When large unilamellar vesicles consisting of sphingomyelin:phosphatidylethanolamine:cholesterol (2:1:1 molar ratio) are treated with sphingomyelinase, production of ceramides in the bilayer is accompanied by leakage of vesicle aqueous contents and by vesicle aggregation in the absence of lipid mixing or vesicle fusion. This is in contrast to the situation of phosphatidylcholine:phosphatidylethanolamine:cholesterol (2:1:1 molar ratio) liposomes when treated with phospholipase C. In that case, in situ generation of diacylglycerol leads to vesicle aggregation followed by vesicle fusion in the absence of leakage (Nieva, J. L., Goní, F. M., and Alonso, A. (1989) Biochemistry 28, 7364–7367). Moreover, when ceramides (5–10 mol %) are included in the formulation of the phosphatidylcholine-containing vesicles, they reduce the lag time of phospholipase C-induced fusion, although they are less active than diacylglycerols in this respect. 31P NMR studies of aqueous lipid dispersions show that diacylglycerols as well as ceramides induce a thermotropic lamellar to non-lamellar phase transition in both phospholipid:cholesterol mixtures under study although sphingomyelin-containing bilayers are more stable than those containing phosphatidylcholine, and ceramide is less active than diacylglycerol in promoting non-lamellar phase formation. These observations are relevant to both the physiological role of ceramides and the current views on the mechanism of membrane fusion.

In recent years, ceramides have been the object of extensive studies from which these lipids have emerged as potential second messengers for extracellular signals of suppression of cell growth and induction of apoptosis (1, 2). At the molecular level, multiple targets for ceramide action have been identified. The structural similarity and metabolic interconnection of ceramides and diacylglycerols have given rise to a number of comparative studies from which these lipids have emerged as potential second messengers for extracellular signals of suppression of cell growth and induction of apoptosis (1, 2). The data suggest that in all cases the corresponding lipid compositions are fluid phases (data not shown).

In order to ascertain that the extrusion procedure does not alter the lipid composition of our system, three lipid mixtures, namely SM:PE:CH (2:1:1), SM:PE:CH:diacylglycerol (47:23:25:10), and SM:PE:CH:ceramide (47:23:25:10), were quantitatively analyzed after the extrusion treatment. For that purpose, the resulting LUV suspensions were extracted with chloroform:methanol (2:1). Each organic phase was concentrated and separated on thin layer chromatography Silica Gel 60 plates, using successively in the same direction the solvents chloroform:methanol:water (60:30:5) for the first 10 cm and petroleum ether:ethyl ether:acetic acid (60:40:1) for the whole plate. After charring with an H2SO4 reagent, the spot intensities were quantified with a dual wave-length TLC scanner CS-930 from Shimadzu Corp. The results of this study are as follows (average of three determinations): for SM:PE:CH:ceramide, the absorption of the 47:23:25:10 mixture was not significantly different from that of the 2:1:1 mixture, indicating that the extrusion procedure does not alter the lipid composition of the system.

Previous research from this laboratory has shown that the catalytic action of phospholipase C induces fusion of large unilamellar vesicles consisting of PC, PE, and CH (4, 5). The onset and completion of the fusion process are regulated by the amount of diacylglycerol in the membrane (6). Recent x-ray and NMR data (7) show that diacylglycerols, in the concentration and temperature ranges at which fusion occurs, induce the transition from lamellar to H2 cubic phases in PC:PE:CH mixtures. On the basis of these data, it has been suggested that phospholipase C induces fusion through the bilayer-destabilizing effect of its reaction product diacylglycerols (7).

The present study is devoted to a comparison of the effects of diacylglycerols and ceramides in bilayer destabilization and fusion. For that purpose, our original system consisting of PC:PE:CH has been replaced by SM:PE:CH, and sphingomyelinase is used instead of phospholipase C. The phase behavior of lipid mixtures containing diacylglycerols or ceramides has also been comparatively examined by 31P NMR. In spite of having similar structures, both families of compounds display very different effects.

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.3) from Bacillus cereus was supplied by Boehringer Mannheim. Sphingomyelinase (EC 3.1.4.12) from B. cereus was from Sigma. Egg PC, egg PE, and diacylglycerol, obtained by phospholipase C hydrolysis of egg PC, were purchased from Lipid Products (South Nutfield, United Kingdom). Egg sphingomyelin, egg ceramide, N-7-nitro-2,1,3-benzoazidol-4-yl-PE (NBD-PE), and N-lissamine rhodamine B-sulfonyl-dioleoyl-PE were from Avanti Polar Lipids (Alabaster, AL). 1-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and NBD-PE, p-xylenebis(pyridinium bromide) (DPX), and octadecylrhodamine B were supplied by Molecular Probes, Inc. (Eugene, OR). CH was from Sigma.

Large unilamellar vesicles (LUV) were prepared by the extrusion method (8) using Nuclepore filters of 0.1 μm pore diameter, at room temperature, as detailed previously (4, 6). Vesicle size was estimated by quasi-elastic light scattering, using a Malvern Instruments, Inc. Zeta-Sizer instrument. SM:PE:CH (2:1:1) vesicles had an average diameter of 160–190 nm. Polarization of diphenylhexatriene fluorescence was routinely measured at 37°C for all the lipid mixtures used in the present study; the data suggest that in all cases the corresponding bilayers are in the fluid phase (data not shown).

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LUV, expected molar ratios 2:1:1, found 2.03:0.96:1.01; for SM:PE:CH: diacylglycerol LUV, expected molar ratios 47:23:25:10, found 47:21:47:25:52:7:88; and for SM:PE:CH:ceramide LUV, expected molar ratios 47:23:25:10, found 47.22:23.03:26.31:10.00. Thus it is concluded that, under our conditions, the extrusion procedure does not significantly modify the lipid composition of the LUV with respect to the original mixture.

For experiments involving sphingomyelinase, LUV were prepared in 10 mM HEPES, 200 mM NaCl, 10 mM CaCl₂, 2 mM MgCl₂, pH 7.0. For experiments involving phospholipase C, or when no enzyme was involved, LUV were prepared in 10 mM HEPES, 200 mM NaCl, 10 mM CaCl₂, pH 7.0. Previous experiments had shown that phospholipase C required >5 mM Ca²⁺, and sphingomyelinase required 10 mM Ca²⁺ and 2 mM Mg²⁺ for optimal catalytic activity under our conditions. All experiments were performed at 37 °C. Unless otherwise specified, lipid concentration was 0.3 mM, and enzymes were used at 1.6 units/ml.

Phospholipase C and sphingomyelinase activities were assayed by determining phosphorous contents (9) in the aqueous phase of an extraction mixture (chloroform:methanol, 2:1) after addition of aliquots from the reaction mixture at different times. The specificity of the enzymes was checked in preliminary experiments by thin layer chromatography.

Liposome aggregation was estimated as an increase in light scattering, measured in an LS50 Perkin-Elmer spectrophotometer with both monochromators set at 520 nm. Lipid mixing was assayed either by the octodecylrhodamine B method (10) or by the resonance energy transfer method using NBD-PE and rhodamine-PE (11), as detailed elsewhere (4, 12). Fusion was assayed, as mixing of aqueous contents, using the ANTS:DPX fluorescent probe system described by Ellens et al. (13).

Vesicle leakage was also measured with the ANTS:DPX system (13), as described previously (4, 6). Calibration of these procedures has also been described in detail in previous papers (4, 6). Lag times for the fusion and aggregation events were computed from the individual time course plots as the time at which the maximum slope line intersects with the “0% effect” base line.

31P NMR spectra were recorded in a KM360 Varian Instrument Business spectrometer operating at 300 MHz for protons. Spectral parameters were 45° pulses (10 µs), 3-s pulse interval, 16-kHz sweep width, and full proton decoupling. 2000 free induction decays were routinely accumulated from each sample; the spectra were plotted with a line broadening of 80 Hz. Samples were equilibrated for 10 min at each temperature before data acquisition.

Human erythrocyte ghost membranes were obtained according to Steck and Kant (14). The membranes obtained from 20 ml of erythrocyte concentrate (as provided by the blood bank) were suspended in a small volume of highly concentrated ANTS:DPX solution (500 mM and 1.8 mM, respectively), and after a few minutes, 20 ml of 10 mM HEPES, 10 mM CaCl₂, 10 mM MgCl₂, 100 mM NaCl, pH 7 (HEPES buffer) were added. Membranes were stabilized and allowed to reseal by incubating them for 1 h at 37 °C. After washing three times by centrifugation in HEPES buffer, the pellet was suspended in 3 volumes of the same buffer, and the resulting membrane suspension was layered over 43% sucrose in 25 mM NaCl, 25 mM Tris, pH 7. After centrifugation for 60 min at 33,000 rpm (TST 55.5 rotor, Kontron) (15). The ressealed ghosts floating on top of the sucrose solution were harvested and washed three times in HEPES buffer. Ghost suspensions were diluted and assayed as described previously to monitor leakage induced by sphingomyelinase.

RESULTS

When large unilamellar vesicles consisting of SM:PE:cholesterol (2:1:1 molar ratio) are treated with B. cereus sphingomyelinase, an initial enzyme activity is detected that cleaves, under our conditions (Fig. 1), ~20% of SM in the first 40 s and then proceeds more slowly until most of that lipid has been hydrolyzed. Preliminary experiments had shown that PE is not degraded by sphingomyelinase under these conditions. The process is accompanied by extensive vesicle leakage, shown in Fig. 1 as release of ANTS:DPX, and by an increase in turbidity (not shown). The latter appears to be due to the formation of large lipid aggregates; quasi-elastick light scattering measurements show particles in the range of 2–6 µm average diameter at the end of the process. Lipid mixing was tested both as octadeylrhodamine B dequenching and by fluorescence resonance energy transfer between NBD-PE and N-(lissamine rhodamine B-sulfonyl)-dioleoyl-PE, always with negative results.

No mixing of aqueous contents (ANTS + DPX) was detected either at any stage of the enzyme treatment.

The situation is very different from the one observed in the system PC:PE:cholesterol + phospholipase C. In the latter case, phospholipid cleavage is accompanied by an increase in turbidity but also by lipid mixing and leakage-free mixing of aqueous contents (4). The main differences between both systems are summarized in Fig. 2, leakage-free fusion with PC and leakage in the absence of fusion with SM.

In phospholipase C-promoted fusion, the relative proportions of lipids in the vesicles were crucial for obtaining leakage-free fusion or, alternatively, various combinations of diacylglycerol production, vesicle leakage, and contents mixing (4). Differences appear to be less marked in the SM-containing system whose behavior is summarized in Table I. Neither lipid nor aqueous content mixing is detected under any of those conditions. As long as SM, PE, and cholesterol are all present, enzyme activity or vesicle leakage is not greatly affected apart from the expected decrease in sphingomyelinase activity when SM is reduced from 50 to 25 mol % in the original composition. However, cholesterol and especially PE appear to be important for leakage. A similar decrease in sphingomyelinase activity upon removal of PE has been described by Tomita et al. (16). Aggregation rates vary irrespective of enzyme activity or vesicle leakage, suggesting a certain independence between the former phenomenon and these latter two.

Varying sphingomyelinase concentration between 1.6 and 8.0 units/ml or varying Mg²⁺ concentrations did not have any qualitative effect in the outcome of the experiment, i.e. aggregation and leakage but not fusion were observed in all cases. Lag times and rates of vesicle aggregation and leakage did vary as expected with enzyme concentration (data not shown).

In the PC-containing system, it was shown (6) that including diacylglycerol in the vesicle membranes prior to enzyme addition had two effects, reducing the lag time and extent of fusion (mixing of aqueous contents). The latter was interpreted as end product retinoihibition (6). Significant leakage-free fusion induced by phospholipase C was observed to occur between ~5 and 20% diacylglycerol in the membranes. A similar experiment has been repeated with PC:PE:cholesterol (2:1:1) and phospholipase C, only substituting some of the phospholipid in
the vesicles with ceramide instead of diacylglycerol, as in our previous study (6). The results with diacylglycerol or ceramide are shown in Table II for vesicle aggregation and fusion (contents mixing). Representative examples of the light scattering and fusion curves are given in Fig. 3. The effects of ceramides on aggregation are similar, though less marked, than those of diacylglycerols, a decrease in lag time and an increase in slope. The extent of aggregation is difficult to determine with accuracy because of extensive non-Rayleigh scattering by the large aggregates; thus slopes of light scattering versus time curves are given instead. In the case of fusion, ceramides do reduce to some extent the lag time (though much less than diacylglycerol) but have no effect on the extent of the process since, unlike diacylglycerols, ceramides are not the end products of the phospholipase C reaction. Note that, in the absence of enzymes, vesicles containing either ceramide or diacylglycerol can be stored for at least 24 h without significant leakage or aggregation.

Phospholipase C has been proposed to induce fusion via the production of diacylglycerols that, in turn, would destabilize the lamellar structure (7). In the SM-containing systems, there are two significant differences with respect to the above; the lipid-soluble enzyme product is ceramide, and their bilayer-perturbing properties may be different from those of diacylglycerol. The bilayer contains SM, and bilayers consisting of SM:cholesterol may be more stable than those containing PC:cholesterol. In order to clarify this situation, the thermotropic properties of both lipid mixtures in the presence and absence of diacylglycerol or ceramide, were studied by 31P NMR. The results are represented in Fig. 4. Previous studies (7) had shown that PC:cholesterol (2:1) formed stable lamellar phases between 20 and 80 °C, while in the presence of 10% diacylglycerol, a lamellar-to-isotropic phase transition centered at ~45 °C was observed, and a pure isotropic signal was already seen at 55 °C. When 10% ceramide is used instead of diacylglycerol, the bilayer-destabilizing effect is much smaller (Fig. 4), and the 31P NMR signal suggests the presence of a lamellar phase even at 60 °C. The SM:cholesterol (2:1) mixture is also lamellar between 20 and 90 °C (not shown). The 31P NMR data in Fig. 4 show that it is also more stable than the PC-containing composition; 10% diacylglycerol elicits an isotropic signal only above 60 °C while ceramide does so only at or above 70 °C, and in the latter case, a purely isotropic signal is not seen even at 90 °C. In summary, both PC:cholesterol and SM:cholesterol mixtures exhibit a lamellar to isotropic phase transition in the presence of 10% diacylglycerol or ceramide on a scale of increasing stability, PC:cholesterol:diacylglycerol (Tm ~45 °C) < PC:cholesterol:ceramide (Tm ~55 °C) < SM:cholesterol:diacylglycerol (Tm ~63 °C) < SM:cholesterol:ceramide (Tm ~75 °C). The midpoint transition temperatures are only approximate because spectra are recorded at 5 °C intervals and because the areas below the “isotropic” and “lamellar” signals may not be exactly proportional to the populations of lipids in one or the other phase. In any case, the 31P NMR results show clearly that SM:cholesterol bilayers are more stable than PC:cholesterol bilayers toward the effect of either diacylglycerols or ceramides and that the former are more potent than the latter in the promotion of non-lamellar phases.

As an additional demonstration of the sphingomyelinase-induced leakage of vesicular systems and also with the aim of extending our observations to the case of cell membranes, “right-side-out” human erythrocyte ghosts were prepared and loaded with ANTS:DPX as indicated under “Materials and Methods.” The plot in Fig. 5 shows that, under conditions similar to those in Fig. 1, sphingomyelinase elicits also fast release of aqueous contents from these vesicles of natural origin.

**DISCUSSION**

The widely accepted consensus that lipids in biomembranes are organized in bilayer form is compatible with the idea that, under non-equilibrium conditions, transient non-bilayer structures may exist. The above results have shown that enzymatically generated diacylglycerols and ceramides may induce two different conditions that do not occur in cell membranes in equilibrium, fusion and leakage, respectively. This raises at

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**TABLE I**

| Lipid composition, SM:PE:CH (molar ratio) | Sphingomyelinase activity | Vesicle aggregation ratea | Vesicle leakage ratea |
|------------------------------------------|--------------------------|--------------------------|---------------------|
| 2:1:1                                    | 12.5 ± 1.8 (4)           | 1.2 ± 0.03 (6)          | 5.9 ± 0.012 (3)     |
| 1:2:1                                    | 6.3 ± 0.18 (4)           | 2.5 ± 0.028 (5)         | 5.9 ± 0.018 (3)     |
| 1:1:2                                    | 4.1 ± 0.61 (4)           | 0.3 ± 0.087 (6)         | 3.7 ± 0.012 (3)     |
| 1:1:1                                    | 8.6 ± 1.14 (4)           | 0.5 ± 0.042 (6)         | 3.7 ± 0.007 (3)     |
| 2:1:0                                    | 7.3 ± 0.24 (4)           | 0.3 ± 0.065 (4)         | 1.4 ± 0.020 (3)     |
| 2:0:1                                    | 4.5 ± 1.04 (4)           | ~0 (3)                  | ~0 (3)              |

a Aggregation rates correspond to the maximum slope of the plot of light scattering versus time.

b Leaksleage rates correspond to the maximum slope of the plots of percent fluorescence versus time. 0 and 100% fluorescence/leakage are determined as described previously (4, 6).
least two questions, what are the non-bilayer structures generated in each case, and why are these structures different in each case? In the case of fusion, there is no clear proof of such a structural intermediate although there are various hypotheses, as well as indirect experimental evidence, of highly bent structures (of geometries reminiscent of the rods of HII hexagonal or Q224 cubic phases) that could generate or be involved in the fusion pore (7, 17–21). Even less is known of the bilayer discontinuity that allows vesicle leakage. Previous studies from this laboratory have shown that surfactants of natural or synthetic origin may induce leakage at subsolubilizing concentrations (22–24) in vesicles that are structurally intact as judged from electron microscopy or 31P NMR observations. The combined data from Figs. 2 and 4 show that leakage may occur from what are, from the point of view of NMR, perfectly stable lamellar structures while our previous studies (7) suggest that

### Table II

Influence of ceramides or diacylglycerols on phospholipase C-induced liposome aggregation and fusion

Ceramides or diacylglycerols were included, as indicated, in the original formulation (PC:PE:CH, 2:1:1). Note that, in the modified formulations, phospholipid but not cholesterol was substituted for diacylglycerols or ceramides. Data are the average values of 4–5 independent measurements, mean ± S.E. ND, not determined.

| Ceramide or diacylglycerol | Aggregation | Fusion |
|---------------------------|-------------|--------|
| Ceramide                  | Diacylglycerol | Ceramide | Diacylglycerol |
| Lag time | Slope* | Lag time | Slope* | Lag time | Extentb | Lag time | Extentb |
| % | s | s | % | s | % |
|---|---|---|---|---|---|---|---|
| 0 | 4.2 ± 0.47 | 5.2 ± 0.72 | 4.2 ± 0.47 | 5.2 ± 0.72 | 9.3 ± 0.14 | 100 |
| 5 | 2.0 ± 0.29 | 6.2 ± 0.88 | 1.4 ± 0.10 | 15.9 ± 2.50 | 6.5 ± 0.15 | 94 ± 2.35 |
| 10 | 1.7 ± 0.23 | 12.1 ± 0.30 | 0.6 ± 0.12 | 14.4 ± 1.63 | 4.1 ± 0.07 | 99 ± 1.80 |
| 15 | 2.3 ± 0.60 | 13.0 ± 0.60 | 0.7 ± 0.20 | 19.1 ± 1.53 | 3.2 ± 0.05 | 106 ± 0.80 | ND | 9 ± 0.2 |

* Maximum slope of a plot of light scattering versus time.

b The extent of fusion after an apparent equilibrium had been reached (after 5 min) is given here in relation to the control experiment (0% diacylglycerol or ceramide).

Fig. 3. Influence of ceramides and diacylglycerols on phospholipase C-induced liposome aggregation and fusion (mixing of aqueous contents). Fusion is assayed as a decrease in ANTS fluorescence. Aggregation is detected as an increase in light scattering. Three different formulations were used, respectively: PC:PE:CH (2:1:1), and the same with the addition of either 10% diacylglycerol (DAG) or 10% ceramide (CER). The time of enzyme addition is indicated by an arrow. u.a., units of absorbance.

Fig. 4. 31P NMR spectra of aqueous dispersions of phospholipid-cholesterol mixtures containing diacylglycerol or ceramide. A, PC:PE:CH (2:1:1) + 10 mol % diacylglycerol (DAG) or ceramide, as indicated on top of the spectra. B, SM:PE:CH (2:1:1) + 10 mol % diacylglycerol or ceramide, as indicated on top of the spectra. Spectra were taken at different temperatures as stated by each curve. Samples were 0.2 m in lipid. Chemical shifts are indicated in parts/million relative to inorganic phosphate.
Ceramides and Diacylglycerols in Membrane Fusion

fuson occurs under conditions clearly favoring non-lamellar architectures. It seems therefore that, at a given temperature, enzymatically generated ceramides in SM-containing bilayers have different structural effects than enzymatically generated diacylglycerols in PC-containing bilayers. Unfortunately the reasons for this difference remain unknown particularly because structural studies of systems containing SM, CH, and ceramide are not available. Cholesterol may interact with sphingomyelin more strongly than with PC, as deduced from a variety of physical and biochemical techniques (25–29); other data suggest, on the contrary, that the affinity of cholesterol for dipalmitoylphosphatidylcholine is the same as that for N-palmitoysphingomycin (30).

Enzyme generation of ceramide and diacylglycerol have in common that both give rise to vesicle aggregation. Das and Rand (31) observed that, contrary to the theoretical predictions based on the relative dehydration of the bilayer surface when PC is substituted for diacylglycerol, the presence of the latter lipid did not facilitate bilayer-bilayer contacts. Those authors suggested that, nevertheless, diacylglycerol could facilitate membrane aggregation when distributed asymmetrically in the bilayer (31). In fact, we observed that, when diacylglycerol is generated asymmetrically through the action of phospholipase C, extensive vesicle aggregation ensues (4, 6). We have now observed a similar effect when ceramide takes the place of sphingomyelin through the action of sphingomyelinase, and partial dehydration of the bilayer surface is the most probable reason also in this case. Aggregation appears to play a different role in the PC- and SM-based systems. In the former case, aggregation and fusion always go in parallel (6), and in fact, aggregation is an obvious prerequisite for fusion to occur. However, in sphingomyelinase-treated SM-containing vesicles, aggregation and leakage appear to vary independently (Table I), suggesting that they are in fact independent phenomena.

Recent reports from Wilschut and co-workers (32, 33) indicate that membrane fusion of Semliki Forest virus requires sphingolipids, and only the d-erythro isomers are active in this respect. This may seem at odds with the lack of fusion observed in our SM-containing vesicles, but we are actually dealing with widely differing phenomena since in their case the sphingolipids were acting hypothetically through specific binding to the viral E1 fusion protein. Our results are more relevant to the current hypotheses on the formation of the fusion pore (7, 17–21). Fusion would take place through the transient formation of a structural intermediate (“stalk”), and in the generation of this intermediate, some of the lipids would have to adopt the kind of “negative” curvature that is found e.g. in HII hexagonal or Q23 cubic phases. We have pointed out recently (7) that, for phospholipase C-induced fusion, this phenomenon was observed only under conditions of temperature and composition that allow formation of HII hexagonal and/or Q23 cubic phases. The results in Fig. 4 show that SM:PE:CH bilayers are perfectly stable at 37 °C even in the presence of ceramide. The lack of fusion under our conditions supports the requirement of a certain plasticity in the lipid mixture for fusion to occur.

The observations in this paper may be also significant in relation to the proposed physiological role of ceramides (1–3, 94–36). After agonist activation of certain cell surface receptors, ceramide is generated by sphingomyelin hydrolysis. Ceramide then activates membrane-bound or cytosolic enzymes, and the physiological effect ensues. The mechanism by which ceramide exerts activating roles is not known. In some cases, a direct binding of ceramide to the corresponding enzyme may be envisaged, but other activation pathways can also be considered. According to our results (Figs. 1 and 5), it is conceivable that ceramide production under physiological conditions would induce a local impairment of the membrane permeability barrier. The resulting abnormal ion fluxes (e.g. Ca2+ entry) and/or changes in membrane potential could well be the factors leading directly to enzyme activation. Alternatively, as suggested recently by Huang et al. (37), membrane-bound enzymes could be activated through ceramide-induced changes in membrane physical properties.

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Different Effects of Enzyme-generated Ceramides and Diacylglycerols in Phospholipid Membrane Fusion and Leakage

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