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SOME ASPECTS OF THE PHASE BEHAVIOUR OF CHARGED LIPIDS

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Based on their swelling in H_2O lipids forming smectic phases may be grouped into two classes: (i) uncharged or isoelectric lipids which show no or limited swelling in H_2O (the thickness of the water layer is less than or equal to 25 Å); (ii) lipids with a net charge, which show continuous swelling. Up to a certain threshold concentration all the water is intercalated between the lipid bilayers: there is a single lamellar, swelling phase. Above this concentration two phases coexist: fully-hydrated unilamellar vesicles and excess water. Continuous swelling and the formation of unilamellar vesicles in excess water is observed with pure charged lipids and lipid mixtures. It is important to note that neutral bilayers doped with only a few percent of a positively or negatively charged lipid exhibit this behaviour. The continuous swelling is a purely electrostatic phenomenon as indicated by the reversible interconversion between continuous and limited swelling behaviour:

in the one-phase domain:

$$\boxed{\begin{array}{c}\textbf{multilamellar}\\ \textbf{continuous swelling}\end{array}}\ \underset{\textbf{removal of counterion}}{\overset{\textbf{excess counterion}}{\rightleftharpoons}}\ \boxed{\begin{array}{c}\textbf{multilamellar}\\ \textbf{limited swelling}\end{array}}$$

in the two-phase domain:

$$\boxed{\begin{array}{c}\textbf{large unilamellar}\\ \textbf{vesicles}\end{array}}\ \underset{\textbf{removal of counterion}}{\overset{\textbf{excess counterion}}{\rightleftharpoons}}\ \boxed{\begin{array}{c}\textbf{multilamellar}\\ \textbf{structures}\end{array}}$$

Introduction

The phase behaviour of phospholipids has been the subject of extensive studies in the past. The justification for this is at least two-fold: firstly, phospholipids have been shown to form integral structures of biological membranes, and the appreciation of their structural and functional role in biological membranes requires a good understanding of their physico-chemical properties, particularly their phase behaviour. It is now generally accepted that the bulk of the phospholipid in biological membranes is present as a bilayer. Based upon this concept phospholipid bilayers have been widely used as model membranes and their physico-chemical properties have been studied extensively in the past. Secondly, phospholipids have been used in industry for quite some time as important ingredients of foodstuffs, emulsifiers, lubricants and cosmetics. More recently another possible industrial application of phospholipids has been considered; the use of phospholipids as drug carrier or drug delivery systems [1].

The emphasis of the past research on the phospholipid phase behaviour and their physico-chemical properties in general, has been on neutral or isoelectric phospholipids such as phosphatidylcholine and phosphatidylethanolamine. A great wealth of data has accumulated over the last 10 to 15 years. This is contrasted by our knowledge of charged phospholipids. Relatively little is known about the phase behaviour of pure, negatively charged phospholipids and, with a few exceptions, nothing is known about multicomponent phospholipid mixtures containing negatively charged phospholipids. This is a major omission since the latter phospholipid mixtures are not only more realistic membrane models but also more likely to be used on an industrial scale than pure phospholipids.

In this paper some common features of the phase behaviour of charged lipids are discussed which have important implications in both model membrane studies and in liposome technology.

Experimental

Materials

2,3-Dimyristoyl- and 2,3-dipalmitoyl-D-glycero-1-phospho-L-serine (= 1,2-dimyristoyl- and 1,2-dipalmitoyl-*sn*-phosphatidylserine) were synthesized as described before [2]. The final product was phosphatidylserine in either the protonated form or as the NH_4^+ salt. For conversion of the protonated form to the NH_4^+ salt, sufficient NH_3 was added to the $CHCl_3/CH_3OH$ (60 : 35, v/v) solution and the organic solvent was evaporated on the rotary evaporator at temperatures below 30°C [3]. Egg phosphatidylcholine, egg phosphatidic acid, the sodium salt of ox brain phosphatidylserine, wheat germ phosphatidylinositol and phosphatidylglycerol were purchased from Lipid Products (Surrey, U.K.). 3-Acyl-D-glycero-1-phospho-DL-glycerol (lysophosphatidylglycerol) was purchased from Sigma.

The lipids used were pure by TLC standards, applying 0.3–1 mg as a 1 cm-band on 20 × 20 cm Silica gel 60F-254 plates (Merck AG) and using two solvent systems ($CHCl_3/CH_3OH/NH_3$, (65 : 25 : 4, v/v); $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50 : 30 : 8 : 4, v/v)) [3]. Unsonicated aqueous phospholipid dispersions were made by adding water or buffer to the dry phospholipid film deposited on the glass wall of a round-bottom flask and gently shaking the flask for 5–10 min. The phospholipid film was produced by taking to dryness the $CHCl_3/CH_3OH$ solution of the phospholipid using the rotary evaporator and drying the film under high vacuum. Sonication of aqueous phospholipid dispersions was carried out as described previously [4].

Methods

Samples for X-ray diffraction experiments were prepared and the X-ray diffraction studies were carried out as described previously [3]. X-ray scattering measurements were made using a Kratky slit-collimated camera.

Electron microscopy

Cryofixation of the samples was carried out as follows: a gold grid was dipped into the aqueous lipid dispersion and then placed between two low-mass copper platelets. The assembly was frozen in a propane jet [5] and the cryofixed sample was freeze-fractured in a Balzers BAF 300 at a pressure of 10^{-5} Pa. Contamination of the fracture-surfaces was prevented by starting the evaporation of platinum/carbon prior to fracturing (2.5 nm PtC/ 20 nm C). Replicas were rinsed with $CHCl_3/CH_3OH$ (1 : 1, v/v). Samples for thin-sectioning were cryofixed as described above. The sample placed between the copper platelets was fractured under liquid nitrogen and freeze-substitution was carried out as follows: after fracturing, the platelets were immersed in the substitution medium at −95°C (this consisted of methanol containing 1% OsO_4, 0.5% uranyl acetate and 3% glutaraldehyde); after leaving the sample for 8 h each at −95°C, −60°C and −30°C the substituent was replaced by methanol. Low-temperature embedding and unilamellar vesicle polymerization in Lowicryl HM20 were performed at −30°C [6]. Thin sections were stained with uranyl acetate and lead citrate.

Determination of the fraction of phospholipid molecules present on the inner layer of the bilayer (P_i/P_t)

The ratio P_i/P_t (P_i = number of phospholipid molecules on the inner layer of the bilayer, P_t = total number of phospholipid molecules) was determined by a radiotracer method. The dry phospholipid film was dispersed in water containing approx. 3 nM $^{22}NaCl$ (phospholipid concentration approx. 13 mM). There is a large excess of negatively charged phospholipid and under these conditions practically all $^{22}Na^+$ will be bound to phospholipid. Phospholipid dispersions were then chromatographed on a Sepharose 4B column and phospholipid determined in each fraction as described previously [24]. During the filtration $^{22}Na^+$ bound to the external layer of vesicles will be released and separated from the phospholipid vesicles, the $^{22}Na^+$ entrapped within the vesicle cavity $(^{22}Na^+)_b$ will be eluted with the vesicles. For each fraction eluted from the column the ratio $^{22}Na_b^+/P_t = P_i/P_t$ was determined. This ratio is expected to be 0.5 for large unilamellar vesicles (diameter > 1000 Å) and for small unilamellar vesicles with a diameter between 200 and 1000 Å it will vary between 0.25 and 0.5. Multilamellar liposomes with concentrically arranged bilayers will give P_i/P_t ratios greater than 0.5.

Determination of the internal aqueous volume V_i of phospholipid vesicles

V_i of phospholipid vesicles was determined by $^{35}SO_4^{2-}$ encapsulation. The phospholipid dispersion was made as described above except that the water added to the dry lipid film contained $^{35}SO_4^{2-}$. The determination of V_i is based on the assumption that the concentration of $^{35}SO_4^{2-}$ in the vesicle cavity is the same as in the external medium. To a first approximation this condition is fulfilled with negatively charged phospholipid dispersions. The external vesicle radius was calculated from V_i as discussed previously [24].

Results

The swelling in H_2O of various phosphatidylserines is shown in Fig. 1A. Phosphatidylserines, both in the uncharged, protonated form and as charged salts, have been shown to form smectic phases when dispersed in water [3,7,8]. The X-ray diffraction pattern of phosphatidylserine dispersions in H_2O consists at low angles of a series of equally spaced sharp lines in the ratio of $1:(1/2):(1/3) \ldots (1/n)$, from which the lamellar repeat distance d is derived. This distance is shown as a function of water content for various phosphatidylserine dispersions in Fig. 1A. With NH_4^+ dimyristoylphosphatidylserine, d increases continuously with water content up to at least 50% H_2O. This is true for dispersions at 20°C and 50°C, where the phospholipid is below and above the gel-to-liquid-crystal transition, respectively [3]. At 20°C the wide-angle reflection at $1/4.2$ Å^{-1} is indicative of the lipid being in the gel phase with 'hexagonal' hydrocarbon chain packing. In the gel at 20°C the lamellar repeat distance d increases from about 60 Å to 100 Å as the water content of the gel is raised from 10 to 50%. The swelling in the liquid-crystalline phase at 50°C is less over the same range of water content and is, within experimental error, identical to that of the Na^+ salt of ox brain phosphatidylserine at 20°C (above the gel-to-liquid-crystal transition) (Fig. 1A). For ox brain phosphatidylserine continuous swelling could be observed up to $d = 130$ Å at about 70% H_2O. At higher hydration levels broadening of the low-angle diffraction lines was observed, for both phosphatidylserines, as a result of multilayer stacking disorders and eventually the low-angle reflections were lost. Addition of sufficient NaCl made the fully-swollen gel of NH_4^+ dimyristoylphosphatidylserine (at 50% water, $d = 100$ Å) shrink. Addition of 0.5 M NaCl reduced d to 62 Å, higher concentrations of NaCl having no further effect on the lamellar repeat distance d [9]. Qualitatively similar effects, namely a significant reduction in the lamellar repeat distance were observed when other alkali metal ions were added to aqueous dispersions of NH_4^+ dimyristoylphosphatidylserine. Polyvalent cations, such as the alkaline earth metal ions and the lanthanides, produced the same effect except that the reduction in d occurred at ionic concentrations 10–50 times smaller (data not shown). The continuous swelling of the charged phosphatidylserine is contrasted by the behaviour of uncharged (isoelectric) phosphatidylserines. When dispersed in hydrochloric acid pH $\simeq$ 2 the carboxyl group of phosphatidylserine is fully protonated [10] and hence the phospholipid has no net charge. In aqueous dispersions of dimyristoyl- and dipalmitoylphosphatidylserine at pH = 2 and 20°C (below the gel-to-liquid-crystal transition) the lamellar repeat distance *d* is invariant over the total hydration range studied (see solid lines in Fig. 1A). The average lamellar repeat distances under these conditions are $d = 42.0 \pm 0.5$ Å and 45.7 ± 0.3 Å for dimyristoyl- and dipalmitoylphosphatidylserine, respectively. These values are, within experimental error, identical to the lamellar repeat distances measured for crystalline dimyristoyl- and dipalmitoylphosphatidylserine, respectively, both in their protonated form. This clearly indicates that under conditions where phosphatidylserines lack a net negative charge they do not swell.

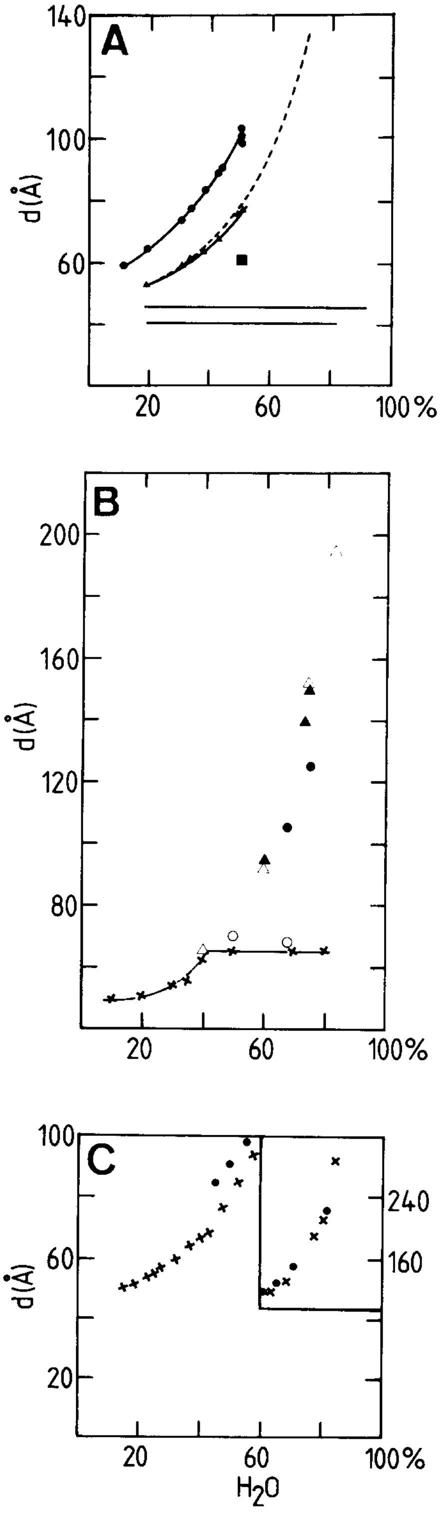


Fig. 1. (A) Lamellar repeat distance *d* (Å) as a function of water content in %. NH_4^+ dimyristoylphosphatidylserine at 20°C (below $t_c = 39$°C, ●) and at 50°C (above t_c, ▲). The lipid was dispersed in 0.025 M ammonium phosphate pH 6.8 (ionic strength 0.05). The data point at $c = 50\%$ (■) gives the lamellar repeat distance of NH_4^+ dimyristoylphosphatidylserine in NaCl solution of concentrations greater than or equal to 0.5 M. The swelling of the Na^+ salt of ox brain phosphatidylserine in water is represented by the dotted line. The solid lines at $d = 42$ Å and $d = 45.7$ Å refer to dimyristoylphosphatidylserine and dipalmitoylphosphatidylserine, respectively, in the protonated form dispersed in 0.01 M HCl. The swelling curves of NH_4^+ dimyristoylphosphatidylserine and ox brain phosphatidylserine were taken from Refs. 3 and 21, respectively.

(B) Lamellar repeat distance *d* (Å) as a function of water content in % for egg phosphatidylcholine and mixed egg phosphatidylcholine dispersions in water. The latter dispersions consist of egg phosphatidylcholine and either cetyltrimethylammonium bromide (CTAB) or sodium oleate. Phosphatidylcholine (×); phosphatidylcholine plus 0.6% CTAB (○); phosphatidylcholine plus 0.9% CTAB (●); phosphatidylcholine plus 1.5% CTAB (△); phosphatidylcholine plus 3.5% sodium oleate (△, adapted from Gulik-Krzywicki et al. [14]).

(C) Lamellar repeat distance *d* (Å) as a function of water content in % for the lipid extract of bovine heart mitochondria at 25°C (×) containing approx. 34% phosphatidylcholine, 29% phosphatidylethanolamine, 10% phosphatidylinositol, 20% cardiolipin, 2% cholesterol and 5% other neutral lipids (adapted from Gulik-Krzywicki et al. [22]). Inset: Lamellar repeat distance *d* for the lipids extracted from human erythrocytes at 0°C (●) containing 26% cholesterol, the phospholipid being distributed between phosphatidylcholine 36%, phosphatidylethanolamine 29%, sphingomyelin 23%, phosphatidylserine 10%, lysophosphatidylcholine 2% (adapted from Rand and Luzzatti [23]).

That the infinite swelling of lipid bilayers bearing a net charge is a general phenomenon is demonstrated in Fig. 1B and C which were made up from data in the literature. In Fig. 1B the swelling of egg phosphatidylcholine in water (x——x) is compared to the swelling of egg phosphatidylcholine doped with small quantities of either cetyltrimethylammonium bromide (CTAB) or sodium oleate. It is seen that pure egg phosphatidylcholine swells with increasing water content reaching an upper limit of $d \simeq 65$ Å at about 40% H_2O. Up to this limit a single, swelling, lamellar phase exists and above that a two-phase system consisting of a fully-swollen, lamellar phase with $d = 65$ Å and excess water [11,12]. A similar behaviour of limited swelling was reported for synthetic phosphatidylcholines with saturated hydrocarbon chains [11] and other neutral and isoelectric lipids, e.g., phosphatidylethanolamine, ethanolamine plasmalogens, sphingomyelins, monoacylglycerols, glycolipids such as cerebrosides, mono and digalactosyl diacylglycerols [13,14]. The swelling behaviour of egg phosphatidylcholine bilayers containing less than 0.6% cetyltrimethylammonium bromide (O——O) is similar to that of pure phosphatidylcholine. However, doping the phosphatidylcholine bilayer with about 1% of this detergent or 3.5% sodium oleate produced a marked change; infinite swelling as with charged phosphatidylserines is observed up to at least 80% water. It should be stressed that the infinite swelling is produced by adding only a few percent of a charged lipid to phosphatidylcholine and furthermore the effect is independent of the nature of the charge. Infinite swelling as shown in Fig. 1A and B has been reported for negatively charged phospholipids other than phosphatidylserine, e.g. phosphatidic acid [15,16] and phosphatidylglycerol [17]. Furthermore, it has been shown for phospholipid mixtures containing negatively charged phospholipids, e.g., egg phosphatidylcholine containing increasing quantities of phosphatidylglycerol [17] and egg phosphatidylcholine containing 10% phosphatidylinositol [17]; it has also been shown for lipids extracted from biological membranes which are known to contain 10% or more negatively charged lipids. The continuous swelling, up to at least 80% H_2O, of the total lipids extracted from bovine heart mitochondria (x) and from human erythrocytes (●) is shown in Fig. 1C.

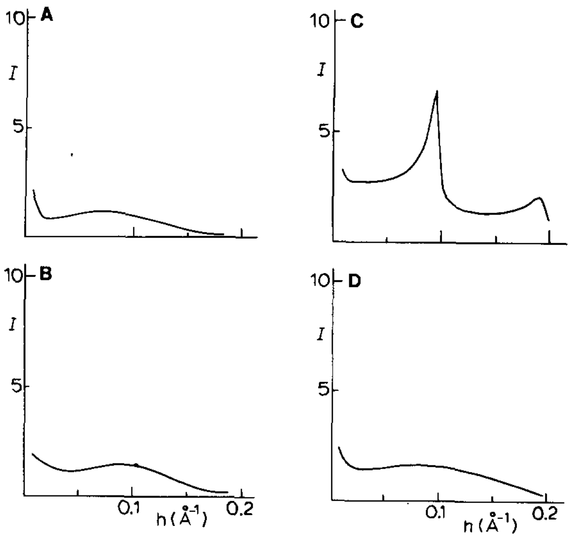


Fig. 2. X-ray scattering curves from dilute aqueous dispersions of Na^+ ox brain phosphatidylserine and egg phosphatidylcholine. (A) 2.5% unsonicated phosphatidylserine; (B) 5% sonicated phosphatidylserine; (C) 5% unsonicated phosphatidylcholine; (D) 5% sonicated phosphatidylcholine. The dispersion in (D) was fractionated by gel filtration on Sepharose 4B. The peak fractions containing small unilamellar vesicles (surrounded by a single bilayer) were pooled and used in the X-ray scattering experiment. Correcting the scattering curves for the effect of the collimation system did not significantly alter the shape of the curves. Therefore I is the experimental scattering intensity; $h = 4\pi \sin \theta / \lambda$ where 2θ is the scattering angle and λ the wavelength (adapted from Atkinson et al. [21]). Unsonicated dispersions were prepared and sonicated as described under Experimental.

A question of considerable interest is the behaviour of smectic phases of charged lipids at dilutions exceeding the range shown in Fig. 1. Evidence for the lipid structure prevailing under these conditions is obtained from X-ray scattering experiments and electron microscopy. As mentioned above the sharp low-angle reflections become diffuse as the water content exceeds 70 or 80% indicating that the stacking order in the swollen lamellar phase decreases at this dilution. Fig. 2 shows that in dilute dispersions of the Na^+ salt of ox brain phosphatidylserine the sharp low angle reflections characteristic of ordered multilamellar structures are replaced by a broad diffuse scattering peak in the range $0.05 < h < 0.15$ (Å)$^{-1}$ (Fig. 2A). This scattering curve from dilute unsonicated phosphatidylserine dispersions resembles that obtained from sonicated Na^+ ox brain phosphatidylserine (Fig. 2B) or sonicated egg phosphatidylcholine (Fig. 2D) known to consist of small unilamellar vesicles. The broad diffuse scattering maxima obtained with sonicated phospholipid dispersions are contrasted by the sharp maxima obtained with a diluted (5%) unsonicated egg phosphatidylcholine dispersion in H_2O (Fig. 2C). The maxima corresponding to 66 Å and 33 Å are the first and second order diffractions from the lamellar repeat distance of the maximally hydrated multilamellar egg phosphatidylcholine structure; the lamellar repeat distance d measured for such diluted unsonicated phosphatidylcholine dispersions is identical to that measured at higher lipid concentrations, i.e. at 50% lipid (cf. Fig. 1B). This clearly indicates that with phosphatidylcholine the fundamental multilamellar packing is maintained in excess water. Therefore, in excess water uncharged (isoelectric) phospholipids are markedly different from phospholipids carrying a net charge. The interpretation of the scattering curves shown in Fig. 2 is based on the computation of scattering curves for spherical, unilamellar vesicles differing in radius. A good fit with the experimental scattering curves (Figs. 2A, B and D) is achieved assuming that the vesicle dispersions are polydisperse. Stacks of bilayers comprising more than a few bilayers can be ruled out on the basis of this computer simulation (for details, see Ref. 21). Furthermore, it can be readily shown that the broad scattering envelopes at $0.05 < h < 0.15$ $(\text{Å})^{-1}$ (Figs. 2A, B, D) results from the one-dimensional Fourier transform of the electron density profile of the bilayer. This strongly suggests that the fundamental structure of charged lipids dispersed in excess water is the unilamellar vesicle.

Evidence to support this conclusion comes from electron microscopy. Figs. 3A and B are electron micrographs of freeze-fractured preparations of a 1% unsonicated dispersion of Na^+ ox brain phosphatidylserine in H_2O. The dispersion appears to consist of unilamellar vesicles of a wide size distribution ranging from 0.1 μm to about 10 μm. Examining several hundred particles revealed that approx. 95% of the spherical particles have diameters between 0.1 to 2 μm. Cross-sections of vesicles frequently show smaller vesicles enclosed in the cavity of larger ones. Occasionally large particles of diameter of more than 1 μm can be seen in cross-section (Fig. 3B), they consist of several lamellae which are stacked in an onion-like fashion reminiscent of the multilamellar packing of unsonicated phosphatidylcholine bilayers. Although an accurate estimate of the layer thickness or repeat distance is difficult to derive from such cross-sectional views, it is still safe to say that it is a multiple of the actual bilayer thickness (Fig. 3B). This suggests that in this instance swelling of the bilayers must have proceeded far beyond the range which can be followed with reasonable accuracy by X-ray diffraction (cf., range presented in Fig. 1). Fig. 3C is an electron micrograph of a freeze-fractured preparation of a 5% unsonicated aqueous dispersion of the sodium salt of phosphatidylinositol. The dispersion consists of spherical unilamellar vesicles of a size distribution ranging from about 300 Å to 1 μm with the majority of particles having diameters between 0.1 and 0.4 μm. Electron micrographs of freeze-fractured samples of dilute (1–5%) unsonicated dispersions of the sodium salt of phosphatidylglycerol and phosphatidic acid were similar to those of Na^+ phosphatidylserine and phosphatidylinositol discussed above (data not shown). Fig. 3D is an electron micrograph of a freeze-fractured preparation of egg phosphatidylcholine containing 10% sodium phosphatidic acid. It is important to note that the addition of the negatively charged phosphatidic acid to phosphatidylcholine produced a dramatic change in the multilamellar packing typical for phosphatidylcholine. Predominantly unilamellar vesicles of a wide particle size distribution are observed ranging from 0.1 μm to several μm. As discussed for Fig. 3A, cross-sections reveal that the particles have an internal solvent-filled cavity that usually contains smaller spherical unilamellar vesicles. Freeze-fractured preparations of 1 to 2% unsonicated phosphatidylcholine doped with 10–30% Na^+ phosphatidylserine or lysophosphatidylglycerol gave electron micrographs very similar to those discussed for Fig. 3D. Thin-sectioning of the samples discussed above provides unambiguous evidence that the vesicles present in dilute unsonicated aqueous dispersions of charged lipids are unilamellar; furthermore that the smaller particles entrapped in the cavity of larger vesicles

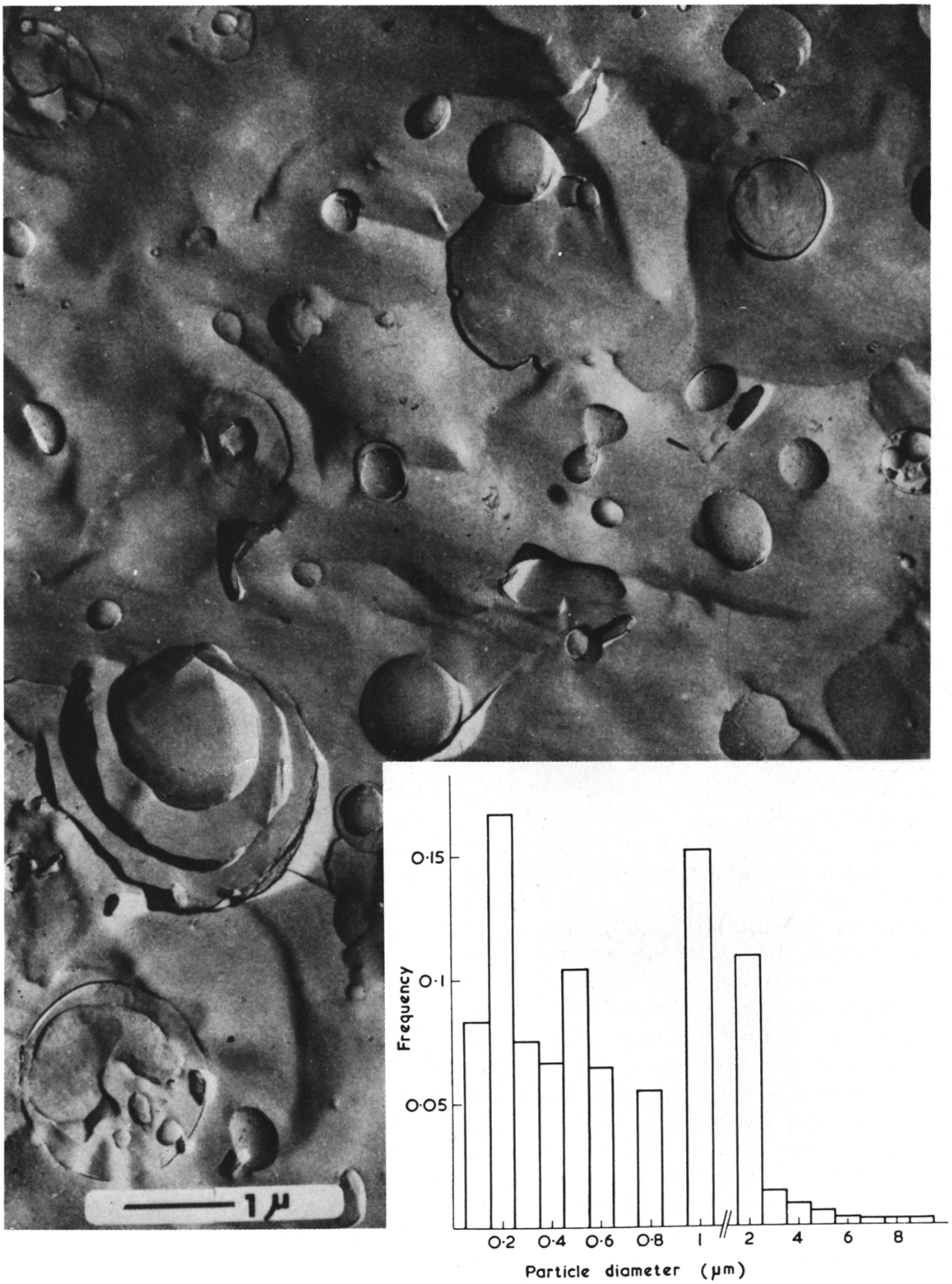
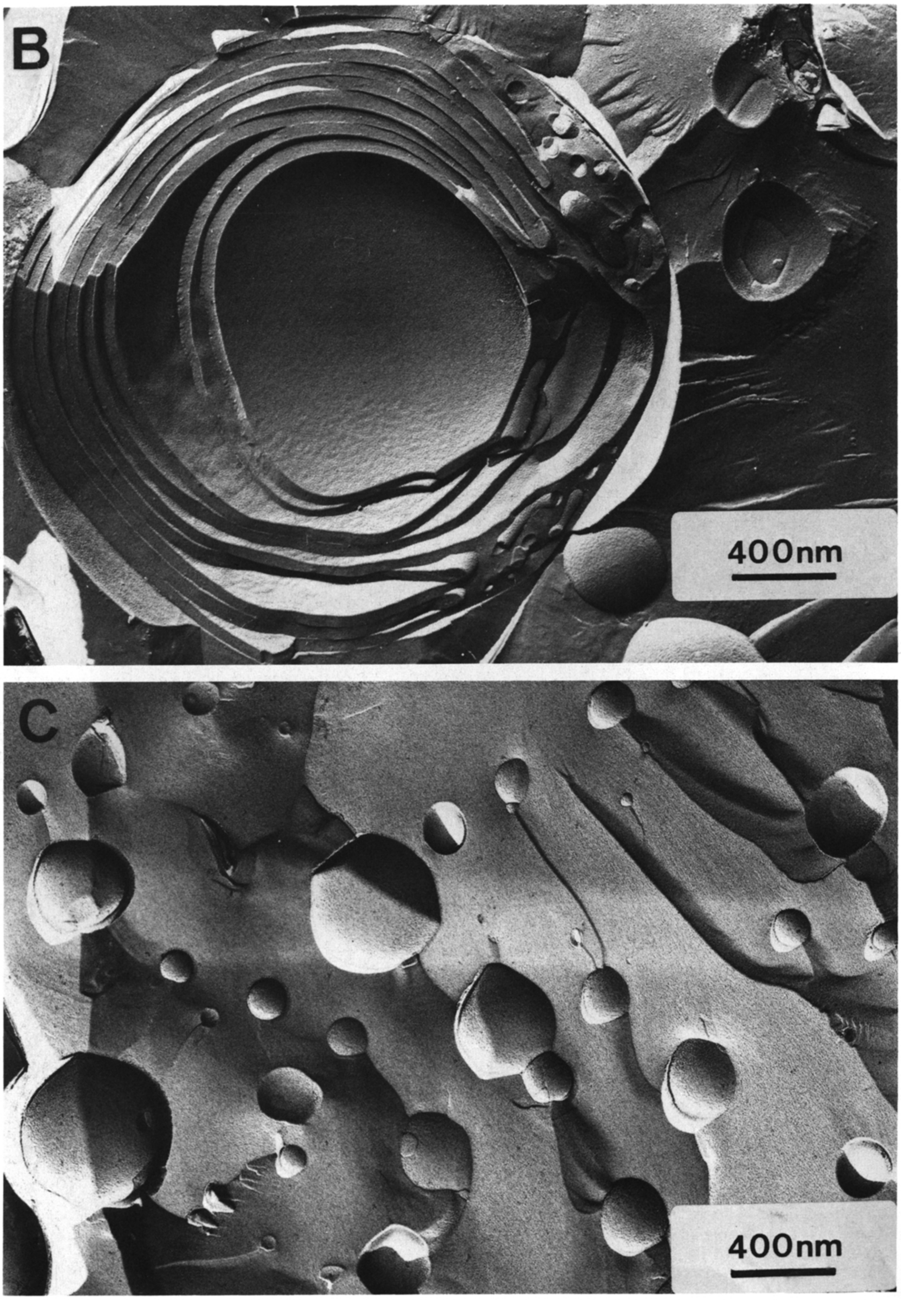


Fig. 3. Electron micrographs of freeze-fractured samples of unsonicated aqueous phospholipid dispersions. (A) 5% Na$^+$ ox brain phosphatidylserine; inset; bar histogram representing the particle size distribution derived from A. The frequency $f_i/\Sigma_i f_i$ is plotted as a function of the particle diameter.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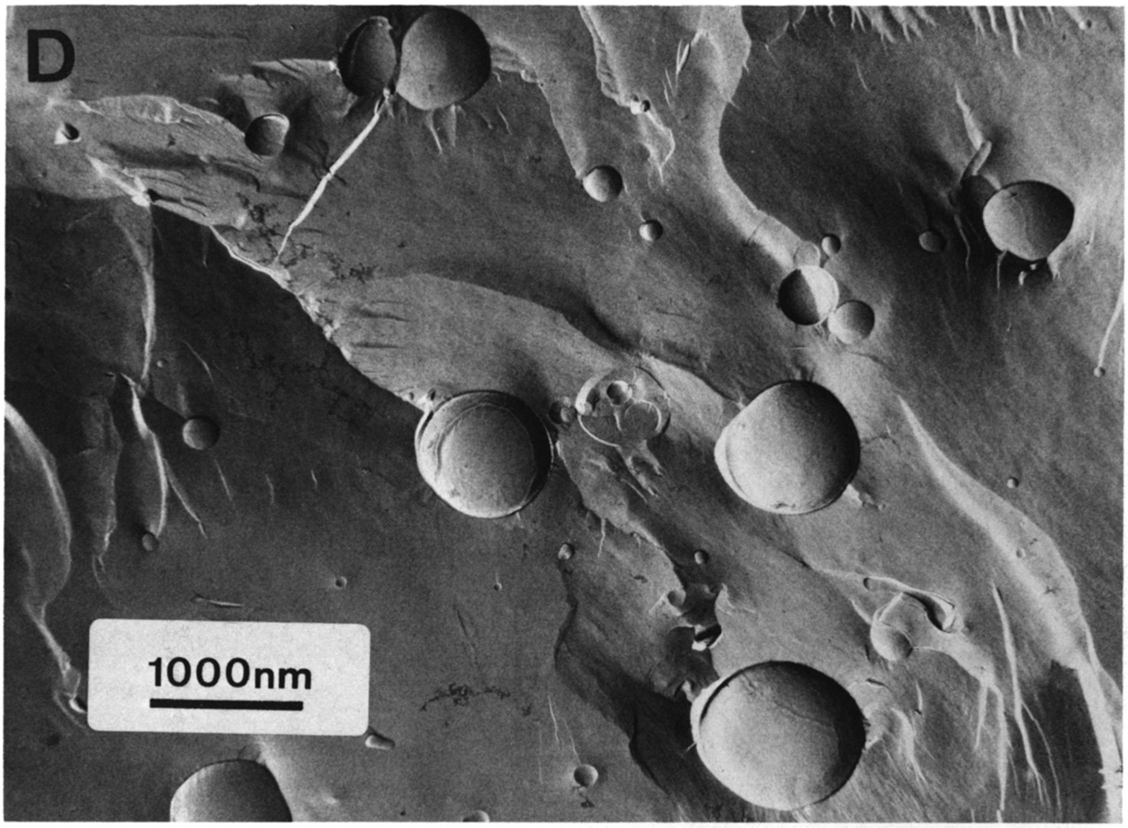
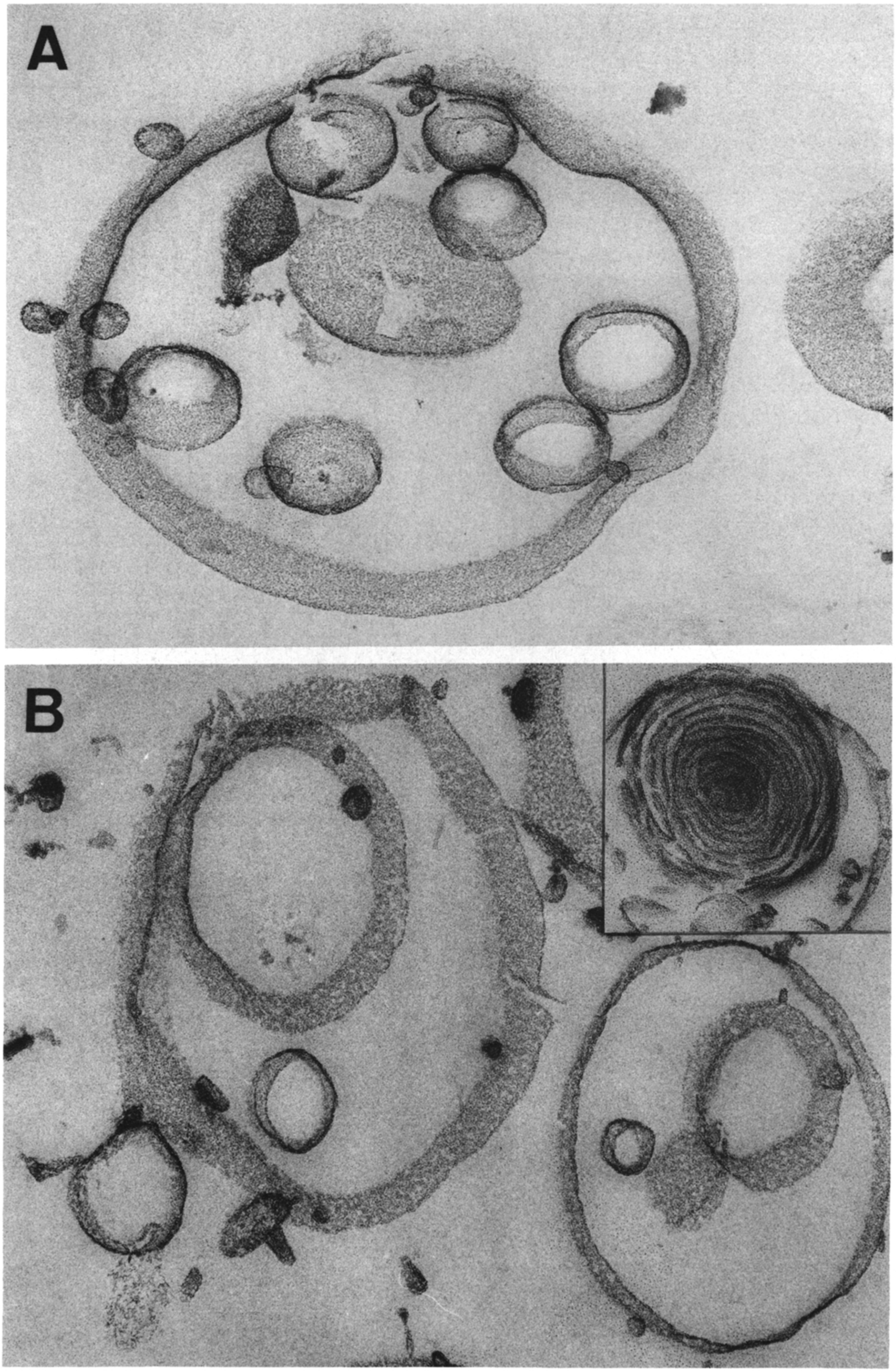


Fig. 3. Electron micrographs of freeze-fractured samples of unsonicated aqueous phospholipid dispersions (B) Cross-section of a large particle present in the dispersion described under (A). (C) 5% Na^+ phosphatidylinositol from wheat germ. (D) 1% egg phosphatidylcholine containing 10% egg phosphatidic acid. The dispersion was transiently exposed to a pH of approx. 12 (less than 1 min) by adding NaOH and then adjusted to pH 7 to 8 with HCl. Unsonicated dispersions were prepared as described under Experimental.

are also unilamellar. Thin-sectioned samples of unsonicated aqueous dispersions of phosphatidylcholine containing 10% and 30% Na^+ phosphatidic acid are shown in Figs. 4A and B, respectively. These electron micrographs confirm the main features discussed in Fig. 3. The cavity of larger unilamellar vesicles is usually filled with smaller ones; in a few exceptions the particles show multilamellar packing (cf. inset Fig. 4B) consistent with freeze-fractured preparations (cf. Fig. 3B). A thin-sectioned preparation of an aqueous unsonicated dispersion of phosphatidylcholine doped with 10% lysophosphatidylglycerol is shown in Fig. 4C.

The ratio P_i/P_t determined as described in Methods is strong evidence for the presence of unilamellar vesicles in the excess water region of the phase diagram of charged lipids. This ratio was determined for unsonicated dispersions of ox brain phosphatidylserine (10 mg/ml) and dispersions of egg phosphatidylcholine containing 10 mol% Na^+ phosphatidic acid. In both cases the ratio P_i/P_t was close to 0.5 as expected for large unilamellar vesicles with a diameter > 1000 Å. For comparison the P_i/P_t value for sonicated phosphatidylserine or phosphatidic acid dispersions varied between 0.19 and 0.32 (average ± S.D. = 0.25 ± 0.04). Further, the determination of the internal aqueous vesicle volume V_i by the method of $^{35}SO_4^{2-}$ encapsulation gives values in reasonable agreement with the vesicle size determined by electron microscopy. For the mixed phospholipid dispersion consisting of egg phosphatidylcholine and 10% Na^+ phosphatidic acid V_i varied between 5 and 15 l/mol lipid, corresponding to a vesicle size of 1500 to 4500 Å. The average vesicle size derived from


A
B

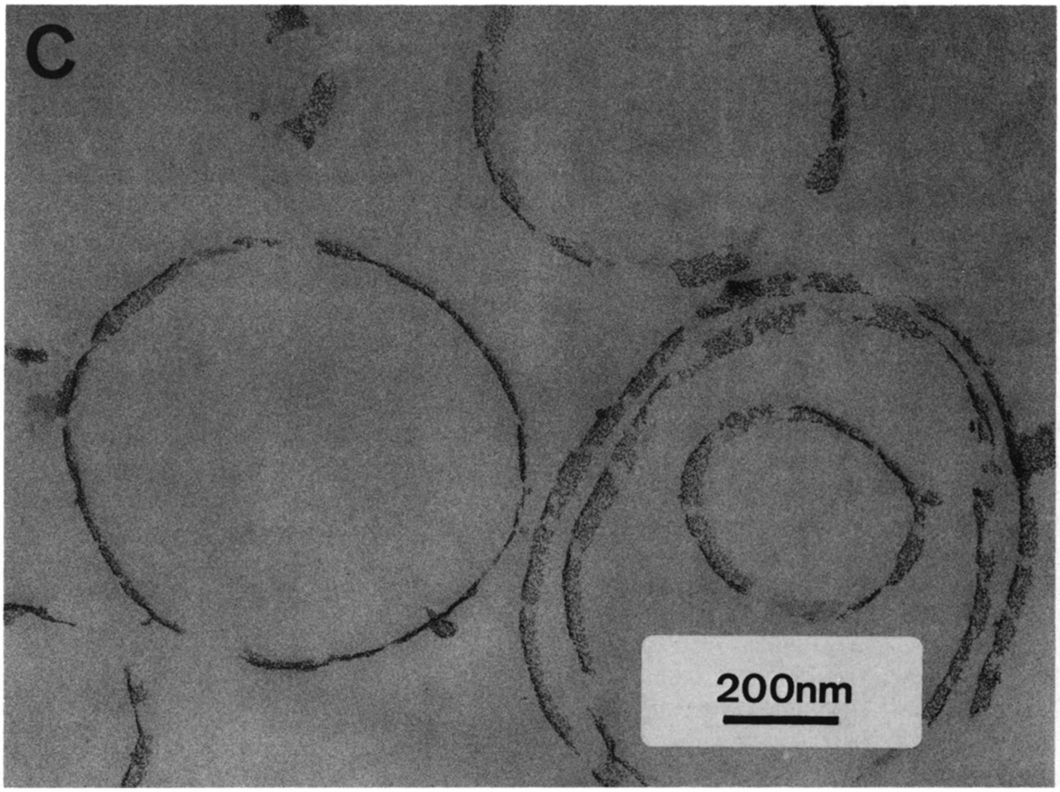


Fig. 4. Electron micrographs of thin-sectioned samples of unsonicated aqueous phospholipid dispersions. 1% egg phosphatidylcholine containing 10% (A) and 30% (B) egg phosphatidic acid. The dispersions were transiently exposed to a pH of approx. 12 by adding NaOH and then the pH was adjusted to 7–8 with HCl. Inset: occasionally multilamellar structures were observed. (C) 1% egg phosphatidylcholine containing 10% lysophosphatidylglycerol pH = 8.

electron micrographs of a freeze-fractured preparation of this mixed lipid dispersion is 3300 Å.

Discussion

On the basis of their swelling properties lipids forming smectic phases may be grouped into two classes: (I) neutral and isoelectric lipids with no net charge, which show no or only limited swelling (the thickness of the water layer is limited to a rather narrow range, usually not more than 25 Å); (II) lipids with a net charge, which show continuous swelling with increasing water content. All the water appears to be intercalated between the lipid bilayers up to relatively high water contents. The exact value has not been determined for any of the charged lipids, but X-ray experiments would suggest that at least up to 70 or 80% H_2O a single lamellar swelling phase exists. X-ray and electron microscopy data presented here show that the thickness of the water layer may be much larger than the lipid bilayer thickness. The question of the state of H_2O intercalated between phosphatidylserine bilayers has been investigated by NMR. From deuterium NMR measurements on the system (^2H_2O-Na^+ ox brain phosphatidylserine) it was concluded that the molecular motion of about the first 20 water molecules (approx. 30% H_2O) is restricted due to interaction with the lipid polar group; this amount of water was therefore designated as bound [18]. Water in excess of this amount (approx. 30%) has motional characteristics identical to free water. Therefore in the lamellar swelling phase at approx. 75% H_2O ($\simeq$ 140 mole H_2O/mole lipid) water in excess of 20 molecules is free water. Despite this fact it is practical to refer to this phase as a single lamellar phase as has been done throughout this paper. Although this terminology seems arbitrary, it is analogous to the one-phase system of neutral and isoelectric lipids. Characteristic of this one-phase system is a well-defined low-angle X-ray diffraction pattern.

X-ray diffraction and electron microscopy provide evidence that at high water content (about 70 to 80%) the stacking order is decreased. Both techniques indicate that at this dilution, which may vary from system to system, the multilamellar structures break up with each lamella sealing off to form closed unilamellar vesicles. We propose that this is termed a two-phase system consisting of fully-hydrated unilamellar vesicles dispersed in excess free water. In Fig. 5 the phase diagram of a pure, negatively charged phospholipid is given showing the general features discussed above. The precise position in the phase diagram of dimyristoylphosphatidylserine where the transformation of the one-phase system into the two-phase system occurs is unknown. In the liquid-crystalline L_α state it is assumed to be in the hatched area of the phase diagram (Fig. 5) at water contents in excess of 70%. Evidence from electron microscopy (cf. Fig. 4 A and B) suggests that there may not be a sharp transition between the one-phase (multilamellar) and two-phase (unilamellar) region, but a gradual one (Fig. 5). Evidence presented in this paper leads to the following generalizations about the phase behaviour of charged lipids: charged lipids forming smectic phases in water show continuous swelling up to high water contents. At a certain dilution or over a range of dilutions, usually greater than 70%, there is a transition from a one-phase to a two-phase system. The fundamental structural unit in the two-phase region is the unilamellar vesicle, a lipid particle surrounded by a single bilayer. The infinite swelling and the formation of unilamellar vesicles in excess water are general features in the phase behaviour of charged lipids that are not observed in the phase behaviour of neutral and isoelectric lipids. The vesiculation occurs in a rather narrow region on the right-hand side of the phase diagram (Fig. 5). The aspects of the phase behaviour of charged lipids discussed here are important in terms of industrial applications of phospholipids, particularly in liposome technology. The 'dispersing' effect of charged lipids has been known for a long time. Bangham and his collaborators [19] were the first to make good use of the repulsive forces and the dispersing effect of charged lipid bilayers.

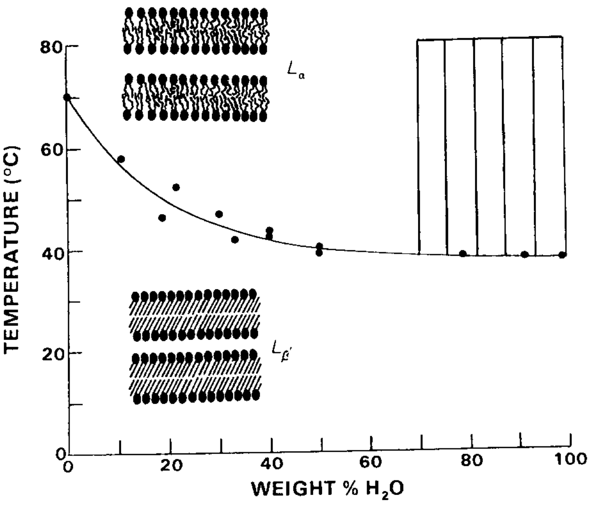


Fig. 5. Phase diagram of NH_4^+ dimyristoylphosphatidylserine in H_2O. The solid circles represent the temperature of the transition from the hydrated gel ($L_{\beta'}$) to the hydrated liquid-crystalline lamellar phase L_α as determined by differential scanning calorimetry [3]. The hatched area on the right of the phase diagram represents the region where the transformation of the one-phase system into the two-phase system occurs, i.e. where the multilamellar packing breaks up and large unilamellar vesicles are formed.

In this context it is important to note that continuous swelling and formation of unilamellar vesicles is a property not restricted to pure charged lipids like phosphatidylserine. It is also observed with uncharged lipid bilayers doped with relatively small quantities of positively or negatively charged lipids or detergents. As shown in Fig. 1B, a few percent of the charged species are sufficient to convert the phase behaviour typical for neutral and isoelectric lipids to that of charged lipids. The conversion apparently occurs at low surface charge densities.

It is clear from the experiments presented that the swelling is essentially independent of the nature of the charge and also the chemical nature of the charged group. That the swelling is a purely electrostatic phenomenon is evident from the effect of cations on swelling. The swelling behaviour and the effect of ions on it can be explained qualitatively and reasonably well quantitatively in terms of an equilibrium between the repulsive forces of the double-layer potential and the attractive van der Waals forces between opposing bilayers (the quantitative treatment of this equilibrium will be the subject of a separate publication). Addition of sufficient cations makes a fully swollen phosphatidylserine dispersion shrink. Cations as counterions accumulate in the electrical double layer and effectively screen the negative surface charges. Water between bilayers is extruded and consequently d decreases. The packing of phosphatidylserine in excess NaCl or salt in general is similar to that of phosphatidylcholine; the swelling is limited (Fig. 1a) and the two-phase system consists of multilamellar structures dispersed in excess water. The addition of 0.5 M NaCl to phosphatidylserine vesicles in excess water (two-phase system) induced aggregation and fusion of the vesicles leading, in equilibrium, to the same multilamellar structure as described above for a 50% phosphatidylserine dispersion in excess NaCl.

The various aspects of phase behaviour of charged lipids discussed here have important implications in several areas: (I) in the use of dilute aqueous unsonicated dispersions of charged phospholipids or mixed phospholipids containing charged lipids as model membranes; (II) in the assembly of artificial membranes reconstituted from membrane proteins and pure or mixed phospholipids carrying a net negative charge; (III) in liposome technology. Regarding the application of charged lipids two aspects of the phase behaviour discussed here seem to be important. (I) The phase behaviour of neutral and isoelectric lipids with the characteristic limited swelling in H_2O can be easily converted to that typical of pure charged lipids; this is achieved by doping the uncharged lipid with small quantities of a charged lipid. (II) Conversely, 'infinitely' swollen bilayers or unilamellar vesicles of charged lipids in excess H_2O (i.e., in the two-phase domain of the phase diagram) can be made to switch reversibly to multilamellar structures with limited swelling. This is demonstrated for phosphatidylserine in Scheme I.

$$\boxed{\text{infinitely swollen multilamellar structures or unilamellar vesicles in excess } H_2O} \underset{\text{removal of cation}}{\overset{\text{addition of excess cation}}{\rightleftharpoons}} \boxed{\text{multilamellar structures exhibiting limited swelling}}$$

Scheme I.

By adding sufficient cations, e.g. Na^+ or Ca^{2+}, unilamellar vesicles are transformed into ordered multilamellar structures. Removal of the ions by dialysis or any other method restores the original system of unilamellar vesicles in excess water. It is easy to verify the above scheme by electron microscopy (data not shown). Papahadjopoulos and his coworkers [20] have described such a scheme using ox brain phosphatidylserine with and without Ca^{2+}.

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