1]$$

Where I_0 is the initial fluorescence intensity of the CF-loaded liposome suspension, I_∞ is the fluorescence intensity after destroying the liposomes by addition of Triton X-100 (60 μL of 10% vol/vol solution); I_T corresponds to the fluorescence intensity at various periods of time due to the CF released, either by spontaneous release or by the addition of surfactants to the liposome suspensions.

RESULTS AND DISCUSSION

Lipid and protein composition of the extracted material from SC. The mixture extracted from SC with different mixtures of chloroform/methanol (0.244 g from 2.506 g dry epidermis) consisted of 228 mg lipids and 16 mg proteins, which corresponded to 93.45% lipid and 6.55% protein by weight.

Application of the TLC/FID technique permitted us to determine the percentages of each lipid in the chloroform/methanol extracted mixture used to form the liposomes (Fig. 1). After obtaining the calibration curves for each standard compound Cer ($Y = 38341.91\ X + 14184.25$); Chol ($Y = 39661.96\ X + 9761.31$); PA ($Y = 16790.92\ X + 5315.51$); Chol-sulph ($Y = 15468.37X + 350.11$); Chol-pal ($Y = 23295.63X + 573.27$); Trig ($Y = 19286.44X + 736.95$), where X = weight of lipid (μg) and Y = integration area of FID signal (Fig. 1), lipid quantitation was performed, and the resulting lipid percentages are ceramides (36.02%), Chol (27.42%), free fatty acids (24.53%), Chol-sulph (4.73%), cholesteryl esters (4.23%),

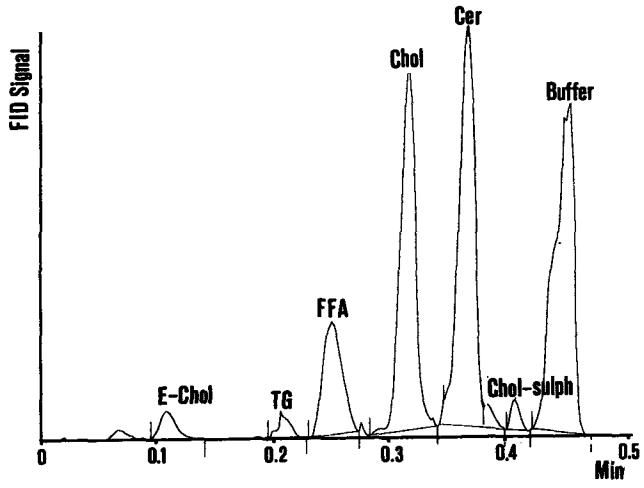

FIG. 1. Thin-layer chromatography/flame-ionization detector chromatograph of proteoliposome suspensions spotted on the rods and developed with chloroform/methanol/water (57:12:0.6) for a distance of 2.5 cm (twice), hexane/diethyl ether/formic acid (50:20:0.3) to 8 cm, and hexane/benzene (35:35) to 10 cm. Abbreviations: ceramides (Cer), cholesterol (Chol), free fatty acids (FFA), cholesteryl sulfate (Chol-sulph), cholesteryl esters (E-Chol), and triglycerides (TG).

and Trig (3.0%). It can be seen that SC lipids consisted mainly of ceramides, Chol, free fatty acids, and small amounts of Chol-sulph, cholesteryl esters, and Trig. The ceramides and Chol constituted the dominant species in the SC lipids (36.02 and 27.42%, respectively), and the analyses showed the absence of phospholipids, in agreement with results reported by Abraham *et al.* (3) for porcine SC.

The proteins that were removed by lipid extraction could be the result of disintegration of corneocytes, given that the chloroform/methanol extractions, besides completely and selectively removing lipids from the cutaneous tissue, could damage the corneocyte/lipid extracellular organization (19,20). The amino acid contents of these proteins (expressed in percentages of amino acids), together with the composition of SC, are indicated in Table 1. Comparison of these data shows significant differences, especially for Glu. These differences may be explained bearing in mind that, as reported by Downing, the links between hydroxyl and/or amine side groups of lipids (mainly ceramides) and the glutamic acid carboxylic groups play an important role in the formation and organization of the corneocyte lipid envelope (24). It is possible that the elevated Glu levels in the extracted material arise from the solvent extraction of Glu residues or Glu-rich peptides attached to the ceramides.

Formation and characterization of liposomes. The mixture of lipid-proteic material extracted from the SC formed bilayer structures when treated according to the methodology described in the Materials and Methods section. In addition to the TEM observations, there was experimental evidence of vesicle formation based on the fact that these structures showed internal volume, as will be indicated in this section. These liposomes will be referred to hereafter as "proteoliposomes," given their lipoproteic nature.

Some specific physicochemical parameters were determined to characterize these proteoliposomes. The phase transition temperature of the extracted mixture (determined by ^1H NMR) showed values that ranged between 60 and 65°C. As a consequence, the preparation of this suspension was carried out at 70°C, i.e., at a slightly higher temperature than that corresponding to mixture phase transition.

TABLE 1
Amino Acid Content (wt%) of the Material Extracted from Stratum Corneum (SC) by Solvent Mixtures and the Complete SC

Amino acids	SC material extracted by solvents	Complete SC
Asp	4.53	7.25
Glu	21.96	14.64
Lys	3.75	6.04
His	3.96	3.62
Arg	7.81	7.38
Ser	11.51	11.07
Gly	8.76	10.12
Thr	5.26	3.98
Ala	6.56	3.70
Pro	6.10	4.46
Tyr	2.70	4.12
Val	3.96	4.52
Met	—	1.51
Cys	1.15	4.02
Ile	2.41	3.51
Leu	5.34	5.76
Phe	4.30	4.31

The proteoliposome vesicle size curve, after preparation and equilibration for 60 min at 37°C, showed a monomodal distribution with a particle size of about 150 nm and a polydispersity index of 0.240, indicating that the vesicle dispersion was reasonably homogeneous. Both the vesicle size distribution and polydispersity index remained constant in the absence of surfactants for 24 h after preparation with a slight increase in the polydispersity index (final value of about 0.280), which indicated that this suspension was stabilized to aggregation during this period.

The internal volume of these vesicles showed a value of 1.2 μL/mg of extracted material. This value contrasts with that obtained for PC unilamellar liposomes, which for a vesicle size of about 100 nm showed an internal volume of 5 μL per mg PC. These variations may be correlated with the nature of the component-building bilayers, which could result in different structural associations leading to the formation of different types of liposomes.

The transmission electron microscopy pictures of proteoliposomes subjected to either negative staining (Fig. 2A) or freeze fracture (Fig. 2B) revealed the presence of structures similar to those exhibited by the liposomes prepared from lipids approximating the composition of SC (7). That is, homogeneous vesicles of about 150 nm were formed, and their size was in line with the aforementioned mean vesicle size distribution values obtained by dynamic light-scattering measurements. However, some smaller structures were detected in both microphotographs despite the fact that the size curve of these vesicles showed a monomodal distribution.

Permeability studies. In surfactant/phospholipid systems, complete equilibrium may take several hours (25,26). However, in subsolubilizing interactions, a substantial part of the surfactant effect takes place within approximately 30 min after its addition to the liposomes (27). Similarly, it could be expected, in agreement with work reported by Downing *et al.* (10), that the addition of surfactants to the proteoliposome suspension results in a competitive distribution of surfactant molecules between bilayers and the aqueous medium, which affects liposome permeability. A kinetic study on the release of CF encapsulated in the interior of proteoliposomes was carried out to determine these changes. To this end, 3 μL of proteoliposomes, containing 1 mg of extracted material per mL, was treated with 150 μL of 10.0 mM buffered solution of different surfactants, corresponding to a total surfactant concentration of 0.47 mM, and the subsequent changes in the permeabilty were studied as a function of time. These results are given in Figure 3A. The permeability changes, caused by the interaction of these surfactants on PC unilamellar liposomes (1 mg PC/mL) under the same conditions are indicated in Figure 3B. The percentages of CF release of Figure 3 are given as a difference between the values obtained in the presence and in the absence of each surfactant to show exclu-

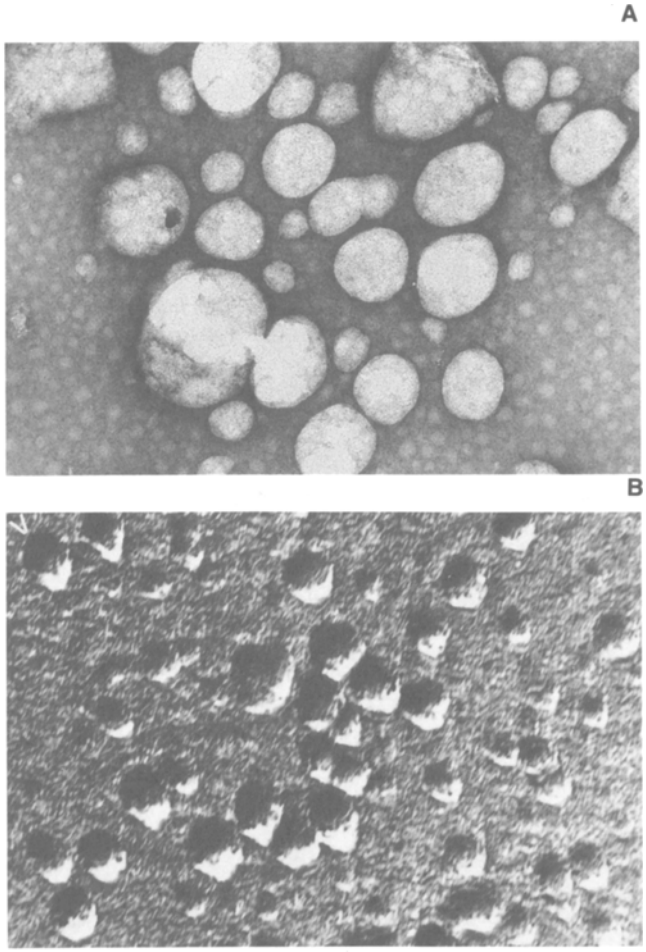

FIG. 2. Transmission electron microscopy microphotographs of proteoliposome suspensions obtained by negative staining (A) and freeze fracture (B). The magnifications are given below each microphotograph, and the direction of shadowing is indicated by the arrow.

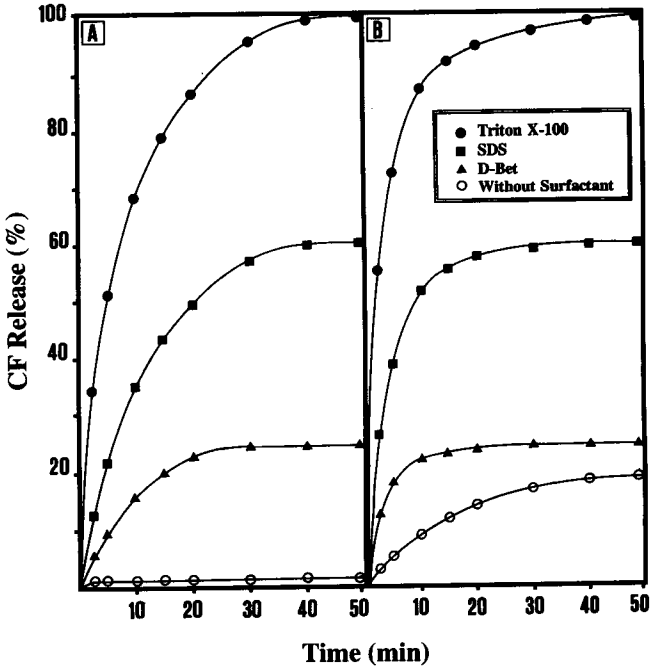

FIG. 3. Variation in the permeability of proteoliposomes (A) and phosphatidylcholine liposomes (B) resulting in the interaction with identical amounts of Triton X-100 (Rohm and Haas, Lyon, France) (●), sodium dodecyl sulfate (SDS)(■), and dodecyl betaine (D-Bet)(▲) as a function of time. The spontaneous permeability of liposome suspensions vs. time is also indicated (○); CF, 5(6)-carboxylfluorescein.

sively the permeability increase caused by surfactants. The spontaneous release of CF from the interior of the vesicles as a function of time in the absence of surfactants has also been indicated in both cases.

The interaction of both types of liposomes with surfactants showed similar tendencies. Thus, the interaction with the nonionic surfactant Triton X-100 led in both cases to the largest increase in the percentage of CF released after 45 min of treatment. However, the presence of the amphoteric surfactant D-Bet resulted in the smallest increase in the percentage of released dye throughout the interaction process. The anionic surfactant SDS showed an intermediate effect, compared with that of the other surfactants tested in the same period of time (45 min). As a consequence, Triton X-100 showed the greatest capacity for altering liposome permeability, whereas D-Bet exhibited the smallest. The proteoliposomes appeared to be particularly resistant to the action of surfactants in the initial interaction steps (period of time ranging between 5 and 15 min). The fact that proteoliposomes showed a negligible permeability in the absence of surfactants with respect to that of PC liposomes can be explained by bearing in mind the differences in lipid composition, and consequently, in their membrane organizations. These differences, due to the different phase transition temperatures of egg PC (below freezing) and pig SC lipids building proteoliposomes (between 60 and 65°C), reduce the spontaneous permeability of proteoliposomes with respect to that of PC liposomes at room temperature. These findings are in line with those reported by Kibat *et al.* (28), who investigated the permeability of liposomes composed of hydrogenated soy lecithin or natural lecithin and cholesterol mixtures. In spite of the different spontaneous permeability of these two liposomes, the presence of identical amounts of different surfactants led in both cases to similar changes in the bilayer permeability 45 min after the addition of these amphiphilic compounds, although with different CF release kinetics.

In conclusion, lipids and proteins extracted from pig SC formed proteoliposomes (vesicle size of about 150 nm), which were stable to aggregation for more than 24 h. Although these liposomes showed negligible permeability in the absence of surfactants with respect to the PC liposomes, interaction with identical amounts of different surfactants resulted in similar permeability changes 45 min after surfactant addition.

ACKNOWLEDGMENTS

The TEM analysis was performed at Barcelona University and we thank Dr. Carmen Lopez for his skillful work in the preparation of

freeze fracture and negative staining samples and TEM observations. We are also grateful to G. von Knorring for expert technical assistance. This work was supported by funds from DGICYT (Dirección General de Investigación Científica y Técnica) (Prog. n° PB94-0043), Spain.

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[Received May 16, 1995; accepted January 10, 1996]