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Surface Activity of Bovine Whey Proteins at the Phospholipid-Water Interface

The interaction of the major whey proteins of cows' milk with phospholipids in a monolayer was studied with a Langmuir Film Balance. The phospholipids were mixtures of phosphatidylcholine and phosphatidylglycerol. The ultraviolet and circular dichroism spectra of transferred films were used to determine the quantity and conformation of proteins interacting with the lipids in the monolayer. The proteins were dissolved in the aqueous subphase and migrated to the phospholipid/water interface where adsorption was controlled by the solution pH and calcium ion concentration. At neutral pH, above the isoionic point of all proteins studied, protein-phospholipid interaction was minimal. Some binding of protein to the lipids was observed when the subphase was at the isoionic pH of the protein. Increased lipid-protein binding was observed when the subphase was below the isoionic pH of the protein; under these conditions a film of protein about one molecule thick formed beneath the lipid monolayer. Calcium in the subphase interfered with lipid-protein binding, but the effect was pH dependent. Circular dichroism spectra of transferred films showed that the secondary structure of the proteins was largely preserved in the lipid-protein complexes. An electrostatic mechanism for lipid-protein interaction is discussed.

Dispersed fat particles in a stable system such as milk are prevented from coalescing by repulsive forces between their surface layers. Phospholipids assist in stabiliz-

ing the lipid phase of milk through formation of a bi-layer fat globule membrane (FGM) on the surface of the oil droplets (1). During milk processing steps such as homogenization, the fat globule particle size is decreased and the oil/water interfacial area is increased. The freshly exposed oil surface is able to adsorb additional surface active agents such as phospholipids and proteins to give a stable emulsion. Since there is enough phospholipid in milk to form a film on a greatly expanded oil/water interface, this lipid undoubtedly plays an important role in stabilizing dairy and other food products which utilize homogenized milk. Although much work has been done on the surface activity of proteins at the oil/water interface, most of the efforts have centered on the interaction of proteins with neutral oil (hydrocarbons, triglycerides) surfaces; interactions with the polar lipids such as the phospholipids have received much less attention. Interaction between phospholipids and proteins depends to a degree on solution pH and mineral (principally calcium) content and on the properties of the lipid and proteins themselves. This of course implies that milk products, when used as a food additive, may exhibit different functional surface properties in different foods. Accordingly we decided to investigate the interaction of proteins with phospholipids over a range of pH's and calcium ion concentrations which were selected to include the conditions found in a variety of foods.

The caseins are the major milk proteins used to stabilize food emulsions, however whey is receiving increased attention as a food additive. This is especially true in acid foods where the whey proteins remain soluble. In such food uses, the end-use environment of whey can vary considerably ie. pH, salt, mineral content, etc. We are studying the interaction of the major whey proteins with film forming substances such as phospholipids under a range of solution conditions typical of food systems. In this work we report on the interaction of phospholipids in monolayers with the major whey proteins, beta-lactoglobulin (β -LG), alpha-lactalbumin (α -LA), and bovine serum albumin (BSA). Mixtures of palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylglycerol (POPG) were chosen to simulate the zwitterionic (POPC) and charged (POPG) phospholipids of milk. The pH of the solutions ranged from 4.4 to 7; the calcium concentration was 0 to 8mM. These conditions include the ranges of pH and calcium ion (free, uncomplexed Ca^{2+}) found in many foods to which milk or dairy products might be added.

Experimental

Materials. The phospholipids were from Avanti Polar Lipids, Birmingham, AL; the proteins were from Sigma, St.

Louis, MO. The spreading solvent for the lipids was ACS Reagent Grade chloroform, treated to remove surface active impurities (2). Walpole's acetate (3) was used for pH 4.4 and 5.2, Tris-HCl for pH 7. A low buffer concentration (1-5 mM) was used to minimize light scattering in the films used for ultraviolet (UV) and circular dichroism (CD) spectroscopy.

The following are some relevant properties of the proteins used in this work. β -LG; molecular weight (MW), 18,300; isoionic point (I_{IP}), 5.1; molar absorptivities (ϵ_M) for the backbone (190 nm) band, 1.56×10^6 ; mean residue absorptivity for the 190 nm band (ϵ_R), 9600 (7); α -LA; MW, 14,200; I_{IP} , 4.4; ϵ_M , 1.07×10^6 ; ϵ_R , 8700; BSA; MW, 66,300; I_{IP} , 5.2; ϵ_M , 5.06×10^6 , ϵ_R , 8700. UV absorptivities for BSA and α -LA were determined in the authors' laboratory (unpublished observations).

A miniature Teflon trough (15 X 11 X 0.7 cm) with a dipping well (total depth 2 cm) was used for film transfer in this work. A magnetic stirrer was used to mix the protein in the subphase. The miniature trough was mounted in the cradle of a previously described (2) Langmuir balance from which the large trough had been removed. The barrier drive, Wilhelmy plate, constant-pressure control, and film transfer were all provided by the large film balance system. The experimental setup is illustrated schematically in Figure 1. A complete discussion of film balance techniques has been given (4).

Methods. The principle objective of this work was to estimate the amount and conformation of protein interacting with phospholipids at the lipid/water interface. The protocols for monolayer film and protein solution preparation, film and solution surface pressure measurements, and transfer of the monolayers for spectroscopic determinations have all been given in extenso (2, 5, 6); the following is a summary. The surface pressures (surface tension lowering) of the proteins were determined by the Wilhelmy plate technique on stirred solutions using thoroughly cleaned glassware. Techniques involving the formation and handling of lipid monolayers were carried out on the miniature film balance. A series of preliminary experiments were run to determine the optimum film pressure, protein concentration, etc., for UV and CD spectroscopy as follows:

- (1) The surface pressures of β -LG, α -LA, and BSA were determined as a function of concentration (up to about 80 mg/L) on buffered solutions. This established the maximum surface pressure that the proteins could achieve over the range of concentrations studied.
- (2) Using the miniature film balance, monolayers of POPG/POPC (35/65 mol%) were formed by spreading the premixed lipid from chloroform solvent onto a

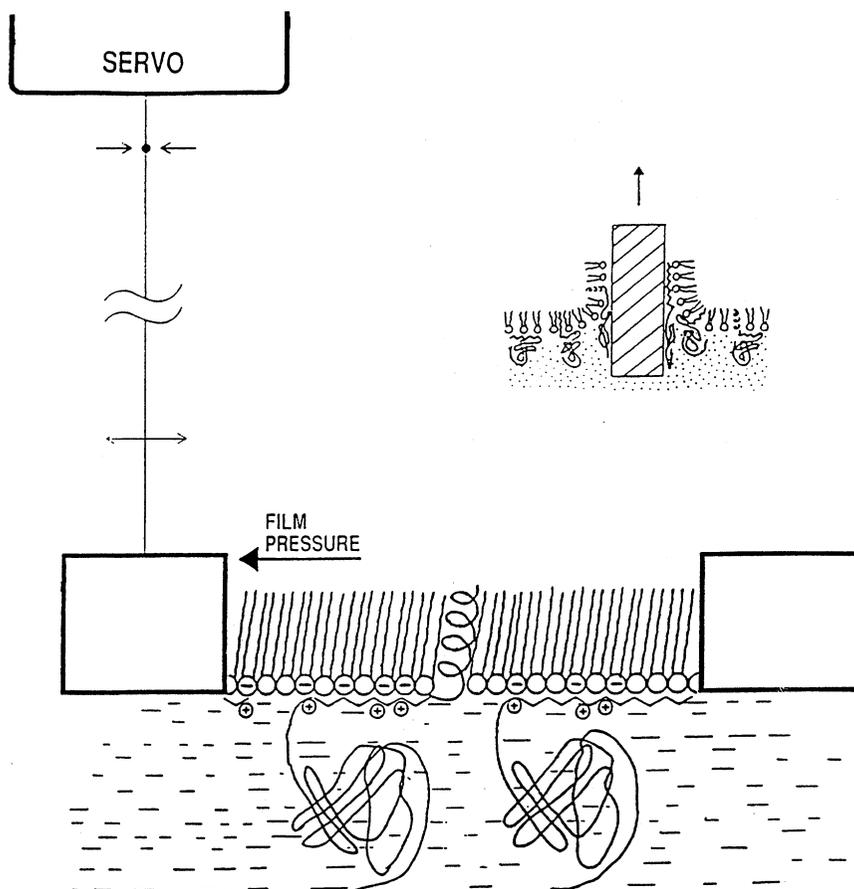


Figure 1. Schematic diagram of a Langmuir-Blodgett film balance showing proteins adsorbed from solution onto the head groups of phospholipid monolayers. The inset shows a lipid-protein monolayer being transferred to the surface of a quartz plate for ultraviolet or circular dichroism spectroscopy. Eight plates were used which gave a total of 16 monolayers for spectroscopic investigations.

buffered subphase. After the spreading solvent evaporated (15 min), the film was compressed to a pressure greater than that achieved by the proteins at the air/water interface in experiment (1) above. Then, with the compression barriers stopped, protein was injected into the subphase beneath the lipid monolayer and the film pressure was recorded for 30 min. Any pressure changes in the film usually occurred during the first few minutes of a run with little if any change noted towards the end of the period. A series of such runs at varying protein concentration established a pressure rise-protein concentration profile for that particular lipid monolayer/subphase combination. These profiles were usually in the shape of a sharply rising pressure at low protein concentration followed by a plateau at higher concentration. The knees of the plateaus generally occurred between 2-3 mg/L protein concentration.

- (3) The pressure rise in a lipid monolayer observed upon injecting protein into the subphase was recorded for a series of lipid monolayers with different initial pressures. Sufficient protein was injected into the subphase to give a protein concentration of 5 mg/L, which was well into the plateau region established in experiment (2) above. These experiments gave the pressure rise in the lipid film (upon injection of protein into the subphase) as a function of initial lipid film pressure (just before injection of the protein). This allowed us to estimate a "critical" initial lipid pressure, above which no change in the monolayer pressure was observed upon injection of protein into the subphase.
- (4) In all subsequent experiments, the initial lipid pressure was kept above the "critical pressure" to prevent the protein molecule from inserting a portion of itself into the monolayer. The effect of solution (subphase) pH and calcium concentration on lipid-protein binding in the monolayers was studied by spreading lipid onto subphases of different composition, injecting the protein and then transferring the film to quartz plates for UV and CD analysis. Eight quartz plates were partially immersed in the subphase prior to spreading the lipid. After spreading the lipid, injecting the protein, and equilibrating the film for about 30 min., the monolayers were transferred to the quartz plates (at constant film pressure) and mounted in the UV or CD instrument for spectroscopic analysis. The spectral range was 180-350 nm for UV spectroscopy and 180-260 nm for CD spectropolarimetry. Control experiments showed that only proteins contributed significantly to either the UV or the CD spectra over the ranges studied. Additional details about equipment and methods for CD (5) and UV (7) spectroscopy have been given.

Adsorption of the proteins to the quartz plates used for film transfer and to the zwitterionic choline was anticipated. Since we were interested in adsorption only to the charged lipid POPG, control experiments were run and contributions to the UV spectra from protein adsorbed to the quartz plates and POPC was subtracted from the UV absorbance. For every UV experiment using POPC/POPG (65/35 mol%) there was a control run using identical procedures (subphase pH, protein concentration etc.) except for the lipid film which was a monolayer of POPC. In the case of calcium free systems, the UV absorption observed with the control films was about 20-30% of that observed with monolayers containing POPG; this contribution was subtracted before calculating the POPG-protein binding. Light scattering was noticeable in films transferred from subphases containing 10 mM sodium chloride; we employed a log-log extrapolation procedure to correct for scattering contribution to the 190 nm protein absorption band (8). Contributions from this source were estimated to be about 20-30% of the total UV absorption signal observed at 190 nm with 10 mM sodium chloride in the subphase.

Results and Discussion

The results discussed here were first published separately for β -LG (6) and for α -LA and BSA (Cornell, D. G.; Patterson, D. L.; Hoban, N.; Colloid Interface Sci., 1990, in press.) Together these two reports give a picture of a lipid-protein-calcium interaction scheme which follows mass action principles and involves an electrostatic mechanism. Of particular importance are the electrical properties of the proteins in relation to the solution pH as discussed below.

The purpose of steps 1-3 above was to establish conditions under which we were confident that the proteins were interacting with only the head groups of the lipids while at the same time achieving maximum lipid-protein complexation. Step 1 established the maximum surface pressure the various proteins could support at the air/water interface over the concentration range studied. These results are shown in Figure 2. In step 2, where protein was injected beneath a lipid monolayer, the initial lipid film pressure was higher than the pressure achieved by proteins at the air/water interface. This effectively suppressed the formation of patches of pure proteins in a monolayer of lipid. Any film pressure rise observed upon injecting protein into the subphase was taken as evidence of insertion of a portion of the protein molecule into the lipid monolayer in an intimate lipid protein mixture. The results are shown in Figure 3. In this step we were also able to establish the

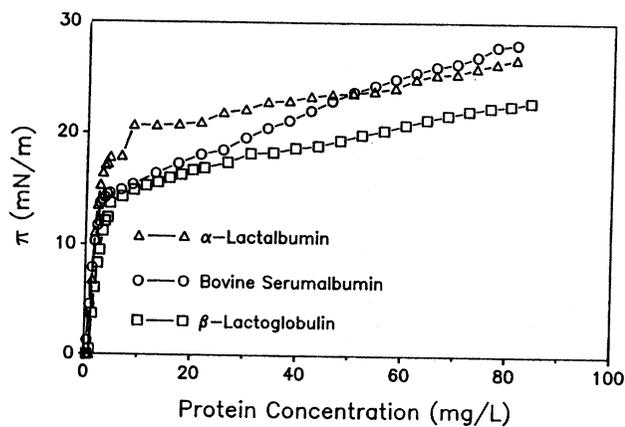


Figure 2. The surface pressure (surface tension lowering) of proteins in Walpole's acetate pH 4.4 as a function of concentration.

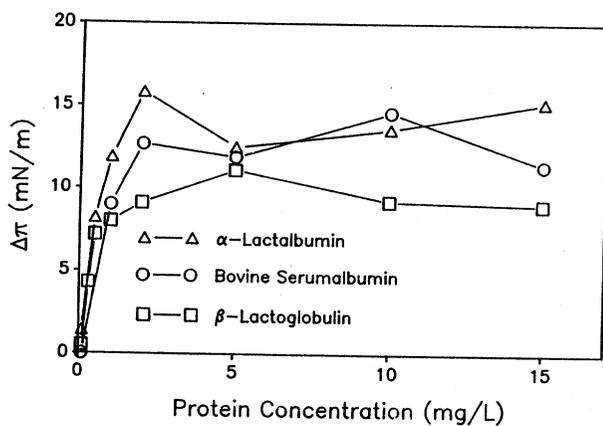


Figure 3. The rise in surface pressure of a phospholipid (POPC/POPG, 65/35 mol%) monolayer upon injection of protein into the underlying subphase. The initial pressure of the lipid films was 22 mN/m. Subphase; Walpole's acetate pH 4.4.

"saturation" concentration for the proteins; the concentration (or narrow range of concentrations) above which no further evidence of lipid-protein interaction was observed. Despite the variability in the pressure rise shown by the three proteins in Figure 3, "saturation" concentration appears to occur between 2 to 5 mg/L protein concentration in the subphase for all proteins. In all subsequent experiments, sufficient protein was injected into the subphase to achieve a concentration of 5mg/L.

The magnitude of the pressure rise observed in the lipid monolayer upon injection of protein into the subphase depended upon the value of the initial monolayer pressure. When the initial lipid pressure was above a certain "critical" value, little or no change in the pressure of the lipid monolayer was observed upon injection of protein into the subphase. This was taken to mean that insertion of the protein into the monolayer was effectively suppressed. The "critical pressure" was estimated by extrapolating a plot of pressure rise vs. initial pressure to zero pressure rise as shown in Figure 4. While the penetration of protein into the lipid portion of the oil/water interface is certainly an interesting mechanism for the stabilization of emulsions, we wished to study electrostatic interactions at the oil/water interface without the complications of protein insertion. For all subsequent experiments reported here 35 mN/m was the initial lipid pressure. In all these cases the change in the film pressure (\pm) after injection of the protein was less than 1/2 mN/m for the duration of each run, about 30-45 min.

The amount of protein interacting with the phospholipids in monolayers at various subphase pH's was determined from the UV spectra of the transferred monolayers using what is essentially Beer's Law for films, namely:

$$A = n\epsilon_m\Gamma/6.02 \times 10^{20} \quad (1)$$

where A is the absorbance of n monolayers, ϵ_m is the molar absorptivity of the protein, and Γ is the number of protein molecules per square centimeter in a single monolayer. The factor $n\Gamma/6.02 \times 10^{20}$ has the same dimensionality (mass per squared length) and numerical value as the lc product (path length in centimeters times concentration in moles per liter) of the classical Beer's law for solutions. A straightforward calculation then gave the surface concentration in milligrams of protein per square meter of monolayer as presented in Table I.

The results in Table I clearly show the effect of pH on protein binding to the monolayer lipids and supports an electrostatic mechanism for lipid-protein interaction. At acid pH's the proteins carry a significant positive charge over their surface which can bind to the negatively charged lipid. At neutral pH where both the lipids

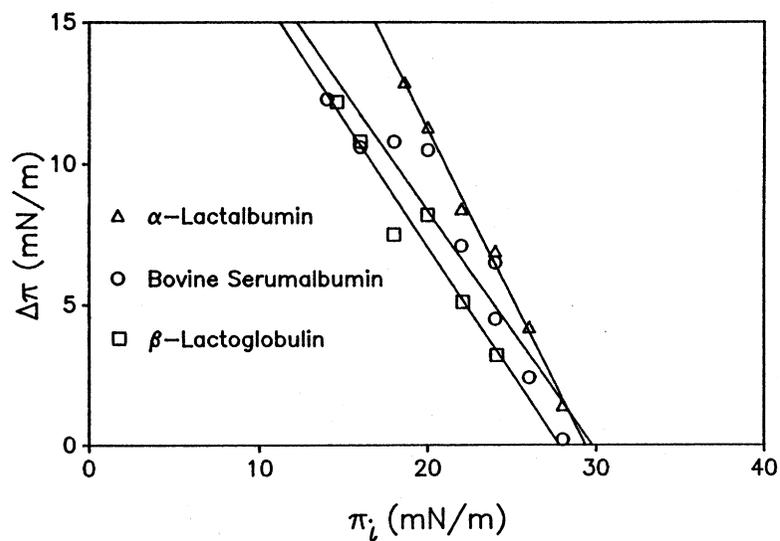


Figure 4. The increase in lipid monolayer pressure upon injecting protein into the subphase as a function of the initial film pressure. Protein concentration in the pH 4.4 subphase, 5 mg/L.

Table I. Protein Concentration in Phospholipid-Protein Monolayers

Protein		BSA	α -LA	β -LG
	Concentration			
Subphase	Units ²			
	mg/m ²	0.09 [.05] ¹	0	0.4 [.1]
Tris pH 7.0	molec./cm ²	8 ² X 10 ¹⁰	0	1.3 X 10 ¹²
Walpole's Acetate pH 5.2	mg/m ²	1.1 [.2]	-	-
	molec./cm ²	1.0 X 10 ¹²	-	-
Walpole's Acetate pH 4.4	mg/m ²	3.1 [.3]	2.6 [.6]	3.0 [.2]
	molec./cm ²	2.8 X 10 ¹²	1.1 X 10 ¹³	1.0 X 10 ¹³
Walpole's Acetate pH 4.4 10 mM NaCl	mg/m ²	1.9 [.4]	0.9 [.3]	-
	molec./cm ²	1.7 X 10 ¹²	3.7 X 10 ¹²	-

¹ Numbers in brackets are standard errors of 3-4 replicates.

² Errors in the molec./cm² units have been omitted for clarity but are proportionally identical to the errors for the corresponding mg/M² units.

and proteins carry a net negative charge there is electrostatic repulsion and a reduced tendency to interact. BSA shows this tendency most clearly. At pH 7, little if any lipid-protein complex is formed (.09 mg/M² is of doubtful statistical significance). At the isoionic point of BSA, pH 5.2, it is clear that the protein is interacting with the lipid. At a more acid pH, 4.4, the coverage has reached 3.1 mg/M². A concentration this high suggests the formation of a contiguous film of protein in its native (globular) state one molecular layer thick. Values of this order have been reported for many proteins adsorbed at the air/water interface (migrating from bulk solution), where the measurement techniques were ellipsometry (9) and surface radioactivity (10). Spread films, formed by direct application of the protein from solution onto the surface of water, generally have lower surface concentrations than adsorbed films at equivalent surface pressures (11). It is thought that some degree of unfolding occurs in a protein molecule when it is applied to the surface of water, subject to constraints such as intramolecular disulfide

cross linking (11). In earlier work, an increase in beta sheet was observed in β -LG spread at the air/water interface (2). In the current work, no such change in the secondary structure of any of the proteins was found. The CD spectra of the proteins in the lipid-protein monolayers (Figure 5) were similar to their spectra in solution as determined in the authors' laboratory. This suggests that there was no gross changes in any of the proteins secondary structure upon interacting with the phospholipids in the monolayers. This is consistent with the picture of globular proteins adsorbing (without unfolding) to the phospholipids in a monolayer.

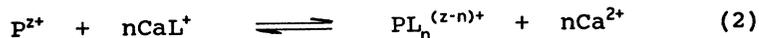
Perhaps the most interesting finding presented in Table I is the amount of complexation that occurs with a lipid by a protein in its zwitterionic state. This is shown by the film concentrations for BSA at pH 5.2 and α -IA at pH 4.4. At first sight this observation seems to run counter to an electrostatic mechanism for lipid-protein interaction, since a protein at its isoionic point has a net neutral charge. If the charge distribution is homogeneous over the surface of such a protein, then the molecule will appear to be neutral even at very close range. As the three dimensional structure of more and more proteins are determined however, it is becoming increasingly clear that the charge distribution over the surface of most proteins is anything but homogeneous. Consistent with this are electrostatic field calculations (12-15) which show that clusters of basic and acidic residues commonly form large potential envelopes over the protein surface. The patches of positive and negative charges will cause significant interaction when they are brought into close enough proximity to a charge of opposite sign. Lowering the pH of the aqueous phase below the isoionic point of the protein will give it a net positive charge, and the total area of the positive patches will grow. This should facilitate docking of the globular proteins to the negatively charged lipids, resulting in increased adsorption of the proteins at the oil/water interface. All of this is consistent with the results presented in Table I.

Calcium ions can also bind to negatively charged phospholipids and thus compete with proteins in a lipid-protein-calcium interaction scheme. Since calcium is a major component of milk and many other foods, knowledge of the role it plays in the adsorption of proteins at the oil/water interface is essential to the understanding of emulsion stabilization in such complex systems. We have studied the effect of calcium on lipid-protein interaction in several of the systems listed in Table I simply by adding calcium to the subphase buffer before spreading the lipid. Except for calcium in the buffers, all other conditions and procedures were as in step 4 above under methods. The amount of protein complexing with lipids in the presence of calcium was determined from the UV spec-

tra of transferred films and formula 1 above. The quantity of protein so obtained was compared to the values obtained for the same system in the absence of calcium (Table I), and plotted as the percent POPG/protein complex vs. calcium concentration as shown in Figure 6. The solid lines represent the best fit to the discrete data points based on the assumption of saturation binding over the entire range of calcium concentrations studied. In the case of BSA at pH 4.4 this resulted in a relatively poor fit at the lowest concentrations. Due to the variability in the data, the significance of this "poor fit" is uncertain. Because of this we have chosen to discuss the data only in terms of overall trends (see below).

When either α -LA or BSA was injected into a subphase near the respective proteins' isoionic point, calcium ions at the micromolar level interfered with lipid-protein binding. This is shown by the steep curves near the ordinate in Figure 6 where the subphase buffer is pH 4.4 for α -LA and pH 5.2 for BSA. These results imply that the apparent dissociation constant of the lipid-calcium complex is of the order of micromoles or less. When the pH of the subphase was below the isoionic point of the protein, millimolar levels of calcium were required to suppress the lipid-protein interaction. This is shown by the upper curves in Figure 6 where the subphase buffer is pH 4.4 for both β -LG and BSA. These results in turn imply that when the protein carries a net positive charge, it is better able to compete with the calcium ions for complexation to the lipid.

Assuming that calcium binds to POPG in a 1/1 complex as found by Lau et al. (16), we can summarize the binding of protein and calcium to monolayer lipid by Equation 2,



where P is protein carrying charge z, Ca is calcium, L is the lipid POPG⁻, and n represents the number of POPG lipids bound per protein molecule. The protein P^{z+} and calcium Ca²⁺ are in solution; the protein-lipid and calcium-lipid complexes PL^{(z-n)+} and CaL⁺ are components of the monolayer. The formalism for expressing the interaction between species in solution and components of a monolayer is essentially the same as that for the mass action law in solution (16).

Clearly what is being proposed here is an electrostatic mechanism for calcium-protein-lipid interactions in monolayers. If this mechanism is correct, then salt should have an effect on lipid-protein binding. This is borne out by the results with 10 mM NaCl in the subphase as shown in Table I. Binding of both α -LA and BSA is slightly reduced in this media compared to the corresponding salt free system.

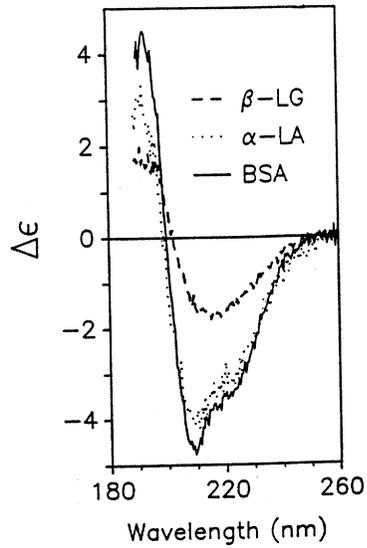


Figure 5. The circular dichroism spectra of lipid-protein monolayers; sixteen monolayers for each lipid-protein set. The proteins were adsorbed from pH 4.4 Walpole's acetate onto POPC/POPG monolayers.

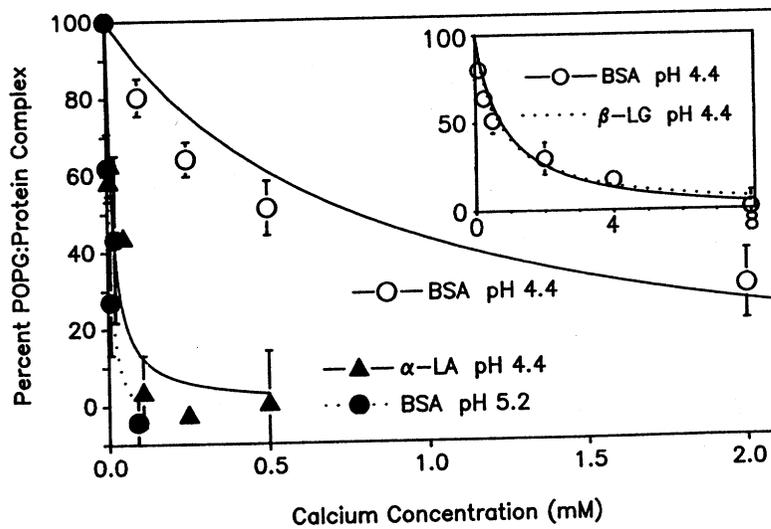


Figure 6. The effect of calcium concentration and pH on the adsorption of proteins to POPG in monolayers.

If expression 2 is correct one would expect that the equilibrium could be driven to the right, in the direction of increased protein-lipid binding and reduced calcium-lipid binding, simply by increasing the protein concentration in the subphase. A small shift of this sort was found with β -LG. Increasing the protein concentration by a factor of 10 (to 50 mg/L) at a calcium concentration of 2 mM increased the amount of lipid-protein complex observed from 25% to about 36% (9). Assuming that equilibrium 2 is for n identical noninteracting binding sites, it can be shown by a simple mass action expression that this relative insensitivity to protein concentration implies that n is "large". The assumption of n identical and independent binding sites is not generally applicable however, and the determination of multiple equilibria constants can be rather involved (17, 18). Furthermore, the scatter in the data of Figure 6 is too great to allow an analysis in terms of multiple equilibria. The protein concentration used in most of this work was quite low (5 mg/L or 0.0005%); increasing this value to levels more typical for many foods (3 g/L for β -LG in milk for example (19)) should increase protein binding to charged lipids.

Conclusion

The colloid stability of foods depends on the complex interplay of many factors. Electrostatic as well as hydrophobic interactions are important stabilizing influences. The aqueous milieu, its pH, mineral content, etc., help determine the electrical forces between surfaces which play such a vital role in colloid stability. In this work we have shown how the interrelationship between the pH of a solution and the isoionic pH of a protein determines its ability to compete with calcium for adsorption onto negatively charged phospholipid surfaces. This suggests that the electrical properties of the individual proteins are among the many factors that must be considered when the development of a new food is being contemplated.

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