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PHOSPHOLIPID MODEL MEMBRANES

I. STRUCTURAL CHARACTERISTICS OF HYDRATED LIQUID CRYSTALS

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SUMMARY

Phospholipid liquid crystals and related (vesicular) structures were prepared from naturally occurring, purified phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, and phosphatidylinositol, and various mixtures of these compounds, swollen in aqueous salt solutions. The structural characteristics of the above particles prepared by various methods were investigated by optical, electron microscopy and X-ray diffraction methods. Large myelinic figures, as well as small vesicle-type particles, were observed. The size and shape of the particles as well as the degree of birefringence and the extent to which negative stain penetrates between the lamellae during electron microscopy were characteristic of the various phospholipid species used. Phosphatidylethanolamine was exceptional in its tendency to form aggregated particles of high 'porosity' in isotonic salt solutions. X-ray diffraction showed the presence of multi-lamellar structures with repeating distances varying from 54 Å for phosphatidylethanolamine, to 75 Å for phosphatidylserine and 125 Å for phosphatidylserine–cytochrome *c* complex. Ultrasonication produced small (approx. 500 Å diameter) sac-like vesicles. Similar structures were formed without sonication, from phosphatidylcholine and phosphatidylcholine–phosphatidylethanolamine mixtures, when a small amount of cytochrome *c* or albumin were present. The above structural characteristics are discussed in terms of the permeability properties in the following article.

INTRODUCTION

Recent advances in the physiology and biochemistry of cell membranes[1] have been accompanied by increased interest in model membrane systems[2–6]. Such systems provide an opportunity to investigate the characteristics of individual membrane constituents and to relate chemical structure and physical properties to physiological

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functions. MUELLER *et al.*[7] have developed an important technique in which a phospholipid membrane of bimolecular thickness ('black film') separates two aqueous compartments. This system is ideally suited to electrochemical studies relevant to the physiology of excitable tissues[8–10]. However, the extent to which the system can be used for biochemical investigations is somewhat limited by the small area of membrane involved, necessitating the use of sensitive analytical methods for any study of transport phenomena. An alternative system, utilizing the ability of natural phospholipids to form liquid crystals which incorporate water and low molecular weight solutes between bimolecular lamellae, was recently described by BANGHAM, STANDISH AND WATKINS[3].

Evidence derived from X-ray diffraction[11,12], electron microscopy[13,14], and optical birefringence[15] indicates that when phospholipids are equilibrated with excess water or aqueous salt solutions, they form liquid crystals of the smectic mesophase type, composed of bimolecular lipid lamellae separated by aqueous layers. BANGHAM, STANDISH AND WATKINS[3] presented evidence suggesting that the aqueous compartments within the liquid crystals are completely enclosed by the bimolecular phospholipid membranes. Although not readily amenable to study by electrical techniques, this system presents excellent opportunities for investigating the diffusion rates of ions and other compounds through phospholipid membranes of well defined chemical structure and to study the influence of various physical and chemical agents on such diffusion.

The work reported here constitutes a further study of the structural characteristics of phospholipid liquid crystals with special reference to their applicability as models for membrane structure and function. A number of naturally occurring phospholipids have been isolated in a purified form and their tendency to 'swell' when in contact with water was investigated. Parallel studies on the morphology of resulting particles prepared under varying conditions were made with the aid of the polarizing light microscope, the electron microscope, and X-ray diffraction data. The permeability properties of the above liquid crystalline particles are presented in the accompanying article[16], where correlations have been attempted between these properties and the structural characteristics described here.

MATERIALS AND METHODS

Materials

All reagents were A.R. grade and were used without further purification. The water was double glass-distilled over $KMnO_4$. Aluminium oxide was obtained from Hopkin and Williams (Brockmann activity 1, 100–200 mesh); silicic acid from Mallinckrodt (100 mesh); hyflo super-cel from Koch–Light laboratories; butylated hydroxytoluene (Puriss) from Koch–Light laboratories; silica gel H from E. Merck A.G.; cytochrome *c* (horse heart, type III) from Sigma Chemical Co.; bovine plasma albumin ('crystallized') from Armour Pharmaceutical Co.

Egg yolk phospholipids

Egg yolks were extracted twice with acetone at room temperature. The acetone-insoluble fraction was extracted twice with chloroform–methanol (1:1, v/v). The extracted material was dried under vacuum, dissolved in petroleum ether (60–80°

fraction) and precipitated with acetone. The precipitate (crude egg phospholipid) was stored dry under N_2 at −20° until further purification, using minor modifications of earlier methods[17,18].

Phosphatidylcholine

Crude egg phospholipid was passed first through aluminium oxide (elution with chloroform–methanol, 1:1, v/v). The crude phosphatidylcholine thus obtained was further purified on silicic acid (elution with chloroform–methanol, 2:1, v/v). The final product was stored at −20° in chloroform.

Phosphatidylethanolamine

Crude egg phospholipid was passed through silicic acid–hyflo super-cel (2:1, w/w) and eluted with chloroform–methanol (6:1, v/v). Most of the yellow colour was eluted with the solvent front, followed by pure phosphatidylethanolamine. This compound is very susceptible to oxidation as indicated by an increase in the absorption at 230–280 mμ of a sample of phosphatidylethanolamine left at room temperature in petroleum ether. This change can be slowed down considerably by addition of a small amount of an antioxidant (0.02 μmole butylated hydroxytoluene/μmole phosphorus) or simply by storing the sample in a sealed ampoule under N_2 at −20°.

Phosphatidic acid

The choline moiety was removed from egg yolk phosphatidylcholine by enzymatic hydrolysis using extracts from savoy cabbage. The preparation of cabbage extracts and the reaction mixture were essentially as described by DAVIDSON AND LONG[19]. Approx. 5 g of crude phosphatidylcholine were dispersed in 500 ml of 0.1 M acetate buffer (pH 5.6) and mixed with 350 ml of crude enzyme solution, 100 ml of 1 M $CaCl_2$ and 400 ml diethyl ether. The mixture was agitated at 25° for approx. 3 h. The phases were allowed to separate and the upper (ether) phase, together with the thick white interface, were centrifuged at 10 000 × *g* for 1 h at 4°. This resulted in a clear upper (ether) phase, a central white precipitate, and a clear aqueous phase at the bottom. The ether phase contained very little phosphorus and was discarded along with the lower aqueous phase. The white precipitate, consisting mostly of the Ca^{2+} salt of phosphatidic acid, was converted to the acid form and then to the sodium form by successive washings with 0.1 M HCl and 0.1 M NaCl in the chloroform–methanol–water system of FOLCH, LEES AND SLOANE-STANLEY[20]. The washed product contained approx. 85% of the original phosphorus and was further purified by passage through silicic acid and elution with chloroform–methanol (9.6:0.4, v/v). It was stored as a solution in chloroform at −20°.

Beef brain phospholipids

Brains were obtained immediately after slaughter and were kept in ice for approx. 30 min until extraction could be commenced. About 800 g (2 or 3 brains) were processed in one batch, essentially as described by FOLCH[21] for the preparation of brain cephalin. Minor modifications were made when necessary to avoid air oxidation during the procedure. The final product (12 g) represented the fraction which was insoluble in acetone and ethanol, soluble in petroleum ether and diethyl ether. Thin-layer chromatography indicated the presence of phosphatidylethanolamine,

phosphatidylserine, and phosphatidylinositol as the main constituents along with some cerebrosides and sulfatides. The fraction was kept as a chloroform solution at −20°.

Further fractionation was performed on DEAE-cellulose following the method described by ROUSER *et al.*[22]. The procedure was improved considerably by the use of 'microgranular' Whatman DE 32 cellulose. This material packed better than older products, thus eliminating the problem of channelling and making possible the use of large columns (100 g of cellulose in chloroform–methanol (7:3, v/v) give a column (4.2 cm × 36 cm) and 500 ml total bed volume). The loading factor was also improved (2.5–3 mg phosphorus per g of cellulose). Chromatography was begun with chloroform–methanol (7:3, v/v), which eluted the cerebrosides and phosphatidylcholine, followed by pure phosphatidylethanolamine. The column was then washed with methanol which eluted some ninhydrin-reacting material not containing phosphorus. Glacial acetic acid was then used to elute phosphatidylserine. The solvent was removed by freeze-drying. The residual white powder was taken up in chloroform and was shown to contain pure phosphatidylserine. Finally, phosphatidylinositol and sulfatides were eluted with chloroform–methanol–conc. ammonia, sp. gr., 0.91 (2:1:0.06, v/v/v), containing ammonium acetate (0.05 M). The combined fraction was washed[20] to remove ammonia and ammonium acetate. Subsequently phosphatidylinositol was separated from sulfatides on a small DEAE-cellulose column with chloroform–acetic acid (3:1, v/v), containing different amounts of ammonium acetate[22]; phosphatidylinositol was eluted with an ammonium acetate concentration of 0.02 M, and sulfatides when this concentration was raised to 0.2 M.

All acidic phospholipids (phosphatidylserine, phosphatidic acid, phosphatidylinositol) were washed again after column chromatography to ensure that all contaminating higher valency metals and water soluble materials were eliminated. This involved conversion to the acid form (equilibration in chloroform–methanol–0.1 M HCl, 2:1:0.6, v/v/v) and subsequent conversion to the desired salt form (equilibration in chloroform–methanol–0.1 M NaCl or KCl containing calculated amounts of NaOH or KOH to neutralise the acid groups). Finally each fraction was dried under vacuum, dissolved in chloroform and stored at −20° under N_2.

Characterization

The different components present in each phospholipid fraction were identified

TABLE I

FATTY ACID COMPOSITION OF SOME PURIFIED PHOSPHOLIPIDS

Each fatty acid is expressed as percentage of total fatty acids in each phospholipid. Identification of each peak was on the basis of retention time relative to palmitate, on polyethylene glycol adipate columns at 180°. Phosphatidylcholine (a) represents the early part of the lecithin peak during silicic acid chromatography and phosphatidylcholine (b) the later part of the same peak.

Lipid	*16:0*	*18:0*	*18:1*	*18:2*	*20:1*	*20:4**,+	*22:6**,§
Egg phosphatidylcholine (a)	33.6	20.3	25.8	11.2	—	7.5	2.2
Egg phosphatidylcholine (b)	36.2	13.4	36.9	10.8	—	1.8	0.8
Egg phosphatidylethanolamine	22.0	27.0	24.0	7.3	—	2.5	16.6
Brain phosphatidylserine	—	48.9	37.2	—	4.8	4.0	3.8
Brain phosphatidylinositol	6.1	43.8	12.5	—	—	28.5	8.0

* Tentative identification only. The percentages shown in these columns include minor peaks appearing: + between 20:1 and 20:4 and § between 20:4 and 22:6.

by thin-layer chromatography. Silica gel H (no binder) was used for support. The solvent was chloroform–methanol–7 M aq. ammonia (230:90:15, v/v/v)[23]. The use of an 'S-chamber'[24] improved the compactness of the spots and decreased the time of development. This method proved to be particularly successful for the separation of acidic phospholipids. After development, the plates were air-dried, sprayed with Rhodamine 6G, 0.005% aq., and viewed under ultraviolet light. The position of each fluorescent spot was marked. The plates were then dried again, sprayed with ninhydrin and heated at 100° for 5 min for detection of free amino groups. They were then sprayed with molybdenum blue reagent[25] for the detection of phosphate groups. Cerebrosides and sulfatides were detected by the presence of reducing sugars, phosphatidylcholine and sphingomyelin by the presence of choline, and total organic material by spraying the plates with 50% sulfuric acid and heating at 110° for 16 min. Fig. 1 is a photograph of a thin-layer plate developed by the above method, illustrating the relative positions of individual phospholipids. The purity of the isolated phospholipids was also checked by the successive hydrolysis technique* of DAWSON, HEMINGTON AND DAVENPORT[26]. The fatty acid composition of some of these compounds is shown in Table I*.

Optical microscopy

For observations under the light microscope, a small amount of the lipid in chloroform was dried on a glass cavity slide under a stream of N_2 or air. The dry material was then covered with salt solution, a cover slip sealed into position, and the preparation allowed to equilibrate for a few hours at room temperature. Crossed polaroids were used with a first-order red compensator so that birefringent and non-birefringent material could be clearly differentiated. Dispersions produced after vigorous agitation of dry phospholipids in aqueous salt solution, appropriately diluted, were also examined.

Electron microscopy

The negative staining technique[14] using ammonium molybdate was as follows: A total of 2 μmoles of phospholipid, or of phospholipid containing one or more of the various lipophilic additives, was dispersed in 1 ml of ammonium acetate (260–300 mosM, pH 7.0), using one of the dispersion methods described in the accompanying article[16]. The use of ammonium acetate, a volatile salt instead of the KCl or NaCl used in the permeability studies[16], avoided crystallization of inorganic salts on the microscope grids while preserving the same ionic strength during preparation of the dispersions as for the diffusion measurements. Where cytochrome or bovine plasma albumin were included in the aqueous medium, the protein concentration was 0.4 mg/ml. Immediately prior to their application to the grid, phospholipid dispersions were negatively stained by the addition of 0.3–1 vol. of ammonium molybdate solution (pH 6.8) of osmotic strength equal to that of the ammonium acetate. Phosphomolybdate has been shown superior to the previously described phosphotungstate in preserving the integrity of phospholipid particles (R. W. HORNE, personal communication). An AEI electron microscope, model EM 6B, was used.

* The fatty acid and successive hydrolysis analysis was courteously provided by Drs. P. KEMP and A. SHELTAWY of the Department of Biochemistry.

X-ray diffraction

A total of 2–5 μmoles of phospholipid was suspended in 0.5 ml of an aqueous solution of the appropriate salt (usually 145 mM KCl or 130 mM KCl/15 mM Tris–HCl, pH 7.4). The suspension was left to equilibrate for a few hours at room temperature. It was then centrifuged and the resulting loose white pellet transferred to a quartz tube (0.7 mm diameter). The apparatus was a Raymax 60 analytical X-ray diffraction unit. Radiation CuK_a nickel-filtered, 8 mA average tube current, 25 kV. The camera was built after a design by D. P. RILEY. Specimen to film distance, 13 cm. Exposure time, usually 15 h. Film: 45–150 ESTAR, Dental film.

RESULTS AND DISCUSSION

General characteristics of phospholipid dispersions

All of the phospholipids used in this study 'swell' spontaneously when the dry material is brought into contact with an aqueous solution at room temperature. The term 'swell' is used to signify the formation of a hydrated liquid-crystalline phase[27]. The degree of swelling and the size, shape, and general configuration of the resulting liquid crystals depend on the particular phospholipid used, as well as on the ionic strength, the valency of the ionic species, and the pH of the aqueous medium. Mechanical agitation promotes fragmentation of the liquid crystals, producing spheroidal particles of varying sizes, generally within the range 5–50 μ. During the shaking, the suspension is initially frothy, but when all the dry phospholipid forming a film on the glass walls has been suspended, no more frothing can be observed. This generalization does not apply, however, to dispersions of phosphatidylethanolamine or when protein is present. The absence of frothing upon completion of the phase change (from the coagel, or dry liquid crystal, to the smectic mesophase of the swollen particles) indicates a very low concentration of free molecules available to adsorb at the air–water interface. This is in accordance with the reported very low critical micelle concentration of phosphatidylcholine[28]. The absorbance of the resulting suspension varies with the size of individual particles and the interlamellar distance within each particle. The latter parameter depends on the fixed charge on the surface of each lamella and the ionic strength of the salt solution[29]. Ultrasonication of phospholipid suspensions produces further fragmentation of the particles, but the lamellar arrangement of the individual molecules is preserved. Depending on the surface charge of the phospholipid, the ionic strength of the medium, and the degree of sonication, optically clear suspensions can be produced containing small vesicles or sacs, the walls of which are constituted of one or more bimolecular leaflets (see electron microscopy results below).

Optical microscopy

Egg yolk phosphatidylcholine swells in water or salt solutions to form particles of varying size showing strong positive birefringence[3]. The shape of the particles is usually that of prolate spheroids although tubular myelinics can also be seen in some cases. Cholesterol can be incorporated in molar proportions of up to 50% without apparent change in the general morphology of the particles. A typical example is shown in Fig. 2a and b. The observed birefringence is the sum of the intrinsic component, usually positive in sign and due to the alignment of individual molecules

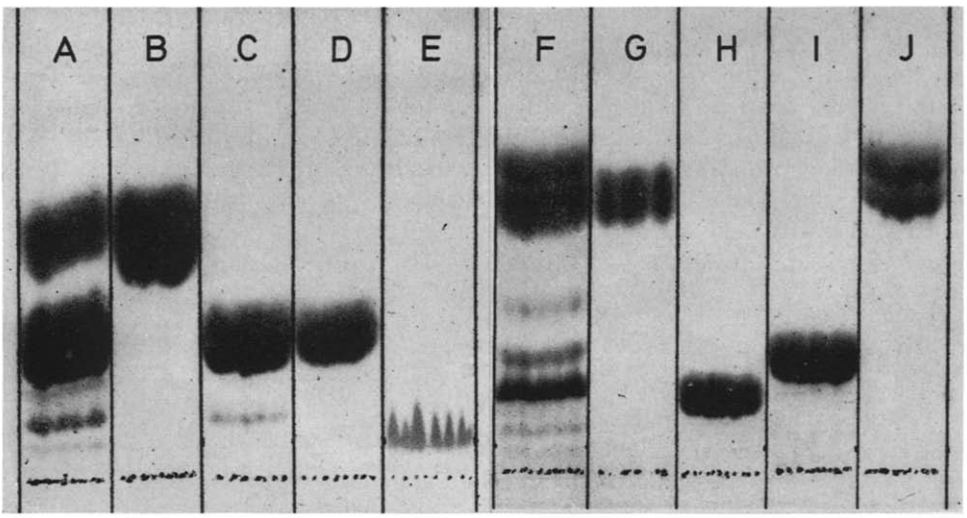

Fig. 1. Thin-layer chromatography of phospholipids. Support, Silica gel H; solvent, chloroform–methanol–7 M ammonia (230:90:15, v/v/v); detection, 50% H_2SO_4 spray. Heating at 110° for 16 min. A, crude egg phospholipid; B, phosphatidylethanolamine; C, crude phosphatidylcholine; D, phosphatidylcholine; E, phosphatidic acid. On a separate plate; F, brain 'cephalin'; G, brain phosphatidylethanolamine; H, phosphatidylserine; I, phosphatidylinositol; J, sulfatides.

within each lamella, and the form component which is negative and due to the stacking of the lamellae[15]. The intrinsic component is usually stronger than the form component since it is caused by a much finer structure of the crystal lattice[15]. When the equilibrium distance between lamellae increases (as would be expected for negatively charged phospholipids due to electrostatic repulsive forces[29]), the intensity of the observed birefringence decreases as a result of the increase in the negative component contributed by form birefringence. The birefringence of phosphatidylcholine and of phosphatidylcholine–cholesterol (Fig. 2b) is independent of the ionic strength or the pH (within the range 3–12) of the swelling solution, indicating no change in the thickness of the water compartments between lamellae under these conditions. This behaviour is a reflection of the internally compensated zwitterionic form of the head-groups of the phosphatidylcholine molecules[30].

The swelling properties of the acidic phospholipids, including phosphatidylserine, phosphatidic acid, and phosphatidylinositol, show a marked dependence on the pH, ionic strength, and the presence of higher valency metals in the swelling medium. In general, no swelling is observed when the ionization of the phosphate group is suppressed by low pH nor when Ca^{2+} is present. When each of the above phospholipids is made to carry one net negative charge per molecule (pH, 5–9 for phosphatidylserine; 3–7 for phosphatidic acid; > 3 for phosphatidylinositol), swelling in 145 mM KCl or NaCl produces prolate spheroidal particles similar to, but generally smaller than, those shown in Fig. 2a for phosphatidylcholine. In contrast to phosphatidylcholine, however, the birefringence is only weakly positive, disappearing completely after a few hours of equilibration. The low positive birefringence is taken as an indication of increased interlamellar spacing (as compared with phosphatidylcholine or phosphatidylethanolamine) due to the presence of the net negative charge.

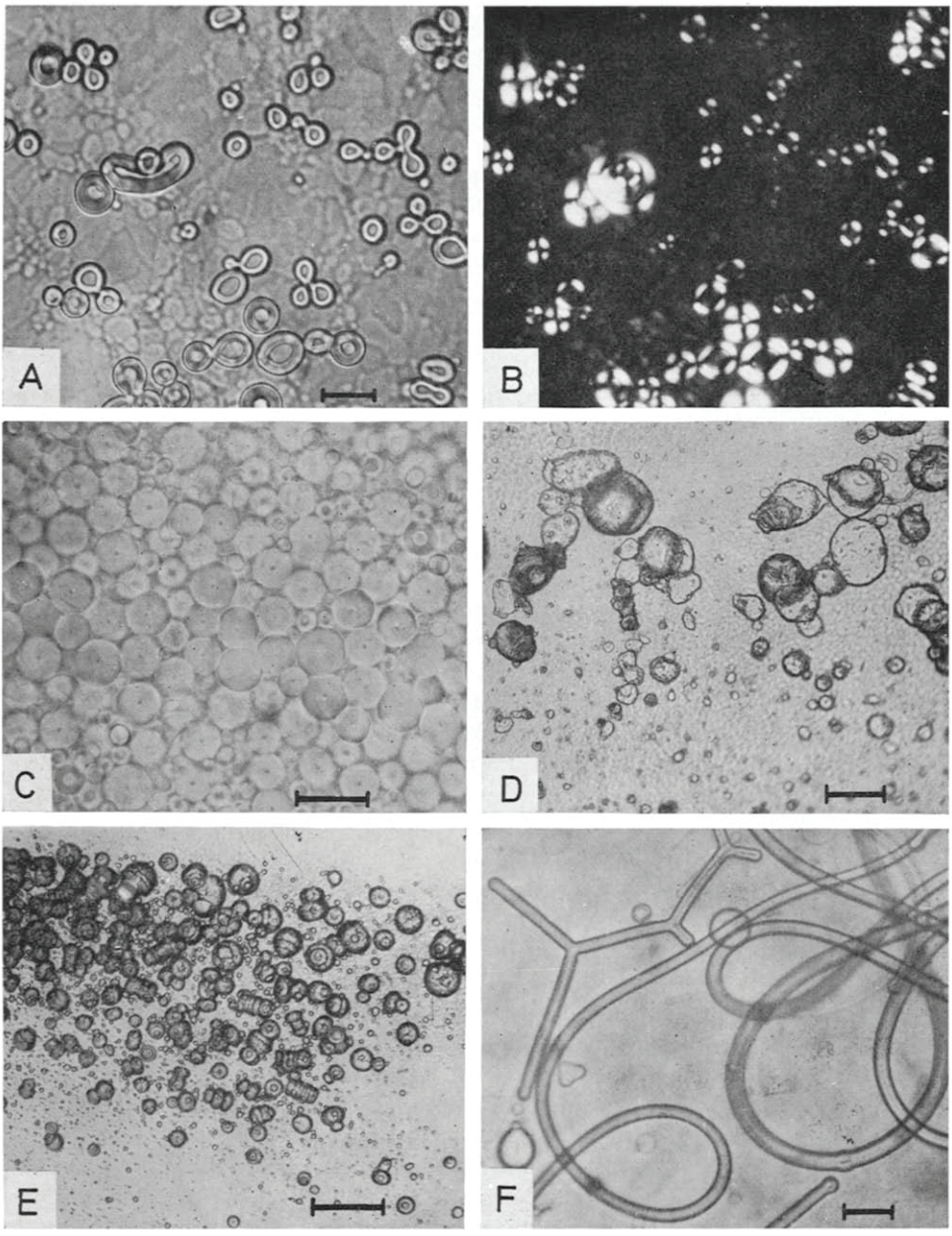

Fig. 2. Optical microscopy of hydrated phospholipid liquid crystals. A, phosphatidylcholine–cholesterol (equimolar) in 145 mM KCl; B, same as A but viewed through crossed Nicol prisms; C, phosphatidic acid in 145 mM KCl–Tris–HCl (pH 8.5); D, freshly prepared phosphatidylethanolamine in 145 mM KCl; E, phosphatidylethanolamine in 145 mM KCl; F, phosphatidylethanolamine in distilled water. Magnification: A, B, C, E: marker = 100 μ. C, F: marker = 20 μ.

In the case of phosphatidylserine, there is a diversity in the size of individual particles, while phosphatidic acid, and especially phosphatidylinositol, show a remarkable

homogeneity in particle size (3–5 μ in diameter). If the same ionic species of the above phospholipids are swollen in water or in salt solution of low ionic strength, the particles take the shape of spheres (Fig. 2c) rather than flat spheroids and exhibit weak negative birefringence. This preference for greater overall curvatures can also be observed at higher ionic strength (145 mM) when molecules carry two negative charges (*e.g.* phosphatidic acid at a pH above 8.0, see ref. 31), and can be interpreted in terms of the increased electrostatic repulsion between individual molecules. When the tonicity of the medium is altered after the particles have formed, similar shape changes take place which, in this case, can be attributed to 'osmotic' swelling and shrinking[32]. Among other factors bringing about changes in the configuration of otherwise stable phospholipid structures are: changes in pH, addition of divalent metals, or mechanical agitation.

The swelling properties of egg yolk phosphatidylethanolamine exhibit some remarkable characteristics which set it apart from the other phospholipids. When phosphatidylethanolamine is allowed to swell in a 145 mM solution of KCl or NaCl at pH 5–6, structures such as those shown in Fig. 2d and e are obtained. Their general characteristics are: (1) 'rough' external surfaces, (2) tendency to form irregular 'spongy' particles (Fig. 2d) and/or elongated aggregates similar to red cell 'rouleaux' (Fig. 2e), and (3) very strong positive birefringence. When phosphatidylethanolamine is dispersed by shaking or sonication in the same salt solution as above, the material settles rapidly in the form of large aggregates. The interpretation of this behaviour in terms of hydration and spatial arrangement of the head-group charges will be discussed in another section. The same phosphatidylethanolamine preparation when swollen in water or a very low ionic strength salt solution (ionic strength less than 2) produces long tubular structures exhibiting weak positive birefringence (Fig. 2f). Tubular formations with low birefringence can also be seen when the pH of the medium (irrespective of ionic strength) is raised above 10. Under the latter conditions the deprotonation of the free amino group on the phosphatidylethanolamine molecule produces a net negative charge, which would tend to increase the equilibrium distance between lamellae. However a certain amount of hydrolysis resulting in the formation of free fatty acids and lysophosphatidylethanolamine cannot be excluded at this pH.

The effect of air oxidation on the swelling properties of phosphatidylethanolamine was also examined. The presence of appreciable quantities of polyunsaturated fatty acid chains (Table I) makes this phospholipid particularly susceptible to autoxidation. For this purpose a sample of phosphatidylethanolamine was prepared under conditions which minimized contact with air. The lack of strong absorption in the region of 230–280 mμ indicated the absence of appreciable quantities of oxidative products[33]. When this material was allowed to swell in 145 mM salt solution only the 'rough' irregular structures shown in Fig. 2d were observed. The same preparation after storage for 1 month in chloroform at $-20°$ displayed a preference towards the elongated swollen aggregates shown in Fig. 2e. Storage of the same sample of phosphatidylethanolamine in chloroform at $-20°$ in the presence of the antioxidant butylated hydroxytoluene exhibited no such change in the swelling characteristics.

Electron microscopy

Earlier work on the ultrastructure of phospholipid aqueous dispersions[13,14]

has demonstrated the presence of lamellar structures. In the present study an investigation has been carried out on the influence of the different head-groups of phospholipids and of different methods of dispersion[16] on the size and shape of the resulting structures.

Vigorous mechanical shaking of phosphatidylcholine suspensions produced smooth-edged particles of varying sizes, mostly within the 500 Å–1 μ range. Most of the particles were completely unpenetrated by the negative stain, but in some, one or two of the outermost lamellae were plainly visible (Fig. 3a). Swelling in the presence of phosphomolybdate allowed greater penetration of the negative stain (Fig. 3b), and sonication decreased the size range of the particles. The smallest sonicated particles (Fig. 3c) probably conform to the dimensions of the phosphatidylcholine particles studied by SAUNDERS[34] although they appear as vesicles rather than 'micelles'.

Phosphatidylserine dispersions contained particles which were similar to those of phosphatidylcholine, specifically in being 'smooth-edged' and almost completely unpenetrated by the negative stain. Sonication produced a greater degree of penetration and the usual decrease in the average size of the particles. The penetrated particles showed the usual concentric lamellar pattern. Dispersions of phosphatidylserine to which Ca^{2+} had been added (in concentration producing increased leakage of captured K^+ or Na^+, see ref. 16) showed large aggregates of unpenetrated particles. Sonication of the aggregated suspensions allowed some penetration of the particles, demonstrating again a lamellar structure.

Prolonged sonication (45 min) of slightly turbid, hand-shaken dispersions of phosphatidylinositol resulted in an optically clear solution. Fig. 3d shows the remarkable homogeneity in particle size obtained by this method. The size range was about 200–1000 Å, with a large proportion being about 300–400 Å in diameter. The particles appear as small vesicles or prolate spheroids consisting of one or two lamellae, each of bimolecular thickness (approx. 50 Å). The general shape is remarkably similar to the prolate spheroids seen under the optical microscope.

Dispersions of phosphatidylethanolamine tended to flocculate with all of the methods of preparation. Particles seen in the electron microscope were usually aggregates. Lamellae were plainly visible, however, and were wavy in appearance (Fig. 3e), a property possibly related to the 'rough' surfaces of phosphatidylethanolamine particles seen under the optical microscope. Particles prepared from a 2:1 mixture of phosphatidylethanolamine and phosphatidylcholine had the general appearance of pure phosphatidylethanolamine; specifically, they were well penetrated by the negative stain and the outlines of the individual particles were often irregular ('rough-edged'). The latter characteristic was not as pronounced with particles made from a 1:1 mixture of phosphatidylethanolamine and phosphatidylcholine.

It is relevant to include here some recent observations of R. W. HORNE and J. C. WATKINS (unpublished) on the effects of protein on the structure of phospholipid particles. They have shown that dispersions of phosphatidylcholine and phosphatidylcholine–phosphatidylethanolamine mixtures made in the presence of cytochrome *c* and, to a lesser extent, bovine plasma albumin, contain a large number of small particles in the range 200–1000 Å (Fig. 3f). These appeared as small vesicles containing a central aqueous compartment (often penetrated by the negative stain)

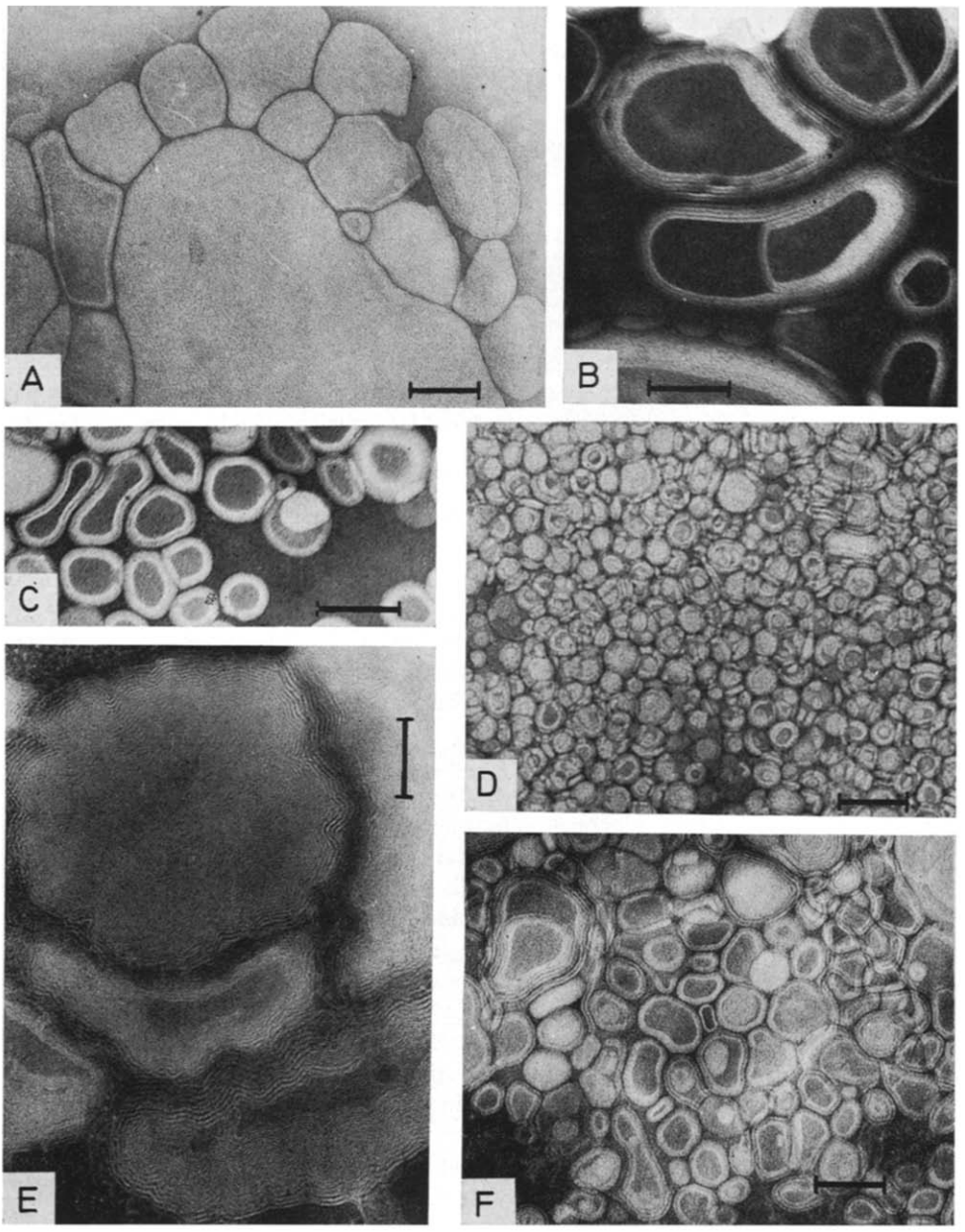

Fig. 3. Electron microscopy of hydrated phospholipid liquid crystals. A, D, E, F dispersed in 145 mM ammonium acetate, subsequently stained with ammonium molybdate. B, C: dispersed in 2% ammonium molybdate. A, B: phosphatidylcholine mechanically shaken; C: phosphatidylcholine sonicated for 20 min; D: phosphatidylinositol sonicated for 50 min; E: phosphatidylethanolamine mechanically shaken; F: phosphatidylcholine–phosphatidylethanolamine (1:1) mechanically shaken in the presence of cytochrome *c* (0.03 μmole/μmole of lipid). Magnification: marker = 0.1 μ (1000 Å).

and enclosed by a lipid shell composed most frequently of two or three bimolecular lamellae although a few single-membrane vesicles could also be seen. Larger structures were also present, especially in the presence of albumin, but many of these were obviously vesicular and bounded only by a few lamellae. All methods of preparation resulted in the formation of similar particles. Evidence as to whether protein adsorbs onto phosphatidylethanolamine–phosphatidylcholine particles will be discussed elsewhere[16]. It was also shown by HORNE AND WATKINS that the presence of cytochrome *c* during the dispersion of phosphatidylserine resulted in the appearance of large aggregates as would be expected for a poly-cation interacting with a poly-anion. Where individual particles could be seen, they exhibited 'rough-edge' boundaries, but lamellae were not clearly defined, probably because of lack of penetration of the negative stain (see X-ray results below). This work on lipoprotein systems is being continued and full details will appear later (HORNE, PAPAHADJOPOULOS, AND WATKINS, in preparation).

Low-angle X-ray diffraction

The long spacings obtained with the different phospholipids were found to vary considerably depending on the particular polar groups involved, the presence of water, of divalent metals and the pH of the aqueous phase. In every case where interferences of higher order were obtained, the Bragg spacings of all the lines were the integral orders of one fundamental spacing (1, $^1/_2$, $^1/_3$, $^1/_4$), indicating that the structure is lamellar[12]. In this case the repeat distance is taken to represent the thickness

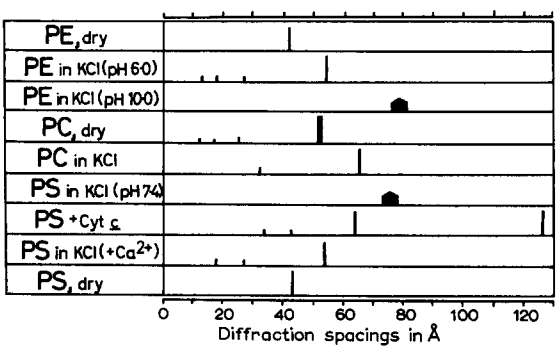

Fig. 4. Low-angle X-ray diffraction of dry and hydrated phospholipid liquid crystals. For details see section on METHODS. The lines shown in figure above represent a qualitative comparison of the relative intensities (represented by line height), and relative sharpness (represented by line width) of diffractions. The position of each line is the spacing in Å, calculated from the Bragg equation. PE, phosphatidylethanolamine; PC, phosphatidylcholine and PS, phosphatidylserine.

of one bimolecular lamella *plus* the distance between two adjacent lamellae. A summary of the X-ray results is given in Fig. 4. A repeating unit of 65 Å was characteristic of egg yolk phosphatidylcholine swollen in 145 mM KCl. The same phospholipid in dried form, obtained by evaporating chloroform solution in vacuum over P_2O_5, had a long spacing of 52 Å. It was not established, however, whether the procedure completely eliminates water of crystallization. Phosphatidylethanolamine gave smaller repeating units as compared with phosphatidylcholine; 42 Å for the

dry material and only 54 Å when it was swollen in 145 mM KCl. This disparity could be ascribed either to a different packing of the hydrocarbon chains of the two phospholipids or to a difference in the arrangement of the head-groups.

The second possibility could easily be explained by the evidence from infrared spectra[35] indicating proton-sharing between the phosphate and primary amino groups of phosphatidylethanolamine. This interaction, which would be expected to reduce the water of hydration, is not possible for phosphatidylcholine. However, CHAPMAN AND MORRISON[36] have recently reported that they could find no evidence for such a hydrogen bond between phosphate and amino groups in phosphatidylethanolamine. It is relevant to note that differential thermal analysis of distearoyl phosphatidylcholine and phosphatidylethanolamine[37] indicates stronger cohesion for the crystalline form of the latter compound. Trans-lamellar interdigitation of the head-groups[38] or their arrangement perpendicular to the axis of the hydrocarbon chains[39] could result in both inter-molecular and inter-lamellar cohesion through ionic and hydrogen bonding. Either arrangement would be easier for phosphatidylethanolamine than for phosphatidylcholine because of the smaller volume of the primary amino groups of phosphatidylethanolamine compared with the trimethyl ammonium group of phosphatidylcholine. When the pH of the swelling medium is high (pH $>$ 10), phosphatidylethanolamine swells easily giving a repeating unit of approx. 80 Å with considerable spreading, presumably due to the deprotonation of the amino group.

The negatively charged phosphatidylserine, swollen in 145 mM KCl–Tris (9:1, v/v) (pH 7.4), exhibits a considerably larger inter-lamellar water compartment, when compared with either phosphatidylethanolamine or phosphatidylcholine at similar pH; (43 Å when dry, 75 Å when swollen in KCl–Tris)*. When phosphatidylserine is swollen in the presence of 0.1 molar ratio of cytochrome *c*, the repeating unit becomes 126 Å, indicating the presence of possibly two layers of cytochrome *c* between lamellae. (The dimensions of cytochrome *c* have been given as 28 Å $\times$ 39 Å, see ref. 40.) The addition of Ca^{2+} to a phosphatidylserine dispersion produces aggregation of the particles and has been shown to increase leakage of the incorporated K^+ (see ref. 41) when 1 equiv of Ca^{2+} is bound to each phosphatidylserine molecule[42]. The repeating unit of the aggregated particles was found to be 54 Å, indicating 'shrinking' of the lamellae within the particles. Second- and third- order spacings of 27 Å and 18 Å, respectively, demonstrated the preservation of lamellar structure (ratios $^1/_2$, $^1/_3$, see ref. 12).

CONCLUSION

The studies reported here, using a variety of purified, naturally occurring phospholipids, confirm and extend previous observations on the structure of hydrated phospholipid particles. Evidently the ability to form lamellar structures is general amongst the various phospholipids, and it is sustained even after prolonged sonication of the larger myelinic-type particles, in the form of small unilamellar vesicles. Nevertheless, several properties characteristic of each individual phospho-

* We are indebted to Dr. I. RABINOWITZ for performing the determinations of interlamellar spacings of phosphatidylserine at the Department of Biophysics, Kings College, London University.

lipid were found which could be important in considerations of biological membrane structure and function. For instance the tendency of phosphatidylethanolamine lamellae to adhere to each other and the ability of Ca^{2+} to decrease interlamellar spacings within phosphatidylserine particles could be important in terms of cell adhesion and communication. The formation of lipid–protein ionic complexes arranged in a lamellar pattern such as that between phosphatidylserine and cytochrome *c* is of obvious relevance to membrane structure. The interlamellar spacing of 125 Å can be compared with the membrane thickness of biological cells including the erythrocyte[43]. On the other hand, the ability of protein to promote the production of sac-like structures under mild conditions of dispersion may well be important in the formation of cellular vesicles. Undoubtedly such artificial vesicles offer special opportunities for the study of structure–function relationships in biological membranes. Some of the permeability properties of the particles whose structure has been described here are discussed in the accompanying article[16].

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