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Effect of concentrations of salts on the formation of protein monolayers

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Effects of salts present in the subphase on the properties of monolayers, *viz.*, molecular areas of proteins and Gibbs elasticities, were studied by the monolayer method.

Key words: proteins, monolayers, molecular areas, elasticity of a monolayer.

The Langmuir—Blodgett (LB) technology is promising for the development of sensors, since it allows one to obtain oriented close-packed layers with specified characteristics.[1] The main difficulty in the preparation of LB polylayers from protein molecules is that they are soluble in the subphase; therefore, the surface pressure of an obtained monolayer is not constant and decreases with time.[2,3] Replacement of water in the subphase by an electrolyte solution (0.05 mol L^{-1} KCl) may lead to stabilization of the surface pressure. This suggests that the quantity of protein that passes to the subphase would decrease, which may finally result in the formation of stable and more close-packed monolayers.

A lot of papers have been devoted to investigation of protein monolayers; however, systematic studies of the effects of subphase salts on molecular areas of proteins are missing.[4—9] The purpose of the present work has been to study the behavior of proteins on the surface of subphases that contain no salts or contain various concentrations of salts, in order to obtain stable close-packed protein monolayers.

Experimental

Monolayers of bull serum albumin (BSA) and mouse immunoglobulin (IgG) were studied. BSA was purchased from "Sigma", and IgG was prepared by a known procedure.[10]

Protein monolayers were studied using a Joyce-Loebl set (UK) and a 200-mL bath with a surface area of 186 cm^2. Tridistilled water with pH 6.9 was used as the subphase. The effects of KCl, KNO_3, and $(NH_4)_2SO_4$ with ionic strengths μ of 0.01, 0.05, 0.1, and 0.15 were studied. Addition of a salt to the subphase resulted in an increase in the pH to 7.3—7.5.

Studies were carried out at pH 7.4, 6.5, and 5.0. For acidification, an acid having a common anion with the salt under study was used; for example, when the effect of KCl was studied, the solution was acidified by HCl (0.01 mol L^{-1}); in the calculations of the ionic strength, the concentration of the acid added was taken into account.

An aqueous solution of protein (0.01—0.05 mL) was sprayed on the surface of a subphase (288 K) and compressed to the pressure of collapse at a rate of 10 mm min^{-1}.

Three rates of compression of a protein monolayer, 10, 25, and 50 mm min^{-1}, were employed. When the rate of compression increased, the isotherms became uniform, since an inflection in the 30—35 mN m^{-1} region, typical of BSA,

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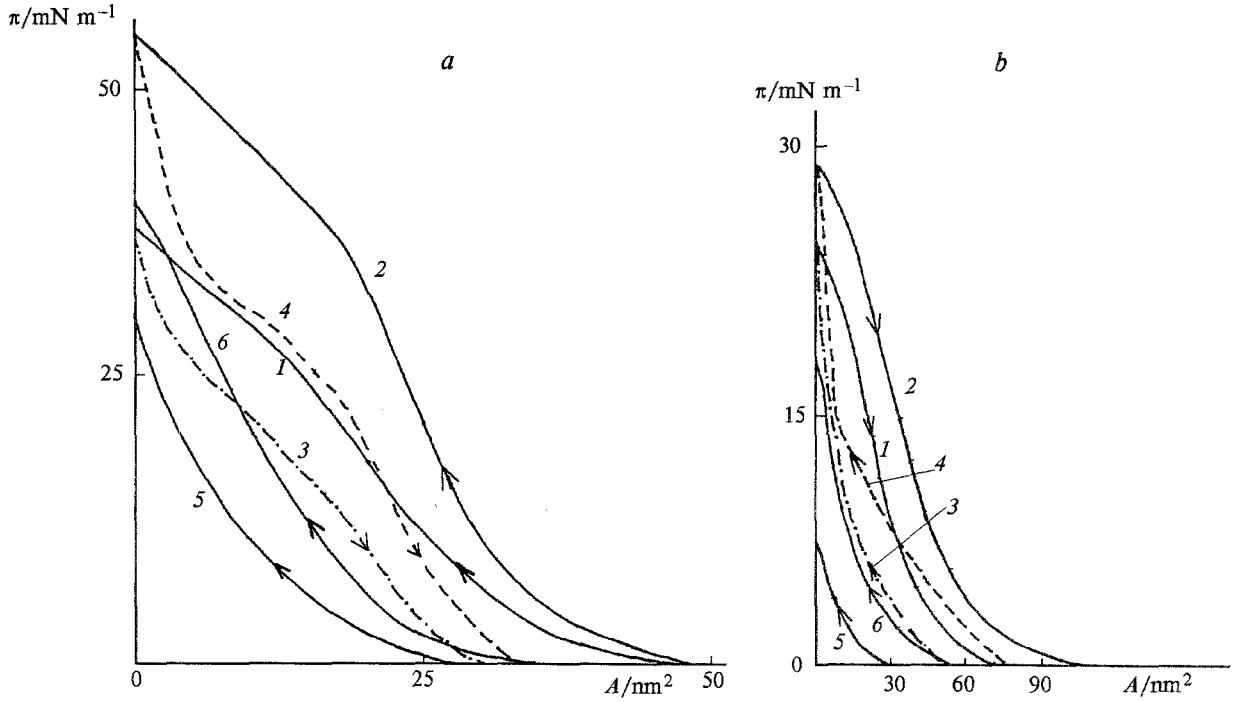

Fig. 1. Forward (*1, 2*), "backward" (*3, 4*), and repeated (*5, 6*) π—*A*-isotherms: BSA at pH 5.0 (*a*); IgG at pH 7.4 (*b*). Subphase: H_2O (*1, 3, 5*) or 0.05 *M* KCl (*2, 4, 6*).

disappeared (Fig. 1, *a*, curve *1*); at these values of surface pressure, collapse of the monolayer, accompanied by a decrease in the surface pressure, also occurred. A similar situation was also observed for IgG. This behavior of protein monolayers at a rate of compression above 10 mm min^{-1} can be explained by either dissolution of protein in the bulk of a subphase or formation of a bilayer structure (protein—protein interactions).

π—*A*-Isotherms of proteins at the three pH values in water or aqueous KCl (μ 0.05) are presented in Fig. 2. The starting protein concentrations were: $1.45 \cdot 10^{-5}$ mol L^{-1} for BSA or $3.4 \cdot 10^{-7}$ mol L^{-1} for IgG.

The molecular areas of the proteins on a monolayer surface were determined graphically by approximating the linear section of the π—*A*-isotherm to the zero pressure. Gibbs elasticity of a monolayer (E/mN m^{-1}) was calculated from the equation $E = -A(\delta\pi/\delta A)_T$, where A/nm^2 is the molecular area, π/mN m^{-1} is the surface pressure, and T/K is the temperature of the experiment. The value of E was calculated for the linear section of the π—*A*-isotherm.

Results and Discussion

Figure 1, *a* and *b* present the π—*A*-isotherms for IgG and BSA (at the pH values corresponding to their isoionic points) on the surface of water containing or not containing KCl. As can be seen from Fig. 1, a protein monolayer on the surface of a salt subphase can be compressed to higher pressures than that on the surface of water. This indicates that the solubility of protein in the salt subphase is lower. The isotherm, obtained during the backward movement of the barrier, exhibits hysteresis having different shapes in the case of

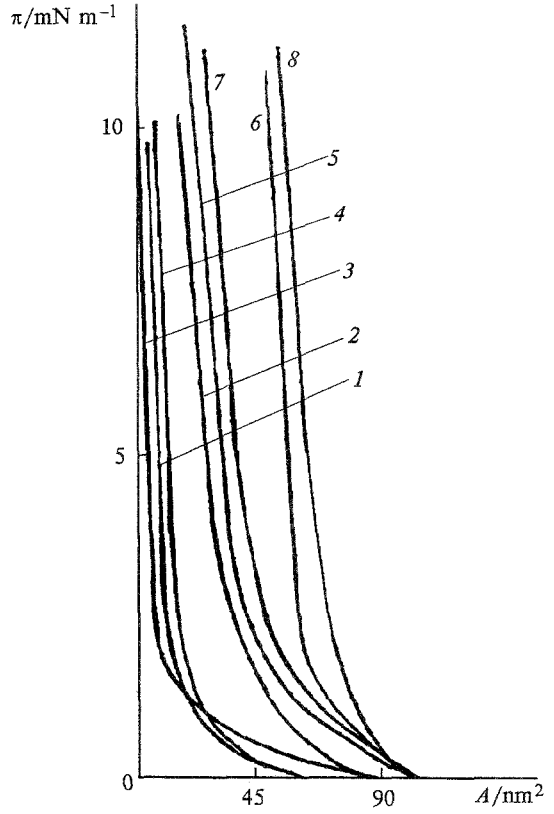

Fig. 2. π—*A*-Isotherms of IgG (*1—4*) and BSA (*5—8*) at pH 7.4 (*1, 2*), pH 6.5 (*3—6*), or pH 5.0 (*7, 8*). Subphase: H_2O (*1, 3, 5, 7*) or 0.05 *M* KCl (*2, 4, 6, 8*).

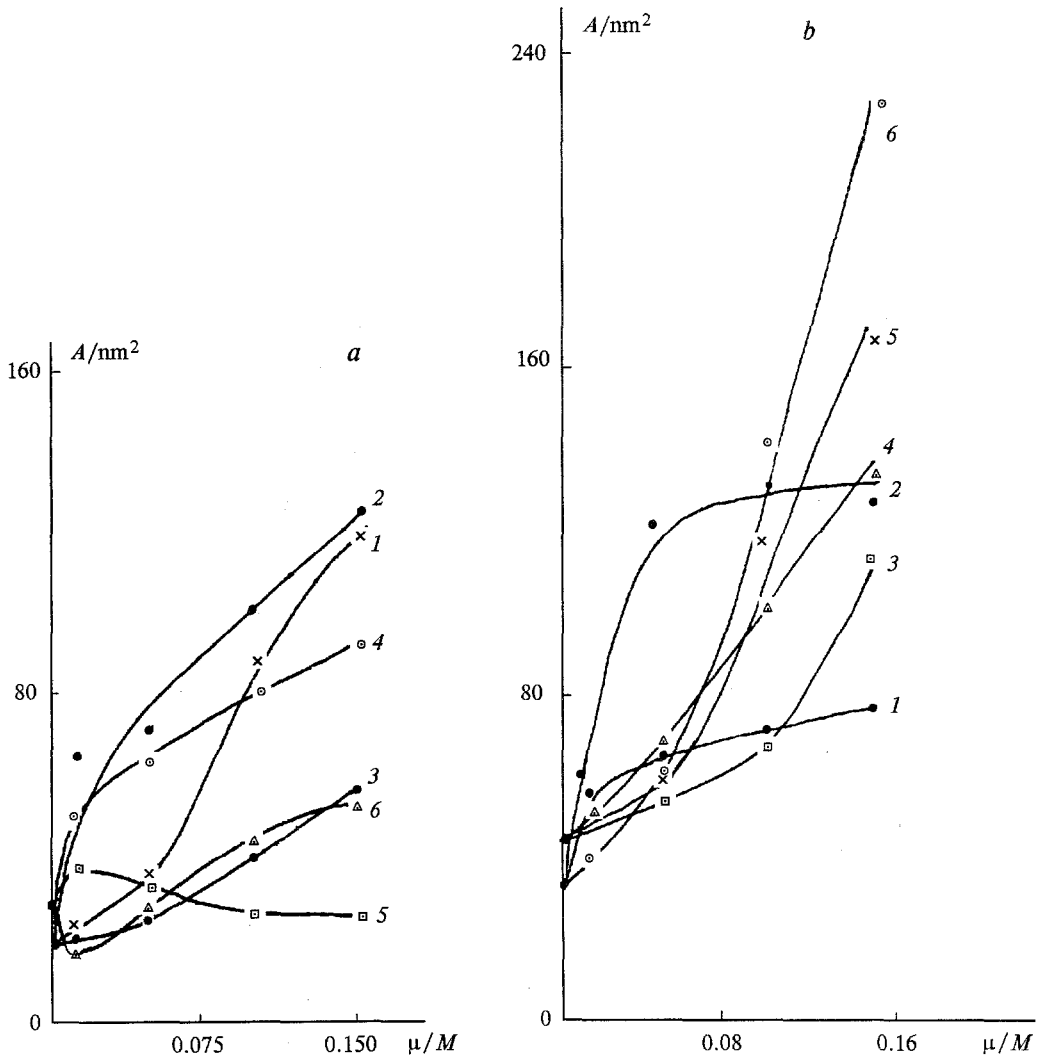

Fig. 3. Effect of ionic strength on molecular area: *a* — IgG at pH 6.5 (*1—3*) or pH 7.4 (*4—6*); subphase: $(NH_4)_2SO_4$ (*1, 5*), KNO_3 (*2, 6*), or KCl (*3, 4*); *b* — BSA at pH 6.5 (*1—3*) or pH 5.0 (*4—6*); subphase: KCl (*1, 4*), KNO_3 (*2, 5*), or $(NH_4)_2SO_4$ (*3, 6*).

water and a salt subphase. For proteins studied on the surface of a salt subphase, the "backward" isotherms have inflection points at a pressure of 15 mN m^{-1} for IgG and at 30 mN m^{-1} for BSA; after the inflection point, an isotherm approaches the initial isotherm. This behavior can be explained by protein—protein interactions that lead, in the presence of a salt, to formation of protein associates on the surface. In the case of proteins on the surface of aqueous subphase, the inflection is weakly pronounced. This shape of the "backward" isotherm is apparently due to a decrease in the concentration of protein at the interface caused by its increased solubility in the subphase bulk. The formation of protein associates in solutions of salts is assigned to reversible denaturation changes of proteins.[13—21]

Figure 2 presents π—*A*-isotherms of the proteins studied at pH 7.4, 6.5, and 5.0 for pure water and for solutions with an ionic strength of 0.05 KCl. The presence of KCl in the subphase results in an increase in the molecular area of a protein, irrespective of its structure and pH.

The pH value has a significant effect on the molecular areas of proteins on the surface of a subphase containing no salts. It should be noted that the molecular area of a protein on the surface of a subphase, whose pH corresponds to the isoionic point of the protein, is larger than that at other pH values. For example, the molecular area of IgG is 26 nm^2 at pH 7.4 (it is the isoionic point of IgG[11]), while at pH 6.5, it is 17 nm^2; for BSA these values are 35 nm^2 at pH 5.0 (isoionic point of BSA [12]) and 20 nm^2 at pH 6.5. Thus, in the electroneutrality point, a smaller quantity of protein passes into the bulk, since it has the minimum total charge.

Figures 3, *a*, *b* and 4, *a*, *b* show the dependences of molecular areas and elasticities of monolayers on the ionic strength in the subphase. An increase in the ionic

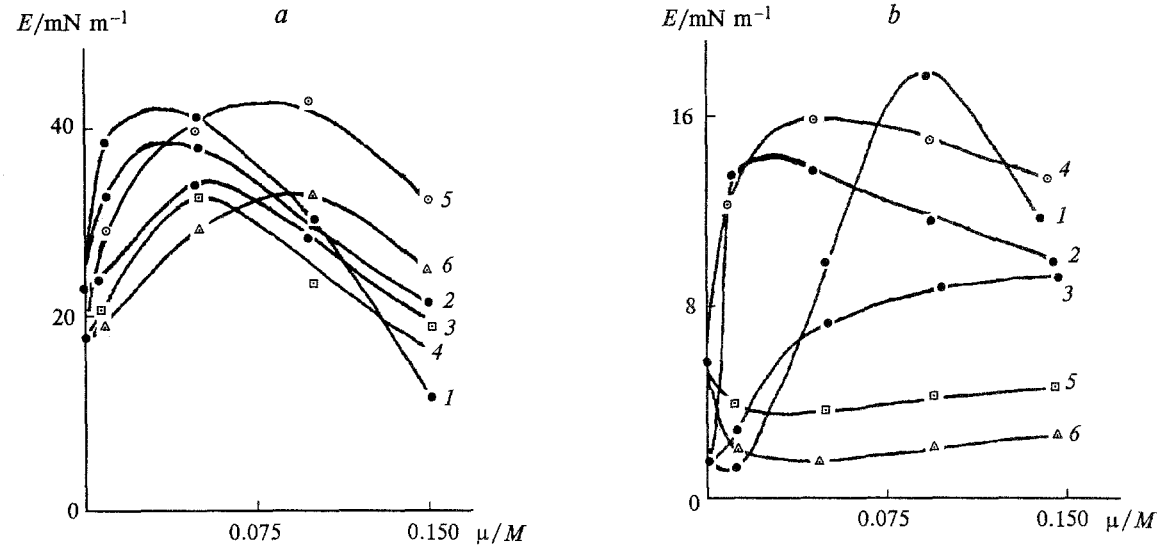

Fig. 4. Dependence of the monolayer elasticity on the ionic strength for BSA (*a*) and IgG (*b*). *a*, BSA at pH 5.0 (*1—3*) or pH 6.5 (*4—6*); subphase: KCl (*1, 5*), KNO_3 (*2, 4*); or $(NH_4)_2SO_4$ (*3, 6*); *b*, IgG at pH 6.5 (*1—3*) or pH 7.4 (*4—6*); subphase: $(NH_4)_2SO_4$ (*1, 5*), KNO_3 (*2, 6*), or KCl (*3, 4*).

strength results in an increase in the molecular areas of proteins, compared to those on the water surface (see Fig. 3).

A different character of dependence is observed for the elasticity of a monolayer. In fact, the dramatic increase in the molecular area with increase in the ionic strength results in a decrease in the Gibbs elasticity of a monolayer (*cf.* Figs. 3 and 4). This is most clear in the case of BSA (see Figs. 3, *b* and 4, *b*). For example, at pH 5.0, with a $(NH)_2SO_4$ solution (μ 0.15) as the subphase the molecular area is almost 7 times larger than that for water (see Fig. 3, *b*, curve *6*), whereas the elasticity of the monolayer diminishes (see Fig. 4, *b*, curve *3*).

The effects of salts on the molecular area of BSA are much more pronounced at pH 5.0 (isoionic point) than at pH 6.5. This may be explained by the fact that at pH 5.0, the total charge of BSA is the minimum, and the increase in the pH to 6.5 leads to an increase in the negative charge of the protein, which has an effect on binding the anions.

The dependence on the elasticity of BSA monolayers on the ionic strength in the subphase passes through a maximum, *i.e.*, at a particular ionic strength, the elasticity of a BSA monolayer is the greatest. Further increase in the ionic strength leads to a decrease in the elasticity, probably due to the formation of a salt form of the protein.

As was mentioned above, formation of a salt form of BSA is a result of its partial denaturation. BSA is prone to form aggregates in the pH region above its isoionic point in the presence of bulky anions such as sulfate or nitrate.[13] A lyotropic series of the effects of ions on aggregation of BSA, $Na^+ > K^+$; $NO_3^- > SO_4^{2-} > Cl^-$, has been reported in the literature.[14] A decrease in elasticities of BSA monolayers on the surface of a salt subphase is likely to be caused by its aggregation.

The effects of salts on the molecular area of IgG at pH 7.4 (isoionic point) is less pronounced than that at pH 6.5. We observed a substantial increase in the molecular area (by a factor of 7) in the presence of KNO_3 or $(NH_4)_2SO_4$ (μ 0.15) at pH 6.5, compared to the area on the water surface (see Fig. 3, curves *1* and *2*). The character of variations of the elasticity of IgG monolayers is similar to that of BSA. Each of these curves also has a maximum at a particular ionic strength (0.05 for KNO_3 and 0.10 for $(NH_4)_2SO_4$), and further increase in the ionic strength results in decrease in the elasticity of a monolayer of IgG.

The effect of salts on the elasticity of an IgG monolayer at pH 7.4 is ambiguous. The presence of KCl (μ 0.01) leads to a threefold increase in the elasticity of the monolayer (see Fig. 4, *a*, curve *4*); as the ionic strength is further increased, the elasticity of the monolayer slightly decreases, while the molecular area continues to grow (see Fig. 3, *a*, curve *4*). In the presence of KNO_3 or $(NH_4)_2SO_4$ (μ 0.01), elasticities of IgG monolayers decrease by a factor of 2.5—3.0 (see Fig. 4, *a*, curves *5, 6*), and subsequent increase in the ionic strength does not result in their variation. As this takes place, the molecular area in the presence of KNO_3 increases, and that in the presence of $(NH_4)_2SO_4$ diminishes, approaching the value of molecular area on the surface of water (see Fig. 3, *a*, curves *5, 6*). It is likely that in the case of $(NH_4)_2SO_4$, salting out of IgG molecules into the bulk of the subphase occurs.

Analysis of dependences of the elasticity of a monolayer and the molecular area on the ionic strength for BSA and IgG (see Fig. 1, *a*, *b* and Figs. 3 and 4) indicates that there are no essential distinctions between the mechanisms of the effects of these salts on these molecules. The substantial increase in the molecular area and also the presence of an inflection on the "backward" isotherm (see Fig. 1) can apparently be explained by association of proteins in the presence of salts. Then it is quite clear, why the elasticity of monolayers decreases as the molecular area increases (see Figs. 3 and 4).

Formation of associates in solutions of various immunoglobulins is a known fact.[15,16] Immunological activities of these partially denatured IgG in salt solutions vary in various manners. In particular, it has been shown that immunoglobulins can acquire an additional affinity to an antigen.[17–21]

Thus, on the surface of a salt-containing subphase, proteins form more close-packed monolayers due to a decrease in the solubility of a protein in the subphase bulk; a substantial increase in the molecular areas of proteins on the surface of a salt solution, accompanied by a drop in the elasticity of the monolayer, is caused by association of proteins. The formation of associates probably occurs as well on the surface of the subphase, but high solubility of protein in the bulk does not allow one to obtain a clear picture. It should be noted that even for a salt subphase the isotherm of a repeated compression (see Fig. 1, *a*, *b*, curves *5* and *6*) differs considerably from the initial isotherm (see Fig. 1, *a*, *b*, curve *2*), and the molecular area of protein calculated from this isotherm is half that obtained from the initial isotherm, since protein associates can pass into the bulk of a salt subphase.

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