[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, STATE UNIVERSITY OF IOWA]

The Effective Electrophoretic Radii of Adsorbed Protein Molecules[1,2]

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The electrophoretic mobilities of bovine serum albumin and of egg albumin absorbed on powdered Pyrex glass have been measured as functions of ionic strength and of the hydrogen ions bound by the protein. The change of the electrophoretic mobilities with binding of hydrogen ions has been calculated for each of a wide range of ionic strengths. From a study of the mobility increments as a function of the ionic strength, it is concluded that the effective electrophoretic radii of the adsorbed protein molecules are very much larger than the radii of the protein molecules in solution.

Recently, we have concerned ourselves with protein monolayers adsorbed at water–Pyrex glass interfaces.[3] Our results indicate that such monolayers have dimensions corresponding at least approximately to those of the native molecules. It is further observed that under appropriate conditions the adsorbed protein monolayers show phase transitions indicating an ordered arrangement of the protein molecules at the glass–water interface.

It has long been known that particles such as those of glass adsorb protein from solution and acquire an electrophoretic mobility which resembles more nearly that of the dissolved protein than of the uncovered glass particles.[4,5]

Electrophoretic mobilities of adsorbed protein was the subject of intensive investigation by Abramson and later by Moyer and both of these workers concluded that under appropriate conditions, the electrophoretic mobility of an adsorbed protein was equal to that of the protein in solution. This conclusion was based principally on a comparison of Abramson's[6] results on the electrophoresis of adsorbed horse serum albumin at an ionic strength of 0.02 in an acetate buffer at 20° using microelectrophoresis with the results of Tiselius[7] on the electrophoresis of dissolved horse serum albumin under the same conditions of *p*H and of ionic strength but employing the moving boundary technique without, however, the refractometric modifications for observation of the boundaries which he later developed. The mobilities by the two methods of electrophoresis were compared from about *p*H 4.2 to 5.4. Close inspection of the figure drawn to show the identity of the mobilities as obtained by the two methods reveals that in the region acid to the isoelectric point, the mobilities of the adsorbed protein are actually about 25% greater than those for the protein in solution. More recently, Luetscher[8] observed that crystalline horse serum albumin shows two electrophoretic boundaries in acetate buffer of *p*H 4 and ionic strength 0.02.

Abramson, Gorin and Moyer[9] cite an additional example to bolster their conclusion that the electrophoretic mobilities of adsorbed and of dissolved protein under the same conditions of temperature, of *p*H and of ionic strength are equal to each other. This protein is horse pseudoglobulin.[10] By present day standards this protein is not well characterized and results obtained with it are not impressive; it easily could have been that the fraction of protein adsorbed was not the fraction whose mobility was measured by the moving boundary method.

Abramson, Gorin and Moyer[9] were of the opinion that since the mobilities of the dissolved and of the adsorbed protein at the same *p*H and ionic strength were, according to them, equal to each other, the adsorbed protein molecules must have an effective electrophoretic size and shape which is the same as that of the dissolved protein molecules. This conclusion follows because the electrophoretic mobility of a particle the size of the protein molecules employed in their study should be significantly smaller than the mobility of a microscopically visible particle provided the two surfaces have the same charge density.

Examination of the evidence upon which the conclusion of Abramson, Moyer and Gorin is drawn does not appear convincing, and it is our opinion that the question of the effective electrophoretic radius of adsorbed protein should be re-examined.

In the size range of many protein molecules the electrophoretic mobility should be a pronounced function of κr where κ is the reciprocal of the Debye–Hückel distance and r is the radius of the particle. κ is directly proportional to the square root of the ionic strength and, accordingly, by a study of the dependence of the electrophoretic mobilities of adsorbed protein on the ionic strength, it should be possible to estimate the effective electrophoretic radius of the adsorbed protein. The most difficult part of such an approach is to maintain a constant surface charge density since the cation and anion binding by the protein would be expected to be a function both of the *p*H and of the ionic strength.

It is the purpose of the present paper to report the mobilities of bovine serum albumin and of hens' egg albumin adsorbed on powdered Pyrex glass as a function of ionic strength after the addition of given quantities of acid or of alkali. From these results the electrophoretic mobilities of the adsorbed proteins at constant surface charge densities have been expressed as a function of the ionic strength and conclusions regarding the effective electrophoretic radii of adsorbed proteins are presented.

(1) Presented before the 132nd meeting of the American Chemical Society, New York, N. Y., September, 1957.

(2) This work was supported in part from a grant from the National Science Foundation.

(3) H. B. Bull, *Biochim. Biophys. Acta*, **19**, 464 (1956); *Arch. Biochem. Biophys.*, **68**, 102 (1957).

(4) W. Pauli and L. Flecker, *Biochem. Z.*, **41**, 470 (1912).

(5) A. Brossa and H. Freundlich, *Z. physik. Chem.*, **89**, 306 (1915).

(6) H. A. Abramson, *J. Gen. Physiol.*, **15**, 575 (1932). See also L. S. Moyer, *J. Phys. Chem.*, **42**, 71 (1938).

(7) A. Tiselius, *Nova Acta Regiae Soc. Sci. Upsaliensis*, **7**, No. 4 (1930).

(8) J. A. Luetscher, THIS JOURNAL, **61**, 2888 (1939).

(9) H. A. Abramson, M. H. Gorin and L. S. Moyer, *Chem. Revs.*, **24**, 345 (1939).

(10) L. S. Moyer, *J. Biol. Chem.*, **122**, 641 (1938).

Methods

The crystalline bovine serum albumin (BSA) was obtained from Armour and Co. and was exhaustively dialyzed in well-washed sausage casing against distilled water. The hens' egg albumin (EA) was prepared by the method of Kekwick and Cannan,[11] recrystallized twice and also dialyzed against distilled water. Concentrations of the mother solutions were determined by drying to constant weight in a vacuum oven at 105°.

A standard suspension of powdered Pyrex glass was used as a stock source of particles. These particles had been carefully fractionated for size by allowing the suspension to settle and decanting; the particles were between 1 and 2 μ in diameter.

Electrophoretic measurements on the particles were conducted in a microelectrophoretic cell of flat design supplied by Arthur H. Thomas and Co. The cell was turned on its side (lateral orientation) as suggested by Hartman, Bateman and Lauffer.[12] This new arrangement of the electrophoretic cell has proven to be a very worthwhile improvement in microelectrophoretic technique. Silver–silver chloride electrodes have been employed using accordion-folded silver foil of relatively large area connected to the electrophoretic cell through 0.5 M potassium chloride salt bridges. Readings were made at the half depth of the electrophoretic cell since this is the level at which the mobility changes least with focus of the microscope. It has been shown that the electrophoretic mobilities can be calculated from the mobilities at the half depth focus provided the walls of the electrophoretic cell are covered with adsorbed protein of the same kind as that adsorbed on the suspended particles[13]; we have reconfirmed this observation. In making this calculation, Komagata's[14] correction for the width relative to the depth of the cell has been applied and the half depth mobilities have been multiplied by 0.6428 to yield the electrophoretic mobilities. Ten determinations of the mobilities were averaged for each experimental point, the polarity of the electrodes being reversed after each individual measurement. The electrophoretic mobilities were determined at room temperature and corrected to 25° by multiplying the measured mobilities by the ratios of the viscosities of water at the temperature of the protein solutions to the viscosity of water at 25°. Conductances were measured at the temperature of the protein solution after electrophoresis.

The solutions were unbuffered except for the protein present, the desired amount of tenth normal hydrochloric acid or of tenth normal sodium hydroxide was added to the protein solution followed by the addition of the required volume of molar sodium chloride to yield the desired ionic strength of the final solution. The ionic strength of the protein solution was assumed to be equal to the sum of the hydrogen ion concentration of the solution and the concentration of the sodium chloride. The necessary volume of water was added to the solution to produce a 1% solution of protein with the desired ionic strength. The final solution contained 22 μg. of glass particles per ml. of solution.

After the electrophoretic mobilities, the conductance and the pH of the solution had been measured, the volume of the protein solution remaining was found by weighing and additional molar sodium chloride solution added to increase the ionic strength and the mobilities, conductance and pH redetermined; these operations were repeated and the mobilities, conductance and pH measured over a wide range of ionic strengths.

The moles of hydrogen ions bound per gram of protein has been calculated in the conventional manner by titrating the protein solution with standard acid and determining the pH with a model GS Beckman glass electrode followed by a blank titration in the absence of protein. The difference in titration required to yield a given pH represents the hydrogen ions bound by the protein. In the pH-range studied nearly all of the added hydrogen ions are bound by the protein.

(11) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).

(12) R. S. Hartman, J. B. Bateman and M. A. Lauffer, *Arch. Biochem. Biophys.*, **39**, 56 (1952).

(13) L. S. Moyer and H. A. Abramson, *J. Gen. Physiol.*, **19**, 727 (1936).

(14) S. Komagata, *Researches Electrotech. Lab. Tokyo*, No. 348 (1933)

Results

Shown in Fig. 1 are the electrophoretic mobilities of the Pyrex glass particles covered with BSA expressed in microns per second per volt per centimeter as a function of the protons bound by one gram of protein (adsorbed plus dissolved protein) at selected ionic strengths. Rather than clutter the figure with too many points about half of the ionic strength curves have been omitted; the omitted curves fall where they would be expected. Shown in Fig. 2 are the results obtained with EA, and again several of the ionic strength curves have been omitted for reasons of clarity.

Discussion

Abramson[6] pointed out that there should exist a direct proportionality between the electrophoretic mobility of a protein and the proton binding of a protein at constant ionic strength and called attention to the close relation between the mobilities of proteins and their titration curves. Longsworth[15] demonstrated a direct relation between the mobility of dissolved egg albumin and proton binding from pH 3 to 12 at an ionic strength of 0.010. A similiar proportionality between the electrophoretic mobility of dissolved β-lactoglobulin and proton binding was reported by Cannan, Palmer and Kibrick[16] at an ionic strength of 0.02 and over a pH range extending from 3.22 to 8.92. Longsworth and Jacobsen[17] also showed a linear relation between these two factors for dissolved bovine serum albumin at an ionic strength of 0.10 and over the limited pH range which they studied.

Inspection of Figs. 1 and 2 of the present paper reveals a linear relation between proton binding of the total protein present and the electrophoretic mobility of adsorbed EA for all the ionic strengths studied. The results for BSA extend to higher proton binding than for EA and at higher proton binding and for the lower ionic strengths the BSA lines have a curvature with decreasing slopes as the positive charge on the protein is increased.

The slopes of the linear parts of the mobility-proton binding curves have been measured for both adsorbed BSA and for adsorbed EA and are shown plotted in Fig. 3 against the factor $(1 + \kappa r_1)/\kappa$ where κ is again the reciprocal of the Debye–Hückel distance and r_1 is the average radii of the small ions and assumed equal to 2.5×10^{-8} cm. Clearly the relation between these factors is linear for BSA and appears to consist of two linear portions for EA both of which pass through the origin.

We can proceed to analyze the results shown in Fig. 3 provided we make these two assumptions.

1. The charge density on the protein is directly proportional to the protons bound even though ions other than protons are bound. The fact that for a given ionic strength the relation between mobility and proton binding is, in fact, linear over the range considered appears to require that the charge density be directly proportional to the proton binding.

(15) L. G. Longsworth, *J. Can. Chem. Process Ind.*, **34**, 204 (1950).

(16) R. K. Cannan, A. H. Palmer and A. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(17) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

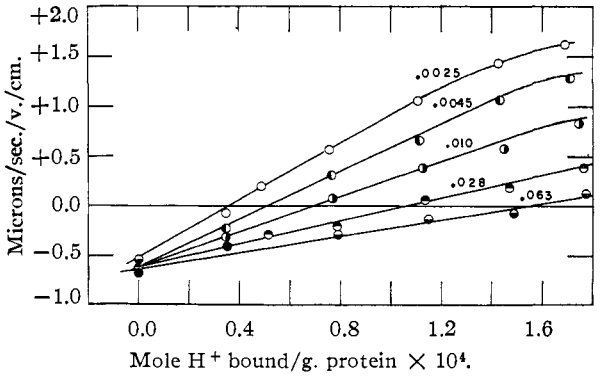

Fig. 1.—Electrophoretic mobility in μ/sec./v./cm. of BSA adsorbed on Pyrex glass particles as a function of the moles of hydrogen ions bound per gram of total protein at indicated ionic strengths.

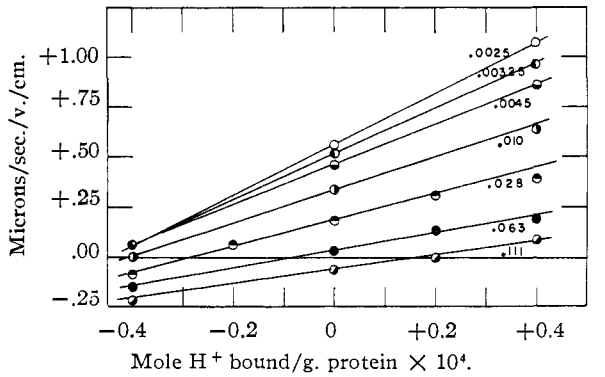

Fig. 2.—Electrophoretic mobility in μ/sec./v./cm. of EA adsorbed on Pyrex glass particles as a function of the moles of hydrogen ions bound per gram of total protein at indicated ionic strengths.

2. The amount of protons bound by unit area of the adsorbed protein surface exposed to the water phase is directly proportional to the protons bound by unit weight of protein in solution and furthermore this proportionality does not vary with ionic strength. This would appear to be a reasonable assumption as long as the adsorbed protein molecules do not alter their orientation at the glass–water interface.

The electrophoretic mobility of a spherical particle is[18]

$$U = \frac{2}{3}\frac{\sigma r}{\eta}\frac{f(\kappa r)(1 + \kappa r_1)}{(1 + \kappa r + \kappa r_1)} \qquad (1)$$

Where r is the radius of the particle, r_1 is the average radius of the small ions, σ is the electrostatic charge per unit area, η is the coefficient of viscosity of the solvent, κ is the reciprocal of the Debye–Hückel thickness of the electrical double layer and is directly proportional to the square root of the ionic strength and $f(\kappa r)$ is the Henry factor,[19] which is itself a function of κr. The Henry factor used by Ambramson, Moyer and Gorin is equal to 3/2 of the original factor as proposed by Henry. When κr approaches 300, the factor as used in eq. 1 approaches 3/2 and as κr approaches 0.5, the factor rapidly approaches unity. It follows from eq. 1 that when κr becomes very large that the electrophoretic mobility reduces to

$$U = \frac{\sigma(1 + \kappa r_1)}{\kappa\eta} \qquad (2)$$

Actually, eq. 2 is valid only if the sinh of the electrophoretic mobility can be replaced by the electrophoretic mobility itself without significant error; this is true of the values of U we are considering.

It is evident from eq. 2 that a plot of the electrophoretic mobility against the function $(1 + \kappa r_1)/\kappa$ should yield a straight line only if the effective electrophoretic radius of the particle is very much larger than is the thickness of the ionic double layer. Since as shown in Fig. 3, a plot of the mobility increment per mole of hydrogen ions bound per gram of BSA is linear, it is concluded that the effective electrophoretic radius of the adsorbed BSA is very much larger than is the radius of the dissolved molecules of this protein, *i.e.*, the radius of curvature of the surface of the adsorbed protein is very much greater than that of the dissolved protein.

Shown plotted in Fig. 3 as a broken line is the mobility of a spherical particle with a radius of 33 Å. and with the same surface charge density as that of the adsorbed BSA as a function of $(1+\kappa r_1)/\kappa$ (33 Å. is the approximate radius of a dissolved BSA molecule).

The results obtained with EA and also shown in Fig. 3 are more complex than those observed with BSA and not as clear cut. It would appear as though the EA molecules underwent a rearrangement at the interface in the interval of ionic strengths 0.00449–0.0156 such that the ratio of protons bound by the adsorbed protein to that bound by the dissolved protein increased as the ionic strength increased; there is a suggestion in

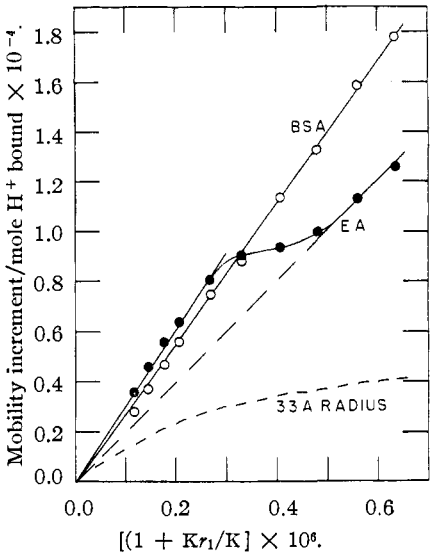

Fig. 3.—Increase in electrophoretic mobility of adsorbed protein per mole of hydrogen ions bound per gram of total protein as a function of the factor $(1 + Kr_1/K$ for BSA (open circles) and for EA (closed circles). Also shown is the theoretical curve for a spherical particle with a radius of 33 Å.

(18) H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," Reinhold Publ. Corp., New York, N. Y., 1942, see eq. 52, p. 123 and replace Q by its equal $4\pi r^2\sigma$.

(19) D. C. Henry, *Proc. Roy. Soc.* (*London*), **A133**, 106 (1931).

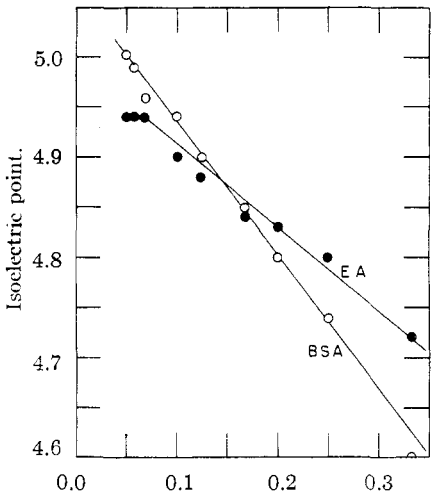

Fig. 4.—The isoelectric point of adsorbed BSA (open circles) and of adsorbed EA (closed circles) as a function of the square root of the ionic strength.

Fig. 2 of some kind of change of the protein surface above an ionic strength of 0.00449. In any event, the plot for EA shown in Fig. 3 is not consistent with the idea that the effective radius of the adsorbed EA is the same as that of the dissolved protein, and it seems probable that the two arrangements of the EA molecules at the glass-water interface both have effective electrophoretic radii which are very much larger than is the thickness of the electrical double layer.

The fact that protein adsorbed on microscopically visible particles behaves electrophoretically as plane plate condensers means a tremendous simplification of the interpretation of electrophoretic measurements on such material. For example, the charge density of such a surface can be calculated from electrophoretic mobilities using the equations for plane surfaces. We are, however, still faced with the problem of the similarity between the mobilities of adsorbed and dissolved protein. It is felt that Abramson, Moyer and Gorin overemphasized the agreement between the electrophoretic mobility of adsorbed and dissolved protein; nevertheless, the fact remains that the mobilities by these two methods do not differ by a large factor; not by the factor to be expected. Thus at an ionic strength of 0.01 and for the same surface charge density the mobility of the adsorbed protein (BSA) should be 2.93 times greater than the mobility of the dissolved molecule.

The only effective explanation for the relatively low observed mobilities of the adsorbed protein is that the charge density per unit area of the adsorbed protein is significantly less than the average surface charge density of the dissolved protein at the same *p*H and ionic strength. It is concluded that when protein molecules are adsorbed on the highly negatively charged glass surface then the protein molecules are oriented and or distorted in such a manner that the ionogenic groups of the protein are preferentially turned inward toward the glass and the charge density per unit area of the protein–water interface suffers a considerable diminution.

The variation of the isoelectric points of the adsorbed protein with ionic strength is not directly related to the problem at hand, but since we now have this information available, we wish to insert it at this point. Shown in Fig. 4 is the variation of the isoelectric points of adsorbed BSA (open circles) and of adsorbed EA (closed circles) as a function of the square root of the ionic strength. As noted by Moyer[20] and confirmed here, the isoelectric point of adsorbed EA is significantly higher than that of the dissolved protein at a given ionic strength. Aoki and Foster[21] report the isoelectric point of dissolved BSA in hydrochloric acid and sodium chloride at an ionic strength of 0.02 of 4.52. The interpolated value of the isoelectric point at this ionic strength of the adsorbed BSA (see Fig. 4) is 4.88; the isoelectric point of adsorbed BSA is also significantly higher than that of the dissolved protein.

(20) L. S. Moyer, *J. Phys. Chem.*, **42**, 71 (1938).
(21) K. Aoki and J. F. Foster, THIS JOURNAL, **79**, 3385 (1957).

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The Kinetics of the Sandmeyer and Meerwein Reactions[1]

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The kinetics of the Sandmeyer and Meerwein reactions of 2,4-dichlorobenzenediazonium chloride have been investigated. The rate of the Sandmeyer reaction was found to increase with increasing cupric chloride and acetone concentrations. The generation of cuprous chloride in acetone solutions of cupric chloride was confirmed, and its catalytic activity established by the correlation of the rate of nitrogen evolution with the rate of cuprous chloride formation. A product analysis showed the Sandmeyer product to be accompanied by arene and chloroacetone in equimolar proportion. In the absence of cupric chloride little nitrogen was evolved, and nitrogen-containing products were isolated. In the Meerwein reaction, the rate was found to vary with unsaturate both in the presence and absence of air. The results for both reactions are interpreted in terms of a comprehensive radical mechanism.

The recent confirmation in two laboratories[2,3] of a cryptic report[4] concerning the formation of cuprous chloride from acetone and cupric chloride has led to the realization[3,5] that the Sandmeyer and Meerwein reactions are initiated by essentially

(1) Presented in part at the 131st Meeting of the American Chemical Society, Miami, Fla., April 8, 1957.
(2) J. K. Kochi, THIS JOURNAL, **77**, 5090, 5274 (1955).
(3) S. C. Dickerman, K. Weiss and A. K. Ingberman, *J. Org. Chem.*, **21**, 380 (1956).
(4) V. Kohlschütter, *Ber.*, **37**, 1153 (1904).
(5) J. K. Kochi, THIS JOURNAL, **79**, 2942 (1957).