THE VALENCE OF CORPUSCULAR PROTEINS*

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The quantity most directly related to the net charge or valence of corpuscular proteins is the electrophoretic mobility. In 1932 Abramson (1) showed that in solutions of the same ionic strength the electric mobility of the same protein at different hydrogen ion activities is directly proportional to the number of hydrogen ions bound or released as determined by titration curves. His comparisons were made with gelatin, serum albumin, egg albumin, and casein. The generality of Abramson's rule has been confirmed by Daniel (2), Moyer and Abels (3), Moyer and Abramson (4), Longsworth (5), and also by Cannan, Palmer, and Kibrick (6). Longsworth's data on egg albumin are especially interesting, for his comparison extends from pH 3 to 12. Inasmuch as the results of Abramson and of Moyer seem to have been misunderstood, it should be emphasized that they deal with comparisons of data on *dissolved* proteins. If the protein in the adsorbed state happens to agree, it is incidental to the argument.

Not only did Abramson show proportionality between the electric mobility and titration curves, but he also attempted to calculate the valence of proteins from the available electrophoretic and titration data. The agreement, however, was incomplete.

Although acid-base titration and membrane potential measurements on protein solutions give results that are related to the electric mobility, the actual determination of the net charge from these measurements is subject to difficulties in interpretation and questionable assumptions. On the other hand, an exact interpretation of electric mobility data in terms of net charge requires independent information concerning the size and shape of the protein molecule and the sizes of the other ions in the solution. Information regarding size and shape is not yet available for any protein. Extreme limits of variation of these quantities, however, may be obtained from sedimentation and diffusion data by the assumption, on the one hand, of spherical shape and enough hydration, or, on the other, of enough asymmetry, to account for the diffusion constant.

The results presented in this communication indicate that when these factors are taken into account, values for the valence calculated from titration, electro-

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phoresis, and membrane potential measurements agree closely. It will be shown that for the cases to be considered the uncertainty about size and shape causes a maximum spread of about 20 per cent in the valence determined from mobility data by using the two extreme models: a hydrated sphere and an unhydrated, elongated rod.

Theoretical

In obtaining the valence of proteins from their electrophoretic mobility and size and shape, dependence is placed upon the Debye-Hückel theory (7), modified to include the effect of the ions in the ion atmosphere, and on Stokes' law, as applied to such systems by Henry (8). Under conditions ordinarily encountered, both the extended Debye-Hückel theory and the hydrodynamic assumptions of Stokes' law should apply quite accurately to systems of the corpuscular proteins. Conditions under which considerable deviations from the simplified Debye-Hückel differential equation should occur in protein solutions, due to the Gronwall-La Mer effect (9), have been investigated by Gorin (10). It was concluded as a result of this work that, in the case of proteins of the size of egg albumin or larger and for ionic strengths greater than 0.01, the Gronwall-La Mer correction is small unless the protein is highly charged (valence greater than 10). At ionic strengths greater than 0.1, the correction is negligible for all values of the net charge likely to be encountered in protein systems. For the systems to be presented here, the Gronwall-La Mer effect may be neglected.[1]

The theory to be outlined below applies equally well to protein systems made up of molecules that all have the same charge at any instant or to systems in which the charge of the molecules fluctuates rapidly with time. In the latter case, the net charge determined by electrophoresis is the charge taken over a time average. The one debatable assumption involved in the application of the Debye-Hückel theory to protein systems is that the net charge over a time average behaves as if symmetrically distributed within the molecule or over its surface. However, from the point of view of physical realities there can be little doubt that this is an assumption that is generally justified.

After the net charge of the protein is obtained, within the limits indicated above, it is then possible to compare these values with those obtained from the interpretation of titration data and of membrane potential measurements. As has been the experience of Abramson (1, 12), Moyer and Abels (3), Longsworth (5), and Cannan, Palmer, and Kibrick (6), reached on the basis of more approximate solutions of the Debye-Hückel differential equation, we too find that

[1] Recently, Tiselius and Svensson (11) have claimed that the simple Debye-Hückel theory can be successfully used over a wide range of ionic strengths to calculate the electrophoretic mobility from the net charge estimated by measurements of membrane potentials. See, however, Gorin (10) and Longsworth (5).

values of the net charge calculated from electrophoretic and titration data, at the same ionic strength, agree poorly. For this reason, we have concluded that the ordinary interpretation of titration data is open to serious question and should be reexamined. Steinhardt (13), working on an entirely different basis, has come to similar conclusions.

In the ideal case, assuming that only protons are bound to protein, the valence, ν, is equal to the number of protons bound (or released) per molecule of protein. When the valence is multiplied by the charge on an electron ($\mathbf{e} = 4.80 \times 10^{-10}$ E.S.U.), the net charge, Q, of the protein molecule, over a time average, is obtained. In other words,

$$Q = \nu\mathbf{e} = hM\mathbf{e}, \tag{1}$$

where h is the number of equivalents of hydrogen bound per gram of protein of molecular weight, M. Equation (1) no longer applies when significant numbers of ions other than hydrogen are bound by the protein and, especially, when this binding is dependent on pH.

The Charged Sphere.—According to the theories of Gouy and others (14), a charged particle in an ionic medium accumulates in its neighborhood a cloud of ions whose net charge, over a time average, is opposite in sign and equal in magnitude to that existing on the particle. The statistical mean distance of the ions from the surface is referred to as the "thickness of the electric double layer" and will depend on the concentration of ions present. These ions are free to move. Close to the surface, however, there can be a film of tightly held water into which they can rarely pass. Over a time average, the statistical thickness of this film of water will determine the position of the surface of shear. Water molecules inside this boundary are carried with the particle to form a single kinetic diffusing unit. The surface of shear is the "effective" surface of the particle as far as electrophoretic and diffusion effects are concerned. Electric charges inside the surface of shear contribute to the charge on the particle, while those outside constitute the ion atmosphere of the particle.

This non-uniform distribution of charges sets up a potential, ψ, extending outward to infinity from the surface of shear. At the surface of shear, ψ assumes the special value, ζ. If a particle of radius, r, is subjected to an applied electric field, it will move with a mobility, v, that is directly proportional to the ζ-potential at its surface. By the theories of Helmholtz, Smoluchowski, and Henry (8), for a particle large enough to obey Stokes' Law,

$$v = \frac{Df(\kappa r)}{6\pi\eta}\zeta, \tag{2}$$

where η is the viscosity of the medium, D is the dielectric constant, and κ is the well known square root function of ionic strength of Debye and Hückel (7). Values of the Henry factor, $6/f(\kappa r)$, are listed in Table I (for the correct ex-

panded form of $f(\kappa r)$, see Gorin (10)). Consequently, v can be calculated if ζ can be related to the net charge and to the thickness of the electric double layer, $1/\kappa$.

Debye and Hückel, following Gouy, combined the Boltzmann and Poisson equations and, with certain simplifying assumptions, arrived at the expression,

$$\nabla^2\psi - \kappa^2\psi = 0, \tag{3}$$

from which, on integration, they obtained for a sphere,

$$\psi_i = \frac{Q}{Dr(1 + \kappa d)}. \tag{4}$$

The essential assumptions have been listed elsewhere (15). Here Q is the net charge of the particle, D, the dielectric constant of the medium, r, the radius of

TABLE I

The Henry Factor

κr	$6/f(\kappa r)$	κr	$6/f(\kappa r)$
0	6.00	5	5.17
1	5.84	10	4.84
2	5.63	25	4.38
3	5.45	100	4.11
4	5.30	∞	4.00

the central particle, and d, a radial distance called the "distance of closest approach." At this distance, ψ assumes a special value that we shall designate ψ_i, rather than ζ, its value at the surface of shear. The situation has been diagrammed in Fig. 1. It is ζ, rather than ψ_i, that is needed here. Only when the mean radius of the counter ions in the double layer is zero will $\psi_i = \zeta$. At ionic strengths commonly used in protein electrophoresis, ψ_i will deviate significantly from ζ. A correction to equation (4) to include the effect of the size of the ions in the ion atmosphere is therefore necessary.

Values of the radii of the electrolyte ions are available from the equilibrium theory of ionic conductance (16). These values and the equations to be presented below involve the assumption that the charge on the ion is concentrated at its center. This assumption should be valid for simple ions, such as halides and those of the alkali metals. For more complex ions, the assumption may be open to question. In general, electrophoretic results obtained with systems in which simple salts like NaCl make the major contribution to the ionic strength are most suitable for interpretation in terms of net charge on the protein. The systems used by Longsworth (5) are especially suitable for this use.

If these notions are introduced into the treatment of Debye and Hückel they lead to the result (15, 16),

$$\zeta = \frac{Q(1 + \kappa r_i)}{Dr[1 + \kappa(r + r_i)]}, \tag{5}$$

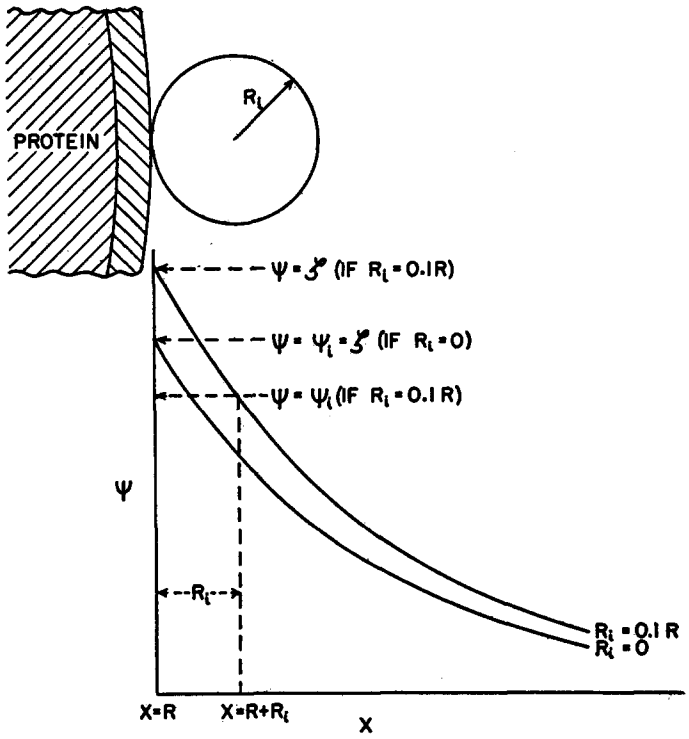

FIG. 1. Diagram of a portion of the surface of a hydrated, spherical protein of radius, r. An ion of radius, r_i, is shown at the distance of closest approach. The upper curve represents the fall of potential, ψ, from the surface of shear as a function of radial distance, x, calculated by equation (5) on the assumption that $r_i = 0.1\ r$. Notice that at the surface of shear $\psi = \zeta$ but that at the distance of closest approach ($x = r + r_i$) $\psi = \psi_i$. It is ζ, rather than ψ_i, that is directly related to the electric mobility. The lower curve, calculated by equation (4) on the assumption that $r_i = 0$, indicates that only in this case does $\psi = \psi_i = \zeta$ at the surface of shear.

where r_i is the mean radius of the ions in the diffuse double layer and r is the radius of the central particle. If the particle is assumed to be hydrated, the effective thickness of its hydration layer (to the surface of shear) must be included in r. This expression departs most widely from equation (4) at high ionic strengths (μ).

On combining equations (2) and (5), we have, after dividing by the electronic charge,

$$v = \frac{6\pi\eta r[1 + \kappa(r + r_i)]}{\mathbf{e} \cdot f(\kappa r) \cdot (1 + \kappa r_i)} v. \tag{6}$$

This spherical model predicts the valence from observed values of the mobility under chosen ionic conditions.

The Unhydrated Charged Cylinder.—Few, if any, proteins are spherical. Consequently, it seemed more realistic to develop the equations for an elongated cylindrical model (17). In this case, the potential, ψ, depends only upon the radial distance, x, from the axis of the cylinder. Equation (3) then becomes

$$\frac{d^2\psi}{dx^2} + \frac{1}{x}\frac{d\psi}{dx} - \kappa^2\psi = 0, \tag{7}$$

a modified Bessel's equation of zero order. When this is solved by the introduction of suitable boundary conditions and by assuming a uniform distribution of surface charge, there is obtained

$$\zeta = \frac{2Q}{D(l + 2a)} \frac{K_0(\kappa a)}{K_1(\kappa a) \cdot (\kappa a)}, \tag{8}$$

where e is the length and a the radius of the cylinder. K_0 and K_1 are special Bessel functions of zero and first order respectively (18). The term, $2a$, in the denominator is in the nature of an end correction for small values of l/a. It was chosen to cause equation (8) to join smoothly with equation (11) as $\mu \rightarrow 0$.

When the effect of the finite size of the ions in the ion atmosphere is included, the complete equation for ζ becomes,

$$\zeta = \frac{2Q}{D(l + 2a)} \left[\frac{K_0(\kappa a + \kappa r_i)}{(\kappa a + \kappa r_i) K_1(\kappa a + \kappa r_i)} + \ln \left(\frac{a + r_i}{a} \right) \right] \tag{9}$$

To relate ζ to v, the factor of proportionality, C, in the general electrophoretic equation,

$$v = \frac{D\zeta}{C\eta}, \tag{10}$$

must be solved for all values of κa. In the case of a suspension containing elongated protein molecules oriented at random by Brownian movement, we assume that over a time average, one-third lie parallel to each cartesian coordinate.[2] Random orientation is justified by the fact that orienting forces involved in electrophoresis are very small when compared with Brownian forces.

[2] This method of averaging is essentially parallel to those used in solving similar physical problems (19, 20).

This method of averaging is simplified by taking one of the coordinates (x-axis) in the direction of the applied field, whereupon only two cases need be solved. These are: the cylinder migrating parallel to the field and the cylinder migrating perpendicular to the field. For each cylinder parallel to the field, two will be perpendicular. For the parallel case, Henry (8) finds $C = 4\pi$ for all values of κa. For the second case, $C = \pi \cdot F(\kappa a)$. By an extension of the work of Henry, this function has been obtained by one of us (M. H. G.) by graphical integration (15).[3] The results are given in Table II.

The function, F', for a randomly oriented cylinder, corresponding to F is

$$F' = \frac{2F + 4}{3}.$$

This is also given in Table II.

TABLE II

The Constants of Proportionality for a Long Cylinder

κa	Orientation	
	Perpendicular	Random
	F	F'
0	8.00	6.67
0.4	7.73	6.49
1.0	7.36	6.26
1.4	6.87	5.91
2.0	6.51	5.67
2.4	6.34	5.56
3.0	6.10	5.40
∞	4.00	4.00

It is somewhat more convenient to define the cylinder in terms of its asymmetry and volume rather than with respect to its length and radius. We define asymmetry as $S = l/2a$, so that

$$a = \sqrt[3]{v/2\pi S},$$

where v is the molecular volume.

The electric mobilities of cylinders of varying asymmetries are compared in Table III with the electric mobilities of a sphere of the same molecular volume and net charge. At $\mu = 0$, the values of the ratios of the mobilities are exactly equal to the corresponding ratios of the diffusion constants, D/D_0, of the cylinder

[3] The function was inadvertently attributed in this paper to a parallel cylinder rather than to a perpendicular one.

and sphere respectively. Since the electrophoretic mobilities for cylinders cannot properly be extrapolated to $\kappa = 0$, the values in the table at $\mu = 0$ are calculated for prolate ellipsoids from Perrin's equation (21),

$$\frac{f_0}{f} = \frac{D}{D_0} = \frac{S^{2/3} \ln \frac{1 + \sqrt{1 - S^2}}{S}}{\sqrt{1 - S^2}}, \tag{11}$$

where S is the ratio of the long to the short axis and f/f_0 is the force factor of the molecule. Notice in the table that the values of the ratios computed by the two methods join smoothly. The values are nearly unaffected by changes in molecular weight between 30,000–150,000. Asymmetry, however, exerts a noticeable effect on the retardation due to the ion atmosphere. It should be emphasized that these calculations are for randomly oriented cylinders of constant net charge but not of constant charge density.

TABLE III

Electrophoretic Mobilities of Cylinders

Asymmetry $l/2a$	Ratio of mobility to that of a sphere of the same molecular volume				
	$\mu = 0$	$\mu = 0.005$	$\mu = 0.02$	$\mu = 0.1$	$\mu = 0.2$
2.5	0.920	0.856	0.774	0.738	0.715
5.0	0.800	0.715	0.653	0.624	0.604
10.0	0.648	0.545	0.503	0.490	0.485
22.5	0.477	0.379	0.361	0.362	0.357
45.0	0.350	0.263	0.254	0.256	0.259

Methods

Serum albumin B was crystallized from horse serum by the methods of Kekwick (22). The dialyzed product was titrated in a nitrogen atmosphere by the use of a micro burette and a glass electrode. The standards of pH employed were those recommended by MacInnes, Belcher, and Shedlovsky (23). To eliminate the effect of protein concentration as much as possible, the measurements were performed at about 0.25 per cent protein. In those titrations that were performed at constant ionic strength, the proper amount of NaCl was added in advance to the HCl and NaOH used in titrating as well as to the protein solution. The mean ionic activity coefficient was taken as that prevailing in the absence of protein at the ionic strength under consideration. Suitable methods for the calculation of the equivalents of hydrogen bound or released have been discussed by Kekwick and Cannan (24) and by Moyer and Abels (3).

RESULTS

Egg Albumin.—Longsworth (5) has found that the diffusion constant, D, of egg albumin is 3.99×10^{-7} cm.^{2}sec.$^{-1}$ at 0°C. If the molecule is assumed to be a

hydrated sphere, its effective radius can be calculated from the Stokes-Einstein equation,

$$r = \frac{kT}{6\pi\eta D} = 27.8 \text{ Å}.$$

Longsworth has also presented moving boundary data for dissolved egg albumin at 0°C. and $\mu = 0.1$. Under these conditions, $\kappa r = 2.87$. From Table I, $6/f(\kappa r)$ is found to be 5.47. On taking the average radius of the electrolyte ions, r_i, as 2.5 Å., κr_i becomes 0.258. If these values are substituted in equation (6), we obtain the valence, ν, in terms of v (in μ/sec./volt/cm.),

$$\nu = 17.5\, v. \tag{12}$$

If, however, the other extreme, an unhydrated cylinder, is assumed, the asymmetry is determined (25) from the diffusion constant of the protein by the use of equation (11). If the molecular weight of egg albumin is 45,000 (26), its unhydrated molecular volume corresponds to that of a sphere of 23.6 Å. The force factor, f/f_0, is thus $27.8/23.6 = 1.18$ and the asymmetry is 4/1 for this rod-like model. We first calculate the valence of the unhydrated sphere for $\kappa r = 2.44$ and $6/f(\kappa r) = 5.55$. This gives $\nu = 13.65\, v$. Reference to Table III shows that a cylinder of this degree of asymmetry should have a mobility equal to 65 per cent of that of a sphere of the same molecular volume. Hence

$$\nu = \frac{13.65}{0.65} v = 21.0\, v. \tag{13}$$

Comparison of equations (12) and (13) shows that a difference of about 20 per cent should be found between a sphere and a cylinder of the same diffusion constant under these conditions. Accurate determinations of the valence from titration data would make possible a decision between the effects of water of hydration and asymmetry.

In Fig. 2 are shown the results discussed above, together with curves for the valence calculated by equation (1) from the recent titration data of Cannan, Kibrick, and Palmer (27) at infinite dilution of egg albumin. The titration data have been shifted vertically so that the curves coincide with the mobility curve at the isoelectric point. The low temperature coefficient of the dissociation of carboxyl groups makes possible a comparison of these curves without temperature correction in the range of pH shown in the figure (5, 27). Although quantitative agreement exists between the mobility data and the titration curve in the absence of added salt, it is evident that the values of the net charge calculated from the titration data at the ionic strength of the electrophoretic measurements are several hundred per cent higher.

Tiselius and Svensson (11) have determined the electrophoretic mobility of

egg albumin in phosphate buffers at 0°C. and $\mu = 0.1$. Under these conditions, the protein is highly negative and r_i can still be taken as 2.5 Å., in spite of the presence of phosphate ions of relatively larger radii. Applying equations (12) and (13) we obtain $\nu = -11.9$ and $\nu = -14.1$ for sphere and cylinder respectively.

Adair and Adair (28) obtained $\nu = -13.8$ for the valence of egg albumin at 0°C. in phosphate buffers at pH 7.1 by means of membrane potential measure-

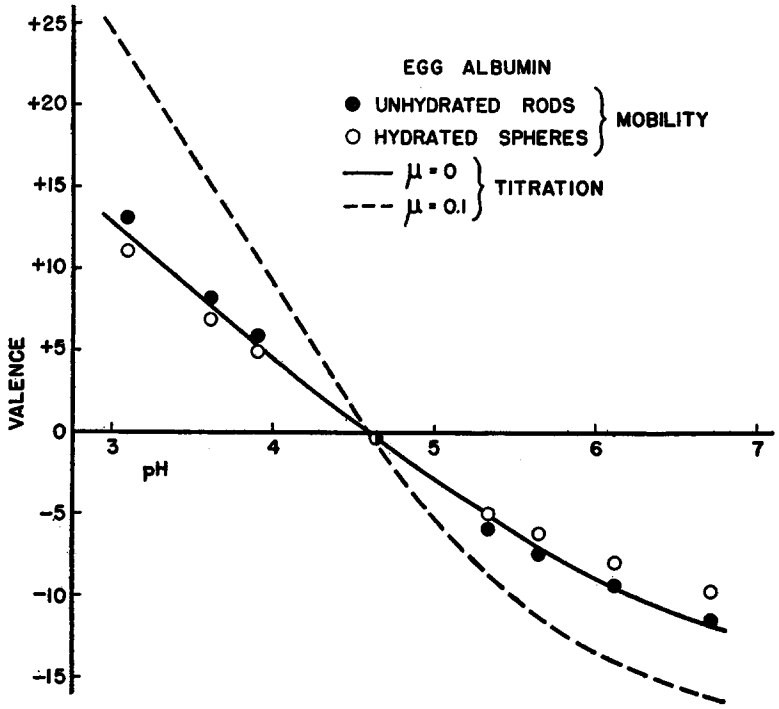

FIG. 2. The valence of egg albumin from titration data of Cannan, Kibrick, and Palmer and electrophoretic results of Longsworth. The curves have been shifted to the isoelectric point at pH 4.60.

ments. Thus the membrane potential measurements of Adair and Adair and the extrapolated titration curve lead to similar results, namely that the agreement is somewhat better for the cylindrical than for the spherical model. By inference, it may be concluded that our method to obtain the net charge from extrapolated titration curves and the membrane potential method should lead to very nearly the same results for the same system. No data are available, as yet, to permit a direct comparison of the two methods with the same system.

The titration method is unreliable for points far removed from the isoelectric point. In addition, the isoelectric point of egg albumin is not accurately known in phosphate buffers.

Calculations from the earlier results of Tiselius (29) for egg albumin in acetate

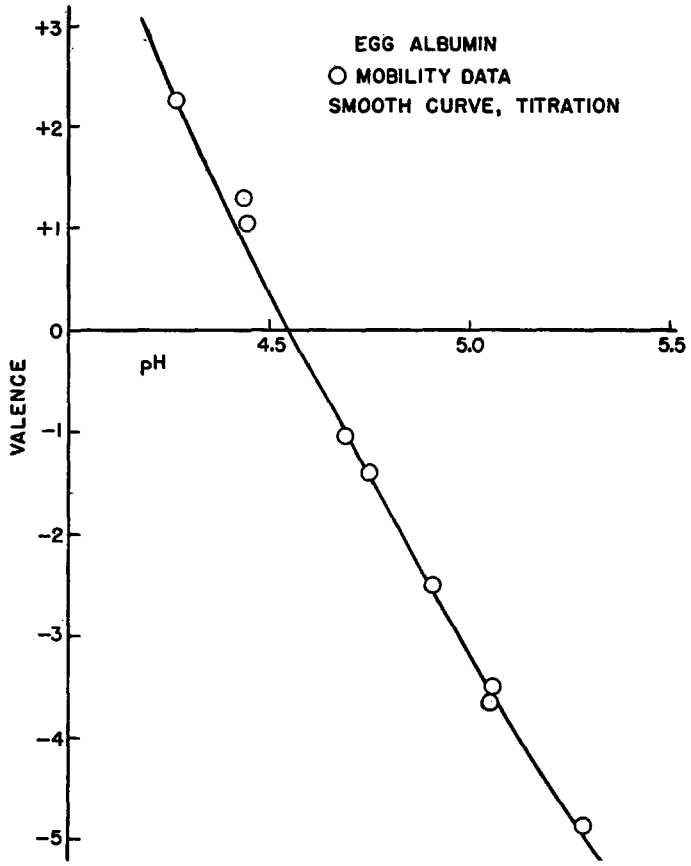

FIG. 3. The valence of egg albumin from titration data of Cannan, Kibrick, and Palmer and electrophoretic results of Tiselius. The isoelectric point lies at pH 4.55 at this ionic strength (0.02). Results for cylinder and sphere have been averaged, as discussed in the text.

buffers at $\mu = 0.02$ and 20°C. are shown in Fig. 3. Under these conditions, we obtain $\nu = 6.9\ v$ and $\nu = 8.0\ v$ for sphere and cylinder, respectively. Since the electrophoretic measurements probably have an error of the order of 10 per cent, no attempt was made to distinguish between the two models in this case. The plotted points are the average values of the charge calculated for a sphere and

cylinder, namely, $\nu = 7.45\ v$. The smooth curve is the valence calculated from the titration curve (27) at infinite dilution, shifted vertically to $v = 0$ at pH 4.545. Notice the excellent agreement between valence from electrophoresis and valence from titration.

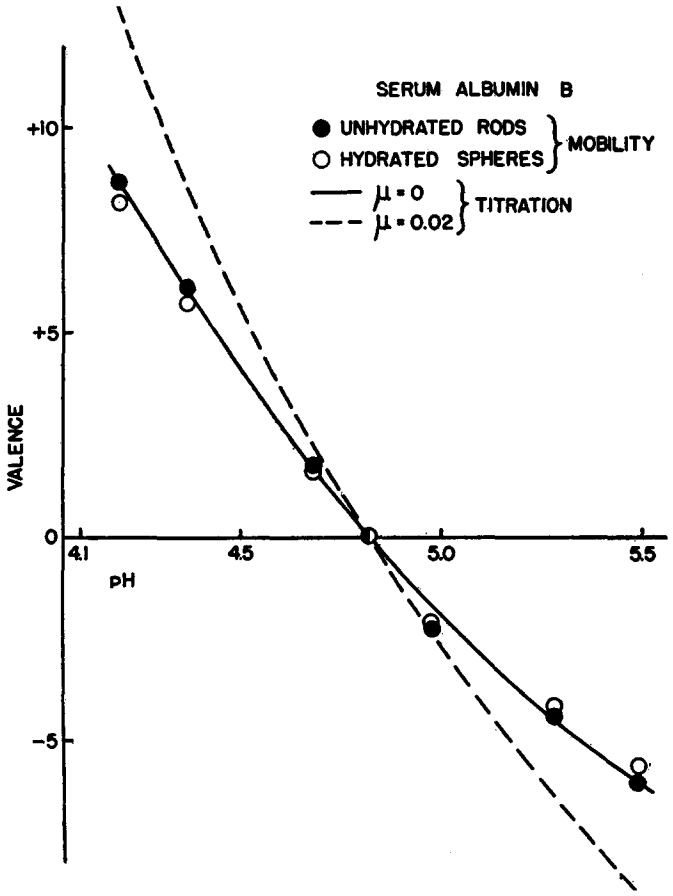

FIG. 4. The valence of serum albumin B from titration data (this paper) and electrophoretic results of Kekwick. The curves have been shifted to the isoelectric point at pH 4.81 ($\mu = 0.02$).

Serum Albumin.—In Fig. 4 are shown values for the valence of serum albumin B calculated from the electric mobility data of Kekwick (22) at 20°C. and $\mu = 0.02$. He finds 70,000 for its molecular weight and 6.10×10^{-7}cm.^{2} sec.$^{-1}$ for its diffusion constant at 20°C. These data lead to an asymmetry of 5/1, if an unhydrated rod is assumed. The titration curves are our own results. Here, $\nu/v = 9.65$ for a sphere or 10.31 for a rod.

Fig. 5 shows a similar comparison for this protein at $\mu = 0.1$. In this instance, the mobility data of Moyer and Moyer (30), for films of serum albumin B adsorbed on microscopic particles, were used. Inasmuch as the electric mobility data at an ionic strength of 0.02 for this protein adsorbed on quartz or

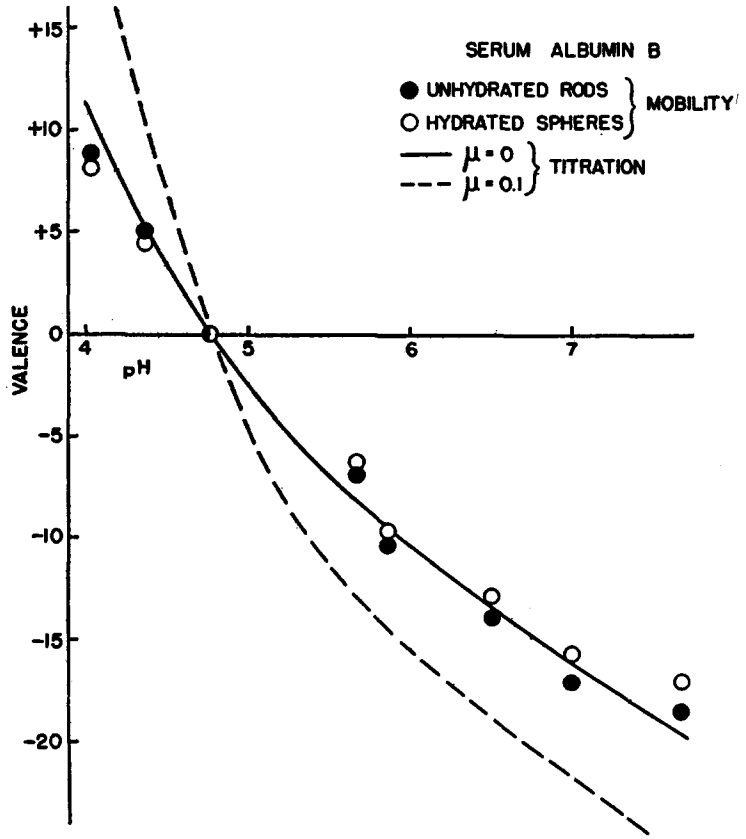

FIG. 5. The valence of serum albumin B from titration data (this paper) and electrophoretic results of Moyer and Moyer. The curves have been shifted to the isoelectric point at pH 4.76 ($\mu = 0.1$).

collodion particles have been shown (30, 31) to be in agreement with moving boundary measurements on dissolved serum albumin B, it is highly probable that the data in Fig. 5 correspond to the behavior of the dissolved protein at $\mu = 0.1$. The titration curves in Figs. 4 and 5 follow a course with respect to the mobility data similar to that found for egg albumin. In this case, $\nu = 12.75\ v$ for a sphere or $13.94\ v$ for a rod.

β-Lactoglobulin.—Similar calculations have been made for β-lactoglobulin, by using the electric mobility data of Pedersen (32) at 20°C and $\mu = 0.02$ and the titration data of Cannan, Palmer, and Kibrick (6). Oncley (33) suggests 40,000 as the most probable value for the molecular weight. This figure, combined with the diffusion constant, 7.27×10^{-7}cm.^{2}sec.$^{-1}$, found by Polson (34) at 20°C., yields a force factor of 1.26, which corresponds to an asymmetry of

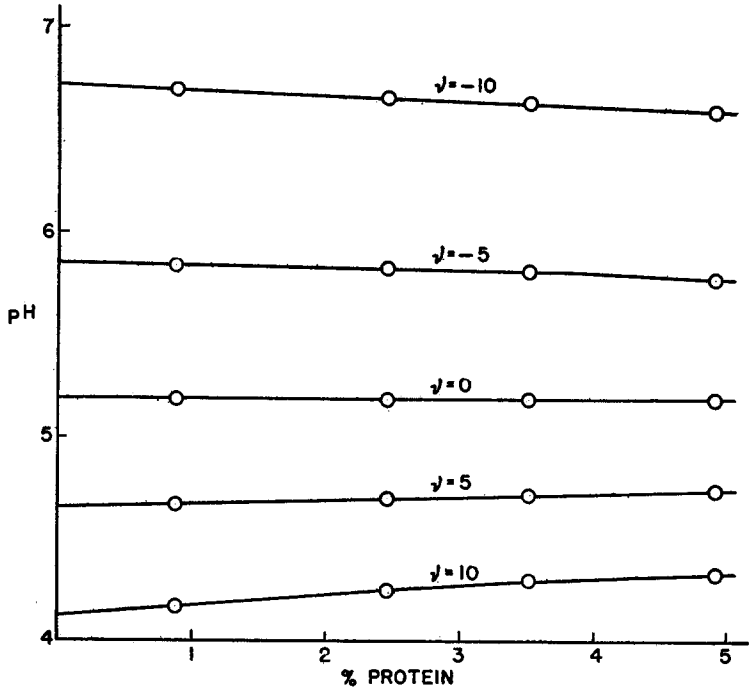

FIG. 6. Extrapolation of the titration data of Cannan, Palmer, and Kibrick on β-lactoglobulin in the absence of salt to infinite dilution of protein.

5.2, if an unhydrated cylinder is assumed. This yields $\nu = 8.11v$. If, however, a hydrated sphere is chosen as the model, $\nu = 7.35\ v$.

Cannan, Palmer, and Kibrick present two sets of titration data for this protein: (1) in the presence of various concentrations of salt at a protein concentration of 0.5 per cent and (2) at various concentrations of protein in the absence of added salt. A curve representing the titration curve at infinite dilution of electrolytes and protein has been obtained by extrapolation of this latter set of results (Fig. 6). At values of pH below 4, the extrapolation becomes less certain; in addition, the steadily increasing concentration of Cl^- begins to con-

tribute to the ionic strength. Hence we have decided to limit our considerations to regions above this value.

Fig. 7 shows our results for this protein. As before, the agreement with the electrophoretic results is good only for the extrapolated titration curve in the absence of salt. Likewise, the correspondence is somewhat better for the cylindrical than for the spherical model.

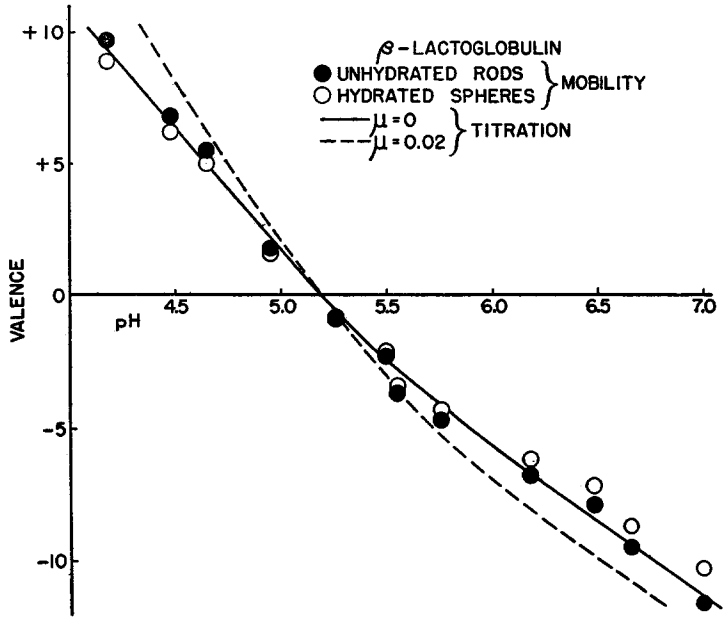

FIG. 7. The valence of β-lactoglobulin from titration data of Cannan, Palmer, and Kibrick and electrophoretic results of Pedersen. Inasmuch as both isoionic and isoelectric points are at pH 5.19, no adjustment of the curves was necessary.

DISCUSSION

The chief difficulty encountered in determining the valence of proteins from titration curves involves the interpretation of titration data. It is unlikely, especially at higher values of ionic strength, that the titration curve represents binding or release of hydrogen ions alone. As a first approximation, the procedure of shifting the titration curve vertically to account for the binding of ions other than hydrogen and hydroxyl appears to be justified for pH values not far removed from the isoelectric point. With a change in ionic strength, the influence of other ions, such as Na^+ or Cl^-, upon the net charge of the protein results mainly in a vertical shift of the net charge curve without much change in its slope; *i.e.*, a shift in isoelectric point (3, 4, 12, 15, 35). Results suggest that

over the pH range investigated the binding of ions other than hydrogen to protein is relatively independent of pH. Two types of interactions of these ions with protein would be expected to be nearly independent of pH. One is adsorption of ions like Na^+ or Cl^- by non-polar parts of the molecule. The other is ion pair formation between the electrolyte ions and the charged groups of the protein molecule (36). Ion pair formation should be independent of pH only in the neighborhood of the isoelectric point, where the net charge of the protein is small compared to the total charge. We have therefore restricted our considerations to this region.

Steinhardt (13), moreover, has obtained results which indicate that undissociated acid, such as HCl, is bound to protein along with hydrogen ion. This is equivalent to saying that ion pairs of $H^+ \cdot Cl^-$ tend to be taken up by the protein molecule. Under conditions where this effect is appreciable, the titration curve would yield too high a value for the valence, since the binding of molecular HCl would remove hydrogen ions from solution and contribute to the experimental titration curve but should not affect the net charge. Hence it would not affect the electrophoretic mobility.

Let us consider the binding of hydrogen ions involving only the acidic and basic groups of the protein molecule. In this case, if we make the assumption that this binding is essentially independent of ionic strength, it is possible to obtain what may be considered the *intrinsic* net charge curve (the titration curve concerned with the binding of hydrogen ions alone), by extrapolating the experimental titration curve to zero ionic strength and zero protein concentration. By hypothesis, this curve would apply at all values of ionic strength. To correct for the binding of other ions, such as Cl^-, the titration curves were shifted vertically, so that the zero point for acid or base bound corresponded with the isoelectric point determined by electrophoresis.

As Abramson (12) has suggested, it seems likely that, as results on additional proteins become available, individuals similar to gelatin, whose isoelectric point is hardly shifted by ionic strength, will be encountered. It is possible that β-lactoglobulin is one of these cases, for its isoionic point is unaffected by salt (Fig. 7) and is identical with its isoelectric point, at least at $\mu = 0.02$. A comparison of the valence curves is possible, therefore, under conditions that are free from any question as to the validity of the vertical adjustment.

The experimental titration curves, however, deviate very markedly from the ideal one at higher values of ionic strength and protein concentration. Cannan, Kibrick, and Palmer (6, 27) have attributed these deviations to the effect of salt on the dissociation constants of the amino and carboxyl groups, combined with an activity effect on the composite protein ion. On the other hand, we prefer, as a first approximation, to attribute these effects to the binding of undissociated acid to the proteins. The magnitude of this binding would depend on the activity product of anions and hydrogen ions in the solution and

would therefore be a function of pH, salt concentration, and protein concentration. In basic solutions binding of pairs, such as K^+ and OH^-, to protein may also occur.

It will be recalled that values of the net charge of protein molecules obtained from electrophoretic data by the use of the two extreme models, the elongated rod and the hydrated sphere, agree within 15 to 20 per cent. Furthermore, in each case they agree very well with the intrinsic net charge curve for values of the valence obtained from titration data by means of our assumptions. On the other hand, the dashed lines, which represent the net charge calculated on the assumption that no molecular HCl is bound to protein, deviate widely from the other results.

The agreement between the rod-like model and the titration data is somewhat better than that for the sphere but it should not be concluded that these proteins are unhydrated rods instead of hydrated spheres. It is probable that the proper model lies somewhere between these extremes; *i.e.*, a less extended rod with some hydration.

SUMMARY

By the use of two extreme models: a hydrated sphere and an unhydrated rod the valence (net charge) of corpuscular proteins can be successfully calculated from electric mobility data by the Debye-Hückel theory (modified to include the effect of the ions in the ion atmosphere) in conjunction with the electrophoretic theory of Henry. As pointed out by Abramson, this permits a comparison with values for the valence from titration data.

Electrometric titration measurements of serum albumin B (Kekwick) have been determined at several ionic strengths. These results, together with the available data in the literature for serum albumin B, egg albumin, and β-lactoglobulin have been used to compare values for the valence calculated from measurements of titration, electrophoresis, and membrane potentials. The results indicate that the usual interpretation of titration curves is open to serious question. By extrapolation of the titration data to zero ionic strength and protein concentration, there results an "intrinsic" net charge curve describing the binding of H^+ (OH^-) ion alone. This curve agrees closely, in each case, with values of the valence calculated from mobility data (which in turn are in close accord with those estimated from membrane potential measurements). The experimental titration curves in the presence of appreciable quantities of ions and protein deviate widely from the ideal curve. It is suggested that, under these conditions, binding of undissociated acid (base) leads to erroneous values for the net charge. This binding would not affect the electrophoretic mobility.

Values of the net charge obtained by the two extreme models from electrophoretic data are in agreement within 15 to 20 per cent. The agreement be-

tween the cylindrical model and the titration data is somewhat better in each case than with the sphere; *i.e.*, this comparison enables a choice to be made between asymmetry and hydration in the interpretation of results from sedimentation and diffusion measurements on proteins. It is concluded that the proteins discussed here are somewhat asymmetric and also hydrated.

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