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# Spontaneous vesiculation of phospholipids: A simple and quick method of forming unilamellar vesicles

(unsonicated phosphatidic acid dispersion/high-resolution $^1$H NMR)

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**ABSTRACT** **Phosphatidic acid dispersions in $H_2O$ vesiculate when the pH is increased transiently (< 2 min) from ≈3 to 10.5–11. The same phenomenon is observed in mixed dispersions of phosphatidylcholine and phosphatidic acid in $H_2O$ when the pH is increased from ≈3 to 7 to 8. With phosphatidic acid, this treatment produces small closed unilamellar vesicles (200–600 Å) from 50–60% of the total phospholipid, with the remainder present as large unilamellar vesicles and multilamellar structures. The extent of vesiculation depends on the pH that the dispersion is exposed to. With mixed phospholipid dispersions, similar vesicles are obtained; in addition to pH, the extent of vesiculation depends on the phosphatidylcholine/phosphatidic acid (wt/wt) ratio; as this ratio increases, the percentage of small unilamellar vesicles formed decreases. The short exposure of the phospholipids to high pH does not cause lipid degradation, as assessed by thin-layer chromatography; hence, the formation of degradation products can be ruled out as being responsible for the spontaneous vesiculation. Ionization of the phosphate group, resulting in a high surface charge density, may be an important factor in the spontaneous vesiculation. The proportion of phospholipid present as small unilamellar vesicles was determined by gel filtration on Sepharose 4B and by $^1$H NMR. The small vesicles give rise to a reasonably well-resolved high-resolution NMR spectrum. A good correlation was found between the proportion of phospholipid giving rise to the high-resolution spectrum, as derived from spectral intensity measurements, and the proportion present as small unilamellar vesicles, as derived from gel filtration.**

There are two main reasons for the growing interest in spherical unilamellar vesicles* (liposomes): they are extensively used as models for biological membranes and, more recently, they have become potentially important for drug encapsulation. The latter use is based on their ability to enclose water-soluble therapeutic molecules such as drugs and enzymes and to direct these compounds to specific sites in the body with the aim of achieving tissue-specific drug absorption (refs. 1–3 and references cited therein). Several methods have been described for the preparation of small unilamellar vesicles (≈250 Å in diameter) (3). They can be produced by ultrasonic irradiation (4–7); controlled removal of detergent [e.g., bile salts (8–12) or Triton X-100 (13, 14)] from aqueous dispersions of detergent/phospholipid micelles by gel filtration (8–10) or dialysis (11–14); injection of phospholipid solution in ethanol (15), or other solvent, into water and removal of the organic solvent by evaporation (16–19); and extrusion of unsonicated aqueous phospholipid dispersions at high pressure through a French press (20, 21). Other methods produce large unilamellar vesicles. In reverse-phase separation (3, 22), vesicles are formed from water-in-oil emulsions under reduced pressure. When phosphatidylserine is dispersed in $H_2O$, large, probably unilamellar, particles of a wide size distribution form spontaneously (23). Another method of forming large vesicles involves the precipitation of sonicated phosphatidylserine dispersions by $Ca^{2+}$ followed by chelation of the $Ca^{2+}$ with EDTA, as described in refs. 3 and 24.

Here we describe a simple and quick method of preparing unilamellar vesicles of phosphatidic acid. The method is based on transiently increasing the pH of the lipid dispersion so that the phosphate group of the lipid is fully ionized. The procedure is also applicable to mixed dispersions of phosphatidylcholine and phosphatidic acid.

## MATERIALS AND METHODS

Egg phosphatidylcholine and egg phosphatidic acid were purchased from Lipid Products (Surrey, UK). The disodium salts of 1,2-dilauroyl-*sn*-glycerol-3-phosphate and 1-lauroyl-*rac*-glycero-3-phosphate were synthesized according to standard procedures (25). The purity of the lipids was monitored by thin-layer chromatography on silica gel 60 F/25 plates (Merck) using $CHCl_3/CH_3OH/H_2O/HOAc$ (65:50:4:1) (26); 0.3–1.0 mg was applied as a 1-cm band, and all samples were pure except for egg phosphatidic acid, which contained ≈10% lysophosphatidic acid.

Unilamellar vesicles were prepared from phosphatidic acid alone or from phosphatidic acid phosphatidylcholine mixtures. A solution of the phospholipids in $CHCl_3/CH_3OH$ (2:1) was rotary evaporated at reduced pressure in a round-bottom flask. Phospholipid mixtures were readily dispersed in distilled $H_2O$ by whirling mixing at room temperature for 5 min with glass beads. Phosphatidic acid was dispersed in distilled $H_2O$ by both whirling mixing and freeze-thawing. Mixed phospholipid dispersions (pH ≈6) were adjusted to pH 7–8.5 with NaOH, and pure phosphatidic acid dispersions (pH ≈3) were adjusted transiently (<2 min) to pH 10.5–11 by adding NaOH and then to pH 7 to 8 by addition of HCl. NaOH and HCl solutions were added to the lipid dispersions from Agla syringes (Wellcome, Beckenham, UK). Thin-layer chromatography of lipid samples exposed to pH 11 for the short periods of time used showed no degradation.

Gel filtration on Sepharose 4B (46 × 0.9 cm) was carried out as described (9). Ultrasonication was carried out under standard conditions (27) using a Branson B12 sonicator with a microtip. $^1$H NMR spectra were recorded at 90 and 360 MHz using Bruker HXE 90 and HXS 360 Fourier-transform spectrometers, respectively.

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* In this paper, the term vesicle is restricted to closed unilamellar structures.

## RESULTS

The particle size distribution of the phospholipid dispersions was monitored by gel filtration on Sepharose 4B (Fig. 1). The elution pattern of a sonicated phosphatidic acid dispersion, pH 7.6, is shown for comparison in Fig. 1A. Two elution peaks† are apparent: the first (I) elutes at the void volume and consists of particles totally excluded from the internal gel space (diameter, >600 Å); the second (II) elutes at 15–25 ml, consists of vesicles 200–600 Å in diameter, and contains the majority of the material. In distinct contrast to a sonicated dispersion of phosphatidic acid, an unsonicated dispersion, pH 7.6, elutes almost entirely in the void volume (Fig. 1B). However, if before column chromatography the pH of the same unsonicated dispersion is increased transiently to 10.5–11 and then returned to 7.6, the majority of the eluted material is found not in the void volume but in peak II (Fig. 1 C and D). An aliquot of the starting material chromatographed for Fig. 1D was centrifuged (160,000 × g for 3 hr), and the supernatant was then chromatographed (Fig. 1E); this treatment eliminated the large particles eluting in the void volume. Fig. 1F is the elution profile of an unsonicated dispersion of phosphatidylcholine/phosphatidic acid, 14:1 (wt/wt), the pH of which was increased to 8. The eluted material is fairly evenly distributed between peak I and peak II.

$^1$H NMR spectra of unsonicated phosphatidic acid dispersions are shown in Fig. 2. For comparison, the spectrum of a sonicated dispersion of phosphatidic acid in $^2H_2O$ is included (Fig. 2A). By using NaOAc as an internal intensity standard, it can be shown that in sonicated dispersions nearly all of the phosphatidic acid molecules contribute to the high-resolution spectrum (Fig. 2A). This is consistent with the gel filtration results (Fig. 1A), which show that >90% of a sonicated phosphatidic acid dispersion is present in the form of small vesicles. It was shown for egg phosphatidylcholine (29) that there is a good correlation between the fraction of the total lipid giving a high-resolution spectrum and the fraction of the lipid present as small unilamellar vesicles. The $^1$H NMR spectrum of an unsonicated phosphatidic acid dispersion, pH ≈3, is shown in Fig. 2B. No high-resolution spectrum is observed (apparently the signals are too broad to be observable on a 10-ppm scale). However, when the pH of such a sample is increased to ≈8 (Fig. 2C), a high-resolution spectrum is obtained with line widths similar to those of sonicated dispersions. As the pH is increased above 8, the spectral intensity increases (Fig. 2D). How much of the phosphatidic acid in unsonicated dispersions contributes to the high-resolution spectra (Fig. 2) is summarized in Table 1. It is seen that, in unsonicated dispersions transiently exposed to pH 10.5–11, 50–60% of the phosphatidic acid contributes to the high-resolution spectrum. This amount correlates well with the fraction of lipid eluted in peak II (see Fig. 1 C and D and Table 1). In unsonicated mixed dispersions of phosphatidylcholine/phosphatidic acid, 10–50% of the lipid contributes to the high-resolution NMR spectrum; the extent of the contribution depends on the lipid (wt/wt) ratio. With increasing phosphatidylcholine content, the fraction of lipid contributing to the NMR spectrum decreased (Table 1).

The particle size analysis from gel filtration on Sepharose 4B was confirmed by electron microscopy. Two examples are shown in Fig. 3. An electron micrograph of a freeze-fractured preparation of an unsonicated egg phosphatidic acid dispersion after the pH had been increased from 3 to 10.5–11 and returned to 8 is shown in Fig. 3A; the elution profile of such a sample chromatographed on Sepharose 4B is shown in Fig. 1 C and D.

† There is a third minor peak eluting close to the total column volume that is probably due to small micelles of degraded lipid.

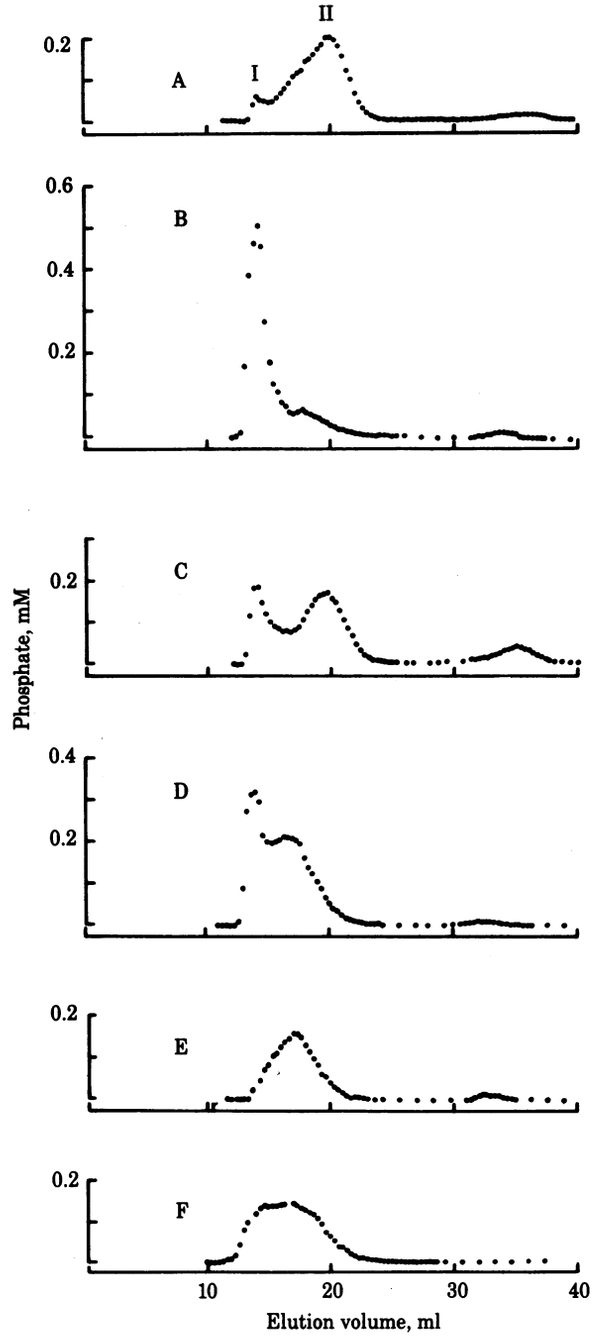


FIG. 1. Elution profiles of various phospholipid dispersions from a Sepharose 4B column. Unsonicated egg phosphatidic acid dispersions in $H_2O$ (pH 3) were prepared first. (A) Sonicated egg phosphatidic acid dispersion. The pH of the unsonicated egg phosphatidic acid dispersion was increased to 7.6 and the dispersion was sonicated for 60 min in the pulsed mode (50% on/off pulse; effective sonication time, 30 min) under nitrogen with ice-water cooling. With increasing sonication time, peak II grew larger at the expense of peak I. A sonicated egg phosphatidylcholine dispersion gave a similar elution profile. (B) Unsonicated egg phosphatidic acid dispersion in $H_2O$, pH 7.6. (C) Unsonicated egg phosphatidic acid dispersion in water after the pH had been transiently increased to 10.6 with NaOH and then returned to 7.6 with HCl (the pH was >7.6 for <2 min). (D) As for C, but a different phosphatidic acid dispersion (the reason for the difference in elution volume of peak II in C and D is uncertain). (E) Supernatant from the dispersion used for D after centrifugation for 3 hr at 160,000 × g. (F) Unsonicated dispersion of egg phosphatidylcholine/phosphatidic acid, 14:1 (wt/wt), in $H_2O$ after the pH had been increased to 8. Samples (0.25 ml) at 10 mg·ml$^{-1}$ were loaded onto the column and eluted with 0.02% $NaN_3$/2 mM Hepes adjusted to pH 7.6 with 2 mM NaOH; the fraction size was 0.3 ml, and the fractions indicated were analyzed for $P_i$ (28).

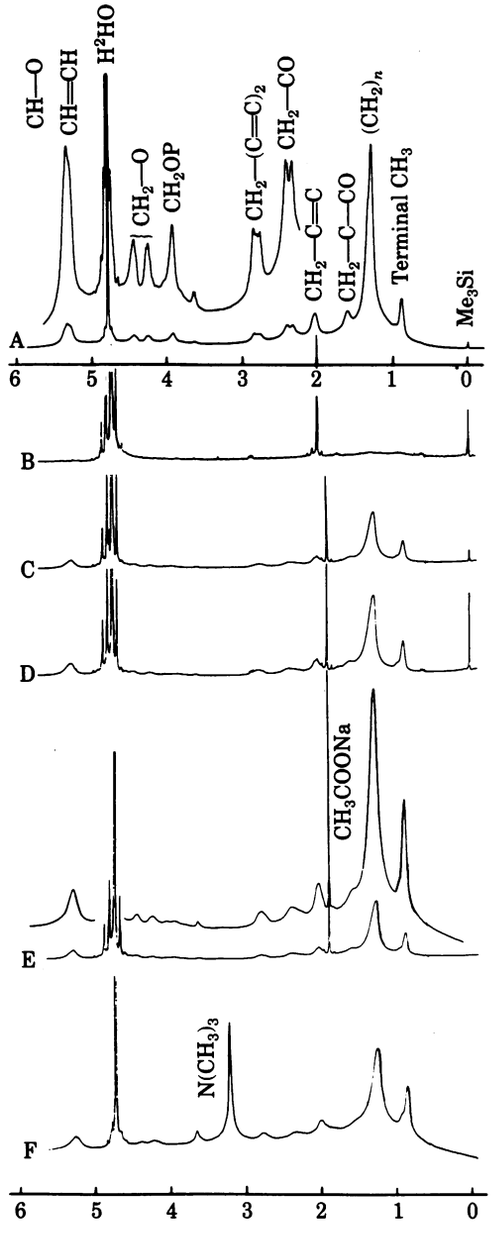


FIG. 2. $^1H$ NMR spectra (360 MHz) of various unsonicated egg phoshatidic acid dispersions (10 mg·ml$^{-1}$ ≈ 0.013 M) in $^2H_2O$. (*A*) For comparison, the spectrum of a sonicated egg phosphatidic acid dispersion in $^2H_2O$, apparent pH 7.5 (14 mg·ml$^{-1}$ ≈ 0.019 M), is shown. Sonication was carried out as described in the legend to Fig. 1. (*B*) Unsonicated dispersion in $^2H_2O$, apparent pH ≈3. (*C*) As for *B*, except that the apparent pH was increased with $NaO^2H$ to 8. (*D*) As for *B*, but the apparent pH was increased to 11. (*E*) As for *D*, but the apparent pH was returned to 8 with $^2HCl$. A vertically expanded version of *E* is also shown. (*B–E*) Signal intensities were determined and normalized against 14.9 mM NaOAc (signal height, 20 cm) as an internal standard. Saturation of the $CH_3$—COONa signal was avoided by using a relaxation time of 25 sec. (*F*) Unsonicated phosphatidylcholine/phosphatidic acid dispersion, 2:1 (wt/wt) in $^2H_2O$; the apparent pH was adjusted to 8.5 with $NaO^2H$. Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl) ($Me_3Si$) propane sulfonate as an internal reference. The assignment is based on published work (29, 30), observed signal intensities, and changes induced by adding $Pr(NO_3)_3$ or by pH titration.

Consistent with the gel filtration pattern, the electron micrograph shows predominantly small unilamellar vesicles of 200–600 Å. This electron micrograph differs from that of the original unsonicated phosphatidic acid dispersion, pH ≈3, which consists almost entirely of large, usually multilamellar, structures (Fig. 3*B*).

## DISCUSSION

The main conclusion from our data is that phosphatidic acid and mixtures with phosphatidylcholine disperse to give a mixture of large particles (peak I, Fig. 1) and small vesicles (peak II). The proportion in each peak is dependent on previous treatment with respect to pH and, for the mixtures, on the lipid ratio (Table 1).

Evidence that small closed single-bilayer vesicles are present in unsonicated dispersions is derived from gel filtration, NMR, and electron microscopy. Gel filtration on Sepharose 4B gives two peaks; one, peak II (Fig. 1), has an elution volume similar to that of sonicated egg phosphatidylcholine dispersions known to consist of small single-bilayer vesicles. The NMR data show that a proportion of the material gives a high-resolution spectrum of line widths similar to those of sonicated dispersions; this proportion is, within experimental error, the same as that eluted in peak II. A similar correlation has previously been found for egg phosphatidylcholine vesicles (29, 32, 33). Further NMR experiments with paramagnetic probes provide evidence that the vesicles are closed; as with sonicated egg phosphatidylcholine vesicles, the addition of paramagnetic ions such as $Pr(NO_3)_3$ and $K_3Fe(CN)_6$ produced a splitting of the polar group signals (data not shown). Increasing the concentration of $Pr(NO_3)_3$ first produced a splitting, due to shifting of approximately two-thirds of each of the polar group signals, and then, at concentrations greater than millimolar, the shifted resonances were broadened until they were eventually lost in the baseline. The induced shifts and broadening are due to shifting or broadening of the resonances from the polar groups on the external surface, which are exposed to the paramagnetic ion, relative to the signals from the polar groups on the surfaces that are inaccessible to it. The invariance of these spectra over several days indicates that the small vesicles are closed and impermeable to these ions.

From thin-layer chromatography analysis, it was found that phosphatidic acid dispersions in $H_2O$ were stable for several hours at pH 11. Therefore, the formation of degradation products during the exposure to high pH can be ruled out as a possible mechanism. However, the egg phosphatidic acid used did contain ≈10% lysophosphatidic acid to start with and the question arises as to whether the diacyl compound or its lyso derivative is the active species. Experiments with phosphatidylcholine and either pure dilauroylphosphatidic acid or 1-lauroyllysophosphatidic acid mixed in an organic solvent and dispersed in $H_2O$ indicate that both compounds are similarly effective in vesiculating multilamellar phosphatidylcholine dispersions. Further, the effectiveness of both compounds is similar to that of egg phosphatidic acid. As to the mechanism of spontaneous vesiculation, preliminary experiments suggest that the ionization of the phosphate group of phosphatidic acid is essential.

It is important to note that maximal vesiculation of phosphatidic acid dispersions requires a higher pH (10.5–11) than phosphatidylcholine/phosphatidic acid mixtures. A possible explanation of this difference is that, in bilayers of pure phosphatidic acid, due to the surface potential, the second pK of phosphatidic acid is significantly higher than that in mixed bilayers with randomly distributed phosphatidic acid. This difference in pH treatment could be important if the procedure were used to assemble proteins into proteoliposomes; exposure of proteins to high pH and high surface charge densities of pure phosphatidic acid bilayers could be detrimental to their activity.

The procedure described here produces small vesicles of var-

Table 1. Fraction of phospholipid giving a high-resolution $^1H$ NMR spectrum

| NMR spectrum* | Sample | Apparent pH | Amount giving a high-resolution $^1H$ NMR spectrum, % | Amount eluted in peak II,† % | Gel filtration experiment† |
|---|---|---|---|---|---|
| *A* | Sonicated phosphatidic acid | 7.5 | 100 | 90 | *A* |
| *B* | Phosphatidic acid | 3 | 2–5 | | |
| *C* | Phosphatidic acid | 8 | 24–46 | | |
| *D* | Phosphatidic acid | 11 | 48–60 | | |
| *E* | Phosphatidic acid | 8‡ | 50–57 | | *C* and *D* |
| *F* | Phosphatidylcholine/ phosphatidic acid (2:1) | 8.5 | 35–45 | | |
| | Phosphatidylcholine/ phosphatidic acid (6:1) | 7 | 13 | | |
| | Phosphatidylcholine/ phosphatidic acid (18:1) | 8.5 | 5 | | |

Unsonicated dispersions of egg phosphatidic acid or mixtures with egg phosphatidylcholine were at 10 mg·ml$^{-1}$ ≈ 0.013 M. pH was adjusted by adding NaO$^2$H or $^2$HCl and the apparent pH in $^2H_2O$ was measured with a combined electrode standardized with aqueous buffers. Intensities of the hydrocarbon chain resonances upfield of the NaOAc signal were measured relative to NaOAc as internal standard. The error of the measurement is estimated to be 10–30%.

* See Fig. 2.

† See Fig. 1.

‡ The sample pH was transiently increased to 11 with NaO$^2$H and then decreased to 8 by adding $^2$HCl.

ious negative surface charge densities. Its advantages are that it is easy, quick, and reproducible; applicable to large volumes and hence large quantities of lipids; and an economic way of producing vesicles requiring only standard laboratory apparatus and avoiding tedious procedures such as sonication or detergent removal. A possible disadvantage is that a mixture of different-sized vesicles, together with some multilamellar structures, is produced. However, the procedure should lend itself well to the encapsulation of drugs, in which a mixed size population may not be a disadvantage. For this purpose, the tendency of the vesicles to aggregate or fuse at high salt concentration would have to be controlled. This tendency is, among other factors, dependent on the phosphatidic acid content, the total lipid concentration, the pH, the ionic strength, and the nature of the ions present.

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1. Gregoriadis, G. (1976) *N. Engl. J. Med.* **295**, 704–710; 765–770.
2. Tyrrell, D. A., Heath, T. D., Colley, C. M. & Ryman, B. E. (1976) *Biochim. Biophys. Acta* **457**, 259–302.
3. Szoka, F. & Papahadjopoulos, D. (1980) *Annu. Rev. Biophys. Bioeng.* **9**, 467–508.
4. Saunders, L., Perrin, J. & Gammack, D. (1962) *J. Pharm. Pharmacol.* **14**, 567–572.
5. Abramson, M. B., Katzman, R. & Gregor, H. P. (1964) *J. Biol. Chem.* **239**, 70–76.
6. Papahadjopoulos, D. & Miller, N. (1967) *Biochim. Biophys. Acta* **135**, 624–638.
7. Huang, C. H. (1964) *Biochemistry* **8**, 344–352.

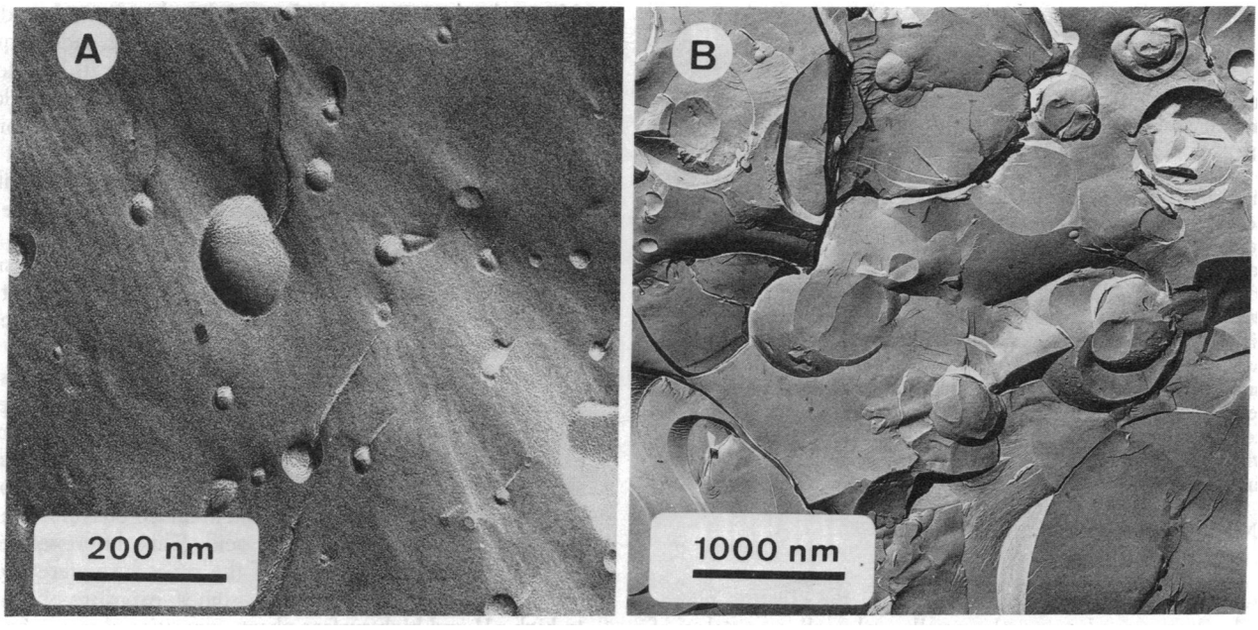


FIG. 3. Freeze-fractured preparations of unsonicated phosphatidic acid dispersions in $^2H_2O$. The samples were prepared for electron microscopy by the method of Müller *et al.* (31). (*A*) pH 8. (*B*) pH ≈3. These are the samples used in Fig. 2 *E* and *B*, respectively.

8. Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem.* **246,** 5477–5487.
9. Brunner, J., Skrabal, P. & Hauser, H. (1976) *Biochim. Biophys. Acta* **455,** 322–331.
10. Enoch, H. G. & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 145–149.
11. Milsmann, M. H. W., Schwendener, R. A. & Weder, H. G. (1978) *Biochim. Biophys. Acta* **512,** 147–155.
12. Zumbuehl, O. & Weder, H. G. (1981) *Biochim. Biophys. Acta* **640,** 252–262.
13. Gerritsen, W. J., Verkley, A. J., Zwaal, R. F. A. & van Deenen, L. L. M. (1978) *Eur. J. Biochem.* **85,** 255–261.
14. Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415,** 29–79.
15. Batzri, S. & Korn, E. D. (1973) *Biochim. Biophys. Acta* **298,** 1015–1019.
16. Chowhan, Z. T., Yotsuyanagi, T. & Higuchi, W. I. (1972) *Biochim. Biophys. Acta* **266,** 320–342.
17. Deamer, D. W. (1978) *Ann. N.Y. Acad. Sci.* **308,** 250–258.
18. Deamer, D. & Bangham, A. D. (1976) *Biochim. Biophys. Acta* **443,** 629–634.
19. Schieren, H., Rudolph, S., Finkelstein, M., Coleman, P. & Weissmann, G. (1978) *Biochim. Biophys. Acta* **542,** 137–153.
20. Hamilton, R. L., Jr., Goerke, J., Guo, S. S., Williams, M. C. & Havel, R. J. (1980) *J. Lipid Res.* **21,** 981–992.
21. Barenholzt, Y., Amselem, S. & Lichtenberg, D. (1979) *FEBS Lett.* **99,** 210–214.
22. Szoka, F., Jr., & Paphadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4194–4198.
23. Hauser, H. & Phillips, M. C. (1973) *J. Biol. Chem.* **248,** 8585–8591.
24. Paphadjopoulos, D., Vail, W. J., Jacobson, K. & Poste, G. (1975) *Biochim. Biophys. Acta* **394,** 483–491.
25. Baer, E. (1963) in *Progress in the Chemistry of Fats and Other Lipids*, eds. Holman, R. T., Lundberg, W. O. & Malkin, T. (Pergamon, London), Vol. 6, pp. 31–86.
26. Hauser, H., Howell, K., Dawson, R. M. C. & Bowyer, D. E. (1980) *Biochim. Biophys. Acta* **602,** 567–577.
27. Brunner, J., Hauser, H. & Semenza, G. (1978) *J. Biol. Chem.* **253,** 7538–7546.
28. Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28,** 1756–1758.
29. Finer, E. G., Flook, A. G. & Hauser, H. (1972) *Biochim. Biophys. Acta* **260,** 49–58.
30. Hauser, H., Phillips, M. C., Levine, B. A. & Williams, R. J. P. (1975) *Eur. J. Biochem.* **58,** 133–144.
31. Müller, M., Meister, N. & Moor, H. (1980) *Mikroskopie* **36,** 129–140.
32. Finer, E. G., Flook, A. G. & Hauser, H. (1972) *Biochim. Biophys. Acta* **260,** 59–69.
33. Penkett, S. A., Flook, A. G. & Chapman, D. (1968) *Chem. Phys. Lipids* **2,** 273–290.