ADSORPTION OF BOVINE SERUM ALBUMIN ON GLASS

by

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The adsorption of proteins from solution on solid surfaces has long been of interest and a large but scattered literature on the subject is available[4]. This work has, in general, suffered from poor information regarding the surface area of the solid.

The present paper describes the adsorption of bovine serum albumin on powdered pyrex glass and the calibration of the surface area of the powder by use of microscopic glass spheres. Knowing the area of the pyrex powder, it is possible to estimate the limiting areas occupied by the bovine serum albumin at the water-glass interface as well as to calculate the minimum affinity of the glass for the protein and to draw force-area curves for the adsorbed monolayers of protein.

EXPERIMENTAL

The crystalline bovine serum albumin was obtained from Armour and Company. It was dissolved in water and dialyzed against distilled water in washed Visking sausage casing. The concentration of the mother solution was determined by drying an aliquot of the solution to constant weight in a vacuum oven at 105° C.

Broken pyrex laboratory glassware was powdered in a ball mill and the smallest and largest particles removed by differential sedimentation. The remaining powder of intermediate sizes was treated with aqua regia over night and exhaustively washed with water, dried and stored.

Sodium acetate buffers at an ionic strength of 0.05 were used except in the extreme acid range where hydrochloric acid was employed to give the desired pH and sodium chloride added to yield an ionic strength of 0.05. All adsorptions were conducted at 30° C.

The microscopic glass spheres used for the calibration of the pyrex powder were obtained from La Pine Company of Chicago and described as 0 to 40 microns in diameter. They were passed through a 325 sieve and the powder passing the sieve was treated with aqua regia over night, exhaustively washed with dilute hydrochloric acid, dried and stored. The diameters of 500 of the spheres selected at random were measured with a filar micrometer attached to a microscope. The ratio of three times the sum of the radii squared to the sum of the radii cubed gave the ratio of the surface area to volume as $2.48 \cdot 10^3$ and since the density of the glass was 2.24, the surface area of the spherical glass particles was 0.111 sq. meters per gram of glass.

The surface area of the pyrex powder was estimated by comparing the amount of protein adsorbed by a gram of pyrex glass to that adsorbed by a gram of spherical glass particles under the same experimental conditions. Since the spherical glass particles are not made of pyrex glass, it was necessary to show that the protein adsorbing character of these particles resemble that of pyrex glass. It was found, for example, that the pH-adsorption curves for the protein on the two glasses resemble each other closely. It was observed, however, that at a given pH the initial slopes of the adsorption-protein concentration curves for the pyrex powder were greater than those for the corresponding curves for the spherical particles. The ratios of the amount of protein adsorbed per gram of pyrex glass to that adsorbed per gram of spherical particles were measured as a function of protein concentration at pH 4.96, at pH 4.66 and at pH 3.95. These ratios at the same protein concentrations agreed with each other so closely for the three selected pH values that average values for the ratios at given protein concentrations are shown plotted in Fig. 1 against the protein concentration. As can be seen in Fig. 1, the limiting ratio of the amount of protein adsorbed per gram of glass for the pyrex glass to the spherical glass

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particles was 3.02 and, accordingly, the surface area of the pyrex powder was estimated to be 0.335 sq. meters per gram.

A typical adsorption experiment was conducted by adding 3 g of pyrex powder to a 15 ml centrifuge tube followed by the addition of 10 ml of protein-buffer solution and the suspension of glass stirred continuously for 30 minutes at 30° C with a beaded stirring rod. The suspension was then centrifuged and the optical density of the supernatant solution measured at 278 mμ and at 320 mμ after appropiate dilution.

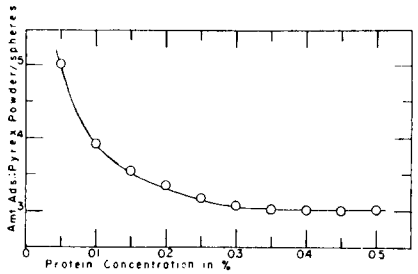

Fig. 1. Average ratio of the amount of protein adsorbed per gram of pyrex powder to that adsorbed by a gram of spherical glass particles as a function of protein concentration in per cent. The ratios for pH 4.96, pH 4.66 and pH 3.95 have been averaged.

It was never possible to remove the suspended glass completely from the protein solution by centrifugation and a glass-correction had to be applied in the estimation of the protein concentration from optical measurements. The ratio of the optical density of the bovine serum albumin at 278 mμ to that at 320 mμ was 62.72. The ratio of the optical density of the glass suspension at 278 mμ to that at 320 mμ was 2.1. It was then possible to obtain the protein concentration by the solution of simultaneous optical density equations.

The rate at which the protein is adsorbed by the glass is very fast. At protein concentrations below about 0.1 % there is evidence that after a rapid uptake of protein there is a slow and gradual uptake of additional protein. Table I shows the influence of time on the extent of adsorption.

TABLE I

MILLIGRAMS OF PROTEIN ADSORBED PER GRAM OF PYREX GLASS POWDER AT pH 5.05 AND AT VARIOUS TIMES

The initial protein concentrations are indicated.

0.037% *protein*		0.185% *protein*	
Time adsorption minutes	*mg protein adsorbed per gram of glass*	*Time adsorption minutes*	*mg protein adsorbed per gram of glass*
5	0.78	5	1.23
15	0.81	10	1.21
30	0.83	15	1.19
		20	1.22
		30	1.21
		45	1.22

Examination of the adsorption data from 0.185 % protein gives an idea of the variability of the experimental data.

RESULTS AND DISCUSSION

Fig. 2 shows a plot of the milligrams of protein adsorbed per sq. meter of pyrex glass surface at pH 4.66 as a function of the protein concentration.

The adsorption isotherms at other pH-values resemble in a general way that shown in Fig. 2. In the more acid range the initial slope of the adsorption-concentration curves becomes progressively larger whereas at the higher pH-values the initial slopes decrease with increasing pH. A plot of the initial slope of the adsorption-concentration curves is shown in Fig. 3.

The increase of the initial slope of the adsorption curves with decreasing pH probably results from the interaction of the electrostatic charges on the protein and on the glass. The isoelectric point of bovine serum albumin in sodium acetate buffer of

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ionic strength is about at pH 4.75 whereas glass is negatively charged throughout the pH-range studied. We are thus in agreement with McLAREN[3] regarding the importance of electrostatic interaction in the adsorption of protein on solid surfaces.

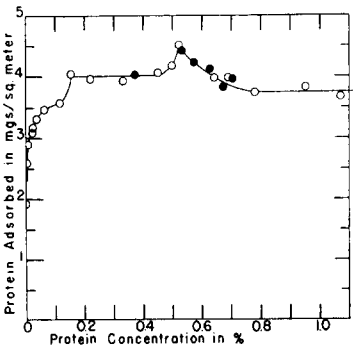

Fig. 2. Plot of milligrams of protein adsorbed per square meter of pyrex glass at pH 4.66 as a function of the protein concentration expressed in per cent. Closed circles indicate adsorption from solution of glass initially exposed to 0.416% protein.

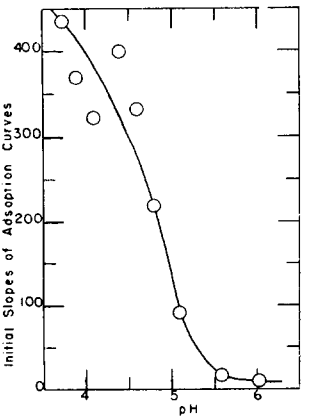

Fig. 3. Initial slopes of the adsorption-concentration curves expressed in mg protein adsorbed per gram of protein in 100 ml of solution per gram of glass as a function of pH.

It can be seen, however, from results at higher protein concentrations the electrostatic factor becomes less important as the protein concentration is increased.

The variation of the amount of protein adsorbed at selected equilibrium protein concentrations as a function of pH is shown in Fig. 4. The amounts of protein adsorbed were interpolated from the adsorption isotherms at the various pH-values.

Examination of Figs. 3 and 4 reveals that the adsorption isotherms above pH 4 show plateau regions between 3.5 and 4.0 mg protein adsorbed per sq. meter of glass. Below pH 4 the plateau region is reached at lower protein concentrations and the level of the plateau is progressively lower as the pH is decreased below pH 4.

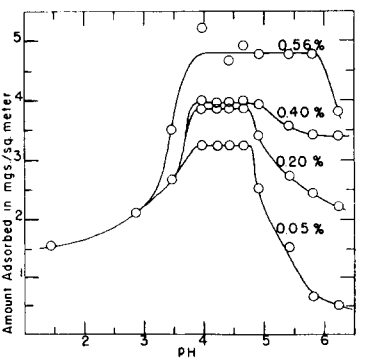

Fig. 4. Protein adsorption in mg per sq. meter of pyrex glass as a function of pH at 0.05%, 0.20%, 0.40% and 0.56% protein concentrations.

A remarkable feature of the adsorption isotherms above pH 4 is a discontinuity observed between protein concentrations of 0.5% to 0.6%. It seems probable that the plateau region noted above corresponds to the completion of an adsorbed monolayer and the additional uptake of proteins in the concentration interval 0.5% to 0.6% can only mean a reorientation of the protein molecules in the adsorbed monolayer or the beginning formation of a duplex layer of protein molecules. Incidentally, the greatest amount of protein adsorbed at the end of this up-swing in the adsorption isotherm was close to 5.0 mg per sq. meter which is insufficient for a completed duplex film.

It is also interesting that at protein concentrations beyond about 0.6%, the amount of protein adsorbed returns to a value approximately equal to that of the

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plateau region before the irregularity in the adsorption isotherm. It is possible that the adsorbed film in this concentration region is an amorphous monolayer due possibly to the rapidity of formation at higher protein concentration. A series of glass samples were exposed to an initial protein concentration of 0.414% at pH 4.66. Half of the volume of clear supernatant solution was removed from these samples and replaced by more concentrated solutions. The adsorptions corresponding to the new equilibrium protein concentrations are shown in Fig. 2 as closed circles. Evidently, the manner in which adsorption is allowed to proceed does not influence the uptake of protein in any notable way.

Above about pH 5, the amount of protein adsorbed becomes a much more critical function of protein concentration; the amount adsorbed being very small at lower protein concentrations and at higher pH-values.

The question arises as to the reversibility of protein adsorption. Two samples of pyrex glass powder were allowed to adsorb protein at pH 5.05. The equilibrium concentration of protein for the samples was 0.0140% and the amount of protein adsorbed was 0.77 mg per gram of protein in both cases. One of these samples was diluted with buffer to give an equilibrium concentration of protein of 0.0065% and the amount adsorbed was found to be 0.78 mg of protein per gram of glass; no protein had been removed from the glass surface by diluting the protein solution. Additional protein was added to the second sample of glass to bring the protein concentration up to 0.034%. The amount adsorbed was then found to be 1.11 mg protein per gram of glass; the addition of protein resulted in increased adsorption. There is thus evidence for hysteresis in protein adsorption.

It is of interest to see if extensive washing of the adsorbed protein can remove the protein from the surface. A series of samples of pyrex glass powder were exposed to 0.45% protein at pH 4.95 and centrifuged. The supernatant solutions were removed and discarded and the glass resuspended in 11 ml of buffer at selected pH-values. These tubes were stirred, centrifuged and this process repeated 5 times. It is estimated that the original protein solution in contact with the glass had been diluted about $5 \cdot 10^5$ times. The washed pyrex particles were finally suspended in 8 ml of one molar sodium acetate solution, stirred, centrifuged and 2 drops of concentrated hydrochloric acid added to the separated supernatant solutions and the protein concentration determined in the usual manner. Assuming that the sodium acetate solution had removed all of the protein remaining on the glass, the amount of such protein remaining on the glass after the washing with the buffers was estimated. The per cent of the amounts originally adsorbed at pH 4.95 from a 0.45% protein solution is shown in Fig. 5.

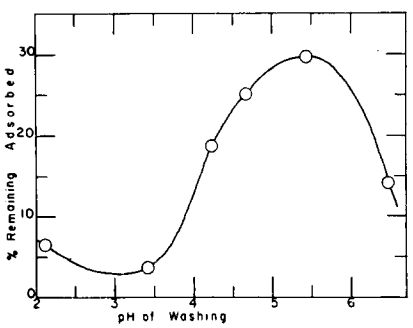

Fig. 5. Per cent of amount of protein adsorbed at pH 4.95 from a 0.45% protein solution remaining on the glass after washing at the indicated pH-values.

As can be seen from Fig. 5, a considerable portion of the adsorbed protein is removed by exhaustive washing of the glass and the variation of the amount of protein remaining on the glass as a function of the pH of the wash solution resembles that found for the variation of adsorption with pH.

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It is clear from the above results that the adsorption of protein on pyrex glass is not strictly reversible in respect to protein concentration and this fact makes the calculation of the free energies of adsorption an arbitrary procedure. Such calculations can, however, have comparative value and do indeed yield minimum free energies and should have much the same meaning as does the determination of the surface tension of compressed monolayers of protein spread at an air-water surface; such compressions also show hysteresis and a degree of irreversibility. These measurements are, however, not without interest.

The free energy of a surface is given by GUGGENHEIM on page 165 in equation 763 of his book *Modern Thermodynamics by the Methods of Willard Gibbs*, Methuen and Company, London, 1933. At constant pressure, temperature, area and at equilibrium this equation reduces to

$$\mathrm{d}F = u_i^{\alpha}\ \mathrm{d}n_i^{\sigma} = RT\ln a\,\mathrm{d}n_i^{\sigma} \tag{1}$$

where u_i^{α} is the chemical potential of the adsorbed solute in solution and a is its activity. n_i^{σ} is the number of moles of adsorbed solute. It is best to integrate eq. 1 by parts and for this purpose eq. 1 can be written

$$\mathrm{d}F = RT\mathrm{d}n_i^{\sigma}\ \ln a - RTn_i^{\sigma}\ \mathrm{d}\ln a \tag{2}$$

and integration leads to

$$\Delta F = RTn_i^{\sigma}\ \ln a - RT\int_0^a n_i^{\sigma}\,\frac{\mathrm{d}a}{a} \tag{3}$$

The first term on the right side of eq. 3 gives the free energy of dilution from unit activity to any arbitrary activity a provided the amount adsorbed is not dependent on the activity of the solute in solution in this activity interval. The second term on the right side of eq. 3 gives the free energy of dilution from activity a to zero activity irrespective of whether or not the amount of solute adsorbed is a function of the activity of the solute.

If we select our standard reference state as the activity of the solute at which the surface is just saturated with solute and dilute from this activity in the presence of the adsorbing surface, the first term on the right side of eq. 3 becomes zero and we can write

$$\Delta F = -RT\int_0^a n_i^{\sigma}\,\frac{\mathrm{d}a}{a} \tag{4}$$

At very low activities of solute in solution, the amount of solute adsorbed is, in general, a linear function of the activity of the solute in solution and n_i^{σ} is equal to Ka where K is an adsorption constant and is shown plotted as a function of pH in Fig. 3. Substituting for the value of n_i^{σ} in eq. 3 and integrating, we have

$$\Delta F = -RTKa = -RTn_i^{\sigma} \tag{5}$$

It is clear from eq. 5 that the free energy of adsorption of one mole of solute is always $-RT$ when the activity of the solute approaches zero and, indeed, this fact is the basis for the method for the determination of the molecular weight of substances spread in monolayers at air-water surfaces and is the condition for an ideal, gaseous film[2].

If we identify the free energy change shown in eq. 4 with the change of the

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interfacial tension of the glass-water interface multiplied by the area of the interface, we have

$$\Delta F = -S(\sigma_0 - \sigma) = -RT\int_0^a n_i^\sigma \frac{da}{a} \qquad (6)$$

where S is the area of the interface. Eq. 6 can be recognized as the integrated form of the Gibbs adsorption equation. Since $(\sigma_0 - \sigma)$ is equal to the so-called film pressure of the adsorbed film, we can calculate the force area curves for the adsorbed protein "spread" at the glass-water interface.

At the small protein concentrations used osmotic pressure measurements show these solutions do not depart greatly from ideality[6] and we have assumed that the activity of the protein is equal to its concentration in solution. To integrate eq. 6 we have plotted n_i^σ/C against C where C is concentration of protein and the area under this curve measured with a planimeter. The molecular weight of the protein was assumed to be 69,000 in the calculation of n_i^σ and the film pressures were expressed in dynes per centimeter. Shown in Fig. 6 are the film pressures plotted against the film areas in square meters per mg of adsorbed protein at pH 3.46, at pH 4.66 and at pH 5.81.

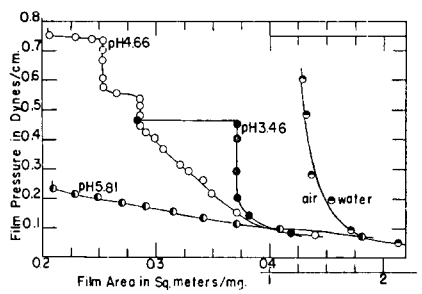

Fig. 6. Force-area curves for bovine serum albumin spread at a pyrex glass-water interface. Also shown on the broken scale is the force-area curve for bovine serum albumin spread at an air-5% ammonium sulfate water surface.

There are several interesting features shown in Fig. 6. The films spread at a glass-water interface are far more condensed than are the spread monolayers at air-water interfaces. Also the protein film at the glass-water interface exhibits several phase transitions not observed in films spread at air-water surfaces.

As can be seen from Fig. 6 the limiting areas of the adsorbed films are between 0.2 and 0.3 sq. meters per milligram with the most compressed films around 0.2 sq. meters per milligram. The surface areas of proteins in the solid state as measured by the adsorption of water vapor and the application of the Brunauer, Emmett and Teller plots[1] were for egg albumin 0.218 sq, meters per milligram, for β-lactoglobulin 0.236 sq. meters per milligram and for horse serum albumin 0.238 sq. meters per milligram. The present value for the limiting adsorbed area for bovine serum albumin provides support for the notion that the adsorption of water vapor on solid protein leads to the completion of a monolayer of water molecules on the protein surface as originally suggested[1] rather than to the binding of water molecules to discrete hydrophilic centers[5].

As shown in Fig. 6, at pH 4.66 there appear to be 3 limiting areas for the adsorbed film which are 0.285, 0.255 and 0.207 sq. meters per milligram. These areas correspond to 3,270, 2,550 and 2,370 sq. A-units per molecule of bovine serum albumin. If the adsorbed protein film has a density of 1.37, these limiting areas correspond to thicknesses of 25.6, 32.8 and 35.3 A-units. These dimensions are, at least, approximately in keeping with the dimensions for the native bovine serum albumin molecules in solution. Below pH 4, the protein molecules appear to expand progressively at the interface and at pH 1.5 the limiting area becomes 0.64 sq. meters per milligram with a calculated thickness of 11.4 A-units which is about the thickness of a compressed monolayer of protein spread at an air-water surface. The expansion of the

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bovine serum albumin at the interface at the lower pH-values is probably related to the expansion of this molecule in solution in acid solutions[8].

The free energy of adsorption of one mole of protein to saturation of the surface of glass at pH 4.66 is —2,560 calories. If we refer the free energy of adsorption to a hypothetical one molal solution of bovine serum albumin, the total free energy of adsorption becomes equal to the free energy of dilution from a molal solution to the concentration at which the glass surface becomes saturated with protein plus —2,560 calories. This yields —8,250 calories per mole. The free energy of adsorption is surprisingly small when the large number of reactive groups in the protein mole is considered. Actually, this calculated affinity is a minimum figure because of the degree of irreversibility of adsorption discussed above and the adsorption of the protein no doubt also involves some dehydration of both the glass and the protein and this would tend to lower the free energy of adsorption.

We have attempted to compare our results with the interesting experiments of TRURNIT[7] on the adsorption of chymotrypin on deposited layers of bovine serum albumin but the protein concentrations used in the present study very greatly exceed those used by TRURNIT and in other ways the experimental conditions are so different that no useful comparisons can be made.

SUMMARY

1. The adsorption of bovine serum albumin on powdered pyrex glass has been studied as a function of protein concentration and of pH at an ionic strength of 0.05 and at 30° C.

2. The surface area of the pyrex glass powder has been calibrated with the use of microscopic spherical glass particles.

3. The limiting area of the adsorbed bovine serum albumin above pH 4 approaches 0.2 square meters per milligram which is close to the area to be expected for an adsorbed layer of native molecules.

4. The adsorption of the protein exhibits considerable hysteresis in respect to desorption.

5. The interfacial tensions at the glass-water interface have been calculated and the corresponding force-area curves for the adsorbed protein drawn.

6. The free energy of adsorption of the bovine serum albumin on the glass has been estimated at pH 4.66.

RÉSUMÉ

1. L'adsorption de la sérumalbumine de boeuf sur de la poudre de verre pyrex a été étudiée en fonction de la concentration en protéine et du pH à une force ionique de 0.05 et à 30°.

2. L'aire de la surface de la poudre de verre pyrex a été calibrée à l'aide de particules de verre sphériques microscopiques.

3. L'aire limite de la sérumalbumine de boeuf adsorbée au-dessus de pH 4 est voisine de 0.2 mètres carrés par milligramme. Cette valeur est voisine de celle que l'on peut prévoir pour une couche adsorbée de molécules natives.

4. L'adsorption de la protéine présente une hystérésis considérable vis à vis de la désorption.

5. Les tensions aux interfaces verre-eau ont été calculées et les courbes force-aire correspondantes pour la protéine adsorbée ont été construites.

6. L'énergie libre d'adsorption de la sérumalbumine de boeuf sur le verre a été évaluée à pH 4.66.

ZUSAMMENFASSUNG

1. Die Adsorption von Rinderserumalbumin auf zerstäubtem Pyrexglas wurde als eine Funktion der Proteinkonzentration und des pH-Wertes, bei Ionenstärke 0.05 und 30° C untersucht.

2. Die Oberfläche des zerstäubten Pyrexglases wurde mit Hilfe von mikroskopischen sphärischen Glaspartikeln kalibriert.

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3. Die Oberfläche, welche die Menge des adsorbierten Rinderserumalbumins begrenzt, nähert sich, bei pH-Werten über 4, 0.2 Quadratmetern pro Milligramm; diese Zahl durfte bei einer adsorbierten Schicht von nativen Molekülen erwartet werden.

4. Die Proteinadsorption zeigt eine erhebliche Desorptions-Hysteresis.

5. Die Spannungen an der Berührungsfläche zwischen Glas und Wasser wurden berechnet und die entsprechenden Kraft-Flächen-Kurven für das adsorbierte Protein hergestellt.

6. Die freie Energie der Adsorption des Rinderserumalbumins auf Glas wurde bei einem pH-Wert von 4.66 schätzungsweise berechnet.

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