# INFLUENCE OF ELECTROLYTES ON THE THICKNESSES OF THE PHOSPHOLIPID BILAYERS OF LAMELLAR LECITHIN MESOPHASES

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ABSTRACT Over a wide range of water contents, aqueous lecithin-water mixtures are mesophases in which lecithin bilayers alternate with water layers. This paper reports on low-angle X-ray diffraction measurements of the effects of electrolytes, at 1.0 N concentration, on the thicknesses of the bilayers in mesophases formed by the synthetic lecithin: 1-octadec-9-enyl-2-hexadecylglycerophosphocholine. With solutions of LiCl, NaCl, $Na_2SO_4$, KCl, and CsCl, the bilayer thicknesses are *less* than with pure water. The maximum reduction in bilayer thickness with these electrolytes is about 10% and occurs with mesophases of high content of KCl and CsCl solutions. With HCl solutions the bilayer thicknesses are about 5% *greater* than with pure water, and with $CaCl_2$ solutions the bilayer thicknesses are about the same as with pure water. The maximum amount of solution which can be mixed with lecithin before a second, purely aqueous phase is formed is also affected by electrolytes, the order for the various 1.0 N solutions being CsCl = KCl > NaCl > $Na_2SO_4$ > (pure water) = LiCl > $CaCl_2$.

## INTRODUCTION

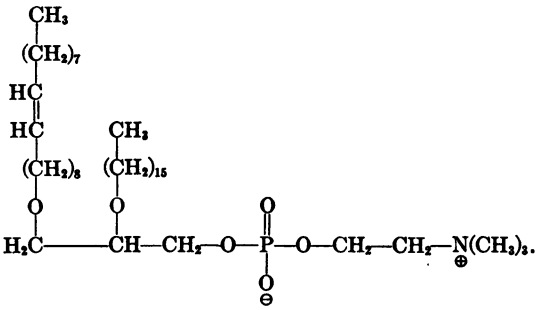
Phospholipids, like most amphipathic substances, form mesophases, i.e. liquid-crystalline phases, when mixed with water (1). Over a wide range of temperature and composition, these mesophases have the well-known lamellar structure in which phospholipid bilayers alternate with water layers. The properties of the phospholipid bilayers of the lamellar mesophases are of particular interest from the point of view of the Davson-Danielli (2), Robertson (3) model of the cell membrane, which pictures the major portion of the membrane as a phospholipid bilayer whose surfaces are covered by protein molecules. Chapman (4) has summarized the extensive studies which have been made of phospholipid-water mixtures.

It seems probable that the properties of a Davson-Danielli, Robertson-type structure would be influenced by interactions between the protein and the phospholipid.

These interactions are undoubtedly quite complex, since all of the various types of functional groups of the protein, i.e. ionic, polar but nonionic, and apolar may be involved. To assess the influence of interactions between the ionic groups of the protein and the phospholipid on bilayer properties, we have made an exploratory study of the effects of various simple electrolytes on the *thicknesses* of the bilayers in lamellar mesophases formed by the phospholipid lecithin. The bilayer thickness, which can be computed from low-angle X-ray diffraction measurements (1), was chosen as the particular bilayer property to be examined, since it is known to be sensitive to such parameters as mesophase water content (1, 5–9) and temperature (1), and since Parsegian's (10, 11) successful theoretical treatment of the effects of water content provides a basis for interpreting thickness changes.

A review of earlier work on bilayer thicknesses in mesophases and of Parsegian's theory is postponed for the Discussion. We are not aware of related work on the effects of low molecular weight substances on the thickness, or indeed on any property, of phospholipid bilayers. Several papers, however, have appeared on low-angle X-ray studies of the inherently more complex protein-phospholipid-water mixtures (12–16).

Since only limited amounts of water (1) and, as will be seen, electrolyte solutions can be mixed with lecithins, a relatively high electrolyte concentration, 1.0 N, was used in order to obtain comparable molar amounts of lecithin and electrolytes in the mesophases. The lecithin was a synthetic material, 1-octadec-9-enyl-2-hexadecyl-glycerophosphocholine:[1]



*Some Considerations Regarding Bilayer Thicknesses of Lamellar Mesophases*

Fig. 1 schematically shows the presumed molecular arrangement in a lamellar mesophase; $\delta_L$ is the bilayer thickness, and $\delta_A$ the thickness of the aqueous layer separating bilayers. The hydrocarbon chains of the phospholipid are believed to be flexible and to be tilted with respect to the plane of the bilayer (4).

[1] This material is more properly classified as a plasmalogen (17) since the hydrocarbon chains are joined to the glycerol moiety by ether rather than ester linkages as is the case with lecithins. Also, the unsaturated and saturated hydrocarbon chains are attached, respectively, to the 1 and 2 carbon atoms of the glycerol, while the reverse is the case with most natural lecithins (4).

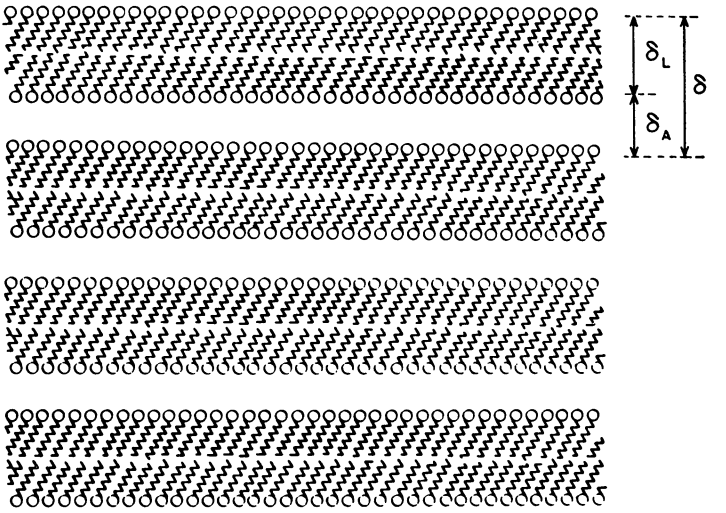



FIGURE 1 Presumed structure for lamellar lipid-water mesophase. $\delta$ = fundamental repeat spacing as obtained from low-angle X-ray diffraction pattern, $\delta_L$ = bilayer thickness, $\delta_A$ = thickness of water layers separating bilayers.

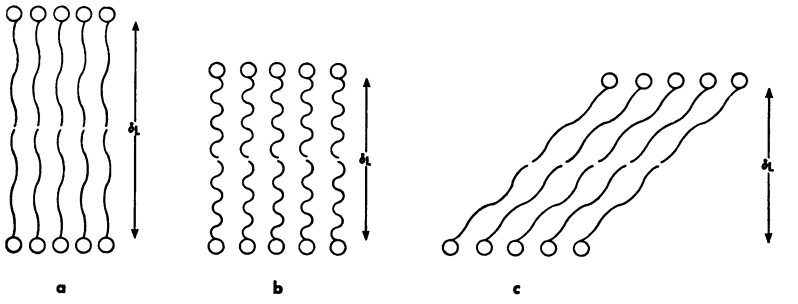



FIGURE 2 Schematic illustration of how changes either in the degree of coiling of the hydrocarbon chains or in their angle of tilt result in changes of bilayer thickness. (*a*) Relatively uncoiled hydrocarbon chain, perpendicular to plane of bilayer. (*b*) More highly coiled hydrocarbon chain, perpendicular to plane of bilayer. (*c*) Relatively uncoiled hydrocarbon chain, tilted with respect to plane of bilayer.

Both the degree of coiling of the hydrocarbon chains and their angle of tilt affect the thickness of the bilayer $\delta_L$. Fig. 2 illustrates this, starting with a hypothetical case in Fig. 2 *a* in which the hydrocarbon chains are relatively uncoiled and are perpendicular to the plane of the bilayer; Fig. 2 *b* shows the effects on $\delta_L$ of coiling of the chains, maintaining the chain axis perpendicular to the bilayer plane; Fig. 2 *c* shows the effects on $\delta_L$ of tilting the chain axis, maintaining the chains relatively uncoiled. The illustrations in Fig. 2 were drawn with the assumption that the density of the bilayer-forming molecules is the same in Figs. 2 *a*, *b*, and *c*, and that therefore the decrease of $\delta_L$ in Figs. 2 *b* and *c* in comparison with that in Fig. 2 *a* must be compensated for by an increase in the lateral distances between lecithin molecules.

The low-angle X-ray diffraction patterns of lamellar mesophases consist of a series of concentric rings, whose diameters correspond to $d$ spacings in the simple ratio 1:2:3:4 . . . (1). The fundamental repeat spacing, i.e. the distance between repeating units of the structure, is computed from the diameters of the rings using standard procedures (18). Referring to Fig. 1, the fundamental repeat spacing, to be referred to as $\delta$, is equal to the sum of the bilayer thickness $\delta_L$ and the thickness of the aqueous interbilayer region $\delta_A$ . Therefore, $\delta_L$ can be computed by multiplying the fundamental repeat spacing $\delta$ by the volume fraction of the material in the mesophase which is present as bilayers. With $\theta_L$ as the volume per cent of material present as bilayers,

$$\delta_L = \frac{\theta_L \delta}{100}. \tag{1}$$

An arbitrary assumption must be made in computing $\theta_L$ from the composition of the lecithin-water (or solution) mixture, since the extent to which the polar groups of the bilayer-forming molecules are located in the aqueous interbilayer regions is not known. The particular assumption made is not important for the present study, since only a comparison of bilayer thicknesses in pure water and in the presence of various electrolytes is involved. Following the previous workers (5–8), we will assume that the entire phosphocholine group of the lecithin is in the aqueous interbilayer region, and that the bilayer, therefore, includes only the remainder of the lecithin molecule. Accordingly, the quantity $\theta_L$ can be computed from the volume fraction of lecithin in the mesophase, to be referred to as $\Phi_L$ , using the expression

$$\theta_L = \frac{\alpha_1 \rho_2}{\rho_1} \Phi_L , \tag{2a}$$

where $\alpha_1$ is the fraction by weight of the lecithin molecule assumed to be in the bilayer, $\rho_1$ is the density of this portion of the lecithin molecule, and $\rho_2$ is the density of lecithin. For the lecithin used here, $\alpha_1 = 0.755$, $\rho_1$ is computed (8) to be equal to 0.93, and $\rho_2$ is equal to 1.04 (8). Accordingly,

$$\theta_L = 0.85\, \Phi_L . \tag{2b}$$

From equations 1 and 2 $b$, the bilayer thickness $\delta_L$ is computed from the measured X-ray repeat spacing $\delta$ by

$$\delta_L = 0.85\, \Phi_L \frac{\delta}{100} . \tag{3}$$

The volume per cent lecithin $\Phi_L$ is obtained from the composition of the mesophase

with the assumption that the densities of the phospholipid and the water (or electrolyte solution) in the mixture are the same as those of the pure materials.[2]

The distance $\delta_A$ between bilayers is readily computed from the fundamental repeat spacing $\delta$ and $\delta_L$ :

$$\delta_A = \delta - \delta_L . \tag{4}$$

As mentioned only a limited amount of pure water (1, 5–9) and of electrolyte solutions can be mixed with lecithin before a second, purely aqueous phase is formed. Therefore the fundamental repeat spacing $\delta$ increases with $\Phi_A$ , the aqueous volume per cent, only until the latter reaches a value corresponding to the maximal aqueous content, to be referred to as $\Phi_A^{max}$. Obviously bilayer thicknesses can only be computed for $\Phi_A < \Phi_A^{max}$.

## EXPERIMENTAL

### *Materials*

The lecithin was obtained from Calbiochem, Los Angeles, Calif., who reported that it had been prepared by the method of Hirt and Berchtold (19). Thin layer chromatography with iodine detection showed a single spot. Elemental analysis: C, 67.8%; H, 11.76%; N, 1.94%; P, 4.21%; theoretical: C, 68.9%; H, 11.9%; N, 1.91%; P, 4.23%. The material, as supplied, was dissolved in chloroform and refrigerated during storage. X-ray studies of mixtures of lecithin with about 50% water or 1.0 N $CaCl_2$ repeated during the course of the study, gave no indications of changes in the material in storage.

The electrolytes were reagent grade materials, used without further purification.

### *Procedures*

All sample preparations and measurements were made at room temperature, 23 ±2°C.

*Sample Preparation.* A sample of stock $CHCl_3$-lecithin solution, containing 25–50 mg lecithin, was dried under vacuum at room temperature, and the appropriate quantity of water or electrolyte solution added. The volume per cents of the lecithin and of the water, or aqueous solution, were computed from their respective weights, using 1.04 for density of lecithin (9) and literature values for the solution densities (20). The mixtures were stirred both 8 hr after preparation and immediately before insertion into the X-ray cell (see below). After preliminary experiments established that constant X-ray spacings were attained within 24 hr, this period was taken as the minimal equilibration time.

*X-Ray Diffraction Measurements.* The sample was placed in a 2.3 mm diameter hole in a 1.3 mm thick lucite holder and sealed between 7.5-$\mu$ thick Mylar film windows. A modified Rigaku-Denki small-angle scattering apparatus was used. The sample-to-film distance was 95 mm; the region between the sample and film was evacuated; nickel-filtered

[2] Dr. D. Small (private communication) and also we (unpublished observations) have verified that at high water contents, where accurate density measurements can be made, the densities of lecithin-water mixtures are essentially as computed from the densities of the individual components.

copper radiation ($\lambda$ = 1.542 A) was employed, and exposure times ranged from 6 to 22 hr. The X-ray diffraction photographs were recorded on flat film with the film face perpendicular to the incident X-ray beam.

With the exception to be noted, the X-ray diffraction patterns consisted of a series of concentric rings, having diameters in the 1:2:3... ratio characteristic of a lamellar structure. For fundamental repeat distances up to 65 A, the first four orders were obtained; only first and second order rings were consistently obtained at greater fundamental repeat spacings. The ring intensity decreased with increasing order. Fundamental repeat spacings were computed from the diameters of the second order rings, which could be measured with an accuracy corresponding to $\pm$0.25 A. With CsCl solutions, only first order rings were obtained and these were weak. This was undoubtedly a result of the high X-ray absorption by $Cs^+$ ions. The fundamental repeat spacings in the CsCl systems are believed accurate to $\pm$0.5 A, except for the two samples of greatest volume per cent CsCl solution, where the uncertainty is considerably greater.

The inaccuracies in the measurement of ring diameters lead to a maximum error in $\delta_L$, the bilayer thickness, of only $\pm$0.15 A. The major source of error is believed to arise from evaporation of water from the samples during stirring, transferring, etc., which would make the computed $\delta_L$ values erroneously low. Control measurements of water loss indicate that the maximum error in $\delta_L$ from this source was 0.4 A. Accordingly, only differences in $\delta_L$ values greater than about 0.5 A will be considered as significant.

## RESULTS

### *Lecithin-Water Mixtures*

*Fundamental Repeat Spacings* $\delta$. Table I lists the fundamental repeat spacings measured with pure water as a function of $\Phi_A$, the volume per cent water (i.e., $\Phi_A = 100 - \Phi_L$). For purposes of comparison, these data are plotted in Fig. 3 along with the results of earlier workers (5–9) who used lecithins of natural origin.

As seen from Fig. 3, in the range $\Phi_A$ = 0–13% the measured fundamental repeat spacing does not increase with $\Phi_A$, as would be expected from increased thicknesses of the water layers separating the bilayers. Also the measured fundamental repeat

TABLE I

FUNDAMENTAL X-RAY REPEAT SPACINGS $\delta$ FOR MIXTURES OF LECITHIN WITH WATER

| $\Phi_A$ volume per cent water | $\delta$ repeat spacing | $\Phi_A$ volume per cent water | $\delta$ repeat spacing |
|---|---|---|---|
| | *A* | | *A* |
| 0 | 59.0 | 27.1 | 55.0 |
| 8.1 | 60.9 | 30.0 | 56.9 |
| 9.8 | 60.7 | 36.8 | 63.3 |
| 13.3 | 61.0 | 41.1 | 64.8 |
| 18.0 | 53.2 | 50.3 | 64.6 |
| 22.6 | 53.9 | 50.5 | 64.8 |

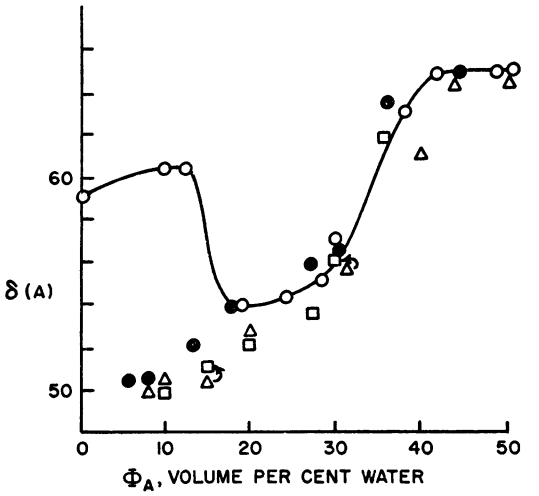



FIGURE 3 Fundamental repeat spacing $\delta$ as a function of the volume per cent water in lecithin-water mesophase. ○, this work; △, data from references 5–7; □, data from reference 8; ●, data from reference 9.

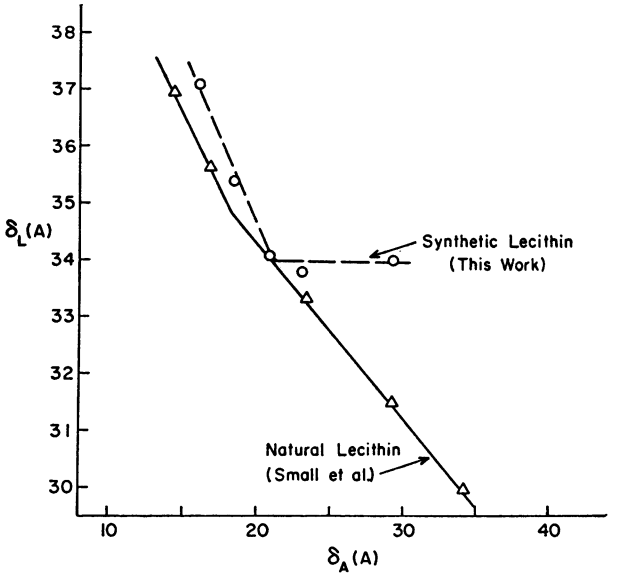



FIGURE 4 Bilayer thickness $\delta_L$ as a function of distance between bilayers $\delta_A$ . ○, this work; △, data from references 5–7.

spacings are much greater than those for the natural lecithins. As also seen from Fig. 3, at a value of $\Phi_A$ between 13 and 18%, $\delta$ for the synthetic lecithin decreases sharply, and at $\Phi_A$ greater than 18%, the results for the synthetic lecithin are essentially similar to those with the natural lecithins. Conventional wide-angle X-ray diffraction

measurements (unpublished observations) showed that at $\Phi_A < 13\%$ the synthetic lecithin is in the crystalline (1, 4), as opposed to liquid-crystalline, state. Accordingly the sharp decrease in $\delta$ at $\Phi_A$ between 13 and 18% is a reflection of the transition between the two states; with the natural lecithins the corresponding transition takes place at $\Phi_A = 5\%$ (5–7). The close numerical agreement at $\Phi_A > 18\%$ between the data obtained for the synthetic lecithin and those for the natural lecithins is particularly noteworthy in view of the chemical differences mentioned in footnote 1.

*Bilayer Thicknesses $\delta_L$.* Fig. 4 shows $\delta_L$, computed from the data of Table I using equation 3, as a function of $\delta_A$, the distance between bilayers, computed using equation 4. (For obvious reasons, bilayer thicknesses for $\Phi_A < 13\%$ are not included.) The analogous data of Small et al. (5–7) for natural lecithin are also shown. The two sets of data are in essential agreement at interbilayer distances less than about 21 A. They differ at higher values of $\delta_A$, where $\delta_L$ reaches a limiting value for the lecithin used in this work, whereas it continues to decrease for the natural lecithins. The results of the other earlier workers with natural lecithin (8, 9), also show a continuous decrease of $\delta_L$ with increasing $\Phi_A$. The question of a limiting bilayer thickness will be considered further in the Discussion.

### *Lecithin-Electrolyte Solution Mixtures*

*Fundamental Repeat Spacings $\delta$.* Table II lists the fundamental repeat spacings $\delta$ for mixtures of the synthetic lecithin with the 1.0 N solutions of the various electrolytes. Wide-angle X-ray measurements of the mixtures with lowest solution contents for each electrolyte verified that the lecithin was in the liquid-crystalline

TABLE II

FUNDAMENTAL X-RAY REPEAT SPACINGS $\delta$ FOR MIXTURES OF LECITHIN WITH 1.0 N SOLUTIONS OF VARIOUS ELECTROLYTES

| LiCl | | NaCl | | KCl | | CsCl | | HCl | | $CaCl_2$ | | $Na_2SO_4$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Phi_A$ | $\delta$ | $\Phi_A$ | $\delta$ | $\Phi_A$ | $\delta$ | $\Phi_A$ | $\delta$ | $\Phi_A$ | $\delta$ | $\Phi_A$ | $\delta$ | $\Phi_A$ | $\delta$ |
| % | A | % | A | % | A | % | A | % | A | % | A | % | A |
| 27.0 | 55.6 | 27.5 | 56* | 23.5 | 54.1 | 26.3 | 55.1 | 26.6 | 56.5 | 23.5 | 55.7 | 34.5 | 60.1 |
| 32.4 | 58.6 | 31.6 | 57* | 28.9 | 55.9 | 35.4 | 59.0 | 31.0 | 60.3 | 30.4 | 58.1 | 40.0 | 63.3 |
| 34.4 | 60.3 | 34.0 | 58* | 33.4 | 59.4 | 40.0 | 64.1 | 35.7 | 66.5 | 35.6 | 62.2 | 44.7 | 66.9 |
| 39.1 | 62.5 | 35.5 | 60.5 | 39.4 | 64.4 | 49.4 | 70.8 | 41.5 | 69.3 | 40.7 | 62.8 | 47.6 | 66.4 |
| 50.0 | 63.3 | 40.2 | 65.0 | 43.3 | 66.0 | 59.7 | 75‡ | 48.5 | 68.1 | 49.6 | 62.4 | 52.3 | 67.0 |
| | | 42.9 | 66.7 | 48.1 | 69.5 | 64.8 | 72‡ | 53.4 | 66.5 | 51.4 | 62.0 | | |
| | | 46.9 | 68.5 | 53.6 | 73.0 | | | 58.6 | 63.2 | | | | |
| | | 51.4 | 68.1 | 59.0 | 73.0 | | | | | | | | |

* The accuracy of these $\delta$ values is no better than $\pm 1$ A since the X-ray diffraction rings were either diffuse or doublets.

‡ The diffraction rings were too faint for accurate measurement.

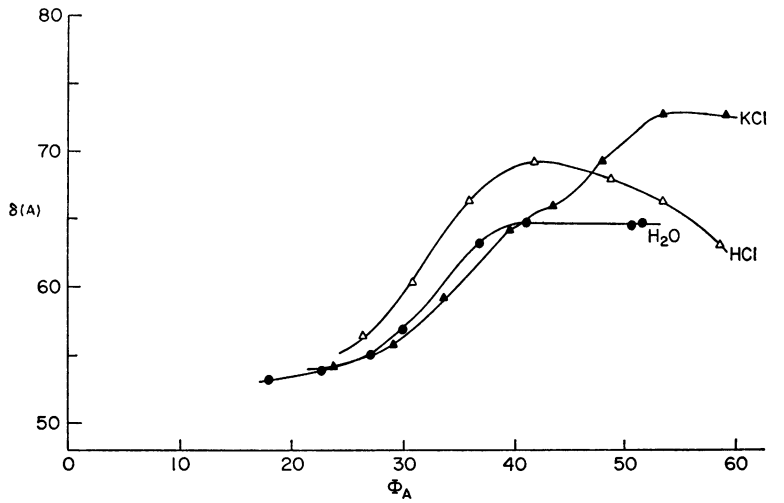



FIGURE 5 Fundamental repeat spacing $\delta$ as a function of volume per cent water or solution in the mesophase. ●, $H_2O$; ▲, KCl solutions (1.0 N); △, HCl solutions (1.0 N).

state. As noted in Table II, the results obtained for NaCl solutions inexplicably differed from those for the other solutions in that at low volume per cent solution, $\Phi_A < 34\%$, diffuse diffraction rings or doublets were obtained; this behavior was not investigated. Also, because of the faintness of the diffraction patterns with CsCl solutions mentioned earlier, the values of $\delta$ for the two CsCl-lecithin mixtures of highest solution content are inaccurate.

Data for two of the electrolytes, HCl and KCl, are shown graphically in Fig. 5 along with the data for lecithin-pure water mixtures, repeated from Table I for comparison. These two particular electrolytes were chosen for illustration of specific points, KCl because it is in most ways typical of the electrolytes studied and HCl because it is atypical and requires separate discussion.

The data of Fig. 5 show that, aside from the actual numerical values of $\delta$, the behavior with KCl solutions is qualitatively similar to that with water, i.e. $\delta$ first increases with increasing volume per cent solution and then becomes constant, indicating the attainment of $\Phi_A^{max}$, the aqueous volume per cent at which the second, purely aqueous phase is formed. The values of $\Phi_A^{max}$ are, however, different for water and for the various electrolyte solutions, with some electrolyte solutions having $\Phi_A^{max}$ greater than that of pure water, and others less. From Tables I and II, the values of $\Phi_A^{max}$ are, in descending order CsCl, 55%; KCl, 52%; NaCl, 44%; $Na_2SO_4$, 43%; pure water, 40%; LiCl, 40%; and $CaCl_2$, 36%.

Fig. 5 also shows that the fundamental repeat spacing $\delta$ for the HCl-lecithin mixtures increases with increasing $\Phi_A$ up to $\Phi_A = 41\%$ but, unlike the behavior with pure water or the other electrolyte solutions, decreases markedly at higher $\Phi_A$. This singular behavior with HCl undoubtedly is caused by the association of $H^+$ ions

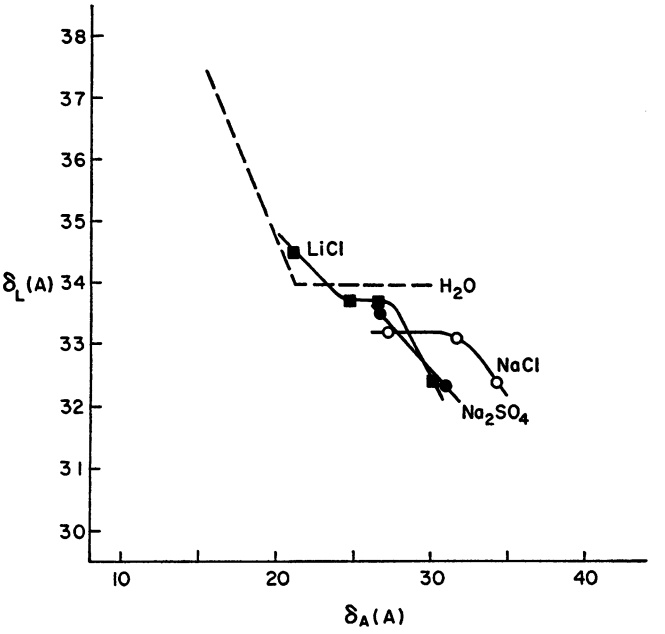



FIGURE 6 Bilayer thickness $\delta_L$ as a function of distance between bilayers $\delta_A$. ■, LiCl solutions; ○, NaCl solutions; ●, $Na_2SO_4$ solutions.

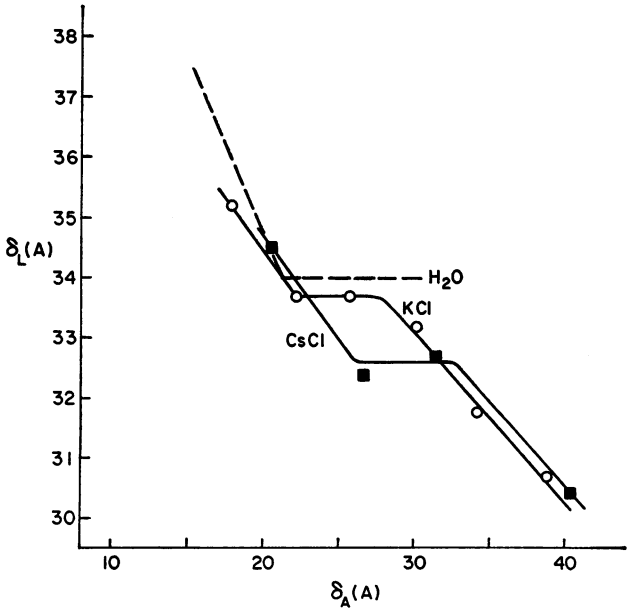



FIGURE 7 Bilayer thickness $\delta_L$ as a function of distance between bilayers $\delta_A$. ○, KCl solutions; ■, CsCl solutions.

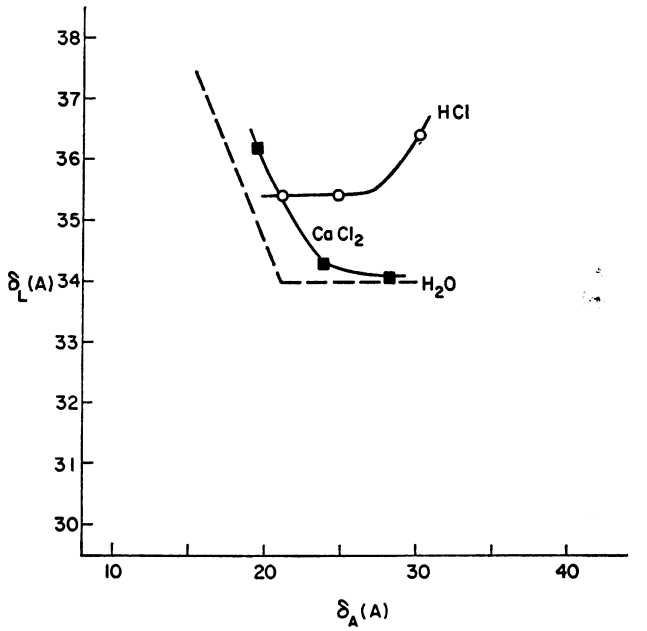



FIGURE 8 Bilayer thickness $\delta_L$ as a function of distance between bilayers $\delta_A$. ○, HCl solutions; ■, $CaCl_2$ solutions.

with the phosphoryl groups of the lecithin. This association would be expected to be essentially complete, assuming that the p$K$ of the phosphoryl group is the same as that for the first dissociation of phosphoric acid, i.e., about 2. We will not go into possible explanations for the maximum in $\delta$ but will only point out that the amount of HCl in the mixture at $\Phi_A = 41\%$ is sufficient to associate about half the phosphoryl groups.[3]

*Bilayer Thicknesses* $\delta_L$. Fig. 6 shows $\delta_L$ computed from the data of Table II as a function of the distance between bilayers $\delta_A$ for the solutions of LiCl, NaCl, and $Na_2SO_4$. The corresponding curve for pure water is repeated from Fig. 4 for comparison. Analogous plots for KCl and CsCl solutions are given in Fig. 7, and for HCl and $CaCl_2$ solutions in Fig. 8. Bilayer thicknesses are not included for the systems not understood, i.e. HCl solutions for $\Phi_A > 41\%$ where $\delta$ *decreases* with $\Phi_A$, and NaCl solutions for $\Phi_A \leq 34\%$.

## DISCUSSION

Before discussing our results, we will review (*a*) the experimental results of earlier workers on bilayer thicknesses in aqueous (pure water) mesophases formed by

[3] Following a referee's suggestion, the possibility that the HCl results were due to hydrolysis of the lecithin was checked. Samples, exposed to 1.0 N HCl for twice the time as, and at a 10°C higher temperature than were the cases in the X-ray experiments, showed no indications of hydrolysis on examination by thin layer chromatography.

several types of amphipathic substances and (*b*) the theory of Parsegian (10, 11) which successfully accounts for these results of these workers.

### *Earlier Work*

Earlier workers have reported low-angle X-ray diffraction measurements for lamellar mesophases formed by mixing water with ionic lipids (e.g., salts of long chain fatty acids) (21), with nonionic lipids (e.g., polyoxyethyleneglycols) (21), and with mixed natural phospholipids (22), as well as with the earlier mentioned natural lecithins (5–9). Only the thicknesses of the bilayers of the nonionic lipids were independent of the water contents. The thicknesses of the bilayers of the ionic lipids, of the mixed phospholipids, and of the lecithins decreased markedly with increasing water content.

### *Theory of Parsegian*

Parsegian's theory of the forces determining bilayer thicknesses in lamellar mesophases is based on the calculation of the bilayer thickness at which the free energy of the mesophase as a whole, i.e. that of the bilayers plus that of the intervening water layers, is a minimum. Parsegian first considers mesophases formed by ionic lipids, e.g., the $Na^+$ salts of fatty acids (10). The $Na^+$ ions are assumed to be located in the water layers separating the bilayers and to behave as freely dissociated counterions to the negatively charged carboxylate groups which are attached to the bilayer proper.

Parsegian's results are stated in terms of (*a*) the distance between the bilayers $\delta_A$ and (*b*) the area of the bilayer-water interface, which, in the absence of density changes, is inversely proportional to the thickness of the bilayer. (*i*) The major force *opposing* expansion of the bilayer-water interface (and thus *favoring thick bilayers*) results from the increase in hydrocarbon-water contacts accompanying the expansion. This force is independent of the distance between bilayers. (*ii*) The major forces *favoring* expansion of the bilayer-water interface (and thus *favoring thin bilayers*) are the repulsive forces between neighboring carboxylate groups. According to Parsegian's calculations these forces increase sharply with increasing distance between bilayers, as a consequence of the increased separation of the $Na^+$ ions from the bilayer proper.

Since the major force favoring thick bilayers is independent of $\delta_A$, while that favoring thin bilayers increases with $\delta_A$, a net decrease of bilayer thickness with $\delta_A$ (i.e., with water content) is predicted. Using a single adjustable parameter $\gamma$, the hydrocarbon-water interfacial energy, Parsegian obtained almost quantitative agreement with the experimental data. Values for $\gamma$ were in the range 18–20 erg-cm$^2$ for the various ionic lipids considered.

Parsegian then considered lecithin-water mesophases (11), where the dependence of the bilayer thickness on water content is qualitatively similar to that for the ionic

lipids. Although in lecithin the negatively charged phosphoryl groups and the positively charged quaternary ammonium groups are linked by the —O—$CH_2$—$CH_2$— chain, Parsegian applied the same theory as was used for the ionic lipids. His justification for this was based on the flexibility of the —O—$CH_2$—$CH_2$— chain, which, he assumes, permits the quaternary ammonium groups to behave as though freely dissociated. Almost quantitative agreement with the experimental data was obtained using a value of 10 erg-cm$^2$ for $\gamma$.

Parsegian does not discuss possible effects of electrolytes on bilayer thickness. From the factors he considered, it would seem that the major effect of electrolytes would be the reduction, by electrostatic screening, of the repulsive forces between the charged groups fixed to the bilayer. Since these repulsive forces favor thin bilayers, it would be expected that electrolytes would increase the bilayer thickness.

### *Experimental Results of This Paper*

To discuss our experimental results, it is convenient to consider separately the bilayer thicknesses with (*a*) pure water, (*b*) the alkali chlorides and $Na_2SO_4$ solutions, (*c*) HCl solutions, and (*d*) $CaCl_2$ solutions.

(*a*) As seen from Fig. 4, with pure water the bilayer thickness $\delta_L$ decreases with increasing interbilayer separation, $\delta_A$, until a limiting thickness is reached; $\delta_L$ then remains constant up to the maximum interbilayer separation attainable.

As noted earlier, this behavior disagrees with that found by the earlier workers for the natural lecithins, where $\delta_L$ decreased continuously with $\delta_A$ . Our results, i.e. those for the synthetic lecithin, would be qualitatively expected from a refinement of Parsegian's approach. Although Parsegian assumes that the quaternary ammonium groups behave as though truly dissociated, it is clear that the distance between these groups and the phosphoryl groups cannot exceed the length of a fully extended —O—$CH_2$—$CH_2$— chain. Therefore, the considerations which predict the dependence of $\delta_L$ on $\delta_A$ would only apply to interbilayer distances at which the —O—$CH_2$—$CH_2$— chain is less than fully extended. At greater interbilayer spacings $\delta_L$ should be independent of $\delta_A$ . Since the length of a phosphocholine [—O—P_$O_2$—O—$CH_2$—$CH_2$—$N_+(CH_3)_3$] group with a fully extended —O—$CH_2$—$CH_2$— chain from bond length data (20) is about 11.5 A, and since the interbilayer separation must be large enough to accommodate fully extended phosphocholine groups from two opposing bilayers, the limiting value of $\delta_L$ should occur at an interbilayer distance of about 23 A. This is quite close to our experimental value of 21 A for the interbilayer distance at which a limiting bilayer thickness is reached. We offer no explanation for the absence of a limiting thickness in the case of the natural lecithins.

(*b*) As seen from Figs. 6 and 7, the results with the alkali chloride and $Na_2SO_4$ solutions in some respects parallel those with pure water but in general are significantly different. With the electrolyte solutions $\delta_L$ first decreases with increasing $\delta_A$ and then becomes essentially constant for $\delta_A$ between about 25 A and 30 A, i.e.,

over about the same range of interbilayer distances as that at which constant bilayer thicknesses occur with pure water. The bilayer thicknesses in this range are, however, somewhat less than with pure water. The behavior with $Na_2SO_4$ is not markedly different from that with the uni-univalent electrolytes.

The behavior with electrolyte solutions differs most from that with pure water at the higher interbilayer spacings, $\delta_A > \sim 30$ A, where $\delta_L$ decreases sharply. This decrease is particularly large for KCl and CsCl solutions, where the bilayer thicknesses are about 10% less than the limiting values in pure water.

As indicated earlier, from Parsegian's theory it would be expected that electrolytes would *increase* bilayer thickness, i.e., would have an effect in the opposite direction from that found experimentally. It is clear that factors other than those considered by Parsegian must be involved.

The fact that the electrolyte effects are greatest at high $\delta_A$ may possibly be a reflection of the greater quantity of the electrolyte in the mixture, since the values of $\delta_A$ of necessity parallel $\Phi_A$, the volume per cent solution. For example at $\Phi_A = 30\%$, where the bilayer thicknesses with electrolyte solutions are only slightly smaller than with pure water, only about 0.2 moles of electrolyte are present for each mole of lecithin, and at $\Phi_A = 50\%$ where with KCl and CsCl solutions, the bilayer thicknesses are about 10% less than with pure water, 0.8 moles of electrolyte are present for each mole of lecithin. Therefore the particularly small bilayer thicknesses with KCl and CsCl solutions at large values of $\delta_A$ are not necessarily a result of specific effects of $K^+$ and $Cs^+$ ions on the bilayer thickness, but may merely reflect the greater values of $\Phi_A^{max}$ with KCl and CsCl solutions. Additional studies, using electrolyte solutions of a range of concentrations, are required to resolve these questions and are planned.

(*c*) Fig. 8 shows that with the HCl solutions, the bilayer thicknesses are greater than those with pure water. The effect of HCl is therefore opposite to that of the previously mentioned electrolytes. An increase in bilayer thickness as a result of association of the phosphoryl groups of the lecithin with $H^+$ ions is consistent with Parsegian's concepts, since a decrease in the number of charged phosphoryl groups would be accompanied by a decrease in repulsive forces near the bilayer-water interface.

(*d*) As also shown in Fig. 8 the results obtained with the $CaCl_2$ solutions are similar to those with pure water. Film balance measurements (23) indicate that $Ca^{++}$ ions form weak complexes with the phosphoryl groups of lecithin. Thus the smallness of the effect of $Ca^{++}$ ions on bilayer thickness may be due to a fortuitous canceling of a general electrolyte effect, which as seen from *a* leads to thinner bilayers, by the effects of association which, at least with $H^+$ ions, results in thicker bilayers.

### *Maximal Aqueous Contents of the Mesophases*

Although this study was primarily concerned with bilayer thicknesses, the electrolyte dependence of $\Phi_A^{max}$, the volume per cent solution at which a second, purely aqueous

phase is formed, warrants comment. The maximal aqueous content of the mesophase probably occurs when the interbilayer distance is such that van der Waals's attractive forces between the bilayers counterbalance the osmotic forces favoring incorporation of water (or solution). The large ion specificity of $\Phi_A^{max}$ and the consideration that its values for the alkali chlorides are in a simple order of ionic radii imply specific ionic interactions with the phosphocholine groups. No explanation of the electrolyte effects on $\Phi_A^{max}$ will be attempted, both because of the complicated nature of specific ion effects[4] and because additional data, i.e. the distribution of the various electrolytes between the mesophases and the purely aqueous phases, are required.

## SUMMARY

In summary, depending on the particular electrolyte, electrolytes either decrease, increase, or have little effect on the thicknesses of phospholipid bilayers in lecithin mesophases. Even the largest effect found, a decrease of thickness of about 10% with KCl and CsCl solutions, however, is small. Thus, while our data indicate that the thickness of the phospholipid bilayer in a Davson-Danielli, Robinson-type membrane structure may be influenced by interactions between phospholipid and ionic functional groups of the protein, it is unlikely that the effects would be large enough to directly affect the permeability properties of the membrane. It is of course possible that small changes in the thickness of the phospholipid bilayer can have a major effect on allosteric specific reactions at the membrane.

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