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THE BINDING OF SERUM ALBUMIN TO PHOSPHOLIPID LIPOSOMES

C. SWEET AND J. E. ZULL
Center for the Study of Materials, Case Western Reserve University, Cleveland, Ohio 44106 (U.S.A.)
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SUMMARY

The binding of bovine serum albumin to phospholipid model membrane systems has been studied as a function of pH, ionic strength and membrane charge.

At pH values below its isoelectric point, bovine serum albumin becomes strongly bound to lecithin–cholesterol–dicetyl phosphate liposomes. The dependence of the binding on ionic strength and membrane charge indicates that actually two types of association are possible: electrostatic and hydrophobic binding. The electrostatic interactions seem to be a prior condition for the formation of the apolar associations which are related to the acid expansion of bovine serum albumin occurring at pH 4.0.

It is suggested that the formation of an apolar complex mediated through an initial electrostatic attraction gives rise to the changes in permeability properties of the model membrane which were studied previously.

INTRODUCTION

The problem of lipid–protein interactions in cellular membranes is a central and critical question in the biomembrane field. These interactions and the description of the molecular architecture of membranes have been the subject of a great deal of modern experimental and theoretical study (see refs. 1–3 for reviews of this subject). However, in spite of many recent advances in this area, the description of lipid–protein associations in cell membranes is still a subject of controversy. Many workers have approached this subject with the idea of finding general principles governing lipid–protein interactions from experimental studies of various biological membranes. It is our hope that a detailed study of lipid–protein interactions in a well characterized artificial system will be of aid in this kind of approach.

BANGHAM and co-workers[4,5] have described certain aqueous dispersions of phosolipids as small spherulites (or liposomes) composed of concentric shells of bilayer lipid membranes. Their experiments indicated that such membranes are similar, in many respects, to those found in living organisms. Using this model membrane system, it may be possible to isolate and interpret specific protein interactions with lipid in an arrangement which is structurally analogous to that thought to be present in cellular membranes.

Prior to the work discussed here, we reported[6] that a small amount of bovine serum albumin (1 bovine serum albumin molecule to 10^4 lipid molecules) could bring

about a 4-fold increase in the rate of efflux of [^{14}C]glucose trapped in the liposomes. The effect is dependent on pH, activation occurring as the pH is lowered from 5.5 to 3.5 with a pK^* of 4.3. This information together with the dependence of the effect on temperature, membrane charge and ionic strength indicated that both electrostatic attraction and hydrophobic forces may play a part in the lipid–protein interaction. We were, therefore, presented with an opportunity to study a lipid–protein association in a simple model system with many of the characteristics of real membranes. The present paper reports further studies of the bovine serum albumin–liposome interaction: specifically the binding of protein to lipid is discussed in terms of pH, membrane charge and ionic strength.

MATERIALS AND METHODS

Chemicals

All reagents were A.R. grade and used without further purification. Distilled, deionized water was used. Egg lecithin was prepared by a modification of the method of SINGLETON *et al.*[7]. Bovine serum albumin was obtained from Pentex, Inc., and its [2-^{14}C]acetamidinyl derivative was prepared from the imido ester by the method of WOLFSY AND SINGER[8]. This procedure brought about the acetamidination of about 85% of the lysyl residues as estimated by the amount of radioactivity incorporated into bovine serum albumin.

Preparation of lecithin dispersions

The liposomes, containing lecithin, cholesterol and dicetyl phosphate (molar ratio 70:10:20), were prepared in a buffered NaCl solution (0.073 M) exactly as previously described[6]. In most cases, an experiment was run a few hours after preparing the lipid suspension.

Removal of unbound protein

Suspensions containing known amounts of lipid and protein were centrifuged at 100000 × g in a Beckman (Model G) ultracentrifuge for 60 min. This procedure concentrated the lipid in a small pellet and allowed sampling of the supernatant and analysis for protein. At some of the higher salt concentrations, the density of the solvent exceeded that of the lipo–protein complex; and a layer formed at the top of the solution. In this instance, the subsolution could be sampled for protein. In some cases, especially where little lipid–protein interaction occurs, it was difficult to remove all of the lipid by a single centrifugation. Therefore a second centrifugation of the supernatant was sometimes employed.

Analysis for protein

Protein binding was determined by comparing the amount of bovine serum albumin originally present in solution with that found after centrifugation of the complex. The solutions were analyzed by measurement of the absorbance at 280 mμ or by reaction with the Folin–Ciocalteu reagent and measurement of the absorbance at 750 mμ. The concentration of [^{14}C]acetamidinated bovine serum albumin was determined by radioactivity measurements in a Picker liquid scintillation counter

* In this paper pK always refers to the midpoint of a pH-dependent process.

using 10 ml of a solution made up of 300 g naphthalene, 13 g 2,5-diphenyloxazole and 0.6 g 1,4-bis-(5-phenyl-2-oxazolyl)-benzene in 3 l dioxane.

RESULTS

pH dependence for bovine serum albumin binding to liposomes

It was desirable to demonstrate directly that bovine serum albumin is associated with the liposomes and to determine whether binding could be related to changes in membrane permeability reported earlier[6]. The pH dependence for binding of bovine serum albumin to negatively charged liposomes is shown in Fig. 1. A striking

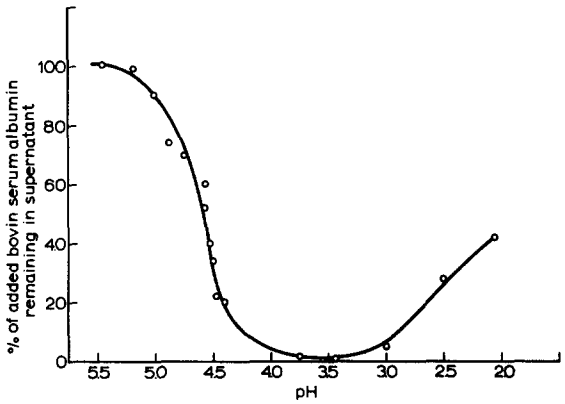


Fig. 1. Binding of native bovine serum albumin to liposomes. Mixtures of bovine serum albumin and liposomes were centrifuged, and the concentration of bovine serum albumin remaining in the supernatant is expressed as a percent of the total bovine serum albumin added as determined by $A_{278m\mu}$. 2 mg of bovine serum albumin were added per ml of liposome suspension (containing 12 mg lecithin, 2.3 mg dicetyl phosphate and 0.9 mg cholesterol).

increase in the amount of bovine serum albumin bound to liposomes was noted below pH 4.6. The isoelectric point of bovine serum albumin under these conditions is approx. 4.5 (see ref. 11); this suggests that electrostatic factors may be involved in the interaction. The decrease in binding at very low pH values (Fig. 1) is also consistent with this conclusion, since negative charge on the liposome phosphates will be titrated at these pH values.

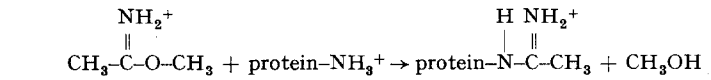
At neutral pH, the surface charge of the liposomes can be changed from negative to positive by substituting an equimolar amount of stearyl amine for dicetyl phosphate in the lipid mixture. Bovine serum albumin had no effect on the permeability of these liposomes even at high pH values[6]. Fig. 2 shows the binding curve for bovine serum albumin to positive liposomes when an equimolar amount of stearyl amine is substituted for dicetylphosphate. Bovine serum albumin is increasingly bound to the liposomes as the pH is elevated with about 40% of the added protein bound at pH 8. However, if the amount of stearyl amine is doubled, complete binding can be attained above pH 8.

Role of lysyl residues of bovine serum albumin

Since positive sites on the protein may be involved in the interaction with the

negative lipid system, it seemed of interest to modify the lysyl residues on bovine serum albumin. These residues contribute most of the positive charge of this protein.

In our laboratory we have used labeled imido esters to produce radioactive protein derivatives[13]. The reaction between an imido ester and the lysyl group of a protein results in a modified residue which still carries a positive charge.



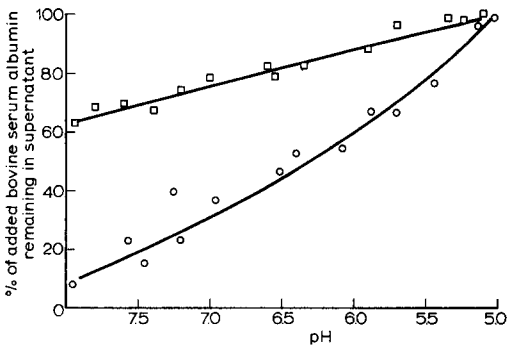


Fig. 2. Binding of native bovine serum albumin to positively charged liposomes. Liposomes were made with stearyl amine in place of dicetyl phosphate. Mixtures of bovine serum albumin and liposomes were centrifuged and the bovine serum albumin remaining in the supernatant is expressed as a percent of the total bovine serum albumin added as determined by $A_{278m\mu}$. The bovine serum albumin concentration was the same as in Fig. 1. □—□, molar ratio of stearyl amine to dicetyl phosphate, 1:1; ○—○, molar ratio, 2:1.

The modified bovine serum albumin is electrostatically similar to the native molecule, with some alteration in steric and bulk factors. Other workers[14] have demonstrated the similarity of acetamidinated protein derivatives to native protein, and the use of the radioactive label provides a very accurate and convenient method for measuring protein concentrations.

Fig. 3 shows the pH dependence for binding acetamidinated bovine serum albumin to negative liposomes. The pK for binding of acetamidinated bovine serum

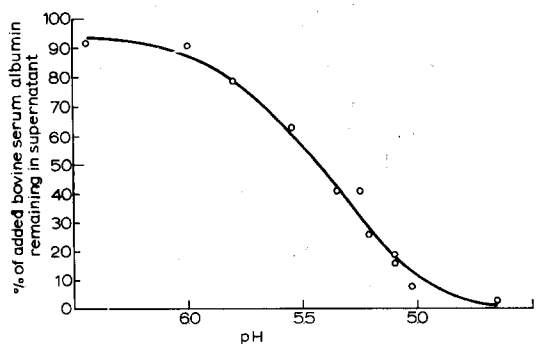


Fig. 3. Binding of acetamidinated bovine serum albumin to liposomes. Mixtures of methyl [2-^{14}C]acetamidinated bovine serum albumin and liposomes were centrifuged at 100000 × g. The radioactivity in the supernatant is expressed as a percent of the total radioactivity added.

albumin is about 5.4, a value, somewhat above the isoelectric point of the protein (such a modification of the lysyl residues of an acidic protein like bovine serum albumin does not affect the p*I* to any great extent). The effect of amidinated bovine serum albumin on the permeability of the liposomes to glucose was also determined, using the methods described previously[6]. The bovine serum albumin derivative was found to be almost identical to native bovine serum albumin in this

TABLE I

THE EFFECT OF ACETAMIDINATED BOVINE SERUM ALBUMIN ON GLUCOSE DIFFUSION FROM LIPOSOMES

See ref. 6 for methods. Acetamidinated and native bovine serum albumin were added to liposomes (molar ratio of bovine serum albumin to lecithin, 0.0004) and the rate of [^{14}C]glucose efflux determined. Rate is expressed as the percent of total trapped glucose that diffuses from the liposomes per h.

Protein	*Number of experiments*	*Diffusion rate for [^{14}C]glucose (% capture/h)*	
		pH 5.0	*pH 3.5*
Acetamidinated bovine serum albumin	2	7	16
Native bovine serum albumin	5	6	18

respect (see Table I). Thus, although almost total binding of the modified protein occurs at pH 5.0, there is no activation of solute diffusion at this pH. However, diffusion can be activated by lowering the pH to 3.5.

Effects of ionic strength

Our earlier work suggested the existence of a bovine serum albumin–liposome interaction which is stable to high ionic strength. This was tested directly by measurement of binding of bovine serum albumin to lipid as a function of ionic strength.

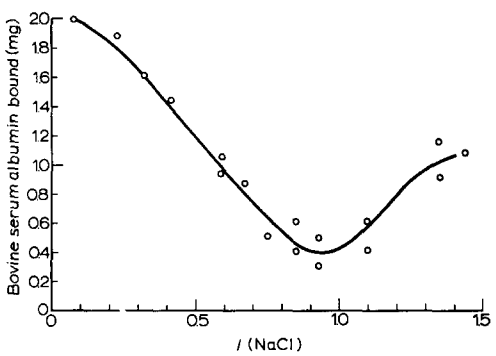


Fig. 4. The dependence of bovine serum albumin–liposome binding on ionic strength. Enough NaCl was added to a mixture of bovine serum albumin and liposomes to give the desired ionic strength. After centrifugation, the protein concentration in the supernatant or subsolution was determined and the amount of bovine serum albumin complexed to lipid calculated by difference. The amount of bovine serum albumin is expressed as mg of bovine serum albumin per ml of liposome suspension (containing 12 mg lecithin, 2.3 mg dicetyl phosphate and 0.9 mg cholesterol), pH 3.5.

The data in Fig. 4 indicate that the sensitivity of binding to an increase in the NaCl concentration is somewhat anomalous. In the range from 0 to 1 M, bovine serum albumin is uniformly displaced by increasing the salt concentration; however, over the range from 1 to 1.5 M bovine serum albumin binding actually increases. Since 90% of the bovine serum albumin bound at low ionic strength can be displaced by 1 M NaCl, non-ionic interaction must be responsible for the rebinding of bovine serum albumin induced by high salt concentrations.

The pH dependence for the "high salt" binding is shown in Fig. 5. This curve differs from the "low salt" curve in several significant respects: (a) it has a pK of about 3.7 as compared to 4.6 for low ionic strength binding; (b) binding is not as sensitive to low pH values (below 3) as is the "low salt" binding (Fig. 1); (c) at saturation, only 50% of the protein originally bound at low ionic strength is bound. This

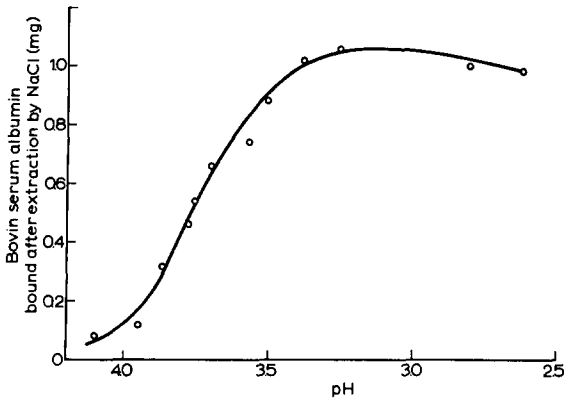


Fig. 5. The effect of pH on salt-stable binding. Enough NaCl was added to a mixture of bovine serum albumin and liposomes to make the concentration 1.4 M. The samples were centrifuged and protein concentration in the subsolution determined. Protein concentration is expressed as mg of protein bound per ml of liposome suspension.

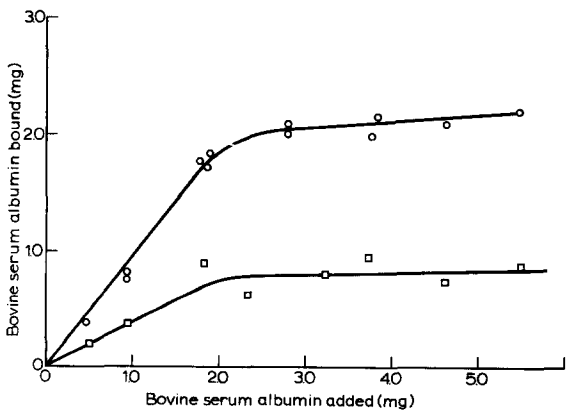


Fig. 6. The concentration dependence of bovine serum albumin–liposome binding. Mixtures of bovine serum albumin and liposomes were centrifuged and the bovine serum albumin remaining in the supernatant determined. The amount of protein bound was calculated by difference. ○—○, NaCl concentration 0.073 M; □—□, enough solid NaCl was added to make the concentration 1.4 M. All results are in terms of mg bovine serum albumin per ml of liposome suspension (containing 12 mg lecithin, 2.3 mg dicetyl phosphate and 0.9 mg cholesterol), pH 3.5.

difference is also demonstrated in Fig. 6 where the amount of bovine serum albumin bound is plotted against the amount added. At low ionic strength, almost all of the added bovine serum albumin is bound until the lipid is saturated at about 2 mg protein per ml of liposome suspension. On the other hand, in 1.4 M NaCl, only half the added bovine serum albumin is bound at saturation.

To determine the extent to which non-electrostatic or "high-salt" binding is involved in the bovine serum albumin–liposome interaction at low ionic strength, the dependence of binding on membrane charge was analyzed. The data are presented in Fig. 7. The amount of bovine serum albumin bound is directly dependent on the amount of dicetyl phosphate (the charged component in our negative membranes) present in the membrane. Thus, there is apparently no charge-independent binding of bovine serum albumin under these conditions. Furthermore, it can be calculated

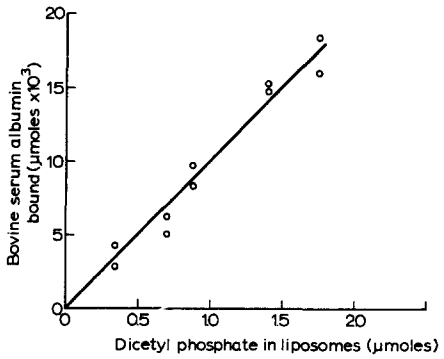


Fig. 7. The dependence of bovine serum albumin–liposome binding on dicetyl phosphate. Liposomes containing increasing amounts of dicetyl phosphate were mixed with bovine serum albumin and then centrifuged. Bovine serum albumin remaining in the supernatant was determined and the amount bound calculated by difference.

from these data (Fig. 7) that the molar ratio for bovine serum albumin to dicetyl phosphate is about 0.01 for all levels of dicetyl phosphate. Since each bovine serum albumin molecule carries about 100 positively charged groups, this further suggests that binding under the conditions of the experiment is predominantly electrostatic.

The previous data as well as the other binding experiments make it important to know how much of the liposome phospholipid is available for interaction with protein. The liposome is a closed structure consisting of layered lipid lamellae and it

TABLE II

BOVINE SERUM ALBUMIN BINDING TO LIPOSOMES PREPARED UNDER DIFFERENT CONDITIONS

Bovine serum albumin was either added to liposomes after formation or directly to the dried lipids in 0.073 M NaCl and in 1 mM phosphate buffer. Liposomes were also subjected to a one minute sonication after the addition of bovine serum albumin. In all cases, 12 mg lecithin, 2.3 mg dicetyl phosphate, and 0.9 mg cholesterol were present in 1 ml of liposome suspension.

Liposome preparation	*Bovine serum albumin bound (mg)*
Standard preparation	2.4
Sonicated liposomes	2.3
Dried lipids	2.5

is not clear that bovine serum albumin added to the outside can interact with the inner lipid layers. Table II contains data relevant to this point. From this table, it can be seen that there is little or no change in the maximum amount of protein bound by liposomes prepared either by sonication or by addition of protein directly to the dried lipid prior to dispersion. In both of these cases, more lipid than normal would be available for interaction if binding occurs only at the surface of the liposome. Thus, the amount of protein bound appears to be independent of the surface area of lipid to which it is exposed. Although somewhat surprising, this result was highly reproducible and it suggests that the ability of the protein to bind to liposomes may be related to a mechanism for actual penetration of the membrane by protein.

DISCUSSION

GREEN AND FLEISCHER[9] have carried out extensive studies on the interaction between mitochondrial proteins and phospholipids. They found that two basic types of binding could be characterized, electrostatic interactions and hydrophobic or nonpolar associations, and offered diagnostic tests to differentiate between the two possibilities. They also considered the possibility that both types of interaction could be invoked in a single lipid–protein system. However, only one of the two kinds of binding seemed to be present in any single lipid–protein system they studied.

Initial consideration of bovine serum albumin and the lecithin–dicetyl phosphate dispersion, suggested that electrostatic interactions were likely to cause lipid–protein binding. A water soluble, globular protein like bovine serum albumin is usually considered to have its ionized, polar side chains directed toward the external solution, and its nonpolar side chains forming an isolated hydrophobic core. For thermodynamic reasons[10], lipid molecules in water also direct their charged groups toward the external solution forming bimolecular leaflets with nonpolar interiors. Therefore, electrostatic interactions would seem to be favored in this situation. In the case of a negatively charged membrane, electrostatic interactions will be more probably near or below the isoelectric point of bovine serum albumin where the negative charge of the protein is diminished and charge–charge repulsions are reduced.

The explanation of bovine serum albumin–liposome binding most consistent with the observations reported here, is an electrostatic association between the positively charged residues of the protein and the negative lipid phosphate groups. Binding is greatly inhibited as the NaCl concentration approached 1 M (Fig. 5), which strongly suggests electrostatic interactions. A lowering of the pH to around 2 also reduces binding, implicating the lipid phosphate groups which are titrated in this region. Finally, extensive acetamidination of the lysyl residues on bovine serum albumin results in a shift in the protein–lipid binding curve to pH 5.4 where bovine serum albumin has a net negative charge. It may be that the extension of the positive charge two bond lengths further from the peptide backbone would make these groups more susceptible to attraction from the negative membrane with less interference from the electrostatic repulsions due to negative groups on the protein.

When stearyl amine is the charge-carrying lipid component, binding takes place over a much broader pH range. This is probably due to the fact that bovine serum albumin does not have many groups which are titrated between pH 5 and 8, so the net charge on the protein will not change dramatically in this pH range. It is interesting that the bovine serum albumin-binding capability of positive liposomes increases

considerably more than twice when the molar concentration of the charged component is doubled. This effect could be due to both the higher charge density and to a spatial arrangement of positive charge which more nearly duplicates the arrangement of negative charges on the surface of the protein.

It is apparent, then, that electrostatic associations are of primary importance in the description of binding between liposomes and bovine serum albumin. However, they cannot be invoked to explain our earlier results on the effect of bovine serum albumin on the permeability of liposome membranes[6]. Electrostatic associations between bovine serum albumin and liposomes containing stearyl amine result in little or no enhancement of membrane permeability. In addition, there is a small but significant difference in the pH dependencies of low ionic strength binding and membrane permeability change. The pK for binding is about 0.3 of a pH unit higher than the pK for activation of diffusion. This pH difference is even greater in the case of acetamidinated bovine serum albumin where the protein is bound to the liposomes at a pH well above that where any substantial change in membrane permeability takes place (see Table II). In line with these observations, PAPAHADJOPOULOS AND WATKINS[12] have found that while cytochrome c is bound to liposomes electrostatically, it has no effect on permeability. It seems probable, then, that some other factors are involved in causing the permeability changes seen in this system.

There are two likely ways for trapped solute to be released from the liposomes. One is a gross disruption of the lipid lamellae with the instantaneous release of the liposome contents, and the other is an alteration of the liposome membranes in such a way that their permeability to solutes is increased. In our earlier work, we discounted the first alternative because both the time course of diffusion and the temperature dependence of bovine serum albumin-activated diffusion indicate that intact lipid membranes are present. This conclusion is supported by subsequent experiments, which have indicated that activated diffusion is not instantaneous and that the bovine serum albumin–liposome complex responds to osmotic stress[15]. If the membranes remain intact and bovine serum albumin is able to penetrate the liposome structure as is indicated by the data in Table II, a complex of at least a transitory nature, is suggested in which bovine serum albumin extends into and across the bimolecular leaflet. In this regard, CAMEJO *et al.*[16] have reported an increase in the surface pressure of phospholipid monolayers in the presence of serum albumin at low pH. This suggests the insertion of protein into the hydrophobic portions of the lipid layer.

The forces which give rise to the penetration of lipid membranes by bovine serum albumin have not been determined. However, the formation of apolar associations seems to be a likely possibility. AOKI AND FOSTER[17] have studied the behavior of bovine serum albumin in acid solutions extensively. Around pH 4 this protein undergoes a conformation change which involves the exposure of hydrophobic areas of the protein to solvent. This is followed by a general expansion of the protein at pH values below 3. Binding of bovine serum albumin to liposomes induced by 1.4 M NaCl has a pH dependence similar although not identical to this conformation change. The fact that the association is stable at high salt concentration and at low pH values and parallels an increase in the hydrophobicity of the protein imply nonpolar interactions. Lipid and protein could associate hydrophobically during the movement of apolar areas of the protein through a lipid bilayer. Of course, there is no

direct evidence that these associations are also present at low salt concentrations; however, they cannot be ruled out. Under conditions of high ionic strength where electrostatic interactions are inhibited, membrane permeability is still enhanced. Presumably, under these conditions diffusion is activated by the associations stabilized at high salt concentrations. However, if these interactions do exist at low ionic strength they must be considerably weaker than the electrostatic associations responsible for binding.

Another mechanism for penetration is suggested by the data in Fig. 7, which shows a linear relationship between the amount of bovine serum albumin bound and the amount of dicetyl phosphate in the membrane. The negative charges on the phospholipid are almost exactly balanced by positively charged functional groups on the protein. If bovine serum albumin is able to concentrate a large number of charge-carrying lipids at one point on the membrane surface, it could result in a localized disruption of membrane structure and allow protein to penetrate and solute to escape.

In conclusion, our picture of the liposome–bovine serum albumin interaction involves an initial binding of bovine serum albumin to the polar groups of the lipid through electrostatic interactions. If the protein exists in the more hydrophobic conformation, it then penetrates the apolar interior of the liposome membrane. This movement creates an area through which polar molecules can move and enhances their diffusion rate. Finally, continuing weak hydrophobic associations between the lipids and apolar areas of the protein or the concentration of membrane charges may help mitigate this process.

It is unfortunate that bovine serum albumin has no known enzymatic activity and little biological relationship to the proteins found in cell membranes. However, this model membrane system remains a valuable tool in the study of lipid–protein interactions in general. It is interesting to note that in the biological synthesis of mitochondria in yeast[18], it has been suggested that a phospholipid framework is laid down before the synthesis of mitochondrial proteins. The test-tube duplication of this process using model membrane systems does not seem at all unlikely.

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