*Biochimica et Biophysica Acta*, 550 (1979) 157—173


BBA 78236

# FUSION OF PHOSPHATIDIC ACID-PHOSPHATIDYLCHOLINE MIXED LIPID VESICLES

MEI-JUNE LIAO and JAMES H. PRESTEGARD

*Chemistry Department, Yale University, New Haven, CT 06520 (U.S.A.)*

(Received June 19th, 1978)

*Key words: Vesicle fusion; Phosphatidylcholine; NMR; Phosphatidic acid;* $Ca^{2+}$; $^{113}Cd^{2+}$

## Summary

$Ca^{2+}$-induced transformation of phosphatidylcholine-phosphatidic acid vesicles to larger bilayer structures has been examined using nuclear magnetic resonance, electron microscopy, gel permeation and radioisotope tracer techniques. For concentrated vesicle preparations where phosphatidic acid content remains less than 50% of total lipid, transformation to larger well defined unilamellar structures can be induced. The size of the product formed is dependent on phosphatidic acid content and on $Ca^{2+}$ content when $Ca^{2+}$ levels are between 0.3 and 1.0 mol ratios with respect to phosphatidic acid. During transformation bilayer composition remains unchanged and internal contents are retained in the final structure. These properties are indicative of concerted two vesicle and multiple vesicle fusions. The controllable and concerted fusions make the phosphatidic acid system suitable for further mechanistic studies.

## Introduction

Membrane fusion has long been recognized as an important event in a variety of cellular activities [1]. It has more recently become of concern in efforts to use synthetic bilayer membranes as transport vehicles for drugs in chemotherapeutic applications [2]. As a result, a great deal of effort has been placed in the study of compositionally simple, pure lipid systems which exhibit some of the fundamental aspects of membrane-membrane fusion [3—6]. The ultimate goal in studying these systems would be the elucidation at a molecular level of the basic prerequisites for fusions in natural systems. In order to model natural systems closely, these models should not fuse indiscriminately but be controllable. Fusion should entail merging of bilayer lipids in a single continuous step as opposed to exchange of small numbers of lipid molecules during

Abbreviation: MES, 2-(*N*-morpholino)ethanesulfonic acid.

collisions and fusion should result in retention of internal contents of both fusion partners in the final structure.

Most models presently under study fail to satisfy one or more of the above conditions. For example, the homogeneous phosphatidylcholine-free fatty acid vesicle system studied in our laboratory exhibits controlled fusion in that vesicles fuse only when temperature is adjusted to that of the gel to liquid crystalline phase transition, but once initiated, further fusions follow indiscriminately leading to large, poorly defined structures [5]. Vesicle contents are also lost during the process [7].

Model vesicle systems incorporating anionic lipids are more promising in that they exhibit a control mechanism more akin to natural systems where variation of $Ca^{2+}$ concentrations seems to play a role. Some evidence that anionic lipid vesicles undergo controlled, conservative fusion when incubated with whole cells also exists [8]. However, $Ca^{2+}$-induced fusion among anionic lipid vesicles themselves, which is more amenable to study at a molecular level, proceeds uncontrollably to large structures with loss of contents [9]. The fusion process for the pure phosphatidylserine vesicle system has been described as one in which vesicles break open to become disk-like structures before merging to form cochleate cylinders, necessitating loss of contents [10]. Studies on pure phosphatidylglycerol vesicles suggest similar properties for other pure anionic lipid systems [11].

Because the anionic lipid-$Ca^{2+}$ pair seems so promising an analog for natural fusion sites, it seems reasonable not to abandon these systems entirely but to attempt moderation of the less desirable aspects of the transformation process. Phosphatidylcholine vesicles above their phase transition temperature are very stable and insensitive to $Ca^{2+}$ addition. Hence, phosphatidylcholine would seem a suitable moderator. In fact, Verkleij et al. [11] have presented electron microscopy evidence for a mixed phosphatidylcholine-phosphatidylglycerol system which shows formation of smooth large vesicles and not cochleate cylinders on $Ca^{2+}$ addition. We wish to present here an investigation of the structural transformations which occur on addition of $Ca^{2+}$ solutions to the functionally similar phosphatidic acid-phosphatidylcholine mixed lipid system with the object of verifying its suitability as a model for further study of fusion mechanisms. Transformation is qualitatively evaluated using proton magnetic resonance techniques, vesicle sizes are monitored by gel permeation chromatography or electron microscopy, and internal contents are monitored by radioisotope tracer techniques. Results will show that both control of the size of a transformation product and retention of vesicle contents can be achieved with the phosphatidic acid-phosphatidylcholine-$Ca^{2+}$ system.

## Materials and Methods

*Materials.* Phosphatidylcholine was extracted from egg yolks as previously described [12] and phosphatidic acid was synthesized from it by the action of partially purified phospholipase D from cabbage following the procedures of Papahadjopoulos and Miller [13]. The primary exception was that sonicated vesicles rather than ether-water dispersions were used as substrates. The phosphatidic acid was purified by elution on silicic acid with methanol/chloroform

solutions increasing stepwise in methanol content. The pure egg yolk phosphatidic acid gave a single spot at $R_F$ 0.25 ± 0.05 on silica gel thin layer chromatography plates developed with chloroform/methanol/ammonium hydroxide (5 : 3 : 1, v/v). The purity was also checked by $^{31}P$ NMR spectroscopy.

2-(*N*-Morpholino)ethanesulfonic acid (MES), used as a buffer throughout, was purchased from Calbiochem (San Diego, Calif.). Sepharose 2B and Sephadex G-50, used as gel permeation media, were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). [1-$^{14}$C]Tetraethylammonium bromide with an activity of 3.0 mCi/mmol and [U-$^{14}$C]sucrose with an activity of 4.9 mCi/mmol were obtained from New England Nuclear Corporation (Boston, Mass.). CdO, enriched to 96.30 ± 0.05% in $^{113}$Cd was purchased from Oak Ridge National Laboratory (Oak Ridge, Tenn.) and was converted to $CdCl_2$ before use by adding concentrated HCl and drying on a steam bath.

*Vesicle preparation.* Vesicles were prepared to be 6% (W/V) total lipid in a pH 6.0 ± 0.1 buffer containing MES (10 mM), NaCl (100 mM) and $^2H_2O$ (10%). Weighed amounts of phosphatidic acid and phosphatidylcholine were premixed in chloroform and the chloroform was removed under vacuum (12 h) to assure homogeneous composition of vesicles. Molar composition was calculated assuming molecular weights of phosphatidylcholine and phosphatidic acid to be 750 and 664, respectively. The resulting mixtures were dispersed in buffer solution by vortexing and vesicles prepared by sonication with a Branson Model E bath sonicator at temperatures not over 35°C. The pH of the solution was checked and readjusted when necessary to pH 6.0 during sonication to compensate for pH changes due to the ionization of phosphatidic acid. After sonication for 4—5 h, the vesicle solution was centrifuged at 35 000 rev./min (107 000 × *g*) for 30 min at 10 ± 3°C. For some experiments, $Ca^{2+}$ was included in the sonication buffer. In these cases visible amounts of large multilamellar or $Ca^{2+}$ salts were removed by the centrifugation step. The clear homogeneous supernatants were employed in the studies to follow.

Transformation of vesicles was induced by adding solutions prepared from phosphatidylcholine and phosphatidic acid in 1 : 1, 2 : 1, and 4 : 1 mol ratios, to equal volumes of $Ca^{2+}$ solution at concentrations sufficient to achieve final $Ca^{2+}$ : phosphatidic acid mol ratios ranging from 0.2 to 1.2. The samples were typically incubated in a water bath at 37°C for one half hour. A slight molar excess of EDTA was then added.

*Monitoring of transformation.* The progress of transformation was monitored using line widths of $^1H$ NMR spectra. The spectra were taken on a Perkin Elmer R32 Spectrometer equipped with a TT7 Fourier Transform accessory. $^2H_2O$ analogs of the samples described above were used.

Vesicles sizes were determined on Sepharose 2B using a 1.5 × 30 cm column. The packed column was equilibrated with buffer (2 mM MES/1 mM EDTA/ 0.02% $NaN_3$, pH 6.1) for 2 days and saturated with lipids by passing 0.5 ml of a 5% egg yolk phosphatidylcholine vesicle solution. 0.6-ml samples were placed on the column and eluted with the same buffer. The elution volume of the vesicle preparation was determined by differential refractive index measurement using a Waters Associates Differential Refractometer. The column void volume and total volume were taken to be maxima due to a small percentage of

multilamellar product and $^2H_2O$, respectively. Calibration constants were determined in previous studies for similar columns by using egg yolk phosphatidylcholine vesicle preparations and proteins of known dimension as markers. Pure egg yolk phosphatidylcholine sonicates were run periodically on columns to monitor performance. Elution volume of the refractive index maximum was converted to vesicle size using the equation of Ackers [14]. Measurements are reproducible to ±5%.

Vesicle sizes for representative transformed and non-transformed samples were also checked by electron microscopy. Vesicles prepared from phosphatidylcholine and phosphatidic acid in 2 : 1 ratio and the transformation products produced by addition of 10 mM $Ca^{2+}$ as described in the section on vesicle preparation, were frozen in Freon 22 before being stored in liquid nitrogen. Samples were cleaved and replicated with platinum and carbon in a Balzer's BAF 300 Freeze-Etch apparatus. Replicas were cleaned by floating them on a graded series of Clorox solutions followed by distilled water and were picked up on uncoated 200 mesh copper grids. Replicas were examined in a Philips 300 electron microscope.

*Redistribution of $Ca^{2+}$.* Entrapment of $Ca^{2+}$ during transformation was also monitored. A transformed sample having a 2 : 1 phosphatidylcholine-phosphatidic acid ratio was eluted after addition of EDTA from a 1 × 12 cm Sephadex G-50 column to remove the bulk of the calcium ions and then the vesicle-containing fraction loaded onto a Sepharose 2B column and eluted as in the size determinations. Fractions were collected and analyzed for $Ca^{2+}$ by atomic absorption spectroscopy. Each 1.5 ml fraction was diluted with 0.5 ml methanol to insure homogeneity and a 10 λ sample injected into a Perkin-Elmer atomic absorption spectrophotometer (Model 3058). Untreated vesicles, vesicles sonicated in the presence of $Ca^{2+}$ and vesicles treated with $Ca^{2+}$ at a low temperature (10 ± 1°C), where transformation does not occur, were run as controls.

$^{113}Cd$ NMR of vesicles transformed with $Cd^{2+}$ in place of $Ca^{2+}$ was used as a second means of monitoring ion redistribution. Samples 1 ml in volume were typically 15 mM phosphatidic acid with a 1 : 0.7 phosphatidic acid to $Cd^{2+}$ ratio and a 2 : 1 phosphatidylcholine to phosphatidic acid ratio. Spectra were run on an extensively modified Bruker HFX 90 spectrometer in pulse Fourier transform mode at room temperature and 19.96 MHz. An internal deuterium lock ($^2H_2O$) was used for field-frequency stabilization. Approximately 50 000 90°C pulses at 0.2 s intervals were required to achieve adequate signal to noise ratios for enriched 10 mM $^{113}Cd$. Chemical shifts are referenced to 1 M $Cd(ClO_4)_2$ run separately but also locked to an $^2H_2O$ resonance. Shifts are accurate to ±0.5 ppm.

*Retention of composition.* The heterogeneity of both sonicated preparations and transformation products necessitates a post-transformation check for possible redistribution of phosphatidic acid among small and large structures. Composition can be monitored by $^{31}P$ NMR spectroscopy. Samples were prepared in $C^2HCl_3$ in order to avoid difficulties due to differences in relaxation behavior for small vesicle and large multilamellar products. A vesicle solution treated with $Ca^{2+}$ and EDTA was centrifuged as described before. The supernatant was decanted and the precipitate was suspended in distilled water. Both

fractions were dialyzed against distilled water at room temperature for 1 day changing the water every 4—5 hours. The solutions were lyophilized and 1 ml of chloroform (with 50% $C^2HCl_3$) was added. $^{31}P$ NMR spectra were run on the Bruker HFX-90 spectrometer at a $^{31}P$ frequency of 36.4 MHz. The proton resonances were continuously decoupled with a noise-modulated frequency at 10 W of power and 90 MHz. The resulting solutions gave sharp, well resolved resonances for both lipids.

*Retention of contents.* 1.5 ml of phosphatidylcholine-phosphatidic acid 2 : 1, 6% (w/v) vesicle solution was resonicated in the presence of 2.5 $\mu$Ci [1-$^{14}$C]-tetraethylammonium bromide or 5 $\mu$Ci [U-$^{14}$C]sucrose in order to test the vesicles ability to retain internally trapped solutes during fusion. Sonicated samples were loaded into a Sephadex G-50 column of 1 $cm^2 \times 10$ cm and eluted with buffer. The vesicles containing internal [$^{14}$C]tetraethylammonium bromide or [U-$^{14}$C]sucrose were collected at the void volume and divided into two parts, one for a control and the other for vesicle transformation. Transformation was induced as described previously using a $Ca^{2+}$ concentration sufficient to bring the final phosphatidic acid : $Ca^{2+}$ ratio near 1 : 1.

After adding EDTA, the vesicle solution was passed through a Sepharose 2B column, as in the determination of vesicle size, except that the elution profile was determined by phosphate analysis rather than refractive index because of the low vesicle concentrations involved. The phosphate analysis of a 0.3 ml aliquot in each 2 ml fraction was undertaken using the procedure of Yang et al. [15]. The radioactivity of a 1 ml aliquot from each fraction was determined by adding 3 ml Bray's solution and counting in a Packard, Tri-Carb Liquid Scintillation Spectrometer (Model 3320).

## Results

*Size distribution changes.* It is easy to qualitatively verify that vesicle size distributions change when the $Ca^{2+}$ concentration is raised in a preparation of sonicated vesicles. It can be done using proton nuclear magnetic resonance ($^1H$ NMR) techniques which rely on the fact that small unilamellar vesicles (300 Å in diameter) give well resolved spectra with narrow lines, while lines broaden and become more difficult to observe for more extended bilayers [16]. Linewidths, as defined by the weight average of a two component Lorentzian fit, vary linearly with vesicle diameter over the 200—600 Å range for the methylene resonance. Widths for the methyl resonance also vary, but to a lesser extent, making these resonances appear more pronounced for larger vesicles. $^1H$ NMR techniques offer an advantage over other spectroscopic methods in that the aggregation of small vesicles which may occur simultaneously with vesicle transformation has a small influence on the line width. Spectra showing the methylene (1.26 ppm), chain terminal methyl (0.88 ppm) and choline methyl (3.3 ppm) regions of the spectrum before and after addition of 15 mM $Ca^{2+}$ to phosphatidylcholine-phosphatidic acid 2 : 1 vesicles are presented in Fig. 1. Lines broaden significantly but lose little area implying that vesicle size changes occur without proceeding to the large cochleate structures of systems with high anionic lipid content. It is also significant that the added $Ca^{2+}$ can be sequestered by addition of more than equimolar amounts of EDTA

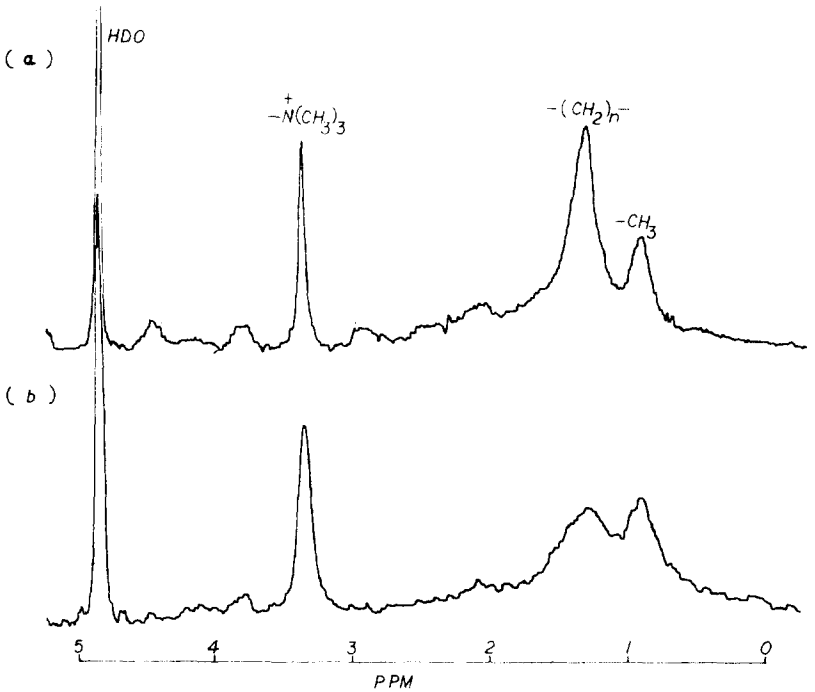



Fig. 1. $^1H$ NMR spectra of 10% phosphatidylcholine-phosphatidic acid 2 : 1 vesicles in $^2H_2O$ buffer at 90 MHz (a) before and (b) after addition of 15 mM $Ca^{2+}$.

without substantial reversal of line width changes. This implies that induced size variations are not easily reversed and $Ca^{2+}$ can be removed without concern for the relevance of more quantitative analytical techniques applied after $Ca^{2+}$ removal.

The time dependence of vesicle transformation was also studied by NMR to evaluate the time span during which further analysis could be undertaken. Data are presented in Fig. 2 for a case in which phosphatidylcholine-phosphatidic

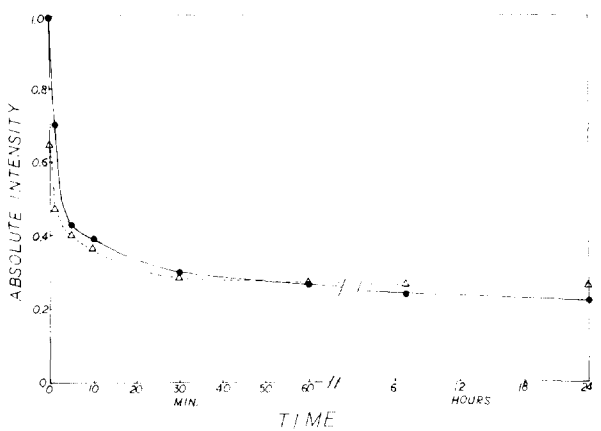



Fig. 2. Absolute intensities of methyl (△) and methylene (●) resonances on $^1H$ NMR spectra vs. time for phosphatidylcholine-phosphatidic acid 2 : 1 vesicles in the presence of 10 mM $Ca^{2+}$ at a 0.7 : 1 $Ca^{2+}$ : phosphatidic acid level.

acid 2 : 1 vesicles were incubated in the presence of 10 mM $Ca^{2+}$ at 35°C for varying lengths of time. Spectra were taken immediately after addition of a slight molar excess of EDTA and changes in amplitudes of methylene and chain terminal methyl resonances were determined. Amplitudes are, to a first approximation, linearly related to the inverse line widths and decrease as vesicle diameter increases. The data appear to show a bimodal process. A rapid change accounting for about 60% of the methylene intensity is over in 5—10 min; a very gradual change continues over the next 24 h. A similar but slightly slower process is noted with 5 mM $Ca^{2+}$. With higher phosphatidylcholine-phosphatidic acid ratios, and with lower temperature, transformation occurs more slowly. For a 4 : 1 system with a 1 : 0.7 phosphatidic acid : $Ca^{2+}$ ratio the rapid phase is over in 20—30 min. Incubation of the 2 : 1 system at 10°C for 30 min shows less than a 10% change in amplitude.

For more quantitative analysis of size distribution changes, gel permeation chromatography was used. $Ca^{2+}$ was sequestered by EDTA addition after 0.5 h incubation and the column run within 48 h. Elution which required about 6 h per sample was monitored with a refractive index detector. Output has been shown to be linear in weight lipid over the accessible size range by comparison to phosphate analysis and therefore the vesicle size indicated by the maximum in the elution profile can be interpreted as the most prbable vesicle size in which a lipid molecule can exist. Only this size is reported.

At low $Ca^{2+}$ levels the distribution of lipids among vesicles of various sizes shifts toward larger sizes as the $Ca^{2+}$ concentration is increased. This is shown in Fig. 3. Little increase in the percentage of lipid at the void volume is noted. This again indicates that the small vesicles are not transforming directly to

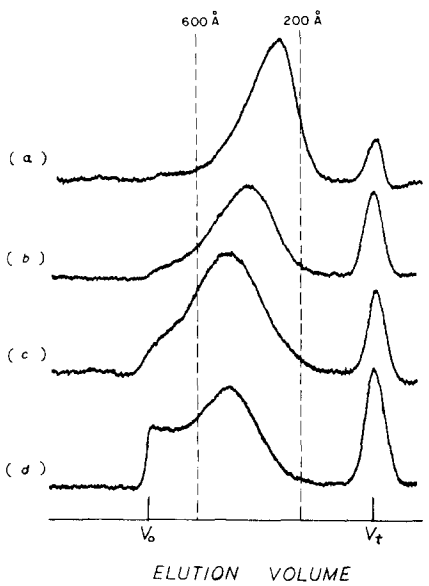
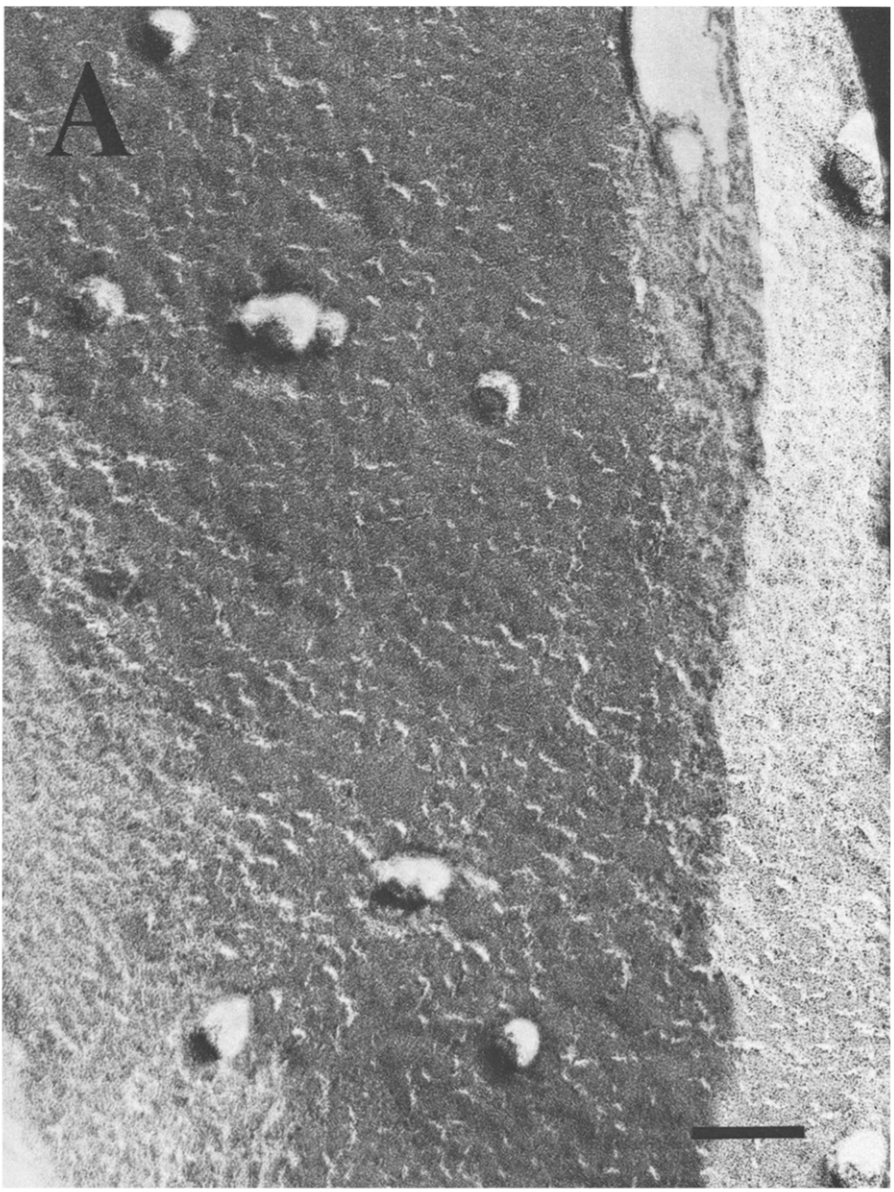



Fig. 3. Elution profiles from Sepharose 2B for 3% phosphatidylcholine-phosphatidic acid 2 : 1 vesicles incubated in the presence of (a) 5 mM $Ca^{2+}$ ($Ca^{2+}$ : phosphatidic acid 0.33 : 1), (b) 7.5 mM $Ca^{2+}$ ($Ca^{2+}$ : phosphatidic acid 0.5 : 1), (c) 10 mM $Ca^{2+}$ ($Ca^{2+}$ : phosphatidic acid 0.66 : 1), (d) 15 mM $Ca^{2+}$ ($Ca^{2+}$ : phosphatidic acid 1 : 1).

multilamellar structures but to unilamellar vesicles of intermediate size. Widths of distributions tend to increase as $Ca^{2+}$ concentration increases but no indication of bimodal distributions between two sizes of small vesicles can be reproducibly resolved. Failure to do this may simply result from the breadth of the initial distribution of sizes and should not be taken as evidence against discrete fusion steps. The second peak at low elution volumes in Fig. 3d does not necessarily indicate a second group of vesicles but can result from the highly non-linear dependence of elution volume on vesicle size near the void volume of the column.



Electron microscope data presented in Fig. 4 confirm observed size distribution changes. The untreated vesicle preparation presented in Fig. 4A shows an average size of 300 ± 50 Å. The 2 : 1 sample transformed with $Ca^{2+}$ at a 0.7 : 1 $Ca^{2+}$ : phosphatidic acid ratio shows an average size of 450 ± 100 Å. Both show the presence of a few multilamellar structures but numbers are not large enough to warrant description as a bimodal distribution.

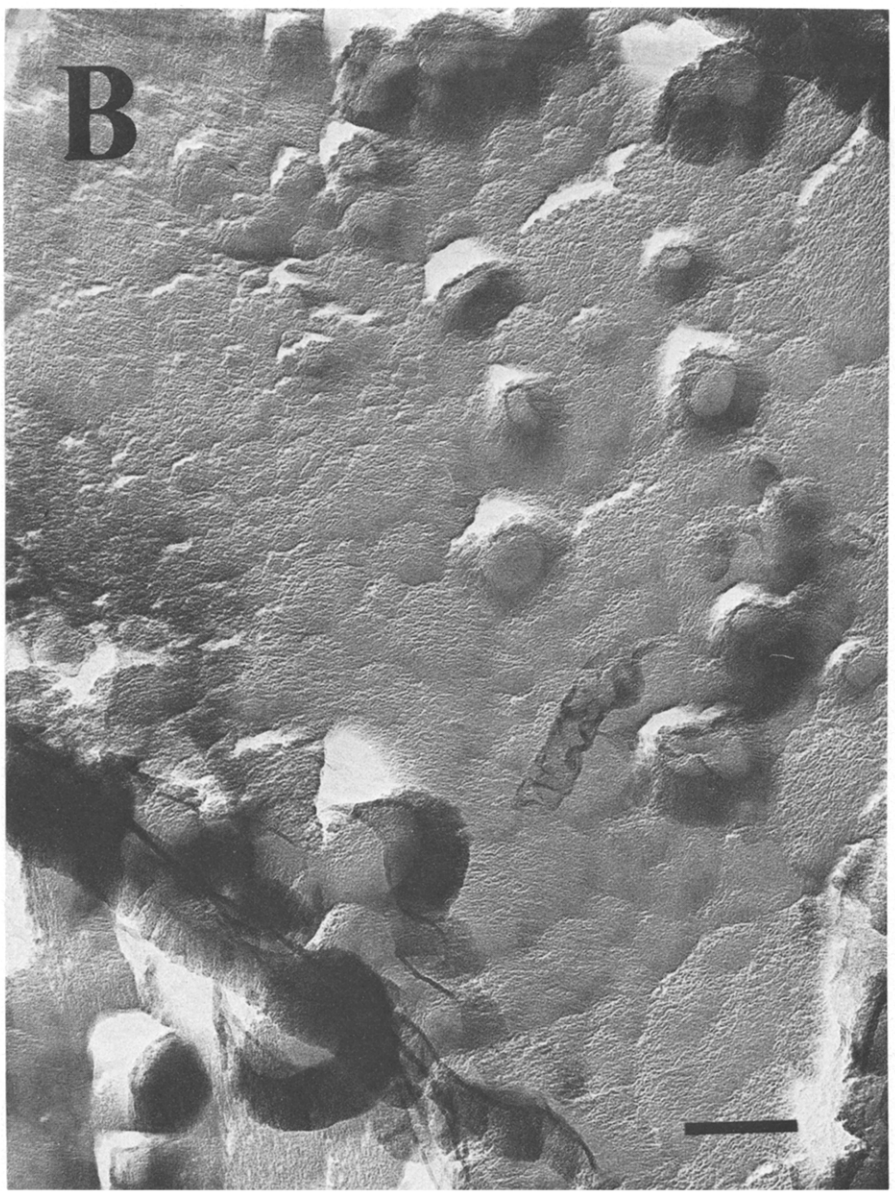



Fig. 4. Freeze-fracture electron micrographs of (A) sonicated phosphatidylcholine-phosphatidic acid 2 : 1 vesicles and (B) the transformation products after incubation in the presence of 10 mM $Ca^{2+}$ for 0.5 h at 37°C and addition of equimolar EDTA. Scale bar = 1000 Å.

As it is obvious that both $Ca^{2+}$ levels and phosphatidic acid levels may be influential in determining ultimate size distributions, the effect of $Ca^{2+}$ addition on samples of three different phosphatidylcholine to phosphatidic acid ratios was studied in detail. Since preparations have slightly different initial sizes (260 ± 10 Å for 4 : 1, 285 ± 10 Å for 2 : 1 and 255 ± 10 Å for 1 : 1), the results are presented in Fig. 5 as a change in vesicle size. The size dependence on $Ca^{2+}$ concentration is, in all cases, sigmoidal. Initiation of size change, and presumably fusion, can be said to correlate with a $Ca^{2+}$ concentration equal to approximately one third of the total phosphatidic acid in the system; 4 mM for the 4 : 1 sample, 5 mM for the 2 : 1 sample and 6 mM for the 1 : 1 sample. The sigmoidal curves plateau near a $Ca^{2+}$ concentration equal to the phosphatidic acid concentration for the 4 : 1 and 2 : 1 samples; 10 mM and 15 mM respectively. A similar phenomenon may occur for the 1 : 1 sample but the ultimate vesicle size cannot be determined on Sepharose because of the extensive profile overlap at void volume for structures above 600 Å in diameter. Between initiation points and plateaus, it seems that transformation may be induced, and ultimate vesicle size controlled, by varying $Ca^{2+}$ concentration.

There is a tendency to shift the limiting size of transformation products to larger values as the mol fraction phosphatidic acid in the vesicle preparation is increased. This tends to extend the range over which size may be controlled. For the 4 : 1 preparation the limiting size at high $Ca^{2+}$ concentration is a

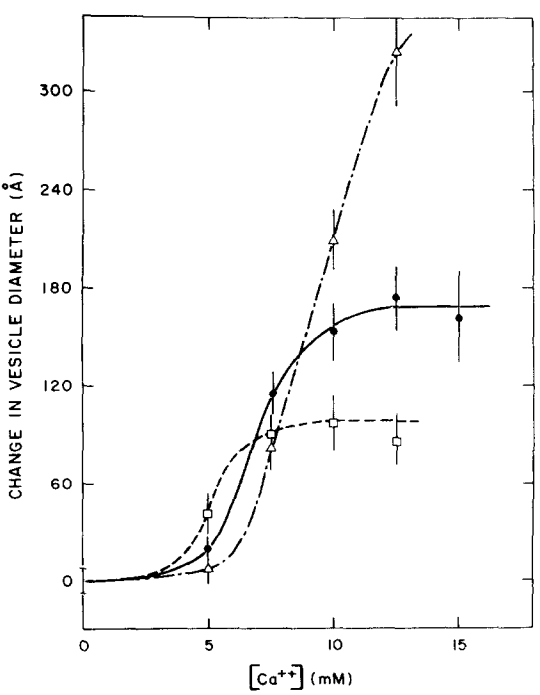



Fig. 5. The effect of $Ca^{2+}$ addition on the change in vesicle sizes of phosphatidylcholine-phosphatidic acid 1 : 1 (△), 2 : 1 (●) and 4 : 1 (□) vesicles. The phosphatidic acid concentration in preparations is 22 mM for 1 : 1, 15 mM for 2 : 1 and 10 mM for 4 : 1. The error bars represent maximum and minimum sizes allowing 10% variation in void volume, total volume and vesicle peaks in elution.

vesicle of 360 Å in diameter, for 2 : 1, 450 Å and for 1 : 1, greater than 580 Å. Induced size changes for the low phosphatidic acid vesicles are such that the predominant process would be consistent with 2 vesicle fusion. For 2 : 1 and 1 : 1 compositions several fusions would have to take place to produce limiting sizes.

It is of interest to compare size distributions to those obtained by sonication in the presence of $Ca^{2+}$. 4 : 1 and 2 : 1 phosphatidylcholine-phosphatidic acid preparations were sonicated in the presence of sufficient $Ca^{2+}$ to maintain a 0.7 : 1 $Ca^{2+}$ : phosphatidic acid ratio. The sizes obtained were 300 ± 10 Å and 350 ± 10 Å for the two preparations. The mere presence of $Ca^{2+}$ does, therefore, lead to increased vesicle size but it is noteworthy that in all cases, size does not reach that obtained by $Ca^{2+}$-induced transformation.

To check the susceptibility of vesicles prepared in the presence of $Ca^{2+}$ to further transformation the 2 : 1 sample was run down a Sephadex column to remove exterior $Ca^{2+}$. The sample was subjected to addition of $Ca^{2+}$ to a level of 0.7 : 1 $Ca^{2+}$ : phosphatidic acid for 30 min at 37°C and the size distribution redetermined. The maximum in the elution profile showed a size of 350 ± 15 Å indicating a lack of susceptibility to transformation despite the fact that vesicles are some 100 Å short of sizes obtained by transformation of $Ca^{2+}$-free preparations.

*Redistribution of calcium and cadmium during transformation.* The above result suggests that internalized $Ca^{2+}$ may influence the course of transformation. Thus, entrapment of $Ca^{2+}$ during transformation was investigated in more detail. Four 2 : 1 samples were prepared; one was sonicated in the presence of enough $Ca^{2+}$ to show a 0.7 : 1 ratio with respect to phosphatidic acid, one was transformed by addition of the same amount of $Ca^{2+}$ at 37°C, one had $Ca^{2+}$ added but was incubated at 10°C where transformation is very slow, and one was retained as an untreated control. EDTA was added to each and each was run down a short Sephadex G-50 column to remove most exterior $Ca^{2+}$. Then each was eluted from Sepharose 2B column and the vesicle maximum fraction analyzed for total phosphate and $Ca^{2+}$ contents. The sonicated sample showed retained $Ca^{2+}$ to be at a 0.12 ± 0.01 : 1 level with respect to total phosphate. The transformed sample retained $Ca^{2+}$ at a 0.10 ± 0.01 : 1 level. The low temperature incubation showed only a small $Ca^{2+}$ retention (0.03 ± 0.01 : 1) and the control showed the $Ca^{2+}$ : total phosphate ratio to be 0.01 ± 0.01. It is clear that transformation entails retention of $Ca^{2+}$ in large amounts and that sonicated vesicles, even though of a size less than the transformation limit, have a $Ca^{2+}$ level consistent with structures at the transformation plateau.

It is most probable that retained $Ca^{2+}$ is internalized within the vesicle structure. This is dramatically demonstrated by examination of the NMR spectrum of $^{113}Cd^{2+}$ used in place of $Ca^{2+}$ to induce vesicle transformation. These two ions are of nearly identical ionic radius, 0.97 Å vs. 0.99 Å, but $^{113}Cd$ offers the well resolved NMR spectrum of a spin one half nucleus of moderately large magnetic moment. That $Cd^{2+}$ substitutes for $Ca^{2+}$ in inducing transformation is confirmed by the fact that vesicle size versus cation concentration curves such as those in Fig. 5 superimpose for the two ions at least for 2 : 1 phosphatidylcholine-phosphatidic acid vesicles.

The $^{113}Cd$ spectrum of a sample of 2 : 1 phosphatidylcholine-phosphatidic

acid fused vesicles is shown in Fig. 6A. Two resonances with a 2 : 1 area ratio are present at +0.8 ppm and −9.5 ppm, respectively. Both are upfield of, and broader than, the $^{113}Cd$ resonance in an equivalent buffer not containing vesicles (+19.2 ppm). This suggests a phosphate interaction. That the downfield resonance can be associated with exterior $Cd^{2+}$ is illustrated by addition of normally impermeant EDTA. This shifts and broadens the downfield resonance leaving the upfield resonance unperturbed (Fig. 6B). The relative areas indicate that about one third the $Cd^{2+}$ is internally confined. This result is consistent with the amount of retained $Ca^{2+}$ in the elution experiments.

*Retention of vesicle composition.* It would be instructive to relate amounts of internalized $Ca^{2+}$ to amounts of phosphatidic acid rather than total phosphate in the vesicles. We could simply assume that phosphatidylcholine-phosphatidic acid ratios do not change during transformation but it is safer to independently determine this ratio in transformation products. Such a determination can also help to distinguish various modes of vesicle transformation. Interaction of $Ca^{2+}$ with vesicle containing negatively charged lipids is known to induce phase separation within lipid bilayers when experiments are conducted under appropriate thermal conditions [9]. If contact transfer between the specific domains were to occur, it is probable that compositions of the larger structures would vary from those of small vesicles. If diffusive transfer were to occur, it is also probable that various lipids would transfer at different rates.

Mol ratios of phosphatidylcholine : phosphatidic acid were determined for two fractions taken from a transformed sample of 2 : 1 initial composition which had $Ca^{2+}$ added to a 1 : 1 $Ca^{2+}$ : phosphatidic acid mol ratio. Isolatable

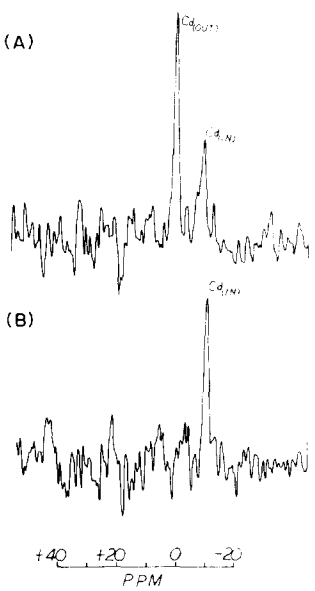



**Fig. 6. $^{113}Cd$ NMR spectra of $Cd^{2+}$ at outer and inner 2 : 1 phosphatidylcholine-phosphatidic acid vesicle surfaces (A) after transformation and (B) after transformation and adddition of equimolar EDTA. Shifts are relative to 1 M $Cd(ClO_4)_2$.**

amounts of multilamellar products are formed under these conditions. The multilamellar fraction can be separated from a vesicle fraction by ultracentrifugation as described in Materials and Methods. $^{31}P$ NMR spectra of the resulting chloroform solution of extracts were examined. The resonance of phosphatidylcholine occurs 0.94 ppm upfield of that in phosphatidic acid where it can be easily integrated. The measured area ratios for the two samples were 2.3 and 2.2, respectively. The small difference which is within limits of error (±5%) indicates no variations in composition between these multilamellar and vesicle fractions. Failing to observe variation at the extremes of the size distribution, it is improbable that composition varies within vesicle fractions of intermediate size. $Ca^{2+}$ to total phosphorus ratios in the previous section can therefore be scaled by a factor of 3 to obtain $Ca^{2+}$ to phosphatidic acid ratios, and no evidence for contact or diffusive transfer is found to exist. An important source of systematic error (due to possible compositional specificities of Sepharose) can also be eliminated from the gel permeation size determinations.

*Retention of internal contents.* A good model for membrane fusion should not only entail merging of bilayers but should also lead to substantial retention of internal contents. It is not at all obvious that one follows from the other. In fact, $Ca^{2+}$-induced transformation of phosphatidylserine vesicles to cochleate cylinders is believed to be accompanied by a substantial increase in permeability to small ions and hydrophylic solutes [9]. We have examined the permeability changes of phosphatidylcholine-phosphatidic acid vesicles undergoing transformation using two normally impermeable carbon-14-labeled solutes: tetraethylammonium bromide and sucrose.

The elution profile from Sepharose 2B for a 2 : 1 vesicle having tetraethylammonium ions entrapped during sonication and most external labeled ions removed by Sephadex treatment is presented in Fig. 7A. Note that curves showing radioisotope tracer and curves showing phosphate content superimpose for the vesicle fraction. The relatively large tracer peak at total volume represents a very low external tracer concentration. It is large only because of the greater than 100 : 1 external to internal volume ratio. If we assume the internal label to be unassociated with the membrane but merely trapped by encapsulation, any breakage of vesicles with a substantial life time or any increase in permeability on $Ca^{2+}$-induced transformation should release the label so that less than 1% remains with the vesicle fraction when eluted on Sepharose. Elution after transformation is induced by adding $Ca^{2+}$ to a 1 : 1 $Ca^{2+}$ : phosphatidic acid mol ratio is presented in Fig. 7B. If transformation is occurring by fusion, size changes observed would require the average vesicle to have undergone more than one fusion. Clearly, the correlation of phosphate and label concentration in the transformation products indicates that more than 85 ± 5% of the label remains confined.

Since tetraethylammonium ion is both positively charged and slightly hydrophobic, there is some concern about the possible association of tetraethylammonium ion with the negatively charged bilayer surface as opposed to uniform distribution throughout the interior volume. Strictly speaking, the isotope profile in Fig. 7A, if indicative of internal volume, should not overlap exactly that of the phosphate analysis. The isotope curve should be skewed slightly to larger volumes as the volume to surface ratio increases with vesicle

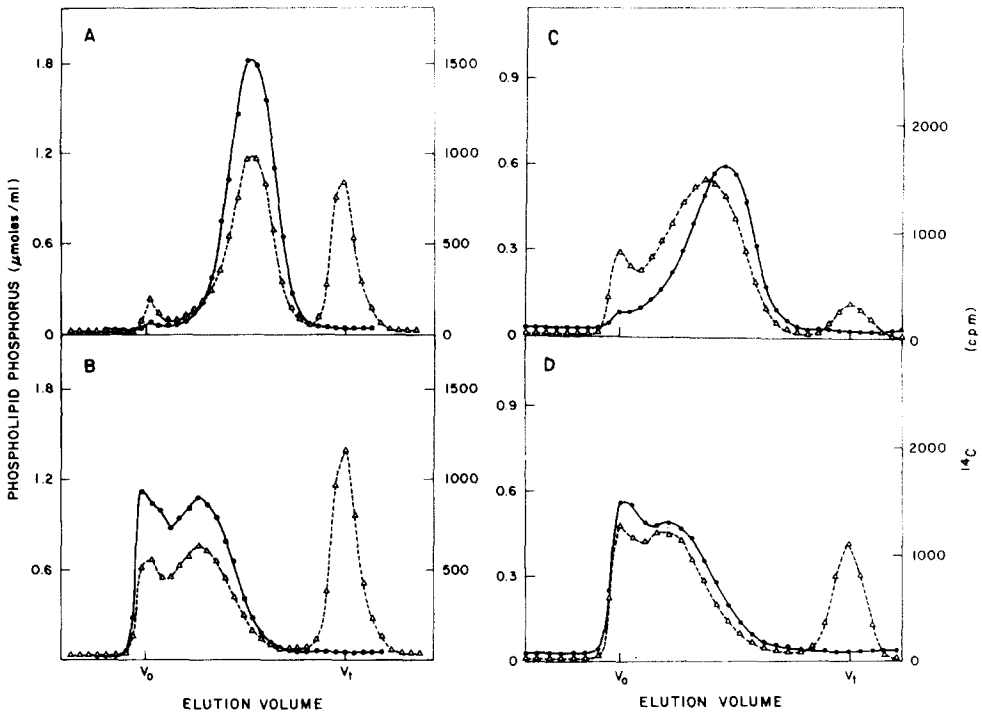



Fig. 7. Elution profiles showing entrapped solutes. 2 : 1 phosphatidylcholine-phospahtidic acid vesicles were eluted from Sepharose 2B. A, [$^{14}$C]tetraethylammonium bromide in the absence of $Ca^{2+}$; B, [$^{14}$C]-tetraethylammonium bromide after addition of a $Ca^{2+}$ to a 1 : 1 phosphatidic acid to $Ca^{2+}$ level; C, [$^{14}$C]sucrose in the absence of $Ca^{2+}$; D, [$^{14}$C]sucrose after the addition of $Ca^{2+}$ to a 1 : 1 level. Solid lines represent the phosphate content and dashed lines internally trapped label content.

size. The skewing in Fig. 7A is not as large as expected. Resonication of non-transformed vesicles after removal of exterior tetraethylammonium bromide on Sephadex also shows some retention of label within the vesicle fraction (6 ± 2%) suggesting some degree of surface binding.

Experiments were therefore repeated using sucrose, a neutral but somewhat larger label. The results are presented in Fig. 7, C and D. The expected skewing is present in the initial preparation and retention of contents after transformation is still greater than 80%. Phosphate and radioisotope curves for the transformation product again overlap closely showing no preference for transformation among small as opposed to large vesicles.

When the size distribution of transformation products is shifted toward larger values by the addition of more $Ca^{2+}$ or the use of lower phosphatidylcholine : phosphatidic acid ratios, leakage becomes more pronounced (approx. 50% for $Ca^{2+}$ : phosphatidylcholine : phosphatidic acid, 1 : 1 : 1). It is possible that the properties of these larger structures are more reminiscent of the phosphatidylserine systems which show increased permeability in the presence of $Ca^{2+}$. The increased permeability may arise from a structural difference or simply from having induced not one but several transformation steps 15—20% being lost at each one.

## Discussion

From the results presented it is clear that the phosphatidylcholine-phosphatidic acid-$Ca^{2+}$ system possesses most properties prerequisite for a model of bilayer fusion. As long as the phosphatidylcholine-phosphatidic acid ratio is kept above 1 : 1 and $Ca^{2+}$ to phosphatidic acid ratio below 1 : 1, transformation stops short of an extended structure of indeterminant dimension. The fact that solutes can be confined internally and the fact that products elute from Sepharose 2B at sizes less than 600 Å suggest the products remain as unilamellar vesicles. The ultimate size distribution of the transformation product can be regulated over the range 250—600 Å diameter by increasing the phosphatidic acid content or $Ca^{2+}$ concentration used in inducing transformation.

The transformation very likely involves merging of bilayers in a single step. This is one of the only conceivable means whereby a change in product composition is not likely and retention of internal contents of fusion partners can occur. Moreover, transformation by merging of bilayers has been demonstrated calorimetrically for the closely related $Ca^{2+}$-phosphatidylserine-phosphatidylcholine system [4]. We therefore feel confident in describing the observed transformations as fusions.

Substantial retention of contents and the small, controllable number of fusions are the main points which distinguish this system from those studied previously. The observation of contents retention does not necessarily contradict demonstrated $Ca^{2+}$-induced leaking in other anionic lipid systems [9]. These systems have in general used anionic lipids other than phosphatidic acid, used anionic lipid to neutral lipid ratios greater than those used here, or operated at $Ca^{2+}$ to anionic lipid levels greater than 1 : 1. Any of these differences may account for the apparent discrepancy.

The molecular mechanism of fusion is of course of primary interest. Open disk-like structures, such as those in the mechanism proposed by Papahadjopoulos, et al. [10] for the phosphatidylserine system, cannot persist in the system discussed here since contents are retained. It is possible, however, that mechanisms differ more in degree than in fundamental nature. Temperature does produce a dramatic effect on fusion rate in both systems, and observed stoichiometry for initiation of fusion is similar.

Assuming that $Ca^{2+}$-phosphatidic acid association constants are high [17—19] and $Ca^{2+}$ only to have access to the outer vesicle surfaces, initiation values in Fig. 3 are indicative of a 0.5 : 1 stoichiometry. Similar stoichiometries have been suggested to initiate cooperative phase transitions in other anionic lipid systems [20]. This transition could play a role in starting fusion in both systems. Existing bilayers must rupture before new junctions can form between fusing vesicles. Such ruptures are probable at phase transitions [7].

Normally ruptures of bilayers would lead to loss of contents. But, if extensive bridging between bilayers preceded rupture, any break may be too small or of too short duration for significant loss of contents in a single fusion event. Bridging of bilayers by $Ca^{2+}$ has been envoked as a primary difference between $Mg^{2+}$ and $Ca^{2+}$ in other anionic lipid systems [21].

Termination of fusion after a small number events is more difficult to

explain than retention of contents. Entrapment of $Ca^{2+}$ on the inside of the vesicle during fusion is, however, the most noticeable factor which could limit numbers of fusions. We have shown that vesicles prepared by sonication in a 0.7 : 1 $Ca^{2+}$-containing solution entrap $Ca^{2+}$ at a level approximately 0.3 : 1 with respect to total phosphatidic acid or 0.9 : 1 with respect to internal phosphatidic acid. These vesicles cannot be induced to fuse despite the fact that their size is below the limiting value. In Fig. 5, limiting vesicle sizes for 4 : 1 and 2 : 1 vesicles are reached at 10 and 15 mM $Ca^{2+}$. These values are consistent with a $Ca^{2+}$ to total phosphatidic acid ratio of 1 : 1, and since we have demonstrated both $Ca^{2+}$ and $Cd^{2+}$ to be internalized on fusion, these values are consistent with an internal $Ca^{2+}$ to internal phosphatidic acid ratio of 1 : 1.

Dependence of fusion product size on the phosphatidic acid content may reflect a limitation to the amount of $Ca^{2+}$ entering the vesicle at each fusion. The amount of internalized $Ca^{2+}$ required to reach a 1 : 1 $Ca^{2+}$ : phosphatidic acid ratio for a 1 : 1 phosphatidylcholine : phosphatidic acid vesicle is clearly larger than that required to reach a 1 : 1 $Ca^{2+}$ : phosphatidic acid ratio in a 2 : 1 vesicle. Therefore, a large number of fusions will occur.

The fact that distribution of $Ca^{2+}$ to the inside of the bilayers reduces susceptibility to fusion has analogs in other systems. For example, increased permeation of black film lipid membranes on addition of $Ca^{2+}$ is known to occur only when $Ca^{2+}$ is added to one side of the structure [22,23]. It is possible that $Ca^{2+}$-induced phase changes on one half of the bilayer (the outer half), but not the other, cause geometric incompatibilities that result in increased permeability in lipid films and external defects which act as fusion sites in vesicles. The role of bilayer defects in stimulating vesicle fusion has been noted in other systems and is worthy of further experimental investigation [24].

## Acknowledgements

This work was supported by grants from the National Institutes of Health, GM19035 and GM22797. We would also like to thank Professor Ian Armitage for assistance in obtaining NMR spectra and Dr. Harry L. Malech and Dr. John Albert for obtaining electron microscope data.

## References

1 Poste, G. and Allison, A.C. (1973) Biochim. Biophys. Acta 300, 421—465
2 Poste, G., Papahadjopoulos, D. and Vail, W.J. (1976) in Methods in Cell Biology (Prescott, D.M., ed.), Vol. 14, pp. 33—71, Academic Press, New York
3 Martin, F.J. and MacDonald, R.C. (1976) Biochemistry 15, 321—327
4 Papahadjopoulos, D., Vail, W.J., Pangborn, W.A. and Poste, G. (1976) Biochim. Biophys. Acta 448, 265—283
5 Kantor, H.L. and Prestegard, J.H. (1975) Biochemistry 14, 1790—1795
6 Day, E.P., Ho, J.T., Kunze, Jr., R.K. and Sun, S.T. (1977) Biochim. Biophys. Acta 470, 503—508
7 Kantor, H.L. and Prestegard, J.H. (1978) Biochemistry 17, 3592—3597
8 Poste, G. and Papahadjopoulos, D. (1976) Proc. Natl. Acad. Sci. U.S. 73, 1603—1607
9 Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579—598
10 Papahadjopoulos, D., Vail, W.J., Jacobson, K. and Poste, G. (1975) Biochim. Biophys. Acta 394, 483—491

11 Verkleij, A.J., de Kruijff, B., Ververgaert, P.H.J.Th., Tocanne, J.F. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 432—437
12 Singleton, M.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53—56
13 Papahadjopoulos, D. and Miller, N. (1967) Biochim. Biophys. Acta 135, 624—638
14 Ackers, G.K. (1967) J. Biol. Chem. 242, 3237—3238
15 Yang, S.F., Freer, S. and Benson, A.A. (1967) J. Biol. Chem. 242, 477—484
16 Gent, M.P.N. and Prestegard, J.H. (1974) Biochemistry 13, 4027—4033
17 Barton, P.G. (1968) J. Biol. Chem. 243, 3884—3890
18 Abramson, M.B., Katzman, R. and Curci, R. (1965) J. Colloid Sci. 20, 777—787
19 Abramson, M.B., Katzman, R., Gregor, H. and Curci, R. (1966) Biochemistry 5, 2207—2213
20 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237—281
21 Pangborn, W.A. and Papahadjopoulos, D. (1978) Abstr. Joint Meet. Biophys. Soc. Am. Phys. Soc., Washington, D.C. p. 194a
22 Papahadjopoulos, D. and Ohki, S. (1969) Science 164, 1075—1077
23 Laclette, J.P. and Montal, M. (1977) Biophys. J. 19, 199—202
24 Lawaczeck, R., Kainosho, M. and Chan, S.I. (1976) Biochim. Biophys. Acta 443, 313—330