CCA-745

541.183.2:547.96
Conference Paper

Biopolymer Adsorption, with Special Reference to the Serum Albumin—Polystyrene Latex System*

J. Lyklema and W. Norde

Laboratory for Physical and Colloid Chemistry of the Agricultural University, De Dreijen 6, Wegeningen, Netherlands

Received November 13, 1972

A study has been made of the adsorption of human serum albumin (HSA) on emulsifier-free, negatively charged polystyrene (PS) latices. The adsorption has been followed directly and microcalorimetrically. Important variables are: pH, temperature, c_{salt} and the surface charge σ_0 of the latex.

Although all adsorption isotherms have a platform, the Langmuir theory is inadequate to account for them. Distinction must be made between the initial stages of adsorption, solely determined by the HSA-PS interaction and the later stages, where lateral interaction between adsorbed HSA molecules plays also an important role.

The adsorption platform as a function of pH is maximal in the isoelectric point. Here the adsorbed amount is roughly compatible with side-on native HSA molecules. Both below and above the i. e. p. adsorption proceeds in a more unfolded conformation but a spread monolayer is never reached.

The adsorption is largely driven by a net entropy gain, both in the initial and later states of the process. Besides this, there is a recognizable influence of the electrostatic attraction between the latex surface and local positive excesses inside the molecule.

INTRODUCTION

The properties of biopolymers at solid—liquid interfaces is a matter of increasing interest. Knowledge on it is for example mandatory for handling articifial implants and immunochemical tests. It is also important for several aspects of enzymology.

In this study we shall deal with some colloid-chemical aspects of biopolymer adsorption, particularly the adsorption of human serum albumin (HSA) on polystyrene (PS) latex particles. Some trends, observed with this system will be compared and analyzed against a background of adsorption information obtained with simpler systems.

With this in mind it is expedient to make first a comparison between the factors involved in the adsportion of simple molecules, polymers and biopolymers, that is in the order of increasing complexity.

The adsportion from solution of simple, low-molecular weight molecules is in general relatively well understood, although on close inspection interpretational difficulties can arise because such an adsorption is always a

* Based on a lecture presented at the *III International Conference on the Chemistry at Interfaces,* Rovinj, Yugoslavia, June 27—30, 1972.

competitive process. If solute molecules adsorb, an equivalent number of solvent molecules must desorb[1]. The adsorption enthalpy is the sum effect of this exchange. If the adsorption proces is written as

$$n_A A_{solution} + n_B B_{surface} \rightleftarrows n_A A_{surface} + n_B B_{solution} \quad (1)$$

then the adsorption enthalpy per cm^2 h_{ads} follows from

$$h_{ads} = n_B (h_{B,\,sol} - h_{B,\,surf}) - n_A (h_{A,\,sol} - h_{A,\,surf}) \quad (2)$$

where $h_{B,sol}$ is the enthalpy per molecule of B in solution, $h_{B,surf}$ the same property on the surface and $h_{A,sol}$ and $h_{A,surf}$ indicate the corresponding quantities for A. n is the number of molecules per cm^2. If A and B have the same molecular cross-section $n_A = n_B$. In ideal solutions h_{sol} is independent of concentration and comparable with (or identical to, depending on the reference state chosen) the molecular solvation enthalpy. On homogeneous surfaces without lateral interaction h_{surf} is also constant. h_{surf} becomes dependent on the adsorbed amount if there *is* lateral interaction between the adsorbed molecules or if the surface is heterogeneous. It follows that three conditions must be fulfilled in order to make h_{ads} constant.

For physical adsorption, the entropy of adsorption s_{ads} is almost entirely determined by the change in the number of configurations in exchanging a solvent molecule by a solute molecule according to (1). The way in which s_{ads} depends on the adsorbed amount and on the bulk concentration determines to a large extent the shape of the adsorption isotherm.

The simplest adsorption isotherm equation is the one-parameter equation $n_A = k_1 c_A$ (Henry), where c_A is the bulk concentration of A. It applies strictly only for low surface coverage Θ and mobile adsorption, but is sometimes also observed at low c_A and localized adsorption. The constant k_1 reflects the interaction between adsorbate and adsorbent and is related to h_{ads} which in this case can be written according to (2).

As soon as the condition $\Theta \ll 1$ is no longer fulfilled, a second parameter k_2 is needed to account for the limited availability of the adsorbent surface. The most familiar two-parameter equation is that of Langmuir. It predicts a platform value to be attained at high c_A, but this does not mean that any isotherm with a platform is a Langmuir isotherm.

If lateral interaction may not be neglected, a third parameter w (or still more, depending on the complexity of this interaction) is needed for a full description of the isotherm. The contribution of this interaction increases progressively when Θ approaches unity. Examples of such three-parameter isotherm equations are the Fowler-Guggenheim and Frumkin equation. In a sense the Stern equation for ion adsorption belongs also to this category because the electrostatic potential accounts for the interionic interaction.

One important conclusion, to be drawn from this is: the interaction between adsorbate and adsorbent manifests itself most clearly at low Θ whereas the mutual interaction between adsorbed molecules is relatively important at high Θ. In broad lines, this conclusion remains valid for the adsorption of (bio)polymers.

It is clearly impossible to describe the adsorption of man-made polymers satisfactorily with as few as two or three parameters. Taking this to the extreme, 3i parameters are needed to specify completely the arrangement of the

i segments of an isolated adsorbed polymer chain over loops, tails and trains (sequences of segments in actual contact with the surface). However such an »instant« picture has little practical sense, since the polymer constantly changes its conformation. Only a statistically averaged segment distribution $\varrho(x)$ retains physical reality.

In special cases the distribution can be evaluated. Relatively simple is the completely random distribution of atactic homopolymers. Among the adsorption isotherm equations the Hoeve-isotherm[2] may be mentioned because it is in principle a two-parameter isotherm, although the constants have a composite character. In this picture, the $\varrho(x)$ relationship has a discontinuity after the first layer of adsorbed segments, beyond which the polymer segment density decays exponentially. Such a distribution is akin to the countercharge distribution in a Stern-Gouy double layer. Another case that has been successfully treated is that of terminally adsorbed tails[3]. In this case $\varrho(x)$ is Gaussian. For more complex cases, *e. g.* polymers with non-negligible intrasegmental attraction, theory has not yet provided complete solutions. By way of recent example, Hesselink's work on polyelectrolyte adsorption can be mentioned[4].

As far as h_{ads} reflects the interaction with the adsorbent, it stems in the first place from the interaction with the segments, adsorbed in the first layer. Segments in the second and following layers are as a rule too far away to contribute significantly to h_{ads}. Other contributions can be visualized: torsional enthalpies due to obstructed rotation after adsorption of some segments of a chain and intrasegmental enthalpies if the average distance between the segments alters upon adsorption. It can be imagined that in some special systems attachment of segments onto the surface is entropically driven (hydrophobic bonding), in which case the contribution of first layer interaction to h_{ads} can be relatively small.

With random polymers, entropic factors play in general a much more pronounced role than with low molecular weight solutes. That almost any polymer adsorbs at any surface is mainly due to the fact that in its adsorbed state a polymer molecule is still to a large extent in contact with the solvent and retains a relatively high molecular entropy. The loss of translational entropy plays only a minor role. Moreover, one polymer molecule, adsorbing with many segments on a substrate liberates several solvent molecules with a concomitant gain of translational entropy. In view of all of this, a positive s_{ads} can be the driving force of polymer adsorption, especially if hydrophobic bonding between polymer segments and the substrate occurs. The possibility of an endothermal polymer adsorption process ($h_{ads} > 0$) is thereby indicated.

One other feature, characteristic for polymer adsorption deserves attention, namely that irreversibility of the adsorption is the rule rather than the exception. The explanation must be sought in the unlikeliness to desorb all segments of a long train simultaneously, a process that would be needed to rearrange the three-dimensional adsorbed coil on its way towards equilibrium. This irreversibility has important consequences for practice and interpretation. For example, the actual state of the adsorbate will depend on the way in which it has been formed (slow or fast, in one or in two steps). The applicability of thermodynamic laws (Gibbs, Clapeyron) that are based on equilibrium becomes questionable, this in addition to the problem whether these laws must be applied to whole molecules or to segments.

As a rule, experimental adsorption isotherms of random polymers have a high-affinity character. The first arriving molecules adsorb so tenaciously that the amount remaining in solution falls below detectability. Hence the initial part of the isotherm merges with the ordinate axis. A platform is not usually attained, the adsorption keeps increasing with increasing concentration in solution.

From random macromolecules to biopolymers is still a long way to go. Statistical averaging of segment densities would do no justice to the subtleties of the conformation constituting the very basis of physiological activity. At present, the number of biopolymers for which the three-dimensional structure in aqueous solution or in the crystalline state is fully known is very limited. Much further away seems the complete solution of the conformation of biopolymers if perturbed by an adsorbing surface (except perhaps in the two limiting cases of (*a*) one segment thick, completely extended adsorption, and (*b*) adsorption in its native state, that is: unperturbed, being cases where the conformation in the adsorbed state may be simpler than or equally »simple« as in solution). Experimental quantities such as adsorbed amount and ellipsometric thickness are far too crude to specify completely the desired three-dimensional conformation.

The properties of biopolymers at interfaces being so important for biological and medical sciences, all serious attempts to learn more about them deserve attention. For this reason, we decided to undertake a systematic study of the adsorption of HSA on polystyrene latex. It was hoped that such a study would constructively contribute to our insight especially since the following experimental conditions were met:

1. The latex has been made without emulsifier. This means that no spurious surface active substances detract from the value and significance of the results. Also the use of buffers has been avoided. It was so to say a clean system.

2. By changing the pH, the charge on the albumin is varied in a known way. By varying the polymerization conditions also the surface charge on the latex could be varied and controlled. Hence, by bringing together HSA and latex under different conditions of charge, insight could be gained in the electrostatic component of the interaction.

3. The adsorption process has been followed microcalorimetrically. In doing so, information is gained on the thermodynamics of the adsorption process including the concomitant conformation changes.

As will be shown below, some trends about the unfolding of the HSA molecule on adsorption emerge. Some surprising results have also been obtained. Since it is the purpose to interpret the results in terms of alterations in the conformation upon adsorption a review on the factors determining the secondary and tertiary structure of HSA will be given first.

Properties of Biopolymers with Special Reference to HSA

In this section the structure of biopolymers in general and of HSA in particular will he discussed. Special attention will be paid to the aspects in which biopolymers distinguish themselves from synthetic polymers.

In contrast to most synthetic (homo) polymers native biopolymers possess a definite and for every species typical structure, which is more or less conditionary for its physiological activity.

First of all we have to deal with the amino acid composition and -sequence in the peptide chain, *i. e.* the primary structure.

Not all spatial arrangements of the peptide chain are energetically equal. In this respect one may distinguish between the extremes α-helix, β-structure and random coil. These spatial arrangements constitute the secondary structure.

Specific interactions between special segments, hydrophobic bonding and the presence of S—S bridges cause a certain folding of the biopolymer molecule, leading to its tertiary structure.

Native serum albumin contains about 55% α-helix and 45% random coil. It is stabilized mainly by intramolecular H-bonds, hydrophobic interactions and by S—S bridges.

A change in the conditions (*e. g.* pH, ionic strength, solvent, temperature, *etc.*) will generally affect at least some of the various interactions in the system and therefore will possibly influence the secondary and/or tertiary structure.

Whether or not structural changes in HSA occur upon adsorption (and, if so: to what extent) is one of the objects of our investigation. As the forces involved depend strongly on the nature of the specific system under consideration, a comparative review of what has been found by other authors and with other systems (*e. g.* with monolayers) is only of little help.

To understand spatial changes in the HSA molecule it is diserable to know the factors that are responsible for its structure.

The following considerations mostly concern bovine serum albumin (BSA), but it is generally accepted that the structure and physical behaviour of BSA and HSA are very similar[6,8,12].

a) Amino acid composition[5-9]. — Serum albumin consists of a single polypeptide chain of *ca.* 590 amino acids and it has a molecular weight of about 69,000. The amino acid sequence in the polypeptide chain has not yet been established, although the composition is known. There are indications that the distribution is rather uniform[9]. 23% is acid, 15% contains an ε-NH_2-group. Together with 3% imidazole and minor amounts of other dissociable groups this determines the charge Z as a function of pH. The molecule contains 17 cystine S—S bridges, 22% hydrophobic side chains of the size $-C_3H_7$ or larger, 3% aromatics, 3.4% heterocyclic groups, 10.5% hydroxyl groups and 5.3% of proline. The last mentioned building brick, by virtue of its molecular structure is usually found in stiff bends in the molecule.

Randomization of disulphide bridges may occur above pH 7[10].

Upon adsorption the primary structure is not altered.

b) Charge. — The charge can be determined by titration. For HSA, if Z is expressed in unit charges per molecule and the isoelectric point (pH *ca.* 4.8) taken as the reference, Z is about +90 at pH 2 and —90 at pH 12[6,11]. Z is higher if the ionic strength ω is increased, a feature that all polyelectrolytes have in common with hydrophobic colloids. Although at the i. e. p. $Z \cong 0$* this does not mean that there are no charged groups on the molecule at that pH. It is likely that there are several positive and an equal number of negative groups present in the molecule[6]. This is important for the structure. As the charge is relatively uniformly distributed, electrostatic attraction pre-

* Z = 0 in the isoionic point

vails over repulsion and may well be one of the reasons for the relative compactness of the molecule at the i. e. p.

The net charge on the protein molecule for pH $\neq$ i. e. p. induces the molecule to swell. Experimentally this expansion can be followed by light scattering and viscosimetry. It appears that with serum albumin except for this relatively minor »physical« expansion also two more definite structural transitions are observed, *viz.* the so-called N—F transition at pH 4.5—3.5[12] and the neutral transition around pH 7—9[9]. The N—F transition is reflected in the titration behaviour (relative increase of Z, about 40 titrable groups that were formerly »buried« and inaccessible become now exposed), $[\eta]$, specific rotation, a decrease of the α-helix content and other properties. It is probable that by this transition subunits of the albumin molecule are reoriented. The neutral transition is less dramatic, involving no significatnt changes in $[\eta]$ or in the α-helix content.

It is certain that the charge of the molecule must play an important role in its adsorption behaviour. In the first place because it affects the intrinsic stability of the molecule and therefore its liability to unfold if an adsorbing substrate is presented. Secondly, there is the net electrostatic interaction between protein and latex (attractive for pH $<$ i. e. p. and repulsive for pH $>$ i. e. p.) with the interesting quantitative question whether or not the electrical term outweighs the other ones, a point to be discussed below.

c) *Hydrogen- and hydrophobic bonding* constitute two other important contributions to the secondary and tertiary structure-formation of biopolymers. The driving force of the first one is energetic, that of the second entropic (gain in translational and rotational entropy of water molecules, liberated if hydrophobic groups associate with each other). Hydrogen-bonding and hydrophobic bonding are typical short-range effects, in contradistinction to the long-range Coulombic forces. Their influence on the structure of the molecule is therefore more subtle, more specific and perhaps also more elusive. The bond energy of H-bridges is about 25 kJ. The bond energy of a hydrophobic bond is very small, usually in the range of 1—6 kJ per bond, but in this case $T\Delta S$ amounts to 2—14 kJ per bond, depending on the nature of the hydrophobic group[13].

It is sure that upon adsorption of HSA on latex alterations in the extents of both of these types of binding occur. The adsorbent offers both hydrophilic (—OSO_3^-) and hydrophobic (exposed parts of the polystyrene chain) attachment sites. As in native HSA the hydrophobic parts of the molecule are more to the inside, unfolding must occur if hydrophobic bonds with the latex surface are formed. Whether this will happen — and to what extent — depends on the contribution of the other forces. In view of the decreasing coherence of the HSA molecule with increasing distance from the i. e. p., it may be expected that then also unfolding becomes more likely.

The insight concerning the size and shape of the serum albumin molecule is apparently not yet settled, although many authors think that several physical quantities (light scattering, sedimentation, diffusion, low-angle X-ray scattering) are satisfactorily accounted for by assuming the molecule to be an ellipsoid of revolution. The actual values presented for the axial lengths depend within certain limits on the author and also on the method of measurement. Examples: Riddiford *et al.* find major and minor axes of resp. 11.6 and 2.7 nm by small angle X-ray diffraction[14] and Squire *et al.* give for the axes values of 14 and 4 nm from sedimentation and diffusion[15]. Low rather thinks in terms of a

prism with axes of about 14.5, 5 and 2.2 nm[16]. Methodical discrepancies, such as the lower value found from X-ray analysis as compared with those obtained kinetically can perhaps be traced to the either or not inclusion of hydration water. The discrepancies between different authors seem for the time being greater than the size variation with pH; most noteworthy, the lowest value quoted (11.6, 2.7 and 2.7 nm) applies to pH 3.6, where the molecule must be expanded.

Adsorption studies, if interpreted against the known dimensions of the free molecule in solution can be helpful in deciding whether or not a molecule unfolds. If for example an adsorbed amount would be found corresponding to a thickness far below the length of the smallest axis, the molecule could well be unfold in its adsorbed state. However, the possibility that it does not unfold but does not occupy the entire surface area should not a priori be excluded.

As a consequence of the subtle interplay and force balances in biopolymers a great variety of physiological functions becomes possible.

For serum albumin in particular holds that the molecule adapts itself readily to slight alterations in conditions. It is, so to say a »nervous molecule«. This may be related to its many biological functions (*e. g.* transport of metal ions, fatty acids, steroids, hormones, regulation of pH and osmotic pressure, source of amino acids). Brought at interfaces, an intricate sum-effect of many simultaneous processes is anticipated and, in fact, also found.

EXPERIMENTAL

Latex: — The preparation of the latex has been described[18 17]. No emulsifier has been used, so that no interference of surfactants with the HSA molecule occurs. The latices are homodisperse with $M_W/M_n < 1.02$. The radii are between 250 and 300 nm for the various samples. The surface charge σ_0 consists entirely of $-OSO_3^-$ groups and ranges between -1 and -8 $\mu C/cm^2$ (after ion exchange).

Albumin. — Electrophoretically pure HSA was obtained from the Sigma Chemical Company and dialysed against pure water prior to use. Native SA contains 1—2 moles of fatty acid per mole[19], which has a structure stabilizing function. No attempts have been made to remove these because we wanted to approach the properties of the native molecule closely.

Adsorption measurements have been described elsewhere[20]. It be recalled that no buffers have been used, latex and albumin were brought to the same pH and KNO_3 concentrations before adding them together. In some other experiments we found that buffers exert a definite influence on the HSA adsorption which is probably attributable to their competitive accumulation at the interface. Sometimes a variation of pH was observed upon mixing HSA and latex.

Microcalorimetry. — The adsorption enthalpy h_{ads} has been measured using an LKB 10700-2 batch microcalorimeter. Before mixing HSA and latex these colloids were brought to equilibrium by dialyzing both against the same equilibrium solution. It is necessary to correct for the heat of dilution of the HSA and the latex. The obtained enthalpies were found to be proportional to the total latex surface, which shows that an interfacial effect is measured and not a bulk effect.

Platform Adsorption

If the adsorbed amount is plotted as a function of the concentration of the HSA remaining in bulk an adsorption isotherm is obtained with a platform. This, by the way, is already a point of difference with synthetic macromolecules. The bulk concentration at which this platform is reached depends

somewhat on the conditions (σ_0, pH, T, c_{salt}) but is usually below 0.5 g/dm^3, which is far below physiological concentrations (several tens of g/dm^3, depending on the genus). Fig. 1 exemplifies the influence of pH and σ_0 (*i. e.* the influence of the charges on albumin and latex) on the platform adsorption.

A graph is obtained that is more or less symmetrical with respect to the i. e. p. The more negative the latex, the more adsorbs, even if pH $>$ i. e. p. where both colloids are negatively charged. This last point is surprising and indicates that it is not the over-all electrostatic interaction between latex and substrate that determines the adsorption. The symmetry with respect to the i. e. p., on the other hand, shows that the adsorbed amount is determined by the charge on the protein, either directly or indirectly. A possible indirect charge effect could be that the resistance against unfolding decreases with increasing distance from the i. e. p. (p 71, point *b*). The maximum at the i. e. p. would then be explainable as due to HSA adsorption more or less in its native state, whereas the progressively lower adsorption with increasing distance from the i. e. p. would indicate progressive unfolding. This constitutes a first working hypothesis, in agreement with the general features observed for HSA, notably with its marked conformational adaptability. An alternative explanation would be that the HSA molecule retains its bulk shape and size, but becomes less densily

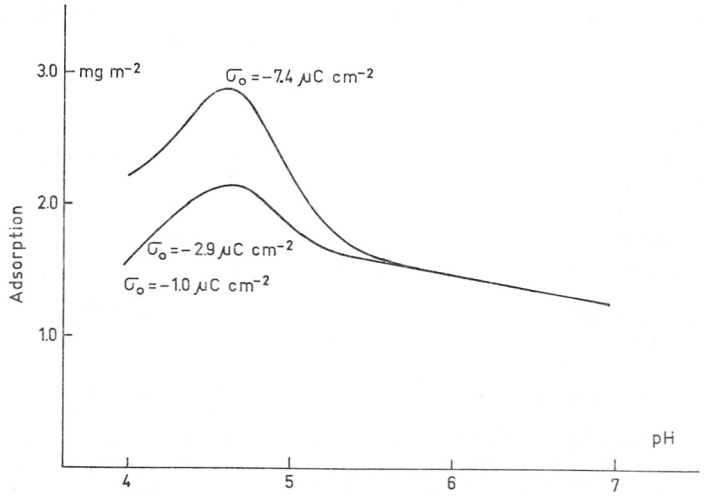


Fig. 1. Adsorption of HSA on polystyrene latices. Platform values. Electrolyte: 10^{-2} M KNO_3, T = 22 °C.

packed if the pH is further from the i. e. p. because of intermolecular electrostatic repulsion between adsorbed molecules. A third possibility is that the molecule is the more swollen the further away one is from the i. e. p. and therefore gives rise to a more tenous adsorbed layer. In the fourth place one could consider the possibility that around the i. e. p. the molecule adsorbs end-on, but undergoes an increasing extent of tilting with increasing distance from the i. e. p.

In order to decide which of these four hypotheses is the most likely (accepting the possibility that still another explanation could be put forward) further analysis is clearly wanted.

The next obvious step is to compare the adsorbed amount with the amount that would follow from adsorption of native molecules. Fig. 2 depicts this

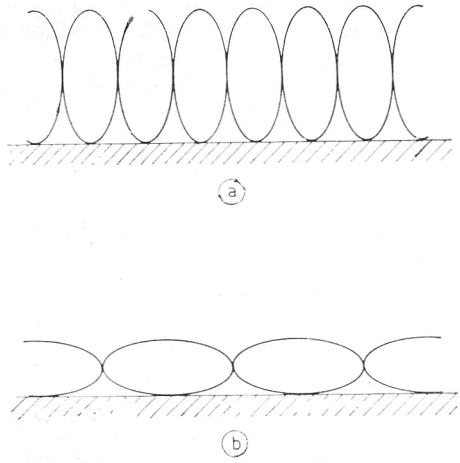


Fig. 2. Adsorption of native HSA molecules. (a) end-on (b) side-on

schematically. Note in the first place that in situation (a) the contact between the molecule and the substrate is considerably less than in situation (b). If we accept the extension of this contact as a measure for the affinity, we arrive at this paradoxal conclusion: the higher the affinity, the lesser the adsorbed amount in the platform.

Taking the same density ($\varrho = 0.74$ gm/cm^3) for HSA in its adsorbed and bulk state and a layer thickness d of 13.5 or 3.5 nm (representative for the situations of Figs. 2 (a) and (b) respectively), an adsorbed amount of 2.4 and 10.0 mg/m^2 is calculated respectively. Even if we allow for an uncertainty of 10—20% in this calculation because of the uncertainty in the values chosen for ϱ and d it is immediately evident that an upright orientation of adsorbed HSA molecules must be rejected because it is at variance with experiment (except if we could accept large gaps between the adsorbed molecules, which is irrealistic). By consequence, the fourth alternative, forwarded above must be discarded. On the other hand the adsorption of a complete monolayer of side-on native molecules in the i. e. p., as in Fig. 2 (b) could well explain our data, especially at high σ_0. The decrease of the adsorbed amount with increasing distance from the i. e. p. must then be accounted for by one of the three mechanisms forwarded above.

It is easily verified that the amount adsorbed in a spread flat monolayer with all the amino acids in contact with the adsorbent is much too low to account for our experiments. (If for example 0.3 nm^2 per residue is taken, which seems a minimum value, one arrives at only about 0.6 mg/m^2). For the further discussions this possibility can be ignored.

Another quantitative argument pleads strongly against the third alternative proposed above. The decrease in adsorbed amount in changing pH from 4.8 to 7 far exceeds the decrease that would be found if caused by swelling

of the bulk molecule, as judged from the relatively insignificant increase of intrinsic viscosity [η] over this pH range[21].

This means that we are left with the first and second alternative: adsorption more or less in its native state around the i. e. p. and either increased unfolding or greater intermolecular distances the further the pH differs from the i. .e. p. We may note that, since the adsorbed amount in the i. e. p. depends on σ_0, it is not probable that the HSA molecule adsorbs in exactly its bulk conformation. Deželić *et al.*[22,23] noted that upon adsorption the physiological activity remains unaffected, also outside the i. e. p. From this it is difficult to deduce to what extent conformational changes occur.

The influence of the surface charge σ_0 of the latex on the adsorption is another interesting variable. This influence is absent as long as σ_0 remains below about 3 μC/cm². In explaining this it is instructive to look at the detailed configuration of the charges. It has been stated that even in the isoelectric HSA molecule some 50 positive and an equal number of negative groups are present, that is 100 unit charges in total. Let the adsorbed molecule be 3 nm thick and let us assume that 0.5 nm of this is so close to the latex surface that its influence is directly felt (the »Stern-layer«). This layer would contain 100/6 unit charges on an area of about 15 × 3 nm, corresponding to *ca.* 13 μC/cm², composed of 3.25 μC/cm² positive and 3.25 μC/cm² negative charge. In comparison with this, it is observed that a surface charge of $\leq$ 3 μC/cm² is too low to be really effective. Latices with such low surface charges behave as if they are virtually uncharged. Only if σ_0 becomes higher than about 5 μC/cm², the charge density on the latex can compete with the volume charge density of the protein and appreciably affect its adsorption.

The argument given is not better than semiquantitative. We do not know, for example whether the charge in the HSA molecule is evenly distributed. Perhaps, continuing this line of thought it is imaginable that local excesses of either positive or negative charge inside the protein molecule turn themselves to the latex surface, the more so if this charge is higher. This would then constitute one of the driving forces for unfolding. It is inherent to this picture that it is not so much the total charge on the HSA molecule that counts, as well as how it is distributed, — or, for that matter, how easily it can redistribute itself —.

Some support for this picture is derived from dielectric studies with HSA[24], from which it has been deduced that the HSA molecule has a net dipole moment in the direction of its short axis. This dipole moment increases with increasing pH from 4.8—7. Thus local electric excesses inside the HSA molecule could be one of the factors responsible for adsorption even if the over-all electrostatic interaction between adsorbent and adsorbate is repulsive.

Comparison with Other Results

In view of the conclusion that over-all electrostatic interaction between HSA and PS plays no major role with respect to the adsorbed amount, whereas the intra- or intermolecular forces of the HSA are relatively important, it could be expected that the adsorption of HSA is not so strongly dependent on the detailed nature of the sorbent, and hence would on other substrates exhibit similar features as a function of pH as found by us. It is therefore worthwhile to have a look at work by other authors, working with comparable systems.

Although there is a fair amount of literature available on biopolymer adsorption, examples that are close enough to warrant mutual comparison are limited. The relatively large amount of work on spread monolayers and that done in connection with blood clotting concern in general too different systems. (For a recent review see ref. 25). That the adsorption of HSA and comparable proteins is maximal in or close to the i. e. p. has been found by a number of other authors. McLaren[26] concludes this for pepsin, ovalbumin, trypsin, lactoglobulin and other proteins on kaolinite, Bull[27] for BSA on pyrex glass, Reed and Rosal[28] for HSA on glass, Mac Ritchie[29] for BSA on two types of Aerosil, Hummel and Anderson[30] for ribonuclease on glass and Curme and Natale[31] for gelatin on AgBr. This last result seems to be confirmed by ellipsometric measurement of the (effective) adsorbed layer thickness[32]. One should realize that exact coincidence of the adsorption with the bulk-i. e. p. must not be expected because this i. e. p. can be somewhat different in the adsorbed state. There are also literature examples where the above rule is not followed, or at least where it is obscured by other factors. For example, Borisova *et al.*[33] observe for serum albumin on macroporous SiO_2-gels no substantial decrease from pH 4.8 to 6.98, although there is a definite decrease from pH 4.8 to 3.4. The porosity of the sample, in conjunction with the penetrability of the albumin molecule seems to be an important factor in this case. In experiments by van Oss and Singer[34] no pH-effect was found for globulins on latices, although the pH did exert an effect on the HSA-adsorption.

It seems safe to state that maximal adsorption close to the i. e. p. is the rule rather than the exception. The fact that this trend is independent of the nature of the adsorbent, supports the idea that the decrease of the adsorbed amount with increasing distance from the i. e. p. is due to a property of the HSA molecule only, *e. g.* its internal coherence and/or its total charge. In this connection experiments by Ishii and Muramatsu[35] may be recalled, who investigated the ageing of spread layers of ovalbumin at the air-water interface. They found no time-effect at the i. e. p. and an increase of the surface pressure with time for pH $\neq$ i. e. p. It is not sure to what extent these results may be compared with ours, but they point again to minimal conformational adaptibility around the i. e. p., even in relatively thin layers.

A comparison of the adsorbed amounts between different authors and different conditions reveals a certain spread. This may be anticipated since it is dependent on many factors [buffers, electrolytes, pH, surface charge (see Fig. 1)]. However, it seems that most results that have been obtained with systems comparable to ours also lead to adsorptions of the same order of magnitude (1.5—3 mg/m^2). The recent work by Deželić *et al.*[23] for HSA on PS latex at pH 8 in a trisbuffer yields results that are about 30% lower than ours, which may be attributable to their using a buffer. Ellipsometric measurements generally tend to give outcomes that are somewhat higher but still of the same order of magnitude[36]. Very low adsorptions ($\leq$ 0.6 mg/m^2) indicative of complete spreading are seldom or never found. On the other hand, in incidental cases very high adsorptions are observed, for example on hydrophobic Aerosil due to interfacial coagulation[29]. The rule is apparently that HSA molecules adsorb side-on.

Our tentative conclusion that the charge distribution over the protein molecule is important for its adsorption behaviour is supported by measurements by Messing[37] on the initial rates of adsorption of RNAse, cytochrome C, pepsin

and other proteins on porous glass as a function of the amount of positive charges in the molecule.

The conclusion of this review is that our results are in general agreement with the trends observed by other workers. All of this is indicative of the similarity in the factors responsible for adsorption on different substrates and points to a common cause in the HSA molecule itself.

Adsorption Isotherms

One way to analyze further the factors responsible for adsorption is to look at the shapes of the adsorption isotherms. If an isotherm is well described by a certain isotherm equation it would seem a logical step to interpret the adsorption data in terms of the parameters of the theory underlying this equation.

However, such an approach is not without danger because many experimental isotherms seem to fit a given equation although the premises of this equation are clearly not met. Interpretation then conveys a false sense of understanding. Most notably this reserve applies to the indiscriminative use of the Langmuir equation. The basic assumptions are: reversible physical adsorption on localized sites without lateral interaction. It follows from the discussion (see p. 70—73) that, except, for the fact that the adsorption is mostly physical, none of the premises is usually realized. To this it may be added that not an individual but a composite isotherm is measured. It is fortuitous that the most general shape of biopolymer adsorption isotherms (initial steep rise followed by leveling off to a platform) resembles the isotherm of the Langmuir equation. It is also without further physical consequence that these experimental isotherms can be linearized to one or two straight lines. We feel therefore that any reference to the Langmuir theory would lead us on the wrong track. A similar reserve applies to the Scatchard plot, which is phenomenologically identical to a Langmuir plot, although in this case some rationale can be derived from the adsorption reversibility per segment. In fact, since no adequate biopolymer adsorption isotherm equation is available and since the application of the adsorption theory for random homopolymers[2] is unwarranted we can only make some qualitative observations.

Fig. 3 gives some of our results. (It is our intention to report more extensively on these aspects in a forthcoming paper[38]). It is noted that, depending on pH and σ_0, drastic differences in these curves occur. Sometimes the curve is smooth, in other cases there is a distinct step. All isotherms approach a platform value. Below, the following properties of these isotherms are successively discussed: 1) the initial slopes, 2) the final slopes, 3) the overall shapes. The platform values have already been extensively described earlier in this paper.

The initial slopes, *i. e.* the slopes at very low adsorption respond most unambiguously to the albumin-PS affinity, because under these conditions any limitation of the adsorbed amount due to packing or crowding on the surface is absent. In other words, the lateral interaction plays no role. For a detailed discussion we refer to ref. (38), but at this instance we report already that the initial slopes are of the Henry type (see Introduction) and reflect very well the over-all electrostatic interactions in that the curves are the steeper, the stronger the charge contrast is between HSA and latex. Another feature is that both for pH $<$ i. e. p. and pH $>$ i. e. p. the adsorption increases with T,

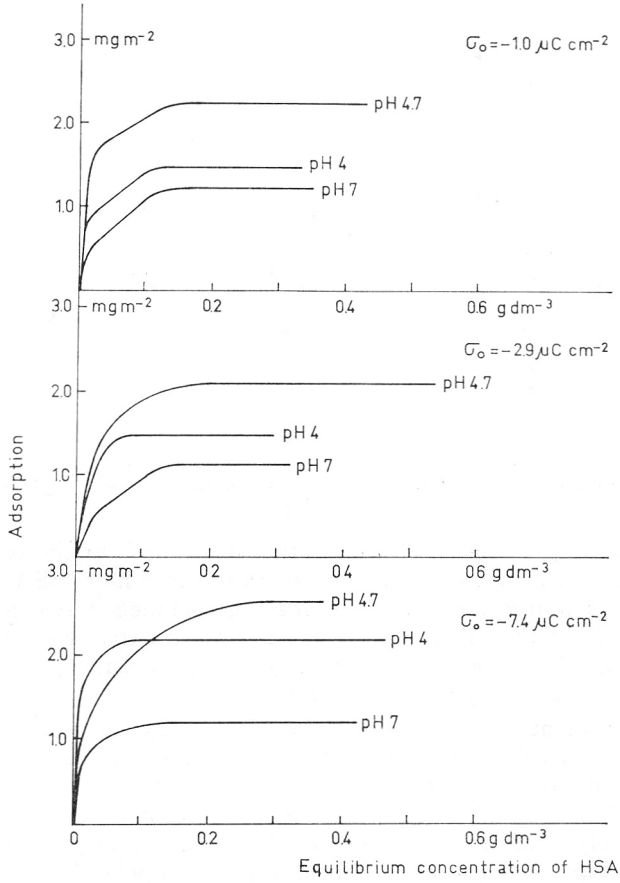


Fig. 3. Examples of adsorption isotherms of HSA on polystyrene latices. Influence of pH and surface charge. Electrolyte: 10^{-2} M KNO_3, T = 22 °C.

indicating a substantial entropic contribution to the adsorption Gibbs free energy. The temperature-influence is absent in the isoelectric point. This athermal behaviour points to a differing adsorption mechanism in the i. e. p.

The final slopes, *i. e.* the slopes of the isotherms close to saturation reflect an entirely different aspect, namely the energetics of finding an available site for a newly arriving molecule on a surface that is already largely covered. In other words, it is now almost exclusively the lateral interaction that counts. By way of example we have plotted in Fig. 4 the concentration at which 90% of the platform adsorption has been attained. This quantity can serve as a measure for the lateral interaction of HSA molecules in the adsorbed state. A continuously increasing value is found (the »baseline«) superimposed on which a maximum occurs around the i. e. p. The course of the baseline indicates that it cannot be the net electrostatic repulsion between the adsorbed HSA molecules that accounts for the lateral repulsion, since in that case a pseudo-parabola with its apex in the i. e. p. ought to be found. It must be something else, that is apparently related to the extent of interaction with the substrate.

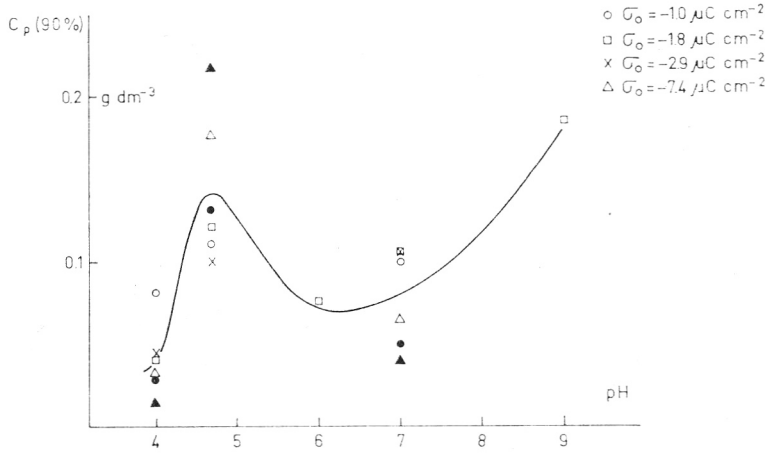


Fig. 4. Adsorption of HSA on polystyrene latices. Bulk concentration at which 90% of the platform adsorption has been reached. Electrolyte: 10^{-2} M KNO_3 (closed symbols 5 × 10^{-2} M KNO_3), T = 22 °C.

This argument pleads against one of the working hypotheses forwarded in Section *Platform Adsorption* viz. the hypothesis in which HSA was postulated to adsorb in its native state with increasing distance between the molecules when the pH is further from the i. e. p. It appears that we are left with the alternative that the decreasing adsorption with increasing distance from the i. e. p. (Fig. 1) is due to progressive unfolding.

Other arguments support this view. The very fact that the isoelectric region in Fig. 4 exhibits typical properties is also indicative for a different mechanism. Note that c_p (90%) is very high at the i. e. p., suggesting strong lateral repulsion where the molecules as a whole are uncharged. In our view it is more likely that it is the way in which the charge is distributed over the HSA molecule that counts. At the i. e. p. the dipoles in the adsorbed HSA molecules are parallel and hence repel each other strongly. When the molecules are flatter due to unfolding, the sites of the dipoles become further apart and this repulsion is reduced. Further it be recalled that also the initial slopes indicated an adsorption mechanism differing between the i. e. p. and outside this point. It underlines that the mode of adsorption depends on pH and pleads against a mechanism by which HSA molecules adsorb in the same conformation at any pH.

Bearing in mind that the factors responsible for adsorption are different between the initial and final ranges of the isotherms it is not surprising that transitions can occur in the range of intermediate adsorptions. These transitions are probably responsible for the inflection points or steps that are sometimes observed (Fig. 3). In this connection we note that Chattoraj and Bull[39] have observed irregularities in the electrophoretic behaviour of HSA and ovalbumin on glass, nujol and paraffin wax. One of these irregularities coincided with steps in the adsorption isotherm and occurred around similar c_p 's as observed by us (Fig. 3). These authors also forwarded an explanation based on structural rearrangements. For our system electrophoretic measurements are not yet available and we would rather postpone a detailed discussion[38]. Still it can be noted that also the isoelectric isotherms display a discontinuity. From this it

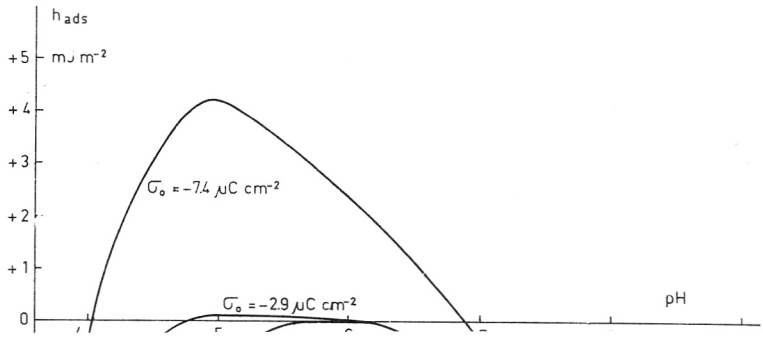
is concluded that either at low or at high adsorption at the i. e. p. (or at both) the adsorbed HSA molecule is not exactly in its native state. Finally we recall that all isotherms do attain a platform. In this respect they differ clearly from synthetic macromolecules whereby the adsorbed amount keeps increasing with c_p. Such an increase is only possible in the absence of intramolecular attractions. It constitutes a pricipal point of distinction between biopolymers and random synthetic homopolymers.

The conclusion is that the isotherms contain several interesting features, many of them being tentatively understood. Interpretation along the lines of a Langmuir plot would obscure several subtleties and hence must be regarded as too crude an approach.

Adsorption Enthalpies

Directly measured adsorption enthalpies constitute another piece of information that is helpful in unraveling the adsorption mechanism. The microcalorimetry of synthetic macromolecule adsorption is known in literature[40], but its application to biopolymers is novel as far as we are aware. Preliminary accounts of our experiments have been published[20,41].

Fig. 5 gives our results for the platform adsorption under the same conditions as those of Fig 1. A set of curves, each with a maximum around the i. e. p. or to the right side of it is obtained. (Note that $h_{ads} > 0$ means that the process is endothermal). The over-all shapes of these curves do certainly not reflect the overall electrostatic interaction between adsorbate and substrate. In fact, after all that has been said above, this could not be anticipated.



In discussing what the curves do mean it is good to state at the very onset that h_{ads} is the compounded enthalpy change due to all the conformational alterations the HSA molecule undergoes upon adsorption, such as breaking and/or recombination of hydrogen bonds, breaking and/or reformation of hydrophobic bonds, increasing dissociation and/or association, changes in dispersion forces. The adsorption enthalpy of protein trains coming in actual touch with the latex surface is one of these many items. Since neither in bulk nor in its adsorbed state the detailed conformation of HSA is known it is impossible to predict from an *a priori* reasoning how h_{ads} is composed. The best thing that can be done is to work the other way round and try to find what kind of factors could lead to the obtained results. In doing so indirect information on the conformation in the adsorbed state and on the forces responsible for adsorption can be inferred.

In the preceding sections we arrived at the idea that local charge excesses in the HSA molecule would play a role in the sorption process. This idea is not incompatible with h_{ads} becoming more exothermal at high and at low pH (Fig. 5), reasoning that at those places more unfolding takes place so that more positive groups can come close to the surface. Superimposed on this are the (apparently weaker) enthalpies of the electrostatic over-all repulsion at pH 7 and attraction at pH 4 and the enthalpy effect of the variation in the dissociation state of the albumin due to the changed interionic distances.

Since electrolytes reduce both the attractive and repulsive electrostatic contributions to h_{ads}, their influence (not shown in Fig. 5) is also the sum-effect of at least three sub-contributions. The general trend is to make h_{ads} more exothermic. Apparently the loosening of internal bounds with subsequent increase of electrostatic attraction with the latex is the leading term. In view of the other contributions it is not surprising that their quantitative influence depends on σ_0 and pH[38].

Another striking feature of Fig. 5 is that $h_{ads} > 0$ in the isoelectric region, the more so when σ_0 becomes more negative. As the adsorption process is spontaneous this must mean that $s_{ads} > 0$ over this region and perhaps also beyond it. Again, several factors can be imagined that would contribute to s_{ads}. We are not yet in a position to unravel s_{ads} into its constituent parts, especially so since the influence of temperature is ambiguous. The process is apparently very complex. However, it may be recalled that from the temperature dependence of the initial slopes also an entropy gain was found to be the driving force, at least at pH 4 and 7 and perhaps also in the isoelectric point. Combining these two findings, obtained under quite different conditions and by differing means it seems safe to conclude that the entropy gain plays an important role in the sorption process.

REFERENCES

1. J. J. Kipling, *Adsorption from Solution of Non-Electrotyles,* Academic Press, London-New York, 1965.
2. C. A. J. Hoeve, *J. Polymer Sci.* (C) 34 (1971) 1.
3. F. Th. Hesselink, *J. Phys. Chem.* **73** (1969) 3488.
4. F. Th. Hesselink, *J. Electroanal. Chem.* **37** (1972) 317.
5. R. A. Phelps and F. W. Putman in *The Plasma Proteins,* Vol. I, F. W. Putman (Editor), Academic Press, New York (1960) p. 143.
6. C. Tanford, S. A. Swanson, and W. S. Shore, *J. Am. Chem. Soc.* **77** (1955) 6414.

7. H. E. Schultze, N. Heimburger, and G. Frank, *Biochem. Z.* **336** (1962) 338.
8. P. F. Spahr and J. T. Edsall, *J. Biol. Chem.* **239** (1964) 850.
9. W. G. M. Braam, *Thesis*, University of Nijmegen, 1972.
10. M. Sogami, H. A. Petersen, and J. F. Foster, *Biochemistry* **8** (1969) 49.
11. G. A. J. van Os, S. H. de Bruin, and L. H. M. Janssen, *J. Electroanal. Chem.* **37** (1972) 303.
12. J. F. Foster in *The Plasma Proteins* Vol. I, F. W. Putnam (Editor), Academic Press, New York, 1960, p. 179.
13. H. A. Scheraga, G. Némethy, and J. Z. Steinberg, *J. Biol. Chem.* **237** (1962) 2506.
14. C. L. Riddiford and B. R. Jennings, *Biochim. Biophys. Acta* **126** (1966) 71.
15. P. G. Squire, P. Moser, and C. T. O'Konski, *Biochem. J.* **7** (1968) 4261.
16. B. W. Low, *J. Am. Chem. Soc.* **74** (1952) 4830.
17. A. Kotera, K. Furusawa, and Y. Takeda, *Kolloid-Z. Z. Polymere* **239** (1970) 677.
18. K. Furasawa, W. Norde, and J. Lyklema, *Kolloid-Z. Z. Polymere* **250** (1972) 908.
19. M. Rosseneu-Motreff, V. Blaton, B. Declerq, D. Vandamme, and H. Peeters, *J. Biol. Chem.* **68** (1970) 369.
20. W. Norde, K. Furusawa, and J. Lyklema, *Proc. 6th Int. Congress Surface Active Substances*, Zürich, 1972, in press.
21. C. Tanford, J. G. Buzzell, D. G. Rands, and S. A. Swanson, *J. Am. Chem. Soc.* **77** (1955) 6421.
22. Gj. Deželić, N. Deželić, H. Muić, and B. Pende, *Eur. J. Biochem.* **20** (1971) 553.
23. Gj. Deželić, N. Deželić, and Z. Telišman, *Eur. J. Biochem.* **23** (1971) 575.
24. A. G. M. van der Drift, private communication, 1972.
25. J. L. Brash and D. J. Lyman in *The Chemistry of Biosurfaces*, M. L. Hair (Editor), Marcel Dekker 1971, Ch. 5.
26. A. D. McLaren, *J. Phys. Chem.* **58** (1954) 128.
27. H. B. Bull, *Biochim. Biophys. Acta* **19** (1956) 464.
28. G. W. Reed and R. E. Rossall, *Radioisotopes Sci. Res.*, Proc. Int. Conf. Paris, 1957, Vol. 2, 502; *Chem. Abstr.* **54** (1958) 655 c.
29. F. MacRitchie, *J. Colloid Interface Sci.* **38** (1972) 484.
30. J. P. Hummel and B. S. Anderson, *Arch. Biochem. Biophys.* **112** (1965) 443.
31. H. G. Curme and C. C. Natale, *J. Phys. Chem.* **68** (1964) 3009.
32. T. Maternaghan, private communication, 1972.
33. M. A. Borisova, L. I. Nekrasov, N. I. Kobozev, A. V. Kiselev, and Yu. S. Nikitin, *Russ. J. Phys. Chem.* **49** (1969) 291.
34. C. J. van Oss and J. M. Singer, *J. Reticuloendoth. Soc.* **3** (1966) 29.
35. T. Ishii and M. Muramatsu, *Bull. Chem. Soc. Japan* **43** (1970) 2364.
36. R. Stromberg, private communication, 1972.
37. R. A. Messing, *J. Am. Chem. Soc.* **91** (1969) 2370.
38. W. Norde and J. Lyklema, to be published.
39. D. K. Chattoraj and H. B. Bull, *J. Am. Chem. Soc* **81** (1959) 5128.
40. E. Killman and R. Eckart, *Makromol. Chem.* **144** (1971) 45.
41. W. Norde and J. Lyklema, *XX. Colloq. Protides of the Biological Fluids*, Brugge, 1972, Proc. Pergamon Press, Oxford, in press.

IZVOD

Adsorpcija biopolimera s posebnim osvrtom na serum albumin-polistiren lateks sistem

J. Lyklema i W. Norde

Proučavana je adsorpcija ljudskog serum albumina na negativno nabijenim lateksima polistirena (slobodnim od emulsifikatora). Adsorpcija je mjerena direktno analitički i praćena mikrokalorimetrijski u gradijentu pH, koncentracije soli, koncentracije albumina, te površinskog naboja lateksa.

Iako adsorpcijske izoterme imaju plato, nisu Langmuirovog tipa, i ta je teorija adsorpcije neadekvatna za interpretaciju rezultata. Adsorpcija je u početnom dijelu

uvjetovana isključivo afinitetom između albumina i lateksa, bez utjecaja lateralne interakcije, a sa znatnim entropijskim doprinosom slobodnoj energiji adsorpcije. U konačnom dijelu adsorpcijske izoterme prevladava lateralna interakcija. U izoelektričkom području, pH između 4,5 i 5,5, postoji maksimum adsorpcije, a entalpija adsorpcije je pozitivna. Izvan izoelektričkog područja, ovisno o naboju lateksa, entalpija adsorpcije je negativna. Utvrđeno je, da je u svim područjima pH i koncentracije, znatan entropijski doprinos energiji adsorpcije, ali je utjecaj elektrostatskih interakcija između negativne površine lateksa i suviška pozitivnih naboja u unutrašnjosti molekule albumina također značajan.

LABORATORY FOR PHYSICAL AND COLLOID CHEMISTRY
AGRICULTURAL UNIVERSITY
WAGENINGEN, THE NETHERLANDS

Primljeno 13. studenoga 1972.