Adsorption of Proteins at the Liquid/Air Interface

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Received: July 30, 1997; In Final Form: October 21, 1997[⊗]

Thermodynamic equations for describing protein adsorption layers at liquid/fluid interfaces are derived as a generalization of a theory published recently [*J. Colloid Interface Sci*. **1996**, *183*, 26]. In this new theory the nonideality of enthalpy (Flory−Huggins' parameter) and entropy of mixing are taken into account, and also the effect of the electric charge of the protein molecules on surface pressure is considered. The model is verified by experimental dynamic and equilibrium surface tension data for HSA solutions obtained from pendent drop experiments (ADSA). The derived isotherm is in good agreement with the experimental data. The values of the isotherm parameters surface area per molecule, electric charge of the HSA molecule, and the adsorption layer thickness are close to values obtained by other methods in literature. It follows that HSA molecules undergo almost no denaturation at the solution/air interface and occupy a surface area of about 50 nm^2, independent of the packing in the adsorption layer, which is in agreement with the concept of a triple-domain structure.

1. Introduction

The great practical significance of the adsorption of polyelectrolytes and, in particular, proteins at fluid interfaces stimulates the development of new theoretical models to describe the equilibrium and dynamic behavior of their adsorption layers. The properties of protein adsorption layers differ in many aspects from those characteristic of surfactant monolayers. First, for proteins surface denaturation can take place, leading to an unfolding of protein molecules within the surface layer, mainly at low surface pressures. The partial molar surface area for proteins, in contrast to surfactants, is large and can vary. This property, and also the large number of configurations of adsorbed protein molecule, which exceeds significantly that in the bulk, leads to a significant increase in the nonideality of the surface layer entropy. This makes it impossible to apply the most simple isotherm models (Henry, Langmuir) for the description of protein adsorption layers.

Thermodynamic models for the protein adsorption at liquid interfaces were recently proposed in refs 1−5. The interrelation between protein denaturation processes within the surface and the activity of the solvent molecules (water) was demonstrated by Ter-Minassian-Saraga,[2] while Joos[1] showed that the degree of surface denaturation decreases with increasing surface pressure. Lucassen-Reynders[3] analyzed the effect of the size of mixed molecules on the entropy of protein surface layers. Joos and Serrien[4] were the first to derive a relationship between the adsorption of proteins possessing two modifications with different partial molar area. From this relationship it follows that the surface pressure controls both the composition and the thickness of a protein surface layer. In particular, the part of molecules with a minimal surface area demands increases with increasing surface pressure Π. The concept proposed by Joos and Serrien[4] was further developed for an arbitrary but discrete number of different conformations of protein molecules at the surface.[5] The resulting equation of state and adsorption isotherm agree satisfactorily with experimental tensiometric, ellipsometric, and radioactivity data in the literature.

In the present study the theoretical model developed in ref 5 is generalized to allow for a continuous change in the molar area of protein molecules within the surface layer (continuous-state model). Also, the effect of the electric charge of the protein molecules and counterions is taken into account. To verify this new theoretical model, results obtained from ellipsometric, tensiometric, and rheological experiments for human serum albumin solutions are discussed.

Theory

Surface pressure and adsorption isotherms for proteins at a liquid/fluid interface can be derived from Butler's equation[6] for the chemical potential μ_i^s of the ith state of a protein molecule within the surface layer,

$$\mu_i^s = \mu_i^{0s} + RT \ln f_i^s x_i^s - \gamma\omega_i \quad (1)$$

and the corresponding equation for the chemical potential within the solution bulk, μ_i^α,

$$\mu_i^s = \mu_i^{0\alpha} + RT \ln f_i^\alpha x_i^\alpha \quad (2)$$

where μ_i^{0s} and $\mu_i^{0\alpha}$ are the standard chemical potentials, R is the gas constant, T is the absolute temperature, γ is the surface tension, ω_i are the partial molar areas, f_i are the activity

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S1089-5647(97)02503-0 CCC: $15.00
Published on Web 01/08/1998

coefficient, $x_i = N_i/\sum N_i$ are the molar fractions, and N_i are the numbers of moles of the ith state. Here the superscripts "s" and "a" refer to the surface (interface) and the bulk. For ideal bulk phases from eqs 1 and 2 it follows that[5]

$$\Pi = -\frac{RT}{\omega_0}[\ln(1 - \sum_{i\geq 1}\theta_i) + \ln f_0^s] \tag{3}$$

$$K_i c = \frac{\theta_i f_i^s}{(1 - \sum_{i\geq 1}\theta_i)^{n_i}(f_0^s)^{n_i}} \tag{4}$$

where $\Pi = \gamma_0 - \gamma$ is the surface pressure, γ_0 is the surface tension of the solvent ($i = 0$), $\theta_i = \Gamma_i\omega_i$, Γ_i are the adsorptions, $\theta_0 = 1 - \sum_{i\geq 1}\theta_i$, $K_i = (x_i^s/x_i^\alpha)_{x_i^\alpha \to 0}$ for $i \geq 1$ are the distribution coefficients of the states at infinite dilution, $n_i = \omega_i/\omega_0$, and c is the protein bulk concentration. It was assumed in refs 1−3 that ω_0 is close to the area of a water molecule, and therefore the adsorption of protein molecules leads to a desorption of $n_0 = \omega_1/\omega_0$ water molecules. This is however only true when the adsorption layer is comprised of water molecules; thus, the adsorption layer is about 0.3 nm thick. Real adsorption layers of proteins are much thicker. Moreover, their thickness increases with protein adsorption. Thus, from the theoretical point of view, the procedure employed in ref 7, where the real thickness of the protein layer was taken into account, seems to be more reliable. In this case the portion of water molecules within the surface increases, while the number of desorbed water molecules per protein molecule becomes significantly larger than ω_1/ω_0. In ref 5 it was assumed that $\omega_0 = \omega_\Sigma$, which coincides with a dividing surface as defined by Lucassen-Reynders,[8,9]

$$\sum_{i=0}^{n}\Gamma_i = 1/\omega_\Sigma \tag{5}$$

Here ω_Σ is the mean partial molar area of all states:

$$\omega_\Sigma = (\sum_{i\geq 1}\Gamma_i\omega_i)/(\sum_{i\geq 1}\Gamma_i) \tag{6}$$

This definition of the dividing surface ensures that for each state the relation $\theta_i = \Gamma_i\omega_\Sigma$ holds. As ω_Σ is the same for all states, $\Gamma_i\omega_\Sigma$ is the molar fraction of the respective adsorption state. Therefore the transformation of eqs 1 and 2 into eqs 3 and 4 with the introduction of θ_i instead of x_i^s is a rigorous procedure. Another important advantage following from the definition of the dividing surface by eq 5 and ω_Σ by eq 6 is that there is no contribution of nonideality of entropy of mixing in the solvent activity coefficient.[5] Finally, using the Lucassen-Reynders' dividing surface, one can exclude the adsorption layer thickness from the consideration so that the actual number of water molecules displaced from the adsorption layer by adsorbing protein molecules does not need to be accounted for.

The activity coefficients in eqs 3 and 4 can be represented in a form that accounts for the enthalpy and entropy of mixing, denoted by superscripts H and E, respectively[3,5]

$$\ln f_i^s = \ln f_i^{sH} + \ln f_i^{sE} \quad i \geq 0 \tag{7}$$

$$\ln f_i^{sH} = a(1 - \Gamma_\Sigma\omega_\Sigma)^2, \quad i\geq 1 \tag{8}$$

$$\ln f_i^{sE} = 1 - \omega_i\sum_{j\geq 0}\Gamma_j = 1 - n_i, \quad i\geq 1 \tag{9}$$

$$\ln f_0^{sH} = a\Gamma_\Sigma{}^2\omega_\Sigma{}^2 \tag{10}$$

$$\ln f_0^{sE} = 1 - \omega_0\sum_{j\geq 0}\Gamma_j = 0 \tag{11}$$

Here a is the intermolecular interaction constant, and $\Gamma_\Sigma = \sum_{i=1}^{n}\Gamma_i$. For simplicity it can be assumed that the nonideality of surface enthalpy is independent of the state of the molecules within the surface and depends on the total adsorption only.[5]

Proteins are polyelectrolytes; that is, they contain ionized groups. At the isoelectric point both hydroxyl groups and amino groups possess equal degrees of ionization, and thus the whole molecule is electroneutral. In strong acidic media the hydroxyl groups become neutral and the molecules acquire an excessive positive charge, while a neutralization of the amino groups in strong alkaline media results in a negative net charge of the protein molecule. Therefore the maximal total charge of a protein molecule in acidic or alkaline media can be equal to the number of amino or hydroxyl groups, while at the isoelectric point at complete ionization of hydroxyl and amino groups the charge can be equal to the total number of all groups. Thus the charge of protein molecules is more or less bound by counterions. Polyelectrolyte molecules in a semidilute solution can be regarded as a random walk of electrostatic blobs.[10] For polyelectrolytes the blob charge not bound by counterions usually amounts to several units. It can be supposed that at the isoelectric point the charges of different blobs possess opposite signs. As the total number of blobs is rather high, the entire protein molecule appears electroneutral. The degree of counterion binding both in separate blobs and in the whole protein molecule is about 90%, which corresponds to ionic micelles, and the number of unbound unit charges of a protein molecule remains sufficiently large and can amount to tens or hundreds. The interaction between unbound charges has to result in a strong repulsion between polyelectrolyte chains, as shown in ref 11.

Davies[12] derived an adsorption isotherm and an equation of state for charged surfactant molecules based on the Gouy−Chapman theory. The same electric double layer model (DEL) was used by Borwankar and Wasan,[13] where the nonideality of the surface layer was taken into account. Combining the results obtained in ref 13 with eqs 7, 10, 11, and 13 and the condition $\omega_0 = \omega_\Sigma$, one can transform eq 3 into

$$\Pi = -\frac{RT}{\omega_\Sigma}[\ln(1 - \Gamma_\Sigma\omega_\Sigma) + a(\Gamma_\Sigma\omega_\Sigma)^2] + \frac{4RT}{F}(2\epsilon RTc_\Sigma)^{1/2}[\mathrm{ch}\varphi - 1] \tag{12}$$

where F is the Faraday constant, ϵ is the dielectric permittivity of the medium, c_Σ is the total concentration of ions within the solution, and $\varphi = zF\psi/2RT$, where z is the number of nonbound unit charges in the protein molecule and ψ is the electric potential. Substituting the chemical potential by the electrochemical potential,[13] the following expression can be obtained instead of eq 4:

$$K_i c = \frac{\theta_i f_i^s}{(1 - \sum_{i\geq 1}\theta_i)^{n_i}(f_0^s)^{n_i}}\exp(2\varphi) \tag{13}$$

The electric potential is determined by the surface charge density:

$$sh\varphi = \frac{z\Gamma_\Sigma F}{(8\epsilon RTc_\Sigma)^{1/2}} \tag{14}$$

The analysis of eq 12 shows that for 1:1 ionic surfactant at low ion bulk concentration the approximate relation $\varphi \gg 1$ holds.[14] This approximation leads to a linear dependence of Π on Γ_Σ in the electrostatic term of eq 12. For protein solutions the situation is quite different. At high ion concentration the Debye length $\lambda = (\epsilon RT/F^2c_\Sigma)^{1/2}$ is small; for example, for $c_\Sigma = 0.1$ mol/L the value of λ is 1.3 nm. This means that for protein solutions the DEL thickness can be smaller than the adsorption layer thickness. Therefore the concentration of ions in eqs 12 and 14 is just their concentration within the adsorption layer, which can exceed 1 mol/L due to the ionization of hydroxyl and amino groups, and the contribution of counterions. It follows from eqs 12 and 14 that for large c_Σ the approximation up $\varphi \ll 1$ can be used. Thus, introducing the series expansions

$$sh\varphi = \varphi + \varphi^3/3! + ... \tag{15}$$

$$ch\varphi = 1 + \varphi^2/2! + ... \tag{16}$$

into eqs 12 and 16, one obtains an equation of state for nonideally charged protein surface layers:

$$\Pi = -\frac{RT}{\omega_\Sigma}[\ln(1 - \Gamma_\Sigma\omega_\Sigma) + (a - a_{el})(\Gamma_\Sigma\omega_\Sigma)^2] \tag{17}$$

where $a_{el} = z^2F/\omega_\Sigma(8\epsilon RTc_\Sigma)^{1/2}$. Using eqs 7−9, 15, and 16, and the relationship

$$\exp(2\varphi) = (ch\varphi + sh\varphi)^2 \tag{18}$$

eq 13 yields the protein adsorption isotherm for any ith states of the molecule.

$$b_1c = \frac{\Gamma_i\omega_\Sigma \exp\left[-a\Gamma_\Sigma{}^2\omega_\Sigma{}^2\left(i\frac{\omega_1}{\omega_\Sigma} - 1\right) - i\frac{\omega_1}{\omega_\Sigma} - 2a\Gamma_\Sigma\omega_\Sigma + 2\frac{a_{el}}{z}\Gamma_\Sigma\omega_\Sigma + \left(\frac{a_{el}}{z}\Gamma_\Sigma\omega_\Sigma\right)^2\right]}{i^\alpha(1 - \Gamma_\Sigma\omega_\Sigma)^{i\omega_i/\omega_\Sigma}} \tag{19}$$

α is a constant that determines the variation in surface activity of the protein molecule in the ith state with respect to state 1 characterized by a minimum partial molar area ω_1, $b_i = b_1i^\alpha$. i can be either an integer or fractional, and the increment is defined by $\Delta i = \Delta\omega,/\omega_1$. For $\alpha = 0$ one obtains $b_i = b_1 = $ const, while for $\alpha > 0$ the value of b_i increases with increasing ω_i. Now the electrostatic constant a_{el} has to be evaluated. Assuming $\omega_\Sigma = 6 \times 10^6$ m^2/mol (i.e., 10 nm^2 per one protein molecule) and $z = 30$, for $c_\Sigma = 2$ mol/L one obtains $a_{el} = 100$.

In the Flory−Huggins' theory of amorphous polymer solutions[15,16] the constant a (otherwise called the Flory parameter χ) is on the order of unity. Thus one can neglect this term in eq 17 as compared to a_{el}. Moreover, for high a_{el} values the logarithmic term in eq 17 can also be neglected, which leads to the approximation $\Pi \approx \Gamma_\Sigma{}^2$, which is in good agreement with the scaling theroy $\Pi \approx \Gamma_\Sigma{}^{9/4}$.[7,17] Taking into account the protein molecular charge,[18] a quadratic dependence of the osmotic pressure on z and the protein concentration results. The consideration of the protein molecular charge leads to a significant simplification of the adsorption isotherm (eq 19). First, for all states of the protein molecule the parameter b_1 is constant and $i = 1$ can be assumed in eq 19. Moreover, $\omega_1 \leq \omega_\Sigma$, $a = \chi$, and a_{el}/z have values of the same order with opposite signs. It will be shown below that for large a_{el} the surface layer coverage $\Gamma_\Sigma\omega_\Sigma$ remains low even for large Π. In conclusion, the exponential term in eq 19 is close to unity and does not depend on $\Gamma_\Sigma\omega_\Sigma$ and can therefore be omitted, which simultaneously excludes two insufficiently defined parameters (z and a). These simplifications allow the transformation of the equation of state for protein surface layers and the adsorption isotherm equation into the simple form

$$\Pi = -\frac{RT}{\omega_\Sigma}[\ln(1 - \Gamma_\Sigma\omega_\Sigma) - a_{el}\Gamma_\Sigma{}^2\omega_\Sigma{}^2] \tag{20}$$

$$b_1c = \frac{\Gamma_1\omega_\Sigma}{(1 - \Gamma_\Sigma\omega_\Sigma)^{\omega_1/\omega_\Sigma}} \tag{21}$$

The total adsorption in these equations can be expressed via the adsorption in state 1.[5]

$$\Gamma_\Sigma = \Gamma_1\sum_{i=1}^{n} i^\alpha \exp\frac{\omega_1(i-1)}{\omega_\Sigma}\exp\left[-\frac{(i-1)\Pi\omega_1}{RT}\right] \tag{22}$$

The first exponential factor arises due to the nonideality of entropy of mixing. This factor and also i^α correspond to a relative increase in adsorption in the states with $\omega_i > \omega_1$. To simplify eq 22 and subsequent expressions, this factor can be omitted when a value $\alpha = 0.5$ is used, which can compensate its effect in the case of proteins (see below). We consider that the use of the ratio b_i/b_j as a preexponential factor is more general. In addition, the presence of the variable ω_Σ in the exponential term makes the equation more general.

Finally, the mean partial molar area of all states and the adsorption in any ith state can be expressed by[5]

$$\omega_\Sigma = \omega_1 \frac{\sum_{i=1}^{n} i^{(\alpha+1)} \exp\left(-\frac{i\Pi\omega_1}{RT}\right)}{\sum_{i=1}^{n} i^{\alpha} \exp\left(-\frac{i\Pi\omega_1}{RT}\right)} \quad (23)$$

$$\Gamma_i = \Gamma_\Sigma \frac{i^{\alpha} \exp\left[-\frac{(i-1)\Pi\omega_1}{RT}\right]}{\sum_{i=1}^{n} i^{\alpha} \exp\left[-\frac{(i-1)\Pi\omega_1}{RT}\right]} \quad (24)$$

The adsorption model described above assumes different discrete states of protein molecules within the surface layer, and neighboring states differ from one another by the molar area increment $\Delta\omega$. From the viewpoint of scaling analysis, $(\Delta\omega)^{1/2}$ has to be close to the size of an electrostatic blob. In the adsorption layer of proteins the flexibility of chains increases due to the high concentration of both protein and inorganic electrolyte.[10] This allows one to consider, instead of discrete states, an infinitesimal change in the molar areas by $d\omega$. One of the advantages of such a continuous state model is that $\Delta\omega$ can be excluded from the equations. Note that an increment $\Delta\omega = \omega_1$ also leads to the elimination of this parameter from the equations, which then describes the behavior of rather inflexible chains. To perform the transformation from the discrete to the continuous model, one has to replace formally the summations in eqs 22−24 by an integration. For example, eqs 22 and 24 transform into

$$\Gamma_\Sigma = \frac{1}{\omega_{max} - \omega_1} \int_{\omega_1}^{\omega_{max}} \left(\frac{\omega}{\omega_1}\right)^{\alpha} \exp\left[-\frac{\Pi(\omega - \omega_1)}{RT}\right] d\omega \quad (25)$$

In some cases simple analytical expressions can be obtained instead of the cumbersome eqs 22−24. For example, for $\alpha = 0$ and $\omega_{max} \gg \omega_1$ (in fact, $(\omega_{max} \to \infty)$ instead of eq 23 one obtains

$$\omega_\Sigma = \omega_1\left(1 + \frac{RT}{\Pi\omega_1}\right) \quad (26)$$

For small Π the results obtained from eq 26 agree well with data calculated from eq 23. For the continuous-state model instead of eq 22 at $\alpha = 0$ one obtains

$$\Gamma_\Sigma = \frac{RT\Gamma_1}{\Pi(\omega_{max} - \omega_1)}\left[1 - \exp\left(-\frac{\Pi(\omega_{max} - \omega_1)}{RT}\right)\right] \quad (27)$$

To illustrate the proposed theory for the proteins adsorption and to compare the results with experimental data, numerical calculations according to eqs 20−24 have been performed. The transition from the discrete to the continuous model was made by decreasing the increment $\Delta\omega$, until the calculated results became independent of $\Delta\omega$. As an example, the dependence of surface pressure of a protein solution (molecular mass $M =$ 24 000 g/mol, $\omega_1 = 2$ nm^2, $(\omega_{max} = 60$ nm$^2)$ on the surface coverage of the adsorption layer $\theta = \Gamma_\Sigma\omega_\Sigma$ is shown in Figure 1. For $\Delta\omega = \omega_1$ the curve differs from that calculated for the continuous-state model. However, already for $\Delta\omega = 0.1\omega_1$ the two results are close to each other. For $\Delta\omega = \omega_1$ eq 24 yields that at $\Pi > 10$ mN/m all states with $i > 1$ disappear from the surface, and the curve $\Pi = \Pi(\theta)$ coincides with that calculated for $n = 1$, that is, when only one adsorption state with $\omega_i = \omega_1$ exists.

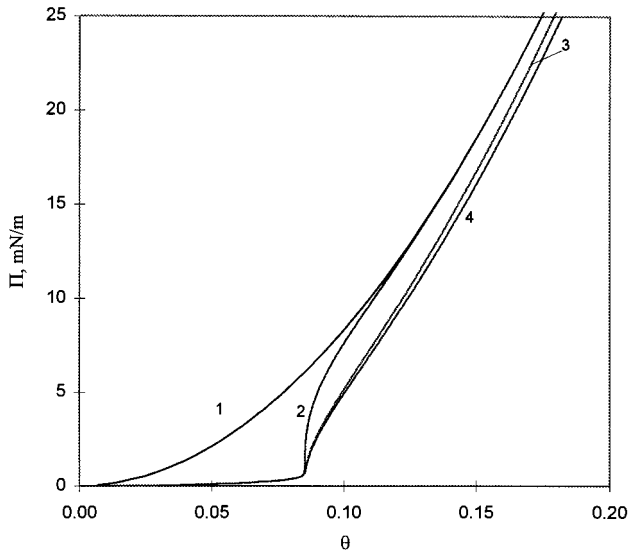

Figure 1. Dependence of surface pressure on the adsorption layer occupation degree, calculated for the protein solution at $M = 24\,000$, $\omega_1 = 2$ nm^2, $\omega_{max} = 60$ nm^2, $\alpha = 2$, and $a_{el} = 400$. Curve 1 calculated for $n = 1$, curve 2 for $\Delta\omega = \omega_1$, curve 3 for $\Delta\omega = 0.1\ \omega_1$, and curve 4 for continuum model ($\Delta\omega < 0.02\omega_1$).

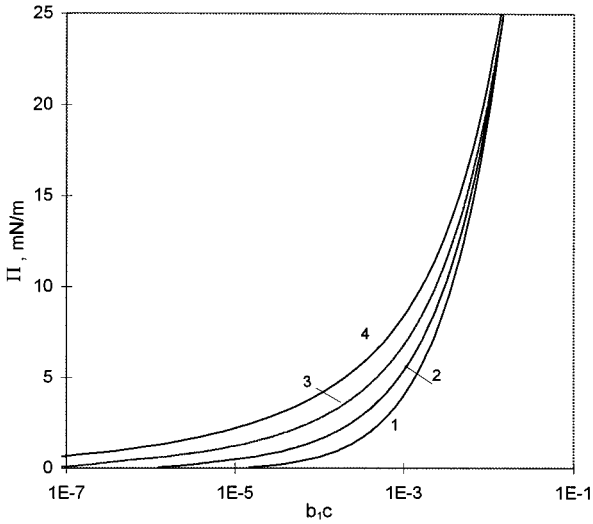

Figure 2. Dependence of adsorption on the reduced concentration b_1c for the protein solution ($M = 24\,000$, $\omega_1 = 2$ nm^2, $\omega_{max} = 60$ nm^2, and $a_{el} = 400$) for $\alpha = 3$ (curve 4), 2 (curve 3), 1 (curve 2), and 0 (curve 1).

Let us consider now the effect of the parameters in the equation of state (eq 20) and adsorption isotherm (eq 21) on the equilibrium protein adsorption and surface pressure. The effect of the coefficient α, which accounts for the adsorption activities of different states and the entropic nonideality of the surface layer, is illustrated in Figure 2. Here all curves are normalized such that all curves meet at $\Pi = 25$ mN/m. One can see that increasing α leads to an increase in the surface pressure and adsorption at very low protein concentrations. This is the consequence of the high adsorption activity of the states possessing large ω_i. In this case, however, the sharp increase of Γ_Σ and Π within a narrow concentration range, characteristic of proteins, disappears. It seems that the actual value of the coefficient a should not exceed 1, restricted to the contribution of the nonideality of entropy, which corresponds *to* $\alpha \approx 0.5$.

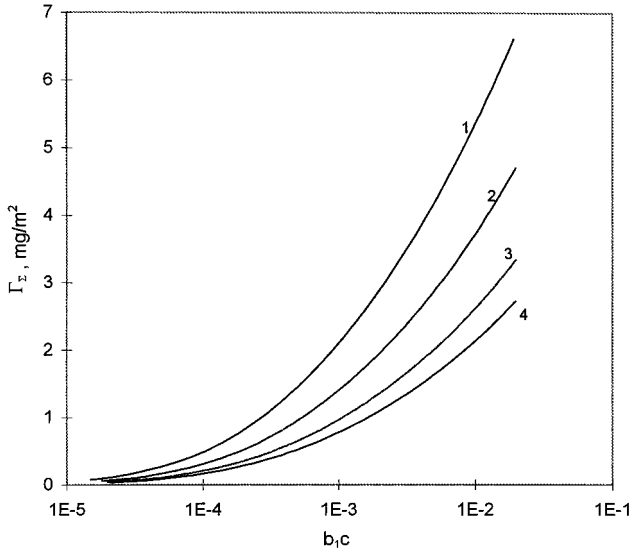

Figure 3. Dependence of surface pressure on b_1c value for the protein solution ($M = 24\,000$, $\omega_1 = 2$ nm^2, $\omega_{max} = 60$ nm^2, and $\alpha = 0$) for $a_{el} = 100$ (curve 1), 200 (curve 2), 400 (curve 3), and 600 (curve 4).

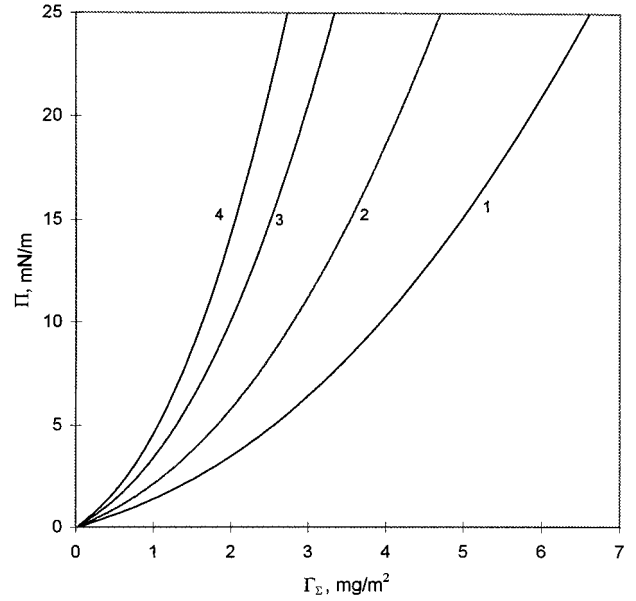

Figure 4. Dependence of surface pressure on adsorption. The values of the parameters and the notation are the same as in Figure 3.

As the curves for $\alpha = 0$ and $\alpha = 1$ in Figure 2 are hardly distinguishable from one another, one can assume $\alpha = 0$ for proteins. This reduces the number of parameters involved in eqs 20 and 21. Thus the adsorption of proteins in the framework of the continuous-state model can be described by a set of four independent parameters: ω_1, ω_{max}, a_{el}, and b_1. Note that for the discrete model for $\omega_1 = \Delta\omega$ the number of independent parameters is also four.

The effect of the repulsion constant a_{el} is illustrated in Figures 3 and 4. Decreasing a_{el} leads to sharper dependencies of Γ_Σ on b_1c, while the Π dependence on Γ_Σ, in contrast, becomes more pronounced with increasing a_{el}. For constant surface pressure the increase of a_{el} leads to a decrease in the total adsorption, because the interior repulsion becomes stronger. The intermolecular repulsion of chains leads to a decrease of the surface layer coverage so that it remains less packed even at high surface pressures. The dependence shown in Figure 5 indicates that for $a_{el} = 600$ the adsorption layer coverage does

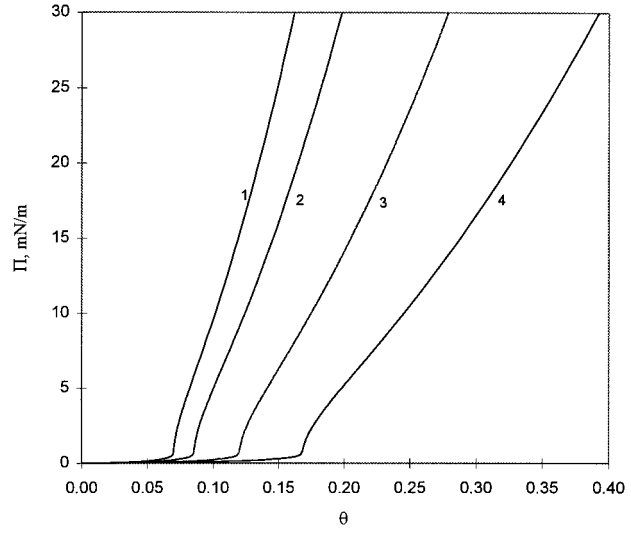

Figure 5. Dependence of surface pressure on the adsorption layer occupation degree for the protein solution ($M = 24\,000$, $\omega_1 = 2$ nm^2, $\omega_{max} = 60$ nm^2, and $\alpha = 2$) for $a_{el} = 600$ (curve 1), 400 (curve 2), 200 (curve 3), and 100 (curve 4).

not exceed 15%. Further increase in the adsorption with a densely packed adsorption layer is possible only at small a_{el}.

The parameters ω_1 and ω_{max} also significantly effect the shape of the dependencies of Γ_Σ on b_1c and Π on Γ_Σ. With the increase in ω_1, the dependencies of Π and Γ_Σ on b_1c become less steep. A similar effect is observed also for ω_{max}. For a fixed Π the adsorption increases with increasing ω_1. The values of ω_1 and ω_{max} are directly related to the molecular mass of the protein, to its physicochemical characteristics, and to the properties of the solvent. It can be argued that $\omega_1^{1/2}$ cannot be smaller than the dimension of an electrostatic blob or the correlation length.[17] The parameter ω_{max} is defined as the maximum area that a denatured protein molecule can occupy within the surface layer.

In Figure 1 as well as Figure 5 the model calculations show that there is a sudden change of Π with increasing θ when a discrete number of molecular conformations and hence molar areas are assumed. This steep change in Π is caused by a replacement of molecules after the surface layer is covered for the first time with molecules having the largest molar surface area demand. While the total surface coverage does not change too much, the layer thickness increases and hence the surface pressure increases significantly.

The main feature of the proposed theoretical model is the self-regulation of both the state of the adsorbed molecules and the adsorption layer thickness by the surface pressure Π. The theory is based on the concept first formulated by Joos and Serrien[4] and differs essentially in the self-regulation mechanism from known thermodynamic, statistical, and scaling models. This mechanism is inherent in the Butler equation,[6] from which all main equations are derived. Of course, the surface pressure cannot be regarded as the only self-regulating parameter, but for the solution/fluid interface this factor is possibly the main one. From eq 24 one can calculate the part of adsorbed molecules existing in the state ω_i. This part is expressed by the distribution function Γ_i/Γ_Σ, which gives the probability density of Γ_i as a function of the partial molar area ω_i at a given Π (cf. Figure 6). For very low Π (0.1 mN/m) all states are represented in the protein adsorption layer; however the number of molecules with maximum area $\omega_i = \omega_{max} = 60$ nm^2 is higher than that for all other states ($\alpha = 2$ was assumed). At $\Pi = 0.5$ mN/m the maximum probability density is achieved for the

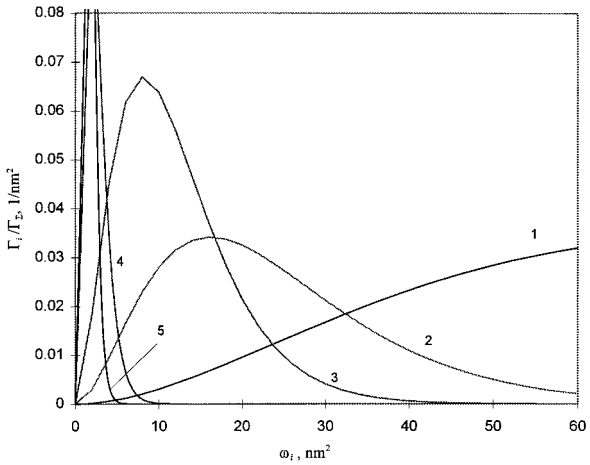

Figure 6. Dependence of distribution function Γ_i/Γ_Σ, for protein solution on ω_i ($M = 24\,000$, $\omega_1 = 2$ nm^2, $\omega_{max} = 60$ nm^2, $\alpha = 2$, and $a_{el} = 600$) for $\Pi = 0.1$ (curve 1), 0.5 (curve 2), 1 (curve 3), 5 (curve 4), and 10 mN/m (curve 5).

molecules with $\omega_i \approx 17$ nm^2, while at $\Pi = 1$ mN/m a maximum is obtained at $\omega_i \approx 10$ nm^2. With further increase in Π the amount of molecules occupying a minimum area increases continuously. For $\Pi \geq 10$ mN/m only a small number of adsorbed molecules occupy an area exceeding $\omega_i = \omega_{min} = 2$ nm^2. Therefore, the equilibrium adsorption layer is characterized by almost complete denaturation at low surface pressure, while for large surface pressure, the equilibrium adsorption layer is built by molecules in a state with minimum surface area demand.

If a_{el} is suffficiently high, the protein adsorption layer coverage in the range $\Pi = 20-30$ mN/m remains very low (Figure 5). The theoretical model described by eqs 20−24 predicts an unrealistically sharp increase of surface pressure at a slight increase of protein concentration and a simultaneous slight increase of the adsorption. This contradicts the experimental data that show that, starting from a certain protein concentration, the Π value remains almost constant, while the adsorption continues to increase, resulting in an increase of the surface coverage up to an almost complete saturation of adsorption layer at high protein concentration. This deficiency of the theoretical model is possibly due to the fact that the a_{el} value does not remain constant. In fact, the number z of unbound ions in the protein molecule can decrease when both adsorption and surface coverage increase. Assuming that, starting from a certain coverage, z is inversely proportional to Γ_Σ, one obtains the approximate dependence $a_{el} \approx (\Gamma_\Sigma)^{-2}$. This is clearly an approximation; however, on the other hand, a relative (per unit polymer concentration) decrease of the osmotic pressure with increasing concentration of the polymer and electrolyte is also predicted by the scaling model.[10]

Let us introduce a critical protein concentration c_c and correspondingly θ_c and Π_c, above which the a_{el} value decreases. Because the surface coverage at $\Pi \approx 25-30$ mN/m is low (see Figure 5), only the second term on the right-hand side of eq 20 can be retained. As for large Π the relation $\omega_\Sigma \approx \omega_1$ al holds, one obtains from eq 20 a relationship between θ_c and Π_c:

$$\theta_c = \left(\frac{\Pi_c \omega_1}{a_{el} RT}\right)^{1/2} \tag{28}$$

Assuming next that for $\theta = \Gamma_\Sigma \omega_1 > \theta_c$ the constant a_{el} is defined by the relation

$$\frac{a_{el}^*}{a_{el}} = \left(\frac{\theta_c}{\theta}\right)^2 \tag{29}$$

where a_{el}^* is the value of the constant a_{el} for $c > c_c$, one obtains from eqs 20 and 21 an equation of state for the surface layer and the adsorption isotherm for concentrated ($c > c_c$) protein solutions:

$$\Pi = -\frac{RT}{\omega_1}[\ln(1-\theta) - a_{el}\theta_c^2] \tag{30}$$

$$b_1 c = \frac{\theta}{1-\theta} \tag{31}$$

It follows from eq 30 that $\Pi \cong \Pi_c$ for $c > c_c$. The calculations performed according to eq 31 show that, in agreement with the experimental data, the adsorption increases significantly for $c > c_c$.

Materials and Methods

Human serum albumin (HSA), A8301, Lot 94H8270, was obtained from Sigma (Germany); its molecular weight was 69.000. The protein was used without further purlfication. The phosphate buffer solution was prepared by mixing appropriate stock solutions of K_2HPO_4 and NaH_2PO_4. The surface tension of the buffer solution at pH 7 was 72.5 mN/m. All experiments were performed at a room temperature of 22 °C.

The adsorption of the proteins was characterized by dynamic surface and interfacial tension measurements using the axisymmetric drop shape analysis (ADSA).[19,20]

Results and Discussion

The curves of dynamic surface tension for HSA solutions with various concentration values are shown in Figure 7. It is seen that the equilibrium is achieved within 4 h time only for concentrated HSA solutions ($c > 10^{-6}$ mol/L). This agrees with the data presented by Gonzalez and MacRitchie,[21] who studied the protein BSA, the structure of which is similar to that of HSA. To estimate the equilibrium surface tension values for less concentrated HSA solutions the curves $\gamma = \gamma(t)$ were extrapolated to $t \rightarrow \infty$. The γ dependencies on $t^{-1/2}$ are presented in Figure 8. For a mixed adsorption mechanism the derivative $d\gamma/dt^{-1/2}$ is defined by the relationship[22]

$$\frac{d\gamma}{dt^{-1/2}} = \frac{RT\Gamma_\Sigma^2}{c}\left(\frac{\Pi}{4D}\right)^{1/2} + \frac{RT\Gamma_\Sigma^2}{c\beta t^{1/2}} \tag{32}$$

where β is the adsorption rate constant. For $\beta \rightarrow \infty$, which corresponds to the diffusion adsorption mechanism, the second term on the right-hand side of eq 32 vanishes, and the expression transforms into the known Joos−Hansen relationship.[22] In fact, the curves presented in Figure 8 possess a linear dependence at large t (which corresponds to small values of $t^{-1/2}$), and therefore the intersection points with the ordinate correspond to the equilibrium surface tension values. The experimental values of $(d\gamma/dt^{-1/2})_{t\rightarrow\infty}$ estimated from Figure 8 however are approximately 100 times higher than those calculated from the Joos−Hansen equation. This has been discussed before elsewhere[20] and can be explained by the fact that the eq 32 is derived for surfactants and cannot take into consideration the particular effects connected with the adsorption kinetics of protein molecules. Assuming another mechanism, that is, neglecting the first term on the right-hand side of eq 32 leads to an extrapolation $d\gamma/dt^{-1}$ as proposed in ref 22. The data of Figure

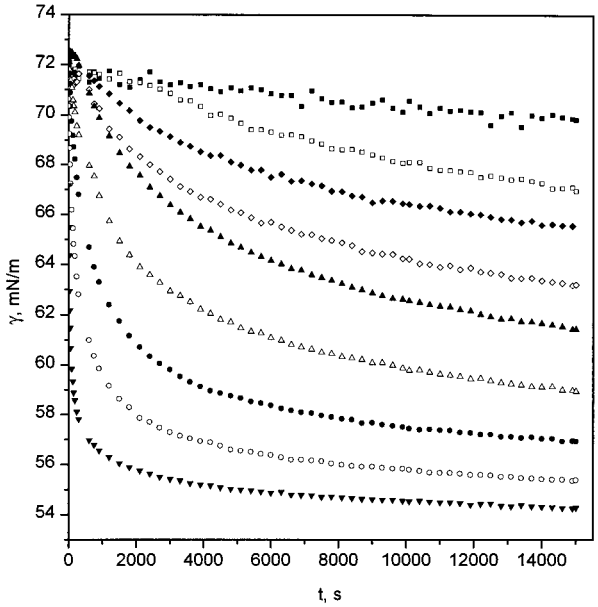

Figure 7. Dynamic surface tension for HSA solutions with various concentration values: 2×10^{-8} mol/L (■), 3×10^{-8} mol/L (□), 5×10^{-8} mol/L (◆), 7×10^{-8} mol/L (◇), 10^{-7} mol/L (▲), 2×10^{-7} mol/L (△), 5×10^{-7} mol/L (●), 10^{-6} mol/L (○), 10^{-5} mol/L (▼).

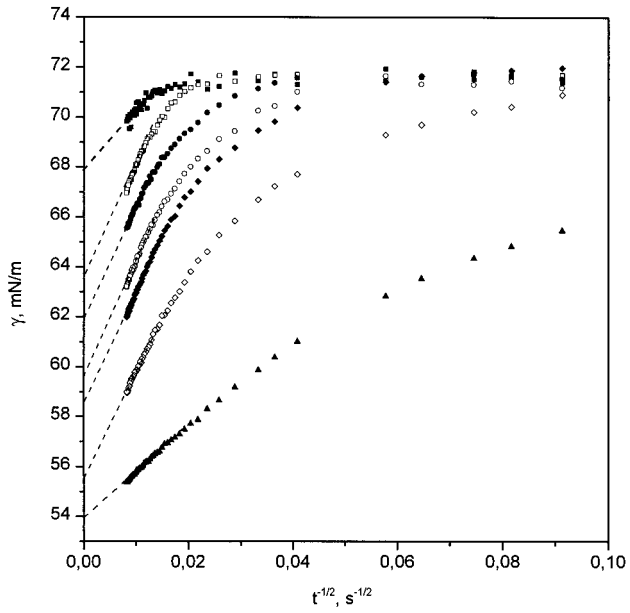

Figure 8. Dynamic surface tension dependencies of HSA solutions on $t^{-1/2}$: 2×10^{-8} mol/L (■), 3×10^{-8} mol/L (□), 5×10^{-8} mol/L (●), 7×10^{-8} mol/L (○), 9×10^{-8} mol/L (◆), 2×10^{-7} mol/L (◇), 10^{-6} mol/L (▲).

8 are replotted in Figure 9 as $\gamma(t^{-1})$. The equilibrium surface tension values obtained from the two different extrapolation procedures are very close to each other and differ by less than 0.5 mN/m. The values averaged between these two extrapolations were used in the following (Figure 10). In the isotherm plot the initial HSA concentrations are used. These initial concentrations can differ from the equilibrium concentration due to HSA adsorption at the drop surface. Note that for $c > 10^{-7}$ mol/L the presented data agree well with those presented by other authors[21,23−25] for BSA, while in the region $c < 10^{-7}$ mol/L our experimental values are lower than those reported by Graham and Phillips.[25] It will be shown below that this can be explained by the decrease of protein bulk concentration due to the adsorption at the drop surface.

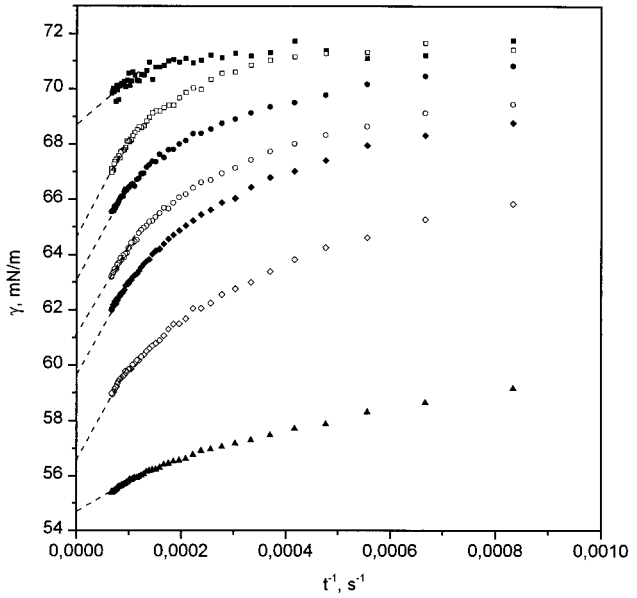

Figure 9. Dynamic surface tension dependencies of HSA solutions on t^{-1}: 2×10^{-8} mol/L (■), 3×10^{-8} mol/L (□), 5×10^{-8} mol/L (●), 7×10^{-8} mol/L (○), 9×10^{-8} mol/L (◆), 2×10^{-7} mol/L (◇), 10^{-6} mol/L (▲).

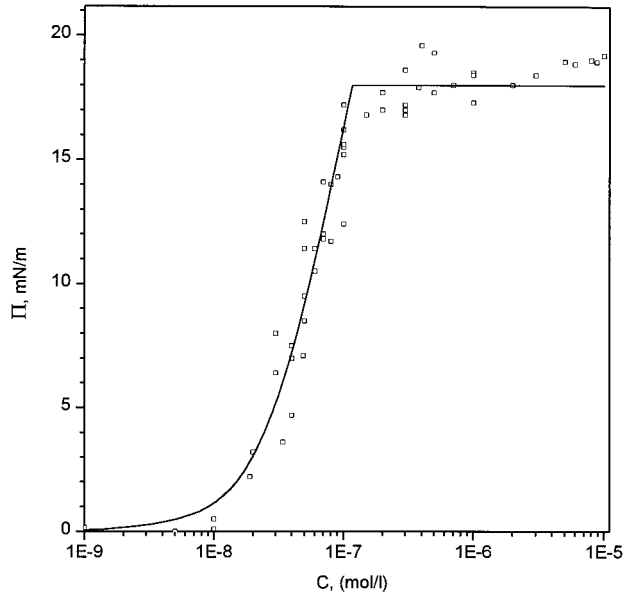

Figure 10. Experimental isotherm for equilibrium surface tension of HSA for pH 7 plotted vs the initial HSA concentration in the solution *c*, and theoretical adsorption isotherm calculated from eqs 20−24, 30, and 31.

In Figure 10 also the theoretical HSA adsorption isotherm is shown calculated from eqs 20−24, 30, and 31 using a fitting program. The parameters of the theoretical equations (ω_1, $\omega_{max} = n\omega_1$, $\Delta\omega$, α, and a_{el}) were varied subject to a maximum HSA adsorption value of about 3 mg/m^2, which corresponds to the data for BSA as reported in refs 25 and 26. The theoretical dependence of Γ_Σ on *c* is shown in Figure 11. For $c > 10^{-7}$ mol/L the calculated dependence agrees well with the data presented in refs 25 and 26, while for $c < 10^{-7}$ mol/L the calculated Γ_Σ values are approximately 40−50% lower that those measured in ref 26. The theoretical curves presented in Figures 10 and 11 have been calculated with the following set of parameters: $\omega_1 = \omega_{min} = 40$ nm^2 (per one HSA molecule), $\omega_{max} = 80$ nm^2, $\Delta\omega = \omega_1$, $a_{el} = 80$, $\alpha = 0$, and $b_1 = 2 \times 10^7$ L/mol. These parameters agree very well with data presented

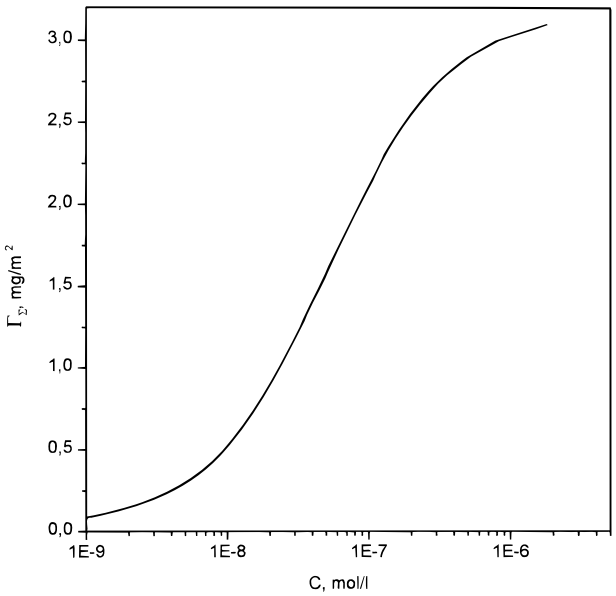

Figure 11. Theoretical Γ_Σ dependence on concentration of HSA in solution, c.

in refs 27−30. In particular, the minimum area per BSA (or HSA) molecule in the monolayer is in fact 40−50 nm^2. In a spread of BSA monolayers the increase of surface pressure becomes appreciable when the area per protein molecule decreases to 150−180 nm^2, which corresponds to a monolayer coverage of ca. 20%; see Figure 11. Note that for $\Delta\omega = \omega_1$ the variation of ω_{max} within the range 40−200 nm^2 does not affect the theoretical dependencies of $\Pi(c)$ and $\Gamma_\Sigma(c)$. For a HSA adsorption of ca. 1 mg/m^2 and an adsorption layer thickness of 4 nm the total concentration of ions within the surface layer can be estimated as 2 mol/L (with a total number of amino acid groups in the HSA molecule of 580; see ref 30). Assuming that the minimum free charge of an albumin molecule $z \cong 20$ (cf. ref 30), one can estimate the value of a_{el} as a few tens (see eq 17), which agrees with the results obtained from the fitting program. The minimum area of one HSA molecule within the monolayer corresponds to a three-domain molecular structure, with each domain composed of nine loops connected by sulfide bridges. At pH = 7 the size of such a molecule is 14 × 4 × 4 nm; see ref 30. This configuration is possibly independent of Π and Γ_Σ, so that HSA molecules do not undergo a denaturation at the liquid/gas interface.

From the theoretical dependence of Γ_Σ on c presented in Figure 11 and also from the data reported in refs 25 and 26, one can estimate the concentration decrease in the drop due to HSA adsorption at the surface. The equilibrium concentration c of the protein within the drop (i.e. the concentration in the adsorption equilibrium state) is related to the initial concentration c_0 via the expression $c = c_0 - \Gamma_\Sigma(S/V)$, where S and V are the area and volume of the drop, respectively. For c_0 in the range 10^{-8}−10^{-7} mol/L the equilibrium protein concentration in the drop is 60−30% lower than the initial concentration.

Therefore the experimental points in Figure 10 should be moved by 60−30% toward lower concentrations. As this shift is larger at smaller Π, the experimental curve becomes less sharp. This correction produces minor effect on the optimum parameters of eqs 20−24, resulting in a decrease in the a_{el} value from 80 to 60 and a 3-fold decrease of b_1. These changes lead to better agreement between our data with the results obtained by Graham and Phillips[25] at low albumin concentrations.

Conclusions

An equation of state for the surface layer and an adsorption isotherm for proteins at the liquid/fluid interface are derived. These relationships reflect the main feature of high molecular electrolytes possessing flexible chains: the capability of changing the partial molar surface area in response to a variation in surface pressure. The new equations describe the case of a nonideal surface layer, taking into consideration the nonideality of enthalpy (Flory−Huggins' parameter) and entropy of mixing. The effect of the electric charge of the protein molecule is considered as a significant contribution to the surface pressure. The model of multiple discrete states of the protein molecules within the surface layer is generalized for the case of an infinite number of infinitesimal states (continuum model). The adsorption behavior of higher concentrated protein solutions is also considered. To verify the equations of state and adsorption isotherms derived, dynamic surface tensions for HSA solutions were measured using the ADSA method. The two theoretically justified extrapolations (t^{-1} and $t^{-1/2}$) lead to similar results for the equilibrium surface tension value. The derived theoretical equations were applied for the description of the HSA surface tension isotherm and show an excellent agreement with the experimental results. Moreover, all principal parameters (area per molecule in the monolayer, electric charge of the HSA molecule, adsorption layer thickness) are close to values obtained in other studies employing other experimental methods. It follows that HSA molecules do not undergo any significant denaturation at the solution/air interface and occupy an area of ca. 40−50 nm^2 in both the diluted and saturated monolayer. This agrees well with the concept of a triple-domain structure of HSA molecules, where the polypeptide chain within the domains exists in loops connected by sulfide bridges.

Acknowledgment. The work was financially supported by projects of the European Community European Community (INTAS 93-2463-ext and INCO PL 96-5069) and the DFG (Wu 187/5). Research grants from the Max-Planck-Institut für Kolloid- und Grenzflächenforschung (V.B.F.) and the Alexander von Humboldt Stiftung (A.V.M., BB-1034737) are also gratefully acknowledged.

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