The substituent effect values may also be used to provide reasonably good initial values of the shifts in ABC systems ($H_2C{=}CHX$). For example, the case of vinyl bromide, although cited above in support of these generalizations, was actually solved by using the chemical shifts of *trans*-bromopropene and 2-bromopropene and computing the effect of removal of the methyl group. The initial shifts so obtained for vinyl bromide were all within 3–4 c.p.s. of the final values, the discrepancy being in the same direction for all protons. Hence the relative positions of all shifts turned out to be correctly predicted to within less than one c.p.s. (see Table VII). This constant deviation of about 3 c.p.s. is what we have termed the "constitutive" effect.

Finally, it may be remarked that since the observed substituent effect of methyl on β-proton shifts is a long-range effect and is roughly independent of wide variations in the polarity of other substituents, it seems reasonable to ascribe its origin to hyperconjugation. It is possible that the constitutive effect arises from variations in the excitation energy, ΔE, of Ramsay's equation.[12] This is, at present, only a speculation, however.

(12) N. F. Ramsay, *Phys. Rev.*, **78**, 699 (1950); **86**, 243 (1952).

TABLE VII

PREDICTED[a] AND OBSERVED CHEMICAL SHIFTS IN VINYL BROMIDE

Proton position	Chemical shift in c.p.s. from TMS internal reference: Predicted[a]	Obsd.
α	−254.1	−254.5
β (*cis*)	−230.6	−230.2
β (*trans*)	−234.3	−234.0

[a] After making the "constitutive" correction of −3.0 c.p.s.

The interpretation of the effect of methyl on α-protons is somewhat more complex because of complications from possible inductive and anisotropy effects.

Acknowledgments.—We wish to acknowledge the support extended to this work by the National Institutes of Health, Research Grant A-2397. The authors are greatly indebted to Dr. E. B. Whipple, Miss Virginia Hall Brinson and Mr. Richard T. Hobgood, Jr., for their valuable assistance in several phases of this study. The computations required were carried out on an LGP-30 Digital Computer provided through a grant from the National Science Foundation.

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Tracer Electrophoresis. V. The Mobility and Charge of Human Serum Albumin at Low Concentrations and Low Ionic Strengths[1]

BY ESTELLA K. MYSELS AND KAROL J. MYSELS

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It is shown that the open tube tracer electrophoresis method yields accurate and precise values for the mobility of human serum albumin if radio-iodine tagged albumin is used as the tracer. The method may be used over a very wide range of protein and buffer concentrations. ζ potentials and charges are calculated from the data and are found to agree well with titration charges (pH = 8.6, veronal buffer) if a radius of 34 Å. is assumed for the hydrodynamically equivalent sphere, provided a moderate expansion of the sphere at very low ionic strength is allowed for. Evidence for such expansion from viscosity data is presented. In the more concentrated buffer solutions the electrophoretic charges agree very closely with the titration charge. However, it is much lower than the net charge of the protein if one takes into account the anion adsorption. The possible role of sodium ion adsorption in this compensation of charge is discussed briefly.

Introduction

The electrophoretic mobilities of proteins have been studied widely over the years and the results have been used extensively for analytical and also for preparative purposes.[2] On the other hand, some difficulty has been experienced in their theoretical interpretation.[3] In particular it has been difficult to correlate the charge of the protein as obtained by titration and ion binding studies with the values calculated from electrophoretic mobilities. A serious stumbling block has been the dearth of data in sufficiently dilute solutions, both with respect to protein and to buffer concentrations.[3] The required data are difficult or even impossible to obtain by the conventional methods. However, the tracer method first developed by Hoyer, *et al.*,[4] readily permits determination of electrophoretic mobilities over a very wide range of conditions, provided only that the protein may be suitably tagged. Human serum albumin is available tagged with iodine-131 and this material behaves essentially as the non-iodinated one with respect to its electrophoretic properties. It was therefore decided to study the electrophoretic behavior of this protein in some detail. Veronal buffer at pH 8.6 was chosen as the medium for most experiments.

Experimental

The experimental method was essentially that described in detail previously.[4,5] Briefly, a horizontal capillary tube (central compartment) was filled with the protein solution of known radioactivity and the anode and cathode compartments were filled with an identical but untagged solution

(1) This investigation was supported by Research Grants RG 4013 and E 1422 from the National Institute of Allergy and Infectious Diseases, Public Health Service. The results were presented in part at the American Chemical Society meetings in New York, September, 1957, and Atlantic City, September, 1959.

(2) See for example, M. Bier, "Electrophoresis," Academic Press, Inc., New York, N. Y., 1959.

(3) J. Th. G. Overbeek, "Advances in Colloid Science," **3**, 97 (1950); see also ref. 2, chapters 1 and 8.

(4) H. W. Hoyer, K. J. Mysels and D. Stigter, *J. Phys. Chem.*, **58**, 385 (1954).

(5) K. J. Mysels and C. I. Dulin, *J. Colloid Sci.*, **10**, 461 (1955).

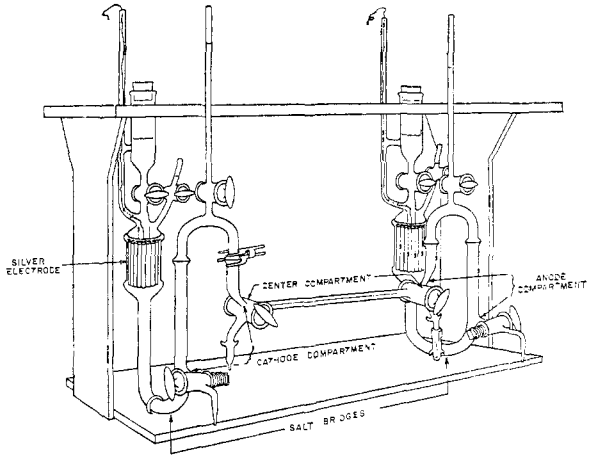

Fig. 1.—The electrophoretic apparatus; details such as braces and clamps have been omitted.

and were connected by salt bridges to Ag/AgCl electrodes. After a measured amount of current had passed through the cell, the compartment contents were analyzed separately for radioactivity and the mobility, μ, was calculated according to the relation

$$\mu = \frac{kv}{it} \qquad (1)$$

where it is the amount of electricity which passed in time t through the solution, k the conductivity of the solution and v the volume swept by the tracer.[5]

Some details and modifications of procedure required by our present study are given below.

Electrophoretic Method.—Because of the low electrophoretic mobility of the protein the lengths of the capillaries were reduced to about 20 cm. Thus, with a potential of 325 volts applied across the apparatus, the potential drop was about 16 volts/cm.

Since a large volume of tagged solution facilitates analysis, we used a 3 mm. capillary having a volume of 1.725 ml. for the highly dilute solutions, where heat dissipation is not a problem. For more concentrated solutions, a 2 mm. capillary having a volume of 0.761 ml. was substituted. Under these conditions an air thermostat kept at 25.00 ± 0.04° provided adequate heat dissipation for veronal buffer solutions of up to 0.05 *M*. However, for still higher concentrations a water thermostat was required. This necessitated some changes in the cell. The mercury cups used by Hoyer, *et al.*,[4] were dispensed with. Instead, the outlets from the capillary were fused to inner ground glass joints so that they could be protected against contamination by capping with the corresponding outer joint. The ends of the inner joints were drawn out so that they formed a smooth tip. The observation tubes and glass tubing enclosing the lead wires were extended well above the surface of the bath and the cell was rigidly mounted on a Lucite holder. The whole assembly is shown in Fig. 1.

The use of a water thermostat introduced the familiar problem of electrical leakage to the bath. Careful experimental technique, including re-greasing stopcocks and joints with Peters–Van Slyke grease (Fisher Scientific Co.) for every run, was necessary to obtain resistances to the bath sufficiently high to make any current bypass negligible for our purposes. In fact, a water-bath could not be used with the highly dilute solutions because the resistance in the capillary became comparable to that of the unavoidable electrical leaks.

The current was applied for a sufficiently long time to allow about 45% of the original material to migrate out of the capillary. This required 0.5 to 2 hr. depending on conditions. No change in mobility could be detected if the extent of migration was reduced by half, indicating that the radioactive migrating material was electrophoretically homogeneous.

About 200 ml. of protein solution was required for one determination, including the conductivity measurement. The volume of the cell occupied by protein solution was about 35 ml.

Counting.—A well type crystal scintillation counter in conjunction with a Nuclear-Chicago Radiation Analyzer Model 1810 was used for all radioactive counting. Its sensitivity was determined to be 600 counts/sec. for 1.0 μ curies of I^{131} dissolved in 10 ml. of buffer with the instrument operating at a 1 volt window. In practice a 5 volt window usually was used to minimize the effect of drift. Under these conditions the background count was about 1.6 counts/sec. The sample counts ranged from about fifty to several hundred counts/sec. Each sample was timed repeatedly for at least 10,000 counts, so that its activity was known to better than 1%.

The size of the sample was determined by weight and was of the order of 10 g. The effect of the individual test tubes and of the level of solution in each upon counting rate was determined and corrected for. The correction for the decay of I^{131} during counting usually was negligible.

With these precautions, recovery from the electrophoresis apparatus was within 1–2% for the radioactive material, except for a few samples of very low activity, where the discrepancy was about three times as great.

Calculation.—The value of the volume v swept by the tracer was obtained by averaging the four ways of evaluating it as discussed by Mysels and Dulin.[5] This introduced only a minor improvement over the simple calculation using the initial and final activity in the central compartment since the material balance was good and electroösmosis usually negligible, *i.e.*, the activity of the cathode compartment was always small, reaching a maximum of 1.3% of the total for the most dilute buffer.

Materials and Solutions.—The human serum albumin was kindly supplied by the Cutter Laboratories. The bottles were marked "salt-poor" and contained a 25% albumin solution.

The serum albumin tagged with I^{131} was the RISA product marketed by the Abbott Laboratories. The solutions are about 1% with respect to protein. Fresh shipments were received every two weeks. No effect of aging was noted within this time provided appropriate precautions were taken as noted below.

The veronal buffer was prepared from Merck chemicals. Other materials were standard reagent grade.

For a typical run using 0.01% protein concentration, the solutions were prepared as follows:

The original human serum albumin solution was diluted to 0.1% with the desired buffer. Fifty ml. of the resulting solution were dialyzed in the refrigerator against several 450 ml. portions of buffer in a rotating jar, with a glass bead providing for stirring of the inner solution. The dialyzed solution finally was diluted to 0.01% with the same buffer, the final concentration being determined by ultraviolet spectrophotometry when desired. A portion of the solution then was "spiked" with 5% by volume of a RISA solution that had in turn been dialyzed against and been diluted to the appropriate protein concentration with the buffer.

This procedure resulted in a spiked solution essentially identical with the normal one provided adequate precautions were taken against the absorption of CO_2. In the highly dilute solutions, such absorption significantly raises the conductivity even before the pH is greatly affected. Since the solutions are exposed to the atmosphere to a varying degree, this results in different conductivities of the tagged and the untagged solutions. This in turn leads to a continuous variation of the resistance of the apparatus during electrophoresis as one solution replaces the other in the capillary. The difficulty was overcome by handling the buffer solutions under nitrogen or in a flask equipped with an ascarite tube.

Continuous dialysis of the RISA solution proved to be essential, since the solution slowly generates a dialyzable radiactive component of much higher mobility than that of the protein. This component accounts for about 3% of the activity 1–2 weeks after shipment.

Conductivity and pH Measurements.—The pH of all solutions was measured with a Cambridge pH meter and conductivities were determined with a Jones–Dyke bridge. In most cases, the protein solution remaining after the electrophoresis apparatus had been filled was used for this purpose. However, for the very dilute buffer runs (0.004 and 0.001 *M*), we preferred to measure the conductivity using the portion of the contents of the cathode compartment nearest to the capillary after the end of the run. This was accomplished by drawing the solution directly into a spe-

cially constructed conductance cell through the three-way stopcock. The cell had a volume of 1.2 ml. and blank platinum electrodes so that it could be used dry and needed no rinsing. A correction for polarization had to be applied but was quite small. The conductance thus measured could then be used with confidence in the calculations, since the resistance in the capillary changed only slightly during the run, indicating that the conductance was essentially uniform throughout the protein solution in the apparatus and that the electrolyte from the salt bridges did not appreciably alter its composition. Furthermore, in the case of the 0.004 *M* buffer the conductance so measured agreed to better than 0.5% with that of the original solution. In the 0.001 *M* buffer solution the discrepancy was of the order of 5%.

Viscosity Measurements.—An Ostwald type viscometer as modified by Jones and Fornwalt[6] was used in this work. The flow time was about 1040 seconds. The viscometer was always charged with approximately 10 ml. of liquid and the exact final level in the reservoir arm of the instrument was determined with a traveling microscope. The effect of this level was determined empirically and the appropriate correction applied to each run. It amounted to 3.58% of flow time per cm. The thermostat was held at 25 ± 0.002° for most runs. All solutions were filtered through millipore filters before being admitted to the viscometer.

The flow time of the most dilute protein solutions was only about five seconds longer than that of the corresponding buffer solutions. Accurate timing was therefore essential. Each charge was timed at least three times. A stop watch graduated in 0.2 seconds and an electric clock graduated in 0.1 seconds were available for timers. Both were started and stopped manually. Surprisingly, the stop watch gave somewhat more precise results. Evidently, the fluctuations of the 60 cycle state-wide main line frequency exceeded at times several tenths of a second in 1000 seconds.

The most serious difficulty was posed by the requirement that the solutions wet the glass walls efficiently. This was not only necessary to insure complete drainage but also because finite contact angles of the advancing meniscus result in very appreciable changes in driving force and therefore flow time of the liquid.[7] Unfortunately, solutions of veronal buffer appear to have a marked tendency to render glass hydrophobic and very frequent cleaning was required to obtain satisfactory results.

Paper Electrophoresis.—The apparatus used was an E-C electrophoresis apparatus, Model EC 401. The power supply was a Heathkit PS 3. The temperature of the cooling water was near 25° and the temperature of the paper was less than 2° higher as shown by the change in total resistance.

The paper strips were dyed with brom phenol blue following the method given in the E-C manual. They were radioautographed by pinning them to Kodak No-Screen Medical X-ray Safety film for a suitable length of time.

Radioactivity was scanned by means of an improvised recording slit-geiger counter. For the more accurate work the paper strips were cut into narrow sections and each section was counted by means of the scintillation counter already described.

For colorimetric measurements each section of the cut-up strip was eluted with a 2% sodium carbonate solution in 50 % methanol,[8] and the resulting solutions were measured with a Beckman DU spectrophotometer.

In most runs a mixture of five parts of normal albumin to one of the iodinated material was used. The total protein concentration was usually of the order of 2–3%. The total volume varied from 1.5 to 30 μl. depending on the purpose of the experiment.

The voltage drop was usually of the order of 10 volts/cm. The albumin was allowed to migrate about 10 cm. in the majority of runs, but the distance was varied up to 18 cm.

Two filter papers were used: Whatman 3 MM and Munktell "filter paper for electrophoresis" as supplied by the Braun Co. Experiments were done at *p*H = 7.7 (phosphate buffer) and *p*H = 8.6 (veronal buffer).

Results

Electrophoretic Identity of RISA and Human Serum Albumin.—It is essential for the success of the tracer method that the tagged protein migrate at the same velocity as the normal one. Abbott Laboratories states that the electrophoretic pattern of its RISA is not significantly different from that of the albumin from which it has been prepared.[9] On the other hand, Gabrieli, *et al.*,[10] found by paper electrophoresis that the bulk of their sample of RISA migrated considerably faster than unaltered serum albumin and that it also contained slower moving components. We therefore repeated the paper electrophoretic experiments using mixtures of RISA and untagged albumin.

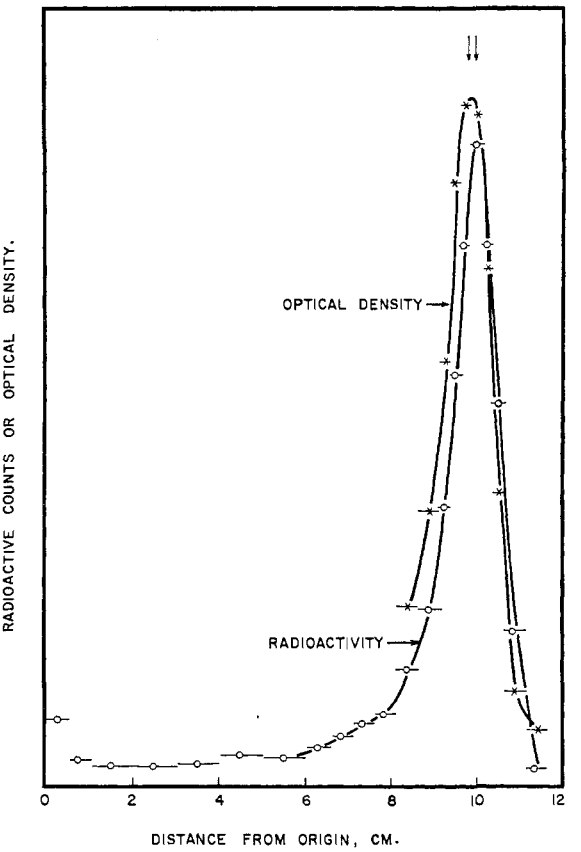

Fig. 2.—Paper electrophoretic migration of human serum albumin and its radio-iodinated analog. The horizontal lines show the widths of the strips analyzed.

All our experiments yielded dyed strips that visually corresponded exactly to their radioautographs, irrespective of buffer system or filter paper used. The only measurable difference was found in two very long runs at *p*H 8.6, one using Munktell paper and the other Whatman 3 MM paper with total migration of 16.5 and 18.2 cm., respectively. Here the radioactive component seemed to be located about 2 mm. ahead of the bulk of the protein. A similar effect was observed in two shorter runs when the paper strips were examined section by section. Fig. 2 shows that here again the peak of radioactivity appears about 1–2% ahead of the peak of optical density.

This 1–2% difference is a great deal smaller than that reported by Gabrieli, *et al.* We also

(6) G. Jones and H. J. Fornwalt, *J. Am. Chem. Soc.*, **60**, 1683 (1938).

(7) H. B. Bull, *J. Biol. Chem.*, **133**, 39 (1940).

(8) J. Hardwicke, *Biochem. J.*, **57**, 166 (1954).

(9) Abbott Laboratories' pamphlet on "RISA," 1954.

(10) E. R. Gabrieli, D. Goulian, Jr., T. Kinersly and R. Collet, *J. Clin. Invest.*, **33**, 136 (1954).

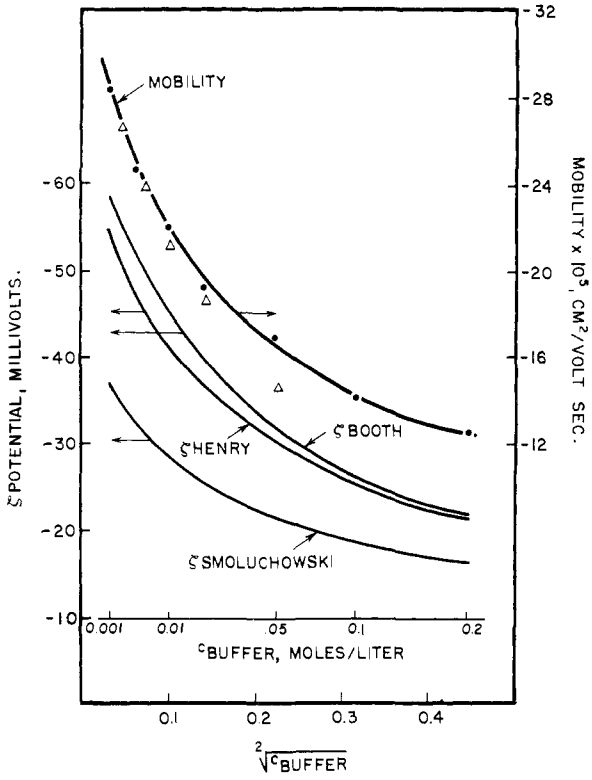

Fig. 3.—The electrophoretic mobility of albumin (right scale) and its ζ potential (left scale) as a function of buffer concentration: ●, our results; △, Möller's extrapolated values[12]; the ζ potentials were calculated according to several theories as indicated in the figure.

found no evidence for the slower moving radioactive component claimed by these authors. There was definite trailing of radioactivity in all our runs, but it extended remarkably evenly over the whole length of the path and seems to be accounted for by the well known trailing of normal albumin.[5]

We concluded therefore that RISA and normal serum albumin are sufficiently close in electrophoretic properties for our purposes since a 2% difference in mobility should have no influence on our conclusions.

It might be noted in passing that we observed marked inhomogeneities of the filter paper with respect to its adsorptive power which were particularly pronounced for the Munktell paper. It is possible that this accounts in part for the differences between our and Gabrieli's results. It should also be noted that Gabrieli worked with normal human blood serum while we used the serum albumin fraction.

Mobilities.—We first investigated the effect of protein concentration upon the mobility at several buffer concentrations. In 0.1 M buffer solution no change in mobility could be detected between 1 and 0.001% of protein concentration. However, in the more dilute buffer solutions, the effect becomes appreciable. Thus, in a 0.01 M buffer solution the mobility of the protein increases by nearly 10% as its concentration is lowered from 1 to 0.01%. On the other hand, even in the most dilute buffer used, 0.001 M, the mobility at a concentration of 0.01% of protein (1.5×10^{-6} M) was only about 1% lower than the infinite dilution value as determined by extrapolation of a plot of the mobility against the cube root of the protein concentration. We therefore concluded that mobilities determined at this experimentally convenient concentration were within experimental error of the infinite dilution values and therefore used it for further work.

Figure 3 shows the mobilities of the albumin (0.01%) as a function of buffer concentration. The changes are pronounced but seem to extrapolate to a finite value when plotted against the square root of the buffer concentration, *i.e.*, of ionic strength.

Figure 3 also shows ζ potentials calculated from experimental mobilities. The lowest line gives the values obtained by the original Smoluchowski equation based on a flat double layer. The next one gives the application of the Henry theory which takes into account the curvature of the double layer and the third line gives the values obtained from the inverse Booth's series as suggested by Stigter.[11] This takes into account also the relaxation effect.

The lowest line in Fig. 4 shows charges calculated from the ζ potential using equation 6 of ref. 11

$$Qe = \beta(1 + \kappa a)\zeta aD \qquad (2)$$

where Q is the number of electronic charges, e; D is the dielectric constant (of water), κ the inverse of the double layer thickness, a the radius of the spherical particle (taken to be 30 Å.) and β a function given by Fig. 4 of ref. 11. It corrects the Debye-Hückel theory for the effect of finite size and high potential and is between 1.02 and 1.05 in our case.

Discussion

Precision and Accuracy of the Method.—With some practice, agreement of ±1% readily is obtained for duplicate determinations of mobility except in the most dilute buffer and protein solutions. In 0.001 M buffer and 0.001% protein the average deviation of 3 determinations was 3.2%.

As to the accuracy of the method, our results may be compared to Möller's[12] extrapolated values for bovine serum albumin (Hittorf method) and published after our experimental work had been completed. As shown by the triangles in Fig. 3, there is close agreement at low ionic strength. Thus at 0.01 M salt we find $\mu = -22.0 \pm 0.3 \times 10^{-5}$ cm.²/sec. volts, while Möeller extrapolates his measurements (lowest protein concentration measured about 0.1%) to -21.1×10^{-5} cm.²/sec. volt. This is certainly within experimental error, particularly if one remembers that our results should be 1–2% too high because we really measure the mobility of the iodinated serum albumin rather than that of the native one.

The increasing spread between the two sets of data as salt concentration increases might be due to the fact that the veronal ion of our buffer is

(11) D. Stigter and K. J. Mysels, *J. Phys. Chem.*, **59**, 45 (1955).

(12) W. J. H. M. Möller, Thesis, Utrecht, The Netherlands, 1959; W. J. H. M. Möller, G. A. J. van Os and J. Th. G. Overbeek, *Trans. Faraday Soc.*, **57**, 312, 325 (1961)

adsorbed more strongly[13] than the chloride ion used by Möller.

At high ionic strength ($\Gamma/2 = 0.2$) we find in phosphate buffers at pH = 7.7 a mobility of $-12.2 \pm 0.1 \times 10^{-5}$ cm.^{2}/volt sec. at 25° and $-5.75 \pm 0.02 \times 10^{-5}$ cm.^{2}/volt sec. at 0°. The latter value is possibly somewhat high since it was determined before the need for dialyzing the iodinated albumin was recognized. It may be compared to a value for human serum albumin of -5.2×10^{-5} cm.^{2}/volt sec. tabulated by Cohen, *et al.*[14] (Tiselius method), which seems to be referred to the conductivity of the buffer. Considering the various uncertainties, this agreement is probably satisfactory. Watanabe, *et al.*[15] (Tiselius method), find a mobility of -5.84×10^{-5} cm.^{2}/volt sec. for horse serum albumin B at 2°. This would correspond to about -5.4×10^{-5} cm.^{2}/volt sec. at 0°. They also find -10.6×10^{-5} (20.8°) corresponding to about -11.8×10^{-5} cm.^{2}/volt sec. at 25°. These values agree well with ours, again remembering the higher mobility of the iodinated material.

Scope of the Method.—The tracer method is applicable over a very wide range of concentration, both of protein and of buffer salt. At the higher salt concentrations it is convenient but contributes little new because the mobilities are essentially independent of protein concentration up to those commonly used in moving boundary electrophoresis, *i.e.*, about 1%. On the other hand, in the range of low ionic strength the method allows measurements at extraordinarily low protein concentrations with acceptable precision, and the limiting factor lies in the difficulty of preparing well-defined solutions rather than in the electrophoretic or analytical procedure.

Furthermore, since the method involves no boundaries between a buffer and a buffer-protein solution, it is not limited as to the ratio of protein to buffer used in contrast to the Tiselius method.[16] For the same reason, any reaction of the protein due to changes of concentration at the boundaries is precluded.

On the other hand, our method gives only an average mobility if several tagged components are present regardless of how rapidly they equilibrate, while the Tiselius method can give separate peaks when the equilibration is slow enough. In our method the only effect would be an earlier decrease in computed mobility with increasing volume v swept by the tracer than expected for a single component (*cf.* Fig. 3 of ref. 4).

ζ Potentials and Electrophoretic Charges.—Theories of electrical conductivity become increasingly complex and unwieldy as concentration and ionic charge increase. The situation is particularly pronounced for colloidal solutions where present theories for the estimation of electrophoretic and relaxation retardation presuppose infinite dilution of the colloidal electrolyte.[17] It is therefore not surprising that rather poor agreement was obtained between charges determined by titration and those calculated from electrophoretic experiments and that the discrepancies decreased at lower ionic strength (about 0.01).

Our results should correspond to infinite dilution of the colloidal electrolyte and they also extend to considerably lower ionic strength than available heretofore. They should therefore be more amenable to theoretical treatment.

Since our charge is calculated from the ζ potential, it is important that the latter be known accurately. Inspection of Fig. 3 shows that the difference between the "Henry" and "Booth" curves is always small, *i.e.*, the relaxation effect is of minor importance throughout the range and does not exceed 8% of the total. Thus, if the Booth expression as modified by Stigter and Mysels[11] is even roughly correct, the potentials should be fairly accurate, especially since they are not likely to be very sensitive to the shape or size of the molecule.

In contrast to the ζ potential, the charge is unfortunately sensitive to the shape and size (see equation 2). We first calculated it assuming a radius of a = 30 A. on the basis of a radius of 27 Å. for the dry protein (mol. wt. = 69,000, ρ = 0.734) plus a monolayer of hydration. If one uses the more commonly accepted value of about 34 Å. for the radius of the hydrodynamically equivalent sphere,[18,19] the curve is raised as shown in Fig. 4. Virtually the same result is obtained if one assumes that the molecule is an ellipsoid with an axial ratio of 4:1 of the same volume as a sphere of radius 30 Å. and with the same surface charge density. It must be stressed that this is merely a crude method of estimating the effect of such a change in shape and that an exact theoretical treatment of an ellipsoidal model still is lacking.

One must also remember that these estimates are based on the assumption that the molecule does not change shape in the range of experimental conditions. There is some evidence from viscosity studies that there is no change down to an ionic strength of 0.01.[18,19] However, there is a very good chance that the molecule eventually will expand or even unfold under the increasing electrical repulsion produced by indefinitely reducing the ionic strength.[20]

Viscosity studies are extremely difficult in this range. One is forced to extend measurements to very low protein concentrations because at the low buffer concentrations protein-protein double layer interaction must be considerable at a protein concentration of 1% by weight. One might therefore expect considerable deviation from linearity in the η_{red} against c plot. In addition, the protein will make a very substantial contribution to the

(13) I. M. Klotz and J. M. Urquhart, *J. Phys. and Coll. Chem.*, **53**, 100 (1949); the relative intrinsic binding constants are 26 and 35 for chloride ion and veronal ion respectively.

(14) E. J. Cohen, W. L. Hughes and T. H. Weare, *J. Am. Chem. Soc.*, **69**, 1753 (1947).

(15) I. Watanaba, N. Ui and M. Nakamura, *J. Phys. Chem.*, **54**, 1366 (1950).

(16) A. Tiselius and H. Svensson, *Trans. Faraday Soc.*, **36**, 16 (1940).

(17) D. C. Henry, *Proc. Roy. Soc.* (*London*), **A133**, 106 (1931); F. Booth, *ibid.*, **A203**, 514 (1950); J. Th. G. Overbeek, *Koll. Beih.*, **54**, 287 (1943); but see ref. 12.

(18) C. Tanford and J. G. Buzzell, *J. phys. Chem.*, **60**, 225 (1956).

(19) M. Champagne, *J. chim. phys.*, **54**, 378, 393 (1957).

(20) We are indebted to Professor J. W. Williams, University of Wisconsin, Madison, Wis., for drawing our attention to this point.

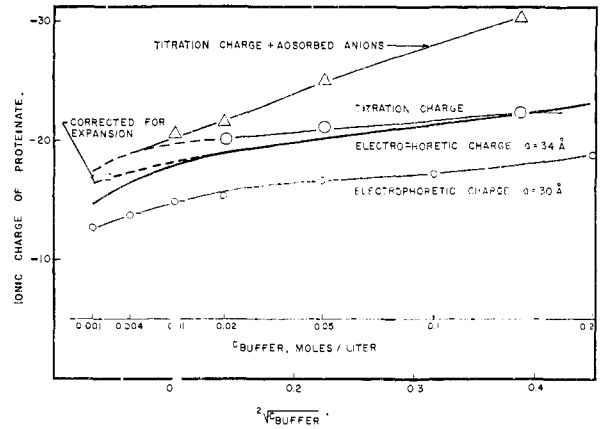

Fig. 4.—The elec rophoretic charge of albumin and its relation to the titration charge.

ionic strength at the concentrations customarily used for viscosity measurements ($1\% = 1.5 \times 10^{-4} M \approx 3 \times 10^{-3} N$ at pH = 8.6). Thus, conditions of our electrophoretic measurements would not be duplicated. For these reasons we worked with 0.1–0.5% protein solutions and have not extended our studies to less than 0.002 M buffer.

Our results are shown in Table I. They indicate

TABLE I

KINEMATIC VISCOSITY, ELECTROVISCOUS EFFECT AND EQUIVALENT RADIUS OF SERUM ALBUMIN IN VERONAL BUFFERS (pH = 8.6)

$\Gamma/2$	$[\eta]_{exp.}$	f	$[\eta]^{\zeta=0}_{calcd.}$	a (Å.)
0.1	0.040	0.03	0.039	[34.0][a]
.01	.047	.14	.041	34.6
.002	.061	.30	.047	36.1

[a] Assigned value.

some swelling though certainly no unfolding even at the lowest buffer concentration.[21] Even if the size of the molecule did not change, the electroviscous effect[22] would increase the experimental intrinsic viscosity according to the relation

$$[\eta]_{exp} = [\eta]_{calcd.}{}^{\zeta=0}(1 + f) \qquad (3)$$

where f is a function of ζ, κa, and the mobilities of the supporting electrolyte. Values of f calculated according to Booth's theory[22] for our systems are listed in the third column of Table I. If KCl were the supporting electrolyte,[18] they would be only about one third as large.

Correcting $[\eta]_{exp}$ (second column, Table I) for the electroviscous effect, we obtain $[\eta]^{\zeta=0}_{calcd.}$ (fourth column) to which the usual considerations apply. In this manner we find that the equivalent radius in 0.002 M buffer is about 6% greater than that in 0.1 M buffer. This increases the calculated charge by about 8% (equation 2, $\kappa a = 0.51$) as plotted in Fig. 4. A similar, presumably somewhat larger, increase of radius and hence charge would be expected at 0.001 M buffer. Thus the electrophoretic charge appears to be nearly independent of buffer concentration, decreasing by some 20% as the ionic strength is decreased one-hundredfold.

Comparison of Electrophoretic Charge and Titration Charge.—Tanford[23] has determined the titration charge of bovine serum albumin as a function of pH for various ionic strengths. He also states that human serum albumin shows very nearly the same titration curve. The small differences are such that they are likely to cancel by the time a pH of 8.6 is reached, so that the charges of the two albumins should become identical. Tanford's data show that near a pH of 8.6 the titration charge decreases by only one or two as the ionic strength is decreased from 0.15 to 0.03. Titration curves at lower ionic strength do not seem to be available in the literature, but Möller[12] states that the protein carries a charge of −20 at a "pH about 8.5" in potassium chloride solutions ranging from 0.05 to 0.002 N, implying that the charge for a given pH is not sensitive to salt concentration down to the 0.002 N range. It is also possible to estimate the charge in this range and even more dilute salt solutions by using Tanford's data for the intrinsic pK's and extrapolate his values for w. This indicates a decrease of charge of about 1.5 units as the ionic strength is decreased from 0.01 to 0.002 and an additional unit as it is decreased to 0.001. The resulting curve is plotted in Fig. 4, the charges being adjusted to a molecular weight of 69,000 from Tanford's value of 65,000 for the sake of consistency.

It is seen that this curve follows remarkably closely that for the electrophoretic charge. This is gratifying for the low buffer concentrations (about 0.01 M or less), where one might expect the theories to hold quite accurately. On the other hand, it poses a curious puzzle in the more concentrated buffer regions, since the well known adsorption of negative ions by serum albumin has not been taken into account. Even at a pH of 8.6 about six chloride ions are thought to be held by an albumin molecule in 0.1 M sodium chloride[24] and veronal ion should be even more strongly adsorbed.[13] Thus in this concentration range the electrophoretic charge would appear to be only about 70% of the "real" charge in substantial agreement with earlier results.

A conceivable explanation for the charge compensation of the adsorbed anions would be the association of sodium ions. We shall call a sodium ion "associated" if it moves with the protein, whether it be merely carried in the hydration sphere, held by electrostatic forces, or adsorbed by specific ones. Most workers in the field were unable to detect association of alkali ions, but Carr[25] as well as Johnson and Doremus[26] have presented evidence for it in solutions much more alkaline than ours. At pH 8.8, Carr does not detect any binding in 0.014 M Na^+. However, on the basis of Fig. 4 we

(21) Champagne observed a similar effect in water but she worked with high protein concentrations. She does not discuss this further. See ref. 19.

(22) F. Booth, *Proc. Roy. Soc. (London)*, **A203**, 533 (1950).

(23) C. Tanford, S. A. Swanson and W. S. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).

(24) See ref. 18 and 23. These values are found by extrapolation of Coleman's data; see also G. Scatchard, J. S. Coleman and A. L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).

(25) C. W. Carr, *Arch. Biochem. Biophys.*, **62**, 476 (1956).

(26) R. H. Doremus and P. Johnson, *J. Phys. Chem.*, **62**, 203 (1958).

would expect only about one sodium ion to be bound per protein molecule under these conditions and this is close to Carr's experimental error. We have made some preliminary measurements of the sodium ion mobility under our conditions using Na^{22}. In a 0.01 *M*–1% protein system an average reduction of the mobility of all sodium ions of about 5% is found, corresponding to a maximum of three sodium ions being associated per molecule of protein. This is reduced to about half if one attempts to take into account the relaxation effect.

From the available data, one estimates that about one veronal ion is adsorbed by the albumin molecule in 0.01 *M* solution and that this number increases to about eight in 0.1 *M* solutions. A similar increase in sodium ion association seems reasonable for a ten-fold increase in concentration. Thus the net charge might well stay nearly constant.

One might expect that a better experimental test for the adsorption hypothesis would be possible at the higher sodium ion concentrations, particularly since the relaxation retardation decreases for our system as the ionic strength is raised beyond 0.05 (compare Fig. 3). Unfortunately, the swamping effect of the excess sodium ions increases, making experimental studies again difficult. It thus remains to be proven whether alkali ion association does play a significant part in the control of the electrophoretic charge of the albumin molecule.

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Studies in Phosphinemethylene Chemistry. IV. The Reaction of Triphenylphosphinemethylene and Triphenylphosphinevinylmethylene with Phenylbromophosphines[1]

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The reaction of triphenylphosphinemethylene with diphenylbromophosphine produces diphenylphosphinomethyltriphenylphosphonium bromide; similar compounds are produced in the reaction of triphenylphosphinemethylene with phenyldibromophosphine and dimethylbromostibine and of triphenylphosphinevinylmethylene with diphenylbromophosphine. The bromination of $[(C_6H_5)_3PCH(CH{=}CH_2)P(C_6H_5)_2]Br$ is of interest, since the initial step apparently is the formation of the insoluble tribromide salt of this cation; the latter then is the active brominating agent which gives the product, $[(C_6H_5)_3PCH(CH{=}CH_2)P(C_6H_5)_2Br]Br_2$.

It has been established previously that triphenylphosphinemethylene reacts with halides of metals of Periodic Groups II and IV to produce organometallic-substituted phosphonium salts.[2] It was expected therefore that the reaction of triphenylphosphinealkylidenes with phosphorus halides would proceed in a similar manner.

This research showed such to be the case. Addition of diphenylbromophosphine to a solution of triphenylphosphinemethylene in ether resulted in the immediate precipitation of diphenylphosphinomethyltriphenylphosphonium bromide.

$$(C_6H_5)_3P{=}CH_2 + (C_6H_5)_2PBr \longrightarrow [(C_6H_5)_3PCH_2P(C_6H_5)_2]Br$$

Attempts to isolate this product as well as all the other phosphonium bromides prepared in this study in the pure crystalline state were unsuccessful; in all cases oils which could not be crystallized resulted when recrystallization was attempted. However, the phosphonium cations formed could be characterized by conversion of the crude bromides to a suitable insoluble derivative, such as the tetraphenylborate or the tri- or tetrabromomercurate. In the case of diphenylphosphinomethyltriphenylphosphonium bromide, an analytically pure tetraphenylborate could be prepared. However, the crude bromide reacted with two moles of mercuric bromide, since two functions capable of complexing mercuric bromide were present, bromide ion and a trivalent phosphorus atom, and the adduct of structure I was formed.

$$\left[(C_6H_5)_3\overset{\oplus}{P}CH_2P(C_6H_5)_2 \rightarrow HgBr_2\right][HgBr_3]^{\ominus} \qquad \text{I}$$

Phenyldibromophosphine reacted with triphenylphosphinemethylene to form a diphosphonium salt, $[(C_6H_5)_3PCH_2P(C_6H_5)CH_2P(C_6H_5)_3]Br_2$, characterized as the bis-tetraphenylborate. Triphenylphosphinevinylmethylene underwent an analogous reaction with diphenylbromophosphine.

These phosphonium salts containing trivalent organophosphorus substituents could be quaternized with methyl bromide to give polyphosphonium salts such as $[(C_6H_5)_3PCH_2P(C_6H_5)(CH_3)CH_2P(C_6H_5)_3]Br_3$ and $[(C_6H_5)_3PCH_2P(C_6H_5)_2CH_3]Br_2$.

Of particular interest was the bromination of $[(C_6H_5)_3PCH(CH{=}CH_2)P(C_6H_5)_2]Br$, since this compound contained at first sight two functions capable of reacting with bromine: the olefinic double bond and the trivalent phosphorus atom. The product of the bromination, which was carried out in methanol, was characterized as the complex with mercuric bromide. The analytical results fit either of two structures: $[(C_6H_5)_3PCH(CH{=}CH_2)P(C_6H_5)_2Br_2][HgBr_3]$ or $[(C_6H_5)_3PCH(CH{=}CH_2)P(C_6H_5)_2Br][HgBr_4]$. In view of

(1) This work was summarized in part at the XVII International Congress of Pure and Applied Chemistry, München, August 30–September 6, 1959; *cf. Angew. Chem.*, **72**, 36 (1960). For Part III, see D. Seyferth, S. O. Grim and T. O. Read, *J. Am. Chem. Soc.*, **83**, 1617 (1961).

(2) S. O. Grim and D. Seyferth, *Chem. and Ind. (London)*, 849 (1959); *J. Am. Chem. Soc.*, **83**, 1610 (1961).