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MEMBRANE FUSION AND MOLECULAR SEGREGATION IN PHOSPHOLIPID VESICLES

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SUMMARY

Fusion between vesicles prepared from individual or mixed phospholipid species was demonstrated by ultracentrifugation and gel-filtration techniques, electron microscopy and differential scanning calorimetry. Variation of the chemical composition of the vesicles permitted evaluation of the effect of surface charge, Ca^{2+}, fluidity and the presence of cholesterol on the fusion reaction and the segregation of lipid species within fused vesicles. Extensive fusion occurred between negatively charged phosphatidylserine vesicles incubated in the presence of $CaCl_2$ (> 1 mM) and in vesicles prepared from greater than 50% phosphatidylserine in phosphatidylcholine in the presence of $CaCl_2$ (> 4 mM) and albumin (0.1 mg/ml). Neutral phosphatidylcholine vesicles showed only a limited capacity to fuse. Vesicles containing lipids that were in a liquid-crystalline state were more susceptible to fusion than vesicles composed of lipids that were in the solid phase at experimental temperatures. Incorporation of equimolar amounts of cholesterol into vesicles composed of lipids in a liquid-crystalline state suppressed their ability to fuse. Calorimetric measurements revealed Ca^{2+} induced segregation of individual lipids to form separate domains within the vesicle membrane (phase separation). The relationship of fusion between vesicles and fusion occurring in natural membranes was discussed.

INTRODUCTION

Membrane fusion is an important event in a wide range of cellular and subcellular activities (for reviews see refs 1–4). Despite its recognized importance in cell biology, there have been few studies on the molecular events accompanying membrane fusion. Most of the available information on this aspect of membrane behaviour has been obtained indirectly from observation of the factors that control cellular and subcellular activities in which membrane fusion occurs [1–3]. The lack of detailed

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Abbreviation: TES, *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

information on events in membrane fusion is due largely to the absence of a suitable experimental system. Investigation of fusion of natural membranes is hindered by the rapidity of the fusion reaction [3, 5–6] and the problem that fusion is usually restricted to small portions of the interacting membranes.

The fusion of cells cultivated in vitro has been proposed as a model for the study of membrane fusion [7]. However, the frequency of spontaneous cell fusion is in general very low [8], and even when increased by treating cells with viruses [9, 10] or chemicals [11, 12] there are still significant drawbacks to the use of cell fusion for elucidating the molecular changes involved in membrane fusion. Although fusion between two cells occurs quickly [5], the number of cells fusing at any given time is very low. Thus, membrane fusion, assessed in terms of the response of the entire cell population, occurs only at a very low level and is extended over several hours. This severely limits the opportunities for biochemical and biophysical investigation of the molecular changes involved.

These difficulties in studying the fusion of natural membranes have prompted the search for more simple systems for investigating the mechanism of fusion. In view of the documented similarities between the properties of phospholipid vesicles and those of natural membranes (for reviews see refs 13 and 14), we have examined the fusion capacity of vesicles prepared from single or mixed phospholipid species in an attempt to develop a simplified model of the complex membrane fusion reaction occurring in natural membranes. In a recent publication [15] we described the use of lipid vesicles to induce cell fusion, and suggested that this required initial fusion between vesicles and the cell membrane. In this communication we have extended these observations to study whether fusion can also occur between individual vesicles. We report here the use of ultracentrifugation and gel-filtration techniques, electron microscopy and differential scanning calorimetry to detect fusion between vesicles and describe some of the factors that influence vesicle fusion.

MATERIALS AND METHODS

Lipids

The phospholipids used in this study were synthesized and characterized in this laboratory using the methods described in detail elsewhere [16, 17]. All lipids were chromatographically pure and the fatty acid ester content was similar to that in previous studies [16]. Phosphatidylcholine was extracted from egg yolk and phosphatidylserine isolated from beef brain. Dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dimyristoylphosphatidylcholine, and dipalmitoylphosphatidylglycerol were synthesized as described previously [17]. Cholesterol (99% pure) was purchased from the Sigma Chemical Co. (St. Louis, Mo.) and recrystallized twice from methanol. All lipids were stored under N_2 in sealed ampoules at -50 °C at a concentration of approximately 10–20 μmoles of phosphate per ml chloroform. A newly opened ampoule was used for each experiment.

Phospholipid vesicles

Multilamellar vesicles were prepared by the method of Bangham et al. [18]. Unilamellar vesicles were made by sonication of multilamellar vesicles as previously

described [16, 19]. The buffer used in all the studies in this paper contained NaCl (100 mM), L-histidine (2 mM), *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (2 mM), EDTA (0.1 mM) and was adjusted to pH 7.4. It will be referred to as NaCl buffer. The temperature of dispersion and sonication was either 24 °C for phosphatidylserine and phosphatidylcholine, or at temperatures a few degrees higher than the T_c of each lipid mixture. In experiments in which unilamellar vesicles containing ^{3}H-labelled phospholipid were used, the ^{3}H-labelled material (usually 1 μCi) was added directly to the original lipid preparation in chloroform (usually 10 μmoles total phosphate) before evaporation and sonication to ensure maximum incorporation of ^{3}H label into the unilamellar vesicles. The phospholipid content of vesicle preparations (usually 5 μmoles/ml) was measured by phosphate assay with molybdate and ELON following $HClO_4$ digestion as described previously [15].

Ultracentrifugation and gel filtration of phospholipid vesicles

The protocol adopted in certain experiments to detect fusion between vesicles, involved incubation of a population of large (multilamellar) vesicles with a population of small (unilamellar) vesicles containing ^{3}H-labelled phospholipid and subsequent detection of the appearanceof the ^{3}H label in the large vesicle population. For descriptive convenience, the mechanism by which the transfer of the ^{3}H label occurred will be referred to as fusion, though as outlined in the results it was necessary to exclude experimentally the role of other mechanisms, such as molecular exchange and vesicle aggregation, in causing this transfer.

The two parent vesicle populations were subjected, before incubation, to an initial ultracentrifugation at 100 000 $\times g$ for 30 min at 24 °C. The multilamellar vesicles were found in the precipitate and were suspended in the NaCl buffer. The unilamellar vesicles were found in the supernatant and were used after appropriate dilution with same buffer. Multilamellar (large) vesicles were incubated with ^{3}H-labelled unilamellar (small) vesicles under the conditions outlined in the results, at concentrations of 1–2 μmoles of phosphate per ml. After incubation for the appropriate times, the mixed vesicle population was incubated for an additional 30 min in the presence of EDTA at concentrations equimolar to the Ca^{2+} that were present during the original incubation. Vesicles containing phosphatidylserine aggregate in the presence of Ca^{2+} but undergo rapid disaggregation when equimolar EDTA is added (visual observation). Thus, the chelation of free Ca^{2+} by the addition of EDTA serves to prevent the complication that any transfer of ^{3}H label to the large vesicle population was due to vesicle aggregation rather than fusion. The mixed vesicle population, still in the presence of EDTA, was then centrifuged at 100 000 $\times g$ for 30 min or passed through a Sepharose 4B column under conditions described before [21]. Finally the amount of the ^{3}H label recovered in the large vesicle fraction separated by these techniques was measured and expressed as a percentage of that present in the original small vesicle fraction.

Vesicles were prepared from either a single lipid species or two phospholipids in defined molar ratios. Except where stated otherwise, the composition of large and small vesicles in any experiment was identical, and equimolar quantities of phospholipid in large and small vesicles were incubated together at a total concentration of 1–2 μmoles/ml.

Measurement of lipid transition temperatures

The transition temperature (T_c) of the phospholipids in vesicle preparations was determined with a Differential Scanning Calorimeter (Perkin-Elmer DSC-2) using a scanning rate of 5 °C/min as described elsewhere [22]. The phospholipids were suspended in 100 mM NaCl buffer as described earlier at concentrations of 4 μmoles/ml. After incubation at specified time and temperature, the suspensions were centrifuged for 100 000 $\times g$ for 30 min at 24 °C, and the wet pellets transferred to the sample pan of the calorimeter. Each sample contained 0.5–1.0 μmole of phosphate in 15 μl.

Electron microscopy

Aliquots of unilamellar vesicle preparations of phosphatidylserine sonicated in 100 mM NaCl buffer, pH 7.4, containing 2 μmoles phosphate per ml were mixed with 1.0% uranyl acetate [23] on 400-mesh copper grids pre-treated with 0.1% bovine serum albumin and examined in a Phillips EM 300 electron microscope operated at 60 kV.

Radioisotopes and other chemicals

[^{3}H]Dipalmitoylphosphatidylcholine was prepared by catalytic hydrogenation of dipalmitoleylphosphatidylcholine by New England Nuclear Corporation. The lipid was synthesized and purified in our laboratory by procedures described earlier [17]. The ^{3}H-labelled lipid was finally purified by silicic acid chromatography. The specific activity of the purified product was 4 Ci/mmole. It was stored as a solution in chloroform at −50 °C. An aliquot containing approximately 1 μCi was used for each experiment and was mixed with the unlabelled lipid before evaporation to dryness and dispersion in buffer. Crystallized human albumin was obtained from Miles Laboratories (Kankakee, Ill.). Sepharose was purchased from Pharmacia(Piscataway, N.J.).

L-Histidine (Sigma grade) and TES were obtained from the Sigma Chemical Company (St. Louis, Mo.). All other chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus.

RESULTS

Centrifugation studies: effect of surface charge and calcium on vesicle fusion

This series of experiments was based on the transfer of ^{3}H-labelled phospholipids from small unilamellar vesicles to unlabelled multilamellar vesicles when incubated together. The small vesicles were labelled with [^{3}H]dipalmitoylphosphatidylcholine and incubated with large vesicles under the conditions outlined below. The mixed vesicle population was then centrifuged at 100 000 $\times g$ for 30 min to separate the small vesicles, which remained in the supernatant, from the large vesicles, which sedimented to form a pellet. The amount of tritium associated with the large vesicle pellet was then measured. For descriptive convenience, we will refer to the transfer of ^{3}[H]dipalmitoylphosphatidylcholine from small to large vesicles as resulting from fusion between the vesicles. Experimental evidence to support this conclusion will be presented in following sections.

The results obtained for the interaction of six types of vesicles are summarized

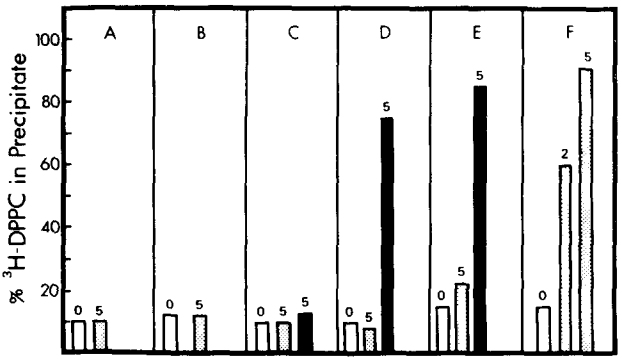


Fig. 1. Transfer of [^{3}H]dipalmitoylphosphatidylcholine from unilamellar to multilamellar vesicles. Large multilamellar vesicles were incubated for 1 h at 37 °C with an equimolar amount of phospholipid in the form of sonicated unilamellar vesicles labelled with [^{3}H]dipalmitoylphosphatidylcholine (^{3}H-DPPC). The incubated mixture was then centrifuged at 100 000 × g and the amount of ^{3}H appearing in the precipitate (multilamellar vesicles) was expressed as a percentage of the total (precipitate plus supernatant). A. Vesicles composed of "pure" phosphatidylcholine (PC). B. Vesicles composed of 10 % phosphatidylserine in phosphatidylcholine. C. 20 % phosphatidylserine in phosphatidylcholine. D. 40 % phosphatidylserine in phosphatidylcholine. E. 60 % phosphatidylserine in phosphatidylcholine. F. "Pure" phosphatidylserine vesicles. [^{3}H]dipalmitoylphosphatidylcholine was present as a trace (less than 1 in 10^3 phosphatidylserine molecules) in all samples labelled as "pure" phosphatidylserine in Figs 1, 2 and 3. Open bars: vesicles incubated in 100 mM NaCl buffer. Dotted bars: vesicles incubated in 100 mM NaCl buffer containing $CaCl_2$. The concentration of Ca^{2+} present is given over each bar, as mmoles/l. Black bars: vesicles incubated in the presence of both Ca^{2+} and albumin (0.1 mg/ml).

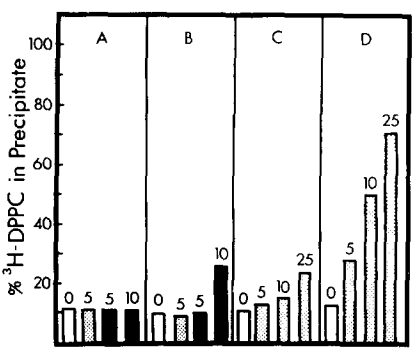


Fig. 2. Transfer of [^{3}H]dipalmitoylphosphatidylcholine (^{3}H-DPPC) from unilamellar to multilamellar vesicles. Experiments were identical to those in Fig. 1 except that vesicles were together for 20 h. A. Vesicles composed of 100 % phosphatidylcholine (PC). B. 20 % phosphatidylserine in phosphatidylcholine. C. 40 % phosphatidylserine in phosphatidylcholine. D. 60 % phosphatidylserine in phosphatidylcholine. The histogram code is the same as Fig. 1.

in Figs 1 and 2. Incubation of large and small egg phosphatidylcholine vesicles for 1 h at 37 °C in the absence of Ca^{2+} resulted in the transfer of only 10% of the ^{3}H label to the large vesicle population (Fig. 1A). This amount of ^{3}H was recovered from the large vesicle pellet even when the vesicles were not incubated together and were merely mixed immediately before final centrifugation. Increasing the incubation time

to 20 h at 37 °C produced only a slight increase in the amount of [^{3}H]dipalmitoylphosphatidylcholine found in the pellet (Fig. 2A). Similarly, incubation of large and small phosphatidylcholine vesicle populations in the presence of $CaCl_2$ (5 or 10 mM) for 1 h (Fig. 1A) or 20 h (Fig. 2A) or in the presence of $CaCl_2$ and albumin (0.1 mg/ml) (Fig. 2A) did not enhance the transfer of ^{3}H-labelled phospholipid from the small to the large vesicles.

We consider that the low level of [^{3}H]dipalmitoylphosphatidylcholine found in the large vesicles represents the background inherent to this technique and the experimental conditions. For example, a small proportion of ^{3}H-labelled small vesicles could be "captured" mechanically within the large vesicle population as a result of the aggregation of vesicles. Alternatively, the recovery of ^{3}H in association with large vesicles could result from low levels of fusion between the large and small vesicles. Some support for this possibility will be presented later in the section on calorimetric measurements on vesicles.

A similar low (10–15%) proportion of the ^{3}H label was found in the large vesicle populations when large and small vesicles of "pure" phosphatidylserine or various mixtures of phosphatidylserine in phosphatidylcholine were incubated in the absence of Ca^{2+} for 1 h (Fig. 1, B–F) or 20 h (Fig. 2, B–D). These results suggest that very little fusion occurs with vesicles of neutral phosphatidylcholine or negative phosphatidylserine incubated in 100 mM NaCl in the absence of Ca^{2+}. In contrast, significant fusion, as indicated by extensive recovery of the tritium label in large vesicles, was found when pure phosphatidylserine vesicles were incubated in the presence of 2 or 5 mM $CaCl_2$ for 1 h at 37 °C (Fig. 1F). Similar experiments indicated that Ca^{2+} were much less effective, however, in promoting fusion between vesicles composed of varying proportions of phosphatidylserine in phosphatidylcholine. Significant fusion occurred between vesicles prepared from 60% phosphatidylserine in phosphatidylcholine after incubation for 1 h (Fig. 1E) or 20 h (Fig. 2D). However, only limited fusion occurred between vesicles prepared from 40, 20 or 10% phosphatidylserine in phosphatidylcholine under the same conditions (Fig. 1, B–D and Fig. 2, B and C). Vesicle fusion in these systems was enhanced significantly by inclusion of albumin (0.1 mg/ml) in the incubation medium (Fig. 1, D and E). The inclusion of albumin alone did not alter the centrifugal properties of the vesicles. The use of albumin was prompted by previous observations [24] on the effect of this protein on the permeability properties of phospholipid vesicles in the presence of Ca^{2+}. However, similar concentrations of albumin were ineffective in promoting fusion between vesicles of 20% phosphatidylserine in phosphatidylcholine (Figs 1C and 2B) or pure phosphatidylcholine (Fig. 2A).

These results indicate that Ca^{2+} can promote the transfer of [^{3}H]dipalmitoylphosphatidylcholine from small vesicles into large multilamellar vesicles, with lipid mixtures containing more than 50% of a negative phospholipid. It is important to stress that the recovery of ^{3}H in the large vesicle pellet in these experiments is not due to simple aggregation of vesicles induced by Ca^{2+}. Equimolar amounts of EDTA were added to the mixture at the end of the incubation period, in order to chelate the Ca^{2+} and to disperse the aggregation which is visually apparent when Ca^{2+} is added to negatively charged vesicles. The presence of EDTA at equimolar concentration is sufficient to reverse the Ca^{2+} effect on the permeability properties of phosphatidylserine vesicles [26] and also on the endothermic transition as determined by Differ-

ential Scanning Calorimetry [15]. We interpret the transfer of phospholipid from small to large vesicles as evidence that fusion can occur between vesicles although alternative mechanisms cannot be excluded at this stage.

Demonstration of vesicle fusion by gel-filtration

The experiments described in the last section do not provide any information on the "net" amount of phospholipid transferred from the small to the large vesicles during the incubation. To clarify this point, and also to demonstrate vesicle fusion using a different technique, we repeated some of the experiments in the last section except that large and small vesicles were separated on the basis of particle size, by gel filtration [20, 21]. Equal amounts of phospholipid in the form of large and small vesicles were incubated together under the same conditions as the earlier experiments. The mixed vesicle population was then eluted through a Sepharose 4B column at room temperature and aliquots of each fraction assayed for their phosphate and tritium content.

Gel filtration of a mixture of large and small vesicles of 10% phosphatidylserine in phosphatidylcholine incubated in 100 mM NaCl buffer at 37 °C for 1 h revealed (Fig. 3A) that the phosphate was divided approximately equally between the early peak of large vesicles (fractions 10–15) and the delayed peak of small vesicles (fractions 16–26). However, only 10% of the ^3H associated with the small vesicles was recovered in the early peak of large vesicles (Fig. 3A). Similar results

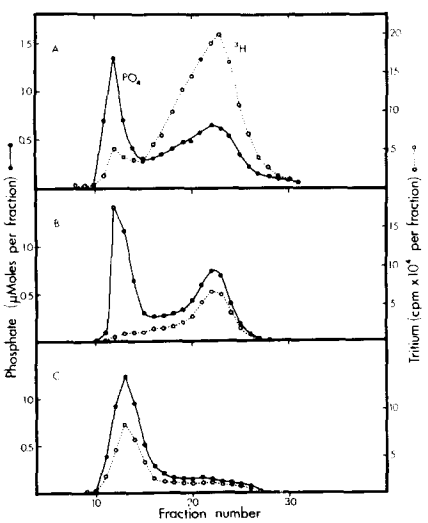


Fig. 3. Transfer of [^3H]dipalmitoylphosphatidylcholine from unilamellar to multilamellar vesicles. Large and small vesicles were incubated together for 1 h at 37 °C under the conditions described in Fig. 1 and in Methods. The mixture was then passed through a Sepharose 4B column and eluted with 100 mM NaCl buffer. The amount of total phosphate (closed circles, solid line) and ^3H (open circles, broken lines) present in each fraction were determined and expressed as μmoles of phosphate and cpm of ^3H. A. Vesicles composed of 10 % phosphatidylserine in phosphatidylcholine B. "Pure" phosphatidylserine vesicles incubated in the absence of Ca^{2+}. C. "Pure" phosphatidylserine vesicles incubated in the presence of Ca^{2+} (2 mM) and then passed through the column after addition of EDTA (2.5 mM). Large vesicles were eluted with the void volume (Fractions 11–14). Small (sonicated) vesicles were eluted in Fractions 16–26.

were obtained when the vesicles were incubated in the presence of 5 mM $CaCl_2$.

A similar elution pattern was also obtained for large and small phosphatidylserine vesicles incubated in the absence of Ca^{2+} (Fig. 3B). However, when the same mixture of phosphatidylserine vesicles was incubated in the presence of 2 mM $CaCl_2$ for 1 h (with equimolar EDTA added before the mixture was run through the column), the bulk of the phosphate and tritium were recovered in the early peak (Fig. 3C). These results indicate that Ca^{2+} can induce large scale association of ^3H-labelled small vesicles into much larger structures which cannot be dissociated by addition of EDTA. This irreversible association, which we interpret as due to fusion between the vesicles, occurs only with vesicles carrying a large negative surface charge. Similar recovery of both phosphate and tritium with the early peak was also obtained in an experiment where only small phosphatidylserine vesicles were incubated with Ca^{2+} and finally with EDTA as in Fig. 3C.

These results are in agreement with those discussed in previous sections, and exclude the possibility that the transfer of ^3H from small to large vesicles is due to exchange diffusion of free molecules rather than vesicle fusion. If exchange diffusion was responsible, the ^3H label would equilibrate between the large and small vesicles rather than be concentrated in either the large (Fig. 3C) or small vesicles (Fig. 3, A and B). A preferential transfer of phospholipid from small to large vesicles via molecular diffusion is also excluded by the lack of transfer between phosphatidylcholine vesicles or phosphatidylserine/phosphatidylcholine vesicles in the absence of Ca^{2+}. Furthermore, large vesicles can be obtained from a population of small ones as was mentioned above.

Electron microscopy of vesicle fusion

Additional evidence for fusion between vesicles was obtained by negative staining electron microscopy of unilamellar vesicles before and after addition of Ca^{2+}. Fig. 4A shows a preparation of small unilamellar vesicles of phosphatidylserine. The vesicles have a diameter of approximately 500 Å and appear either singly or in clusters. The latter probably result from aggregation caused by the interaction of uranyl ions in the stain solution with phosphate or carboxyl groups on the vesicles. Fig. 4B, shows the same phosphatidylserine vesicle population at the same magnification as in Fig. 4A after the vesicles had been incubated in the presence of 2 mM $CaCl_2$ for 1 h at 37 °C before addition of the stain solution. The individual particles are now at least 10 times larger than the phosphatidylserine vesicles shown in Fig. 4A, and we interpret these larger structures as resulting from the fusion of smaller vesicles. Fig. 4C, also at the same magnification, shows the vesicle preparation used in Fig. 4B except that EDTA at a concentration of 2.5 mM was added immediately before addition of the stain solution. The large particles are the same size as those in Fig. 4B but show numerous "folds" and "creases" and give the appearance of collapsed closed bags.

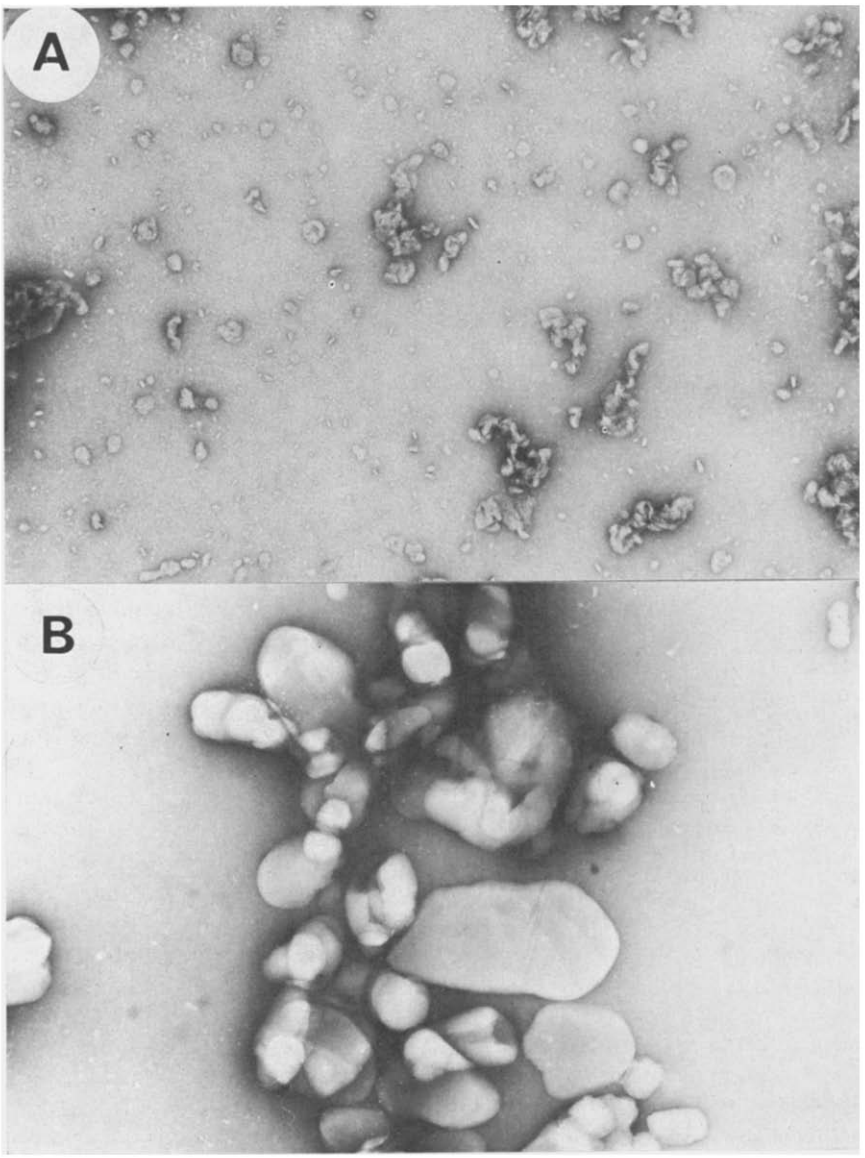
These results indicate that the presence of Ca^{2+} induces an irreversible change in phosphatidylserine unilamellar vesicles to form much larger vesicles. Importantly, the latter are not produced by aggregation of single vesicles but represent new intact structures. We conclude that this change occurs by fusion between vesicles.

Differential scanning calorimetry: molecular mixing during vesicle fusion

The interaction of two populations of small vesicles of similar composition

but of different transition temperatures (T_c) was next investigated in an attempt to identify molecular mixing of the two lipid species in fused vesicles by calorimetry.

Differential scanning calorimetry measurements on vesicles prepared without sonication in 100 mM NaCl from 60% phosphatidylserine in distearoylphosphatidylcholine revealed a broad endothermic peak centering at a temperature of 42 °C (Fig. 5a). In contrast, similar vesicles prepared from 60% phosphatidylserine in dimyristoylphosphatidylcholine displayed a broad peak that centered at approximately 11 °C (Fig. 5B). By mixing two populations of vesicles with such widely differing T_c values it was hoped that fusion between the two vesicle populations could be detected by the melting characteristics of the mixture.



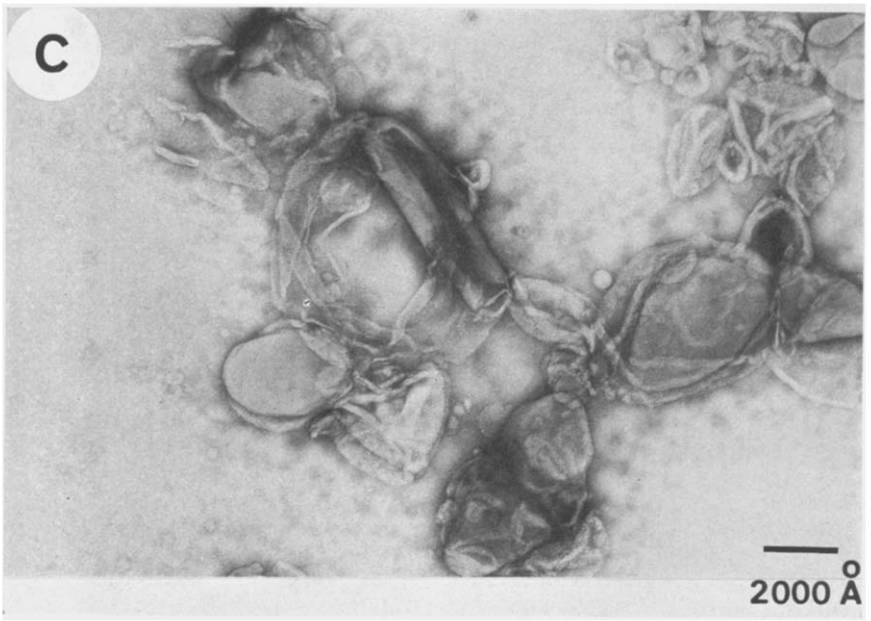


Fig. 4. Electron microscopy of sonicated phosphatidylserine vesicles before and after addition of Ca^{2+}. Vesicles were negatively stained with uranyl acetate as described in Methods. All pictures are at the same magnification. Bar = 0.2 μm (2000 Å). A. Sonicated vesicles of phosphatidylserine in 100 mM NaCl buffer. B. Same vesicles after 1 h incubation in the presence of 2 mM Ca^{2+}. C. Same preparation as in B except for the addition of 2.5 mM EDTA after incubation with Ca^{2+}.

Vesicles of 60% phosphatidylserine in distearoylphosphatidylcholine prepared by sonication were incubated with an equal amount of sonicated vesicles composed of 60% phosphatidylserine in dimyristoylphosphatidylcholine in the presence of $CaCl_2$ (15 mM) and albumin (0.04 mg/ml) for 2 h at 42 °C. Excess EDTA was then added to chelate Ca^{2+} and the mixture centrifuged to form a pellet. The latter was then examined by calorimetry. The results (Fig. 5C) show that in addition to the two peaks at approximately 12 and 42 °C, which correspond to the T_c values of the two types of parent vesicles, the mixture also exhibited a new peak centering at 33 °C, indicating the presence of a new lipid mixture. That this new peak represents mixing of the original lipids following vesicle fusion is supported by the finding that equal proportions of the two lipid mixtures, phosphatidylserine in dimyristoylphosphatidylcholine and phosphatidylserine in distearoylphosphatidylcholine, displayed an endothermic peak centering at 33.5 °C when mixed in chloroform and dried to form mixed lipid vesicles (Fig. 5D). This result indicates that the middle peak in Fig. 5C represents molecular mixing between the lipids in the two vesicle populations, presumably as a result of fusion between the different vesicles. Mixing due to molecular diffusion of single lipid molecules from one vesicle population to the other would not be expected to create such an equimolar mixture together with the persistence of peaks for the two original lipids. Exchange diffusion would instead eliminate the endothermic peaks of the two original lipids completely.

Calorimetric studies on vesicles prepared by sonication from phosphatidylserine in distearoylphosphatidylcholine (Fig. 5A′) and also phosphatidylserine in

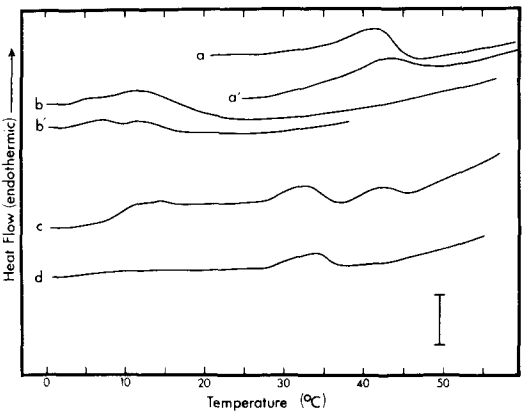


Fig. 5. Differential scanning calorimetry of 60 % phosphatidylserine in phosphatidylcholine vesicles. Phospholipid dispersions were made at 42 °C in NaCl buffer as described in Methods. After 2 h further incubation at 42 °C under the conditions described below, the single or mixed suspensions were centrifuged (100 000 × *g* for 30 min) and the pellet transferred to the calorimeter sample pan for analysis. Each incubation mixture contained 4–8 μmoles of total phospholipid in a total volume of 1–2 ml. A. Non-sonicated vesicles of 60 % phosphatidylserine in distearoylphosphatidylcholine incubated in NaCl buffer; (A') sonicated vesicles of 60 % phosphatidylserine in distearoylphosphatidylcholine, incubated in NaCl buffer containing $CaCl_2$ (15 mM) and albumin (0.04 mg/ml) for 2 h. Excess EDTA (20 mM) was added before centrifugation. B. Non-sonicated vesicles of 60 % phosphatidylserine in dimyristoylphosphatidylcholine incubated in NaCl buffer; (B') sonicated vesicles of 60 % phosphatidylserine in dimyristoylphosphatidylcholine, incubated in the presence of Ca^{2+} and albumin and then treated with EDTA as described in (A'). C. Equal amounts of sonicated dispersions of 60 % phosphatidylserine in distearoylphosphatidylcholine and 60 % phosphatidylserine in dimyristoylphosphatidylcholine were incubated together in the presence of Ca^{2+} and albumin and treated with EDTA as described in (A'). D. Equal amounts of the two mixtures 60 % phosphatidylserine in distearoylphosphatidylcholine and 60 % phosphatidylserine in dimyristoylphophatidylcholine mixed in chloroform and suspended in NaCl buffer as in A and B. Marker, 50 μcal/s.

dimyristoylphosphatidylcholine (Fig. 5B') incubated separately in the presence of albumin and $CaCl_2$ and treated with EDTA at the concentrations described above in Fig. 5C indicate similar transition temperatures to the same mixtures prepared without these treatments (Figs 5A and 5B). The similarity between the respective curves indicates that sonication, Ca^{2+} and EDTA treatment have no overall effect on the calorimetric behaviour of the individual mixture.

The same principle as above, of incubating two populations of vesicles with different T_c values, was also used in the following two series of experiments involving neutral or oppositely charged vesicles. Multilamellar vesicles composed of dipalmitoylphosphatidylcholine (Fig. 6A) exhibit a main endothermic peak centering at 42 °C. Similar vesicles composed of distearoylphosphatidylcholine have a main peak at 55 °C (Fig. 6B). These values are in agreement with earlier studies [25]. When these two vesicle populations were incubated together for 20 h at 24 °C (Fig. 6C) and at 58 °C (Fig. 6D), the two main endothermic peaks were still prominent. Molecular mixing of the two lecithins would have resulted in membranes with a T_c at approximately 50 °C, as shown by vesicles made from a mixture of these two lipids in chloroform (Fig. 6E). The increased amount of heat absorbed in the region

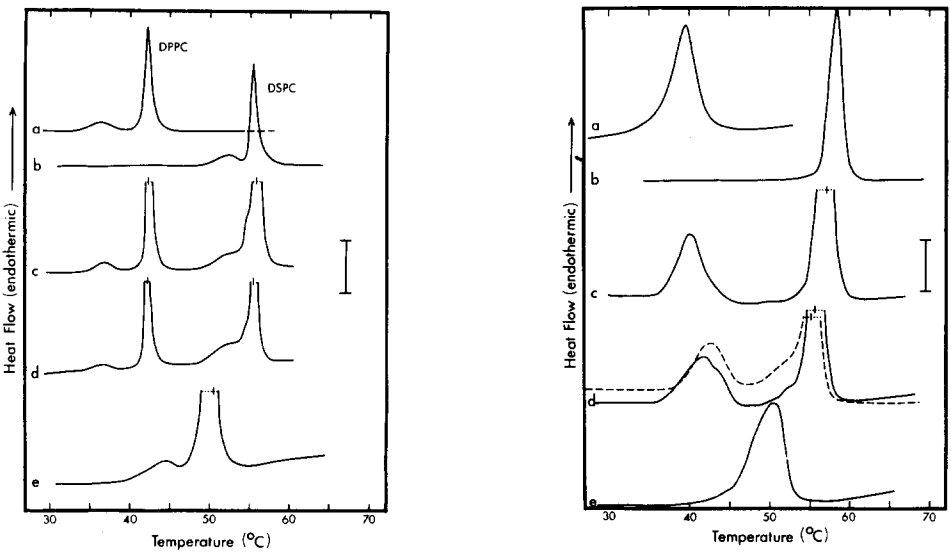


Fig. 6. Differential scanning calorimetry of phosphatidylcholine vesicles. Phospholipid dispersions were made at 45 °C for dipalmitoylphosphatidylcholine (DPPC) (A), and 58 °C for distearoylphosphatidylcholine (DSPC) (B), in NaCl buffer at concentrations of 5 μmoles/ml. Each was incubated at the temperature for 2 h before centrifugation. C. An aliquot of each of the above dispersions containing equal amount of phospholipid was incubated at 24 °C for 2 h, centrifuged at 24 °C as described in Methods, and the pellet placed in the sample pan and incubated further for 20 h at 24 °C. D. Vesicles of distearoylphosphatidylcholine and dipalmitoylphosphatidylcholine were mixed and treated as in C, except that they were incubated at 58 °C for 2 h before and another 20 h after centrifugation. E. Equimolar amounts of dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine were mixed in chloroform and suspended in NaCl buffer at 58 °C as in B. Marker, 100 μcal/s.

Fig. 7. Differential scanning calorimetry of phosphatidylcholine vesicles incorporating positive or negative components. Phospholipid suspensions were made at 60 °C in NaCl buffer, at concentrations of 2 μmoles/ml. A. 20 % palmitoleic acid in dipalmitoylphosphatidylcholine (negatively charged vesicles). B. 20 % stearylamine in distearoylphosphatidylcholine (positively charged vesicles). C. Equimolar amounts of each of the above vesicles, incubated for 2 h at 24 °C. D. Equimolar amounts of each of A and B incubated together for 2 h at 60 °C. The broken line was obtained from the same sample, incubated for 20 h at 60 °C. E. Equimolar amounts of A and B mixed in chloroform and dispersed together in NaCl buffer at 60 °C. All samples were centrifuged at 24 °C following the 2-h incubation period and the pellets used for the calorimetric analysis. Marker: 100 μcal/s.

50–53 °C (Figs 6C and 6D) indicates that a small degree of mixing has taken place especially when the two vesicles were incubated at 58 °C. These results are in agreement with the centrifugation studies indicating that very little fusion occurs under these conditions between vesicles of neutral phosphatidylcholine.

A similar series of experiments were done with populations of negatively charged vesicles (20 mole percent palmitoleic acid in dipalmitoylphosphatidylcholine) (Fig. 7A) and positively charged vesicles (20 mole percent stearylamine in distearoylphosphatidylcholine (Fig. 7B). As can be seen in Fig. 7, incubation of the two types of vesicles for 2 h at 24 °C (Fig. 7C) has no appreciable effect on the two endothermic peaks seen separately in Figs. 7A and 7B. However, following 2 h incubation at 60 °C, each peak becomes broader, with additional shoulders appearing

at temperatures between the two original peaks. Longer incubation (20 h) at this temperature, increases the effect (dotted line) but has no effect at 24 °C. Vesicles composed of the complete mixture of these lipids give a peak centering at 50 °C (Fig. 7E).

If the degree of molecular mixing observed in Figs 6 and 7 is taken as proportional to the extent of fusion between separate vesicles, it can be concluded that only a small percentage of vesicles underwent fusion even after prolonged incubation. Vesicles of opposite charge are in this respect more amenable to fusion than neutral vesicles. Although it is not possible at present to quantitate these results, it is clear that the Ca^{2+}-induced fusion described earlier of negatively charged vesicles is a much more efficient process.

Differential scanning calorimetry: molecular segregation induced by Ca^{2+}

During the course of the above experiments it became important to understand the effect of Ca^{2+} on the melting characteristics of the vesicles used in this study. We have reported previously that the presence of Mg^{2+} increased [22] the T_c of dipalmitoylphosphatidylglycerol by approximately 10 °C and that the addition of Ca^{2+} (more than 1 mM) abolished [15] the phase transition of phosphatidylserine. The effect of Ca^{2+} and Mg^{2+} on the vesicles composed of 60% phosphatidylserine in phosphatidylcholine is shown in Fig. 8. Fig. 8A indicates the endothermic peak of non-sonicated vesicles of 60% phosphatidylserine in distearoylphosphatidylcholine.

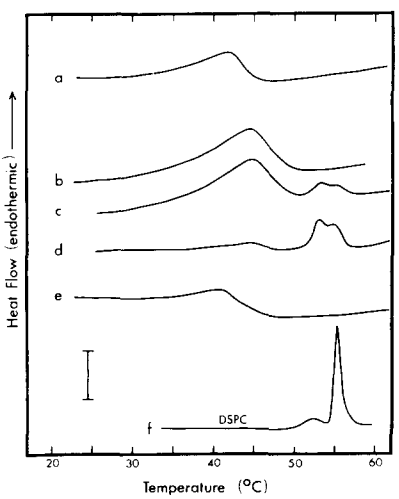


Fig. 8. Differential scanning calorimetry on the effect of Ca^{2+} on mixed lipid membranes. Phospholipid dispersions were made at 42 °C in NaCl buffer at concentrations of 4 μmoles/ml. A. 60% phosphatidylserine in distearoylphosphatidylcholine non-sonicated vesicles. B. 60% phosphatidylserine in distearoylphosphatidylcholine vesicles sonicated for 1 h at 42 °C, then incubated at 52 °C for 2 h in the presence of Ca^{2+} (5 mM). C. 60% phosphatidylserine in distearoylphosphatidylcholine sonicated at 42 °C for 1 h, then incubated at 52 °C for 2 h in the presence of Ca^{2+} (10 mM). D. 66% phosphatidylserine in distearoylphosphatidylcholine sonicated as above, then incubated at 42 °C for 1 h with Ca^{2+} (10 mM). E. 66% phosphatidylserine in distearoylphosphatidylcholine sonicated as above, then incubated at 42 °C for 1 h with Mg^{2+} (10 mM). F. Distearoylphosphatidylcholine suspended and incubated at 58 °C, as in Fig. 6D. Marker: 50 μcal/s.

Fig. 8B shows the calorimetric characteristics of the same vesicles, following sonication and incubation for 2 h at 52 °C in the presence of 5 mM Ca^{2+}. Normally sonication induces a slight shift of the endothermic peak toward lower temperatures. In this case, the peak appears at an approximately 3 °C higher temperature, presumably due to the presence of Ca^{2+}. Fig. 8C shows the results obtained in a similar experiment to Fig. 8B except that the concentration of Ca^{2+} during incubation was increased to 10 mM. The significant result in this case is the appearance of a new double peak at approximately 53 and 55 °C. Fig. 8D was obtained with a 66% phosphatidylserine in distearoylphosphatidylcholine mixture incubated in 10 mM Ca^{2+}. In this case, the double peak at higher temperatures is more pronounced. Not shown here, is a similar experiment as in Figs 8C and 8D, but with a mixture of 50% phosphatidylserine in distearoylphosphatidylcholine. A curve with a broad peak centering at approximately 48.5 °C was obtained, without a high temperature peak.

The above results showing a new peak appearing in the presence of Ca^{2+} suggest that molecular sorting or segregation may be occurring within the vesicles. Fig. 8F shows the calorimetric behaviour of a preparation of pure distearoylphosphatidylcholine vesicles, with a main peak at 55.5 °C and a pre-melt at 52.5 °C. This strongly suggests that the peaks at high temperatures obtained in Figs 8C and 8D represent the melting of pure distearoylphosphatidylcholine domains within the original (phosphatidylserine in distearoylphosphatidylcholine) vesicles.

It is important to note in relation to the mechanism of vesicle fusion that the molecular segregation observed here in Fig. 8 requires a similar Ca^{2+} concentration and occurs at a similar percentage of phosphatidylserine in phosphatidylcholine as the vesicle fusion shown in Figs 1 and 2. It is possible therefore that the two phenomena are related. In any case, the ability of Ca^{2+} to produce molecular sorting and domains within phospholipid membranes is of obvious biological relevance. As shown in Fig. 8E, no molecular segregation was detected under similar conditions in the presence of Mg^{2+}, indicating a significant difference in the possible biological role of these ions. Ca^{2+} and Mg^{2+} differ also in their effects on pure phosphatidylserine and dipalmitoylphosphatidylglycerol membranes. Mg^{2+} increases the T_c by approximately 10 °C (see ref. 22) while Ca^{2+} abolishes the phase transition completely at concentrations greater than 1 mM (see ref. 15). This Ca^{2+} effect is observed at concentrations identical to those causing a large increase in permeability in phosphatidylserine membranes [26, 27] and may be related to the polymeric complex with phosphatidylserine proposed earlier [28].

The effect of membrane fluidity and cholesterol on vesicle fusion

Previous observations in this laboratory on the ability of vesicles prepared from different lipid species to induce cell fusion [15] have indicated that vesicles composed of lipids that were fluid were significantly more effective in promoting fusion than vesicles containing lipids below their T_c. It was considered pertinent therefore to study the possible importance of phospholipid fluidity in fusion between vesicles.

The experiments summarized in Table I were obtained using the ultracentrifugation system described earlier. Populations of large and small vesicles were incubated together at the temperatures and the times shown in Table I. In all cases, the small vesicle population was labelled with [^{3}H]dipalmitoylphosphatidylcholine

TABLE I

THE RECOVERY OF [^{3}H]DIPALMITOYLPHOSPHATIDYLCHOLINE IN A LARGE VESICLE FRACTION (PRECIPITATED BY CENTRIFUGATION) PRODUCED BY FUSION OF SMALL AND LARGE VESICLES OR FUSION OF SMALL VESICLES

Vesicles were prepared and incubated as described in Methods and Fig. 1. PC, phosphatidylcholine from egg yolk; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol. In all experiments except 7 and 8 the large vesicles were incubated with ^{3}H-labelled small (sonicated) vesicles equimolar in phosphate. In Expts 7 and 8 small vesicles only were present. In Expts 9 and 10 DPPG was mixed with equimolar amounts of cholesterol in chloroform and the mixture suspended in NaCl buffer at 42 °C. PC was suspended and sonicated at 24 °C and DPPG at 42 °C.

Expt No.	Sample	Incubation time (h)	$CaCl_2$ (mM)	% ^{3}H counts in precipitate			
				0 °C	24 °C	38 °C	45 °C
1	PC (small)+PC (large)	2	0	–	10	12	
2	PC (small)+DPPC (large)	2	0	6	10	17	17
3	PC (small)+DPPC (large)	20	0	10	21	23	23
4	DPPG (small)+DPPG (large)	2	0	–	42	–	47
5	DPPG (small)+DPPG (large)	2	5	–	66	–	63
6	DPPG (small)+DPPG (large)	2	10	–	87	–	90
7	DPPG (small only)	2	0	–	8	–	11
8	DPPG (small only)	20	0	–	10	–	14
9	DPPG/cholesterol (small) + DPPG/cholesterol (large)	2	0	–	20	–	22
10	DPPG/cholesterol (small)+ DPPG/cholesterol (large)	2	10	–	20	–	20

and the percentage of this label transferred to the large vesicle population was measured as an index of vesicle fusion, as described earlier. The results indicate that there is significantly more fusion between phosphatidylcholine (small) and dipalmitoylphosphatidylcholine (large) vesicles when incubated at temperatures (38 or 45 °C) close to or above the transition temperatures of these lipids than at temperatures (0 or 24 °C) below the T_c (Table I, Expts 1–3). The T_c for dipalmitoylphosphatidylcholine is approximately 42 °C.

The results of Expts 4–6 shown in Table I indicate that there is also considerable fusion between small and large dipalmitoylphosphatidylglycerol vesicles and that this is enhanced in the presence of Ca^{2+}. This result might reflect the inherent instability of the mixture of small and large vesicles due to differences in surface energy. In contrast, incubation of small dipalmitoylphosphatidylglycerol vesicles alone for up to 20 h did not result in a significant percentage of vesicles that could be recovered as a precipitate by centrifugation (Table I, Expts 7 and 8).

When cholesterol was added to large and small dipalmitoylphosphatidylglycerol vesicles in equimolar amounts, the extent of fusion was significantly reduced (Table I, Expts 9 and 10) compared with similar vesicles without cholesterol (Expts 4–6). These results indicate that cholesterol inhibits the fusion of large and small vesicles. This accords with previous findings [15] in which the incorporation of cholesterol into dipalmitoylphosphatidylglycerol vesicles reduced their capacity to induce cell fusion.

The above studies indicate that the fluidity of the vesicle membranes has a significant effect on the rate of fusion between vesicles.

The effect of membrane fluidity was also observed in Figs 6 and 7 where it was shown that very little if any molecular mixing occurs between different vesicles incubated at temperatures below their T_c. Since all the membranes would be solid under these conditions, molecular mixing due to lateral diffusion on the plane of the membranes would be very slow [29, 30]. Therefore in the present experiments, the absence of molecular mixing does not prove that fusion has not taken place. However when the samples shown in Fig. 6C and 7C were examined again after exposure to temperatures above the highest T_c for a few minutes no change in the calorimetric behaviour was observed. If fusion had taken place, a brief exposure to high temperatures should be sufficient to achieve molecular mixing, since lateral diffusion in phospholipid membranes above their T_c is very fast (approx. $1 \cdot 10^{-8}$ cm^2/s or approx. $1 \cdot 10^8$ Å^2/s) [29, 30].

DISCUSSION

The present experiments have demonstrated fusion between phospholipid vesicles. Variation of the chemical composition of the vesicles has permitted investigation of the effect of surface charge and fluidity on the fusion reaction, and the effect of Ca^{2+} on fusion and subsequent molecular mixing of different lipid species within fused vesicles.

The results obtained here indicate that the greatest degree of fusion occurred in the presence of Ca^{2+} between negatively charged vesicles in which the phospholipids were in a "fluid" state. A similar requirement for Ca^{2+} has been demonstrated for optimum fusion of natural membranes [1, 3, 41]. Vesicles composed of neutral phospholipids or of charged lipids that were below their T_c at experimental temperatures showed only a limited capacity to fuse. Similarly, the incorporation of equimolar amounts of cholesterol into vesicles composed of saturated phospholipids significantly reduced their ability to fuse.

These findings on the influence of surface charge, phospholipid fluidity and the effect of cholesterol on the fusion capacity of vesicles agree with previous results from this laboratory on the effect of such factors on the ability of vesicles to induce cell fusion [15]. The present results, together with our previous observations [15], indicate that it is important for membrane lipids to be in a "fluid" condition for fusion to occur. The possible role of membrane fluidity in determining the succesful outcome of the membrane fusion reaction has been discussed in detail elsewhere [3, 15]. An optimum requirement for a "fluid" membrane has also been reported recently for resealing of plasma membrane fragments to form closed vesicles [31]. Similarly, the resealing of erythrocyte ghosts [32] requires incubation at 37 °C, and it is perhaps pertinent that this temperature represents the upper limit for the melting of isolated phospholipids from the erythrocyte membrane [33].

We consider that an accurate definition of fusion between lipid vesicles is possible only if a variety of techniques are used to study their interactions. The combined use of ultracentrifugation, gel filtration, electron microscopy and differential scanning calorimetry in the present experiments has established reasonable evidence in favor of fusion as distinguished from other processes such as vesicle

aggregation or simple diffusion of individual molecules between vesicles, which might account for the association or transfer of labelled lipids between vesicles in certain situations.

In addition to the fusion of lipid vesicles with each other as described here, the capacity of similar vesicles to fuse with the plasma membrane of living cells [34], isolated sarcoplasmic reticulum membranes [35] and artificial black lipid membranes [36] has been proposed recently on the basis of transfer of lipid molecules labelled with radioisotopes [34], spin labels [35] or fluorescent pigments [36] from vesicles into the other membranes. The data in these reports does not, however, permit a clear distinction to be made between the transfer of labelled molecules by simple diffusion or by the incorporation of complete vesicles into the membranes by fusion. However, Grant and McConnell [34] also demonstrated that the transfer of ^{14}C-labelled phospholipids from vesicles into *Acholeplasma laidlawii* membranes was accompanied by a significant decrease in cell buoyant density. This indicates that significant exogenous lipid had been accumulated which is suggestive that fusion, rather than exchange diffusion, was probably the principal mechanism involved. Recent work in this laboratory using mammalian cell cultures has demonstrated similar extensive cellular incorporation of exogenous labelled lipids from vesicles without impairment of cell viability (Papahadjopoulos, D., Mayhew, E. and Poste, G., unpublished).

To the best of our knowledge, fusion occurring between lipid vesicles has not been demonstrated previously. Metcalfe et al. [35] using NMR techniques demonstrated mixing of phospholipids following incubation of saturated and unsaturated lecithin vesicles together. These investigators suggested that the observed mixing resulted from vesicle fusion. However, their data does not allow exclusion of the role of simple diffusion, and exchange of individual lipid molecules between vesicles.

The present experiments have also demonstrated that Ca^{2+} can influence the distribution of lipid species within membranes by inducing segregation of different species to form domains (phase separation). Evidence for Ca^{2+}-induced clustering of lecithin has also been reported recently on the basis of the broadening of ESR spectra of millipore filters coated with phosphatidylserine containing spin-labelled lecithin [37]. The ability of Ca^{2+} to induce molecular segregation of the kind described here is of obvious biological relevance and further studies are required to investigate the ability of Ca^{2+} to induce similar segregation in other phospholipid mixtures.

It is of interest to note that the concentration of Ca^{2+} required to induce segregation, and the ratio of phosphatidylserine in phosphatidylcholine at which segregation occurs, resemble closely the optimum requirements for fusion between vesicles of this composition. It is possible therefore that the two phenomena are related. For example, effective segregation of phosphatidylserine and phosphatidylcholine into domains might allow charged regions of phosphatidylserine in apposed vesicles to preferentially fuse with each other. However, the precise relationship between Ca^{2+}-induced segregation and fusion must await further clarification.

Both these phenomena could be related to the documented effect of Ca^{2+} in causing a decrease in the area per molecule in phosphatidylserine monolayers [28] and an increased permeability in phosphatidylserine vesicles [27, 28]. The latter phenomenon has been shown to be caused by instability of the vesicle membrane

created by the asymmetric distribution of Ca^{2+} across the membrane [27, 38, 39]. It is of considerable interest therefore that independent theoretical considerations of the membrane changes occurring in fusion have attributed a similar role to Ca^{2+} asymmetry across the membrane as the major factor responsible for the transient instability and structural re-organization of the membranes which enable them to fuse [1, 3, 40, 41].

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REFERENCES

1 Poste, G. (1972) Int. Rev. Cytol. 33, 157–252
2 Smith, A. D. (1971) Philos. Trans. R. Soc. London Ser. B 261, 423–437
3 Poste, G. and Allison, A. C. (1973) Biochim. Biophys. Acta, 300, 421–465
4 Novikoff, A. B. (1973) in Lysosomes and Storage Diseases (Hers, H. G. and Van Hoof, F., eds), pp. 1–77, Academic Press, New York
5 Rash, J. E. and Fambrough, D. (1973) Develop. Biol. 30, 166–186
6 Nickel, E. and Potter, L. T. (1971) Philos. Trans. R. Soc. London Ser. B. 261, 383–385
7 Lucy, J. A. (1970) Nature, 227, 815–817
8 Barski, G. (1970) Int. Rev. Exp. Pathol. 9, 151–190
9 Poste, G. (1970) Adv. Virus Res. 16, 303–356
10 Harris, H. (1970) Cell Fusion, Clarendon Press, Oxford
11 Croce, C. M., Sawicki, W., Kritchevsky, D. and Koprowski, H. (1971) Exp. Cell Res. 67, 427–435
12 Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I. and Lucy, J. A. (1972) J. Cell Sci. 10, 769–787
13 Bangham, A. D. (1972) Annu. Rev. Biochem. 41, 753–776
14 Papahadjopoulos, D. (1973) in Form and Function of Phospholipids (Ansell, G. B., Hawthorn, J. N. and Dawson, R. M. C., eds), pp. 143–165, Elsevier, Amsterdam
15 Papahadjopoulos, D., Poste, G. and Schaeffer, B. E. (1973) Biochim. Biophys. Acta 323, 23–42
16 Papahadjopoulos, D. and Miller, N. (1967) Biochim. Biophys. Acta 135, 624–638
17 Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330–348
18 Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965) J. Mol. Biol. 13, 238–252
19 Papahadjopoulos, D. (1970) Biochim. Biophys. Acta 211, 467–477
20 Huang, C. H. (1969) Biochemistry 8, 344–352
21 Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta 266, 561–583
22 Kimelberg, H. K. and Papahadjopoulos, D.(1973) J. Biol. Chem., 249, 1071–1080
23 Brenner, S. and Horne, R. W. (1959) Biochim. Biophys. Acta 34, 103–110
24 Juliano, R. L., Kimelberg, H. K. and Papahadjopoulos, D. (1971) Biochim. Biophys. Acta 241, 894–905
25 Ladbrooke, B. D. and Chapman, D. (1969) Chem. Phys. Lipids 3, 304–367
26 Papahadjopoulos, D. and Bangham, A. D. (1966) Biochim. Biophys. Acta 126, 185–188
27 Papahadjopoulos, D. and Ohki, S. (1969) Science 164, 1075–1077
28 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254
29 McConnell, H. M., Devaux, P. and Scandella, C. (1972) in Membrane Research, 1st I.C.N./U.C.L.A. Symposium on Molecular Biology (Fox, C. F., ed.), pp. 27–37, Academic Press, New York
30 Träuble, H. and Sackmann, E. (1972) J. Am. Chem. Soc. 94, 4499–4512

31 Mason, W. T. and Lee, Y. F. (1972) Nat. New Biol. 244, 143–146
32 Hoffman, J. F., Tosteson, D. C., Whittam, R. (1960) Nature 185, 186–187
33 Jackson, W. M., Kostyla, J., Nordin, J. H. and Brandts, J. F. (1973) Biochemistry 12, 3662–3667
34 Grant, C. W. M. and McConnell, H. M. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1238–1240
35 Metcalfe, J. C., Birdsall, N. J. M. and Lee, A. G. (1972) in Mitochondria-Biomembranes, pp. 197–217, North Holland, Amsterdam
36 Pohl, G. W., Stark, G. and Trissl, H-W. (1973) Biochim. Biophys. Acta 318, 478–481
37 Ohnishi, S-I and Ito, T. (1973) Biochem. Biophys. Res. Commun. 51, 132–138
38 Papahadjopoulos, D. and Ohki, S. (1970) in Symposium on Ordered Fluids and Liquid Crystals (Johnson, J. F. and Porter, R. S., eds), pp. 13–32, Plenum Press, New York
39 Ohki, S. and Papahadjopoulos, D. (1970) in Surface Chemistry of Biological Systems (Blank, M., ed.), pp. 155–174, Plenum Press, New York
40 Poste, G. and Allison, A. C. (1971) J. Theor. Biol. 32, 165–184
41 Poste, G. and Reeve, P. (1972) J. Gen. Virol. 16, 21–28