Görrissen, H., Marsh, D., Rietveld, A., & de Kruijff, B. (1986) *Biochemistry 25*, 2904-2910.
Hartl, F.-U., Pfanner, N., Nicholson, D. W., & Neupert, W. (1989) *Biochim. Biophys. Acta 988*, 1-45.
Hauser, H. (1984) *Biochim. Biophys. Acta 772*, 37-50.
Hennig, B., & Neupert, W. (1981) *Eur. J. Biochem. 121*, 203-212.
Hennig, B., & Neupert, W. (1983) *Methods Enzymol. 97*, 261-274.
Hurt, E. C., & van Loon, A. P. G. M. (1986) *Trends Biochem. Sci. (Pers. Ed.) 11*, 204-207.
Jordi, W., Zhou Li Xin, Pilon, M., Demel, R. A., & de Kruijff, B. (1989a) *J. Biol. Chem. 264*, 2292-2301.
Jordi, W., de Kruijff, B., & Marsh, D. (1989b) *Biochemistry 28*, 8998-9005.
Killian, J. A., Burger, K. N. J., & de Kruijff, B. (1987) *Biochim. Biophys. Acta 897*, 269-284.
Lange, A., Marsh, D., Wassmer, K.-H., Meier, P., & Kothe, G. (1985) *Biochemistry 24*, 4383-4392.
Myer, Y. P. (1968) *Biochemistry 7*, 765-776.
Nicholson, D. W., Köhler, H., and Neupert, W. (1987) *Eur. J. Biochem. 164*, 147-157.
Nicholson, D. W., Hergersberg, C., & Neupert, W. (1988) *J. Biol. Chem. 263*, 19034-19042.
Nicolay, K., Timmers, R. J. M., Spoelstra, E., van der Neut, R., Fok, J. J., Huigen, Y. M., Verkley, A. J., & de Kruijff, B. (1984) *Biochim. Biophys. Acta 778*, 359-371.
Ou, W.-J., Ito, A., Umeda, M., Inoue, K., & Omura, T. (1987) *J. Biochem. 103*, 589-595.
Pilon, M., Jordi, W., de Kruijff, B., & Demel, R. A. (1987) *Biochim. Biophys. Acta 466*, 10-22.
Rietveld, A., & de Kruijff, B. (1986) *Biosci. Rep. 6*, 775-782.
Rietveld, A., Sijens, P., Verkleij, A. J., & de Kruijff, B. (1983) *EMBO J. 2*, 907-913.
Rietveld, A., Ponjee, G. A. E., Schiffers, P., Jordi, W., van de Coolwijk, P. J. F. M., Demel, R. A., Marsh, D., & de Kruijff, B. (1985) *Biochim. Biophys. Acta 818*, 398-409.
Rietveld, A. Jordi, W., & de Kruijff, B. (1986a) *J. Biol. Chem. 261*, 3846-3856.
Rietveld, A., Berkhout, T. A., Roenhorst, A., Marsh, D., & de Kruijff, B. (1986b) *Biochim. Biophys. Acta 858*, 38-46.
Roise, D., & Schatz, G. (1988) *J. Biol. Chem. 263*, 4509-4511.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327-1334.
Rouser, G., Fleischer, S., & Yamamota, A. (1970) *Lipids 5*, 494-496.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105-140.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922-3932.
Sixl, F., & Watts, A. (1982) *Biochemistry 21*, 6446-6452.
Smith, M., Leung, D. W., Gillian, S., Astell, C. R., Montomery, D. L., & Hall, B. D. (1979) *Cell 16*, 753-761.
Sperka-Gottlieb, C. D. M., Hermetter, A., Paltauf, F., & Daum, G. (1988) *Biochim. Biophys. Acta 946*, 227-234.
Stellwagen, E., Rysavy, R., & Babul, G. (1972) *J. Biol. Chem. 247*, 8074-8077.
Tamm, L. K. (1986) *Biochemistry 25*, 7470-7476.
Tamm, L., & Seelig, J. (1983) *Biochemistry 22*, 1474-1483.
van Deenen, L. L. M., & de Haas, G. H. (1964) *Adv. Lipid Res. 2*, 168-229.
Verner, K., & Schatz, G. (1988) *Science 241*, 1307-1313.
Zhou Li-Xin, Jordi, W., & de Kruijff, B. (1988) *Biochim. Biophys. Acta 942*, 115-124.

Spontaneous Formation of Small Unilamellar Vesicles by pH Jump: A pH Gradient across the Bilayer Membrane as the Driving Force†

Helmut Hauser,*,‡ Henry H. Mantsch,§ and Hector L. Casal§

Laboratorium für Biochemie, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland, and Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

Received June 23, 1989; Revised Manuscript Received October 5, 1989

ABSTRACT: ^{31}P NMR and infrared spectroscopic methods have been used to study the formation of small unilamellar vesicles by the pH-jump method. It is shown that increasing the pH of different lamellar phospholipid dispersions (phosphatidic acids and phosphatidylserines) induces a pH gradient. This pH gradient is estimated to be 4 ± 1 pH units, and its direction is such that the inner monolayer of the vesicles is at lower pH. There is spectroscopic evidence for tighter packing of the lipid hydrocarbon chains in the inner monolayer, probably due to the constraints imposed by the high curvature of the small vesicles formed. These results are discussed in terms of the driving force of the spontaneous vesiculation.

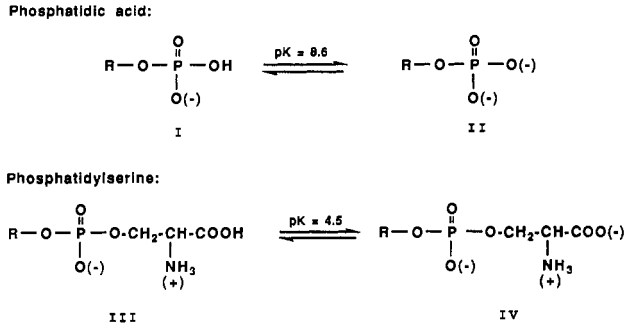
Spontaneous vesiculation is defined as the formation of unilamellar vesicles upon dispersing a dry smectic (lamellar) lipid film in aqueous medium. The term spontaneous implies that vesicle formation occurs without energy being supplied to the dispersion. The phenomenon of spontaneous vesiculation is well documented in the literature (Hauser, 1984, 1985, 1987; Hauser & Gains, 1982; Hauser et al., 1983, 1985, 1986; Haines, 1983; Rydhag et al., 1982; Rydhag & Gabran, 1982; Gains & Hauser, 1983; Aurora et al., 1985; Li & Haines, 1986; Mantelli et al., 1985). A special case of spontaneous vesiculation is the pH-jump or pH-adjustment method originally described by Hauser and Gains (1982).

Raising the pH of a smectic (lamellar) phosphatidic acid dispersion transiently to values between 10 and 12 induces the formation of small unilamellar vesicles of diameter of about 20-60 nm (Hauser & Gains, 1982). Addition of an alkaline solution of pH 10-12 to a dry film of phosphatidic acid deposited on the glass wall of a round-bottom flask has the same effect (Hauser et al., 1983). The alkaline dispersion is then

† Issued as NRCC Publication No. 30762. This work was supported in part by the Swiss National Science Foundation (Grant 3.223-0.85).
‡ Eidgenössische Technische Hochschule.
§ National Research Council of Canada.

0006-2960/90/0429-2321$02.50/0

Scheme I[a]



[a] R = diacyl- or dialkylglycerol; apparent p*K* values are given.

neutralized as quickly as possible in order to prevent lipid degradation. The size of the unilamellar phosphatidic acid vesicles produced by pH jump is similar to that obtained by ultrasonication of phospholipid dispersions (Hauser, 1971; Hauser et al., 1973). The characterization in terms of physicochemical properties of these vesicles has been the subject of recent studies (Hauser & Gains, 1982; Gains & Hauser, 1983; Hauser et al., 1983, 1986). Modification and extensions of the pH-jump method have been described which yield large unilamellar vesicles of diameter larger than 150 nm having a rather homogeneous size distribution (Aurora et al., 1985; Li & Haines, 1986).

The mechanism of the spontaneous formation of small unilamellar vesicles (of diameter smaller than 60 nm) by pH jump is unknown. It is, however, well-known that small unilamellar vesicles of long-chain phosphatidylcholines, i.e., with hydrocarbon chains of 14 and more C atoms, do not form spontaneously. They require the input of energy which is usually supplied in the form of ultrasonic irradiation. It has been pointed out (Hauser, 1971) that the resulting small vesicles are thermodynamically unstable. For instance, small unilamellar vesicles of egg phosphatidylcholine aggregate and fuse at room temperature, forming larger particles including multilamellar liposomes. The formation of small unilamellar vesicles by pH jump was previously defined as spontaneous, i.e., without apparent input of energy. By definition, these vesicles should therefore be thermodynamically stable. That this is not so is the subject of the present work; it is essentially addressed to the mechanism of the formation of small unilamellar vesicles by the pH-jump method. During the pH jump, the pH is raised to 10–12. The effect of this pH increase is to produce a fully ionized phosphate group in those phosphatidic acid molecules exposed to the high pH of the medium (Hauser & Gains, 1982; Hauser, 1989). Two different phospholipids are studied here, phosphatidic acids and phosphatidylserines. Infrared and ^{31}P NMR spectra of these phospholipids show that upon raising the pH to 10–12 a pH gradient is generated across the phospholipid bilayer. In the case of phosphatidic acids, the spectra show that there is coexistence of the monoanionic (I) and dianionic (II) forms while in the case of phosphatidylserines there is coexistence of the neutral or isoelectric (III) and monoanionic (IV) forms as depicted in Scheme I.

Materials and Methods

Egg phosphatidic acid (EPA)[1] and ox brain phosphatidylserine were purchased from Lipid Products (South Nutfield, Surrey, U.K.) and from Avanti Polar Lipids (Birmingham, AL) and used without further purification. 1,2-Dilauroyl-*sn*-phosphatidic acid, 1,2-dimyristoyl-*sn*-phosphatidic acid, 1-lauroyl-*rac*-lysophosphatidic acid (lyso-PA), all as the disodium salts, and 1,2-dihexadecyl-*rac*-glycero-3-phospho-L-serine were synthesized by R. Berchtold (Biochem. Laboratorium, Bern). Cholic acid (highest quality) was purchased from Fluka, Buchs, Switzerland, and Triton X-100 was obtained from Serva, Heidelberg, FRG. Cholic acid was recrystallized several times from acetone/water (4:1 v/v); the sodium salt was made by adding NaOH. Monomethylphosphoric acid [bis(cyclohexylammonium) salt] and *O*-phospho-L-serine were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-L-serine was synthesized as described previously (Hermetter et al., 1982). The phospholipids used in this study were pure by thin-layer chromatographic standard using several solvent systems and by C, H, N, P microanalysis.

Preparation of Phospholipid Dispersions. Unsonicated phospholipid dispersions for ^{31}P NMR measurements were prepared as described by Hauser (1984). Sonication of aqueous phospholipid dispersions was carried out under standard conditions using a Branson B-30 sonicator with a microtip (Hauser, 1971). The purity of phospholipids subjected to sonication was checked by TLC and ^{31}P NMR. In sonicated dispersions of phosphatidic acid, sample degradation was detected sometimes, and these samples were discarded. Unsonicated phospholipid dispersions for infrared measurements were prepared as described previously (Casal & Mantsch, 1984); in all cases, hydration was achieved by adding H_2O or ^2H_2O, by vortexing, and by warming and cooling cycles. The pH was adjusted by adding NaOH (NaO^2H) or HCl (^2HCl).

pH-Jump Method. Spontaneous vesiculation in unsonicated dispersions of phosphatidic acid or phosphatidylserine was induced by the pH-jump method as described in detail before (Hauser & Gains, 1982; Gains & Hauser, 1983; Hauser et al., 1983, 1986). The apparent pH of the phospholipid dispersions was raised transiently in two ways: first, by adding the appropriate amount of NaOH in the form of a 1 M NaOH solution from an Agla syringe to a final pH of 10–12 and immediately neutralizing the alkaline dispersion by adding the appropriate amount of HCl. This procedure was conveniently carried out within 2–3 min. Similar results were obtained by directly dispersing the dry lipid film deposited on the glass wall of a round-bottom flask in the appropriate volume of 1 M NaOH solution to a final pH of 10–12 and neutralizing the alkaline dispersion with HCl.

Phospholipid dispersions for ^{31}P broad-line NMR studies were made in double-distilled H_2O; phospholipids for ^{31}P high-resolution NMR work were dispersed in mixtures of H_2O and ^2H_2O (usually 1:1 by volume) for locking purposes. The apparent pH was the pH meter reading uncorrected for isotope effects.

Proton-decoupled ^{31}P powder NMR spectra were recorded on a Bruker CXP 300 Fourier-transform spectrometer operating at a ^{31}P frequency of 121.47 MHz. Chemical shifts were measured relative to 85% orthophosphoric acid and are expressed as chemical shielding (σ) in ^{31}P powder NMR spectra; i.e., signals upfield with respect to the reference are given positive σ values. Proton-decoupled high-resolution ^{31}P NMR spectra were recorded on a Bruker AC 200 Fourier-transform spectrometer operating at ^{31}P frequencies of 81.02 MHz.

Electron microscopy and gel filtration on Sepharose CL-4B were used to identify the particles present in phospholipid dispersions and to determine the particle size distribution in

[1] Abbreviations: EPA, diacylphosphatidic acid from egg yolk; DHP-SH, 1,2-dihexadecyl-*rac*-glycero-3-phospho-L-serine with a protonated COOH group; lyso-PA, 1-lauroyl-*rac*-lysophosphatidic acid.

these dispersions. Electron microscopy of freeze-fractured samples of phospholipid dispersions was carried out as described previously (Hauser et al., 1977, 1983). Details of the gel filtration method are given elsewhere (Schurtenberger & Hauser, 1984).

Infrared spectra at 2 cm^{-1} resolution were recorded on a Digilab FTS-60 spectrometer equipped with a DTGS detector and purged continuously with dry nitrogen. Usually, 200 interferograms were averaged for each spectrum. The samples, prepared in either H_2O or ^2H_2O, were held in cells of 12-μm path length fitted with CaF_2 or ZnSe windows. The titration curve for lyso-PA (Figure 6B) was constructed as follows: the integrated areas under the PO_2 antisymmetric stretching band (1182 cm^{-1}) and the PO_2 symmetric stretching band (980 cm^{-1}) were normalized to the integrated area under the C-H stretching spectral region (3100-2800 cm^{-1}). This procedure assumes that the integrated intensity of the C-H stretching bands does not change with pH. The integrated area under the C-H stretching bands in the infrared spectra of aqueous micellar dispersions of 1-myristoyl-*sn*-lysophosphatidylcholine was found to be constant over the pH range 3-12. The pH gradient found in the case of EPA was calculated by using the data of this titration curve and taking into account the greater C-H stretching intensity in the spectra of this diacylphosphatidic acid.

Results

^{31}P NMR. The ^{31}P NMR spectrum of an unsonicated EPA dispersion at pH 2-3 consists of a single, broad, asymmetric peak of line width $\Delta\nu_{1/2}$ = 500 Hz rather than the powder pattern typical of unsonicated liquid-crystalline phosphatidate dispersions at neutral pH (Hauser, 1989). Titrating the unsonicated EPA dispersion with NaOH to an apparent pH of 8.5 produces a sharp singlet at about -2 ppm relative to 85% orthophosphoric acid. Upon raising the pH further to 10.5, a doublet is obtained due to part of the resonance at -2 ppm moving further downfield to -4 ppm. The intensity ratio of the downfield to upfield resonances is about 2. When sodium cholate was added to this dispersion to a final concentration of about 2%, the doublet collapsed to a singlet which resonates at the downfield position of the original doublet. The cholate concentration was chosen such that phospholipid bilayer vesicles are solubilized to mixed micelles. The effect of the solubilization by sodium cholate is that all phosphate groups become exposed to pH 10.5 and are therefore shifted downfield. On the basis of the intensity ratio of 2 and the cholate experiment described above, the downfield signal of the doublet can be assigned to molecules located in the outer monolayer and the upfield signal of the doublet to molecules located in the inner monolayer of the EPA bilayer. After back-titration of the alkaline dispersion of EPA to neutrality, a ^{31}P NMR spectrum consisting of a sharp singlet is observed at -0.5 ppm, very similar to that observed at pH 8.5. Reducing the pH of the EPA dispersion further to $\simeq$2 had little effect: a sharp singlet is retained. Upon addition of NaOH to the sample and raising the pH again to 10-11, a doublet is observed identical with that described above, indicating that the sequence of spectral changes is reversible. The pH-jump treatment of unsonicated EPA dispersions apparently generates high-resolution ^{31}P NMR spectra. The line width is similar to ^{31}P high-resolution NMR spectra obtained with sonicated phosphatidylcholine dispersions. This is demonstrated in Figure 1. The ^{31}P NMR spectrum of a sonicated dispersion of the disodium salt of dilauroyl-*sn*-phosphatidic acid in water is shown in Figure 1A. Freeze-fracture electron microscopy and ^1H high-resolution NMR applied to these dispersions reveal

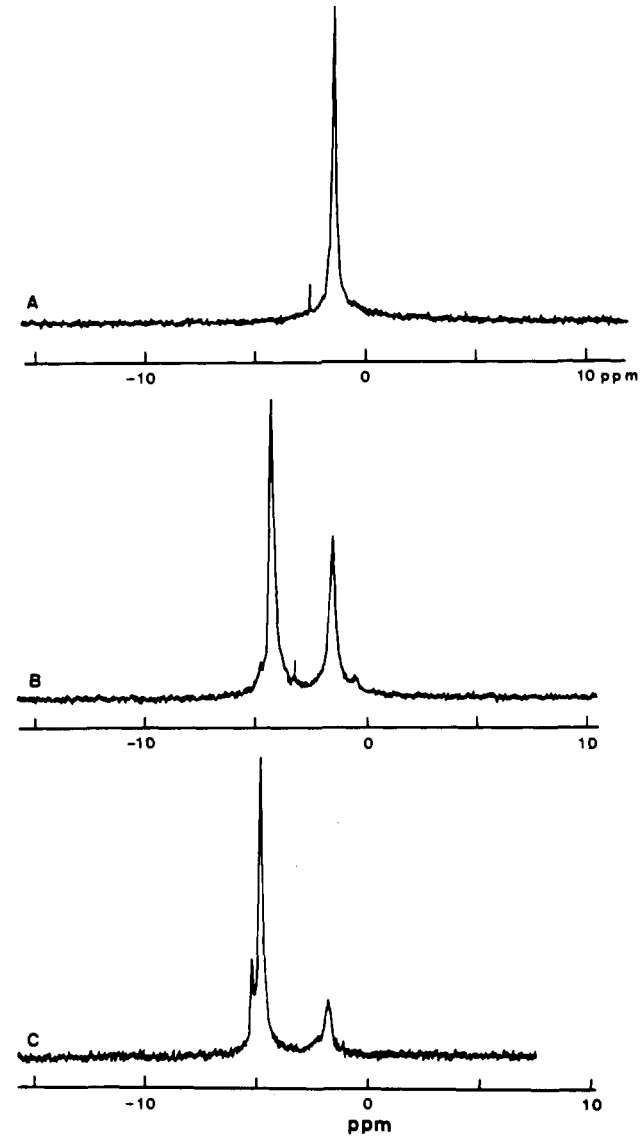


FIGURE 1: Proton-decoupled ^{31}P high-resolution NMR spectra of aqueous phospholipid dispersions recorded at 24 °C and 81.015 MHz on a Bruker AC200 NMR spectrometer. (A) Sonicated dispersion of the disodium salt of 1,2-dilauroyl-*sn*-phosphatidic acid in $H_2O/^2H_2O$ (1:1 v/v), apparent pH 7.7, at 100 mg/mL = 0.17 M. (B) To sample A, 1 M NaOH was added to a final pH of 10.9. (C) Unsonicated dispersion of EPA in $H_2O/^2H_2O$ (1:1 v/v) at 100 mg/mL = 0.15 M adjusted to pH 11.7 by adding NaOH.

the presence of small particles, probably vesicles of diameter 20-60 nm, and some even smaller particles which could be micelles. The ^{31}P NMR spectrum consists of a sharp singlet and is representative for sonicated aqueous dispersions of synthetic and naturally occurring phosphatidic acids such as EPA. The chemical shielding of the singlet (Figure 1A) is -1.4 ppm relative to 85% orthophosphoric acid. The ^{31}P NMR spectrum of the same sonicated dilauroyl-*sn*-phosphatidic acid dispersion after addition of NaOH to pH 10.9 is shown in Figure 1B. The main signal corresponding to phosphatidic acid molecules located in the external layer of the bilayer vesicle is at -4.25 ppm, the minor one corresponding to phosphatidic acid molecules on the inner monolayer is at -1.5 ppm, and the signal intensity is in the ratio 2:1 as expected for small unilamellar vesicles. Titration curves reported previously (Hauser, 1989) relate chemical shielding to the apparent pH of aqueous phosphatidic acid dispersions. According to these titration curves, the chemical shielding of -4.25 ppm corresponds to a pH of 11.5; that of -1.5 ppm of the internal signal corresponds to a pH of 8.0. These values

are in good agreement with the measured pH values. Figure 1C shows the ^{31}P high-resolution NMR spectrum of an unsonicated EPA dispersion subjected to the pH-ump treatment. The spectrum was recorded right after dispersing the dry EPA film in dilute NaOH of an apparent pH 11.7. As expected from the discussion above, the pH-jump treatment produces a good ^{31}P high-resolution NMR spectrum which has similar features as the spectrum shown in Figure 1B. It consists of two sharp signals, a major one at −4.5 ppm and a minor one at −1.5 ppm. The intensity ratio of the two signals is about 2.8:1. Chemical shielding is converted to the apparent pH by using the titration curves. The two resonances assigned to external and internal phosphatidic acid molecules correspond to pH values of 12 and 8, respectively: this means that a pH gradient of ~4 units is generated across the phosphatidic acid bilayer. Upon addition of sufficient HCl to bring the pH of the EPA dispersion to pH $\simeq$ 8, the external signal moves upfield as expected from the titration curve (Hauser, 1989), but it remains separated from the minor signal arising from internal phosphatidic acid molecules.

The pH-jump method was subsequently applied to aqueous dispersions of synthetic phosphatidic acids. Its effect is followed by recording ^{31}P NMR spectra. An unsonicated aqueous dispersion of the disodium salt of dilauroyl-*sn*-phosphatidic acid at neutral pH gives an axially symmetric ^{31}P powder NMR spectrum characteristic of liquid-crystalline bilayers with rapid axial averaging. Superimposed on the powder spectrum is a small isotropic signal at −1.4 ppm (Figure 2A). The width of the ^{31}P NMR spectrum is given by $|\Delta\sigma| = |\sigma_{\parallel} - \sigma_{\perp}| \simeq 45$ ppm which is also a measure of the anisotropy of motion of the phosphate group in dilauroyl-*sn*-phosphatidic acid bilayers. The effect of raising the pH transiently to 12 is shown in Figure 2B. Apparently, the isotropic signal grows at the expense of the axially symmetric powder pattern. Integration gives an approximate estimate of the intensity distribution: the isotropic signal arising from the spontaneous formation of small unilamellar vesicles amounts to ~35%. Furthermore, the isotropic signal shows splitting or at least a clearly discernible shoulder on the high-field side. This is consistent with the data shown in Figure 1. As discussed for this figure, the splitting of the isotropic signal indicates the formation of small unilamellar vesicles in the presence of a pH gradient across the phosphatidic acid bilayer. After the pH of the dilauroyl-*sn*-phosphatidic acid dispersion was raised to pH 12, the pH was brought back to 7.2 by adding HCl. The spectrum recorded from this dispersion is shown in Figure 2C. A single isotropic signal at the chemical shielding position of the shoulder in Figure 2B (see arrow) is retained, and this singlet is superimposed on the axially symmetric powder spectrum. The chemical shielding anisotropy $|\Delta\sigma| = 48$ ppm is similar to that observed in the original dispersion at pH 7 (Figure 2A). The intensity of the isotropic signal is reduced to ~25%, indicating that some of the unilamellar vesicles generated by raising the pH to 12 undergo aggregation/fusion when the pH is returned to neutrality.

The result of the pH-jump treatment applied to unsonicated dispersions of the disodium salt of dimyristoyl-*sn*-phosphatidic acid is quite different from that of the dilauroyl analogue. Unsonicated, aqueous dispersions of dimyristoyl-*sn*-phosphatidic acid at neutral pH give rise to an axially symmetric ^{31}P NMR powder pattern characteristic of liquid-crystalline bilayers. The chemical shielding anisotropy varies between 47 and 54 ppm. In contrast to the dilauroyl analogue, upon raising the pH to ~12, the ^{31}P powder pattern collapses to

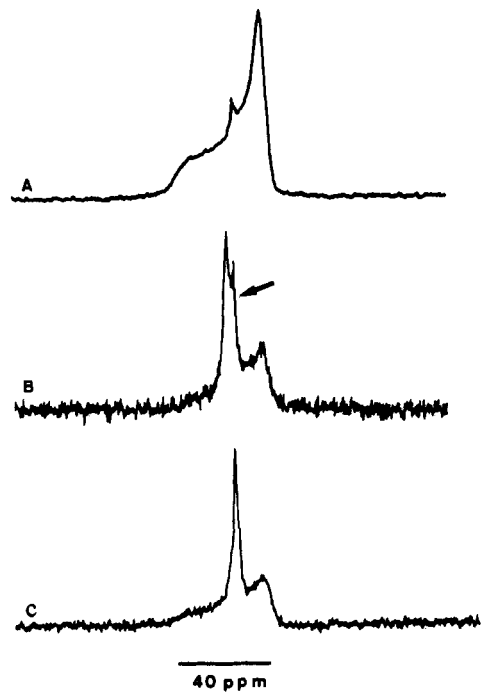


FIGURE 2: Proton-decoupled ^{31}P powder NMR spectra of unsonicated, aqueous dispersions of the disodium salt of 1,2-dilauroyl-*sn*-phosphatidic acid subjected to the pH-jump method. (A) The original dispersion in H_2O, apparent pH ~7, at 100 mg/mL = 0.17 M. (B) The pH of the dispersion (A) was adjusted to 11.9 by adding NaOH. (C) The pH of the dispersion (B) was brought back to neutrality (pH 7.2) by adding HCl. ^{31}P powder NMR spectra were recorded at 121.47 MHz and 25 °C on a Bruker CXP 300 NMR spectrometer. The chemical shielding (σ) was referenced to 85% orthophosphoric acid.

a sharp singlet, and when the pH of the dimyristoyl-*sn*-phosphatidic acid dispersion is returned to pH 8.1, it gives essentially an axially symmetric powder pattern of $|\Delta\sigma| = 55$ ppm (data not shown). This sequence of spectral changes indicates that the small particles undergoing isotropic motion and responsible for the complete averaging of the chemical shielding tensor at high pH are not stable at neutral pH. Upon returning to pH $\simeq$7, they undergo aggregation and/or fusion, giving rise to particles with a diameter greater than $\approx$100 nm. These particles allow only partial averaging of the chemical shielding tensor giving rise to axially symmetric powder patterns.

If the pH gradient is the driving force of the spontaneous formation of small unilamellar vesicles, then this procedure should not be restricted to phosphatidic acid bilayers. To test the general applicability of pH gradients as the driving force inducing spontaneous vesiculation, phosphatidylserine dispersions were subjected to the pH-jump treatment. The effect of this treatment on unsonicated dispersions of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (NH_4^+ salt) is shown in Figure 3. Unsonicated aqueous dispersions of this lipid at pH 6.9 give essentially an axially symmetric powder pattern characterized by a chemical shielding anisotropy of $|\Delta\sigma| = |\sigma_{\parallel} - \sigma_{\perp}| \simeq 80$ ppm. Superimposed on the powder spectrum is a small isotropic component at ~0 ppm (Figure 3A). Upon raising the pH to ~12 by adding NaOH, the powder pattern collapses to a sharp singlet at 0 ppm (Figure 3B). Visually, the milky dispersion of unsonicated dipalmitoylphosphatidylserine clarifies upon raising the pH to ~12. Similarly, when crystals of the acidic form of dipalmitoylphosphatidylserine are dispersed in H_2O, addition of NaOH to an apparent pH of 12.3 causes the crystals to disperse to a translucent suspension. The ^{31}P NMR spectrum of such a

FIGURE 3: Proton-decoupled ^{31}P powder NMR spectra of unsonicated, aqueous dispersions of the NH_4^+ salt of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine recorded at 42 °C on a Bruker CXP 300 NMR spectrometer operating at 121.47 MHz. (A) ^{31}P powder pattern of an unsonicated dispersion (100 mg/mL = 0.133 M) in $H_2O/^2H_2O$ (1:1 v/v), apparent pH 6.9. (B) The above dispersion was made alkaline, pH $\simeq$12, by adding NaOH. (C) Crystals of the protonated form of the same phospholipid were dispersed at 100 mg/mL (0.133 M) in $H_2O/^2H_2O$ (1:1 v/v) at 70 °C to an apparent pH of 3.7. The pH of this suspension was adjusted to 11.2 by adding NaOH, and the spectrum was recorded. (D) The same dispersion as described under (C) after returning the pH to 6.8.

suspension consists of a sharp signal with a shoulder at high field (Figure 3C). The sharp singlets observed at alkaline pH (Figure 3B,C) are true high-resolution spectra, indicating that the pH treatment generates small particles. When a ^{31}P NMR spectrum of the dispersion discussed in Figure 3C is recorded in a high-resolution NMR spectrometer at 36.43 MHz, the sharp singlet has a line width at half-height of 33 Hz. The Brownian tumbling of these particles together with internal modes of motion is apparently such that the chemical shielding tensor is completely averaged out. When the pH of the dispersion is brought back to neutrality, the ^{31}P high-resolution spectrum is essentially retained (Figure 3D). Sometimes, as shown in Figure 3D, a small contribution from a powder spectrum is observed. This indicates that the small particles

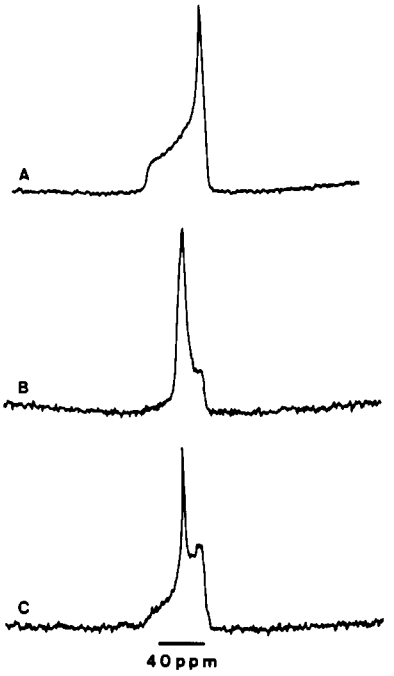


FIGURE 4: Proton-decoupled ^{31}P powder NMR spectra of unsonicated, aqueous dispersions of the sodium salt of ox brain phosphatidylserine recorded at 25 °C on a CXP 300 NMR spectrometer operating at 121.47 MHz. (A) Ox brain phosphatidylserine in H_2O at 100 mg/mL = 0.12 M, apparent pH 7.3. (B) The above dispersion was made alkaline, pH $\simeq$12.9, by adding NaOH. (C) The dispersion described under (B) was titrated back to pH 7.4 by adding HCl.

produced by the pH-jump treatment are stable upon returning the pH to neutrality.

When ox brain phosphatidylserine was subjected to pH treatment at room temperature, it behaved differently. The original ^{31}P powder NMR spectrum characteristic of liquid-crystalline bilayers is shown in Figure 4A. Its chemical shielding anisotropy is $|\Delta\sigma|$ = 52 ppm. Upon raising the pH to 11–12, partial formation of unilamellar vesicles is induced as evident from the ^{31}P NMR spectrum in Figure 4B. A broad isotropic peak is observed indicative of the presence of relatively small particles, but there is still a significant contribution from an axially symmetric ^{31}P powder pattern similar to the original spectrum shown in Figure 4A. Integration provides a crude estimate of the intensity distribution: the isotropic signal amounts to 35–65%. Upon returning the pH to neutrality, the spectrum in Figure 4C is obtained. The ^{31}P NMR spectrum thus obtained is essentially an axially symmetric powder pattern with a chemical shielding anisotropy $|\Delta\sigma|$ = 50 ppm and with a minor isotropic component at ~0 ppm superimposed on it. Integration shows that the isotropic component amounts to ~10–15%. This indicates that there are morphological changes induced by neutralizing the alkaline ox brain phosphatidylserine dispersion.

Infrared Spectroscopy. In order to use infrared spectroscopy to detect a pH gradient across a phosphatidic acid bilayer, characteristic bands of the monoanionic (structure I, Scheme I) and dianionic (structure II, Scheme I) forms of the phosphate group must be selected. Infrared spectra of the disodium salt of monomethyl phosphate were recorded as a function of pH in order to establish pH-induced changes in infrared bands. Representative spectra in the 1300–950 cm^{-1} region at pH 5.1 and 9.8 are shown in Figure 5. The spectra indicate that there are very pronounced changes in all the bands observed in this region. We point out that the broad band at 1182 cm^{-1} is observed below pH 7 (the pK_2 of monomethyl phosphate is ~7) and the band at 980 cm^{-1} is observed above this pH.

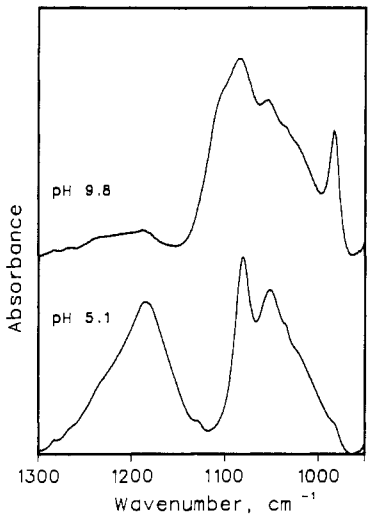


FIGURE 5: Infrared spectra (1300–950 cm^{-1}) of the disodium salt of monomethyl phosphate (0.1 M) at pH 5.1 and 9.8. The pH was adjusted by adding NaOH. The spectra shown are the result of subtracting the spectrum due to the solvent from the original spectra.

There are also changes in the group of bands centered at ~1100 cm^{-1}; however, band overlap makes identification of specific changes in individual bands difficult. Spectra of *O*-phospho-L-serine at various pH values show changes comparable to those observed in the spectra of monomethyl phosphate (data not presented). The bands at ~1182 and 980 cm^{-1} can be used to define the state of ionization of the primary phosphate group and therefore the pH of the sample.

The infrared spectra of lipids containing a primary phosphate group such as phosphatidic acids yield information on the degree of ionization of the lipid and in turn on the pH of the sample. The behavior of the infrared spectra of 1-lauroyl-*rac*-lysophosphatidic acid (lyso-PA) is presented in Figure 6A. These spectra at pH 5.5 and 11 confirm that the 1182 cm^{-1} band is characteristic of the monoionized phosphate group (structure I, Scheme I) and that the 980 cm^{-1} band is due to the fully ionized phosphate group (structure II, Scheme I). These bands are assigned to the antisymmetric (1182 cm^{-1}) and symmetric (980 cm^{-1}) stretching of the O–P–O moiety in accord with previous work (Shimanouchi et al., 1964; Sanchez-Ruiz & Martinez-Carrion, 1988). As in the case of monomethyl phosphate (Figure 5), there are also pH-induced changes in the bands at ~1100 cm^{-1} (Figure 6A). Overlap of several bands precludes their use for diagnostic purposes.

The change in integrated intensity of the diagnostic bands at 1182 and 980 cm^{-1} may be used to construct a titration curve for lyso-PA. Such a curve is shown in Figure 6B. The value of the second p*K* (pK_2) obtained from this curve is 7.3, in excellent agreement with the value of 7.0 derived from ^{31}P NMR (Hauser, 1989).

In the case of EPA, which is a diacylphosphatidic acid, the infrared spectra at alkaline pH are different from those of the monoacyl compound lyso-PA shown in Figure 6A. Infrared spectra of EPA at pH 6.7 and 11.3 are shown in Figure 7. Of particular importance here is the spectrum recorded at pH 11.3; in this spectrum, there is a band at 980 cm^{-1} characteristic of the fully ionized phosphate group (structure II, Scheme I). Furthermore, in contrast to the case of lyso-PA, there is a band at 1182 cm^{-1} characteristic of the monoionized phosphate group (structure I, Scheme I). The changes induced in EPA by increasing the pH (Figure 7) are reversible. If the pH is brought back to 6 from 11.3, the spectrum is the same as that observed at pH 6.7. Furthermore, if excess sodium cholate is mixed with EPA and dispersed at pH 11, the

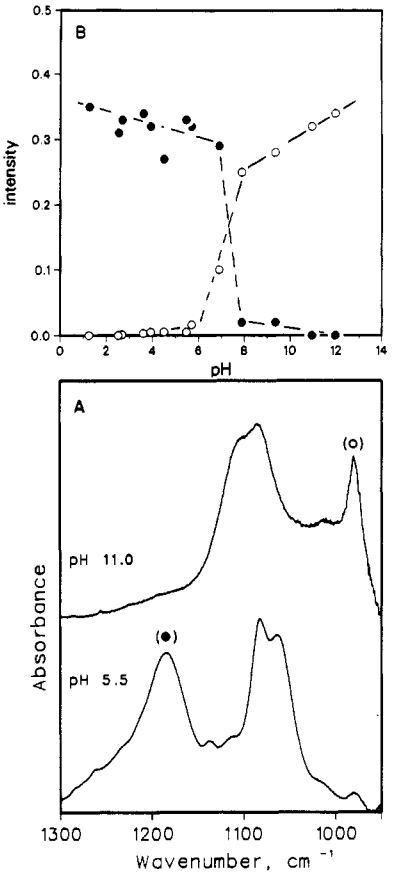


FIGURE 6: (A) Infrared spectra (1300–950 cm^{-1}) of 1-lauroyl-*rac*-lysophosphatidic acid (0.13 M) at pH 5.5 and 11.0. The spectra shown are the result of subtracting the spectrum due to the solvent from the original spectra. (B) pH dependence of the normalized (relative) intensities of the bands at 1182 cm^{-1} (●) and 980 cm^{-1} (○).

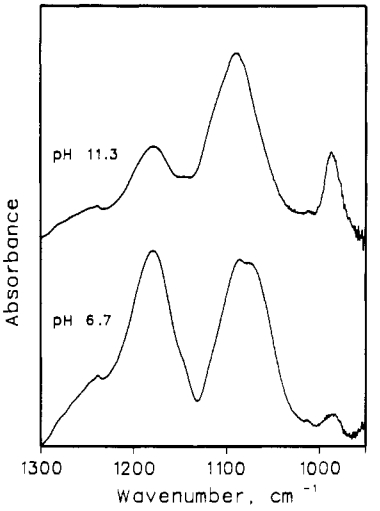


FIGURE 7: Infrared spectra (1300–950 cm^{-1}) of unsonicated EPA dispersions (0.16 M) at pH 6.7 and 11.3.

spectrum obtained is practically the same as that of lyso-PA at pH 11; i.e., there is no band at 1182 cm^{-1}.

Infrared spectra of unsonicated dispersions of DHPSH at different pH values are shown in Figure 8A. In the case of phosphatidylserine, the bands due to the carboxyl group of serine are of interest (Scheme I). The nonionized COOH form (structure III, Scheme I) gives bands in the 1750–1700 cm^{-1} spectral range while the ionized COO^- form (structure IV, Scheme I) gives bands in the 1640–1620 cm^{-1} spectral range. DHPSH was chosen to illustrate the effects of the pH-jump method because DHPSH, lacking ester linkages, gives no ester

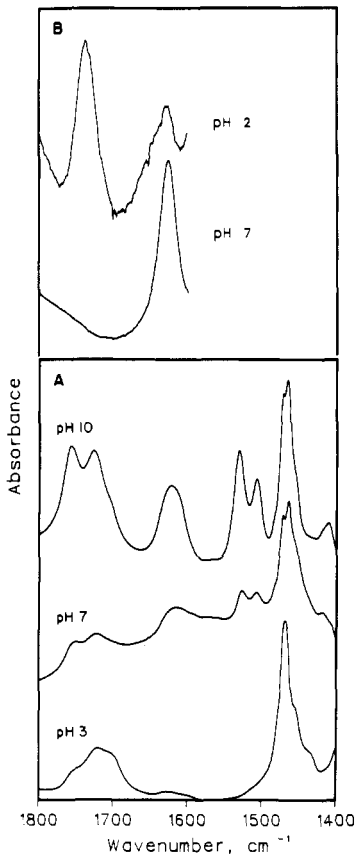


FIGURE 8: (A) Infrared spectra (1800–1400 cm^{-1}) of DHPSH (0.12 M) dispersed in ^2H_2O at pH 3, 7, and 10; the pH was adjusted by adding NaO^2H. (B) Infrared spectra (1800–1600 cm^{-1}) of DHPSH mixed with 1-dodecyl-*rac*-lysophosphocholine (1:6, weight ratio) at pH 2 and 7. The approximate concentrations of DHPSH are 0.06 M at pH 7 and 0.03 M at pH 2.

C=O stretching bands which would overlap with the COOH bands at ~1720 cm^{-1}. At pH 3 (Figure 8A), the spectrum of DHPSH shows bands at 1751, 1721, and 1700 cm^{-1} due to the protonated carboxyl group COOH (structure III, Scheme I). There is also a very weak and broad band at 1622 cm^{-1}, probably due to some remaining ionized COO^- residues. If the pH is increased above the p*K* of the carboxylate group (ca. 4.5; Papahadjopoulos, 1968; Hauser & Phillips, 1979), there should be conversion to the ionized form (structure IV, Scheme I). However, in the spectrum recorded at pH 7 (Figure 8B), there are strong bands due to the protonated COOH residue at 1751 and 1721 cm^{-1} together with a strong band at 1618 cm^{-1} due to the ionized COO^- form. Increasing the pH to 10 does not induce the total disappearance of the protonated form as shown in Figure 8A. With increasing pH, the infrared spectra indicate the coexistence of two environments different in pH. We assign this to the existence of a pH gradient across the bilayer of DHPS vesicles. Control experiments confirm this assignment. In Figure 8B, we show spectra of DHPSH mixed with the detergent 1-dodecyl-*rac*-lysophosphocholine (1:6 weight ratio) at pH 2 and 7. The amount of 1-dodecyl-*rac*-lysophosphocholine is sufficient to solubilize DHPSH into mixed micelles. The COOH band is observed at 1740 cm^{-1} at pH 2 and not at all at pH 7. Correspondingly, the COO^- band at 1625 cm^{-1} increases in intensity in going from pH 2 to 7. The same results are obtained when DHPSH is mixed with sufficient Triton X-100 to solubilize the DHPSH bilayers into mixed micelles. Thus, in the presence of detergents, where all the DHPS head groups are in direct contact with the aqueous bulk medium, all the carboxyl groups are titrated by changing the pH.

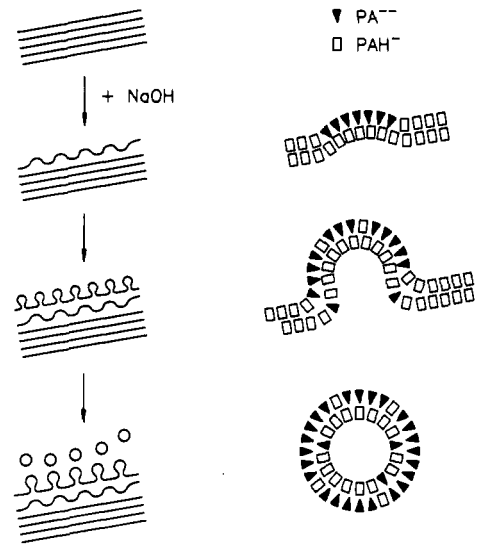


FIGURE 9: Pictorial description of the process of spontaneous vesiculation induced by the pH-jump method. PAH^- and PA^{--} represent mono and fully ionized phosphatidic acid molecules (structures I and II in Scheme I).

Discussion

One prerequisite for the spontaneous formation of small unilamellar EPA vesicles by the pH-jump method is the exposure of the dispersion to a pH >10. The fact that after raising the pH to 10–12 two ^{31}P signals are observed is interpreted to indicate the formation of a transverse pH gradient across the phospholipid bilayer. This interpretation is also based on the observation that small unilamellar phosphatidic acid vesicles produced by sonication give rise to a single sharp ^{31}P NMR signal. The singlet indicates that there is no difference in pH between the external medium and the internal vesicle cavity as would be expected for vesicles produced by sonication. It also means that there is no difference in the molecular packing between molecules in the outer and inner monolayers. Packing constraints within the inner monolayer have been suggested to be responsible for the observed ^{31}P chemical shielding difference between external and internal monolayers of phosphatidylcholine unilamellar vesicles (Lee et al., 1972; Sheetz & Chan, 1972). The singlet observed with phosphatidic acid dispersions (Hauser, 1989) is clear evidence that the phosphate groups on both monolayers are chemically and magnetically equivalent. Therefore, in the case of unsonicated phosphatidic acid dispersions, the observed differences in chemical shielding can be attributed to differences in surface pH. Infrared spectroscopy demonstrates unequivocally that the pH-jump method induces a pH gradient across the bilayer. Furthermore, from the observed intensities of the monoanionic and fully ionized phosphate bands and the titration curve (cf. Figure 6B), the pH gradient may be estimated to be 4 ± 1 pH units, in excellent agreement with the value determined by ^{31}P NMR (Hauser, 1989). Its orientation is such that the pH of the vesicle cavity is more acidic than the pH of the external medium.

On the basis of ^{31}P NMR and infrared spectroscopic evidence, the following mechanism of the formation of small unilamellar vesicles by pH jump may be proposed (cf. schematic diagram in Figure 9). Upon exposure of a multilamellar liposome of phosphatidic acid (in the protonated or monoanionic form, structure I, Scheme I) or a lamellar packet of phosphatidic acid deposited by drying (dehydration) on the glass wall of a flask to high pH (10–12), the outermost layer of phosphatidic acid molecules will become fully ionized, bearing two negative charges per phospholipid molecule. In

contrast, phosphatidic acid molecules on the inner monolayer of the outermost bilayer and of bilayers in the interior of the multilamellar phospholipid packet will be close to neutrality. At this pH, the phosphate group of phosphatidic acid is partially ionized (monoanionic, form I in Scheme I). As a result, a pH gradient across the phospholipid bilayer is produced. There is greater electrostatic repulsion between fully ionized (doubly negatively charged) molecules present in the outer monolayer than between phospholipid molecules present on the inner monolayer of the (first) bilayer. The pH gradient will therefore give rise to a molecular packing gradient. Fully ionized molecules in the outer monolayer occupy a larger volume while molecules in the inner layer exposed to a more acidic pH and only partially ionized are more tightly packed. An imaginary body encasing one phosphatidic acid molecule in the outer monolayer and its counterpart in the inner monolayer will be wedge-shaped, matching ideally the packing requirement of a highly curved lipid bilayer. As a result of this differential electrostatic repulsion and the difference in packing mode between outer and inner monolayers, small vesicles can be envisaged to bud off spontaneously from the first bilayer. The spontaneous vesiculation occurs first in the outermost or first bilayer of a multilamellar phospholipid particle. This process exposes the next bilayer to the high pH of the external medium, generating a pH gradient across the second bilayer. The formation of a pH gradient and the budding off of small vesicles described above for the first or outermost bilayer will now take place in the second bilayer and in this way continue to spread to bilayers located in the interior of the multilamellar lipid particle.

Results obtained previously (Hauser & Gains, 1982; Gains & Hauser, 1983) with a number of physical techniques showed that the pH-jump treatment applied to EPA dispersions produces small vesicles surrounded by a single closed phospholipid bilayer. The diameter of these vesicles ranges from 20 to 60 nm. Ions, fluorochromes, and macromolecules such as proteins can be entrapped in the internal, aqueous cavity. The general applicability of the pH-jump method has been tested by first applying it to saturated phosphatidic acid molecules. Information concerning the morphology of the small particles formed by spontaneous vesiculation in dispersions of saturated phosphatidic acids may be derived from freeze–fracture electron microscopy. Electron micrographs of dilauroyl- and dimyristoyl-*sn*-phosphatidic acid dispersions show the presence of flat discoidal particles (diameter 20–50 nm) which look like collapsed vesicles. Their appearance is markedly different from that of typical spherical, unilamellar egg phosphatidylcholine vesicles of similar size (data not shown) (Huang, 1969; Hauser et al., 1973). Dilauroyl-*sn*-phosphatidic acid dispersions contain also larger particles (diameter >60 nm) which can be identified as large unilamellar vesicles. We propose the small discoidal structures present at alkaline pH to be flattened vesicles rather than micelles. More work is required to resolve this question. The two dispersions of saturated phosphatidic acid differ significantly in their behavior when the alkaline dispersions are neutralized. Electron micrographs of freeze–fractured samples of dilauroylphosphatidic acid show that neutralization leads to partial aggregation of the discoidal particles consistent with ^{31}P NMR. Upon neutralization, the small particles present in dimyristoyl-*sn*-phosphatidic acid dispersions revert back almost 100% to large structures as evident from the axially symmetric ^{31}P powder NMR spectrum. The difference in the behavior of the two saturated phosphatidic acids is probably due to the different gel-to-liquid-crystal transition temperatures: these temperatures are 32 and 52 °C for aqueous dispersions (pH 7) of dilauroyl- and dimyristoyl-*sn*-phosphatidic acid, respectively (H. Hauser, unpublished results).

The mechanism of spontaneous vesiculation discussed above can be expected to be a general phenomenon and should not be restricted to phosphatidic acid. In principle, it should be applicable to bilayer-forming molecules with one or more ionizable groups (Hauser et al., 1986). This hypothesis was put to test by applying the pH-jump method to phosphatidylserine dispersions. Phosphatidylserine contains a secondary phosphate group with a pK value of ca. 1.5 (Garvin & Karnovsky, 1956; Abramson et al., 1964), and the carboxyl group and the amino group of serine which have apparent pK values of 4.6 and 9.9, respectively (Papahadjopoulos, 1968; Hauser & Phillips, 1979). Therefore, at pH 11–12, phosphatidylserine carries two negative charges per molecule. Under these conditions, phosphatidylserine can be expected to behave like a detergent. Like with phosphatidic acid bilayers, raising the pH will generate a pH gradient across phosphatidylserine bilayers, and as a result, spontaneous vesiculation is expected to take place. This is borne out by experiment. The infrared spectra shown in Figure 8A prove that there is indeed a pH gradient in the case of disaturated DHPSH. Also, in these spectra, there are two bands at 1470 and 1466 cm^{-1} due to the CH_2 deformation mode which are indicative of tighter alkyl chain packing (Casal & Mantsch, 1984).

The identification of the small particles produced by the pH-jump treatment of dipalmitoylphosphatidylserine dispersions is complicated by the fact that the resulting dispersion is unstable at room temperature. This is expected, considering that its gel-to-liquid-crystal transition temperature is 53 °C (Browning & Seelig, 1980). The particles present in these dispersions therefore have a tendency to aggregate and/or fuse at room temperature, forming large structures. These structures are apparently trapped on Sepharose 4B columns because only 20–65% of dipalmitoylphosphatidylserine dispersions subjected to the pH-jump method and subsequently applied to the Sepharose 4B column were eluted. Furthermore, a significant proportion of the total lipid (10–25%) was eluted in the column total volume, indicating the presence of very small particles. A possible explanation of this result is that exposure of the lipid dispersion to high pH leads to hydrolysis of phosphatidylserine with the formation of small micelles and water-soluble compounds eluted at or close to the column total volume.

The pH-jump treatment applied to ox brain phosphatidylserine leads to partial vesiculation (Figure 4). The vesicles formed show a wide size distribution. This is evident from ^{31}P NMR showing a broad isotropic signal that is superimposed on an axially symmetric powder pattern. When the dispersion after the pH-jump treatment was subjected to centrifugation at 12000g for 10 min, 80–95% of the lipid remains dispersed. When the supernatant after centrifugation was applied to a calibrated Sepharose 4B column, 70–80% of the lipid was eluted in the void volume, indicating that the majority of vesicles present are greater than ~60 nm. In contrast to dispersions of dipalmitoylphosphatidylserine, the yield of phospholipid eluted from the Sepharose 4B column was 80–90%.

The pH gradient generated across the phospholipid bilayer is postulated to be the driving force for the formation of highly curved, unilamellar vesicles. If correct, the term spontaneous used in previous publications to describe the vesiculation phenomenon (Hauser & Gains, 1982; Gains & Hauser, 1983; Hauser et al., 1983, 1986) is misleading and, strictly speaking,

incorrect. It implies that vesiculation occurs without input of external energy, i.e., without energizing the system. However, the pH gradient across the phospholipid bilayer represents a physical form of energy which is used to drive the vesiculation. In this sense, the vesiculation procedure described here is not spontaneous. The term spontaneous used previously would also imply that the resulting unilamellar vesicles are thermodynamically stable. This issue has not been resolved. To the best of our knowledge, no systematic study pertaining to the question of the thermodynamic stability of phosphatidic acid vesicles of diameter 20–60 nm has been carried out so far. This issue is of considerable interest and will be addressed in a separate study.

It has been shown before that small unilamellar vesicles can form spontaneously in two-component or multicomponent lipid mixtures. Such mixtures consist of at least two types of molecules: molecules with a cylindrical shape and wedge-shaped molecules. As shown in the schematic diagram (Figure 9), vesicles with a high curvature (small radius of curvature) can form by an asymmetric distribution of the two kinds of molecules between the two halves of the bilayer: the wedged molecules are preferentially located on the outer layer of the bilayer, and the cylindrically shaped molecules having the shape of an inverted cone will be on the inner monolayer. Spontaneous formation of small unilamellar vesicles has been predicted to occur with mixtures of neutral (isoelectric) lipids provided the appropriate composition of differently shaped molecules is selected. An example of this type of spontaneous vesiculation was reported recently (Hauser, 1987) for mixtures of egg phosphatidylcholine of approximately cylindrical shape and its lyso compound that is wedge shaped. The molar ratio of the two types of molecules was shown to be a critical parameter: spontaneous formation of small unilamellar vesicles of diameter <80 nm occurs over a very narrow compositional range. The formation of small phosphatidic acid vesicles by pH jump is not an exception, but is consistent with the notion that for spontaneous vesiculation to occur at least two components are required. The pH jump generates the two types of molecules: fully ionized, highly hydrated phosphatidic acid molecules are wedge-shaped while partially protonated and probably less hydrated molecules are more cylindrical.

Conclusions

The pH-jump method induces formation of small unilamellar vesicles of EPA of diameter 20–60 nm. ^{31}P NMR and infrared spectroscopic measurements demonstrate unequivocally that in the unilamellar vesicles formed by the pH-jump method there exists a pH gradient across the bilayer. The magnitude is estimated to be 4 ± 1 pH units, and its orientation is inside more acidic. This pH gradient is proposed to be the driving force for the formation of small unilamellar vesicles. The pH gradient gives rise to a packing gradient upon vesiculation. A mechanism is postulated based on differences in electrostatic repulsion between the outer and the inner layer of the bilayer. The general applicability of a pH gradient as the driving force for spontaneous vesiculation has been demonstrated.

Acknowledgments

We thank Adèle Martin and Jeannette Stäuble for expert technical assistance and Dr. E. Wehrli for communicating the electron microscopy results. We are indebted to Dr. Boris G. Tenchov of the Bulgarian Academy of Sciences for useful discussions.

Registry No. DHPSH, 64563-65-1; lyso-PA, 86594-49-2; 1,2-dilauroyl-*sn*-phosphatidic acid, 55332-91-7; 1,2-dimyristoyl-*sn*-phosphatidic acid, 28874-52-4; 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine, 40290-42-4; 1-dodecyl-*rac*-lysophosphocholine, 70641-52-0; monomethyl phosphate, disodium, 17323-81-8.

References

Abramson, M. B., Katzman, R., & Gregor, H. P. (1964) *J. Biol. Chem. 239*, 70–76.
Aurora, T. S., Li, W., Cummins, H. Z., & Haines, T. H. (1985) *Biochim. Biophys. Acta 820*, 250–258.
Browning, J. L., & Seelig, J. (1980) *Biochemistry 19*, 1262–1270.
Casal, H. L., & Mantsch, H. H. (1984) *Biochim. Biophys. Acta 779*, 381–401.
Gains, N., & Hauser, H. (1983) *Biochim. Biophys. Acta 731*, 31–39.
Garvin, J. E., & Karnovsky, M. L. (1956) *J. Biol. Chem. 221*, 211–219.
Haines, T. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 160–164.
Hauser, H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1049–1055.
Hauser, H. (1984) *Biochim. Biophys. Acta 772*, 37–50.
Hauser, H. (1985) *Chimia 39*, 252–264.
Hauser, H. (1987) *Chem. Phys. Lipids 43*, 283–299.
Hauser, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5351–5355.
Hauser, H., & Phillips, M. C. (1979) *Prog. Surf. Membr. Sci. 13*, 297–413.
Hauser, H., & Gains, N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1683–1687.
Hauser, H., Oldani, D., & Phillips, M. C. (1973) *Biochemistry 12*, 4507–4517.
Hauser, H., Kostner, G., Müller, M., & Skrabal, P. (1977) *Biochim. Biophys. Acta 409*, 247–261.
Hauser, H., Gains, N., & Müller, M. (1983) *Biochemistry 22*, 4775–4781.
Hauser, H., Gains, N., & Lasic, D. D. (1985) in *Proceedings S.I.F.* (Degiorgio, V., & Corti, M., Eds.) Course XC, pp 648–662, North-Holland, Amsterdam.
Hauser, H., Gains, N., Eibl, H.-J., Müller, M., & Wehrli, E. (1986) *Biochemistry 25*, 2126–2134.
Hermetter, A., Paltauf, F., & Hauser, H. (1982) *Chem. Phys. Lipids 30*, 35–45.
Huang, C. H. (1969) *Biochemistry 8*, 344–352.
Lee, A. G., Birdsall, N. J. M., Levine, Y. K., & Metcalfe, J. C. (1972) *Biochim. Biophys. Acta 255*, 43–56.
Li, W., & Haines, T. H. (1986) *Biochemistry 25*, 7477–7483.
Mantelli, S., Speiser, P., & Hauser, H. (1985) *Chem. Phys. Lipids 37*, 329–343.
Papahadjopoulos, D. (1968) *Biochim. Biophys. Acta 163*, 240–254.
Rydhag, L., & Gabran, T. (1982) *Chem. Phys. Lipids 30*, 309–324.
Rydhag, L., Stenius, P., & Oedberg, L. (1982) *J. Colloid Interface Sci. 86*, 274–276.
Sanchez-Ruiz, J. M., & Martinez-Carrion, M. (1988) *Biochemistry 27*, 3338–3342.
Schurtenberger, P., & Hauser, H. (1984) *Biochim. Biophys. Acta 778*, 470–480.
Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.
Shimanouchi, T., Tsuboi, M., & Kyogoku, Y. (1964) *Adv. Chem. Phys. 8*, 435–498.