Sphingomyelin from Milk—Characterization of Liquid Crystalline, Liposome and Emulsion Properties

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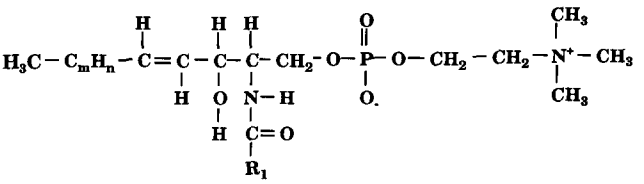
The properties of sphingomyelin obtained from bovine milk were investigated. In particular, the properties of liposomes and emulsions prepared from the spingomyelin, as well as the liquid crystalline behavior, were investigated and compared to those of related phosphatidylcholine systems. Like sphingomyelins from other sources, sphingomyelin from milk contains a large fraction of long and saturated acyl groups, which results in a high gel-to-liquid crystal transition temperature (T_c 35–82°C, depending on the lipid concentration). At high sphingomyelin concentrations, a lamellar phase forms above T_c, while a swollen gel phase is obtained below T_c. The gel phase swells to about 20 wt% water, whereas the swelling continues to about 40 wt% water above T_c. The limiting areas per molecule are 51 and 68 Å^2 below and above T_c, respectively. Sphingomyelin from milk forms liposomes readily in the presence of cholesterol. The liposomes formed have a diameter of about 100 nm and are stable, even at 0.1 M NaCl or HCl. Materials entrapped in the liposomes are released rather slowly (typically 40% over 5 h). A comparison shows that the sphingomyelin liposomes behave similarly to those formed by phosphatidylcholine systems. Furthermore, sphingomyelin from milk forms stable oil-in-water emulsions with soybean oil. The size of the emulsion droplets obtained was about 200 nm. Both the size of the emulsion droplets and its dependence on electrolyte addition correlate closely with those of emulsions formed by the corresponding phosphatidylcholine system. Therefore, it is possible to use sphingomyelin as an alternative for saturated phosphatidylcholines, which may be advantageous for oral and dermal pharmaceutical applications, as well as in cosmetics.

KEY WORDS: Emulsion, lamellar phase, leicthin, liposomes, sphingomyelin.

Milk fat is one of the most complex of all fats, both in terms of composition and of properties. Bovine milk contains approximately 4% fat in the form of globules. These are surrounded by a thin membrane, consisting of a complex mixture of lipids, proteins, enzymes and other components. The lipids of the membrane comprise 1–2% of the total milk lipids, and half of the membrane lipids are phospholipids. Five different phospholipids are present in milk fat, i.e., phosphatidylcholine (34%), phosphatidylethanolamine (33%), sphingomyelin (25%), phosphatidylinositol (5%) and phosphatidylserine (3%). Milk is a convenient source of sphingomyelin (Scheme 1; R_1 denotes the "fatty acid" aliphatic chain) because the alternative raw materials are blood or brain. Of the sphingolipids, sphingomyelins (N-acyl-sphingosine-1-phosphorylcholines) constitute the simplest class and are, in fact, among the major lipid components in mammalian cell membranes. Furthermore, sphingomyelin is an important component of the serum lipoproteins.



SCHEME 1

Sphingomyelins are composed of a phosphorylcholine head group, a sphingoid long-chain base and a fatty acid linked to the amide nitrogen of the base. They differ in the nature of their sphingoid base and in the acyl group linked to the ceramide nitrogen (1). The most commonly occurring base in sphingomyelin from animals is the C_{18} amine diol, usually referred to as sphingosine. The major acyl groups found in most sphingomyelins are $C_{16:0}$, $C_{24:1}$, $C_{22:0}$ and $C_{24:0}$. In brain, however, the main acyl groups are $C_{18:0}$, $C_{24:1}$ and $C_{24:0}$.

Sphingomyelins have a structure similar to that of the phosphoroglycerides (1). However, the hydrophobic region of phosphatidylcholine is composed of two acyl groups esterified to a glycerol backbone. Generally, these acyl groups are of comparable length. For sphingomyelins, on the other hand, this region is composed of one acyl chain, which is linked through an amide bond to the sphingoid base, and one paraffinic hydrophobic region within the sphingoid base. Typically, more than 60% of the naturally occurring sphingomyelins contain saturated chains in the range C_{16}–C_{24}. The paraffinic residue of sphingosine, on the other hand, is generally somewhat shorter, usually of the order C_{13}–C_{15}.

Judging from the chemical composition of sphingomyelin, this substance should, in principle, have several advantages as compared to phosphatidylcholines. For example, the lack of fatty acid ester bonds should make sphingomyelins less susceptible to hydrolysis, catalyzed by either an acidic environment or lipases and phospholipases. Secondly, the low level of unsaturation [the number of *cis* double bonds per sphingomyelin molecule is typically 0.1–0.35, whereas the corresponding figure for phosphatidylcholine is 1.1 to 1.5 (1)] should make sphingomyelins less susceptible to electrophilic attack than natural phosphoroglycerides. This may be advantageous, e.g., for drug delivery to the small intestine, as well as for cosmetic applications. Indeed, metabolic differences between these classes of lipids have been observed (2–5). For example, dietary lecithins are rapidly hydrolyzed in the upper small intestine, predominantly by the action of phospholipase A_2. The products of the hydrolysis (i.e., α-acyl lysolecithin and free fatty acids) are absorbed in parallel, also in the upper small intestine. Sphingomyelin is metabolized in the small intestinal tract, and the sphingosine portion is converted to fatty acids in the mucosal cells. Enzymes localized in the brush borders bring about sequential release of phosphorylcholine and fatty acid moieties from the sphingosine portion of the molecule. No evidence has been found to indicate any uptake ot the intact lecithin and sphingomyelin molecules.

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Extensive use of sphingomyelin in pharmaceutical and cosmetic applications has been limited, mainly due to its high price. Recently, however, sphingomyelin extracted from bovine milk has become commercially available in technical quantities. So far, little has been known about the physicochemical properties of sphingomyelin from milk, and, therefore, this initial investigation was undertaken. In the present study the liquid crystalline, liposome and emulsion properties of sphingomyelin from milk are investigated from a physicochemical point of view. Comparison is made with the physicochemical properties of sphingomyelin from other sources, as well as with those of phosphatidylcholine.

EXPERIMENTAL PROCEDURES

Materials. Sphingomyelin from bovine milk was manufactured by SMR (Lund, Sweden). (A patent on the extracting technique is pending.) The acyl group (fatty acid) and sphingoid base composition [obtained by plasma spray tandem mass spectrometry (6)] are given in Table 1. The L-α-phosphatidylcholine, dimyristoyl (DMPC), synthetic 99%, DMPA (L-α-phosphatidic acid, dimyristoyl, sodium salt), synthetic 99 and cholesterol 99% were all obtained from Sigma (St. Louis, MO). Tween-80, sorbitan monooleate $(EO)_{20}$, was obtained from Atlas Chemicals, (Wilmington, DE) and Epikuron 180 soybean lecithin was obtained from Lucas Meyer (Hamburg, Germany).

Methods. Samples for thermal analysis and x-ray diffraction experiments were prepared by adding sphingomyelin and water to an ampoule, which was subsequently sealed and repeatedly centrifuged (mild centrifugation with a table centrifuge for a few minutes at a time) above crystal transition temperature (T_c). After this, about one week of equilibration was allowed prior to thermal analysis and x-ray diffraction measurements. The thermal analysis was performed with a Mettler low-temperature thermal analysis system TA2000B (Mettler, Grefensee-Zürich, Switzerland) at a heating rate of 2°C/min. The dry sphingomyelin samples showed only a slight (≈3°C) hysteresis on heating and cooling, whereas the gel-to-liquid crystal transition ranged over about 10°C.

For the x-ray experiments, samples were transferred to glass capillaries, which were flame-sealed immediately thereafter. The sample holder was thermostated with a Peltier element to within ±2°C. X-ray patterns were recorded with a low-angle Kiessig camera equipped with a position-sensitive electronic detector (PSD 100; Tennelec Inc., Oak Ridge, TN). The x-ray beam was produced by an Ni-filtered Cu K_α radiation source (λ = 1.54 Å), and the signal was detected simultaneously at angles ranging from 0.12 to 1.7°. The detector was calibrated with crystalline sodium octanoate, showing a repeat distance of 23 Å. The structural parameters were calculated from the measured repeat distance by assuming lamellar symmetry and planar bilayers. Furthermore, the penetration of water in the lipid bilayer and the dissolution of lipid in the water layer were taken as negligible. The repeat distance (d) is separated into a lipid layer thickness (d_l) and a water thickness (d_w) by (7):

$$d_l = \{(1 - c)\nu_l/[(1 - c)\nu_l + c\nu_w]\}d \quad [1]$$

$$d_w = d - d_l \quad [2]$$

TABLE 1

Fatty Acid and Sphingoid Base Composition of Sphingomyelin from Milk

Fatty acid	wt%	Sphingoid base	wt%
$C_{14:0}$[a]	2.6	$C_{16:0}$	3
$C_{16:0}$	34.4	$C_{16:1}$	23
$C_{18:0}$	4.1	$C_{17:1}$	3
$C_{18:1}$	3.3	$C_{18:0}$	3
$C_{18:2}$	<1	$C_{18:1}$	64
$C_{20:0}$	<1		
$C_{20:1}$	<1		
$C_{22:0}$	17.0		
$C_{23:0}$	21.4		
$C_{24:0}$	13.5		
$C_{24:1}$	<1		

[a]Number of carbon atoms:number of double bonds.

where c is the water weight fraction, and ν_w and ν_l are the partial specific volumes of water and lipid, respectively. The latter two were taken to be 1.00 and 1.04, respectively. The area per lipid molecule (A) was obtained from:

$$A = 2M\nu_l d_l N_a \quad [3]$$

where M is the lipid molecular weight (750 g/mol) and N_a is Avogadro's number.

Liposomes were prepared by dissolving either sphingomyelin, cholesterol and Tween (5.2, 5.2 and 0.4 mM, respectively) or DMPC, DMPA, cholesterol and Tween (4.5, 0.7, 5.2 and 0.4 mM, respectively) in a toluene/methanol mixture (1:1). (Note that the surfactant was added before the liposomes were prepared, i.e., before sonication. Therefore, the surfactant remains evenly distributed among the lipid material after liposome formation.) An evaporation was performed with nitrogen after this, resulting in a thin lipid film on the inner wall of the glass vessel. Sonication was performed for 20 min with an ultrasonic probe (MSE Scientific Instruments, Crawley, Suddex, England) after addition of the aqueous solution (1 mM Tris buffer, pH 7.5). The sample was then centrifuged at 3600 × g for 10 min to remove particular impurities, originating from the ultrasonic probe and generated during sonication. The liposome solutions were then filtered once with a 0.2-μm filter (Schleicher & Schuell, Dassel, Germany).

In tracer entrapment experiments (*vide infra*), the aqueous solution used in the sonication contained 10^{-2} M 5(6)-carboxyfluorescein (CF) (Eastman, Rochester, NY). After sonication and centrifugation, the liposome sample was placed on a Sephadex G-50 (Uppsala Pharmacia, Sweden) size-exclusion column (at 5°C) to remove nonentrapped CF.

Because the local concentration of CF in the liposomes is quite high, CF acts as a self-quencher, the fluorescence intensity is initially quite low [nonentrapped CF was removed through gel permeation chromatography (GPC)]. As CF is released from the liposomes, however, the fluorescence intensity increases, due to a reduction in quenching (8–10). We used a Pye Unicam (Cambridge, United Kingdom) SP8-200 UV spectrometer at 330 nm, with a filter that absorbed wavelengths shorter than 475 nm in front of the detector. To obtain an absolute leakage scale, the liposomes were disrupted after about 6 h by addition of 0.25 wt% Triton X-100. The uncertainty in these experiments is estimated to be about ±5%.

Emulsions were prepared by first dispersing sphingomyelin or phosphatidylcholine in soybean oil and then emulsifying the oil phase in water with a high-shear colloid mill (Ultra-Turrax T18/10, Shaft TP10N; Janke and Hunkel GmbH, IKA-Labortechnile, Staufen, Germany) for 2 min at room temperature, followed by high-pressure homogenization in a Microfluidizer (Model TM 110; Microfluidics Co., Newton, MA) for 5 min at a pressure difference of 1000 bar under cooling in a water bath. The final concentrations of soybean oil and lipid in the resulting oil-in-water emulsion were 3 and 0.1 wt%, respectively.

The lipsome and emulsion droplet size was obtained through photon correlation spectroscopy at a scattering angle of 90° with a Malvern Autosizer II (Malvern, Worcs, England). No concentration dependence was observed in the measurements, i.e., the samples were sufficiently dilute to remove obstruction effects.

RESULTS AND DISCUSSION

Table 1 shows that the composition of sphingomyelin from bovine milk is quite complex. Like other sphingomyelins, it contains a large fraction of long acyl groups. Furthermore, it contains primarily saturated acyl groups, with less than 10% unsaturated groups. The dominating acyl groups for sphingomyelin from milk are, in decreasing order: $C_{16:0}$ (34.4%), $C_{23:0}$ (21.4%), $C_{22:0}$ (17.0%) and $C_{24:0}$ (13.5%).

Considering the large fraction of long and saturated acyl groups, a high gel-to-liquid T_c is expected. Therefore, finding T_c-values ranging from 82°C (for the pure sphingomyelin) to 35°C (at sphingomyelin concentrations lower than about 70 wt%) is not surprising (Fig. 1). For this sphingomyelin, a single transition peak (width approximately 10°C) was observed, and, at a heating/cooling rate of 2°C/min, the peaks obtained at heating and cooling were similar to within 3°C. The transition temperatures obtained for the present sphingomyelin agree with those obtained for sphingomyelins from other sources (1,11). Furthermore, the T_c-values agree well with those of comparable saturated phosphatidylcholine systems. For example, for DPPC, $T_c \approx 41.5$°C (19), which is in reasonable proximity of our 35°C. Furthermore, the temperature span between the dry and the hydrated material is about the same for both systems (≈50°C).

The swelling of the sphingomyelin system was investigated by x-ray diffraction experiments (Fig. 2; Table 2). As can be seen, little swelling occurs in the gel phase. Thus, the upper swelling limit occurs at about 20 wt% water, above which the gel phase is in equilibrium with excess water. On going from 10 wt% water to 20 wt% water, the water layer thickness increases from 6 to 12 Å, whereas the area per sphingomyelin remains fairly constant (47 and 51 Å^2 at 10 and 20 wt% water, respectively). Above T_c, however, the swelling is more substantial, and proceeds up to about 40 wt% water. This corresponds to an increase in the water layer thickness from about 5 Å at 10% water to about 25 Å at 40 wt% water, whereas the area per sphingomyelin molecule increases from about 52 Å^2 to about 68 Å^2.

The fact that fairly moderate swelling is observed is consistent with a rather short-range force operating between the sphingomyelin bilayers (12). This force is also observed for a number of other systems, including lecithins, and is usually referred to as the hydration force (12–14). The data suggest that only a small fraction of ionic lipids, if any, is present. It is interesting to note that the swelling is substantially less below T_c. This is what one would

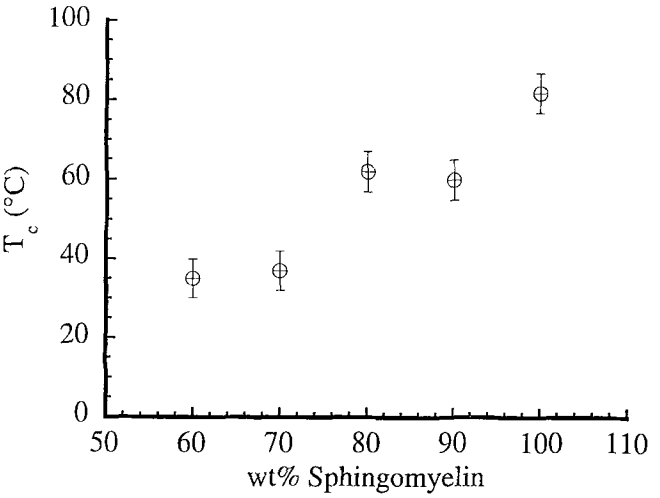

FIG. 1. Gel-to-liquid crystal transition temperature (T_c) of sphingomyelin at different water contents. The heating rate was 2°C per min.

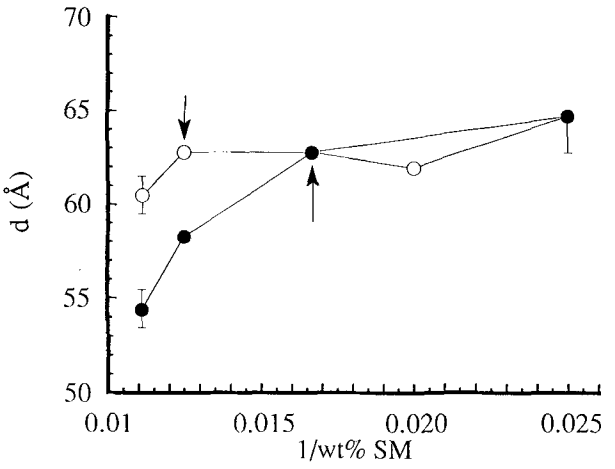

FIG. 2. Repeating distance (d) of the milk sphingomyelin (SM) lamellar phase at different water contents. The measurements were performed at 20°C (open circles) and at T_c + 5°C (filled circles). T_c, crystal transition temperature.

TABLE 2

Phase Behavior of Sphingomyelin from Milk

C_{Lipid} (wt%)	T_c (°C)	d (Å)	d_w (Å)	d_l (Å)	A (Å^2)
100	82	—	—	—	—
90	60	60[a]	6[a]	54[a]	47[a]
		54[b]	5[b]	49[b]	52[b]
80	62	63[a]	12[a]	51[a]	51[a]
		58[b]	11[b]	47[b]	55[b]
70	37	—	—	—	—
60	35	—[a,b]	—[a,c]	—[a,c]	—[a,c]
		63[b]	25[b]	38[b]	68[b]

[a]Temperature (T) = 20°C.
[b]T = T_c + 5°C. T_c, crystal transition temperature.
[c]Above the upper swelling limit.

expect from the protrusion models of the hydration force. We further note that the area per sphingomyelin is somewhat smaller in the gel phase than in the liquid crystalline phase. Finally, the area per sphingomyelin increases significantly with the water content above T_c.

The swelling of bovine brain sphingomyelin has previously been investigated by Shipley *et al.* (11). Our results agree semi-quantitatively with these previous measurements above T_c. For example, the maximum swelling limit was found to be 35 wt% water, which corresponded to a water layer thickness of about 22 Å and a surface area per shingomyelin molecule of about 60 Å^2. [Reiss-Husson (15), on the other hand, obtained 30 Å and 54 Å^2, respectively, at the upper selling limit at 40 wt% water.] These findings should be compared to the present ones of a maximum water content of about 40 wt% and a water layer thickness and an area per sphingomyelin molecule of 25Å and 68 Å^2 at T_c + 5°C, respectively. In comparison, the limiting area per bovine brain sphingomyelin at the air/water interface is 42–45 Å^2, whereas those for dipalmitoyl phosphatidylcholine and egg phosphatidylcholine are 43–46 Å^2 and 56 Å^2 per molecule, respectively (1,16–18).

Although a good agreement was found for the liquid crystalline sphingomyelin, our data on the gel phase are in contrast with those obtained by Shipley *et al.* (11) in that the swelling observed in the present investigation below T_c is much smaller than that observed previously. For example, at 25°C, these investigators found that the gel phase could incorporate 42 wt% of water, corresponding to a water layer thickness and a surface area per molecule of 34 Å and 54 Å^2, respectively. The origin of this difference is not entirely clear, but, in parallel to DPPC, a lower swelling below T_c is expected (19).

The swelling behavior of sphingomyelin from milk is similar to that displayed by comparable systems, such as lecithins. Thus, by comparing the swelling behavior of sphingomyelin and that of, e.g., egg yolk lecithin, one finds that at temperatures just above the phase transitions of the respective lipids, both lipids incorporate about 30–40 wt% water and, at maximum hydration, have similar values of d, d_l, d_w and A (1,11). Furthermore, the swelling of the lamellar phase above T_c is about equal to that observed for DPPC (≈40%) (20). On the other hand, the swelling below T_c is slightly smaller than that observed in the "gel-like" rippled $P_{\beta'}$ phase and the $L_{\beta'}$ phase of DPPC (about 30 and 25%, respectively) (20). Nevertheless, the swelling behavior is still quite similar to that of related phosphatidylcholine systems. This is not unexpected, considering that both sphingomyelin and phosphatidylcholine have zwitterionic choline head groups.

Because sphingomyelin is structurally related to lecithins and seems to interact with water in a similar way, one might expect them to form liposomes, in analogy to the latter substances (12,21,22). Indeed, we found that sphingomyelin from milk formed liposomes (Fig. 3), the size of which could be reduced to about 100 nm. In analogy with phosphatidylcholine, sphingomyeline liposomes are extremely "leaky" on their own, rendering a lasting encapsulation difficult. However, in the presence of cholesterol, a slow release of entrapped CF was observed (Fig. 4). This effect of cholesterol is well known and is used frequently in liposome preparation (10,18,23,24).

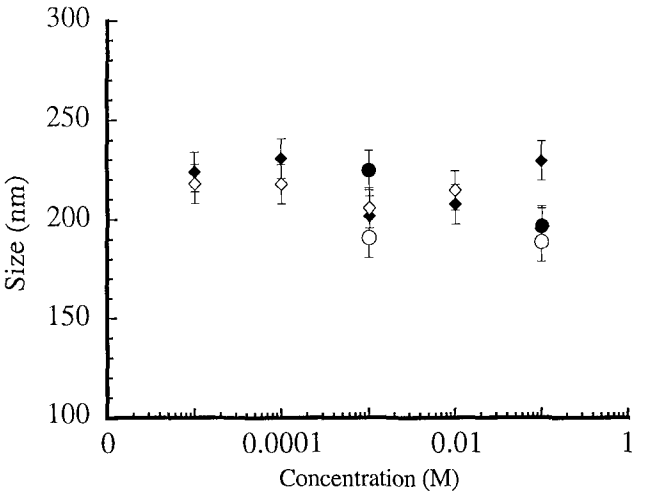

FIG. 3. Size of sphingomyelin/cholesterol/Tween liposomes at different concentrations of NaCl (circles) and HCl (diamonds) after 1 min (filled symbols) and after 60 min (open symbols). The temperature was 20°C. (Note that the liposome size could easily be reduced to about 100 nm by using more efficient sonication.)

To protect the sphingomyelin liposomes from flocculation and fusion, a nonionic surfactant (Tween-80) was incorporated in the liposomes. Tween contains oligo(ethylene oxide) (EO) chains, which protrude from the surface of the liposomes. The EO chains act as so-called steric stabilizers (25) and protect the sphingomyelin liposomes from flocculation and fusion, even at high electrolyte and/or acid concentrations, as indicated in Figure 3. Note, however, that the presence of the nonionic surfactant does not have any negative effect on the release of entrapped CF (Fig. 4), which is consistent with the results of Kronberg *et al.* (10) but at odds with those of Jamshaid *et al.* (26). Indeed, the leakage of CF seems to be even somewhat slower in the presence of Tween, which indicates that liposome collisions contribute to the release rate of CF.

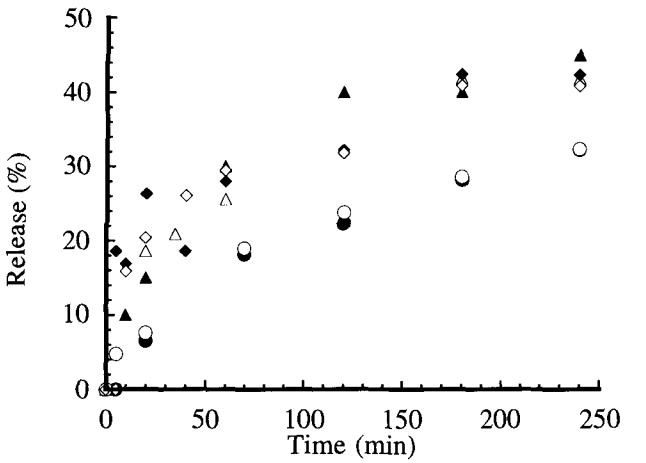

FIG. 4. Release of 5(6)-carboxyfluorescein (CF) from sphingomyelin/cholesterol liposomes in the presence (circles) and absence (diamonds) of Tween vs. time. Also shown is the release from L-α-phosphatidylcholine, dimyristoyl/L-α-phosphatidic acid dimyristoyl, sodium salt (DMPC/DMPA)/cholesterol/Tween liposomes (triangles). Double experiments are shown. The temperature was 37°C.

It is interesting to note that the release of CF from sphingomyelin liposomes is similar to that of DMPC/DMPA liposomes (Figs. 4-6). That the release is somewhat slower for sphingomyelin liposomes might be due to the longer acyl groups of the latter and is consistent with the finding that the permeability of erythrocyte membranes to molecules for which no specific transport system exists decreases with an increasing sphingomyelin content (1). Furthermore, the size of the sphingomyelin liposomes (≈100 nm) is comparable to that of the DMPC/DMPA liposomes (≈70 nm).

No significant effects are observed on sphingomyelin liposome size on addition of even high concentrations of NaCl (Fig. 3). However, on addition of NaCl, there is a pronounced decrease in the release rate of CF from spingomyelin liposomes (Fig. 5a). The origin of this decrease is unclear at present, but it may be related to the presence of small amounts of charged lipids, prohibiting a dense packing in the liposomal bilayer, due to lateral electrostatic repulsions (13,27). [Note that the same effects were observed for DMPC/DMPA (Fig. 5b).] Another explanation is that there is an osmotic pressure gradient across

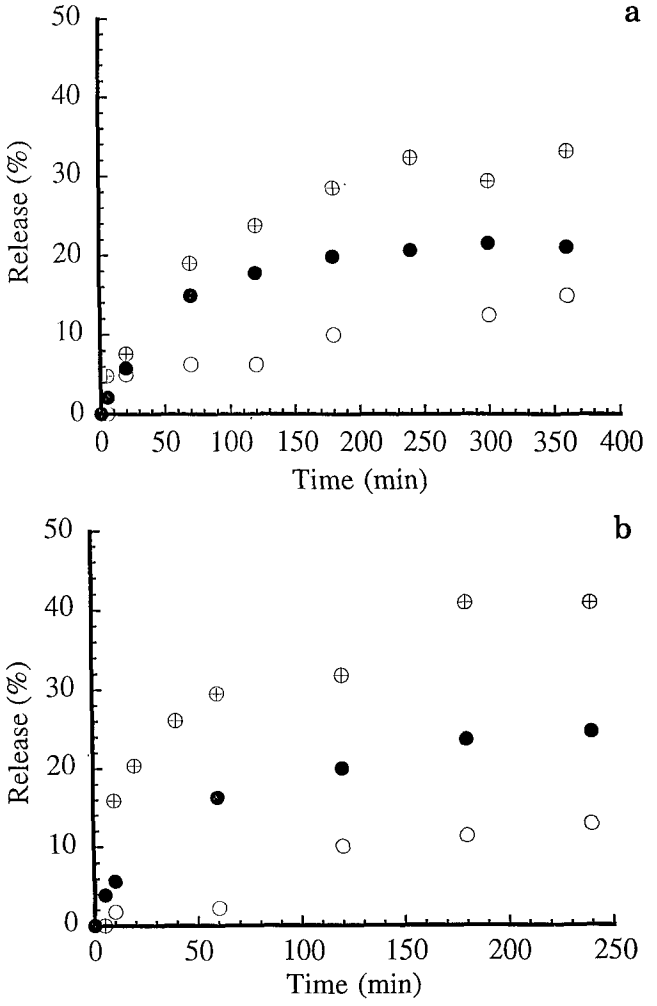

FIG. 5. Release of CF from (a) sphingomyelin/cholesterol/Tween and (b) DMPC/DMPA/cholesterol/Tween liposomes vs. time. The excess NaCl concentrations used were 0 mM (crossed circles), 1 mM (open circles) and 100 nm (filled circles). The temperature was 37°C. Abbreviations as in Figure 4.

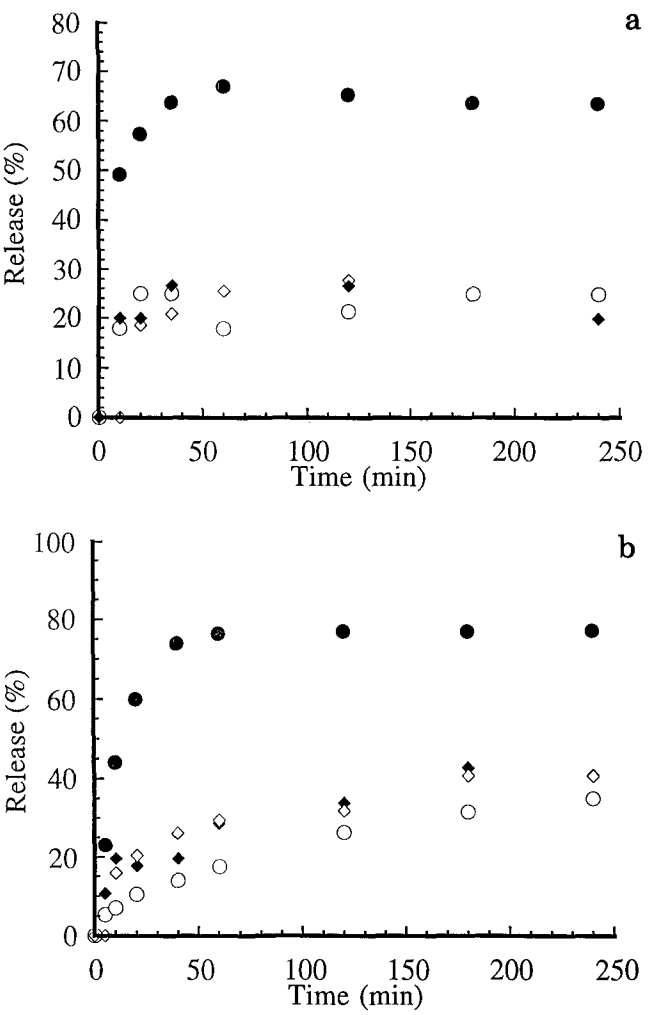

FIG. 6. Release of CF from (a) sphingomyelin/cholesterol/Tween and (b) DMPC/DMPA/cholesterol/Tween liposomes vs. time. The pH values used were 7.5 (open diamonds), 6.4 (filled diamonds), 4.8 (open circles) and 2.9 (filled circles). The temperature was 37°C. Abbreviations as in Figure 4.

the sphingomyelin bilayer because the total internal electrolyte concentration is 11 mM, whereas the external concentration is 1 mM. Adding external NaCl reduces this osmotic strain, which is expected to result in a slower release of entrapped material.

On addition of small amounts of HCl, little happens with the liposome size, as well as with the release rate of CF (Figs. 3 and 6). At higher HCl concentrations, however, an increase in the release rate of CF is observed, both for sphingomyelin and DMPC/DMPA liposomes (Fig. 6). For the latter, an acid-catalyzed hydrolysis of the fatty acid ester bonds is expected (28,29), which should result in an enhanced release rate of entrapped material. No such effects are expected for sphingomyelin, and the origin of the enhanced release at low pHs is uncertain.

Figure 7 clearly shows that sphingomyelin from milk forms good oil-in-water emulsions with soybean oil. The droplet size of the resulting emulsion was about 200 nm. In comparison, emulsions formed by the corresponding phosphatidylcholine system showed a slightly larger droplet size (300 nm). Upon addition of NaCl or HCl, little happens with the emulsion droplet size up to about 0.01

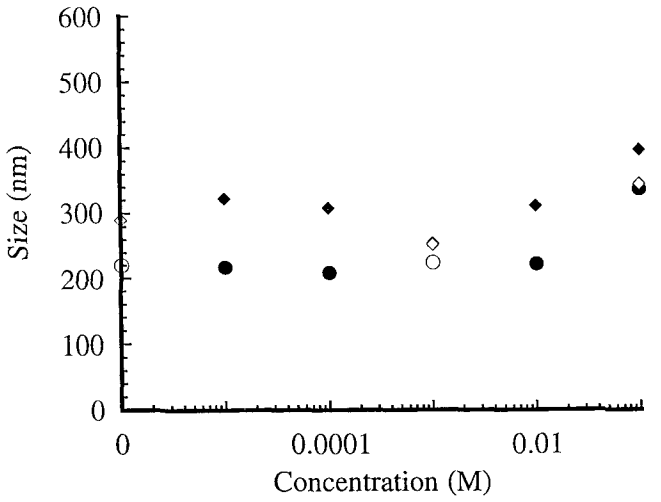

FIG. 7. Size of sphingomyelin/soybean oil (circles) and phosphatidylcholine/soybean oil (diamonds) oil-in-water emulsion droplets vs. [NaCl] (open symbols) and [HCl] (filled symbols). The temperature was 20°C.

M NaCl or HCl. At 0.1 M, however, a slight droplet size increase is observed for both systems. This finding shows that electrostatic interactions have some small effect on the colloidal stabilization in these systems.

In conclusion, the present study shows that the physicochemical properties of sphingomyelin from bovine milk are similar to those of saturated phosphatidylcholines. Therefore, it is possible to use sphingomyelin as an alternative for the latter in emulsions and liposome dispersions, which might be advantageous both for the stability and the biological acceptance of the formulation. In particular, this may be the case for oral administration of drugs to the small intestine and for cosmetic applications.

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