BBA 75714

# STRUCTURAL STUDIES BY X-RAY DIFFRACTION OF MODEL LIPID–PROTEIN MEMBRANES OF SERUM ALBUMIN–LECITHIN–CARDIOLIPIN

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(Received April 8th, 1971)

SUMMARY

X-ray diffraction studies were made on precipitates formed, at pH 3.33, between bovine serum albumin and mixtures of lecithin and the acidic phospholipid cardiolipin of lecithin/cardiolipin mole ratios from 99:1 to 50:50. For lecithin/cardiolipin from 93:7 to 50:50 the precipitates are single lamellar phases, made up of alternating layers of lipid–protein and water, whose repeat distance $d$ changes from 90 to 68 Å. The compositions vary systematically showing little stoichiometry, the weight percent lipid being about 41%, the protein increasing from 10 to 30% and water decreasing from 50 to 30%. Regarding interlamellar interactions, the shrinking is correlated with increasing cardiolipin/bovine serum albumin mole ratio, which reflects decreasing net positive charge in the complex, and when this is close to zero the structure is condensed and the area available per protein molecule in the plane of the layer indicates tight packing. When the net charge on the lipid–protein lamellae is reversed by incorporating more cardiolipin, however, swelling does not occur indicating increased interlamellar attractive forces compared to pure lipid lamellae. Regarding intralamellar interactions, molecular packing shows that there must be hydrophobic interactions between lipid and protein either (i) by spreading apart of the phospholipid molecules which then form a thin continuous bimolecular layer or (ii) by penetration of bovine serum albumin into a normal bilayer: the degree of hydrophobic interaction increases with increasing cardiolipin and bovine serum albumin content. As lecithin/cardiolipin varies from 93:7 to 50:50, if (i) applies then the amount of the surface of the lipid layer that is occupied by non-polar parts of the lipid varies from 8 to 23%; if (ii) applies then the percent volume of the protein that penetrates into the bilayer varies from 36 to 58%.

## INTRODUCTION

It is unreasonable to suggest that the many diverse functions of biological cell membranes are served by a single basic membrane 'structure', several models of which have been proposed[1]. Even within the plasma membrane, for example, of a particular cell a wide variety of specific and independent functions are being performed simultaneously. These functions presumably are served by a variety of different but mutually compatible structures or 'sites' arranged tangentially over the surface of

the membrane, and indeed these may be changing with time and dependent on the environment of the membrane. It is primarily because of the thinness, heterogeneity and lability of membranes that there is as yet little structural basis for their many well characterized functions. Under these circumstances investigations have turned to model systems which are simpler in chemical composition and which hopefully mimic some properties of intact membranes, or of some 'sites' on membranes.

The problem of the structure of biological membranes is that of the structural interactions of the membrane components, primarily lipids and proteins, in aqueous environments. Led primarily by LUZZATI *et al.*[2] many systematic structural studies of simple model systems, lipids and water, have been done and these have been extended to lipid–protein–water systems[3]. The results have shown that a remarkable variety of structures and structural changes can occur in these systems depending on both the specific molecular composition and the experimental conditions of the system. This polymorphism must surely underlie the heterogeneity of intact membrane structure and function, and indeed may well be amplified by the chemical heterogeneity of intact membranes compared to the relatively simpler model systems. However, by studying the latter, the interactions and polymorphism of a specific lipid–protein complex of a specific membrane 'site' in a heterogeneous intact membrane surface may be revealed. More data is required on the structure of specific lipid–protein complexes, particularly using membrane proteins. However, these latter are not well characterized, a requirement for detailed structural investigation, and studies continue with better characterized proteins which are of dubious relevance to membranes but which are interesting in the general problem of lipid–protein interactions.

Beside transport, membranes have the important property of interacting in a very specific way in cell–cell contact and communication. The ability of specific cells to aggregate, form close intermembrane contacts and become immobilized and differentiated, is critical to the problem of tissue development and must result in the first instance by interactions between specific membranes on contact. The problem of contact is basically one of the interaction of lipid – protein lamellae. The technique used in the present study, that of X-ray diffraction, is particularly suited to determining, at low resolution, both intralamellar interactions, which underlie transport properties of membranes, and interlamellar interactions which underlie the problem of cell contact.

The results reported here are on the structural associations that occur, by precipitation, between phospholipids and protein. The phospholipids are lecithin and cardiolipin mixed in varying proportions and representing thereby various ratios of zwitterionic to acidic phospholipids. The protein used is bovine serum albumin. This protein has been used both to study lipid–protein interactions, primarily by its penetration into phospholipid monolayers at air–water interfaces, and to study its effect on the permeability of thin lipid membranes. Penetration into monolayers[4], but not necessarily into the hydrophobic region, is very dependent on surface pressure but increases with increasing length of the fatty acyl chains. Penetration is greatly enhanced when the lipid has a net negative charge and the protein is positively charged, but not *vice versa*. Bovine serum albumin increases the rate at which glucose diffuses from labelled liquid crystals of egg lecithin[5] providing the lipid and protein carry negative and positive charges, respectively, but not the reverse. The effect is very dependent on pH but not on ionic strength. In the present studies the structures were

formed by precipitation, at pH 3.33, of positively charged bovine serum albumin and lipids containing a net negative charge. At this pH bovine serum albumin is expanded or unfolded[8,11] and hydrophobic interactions with lipid might be expected.

## MATERIALS

Lecithin and cardiolipin, extracted from pig liver and beef heart, respectively, were obtained from Serdary Research Laboratories, Canada, and were stored in chloroform–ethanol solution at —20° for a maximum of two months. They were assayed before and after the X-ray experiments by thin layer chromatography. Crystalline bovine serum albumin (Sigma and British Drug Houses) was used without delipidation.

## METHODS

### *Preparation and chemical analysis of X-ray samples*

Chloroform–ethanol solutions of lecithin and of cardiolipin were mixed to yield the desired molar ratio of the two lipids, and were dried by rotary evaporation under nitrogen and finally under vacuum for a minimum of 2 h. The lipids (approximately 18 mg) were then immediately suspended in 2 ml of 0.05 M sodium citrate buffer, (pH 3.33) occasionally using 10–30 sec sonication in an ice bath and under $N_2$, to speed dispersion. Bovine serum albumin (1 mg/ml) was dissolved in the same buffer. The lipid dispersion was then added, dropwise and with vigorous stirring, to enough of the protein solution, usually 6 ml, so that not all the protein was removed in the heavy precipitation that resulted; *i.e.* the precipitation was done in excess protein. The solution was then centrifuged (120000 × *g* for 30 min) yielding a very cohesive precipitate (like putty and often clear) and a supernatant whose protein concentration was measured spectrophotometrically. The precipitate was then washed by resuspending it in buffer and recentrifuging as before. Preliminary control experiments showed that (i) centrifuging the precipitate either gently (desk centrifuge for 2 min) or vigorously (150000 × *g* for 4 h) yielded precipitates whose compositions and X-ray diffraction patterns were identical (ii) no phosphorus could be detected in the supernatants showing insignificant amounts of lipid were uncentrifuged (iii) protein in the supernatants of the washing buffer could be not detected and (iv), further washing of the precipitate up to 4 times yielded no change in its X-ray diffraction pattern. A few precipitates were formed under identical conditions except the protein was all bound and therefore the lipid/protein ratio of the samples varied.

The chemicals analysis of the precipitates was carried out as follows. The lipid/protein ratio was found, in the first instance, from the amount of lipid added to the protein solution and the amount of protein bound from it. After the precipitates were dried slightly under a $N_2$ stream, (see below) the lipid content was determinded by phosphorous analysis[6], using mol. wt. of lecithin as 790 and of cardiolipin as 1480 (the latter was determined by phosphorous analysis of the pure lipid) and assuming the lecithin/cardiolipin ratio was the same as in the premixed lipids. Water content was determined gravimetrically by first weighing the sample, then adding chloroform–methanol and lyophilizing the sample in a Virtis lyophilizer for 24 h. Comparative analytical studies (unpublished) carried out by sonicating the samples in $^2H_2O$ and measuring $H_2O$ by NMR gave the same results as the more convenient gravimetric method. Protein content was determined by difference. The lipid/protein weight ratio of the

precipititates and of the drier X-ray samples were nearly identical and the results show the average.

Control samples of the lipids alone at various lecithin/cardiolipin ratios were prepared by weighing the dried lipids and adding various amounts of buffer. (These lipids, suspended, would not centrifuge into a cohesive enough sediment to handle.)

*X-ray structural determinations*

The structural analysis by X-ray diffraction of these sorts of systems has been described before in detail[2]. It will be described briefly here as it applies to this study. The X-ray camera is of the Guinier type operating *in vacuo* and using a bent quartz crystal monochromator which isolates the $CuK\alpha_1$ line ($\lambda$ = 1.540 Å). The X-ray samples are sealed between mica windows approximately 1 mm apart and their temperature in the camera can be controlled. The only structure of interest in the present study is the lamellar phase which gives 2–5 X-ray reflections, all integral orders of the single repeat distance $d$ of the one dimensional crystal. Unless noted, the X-ray diagrams otherwise give only a broad band at 4.5 Å, typical of the liquid paraffin chains of the phospholipid molecules.

Within the planar lamellae of thickness $d$ must be packed the lipid (l) protein (p) and water (w) in proportion to their respective relative volume concentrations, $\phi_l$, $\phi_p$ and $\phi_w$; these latter being determined by the weight concentrations of the components $c_l$, $c_p$, $c_w$, ($c_l + c_p + c_w = 1$) and their partial specific volumes, $\bar{v}_l$, $\bar{v}_p$, $\bar{v}_w$. As a first hypothesis, if the lipid, protein and water each formed separate layers then the partial thicknesses, $d_l$, $d_p$ and $d_w$ of these layers can be found.

Thus

$$d_l = \phi_l d$$

where

$$\phi_l = \frac{c_l\bar{v}_l}{c_l\bar{v}_l + c_p\bar{v}_p + c_w\bar{v}_w} \quad etc.$$

and

$$d = d_l + d_p + d_w$$

In order to determine the water content of the lamellar structure of the precipitates, which were formed in excess buffer, *i.e.* $c_w$ above, the precipitates were dried with a stream of $N_2$ until the repeat distance $d$ started to decrease. Only at that point was the water concentration determined since only at that point was the drying removing water from the structure and not from pools of excess buffer. In this way $c_w$ of the sample was found and by extrapolating to the $d$ value of the original undried precipitate, $d_w$ of that precipitate was determined.

For the purposes of the results the mol. wt. of cardiolipin is taken as 740, one half that as determined by the phosphorous analysis. This is the weight of each part of the whole molecule that contains one phosphate group, the acidic group that carries one of the molecule's two negative charges, and two fatty acyl chains, and therefore, is comparable in size and weight to one lecithin molecule. Hence the lecithin/cardiolipin ratio as represented here gives the ratio of zwitterions to charges in the lipid mixture. However, it is clear that the cardiolipin molecule is two 740 units covalently

bonded at the polar end and that, therefore, the charged groups on the surface of the lipid bilayer occur in pairs.

The partial specific volumes $v_l$, $v_w$ and $v_p$ used in all calculations are 1.00, 1.00 and 0.734 (ref. 11) $cm^3/g$, respectively and the molecular weight of bovine serum albumin is taken as 67000.

## RESULTS

### *Control lipids*

The control lipids (without protein) form a lamellar phase when mixed with buffer. Assuming, as has been proved a number of times[7], that the lameller structure is formed by alternating layers of water and lipid, (the latter being a bilayer of phospholipid formed by the fatty acyl chains forming a hydrocarbon layer with the polar groups occupying the interface between this layer and the water layer) the thickness of the lipid layer $d_l$ can be calculated. Table I gives the values of $d_l$ for a sampling of lecithin/cardiolipin ratios and for a number of lipid concentrations (dry-weight percent). As with most other systems of natural phospholipids the thickness of the bimolecular layer is approximately 39–41 Å, increasing slightly in thickness at higher concentrations. The surface area available to each phospholipid molecule then is approximately 60 $Å^2$ and consequently, given the lecithin/cardiolipin ratio the surface-charge density, $\sigma_l$, and the area per charge, $Å^2$ charge, are given in Table I.

### *Lipid – protein precipitates*

The conditions, pH 3.33, were chosen so that a clear interaction between lipid and protein would occur, and whenever cardiolipin was present in the lipid an electrostatic interaction of the negatively charged lipids and positively charged protein resulted in heavy precipitation.

Table II is a summary of the results of the chemical and structural analysis of the lecithin–cardiolipin–bovine serum albumin precipitates. The results were obtained from two complete experiments covering the lecithin/cardiolipin ratios and six spot checks at different lecithin/cardiolipin ratios. The results were remark-

TABLE I

VALUES OF $d_l$, THE THICKNESS OF THE LIPID BIMOLECULAR LAYER IN LAMELLAR PHASES FORMED BY MIXTURES OF LECITHIN (L) AND CARDIOLIPIN (CL), AT THE INDICATED LECITHIN/CARDIOLIPIN MOLE RATIOS, IN 0.05 M SODIUM CITRATE BUFFER, pH 3.33, AT THE DRY WEIGHT CONCENTRATIONS SHOWN

$\sigma_l$ and $Å^2$/charge are the surface charge density, and its inverse, calculated on the basis of one phospholipid molecule occupying 60 $Å^2$ on the lipid surface.

| *L/CL* | $\sigma_l \times 10^{-3}$ (*charge*/$Å^2$) | $Å^2$/*charge* | *Concn. (dry wt. %):* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *0.66* | *0.70* | *0.73* | *0.77* | *0.80* | *0.90* |
| L | 0 | — | 39.1 | | | | | |
| 90:10 | 1.66 | 600 | | | | 41.1 | | |
| 87:13 | 2.17 | 460 | | 38.6 | | | 40.6 | 43.0 |
| 75:25 | 4.16 | 240 | | | 39.0 | | | |
| 47:53 | 8.82 | 113 | | | 40.0 | | | |

TABLE II

STRUCTURAL AND CHEMICAL ANALYSES OF BOVINE SERUM ALBUMIN–LECITHIN–CARDIOLIPIN PRECIPITATES

Each column is discussed in turn in RESULTS.

| *L/CL* | $\sigma_l$ $\times 10^{-3}$ | Å²/*charge* | *d* (Å) | $c_l$ | $c_p$ | $c_w$ | *CL/BSA mole ratio* | $d_l$ (Å) | $d_p$ (Å) | $d_w$ (Å) | *Area/BS.* (Å²) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| L | 0 | — | 59.5 | 0.66 | | 0.34 | | 39.1 | 0 | 20.4 | |
| 99:1 | 0.166 | 6000 | 68.5 | | | | | | | | |
| 97:3 | 0.500 | 2000 | — | | | | | | | | |
| 95:5 | 0.83 | 1200 | 90.1 | | | | | | | | |
| 93:7 | 1.16 | 860 | 89.9 | 0.39 | 0.10 | 0.51 | 24 | 35.9 | 6.8 | 47.2 | 12100 |
| 90:10 | 1.66 | 600 | 83.6 | 0.42 | 0.13 | 0.45 | 28 | 36.3 | 8.3 | 39.0 | 9600 |
| 87:13 | 2.17 | 460 | 79.1 | 0.43 | 0.16 | 0.41 | 30 | 35.5 | 9.7 | 33.9 | 8300 |
| 80:20 | 3.33 | 300 | 73.4 | 0.44 | 0.20 | 0.36 | 39 | 34.3 | 11.4 | 27.7 | 7300 |
| 75:25 | 4.16 | 240 | 74.0 | 0.41 | 0.23 | 0.36 | 39 | 32.5 | 13.4 | 28.1 | 6100 |
| 50:50 | 8.3 | 120 | 68.5 | 0.41 | 0.30 | 0.29 | 60 | 30.5 | 15.6 | 21.4 | 5200 |

ably consistent, within less than 1 Å for the repeat distance *d* and within a few percent for the chemical analyses. These results and their interpretation are discussed column by column in the following.

L/CL represents lecithin/cardiolipin mole ratio and, as in the control lipids above, determines the surface charge density, $\sigma_l$, of the phospholipid before interaction with the protein. Both $\sigma_l$, and Å² charge are given in the table and the series represents model membranes of increasing acidic phospholipid content.

*d* represents the repeat distance *d*, in Å, of the lamellar phase of the precipitate. With no acidic lipid, *i.e.* with lecithin alone, no bovine serum albumin is bound and only the maximally swelled lecithin lamellar phase is observed. For lecithin/cardiolipin from 99:1 to 95:5, in spite of heavy precipitation the X-ray diagrams show only ill-defined lamellar reflections, whose *d* spacings are nevertheless shown, superimposed on central scattering. This observation and the large values of *d* near lecithin/cardiolipin = 97:3 indicate a very hydrated precipitate but this region requires further study. For lecithin/cardiolipin from 93:7 to 50:50 a pure lamellar phase, characterized by 2–5 sharp reflections, is formed whose repeat distance *d* consistently decreases from 90 to 68 Å as the proportion of cardiolipin in the lipid increases.

$c_l$, $c_p$, $c_w$ represent the weight concentrations of the three components of the precipitate. As $\sigma_l$ increases the proportion of lipid in the precipitate remains constant but proportionally more protein is bound and less water is present.

CL/BSA represents the mole ratio of cardiolipin to bovine serum albumin or rather the number of negative charges in the lipid mixture per albumin molecule. As $\sigma_l$ increases both more protein is bound and the cardiolipin/bovine serum albumin ratio increases to about 39 at lecithin/cardiolipin of about 80:20. This value, 39, is very close to the number of positively charged sites on the albumin molecule at pH 3.3[8]. In the region of lecithin/cardiolipin from 80:20 to 75:25 this stoichiometry is maintained but at some point thereafter the cardiolipin/bovine serum albumin ratio increases becoming 60 when lecithin/cardiolipin is 50:50. Our interpretation is that although more and more cardiolipin is available to bind bovine serum albumin it would cease

to do so at the point where there was no room left to pack more bovine serum albumin molecules on the lipid layer. This fits with the calculations of the area available to the bovine serum albumin molecule (see below).

Swelling of pure phospholipid lamellar phases depends upon the balance of the electrostatic repulsion between layers resulting from the presence of a net surface charge on the lipid layers, and a long range van der Waals attraction between layers[9,10]. In the present case, the swelling of the lipid–protein lamellar phase, represented by increasing $d$ and $c_w$, is inversely proportional to $\sigma_l$, the charge density of the lipid alone. However, when the bovine serum albumin, positively charged at pH 3.3, is bound to the lipid, the surface charge density on the lipid–protein layer will result from the net charge of cardiolipin and bovine serum albumin. At the lower cardiolipin/bovine serum albumin ratios the large values of $d$ and $c_w$, indicating a more swollen phase, must result from a net surface charge due to the protein. As the cardiolipin/bovine serum albumin ratio increases this net charge is reduced and the structure becomes more compact. As suggested above when cardiolipin/bovine serum albumin is approximately 39 the net charge will be close to zero and the swelling appears to be minimal. However, reswelling does not occur as the cardiolipin/bovine serum albumin ratio increases above 39 (see lecithin/cardiolipin = 50:50 and also Table IV below) where electrostatic repulsion between leaflets would be expected to result from excess negative charges. This will be discussed further.

$d_l$, $d_p$, $d_w$ represent the partial thicknesses of the lipid, protein and water layers if these three components form three separate layers containing only, and all of,

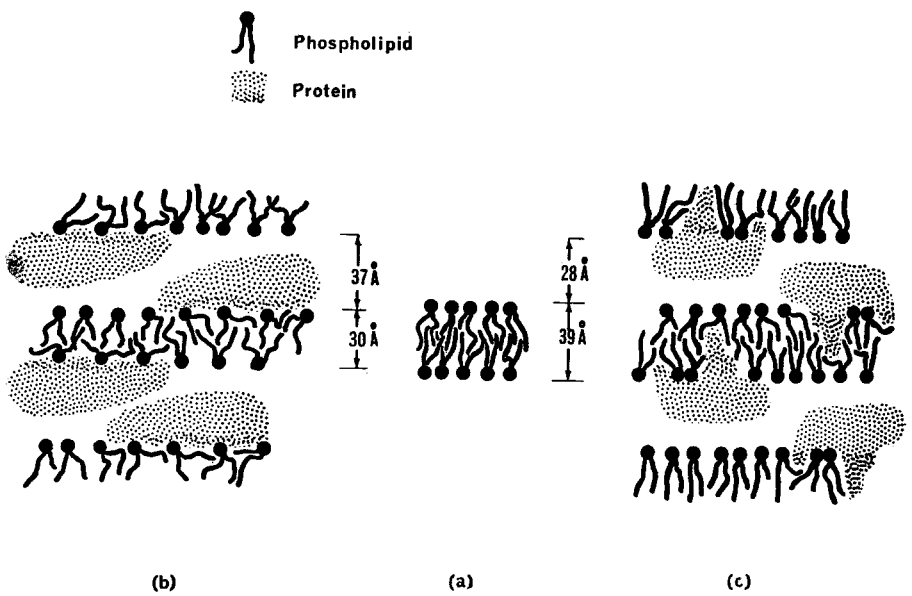


Fig. 1. Section through the lamellar phase of the lecithin/cardiolipin alone (a), or the precipitate formed with lecithin/cardiolipin = 50:50 and bovine serum albumin (b and c). Dimensions of the thicknesses of the layers are for the two models; (b) the lipid layer is continuous and the polar groups must be spread apart, exposing 23% of the surface to non-polar parts of the lipid molecules; (c) the protein (in this case 58% of it) penetrates into the bimolecular lipid layer. In either model hydrophobic interaction (heavier stippling) between lipid and protein is necessary.

the one component. Consideration of $d_1$ alone shows both that it is less than $d_1$ of the control lipid without the protein (Table I) and that it decreases with increasing proportion of protein. This shows that the hydrocarbon layer of a phospholipid bilayer must come in contact with the water–protein layers, presumably the protein. This can be done either by a spreading apart of the polar groups of the phospholipid molecules if a continuous lipid layer still exists (Fig. 1b) or by penetration of the protein into the phospholipid bilayer (Fig. 1c) if the lipid molecules in fact maintain the length they have in a protein-free bilayer. In either case there must be contact between either the water or protein, and most reasonably the protein, and the fatty acyl chains of the phospholipid molecules. As $\sigma_1$ increases this contact increases. GULIK *et al.*[3] have used this measurement as an operational definition of a hydrophobic bond.

It is difficult to differentiate between these two models on the basis of the present evidence, particularly since we have no evidence of the conformation of the protein except that at this pH, and in order to fit the protein into the available space in this study, the protein is largely extended or unfolded and indeed hydrophobic areas of the protein are exposed[8]. In addition, this conformation may well change as $\sigma_1$ and the cardiolipin/bovine serum albumin ratio vary.

However, to illustrate the molecular packing in the two cases we shall apply these two models to the data starting at the lecithin/cardiolipin ratio of 50:50 where $d_1$ has its lowest value. (a) If $d_1 = 39$ Å for the control lipid (Table I) and decreases to 30 Å in the lipid–protein complex (Fig. 1b), the surface area available to each phospholipid molecule on the bilayer goes from 60 to 78 Å$^2$ assuming the lipid molecules do not change in volume. If the polar groups of the lipid molecules still occupy 60 Å$^2$ then 18 Å$^2$/molecule or 23 % of the surface of the lipid layer is occupied by non-polar parts of the lipid and must make contact with the protein. Similar calculations show that this percentage varies from 8 %to 23 % as the lecithin/cardiolipin ratio changes from 93:7 to 50:50. (b) Alternatively, if the lipid molecules have the same length in the lipid–protein complex as in the control lipids, 39 Å, and protein penetrates into the bilayer (Fig. 1c) then for lecithin/cardiolipin = 50:50, 58 % of the volume of the protein penetrates into the bilayer. Similar calculations show that this value varies from 36 to 58 % as the lecithin/cardiolipin ratio varies from 93:7 to 50:50.

It is possible that both (a) and (b) occur to varying extents but hydrophobic contact is made. The presence of a distinct 4.5-Å band in the X-ray diagrams of the precipitates, the negative temperature coefficient of the repeat distance $d$, and the presence of a 4.1-Å line at $-8°$ in these precipitates duplicate the classic results of similar observations with pure phospholipids and indicate that in the precipitates the fatty acyl chains of the phospholipid molecules form continuous regions even with the hydrophobic bonding of protein.

It is unreasonable to assume that water and protein form separate layers (*i.e.* completely dehydrated protein). It is more likely that both occupy the same plane at least to some extent. For example if at lecithin/cardiolipin = 50:50 (a) above holds, then $d_1 = 30$ Å and a protein–water layer is 37 Å thick (Fig. 1b). 37 Å is very close to one-half the diameter of a spherical, hydrated bovine serum albumin molecule, so the bovine serum albumin molecule must be far from spherical in shape. Alternatively, if (b) above holds then the lipid–protein layer is 38Å thick and the protein–water layer is 28 Å (Fig. 1c). At this lecithin/cardiolipin ratio, approximately 50 % of the volume of the protein–water layer is occupied by protein, and assuming the water of

hydration of albumin is 1.07 g water per g protein[11] little more room is available for more protein. This is shown in the next column.

Area/protein gives the area on the surface of the lamellar sheets that is available to each protein molecule. As more protein is bound its surface density increases to a value where, at lecithin/cardiolipin = 50:50, each molecule has approximately 5200 Å$^2$ available to it. The hydrated bovine serum albumin molecule as a sphere would have a cross sectional area of 4800 Å$^2$ and therefore, at lecithin/cardiolipin 50:50 the protein is densely packed on the surface. The increase in cardiolipin/bovine serum albumin ratio as the lecithin/cardiolipin ratio goes from 75:25 to 50:50 is interpreted (see above) as meaning that although cardiolipin was available to bind bovine serum albumin no more room was available to pack more bovine serum albumin molecules into the lamellae.

*Changes on drying the precipitate*

Table III shows the changes that take place when the precipitate, formed with a lecithin/cardiolipin ratio of 87:13, is dried with a stream of $N_2$ and the lamellar repeat $d$ and $c_w$ are observed at various stages of drying. $d_l$, $d_p$ and $d_w$ are calculated as before. As about 25% of the water in the original precipitate is removed ($c_w$ from 0.43 to 0.32) no change in $d_l$ or $d_p$ occurs, the change in $d$ being accounted for by changes in $d_w$. This strongly suggests that as this water is removed the lipid–protein layer does not change and that this water is free in the complex (unbound to protein) and forms a layer the removal of which allows the lipid–protein layers to approach. Further drying on the other hand leads to decreases in both $d_l$ and $d_p$ showing a change in the structure of lipid – protein layer and in a direction that increases the hydrophobic contact with the lipid. (When lipid alone becomes more concentrated $d_l$ increases (Table I).) Therefore at, the point where the repeat distance $d$ goes from 67 to 54Å and the combined protein–water layer is between approximately 32 Å and 22 Å thick ($d_p + d_w$), adjacent lipid–protein layers approach to the point where structural changes occur.

*Precipitation with excess lipid*

If the precipitation is not done in excess protein but rather under conditions

TABLE III

CHEMICAL AND STRUCTURAL ANALYSIS OF A PRECIPITATE OF LECITHIN/CARDIOLIPIN, RATIO 87:13, WITH BOVINE SERUM ALBUMIN AS THE PRECIPITATE IS DRIED UNDER A STREAM OF NITROGEN

$d$, repeat distance of lamellar phase; $c_l$, $c_p$ and $c_w$, weight proportions of lipid, protein and water, respectively; $d_l$, $d_p$ and $d_w$, partial thicknesses of the three components.

| $d$ (Å) | $c_l$ | $c_p$ | $c_w$ | $d_l$ (Å) | $d_p$ (Å) | $d_w$ (Å) |
|---|---|---|---|---|---|---|
| 79.4 | 0.41 | 0.16 | 0.43 | 34.1 | 9.6 | 35.7 |
| 80.0 | 0.41 | 0.16 | 0.43 | 34.3 | 9.8 | 35.9 |
| 75.8 | 0.44 | 0.17 | 0.39 | 34.7 | 9.9 | 30.2 |
| 67.0 | 0.49 | 0.19 | 0.32 | 34.5 | 9.8 | 22.7 |
| 53.8 | 0.56 | 0.21 | 0.23 | 31.8 | 8.7 | 13.3 |
| 47.5 | 0.63 | 0.23 | 0.14 | 31.9 | 8.5 | 7.1 |

where all the protein is bound, then the lipid/protein ratio of the precipitate increases and can be controlled. A lamellar phase is formed whose repeat distance $d$ is variable. Table IV give the data of two such experiments. A fixed amount of lipid of lecithin/cardiolipin ratio 87:13 was added to a varying amount of protein solution. The systematic increase of lipid/protein ratio is accompanied by a systematic decrease of the $d$ spacing at least to 14 mg lipid per mg protein. A measure of the cardiolipin/bovine serum albumin ratios supports the data of Table II that as the cardiolipin/bovine serum albumin ratio increases even to the point where there must be an excess of negative charges, (cardiolipin/bovine serum albumin $> 40$), the repeat distance still decreases.

TABLE IV

CHEMICAL AND STRUCTURAL ANALYSIS OF LECITHIN/CARDIOLIPIN MIXTURES PRECIPITATED WITH BOVINE SERUM ALBUMIN BOTH WITH EXCESS PROTEIN AND EXCESS LIPID

See RESULTS for explanation.

| *L/CL = 87:13* | *mg lipid/mg protein* | *CL/BSA* | *d* |
|---|---|---|---|
| Excess protein | 2.6 | 28 | 80.0 |
| Excess lipid | 3.5 | 40 | 80.0 |
| | 5 | 57 | 79.4 |
| | 7 | 80 | 74.4 |
| | 9 | 100 | 73.5 |
| | 10 | 110 | 71.3 |
| | 14 | 180 | 67.1 |

| *L/CL = 75:25* | *d* | $c_l$ | $c_p$ | $c_w$ | $d_l$ | $d_p$ | $d_w$ | *CL/BSA* |
|---|---|---|---|---|---|---|---|---|
| Excess protein | 74.0 | 0.41 | 0.23 | 0.36 | 32.5 | 13.4 | 28.1 | 39 |
| Excess lipid | 66.7 | 0.49 | 0.18 | 0.33 | 34.2 | 9.4 | 23.1 | 60 |

The same experiment done with lecithin/cardiolipin 75:25 (Table IV) gives the same result and an analysis of $d_l$, $d_p$ and $d_w$, indicates that the change in $d$ is accounted for almost entirely by changes in the protein – water layer. These preliminary data, which need further systematic study and elaboration, indicate that although a particular lecithin/cardiolipin ratio forms a precipitate of fixed lipid/protein ratio in excess protein, this precipitate can accommodate more lipid, and in doing so it structure changes.

## DISCUSSION

### *Intralamellar interactions*

It is expected that in this system, where the bovine serum albumin and lecithin/cardiolipin mixtures have opposite charges, electrostatic interactions would predominate. Nevertheless, it is clear that significant hydrophobic interactions occur in these conditions and indeed if lecithin alone is used no detectable binding of bovine serum albumin occurs. The electrostatic interaction is most likely the first step in getting the two components close enough so that much shorter range polar and hydrophobic interactions can occur causing, presumably, subsequent gross conformatio-

nal changes in the protein. For non-membrane proteins this certainly represents 'denaturation' but for membrane proteins might represent 'renaturation' as they return to their native surface.

It has been amply demonstrated that the degree of lipid–protein interaction depends upon those factors that affect the surface charge density and surface potential of the lipid surface (see, for example ref. 12). With the exception of the cardiolipin/bovine serum albumin ratio of 40 noted above, the present precipitates show little stoichiometry and it is likely that the protein, rather than forming stoichiometric cardiolipin/bovine serum albumin complexes that are bound in the layer, forms rather non-specific and variable lipid–protein contacts, both polar and hydrophobic, that depend on surface potential and distance between negative charges. In fact these results clearly show that as the surface charge density of the lipid increases, even though excess protein in solution is always available to the lipid the amount of protein that is bound increases and the cardiolipin/bovine serum albumin ratio increases. In addition the degree of hydrophobic bonding increases showing, as might well be expected, a variation in the conformation of the protein as the surface charge density increases. The relevance to biological membranes is that the dynamic configuration of membrane proteins might depend on all those factors, including the acidic phospholipid content, that affect surface potential and its variation.

*Interlamellar interactions*

Interaction between adjacent leaflets is important in the context of cell–cell contact. The interaction between pure lipid leaflets depends on a balance of long-range van der Waals attraction and electrostatic repulsion[9,10]. Grossly this same balance appears to operate in the present equilibrium lipid–protein structures where the degree of swelling depends on the charge ratio reflected in cardiolipin/bovine serum albumin, at least until cardiolipin/bovine serum albumin of about 40 at which point the net charge must be close to zero[8]. However, in the case where the cardiolipin/bovine serum albumin ratio increases above 40 (Table II) and reaches high values (Table IV), it might be expected on the same basis that repulsion between leaflets would be shown by swelling, but this does not occur. NINHAM AND PARSEGIAN[13] have indicated that protein materials can greatly increase the interlamellar attractive forces of pure lipid lamellae and this is consistent with the preliminary observations that adding more charged lipid to the lipid–protein layer does not cause swelling. However, further systematic experimental study of the phenomenon is required.

ACKNOWLEDGEMENTS

This research was supported by the National Research Council of Canada, Grant No. A4920. The author gratefully acknowledges the technical assistance of Mrs. J. Joss. and Miss O. Haras.

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