MIXED MONOLAYERS OF β-LACTOGLOBULIN AND PHOSPHATIDES

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SUMMARY

Mixed monolayers of β-lactoglobulin and milk phosphatides were prepared by thoroughly mixing the gaseous films of the film constituents. From the force/area curves of these mixed monolayers the amount of interaction between protein and phosphatide at different pH values was estimated. No appreciable interaction was found at pH 1.0 and 5.9, where the film constituents carry charges of similar sign. Strong complex formation, however, occurred at pH 3.9, where the protein and the phosphatide are oppositely charged. Calculations are given of the electrostatic interaction to be expected in the mixed monolayer and it is shown that the greater part of the experimental cohesion must be ascribed to non-electrical effects.

INTRODUCTION

The interaction between proteins and phospholipids has been studied extensively at air/water and oil/water interfaces, as the survey by FRASER[1] shows. It is thought that such studies may throw some light upon the nature of lipoprotein interaction and upon the physicochemical properties of biological membranes, which are often found to consist of protein and phosphatide components.

DOTY AND SCHULMAN[2] and MATALON AND SCHULMAN[3] were the first to investigate the interaction by means of the spreading technique. They injected various proteins beneath films of different lipids that were spread on a Langmuir trough. The interactions that occurred were then followed by observing either the changes in the surface pressure at constant film area or the area variations at constant pressure. Apart from electrostatic effects between the protein ions and the ionic heads of the film molecules, only weak interactions between the two substances could be detected. The interaction of protein and phospholipid is strongly influenced, however, by their ionic charges. For instance, at pH values slightly below its isoelectric point the positively charged serum albumin vigorously penetrates between the negative ionic heads of a cephalin monolayer. On the other hand, by shifting the pH of the subsolution to alkaline values nearly all of the protein that had penetrated could be ejected from the phospholipid film. The latter effect is clearly due to the electrostatic repulsion between the two negatively charged components. SCHULMAN *et al.* carried out their experiments with phospholipid films that were kept initially at surface pressures beyond the collapse pressure of protein films (*e.g.* > 15 dynes cm^{-1}). In

this way spontaneous spreading of the injected protein on the surface is prevented and the detected surface pressure changes may readily be ascribed to interactions between the protein and the lipids. It must be emphasized, however, that at such high initial pressures the phosphatide film is already rather compressed; consequently the non-polar chains of the phosphatide molecules will, for steric reasons, be partly prevented from interaction with the side-chains of the protein. The difficulty has been avoided in the experiments of ELEY AND HEDGE[4], who injected the proteins at considerably lower surface pressure of the phosphatide film (*e.g.* 2 dynes. cm^{-1}). But under such conditions spontaneous spreading of the protein will occur and it will be difficult to distinguish between spreading of the protein and proper interaction effects. Thus it is still open to question, whether there is any specific interaction between protein and lipid.

A serious objection to the injection technique is that experiments are very difficult to evaluate, as was pointed out by BULL[5]. In fact one is never sure whether the injected protein is completely adsorbed on the phosphatide film since there is a possibility that it may also dissolve in the subsolution.

In the investigation on which we report here we tried to overcome these difficulties by studying the behaviour of mixed monolayers of β-lactoglobulin and milk phosphatides.

In the absence of a suitable spreading solvent for mixtures of these two substances a modified spreading technique for the mixed monolayer had to be applied. By this technique the mixed monolayer is prepared by thoroughly mixing the gaseous monolayers of the constituents, which have been spread separately.

The amount of interaction in the mixed film can easily be estimated from a comparison of the experimental force/area curve with an ideal plot. The latter is calculated from the force/area curves of the pure components, assuming that there is no interaction between them.

MATERIALS AND METHODS

Milk phosphatides were extracted from fat-free butter serum with an alcohol–carbon tetrachloride mixture and purified as described by VAN HANDEL[7] and by KOOPS[8]. The composition of the final product was found by chemical analysis to be: lecithin 30 %, cephalin 45 % and sphingomyelin 25 %. For spreading, the phosphatides were dissolved in a mixture of 80 % petroleum ether (b.p. 60—80°) and 20 % chloroform in a concentration of 0.756 mg/ml.

β-Lactoglobulin was prepared from fresh skim milk according to the method of LARSON AND JENNESS[9]. Because the final product failed to crystallize the protein was dried from the frozen state. When subjected to moving boundary electrophoresis it appeared to be homogeneous. For spreading purposes β-lactoglobulin was dissolved in barbiturate buffer pH 6.5 and ionic strength 0.1. Its concentration was about 0.5 mg/ml and fresh solutions were made every week.

Sodium chloride, used to establish a constant ionic strength in the subsolution, was heated to glowing in a platinum beaker to free it from surface-active impurities.

Acetate buffer solutions were prepared with laboratory distilled water which had passed through an "Amberlite" mixed ion-exchange bed. At the lowest surface pressures measured (*e.g.* 0.15 dynes. cm^{-1}) no surface-active contamination from this

water could be detected. At pH 1.0 the subsolution was prepared from equimolar quantities of hydrochloric acid and sodium chloride.

Force/area curves at the air/water interface were determined with a conventional Langmuir through of the type described by GORTER AND SEEDER[10]. Its dimensions were 60 × 14 × 1 cm^3. Pressure readings were accurate to about 0.15 dynes. cm^{-1}.

For spreading, surface-active material was administered to the surface by means of an "Agla" micrometer syringe mounted on the micrometer of a microscope to permit a cautious approach to the surface.

The ionic strength of the subsolution was 0.2 in all experiments. On such subsolutions it was found that the spreading of β-lactoglobulin was completed within 20 minutes.

Force/area curves were most reproducible when drops of the protein solution were gently touched at the interface[5].

No difficulty was experienced in spreading the phosphatides. Pressure readings in this case were taken after 5 minutes.

We could not find a suitable spreading solvent for mixtures of β-lactoglobulin and phosphatides. Either the phosphatides form sols in the aqueous protein solution or the protein is flocculated by the apolar solvent of the phosphatides. Such "solutions" do not permit reproducible force/area measurements for the mixed monolayers.

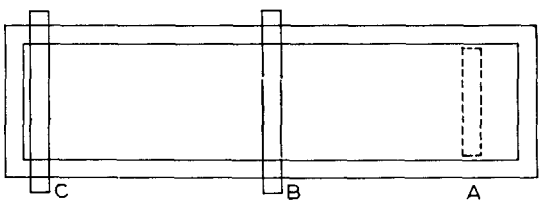

Fig. 1.

We therefore proceeded as follows: With the float of the measuring device, which is normally situated at A (Fig. 1), removed, the trough surface is divided into two equal areas by a paraffin-coated glass barrier B. Now β-lactoglobulin is spread as a gaseous film on the right-hand area. After 15 min a gaseous film of phosphatides is spread on the left compartment. After a further 5 min the barrier B is carefully removed from the surface and placed at the right-hand end of the trough. By slowly sweeping the protein film to the left it is driven into the phosphatide film. When B is again at its initial place the measuring balance is installed at A. By now moving B to the right towards the balance one can check that no surface-active material is left behind to the right of B. Then B is again removed and barrier C at the left is moved to the right to compress the mixture of the films. After several compressions and expansions the mixed monolayer is allowed to stand for a further 5 min before pressure readings are taken. The film is re-expanded and the force/area curves redetermined after different intervals of time. This check is always made and reproducibility of the surface pressures has always been good, even after three hours.

RESULTS

Force/area plots for phosphatides and β-lactoglobulin at pH 5.9, 3.9 and 1.0 are given in Figs. 2 and 3. Each of these and the following plots is the average value of

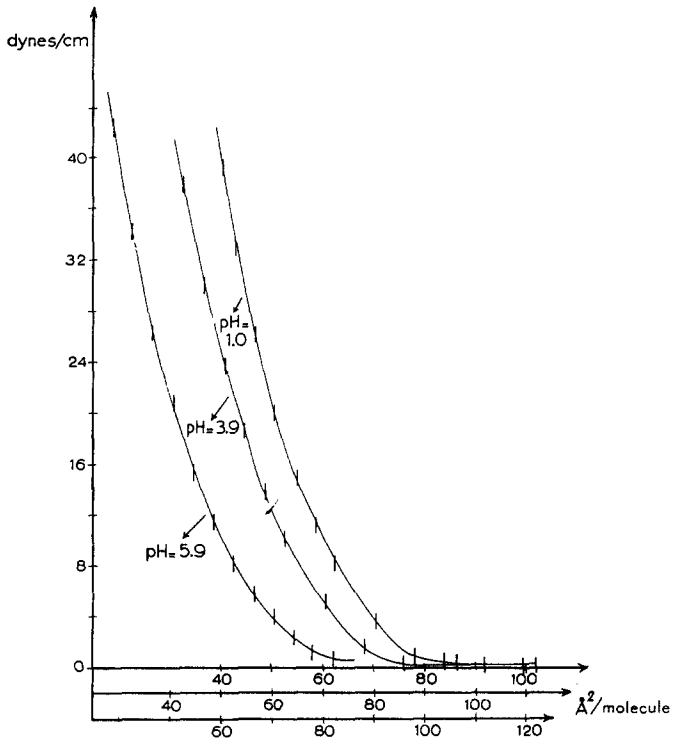

Fig. 2. Force/area plots of milk phosphatides at air/water interface and different pH values. Ionic strength of subsolution: 0.2. Temperature: 16° ± 1°. Upper horizontal axis: pH 1.0. Middle horizontal axis: pH 3.9. Lower horizontal axis: pH 5.9.

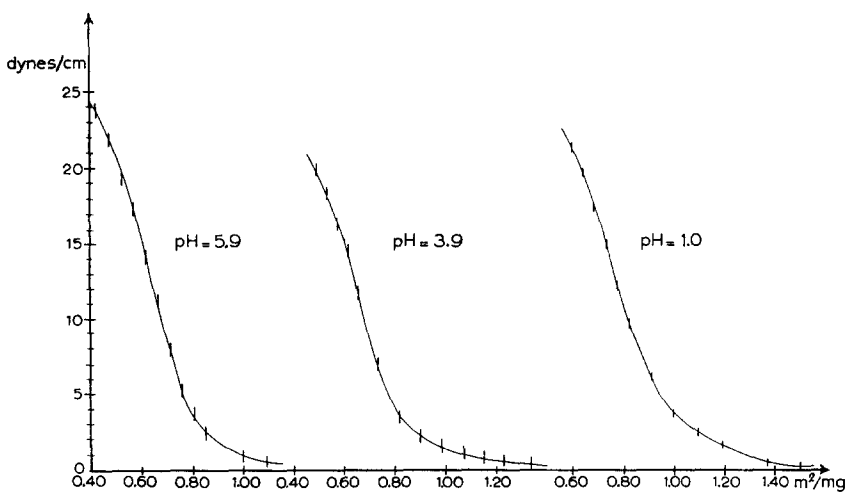

Fig. 3. Force/area plots of β-lactoglobulin at air/water interface and different pH values. Ionic strength of subsolution 0.2. Temperature: 16° ± 1°.

at least five independent measurements. For the calculation of molecular film areas in the phosphatide films of Fig. 2 an average molecular weight of 760 was used. The force/area curves of the phosphatides closely resemble those of TURNER AND WATSON[11]. They are practivally independent of the pH of the subsolution. This may be due to the zwitterion-nature of the ionic heads of lecithin and sphingomyelin and to the high value of the ionic strength of the subsolution, which tends to suppress ionization effects in the film pressure[12].

The high ionic strength also explains why the force/area curves of β-lactoglobulin at pH 5.9 and 3.9 coincide. At pH 1.0, however, the protein monolayer is highly expanded. This effect, as pointed out by CHEESMAN AND DAVIES[6] is due to the combined influence of the breaking of hydrogen bonds between the peptide chains of the film and extreme ionization of the protein at this pH. At surface pressures beyond

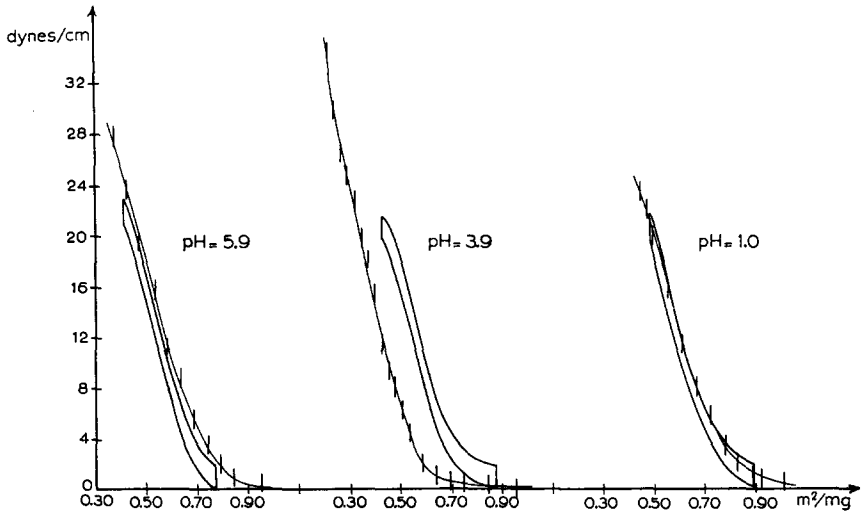

Fig. 4. Force/area graphs of mixed monolayers of β-lactoglobulin and milk phosphatides at air/water interface. Ionic strength of subsolution: 0.2. Temperature 16° ± 1°. Drawn curves: experimental values. Enclosed areas: ideal force/area values for mixed monolayers without interaction on mixing.

20 dynes. cm^{-1} the β-lactoglobulin films become unstable, and irreversible decreases in the surface pressure are observed. At pH 1.0 the protein film is considerably more reproducible than at pH 3.9 and 5.9. Obviously, denaturation by the acid subsolution enhances the protein's ability to spread.

In Fig. 4 the drawn curves represent the force/area curves of mixed monolayers of β-lactoglobulin and phosphatides at the same pH values of 1.0, 3.9 and 5.9. The results at pH 5.9 and 3.9 were obtained by mixing 43 and 57 wt. % of protein and phosphatide, respectively. At pH 1.0 these figures were 40.3 and 59.7 %. At pH 1.0 and pH 5.9 the stability of the mixed monolayer markedly decreases when the surface pressure exceeds the collapse pressure of the β-lactoglobulin film (*e.g.* > 20 dynes. cm^{-1}), although the instability does not rise as rapidly as in the case of the pure protein monolayer. At pH 3.9 the mixed monolayer is much more stable and the film can be compressed to considerably higher surface pressures. This is due, as will be explained in the next section, to the formation of a complex between the oppositely charged

protein and phosphatide constituents of the film. The positively charged β-lactoglobulin is prevented from dissolving in the subphase by the negative cephaline ions.

DISCUSSION

For an estimation of the interactions occurring in the mixed monolayers the experimental force/area curves have been compared with ideal ones in which it is assumed that there is no interaction between phosphatide and protein. The ideal force/area curves were constructed on the assumption that the specific areas of the components in the mixed monolayer would be equal to those of the pure component monolayers at constant value of the surface pressure. For the specific area in the ideal mixed monolayer we may thus write:

$$(A_m)_\pi = (1 - w)\,(A_1)_\pi + w\,(A_2)_\pi\,, \qquad (1)$$

where A is area/mg, π surface pressure and $(1-w)$ the weight fraction of phosphatide in the mixed monolayer.

The resulting ranges of ideal force/area values are represented by the enclosed areas in Fig. 4. The corresponding experimental values are given by the single drawn curves.

It is obvious that strong interaction takes place only at pH 3.9, where the lowering of the experimental force/area curve as compared with the ideal one demonstrates a strong cohesion between phosphatide and protein. At pH 1.0 the experimental and ideal plots coincide, indicating that within the experimental error there is no interaction between the film constituents. A comparison of experimental and ideal plots at pH 5.9 suggests a slight repulsion between protein and phosphatide, though the results are not very conclusive in this respect.

The strong influence of the pH on the interaction between the film constituents indicates that the cohesion at pH 3.9 is of an electrostatic origin. β-lactoglobulin at this pH carries a positive net charge, its isoelectric point being at pH 5.1. It can therefore be expected to interact strongly with negatively charged phosphatide ions. From the investigations of SCHULMAN *et al.*[2,3,13], we know that the phosphatide involved in such interaction will be the cephalin fraction. Owing to its weak base properties its isoelectric point is at approx. pH 2; accordingly the cephalin monolayer was found to behave as a negatively charged film over the pH range 2–14. On the other hand, it was found by SCHULMAN that lecithin did not interact with serum proteins. This may be explained by the fact that it is a zwitterion at this pH and therefore carries no net charge. Nothing is known about the behaviour of sphingomyelin in this respect, but we may expect it to behave as lecithin, since the molecule has the same phosphatidylcholin group. We therefore feel justified in assuming that, in our experiments, it is the negatively charged cephalin fraction that interacts with β-lactoglobulin at pH 3.9.

Thus far the present results parallel the penetration experiments of SCHULMAN and co-workers as regards the influence of the pH of the subsolution. There is, however, a difference in the positions of polar and apolar groups of the protein in the mixed and in the penetrated monolayer. In the latter, the protein is adsorbed under the phosphatide film and consequently its side chains, will for steric reasons, be partly prevented from interaction with the phosphatide molecules. We may thus expect

that electrostatic interactions and possibly Van Der Waals' forces and hydrogen bonding will be more pronounced in the mixed monolayer than in the penetrated one.

To investigate whether the presents results can account for such non-electrostatic interactions, let us analyse more carefully the cohesion at pH 3.9. From Fig. 4 it is evident that at this pH the mixed monolayer is highly condensed, indicating a strong attraction between the negative cephalin and positive protein ions. As a result of the mutual neutralization of the protein and cephalin ions the net charge of the monolayer nearly vanishes and the resulting decrease of the surface pressure can be estimated by the double layer theory of ionized monolayers. To this end we proceed as follows.

According to Fig. 4 the experimental values of the cohesion at a given film area are given by:

$$\Delta\pi = \pi_{1d} - \pi_{exp}\,, \tag{2}$$

where π_{1d} is the surface pressure of the ideal monolayer and π_{exp} that of the experimental one.

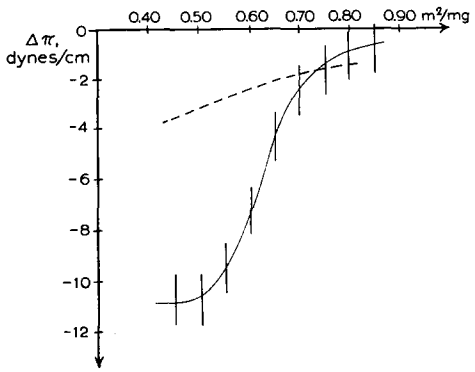

Fig. 5. Excess surface pressures of mixed monolayers of β-lactoglobulin and milk phosphatides at pH 3.9. Air/water interface 16° ± 1°. Drawn curve: experimental values. Dotted curve: charge-effect according to Gouy theory.

Distinguishing between electrical and non-electrical effects in $\Delta\pi$, we can write:

$$\Delta\pi = \Delta\pi_0 + \Delta\pi_{el}\,, \tag{3}$$

$\Delta\pi_0$ being the non-electrical and $\Delta\pi_{el}$ the electrical effect.

A detailed calculation of $\Delta\pi_{el}$ on the basis of the Gouy theory of the electrical double layer is given in the Appendix. The results are shown in Fig. 5, where the experimental values of $\Delta\pi$ are compared with values of $\Delta\pi_{el}$ calculated with the aid of the Gouy theory. Obviously, electrical effects in $\Delta\pi$ constitute only a minor part of it. Considering the difference between $\Delta\pi$ and $\Delta\pi_{el}$ we must bear in mind that the validity of the Gouy theory is rather restricted at the relatively high ionic strength used in this investigation. On the other hand it is known that the deviations occurring, which are due to the finite size of the counter ions and the penetration of these between the film charges, to a great extent cancel each other out[12,15]. This confirms that the calculated values of $\Delta\pi_{el}$ are of the right order of magnitude and that the experimentally found cohesion cannot be due to electrostatic effects alone. A considerable amount of the cohesion at pH 3.9 must therefore be attributed to non-

electrostatic interactions originating from complex formation between protein and phosphatide.

Of the factors that may be important in $\Delta\pi_0$ we mention the non-random distribution of the spread molecules on complex formation and the ample opportunity for short-range interactions (Van Der Waals' forces, hydrogen bonding) in the "lipoprotein" complex. Also the flexibility of the peptide chains may be decreased considerably by the neutralization of the protein charge through the adsorption of the cephalin ions[6]. All such factors tend to increase the cohesion in the mixed monolayer and should be considered when explaining the difference between $\Delta\pi$ and $\Delta\pi_{el}$.

The evidence of our experiments that non-electrostatic interactions between protein and phosphatide take place only at pH 3.9 indicates that the interpretation of penetration experiments at low initial surface pressures and neutral pH must be done with great care. At least part of the observed increases of the surface pressure in such penetration experiments may be due to the spontaneous spreading of the protein at the low initial surface pressures applied to the phosphatide film. In our opinion the present method of mixing the constituent monolayers in order to study interactions between protein and phosphatide permits a more reliable interpretation than the penetration experiments at low initial surface pressures.

APPENDIX

I. *The surface pressure of an ideal mixture of insoluble monolayers*

The specific area of the ideal mixture of monolayers is defined by:

$$(A_m)_\pi = (1 - w)\,(A_1)_\pi + w\,(A_2)_\pi\,, \tag{1}$$

where w is the weight fraction in the mixed monolayer and A_1 and A_2 the area per mg of spread substance in the pure phosphatide and protein monolayers.

According to (1) the surface fraction of component 2 in the mixed monolayer is:

$$y = wA_2/A_m\,, \tag{4}$$

and likewise the surface fraction of component 1:

$$(1 - y) = (1 - w)\,A_1/A_m\,. \tag{5}$$

Gibbs' adsorption theorem for an insoluble monolayer of 2 components reads[16]:

$$\mathrm{d}\pi_m = \Gamma_1\,\mathrm{d}\,\mu_1 + \Gamma_2\,\mathrm{d}\,\mu_2\,, \tag{6}$$

Γ_1 and Γ_2 being the amounts of component 1 and component 2 per unit area of monolayer and μ_1 and μ_2 their respective thermodynamic potentials.

Assuming that the components 1 and 2 form an ideal mixture of constant composition we may write:

$$\mathrm{d}\,\mu_1 = \mathrm{d}\,\mu_1^0\,,$$

and

$$\mathrm{d}\,\mu_2 = \mathrm{d}\,\mu_2^0\,,$$

the superscript ^{0} denoting the pure component monolayers. Hence, applying (6) to an ideally mixed monolayer we obtain:

$$\mathrm{d}\,\pi_{1d} = \Gamma_1\,\mathrm{d}\,\mu_1^0 + \Gamma_2\,\mathrm{d}\,\mu_2^0\,. \tag{7}$$

For the Gibbs' equation of the pure component monolayers we have:

$$\mathrm{d}\,\pi_1^0 = \Gamma_1^0\,\mathrm{d}\,\mu_1^0\,, \tag{8}$$

and

$$\mathrm{d}\,\pi_2^0 = \Gamma_2^0\,\mathrm{d}\,\mu_2^0\,. \tag{9}$$

Substituting (8) and (9) into (7) we obtain:

$$d\pi_{1d} = (\Gamma_1/\Gamma_1^0)\, d\pi_1^0 + (\Gamma_2/\Gamma_2^0)\, d\pi_2^0 = \{(1 - w)\, \Gamma_m/\Gamma_1^0\}\, d\pi_1^0 + \{w\, \Gamma_m/\Gamma_2^0\}\, d\pi_2^0 ,$$

where Γ_m is the total amount of spread substances per unit area of the mixed monolayer.

Since $\Gamma_m = 1/A_m$, $\Gamma_1^0 = 1/A_1$ and $\Gamma_2^0 = 1/A_2$ the latter equation reduces to:

$$d\pi_{1d} = \{(1 - w)A_1/A_m\}\, d\pi_1^0 + \{wA_2/A_m\}\, d\pi_2^0 = (1 - y)\, d\pi_1^0 + y\, d\pi_2^0 \qquad (10)$$

Integrating (10) we obtain for the surface pressure of the ideally mixed monolayer

$$\int_0^{\pi} d\pi_{1d} = \int_0^{\pi} (1 - y)\, d\pi_1^0 + \int_0^{\pi} y\, d\pi_2^0 \qquad (11)$$

From (11) we see that it is possible to express π_{1d} explicitly in the surface pressure π_1^0 and π_2^0 of the pure component monolayers only if the dependence of y on surface pressure is known. For the particular case that y is constant (11) simplifies to:

$$\pi_{1d} = (1 - y)\, \pi_1^0 + y\, \pi_2^0 \qquad (12)$$

In view of the ideally mixed monolayer of β-lactoglobulin and milk phosphatides we may estimate y from the corresponding force/area plots of the ideal and protein monolayers. It is found that as a result of the rather small variations in y the simple equation (12) may be applied at surface pressures beyond 5 dynes. cm^{-1}, using a mean value $y_m = 0.51$. The error introduced in this way does not exceed 5 % of the total area of the integrals in (11). In the lower pressure region values of y_m can be calculated by graphical integrations of the y *versus* π_{1d} plot.

II. *Electrostatic interaction in mixed monolayers of β-lactoglobulin and milk phosphatides*

The cohesive force in the mixed monolayer is defined by:

$$\Delta\pi = \pi_{1d} - \pi_{exp} \qquad (2)$$

The surface pressure of ionized monolayers quite generally is given by:

$$\pi = \pi_0 + \pi_{el}$$

π_0 being the surface pressure of the unionized monolayer and π_{el} the pressure increment due to its ionization. Consequently, we may write for $\Delta\pi$:

$$\Delta\pi = \Delta\pi_0 + \Delta\pi_{el} \qquad (3)$$

From general thermodynamic considerations it was deduced (12) that

$$\pi_{el} = \int_0^{\psi} \sigma\, d\psi ,$$

in which σ is the monolayer charge per cm^2 and ψ the corresponding monolayer potential.

From the expression (12) for π_{1d} deduced in the preceding section $\Delta\pi_{el}$ thus becomes:

$$\Delta\pi_{el} = \left[(1 - y_m)\left\{\int_0^{\psi_1} \sigma_1\, d\psi_1\right\}_{A_1} + y_m\left\{\int_0^{\psi_2} \sigma_2\, d\psi_2\right\}_{A_2}\right] - \left\{\int_0^{\psi_{exp}} \sigma_{exp}\, d\psi_{exp}\right\}_{A_m} . \qquad (13)$$

The monolayer charges in equation (13) are given by:

$$\sigma_1 = -\Gamma_1 e$$
$$\sigma_2 = +\Gamma_2 v e$$
$$\sigma_{exp} = (\Gamma_2 v - \Gamma_1) e$$

where Γ_1 is the number of cephalin ions per cm² of monolayer and Γ_2 the corresponding number of protein ions at the film areas indicated in (13); e is the absolute value of the electronic charge, and v the valency of the protein at pH 3.9.

For an estimation of Γ_2 and v we need to know the molecular weight of β-lactoglobulin and its charge at pH 3.9. For the former we accept a value of 40,000[5,14]; the charge can be estimated from the titration curve to be $+ 22\ e$ at pH 3.9 and ionic strength 0.2[16].

According to the Gouy theory (12):

$$\int_0^{\psi} \sigma \, d\psi = 6.1 \, c^{1/2}[\cosh \{ \text{arc sinh } (28.10^{-6} \sigma c^{-1/2})\} - 1], \qquad (14)$$

where c is the equivalent concentration of electrolyte in the subsolution and σ the monolayer charge expressed in electrostatic units per cm².

Application of eqns. (13) and (14) to the present experiments at pH 3.9. leads to the values of $\Delta\pi_{el}$ represented by the dotted curve of Fig. 5.

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