Phase equilibria and structure of dry and hydrated egg lecithin

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ABSTRACT The behavior of purified egg lecithin in water has been investigated in relation to the quantity of water present and the temperature. The complete binary phase diagram of egg lecithin–water is presented as well as X-ray diffraction data on selected mixtures. Dry egg lecithin is present in at least partially crystalline form until about 40°C. Above this temperature it forms a "wax-like" phase up to about 88°C. From 88 to 109°C it forms a viscous isotropic phase which gives face-centered cubic spacings by X-ray analysis. Above 110°C its texture is "neat" and the structure is assumed to be lamellar until its final melting point at 231°C.

Hydrated lecithin forms (except for a small zone of cubic phase at low water concentrations and high temperature) a lamellar liquid crystalline phase. This phase contains up to 45% water at 20°C. Mixtures containing more water separate into two phases, the lamellar liquid crystalline phase and water. In the melting curve of hydrated lecithin a eutectic is noted at about 16% water and the cubic phase seen when less water is present disappears at this composition of the mixture.

These facts, along with previous vapor pressure measurements, suggest that there is a structural change at about 16% water. X-ray diffraction studies of lecithin at 24°C and calculations from these data suggest that the reason for this may be the presence of a "free water layer" when more than 16% water is present.

KEY WORDS egg lecithin structure, dry and hydrated · binary phase diagram · small angle X-ray diffraction · liquid crystals · membranes · lipid bilayers · myelin figures · phospholipids

LEATHES (1–3), in 1925, published a series of papers discussing the surface and bulk properties of lecithin in water. He demonstrated clearly that lecithin swells in water to form myelin figures and that lecithin was not soluble in water. He proposed that this swollen phase of lecithin was "made up of films two lecithin molecules thick with the hydrophilic groups facing the water on each surface." In other words, in water lecithin forms repeating bimolecular leaflets separated by layers of water. This structure is called the lamellar liquid crystalline phase.

In the present paper, the behavior and structure of dry and hydrated egg lecithin will be discussed with respect to the percentage of water present and temperature.

The binary phase diagrams of McBain (4) proved to be the most valuable way of presenting the behavior of soap molecules with water. The present study presents the binary phase diagram of egg lecithin in distilled water and selected X-ray diffraction data on both the thermotropic mesomorphism of dry egg lecithin and the structure of the lyotropic lamellar liquid crystalline phase.

MATERIALS AND METHODS

The purified egg lecithin employed in the studies is identical with that described in a previous paper (5). The molecular weight was calculated to be 775. The water used was doubly-distilled, low-conductance water stored under CO_2-free nitrogen.

Preparation of Mixtures

Alcoholic solutions of egg lecithin were placed in small vials to give a final quantity of lecithin equal to 100 mg. Each vial was dried at 25°C over phosphorus pentoxide under vacuum until no further weight was lost by the

Abbreviations: V.I., viscous isotropic; CMC, critical micelle concentration.

A major portion of this work was performed in 1964–65 at the Service de Biophysique, Institut Pasteur, Paris, France, under the direction of Dr. D. G. Dervichian.

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sample. An appropriate amount of water was then added to the sample to make a specific percentage of water. The vials were sealed under nitrogen and allowed to equilibrate, with intermittent shaking, over a period of 5–14 days.

Microscopic Examination

The vials were unsealed, and a small amount of the equilibrated mixture was drawn into a fine flattened glass capillary which was sealed under vacuum. The tube was then placed in a small silicone oil-filled chamber built into a microscope heating–cooling stage. The temperature was then raised slowly (1–2°C per min) and the changes were noted under polarized and direct light. After the experiment was performed, the capillary was broken and the dry weight of the lecithin was ascertained on a microbalance to check the weight fraction of water present. This final value was used as the concentration of the experiment. It usually varied less than 3% from the original calculated concentration. Lecithin dried from alcoholic solutions at 25°C over P_2O_5 in an evacuated desiccator was examined between a slide and coverslip. Because lecithin is hygroscopic, it is possible that a trace of water was present in these samples. The growth of myelin figures from dry and hydrated lecithins with the addition of water to a sample placed between slide and coverslip was carried out in a cold room (2°C). Myelin figures grew from both anhydrous and hydrated lecithin at this temperature. Their rate of growth at 2°C was much slower than at 20°C.

X-Ray Studies

Anhydrous lecithin was studied at 20, 40, 60, 80, and 90°C. Hydrated lecithin was studied at 24°C at weight fractions of water (C_w) of 0.07, 0.10, 0.15, 0.20, 0.30, 0.38, 0.44, 0.50 and 0.60. The sample holder has been described previously (6). The X-ray tube has a copper anticathode and functions at 45 kv and 7 ma. The distance from sample to film was 23.5 cm. A Debye-Sherrer camera of 48.0 cm circumference was used to determine the short spacings. A thermostating device on the X-ray cell permitted us to maintain the temperature at any level between 20 and 90°C. The dry weight of the lecithin was determined on a microbalance both before and after X-ray exposure. Diffraction patterns of specimens which lost water during X-ray examination were discarded. The time of exposure was 24 hr. Some of the X-ray data on hydrated egg lecithin have been given briefly in a previous communication (7).

RESULTS AND DISCUSSION

Fig. 1 represents the egg lecithin–water binary phase diagram. Along the abscissa is plotted the percentage of water and along the ordinate, the temperature in degrees centigrade. This diagram bears some resemblance to the soap diagrams of McBain (4) and the monoglyceride diagrams reported by Lutton (8).

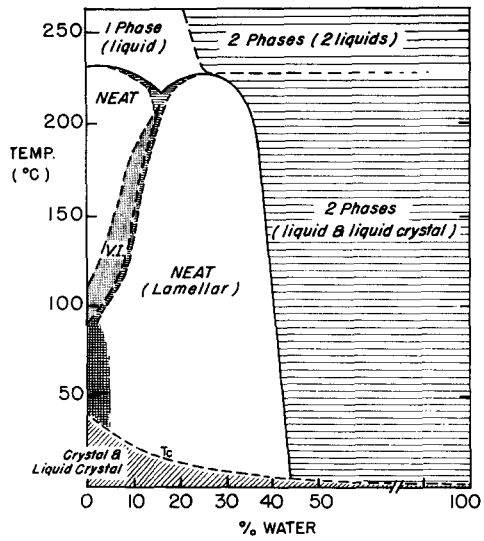

FIG. 1. Swelling of pure egg lecithin in water: the binary phase diagram for egg lecithin and water as a function of temperature. V.I. represents "viscous isotropic" phase (face-centered cubic by X-ray diffraction). *Tc* is the ill-defined boundary of crystal–to–liquid-crystal phase transition. The cross-hatched area between 0 and 5% water from 45 to 90°C represents a poorly defined area in which lamellar liquid crystalline phase may coexist with another liquid crystalline phase. For further explanations see text.

Observations on Dry Egg Lecithin

Along the left side of the diagram are recorded the phase changes in dry lecithin observed by microscope as one proceeds from 2 to 231°C (the true melting point of this egg lecithin). From 2 to 35°C this lecithin is an opaque, partly crystalline, stiff waxy material. Between 36 and 40°C the anhydrous egg lecithin is transformed into a grossly clear viscous material with a "waxy" birefringence. At about 80°C the preparation begins to turn isotropic. Between 88 and 109°C the preparation is completely isotropic and stiff and contains angular air bubbles. The aspect is identical with that of the cubic phase of soaps (9), the viscous isotropic (V.I.) phase of nonionic detergents (10), and the cubic phase described previously for lecithin–bile salt–water systems (5). Abruptly at 110°C a brilliant birefringence develops. It is of the neat soap type (11) and is accompanied by a sharp decrease in the viscosity of the material. The preparation remains birefringent until it melts sharply at 231°C. Just before melting it forms the "batonets" of Friedel (12). These observations are quite reproducible with fresh samples, but there appeared to be a hysteresis effect in samples that were cooled and reheated. This

effect has been noted with cephalins (13) and monoglycerides (8).

The X-ray examination of these particular phases up to 90°C showed the following. At 20°C anhydrous lecithin gave a number of very sharp X-ray lines in both the narrow and wide angle ranges consistent with at least one, and perhaps more, crystalline structures. These persisted at 40°C. At 60°C the short spacings showed one broad band at about 4.6 A and the number of long spacings was reduced to three (44.2 A, moderate intensity; 39.5 A, strong intensity; and 33.0 A, moderate intensity). These spacings probably represent a paracrystalline phase, but the lattice type has not yet been classified. At 90°C the long spacings of a face-centered cubic lattice (a = 67.0 A) became evident. This structure corresponded to the V.I. phase noted by polarizing microscope. Similar changes have been noted for several other synthetic and naturally occurring lecithins, but the transition temperatures depend on the type of fatty acyl chains present. A detailed account of these observations will be presented elsewhere (D.M. Small and D.G. Dervichian, in preparation).

Observations on Hydrated Egg Lecithin

When water is added to lecithin, a number of interesting changes occur. First, the dotted line labeled *T*c (Fig. 1), which represents roughly the phase transformation from crystalline to liquid crystalline, falls from about 36–40°C in the anhydrous state to below 15°C at 12% water. This is analogous to the fall in a *T*c line in soap–water binary phase diagrams. We must stress here that this line represents the clearing of the opaque lecithin specimen and is accurate to only ±3°C. Since this egg lecithin is a mixture of lecithins with different fatty acyl chains (5), one would predict that the *T*c line would be ill-defined. McBain, Vold, and Porter (14), using a mixture of sodium and potassium commercial soaps (containing many different fatty acyl chains), found that the mixture would behave as a two-component system only at high temperatures in the anhydrous or slightly hydrated state, and that the *T*c line was impossible to define in mixtures with only small weight fractions of water. Below 45°C in the dry state or at 24°C with less than 12% water, egg lecithin appears to behave similarly to the mixture of soaps. Like the soap mixtures in this part of the diagram, egg lecithin behaves as a multicomponent system. At 24°C between 0 and 7% water, egg lecithin is a mixture of crystalline and waxy phases. Between 7 and 12% water it is opaque and has a waxy birefringence. From 12 to 45% water a single clear homogeneous phase of neat soap texture (11) and lamellar structure as shown by X-ray diffraction is present. Between 46 and 65% water the mixtures are turbid and islands of water are seen by direct microscopy interdigitated between portions of the liquid crystal phase of lecithin. Increasing the concentration of water increases the amount of water amongst the lecithin and at about 65–70% water myelin figures appear. When the amount of water is increased to 80%, myelin figures and anisotropic droplets are seen floating in the water. Centrifugation of a 5% suspension of myelin figures and anisotropic droplets in water followed by assay of the separated aqueous phase for dry weight shows that there is virtually no lecithin dissolved in the aqueous phase. Lecithin then, in fact, forms in an excess of water a lamellar liquid crystalline phase present as myelin figures or anisotropic droplets in equilibrium with water itself. It should be noted here that myelin figures can be broken down by ultrasonication or other procedures to form polydisperse fragments which are quite stable (15–17). Since these aggregates do not form spontaneously, are not monodisperse, and have no demonstrable CMC (18), they should not be considered analogous to soap micelles.

The line starting at 231°C (the melting point of dry lecithin) across the top of the diagram (Fig. 1) can be considered the melting curve of hydrated lecithin. It delineates the transformation from the neat phase to an isotropic liquid. This curve shows a definite eutectic at about 16% H_2O. At this same concentration of water the V.I. phase, which was readily observed with less water, is no longer seen. Furthermore, this percentage of water corresponds to a striking change in the absorption of water vapor that appears on the curves of Elworthy (19).

At about 25% water there is a maximum in the melting curve. Mixtures containing more than 25% water, when raised to the melting point, appeared to decompose into two immiscible isotropic liquids. This was also true of myelin figures that were heated to above 225°C. Upon cooling, myelin figures did not readily reform.

It is interesting to note that the cubic (V.I.) phase can contain only up to 15% water, unlike the cubic phases found with soaps (9). The V.I. phase probably consists of spherical aggregates of the polar heads of the lecithin surrounding small islands of water packed in a face-centered lattice. The distance between these groups is about 47. 5A. The space between the polar groups would be filled with the liquid hydrocarbon parts of the lecithin molecules.

X-ray studies on hydrated lecithin containing between 10 and 60% water at 24°C showed long spacings of the lamellar type with at least one and often two and three orders. The long spacings, *D*, the thickness of the lipid layer, d_L, and the surface area per lecithin molecule, *S*, (see below) are plotted in Fig. 2 against the percentage of water incorporated into the lecithin. The long spac-

TABLE 1 DIMENSIONS OF THE LAMELLAR

V_L, 1267 A³;

C_L Weight fraction of lecithin	c_W Weight fraction of water	ϕ_L Volume fraction of lecithin	D Long spacing	S Surface area of one lecithin molecule	d_L Thickness of lipid layer	d_w Thickness of water plus phosphoryl layer	d_{wf} Thickness of "free water zone"
			A	A^2	A	A	A
0.85	0.15	0.837	51.0	59.3	35.8	15.2	0
0.80	0.20	0.787	52.5	61.3	34.7	17.8	1.8
0.70	0.30	0.689	56.4	65.2	32.6	23.8	7.8
0.62	0.38	0.610	60.6	68.5	31.0	29.6	13.6
0.56	0.44	0.551	64.1	71.7	29.6	34.5	18.5

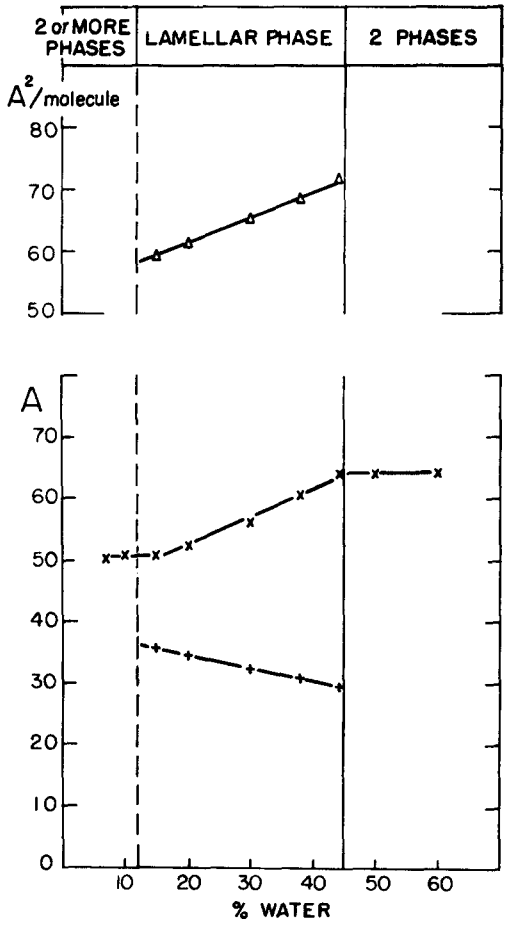

FIG. 2. Dimensions and structural elements of the lamellar phase of egg lecithin at 24°C. X, repeat distance (D) between the layers in A; Δ, average surface area (S) in A²/molecule; +, calculated thickness of lipid layer (d_L). The dotted line at 12% water represents a probable phase change from crystalline to lamellar liquid crystalline. The line at 45% water represents the separation of two phases, (*a*) a lamellar liquid crystalline phase saturated with water, and (*b*) water itself. The lamellar liquid crystalline phase in equilibrium with water has a repeat distance of about 64.1 A.

ings increase in a fairly linear fashion between 15 and 44%. The values at 50 and 60% water are identical with that at 44% water. It should be noted here that the long spacings obtained between 7 and 15% water rose only from 50.5 to 51 A and therefore represent another plateau. The break comes again at about 15% water, which is close to the composition at the eutectic point and the disappearance of the cubic phase noted in the phase diagram (Fig. 1).

The lamellar structure of classical amphiphiles (soaps, etc.) can be divided into two parts, a lipid layer and a water layer. Water is generally thought to be almost completely excluded from the lipid layer and thus to exist between the polar groups of the soaps. Unlike soap molecules, which have a very limited polar zone around the COONa, the lecithin molecule has two strong polar groups separated by a distance of up to 5.0 A.[1] The entire length of the phosphoryl choline group can be taken as about 8 A when extended maximally. In an attempt to correlate the observed changes in the vapor pressure of water for various amounts of water in lecithin (19) with the X-ray structural data, one may roughly distinguish two regions where water is found, the "phosphoryl choline zone" and the "free water zone." The zone extending from the glycerol backbone to the maximum extended length of the phosphoryl choline molecule (8 A) will be called the "phosphoryl choline zone." The zone beyond the 8 A limit will be called the "free water zone."[2] With a few assumptions and knowing the percentage (weight fraction) of water and the corresponding long spacing (D), one can calculate a number of parameters of hydrated lecithin which help to explain its physical properties. The following considerations are taken into account.

(*a*) The anhydrous density of egg lecithin has been calculated by Elworthy (20) and found to be 1.016 g/cm³. The molecular volume in A³/molecule was calculated from the general formula $V_m = 1/P \times M/N \times 10^{24}$, where V_m = molecular volume in A³/molecule, p =

[1] Calculated from Cambridge and Stuart-Breigleb molecular models.

[2] This is, of course, not free water in the strict sense, but only in that it is much less intimately associated with the phosphoryl choline groups.

V_{HC}, 1063 A³; V_{PC}, 204 A³.

V_T Total volume occupied by one lecithin molecule and its water	V_{wT} Total volume available to water per lecithin molecule	M_{wT} Molecules of water per lecithin molecule	V_{wpc} Volume of water in the "phosphoryl choline zone"	M_{wpc} Molecules of water in the "phosphoryl choline zone"	V_{wf} Volume of water in the "free zone"	M_{wf} Molecules of water in "free zone"
A^3	A^3		A^3		A^3	
1513.1	246.6	8.3	270.7	9.1	0	0
1609.3	342.8	11.5	286.5	9.6	56.3	1.9
1838.2	571.7	19.1	317.4	10.6	254.3	8.5
2076.2	809.7	27.1	344.2	11.5	465.5	15.6
2298.5	1032.0	34.5	369.8	12.4	662.2	22.2

density in g/cm³, M = molecular weight, N = Avogadro's number, and 10^{24} = number of A³ per cm³. The molecular volume of this lecithin (V_L) is 1267 A³/molecule.

(*b*) The density of crystalline glyceryl phosphoryl choline is given by Abrahamsson and Pascher (21) as 1.32 g/ml. Its calculated molecular volume is 324.5 A³/molecule. By subtracting the molecular volume (120.5 A³/molecule) of propanediol (calculated from its density)[3] we obtain the figure of 204 A³/molecule for phosphoryl choline. This volume corresponds to a cylinder 8 A long with a cross-sectional area of 25.5 A². These dimensions are quite compatible with Stuart-Breigleb models of phosphoryl choline. The molecular volume of the phosphoryl choline (V_{pc}) is taken as 204 A³/molecule.

(*c*) The partial molecular volume of the glycerohydrocarbon part, V_{HC}, was calculated by: $V_{HC} = V_L - V_{pc} = 1063$ A³. The corresponding density of this part of the molecule is 0.924 g/cm³. This is slightly higher than the value given for 1,3-diolein at 35°C (d = 0.9128) (22), but seems a reasonable estimate.

(*d*) The density of water is taken as 1.0 g/cm³ and its molecular volume 29.9 A³.

With these assumptions (V_L = 1267 A³/molecule, V_{pc} = 204 A³, V_{HC} = 1063 A³, and density of water = 1.0 g/cm³), the following can be estimated.

1. The surface area, S, per lecithin molecule in A²/molecule. $S = V_L/(D/2.\phi_L)$, where $D/2$ is one half the long spacing and ϕ_L equals volume fraction of lecithin (see Fig. 2).

2. The thickness (d_L) in A of the lipid layer. $d_L = 2V_{HC}/S$ (see Fig. 2).

3. The thickness in A of the water layer (d_w) including the phosphoryl choline group. $d_w = D - d_L$.

4. The thickness (d_{wf}) in A of the "free water layer." $d_{wf} = d_w - 16$.

5. The total volume (V_T) in A³ occupied by one lecithin molecule and its quota of water. $V_T = V_L/\phi_L$.

6. The total volume in A³ available to water (V_{wT}) per lecithin molecule. $V_{wT} = V_T - V_L$.

7. The total number of water molecules (M_{wT}) per lecithin molecule. $M_{wT} = V_{wT}/29.9$.

8. The volume of water in A³ (V_{wpc}) in the phosphoryl choline zone. $V_{wpc} = (S \times 8) - 204$.

9. The number of water molecules (M_{wpc}) per lecithin in the phosphoryl choline zone. $M_{wpc} = V_{wpc}/29.9$.

10. The volume of water (V_{wf}) in A³ in the "free zone." $V_{wf} = V_{wT} - V_{wpc}$.

11. The molecules of water (M_{wf}) per lecithin molecule in the "free zone." $M_{wf} = V_{wf}/29.9$.

These findings have been summarized in Table 1. The long spacing, D, increases as the weight fraction of water increases. However, the thickness of the lipid or the hydrocarbon layer, d_L, falls as the weight fraction of water is increased. This finding is also true of the lamellar phase of soaps and detergents (9).

The surface area per lecithin molecule (S) gradually increases as the weight fraction of water increases. It is interesting to note that when the weight fraction of lecithin (C_L) equals 0.85 or at 85% lecithin, the surface area is 59.3 A²/molecule. This area agrees well with that of a similar egg lecithin compressed to its collapse point (43 dynes/cm) at the air–water interface (23). However, when C_L equals 0.56 (56% lecithin), this area has expanded to nearly 72 A²/molecule and corresponds to the area in a monolayer under a pressure of about 22 dynes/cm at the air–water interface (23). Therefore, in the lamellar liquid crystalline state, at 24°C, the surface area of lecithin can vary between 72 and 59 A²/molecule depending upon the degree of hydration. A direct result of this is that more water can be incorporated into the phosphoryl choline zone at high weight fractions of water than at low weight fractions of water (see below).

The thickness of the water plus the phosphoryl choline layer, d_w, increases with increasing hydration, as would be expected. However, there is no water in the "free

[3] The density of propanediol is given by the Handbook of Chemistry and Physics (Chemical Rubber Publishing Co., Cleveland.) 49th edition (d_4^{20} = 1.0361).

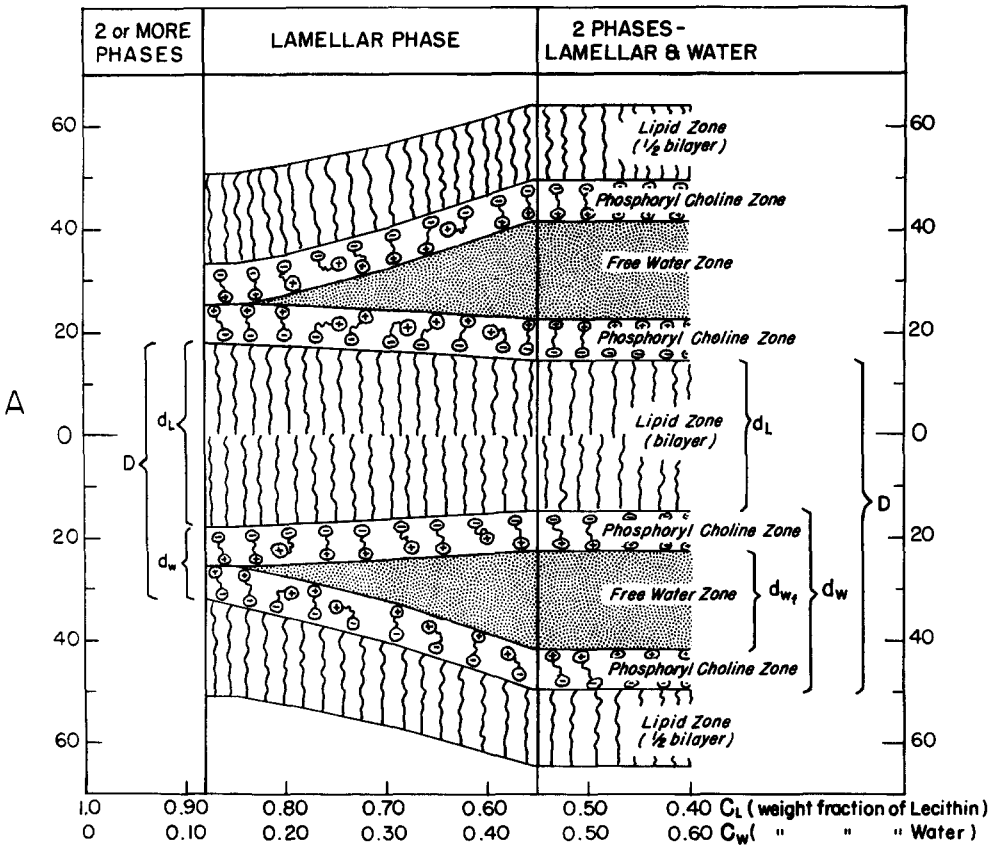

FIG. 3. Diagrammatic reconstruction of the bimolecular lecithin leaflet in relation to the amount of water present. D is the repeat distance; d_L, the thickness of the lipid bilayer; d_w, the thickness of the water layer including the phosphoryl choline zone; and d_{wf}, the thickness of the "free water layer." The hydrocarbon parts of the lipid layer are represented as wavy lines to emphasize their partially liquid-like state. The choline groups of the phosphoryl choline zone are represented in more or less random array within a zone 8 A thick. For further explanation see text.

water zone" (d_{wf}) at $C_L = 0.85$. "Free water" first appears between the weight fractions of 0.85 and 0.80, after which the free water zone increases steadily.

The volume of water, V_{wpc}, in the phosphoryl choline zone gradually increases as the surface area increases with increasing hydration. At $C_L = 0.85$ about 9 molecules of water per molecule of lecithin can be accommodated in this zone. However, at $C_L = 0.56$, 12.4 molecules per lecithin molecule can be located in this zone. Therefore, it might be said that as one dehydrates lecithin, some water shifts from a tightly bound type of water to a nearly free type whose vapor pressure approaches that of free water.[4] It is also clear that even though 9 molecules can be accommodated when $C_L = 0.85$, only 8.3 molecules are present. Therefore, there is no "free water zone" and, in fact, the phosphoryl choline groups must overlap to some degree. It is possible that this overlapping hinders the relatively free motion of the phosphoryl choline groups. Finally, the molecules of water in the free zone begin to appear by extrapolation at $C_L = 0.84$ or about 16% water. That this water is similar to free water is suggested strongly by the fact that it has a vapor pressure very close to the vapor pressure of pure water (19).

A diagrammatic reconstruction of the bimolecular lecithin leaflet as it swells in water has been attempted in Fig. 3. Below 16% water there is no free water zone and the opposing phosphoryl choline groups must encumber one another to some degree. Above 16% water structural changes occur that may be related to the appearance of a "free water layer" and less-hindered motion of the phosphoryl choline groups. A second phase change occurs when $C_L \simeq 0.55$. At this concentration there is about 18–19 A of "free water zone" separating the phosphoryl choline groups. When $C_L < 0.55$, large islands of unbound water are present and the vapor pressure is the same as that of free water (19).

[4] The concept of bound water is supported by recent experiments on hydrated lecithins employing a differential scanning calorimeter (D. Chapman, R. M. Williams, and B. D. Ladbroke, personal communication). The curves obtained show a definite ice peak with mixtures containing more than 20% water, but no ice peak in mixtures with less than 20% water.

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