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Electric Properties of Macromolecules. III. Kerr Constants and Rotational Diffusion of Bovine Serum Albumin in Aqueous Solutions[1,2]

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The treatment electric birefringence method was extended to the submicrosecond region for structure studies of proteins and other polymers in solution. Birefringence relaxation times and specific Kerr constants of bovine serum albumin in aqueous solutions were observed at various *p*H values, ionic strengths and BSA concentrations. The birefringence relaxation time in water at 30° and *p*H 5 was 0.20 μsec., and the specific Kerr constant extrapolated to infinite dilution was 1.3×10^{-8} e.s.u. The calculated rotational diffusion constant was 0.83×10^{6} sec.$^{-1}$, and this increased about 20% at *p*H 2.6. The Kerr constant increased on either side of *p*H 5, the isoelectric point and decreased upon addition of electrolytes. A spherical macromolecular shape is excluded by the results when considered with the known molecular weight, specific volume and degree of hydration. The rotational diffusion constant leads to a maximum dimension of 200 ± 35 Å., assuming either an oblate or prolate ellipsoid of revolution; this corresponds to an axial ratio far from unity in either case. A prolate model is consistent with the relaxation time obtained here and the harmonic mean obtained from fluorescence depolarization. The results are discussed in relation to other known physicochemical properties of BSA.

Introduction

Studies of the magnitude and time dependence of electric birefringence (Kerr electro-optic effect) have yielded information on the size, shape and electrical properties of large, rigid macromolecules during the last ten years. Up to the present time this method has been applicable only to very large, anisometric molecules such as tobacco mosaic virus,[3-6] fibrinogen,[7,8] sodium thymonucleate[9] and high molecular weight preparations of poly-γ-benzyl-L-glutamate.[10,11] The lengths of these molecules are of the order of a thousand ångströms and the birefringence relaxation times are the order of 100 μsec.

It was desired to extend the transient electric birefringence technique to smaller macromolecules, especially the proteins, with lengths of the order of 100 Å. A tenfold size reduction requires a 1000-fold increase in speed of application of the polarizing field and of optical response, other things like macromolecular shape and solvent viscosity remaining constant. Further, a large increase in field strength is required to obtain comparable signals, since the interaction of a molecule with the electric field depends upon its dipole moment which is related to its size and its polarizability which is directly proportional to its size. Since suitable electronic equipment was not commercially available, its development was undertaken and this is reported briefly here. Modifications of the optics also were incorporated, to extend the versatility and sensitivity of the apparatus. These are outlined briefly.

In this investigation, bovine serum albumin (BSA) has been studied as a function of *p*H, ionic strength and a variety of treatments. BSA was chosen as the first protein for investigation because it has been characterized extensively by other techniques. Thus, many of its physico chemical properties are known, although there is disagreement about its size and shape. The present results decisively reject the possibility of a rigid spherical model and are approximately consistent with a rigid prolate ellipsoidal model with an axial ratio of 7. They also suggest, when considered with other data, that the molecule must be less symmetric than an ellipsoid of revolution.

(1) Based upon the thesis submitted by S. Krause in partial fulfillment of the requirements for the Ph.D. in Chemistry, September, 1957.

(2) Presented in part before the Division of Physical Chemistry at the 133rd Meeting of the American Chemical Society in San Francisco, Calif., April, 1958.

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Experimental

Apparatus.—A high speed electronic pulser, capable of delivering rectangular pulses at over a megawatt peak power, was designed and constructed. It employed a 5C22 hydrogen thyratron in a delay-line circuit using RG8/U coaxial cable. The 5C22 was triggered by a similar stage employing a 2D21 thyratron, which was energized by an external pulse generator started with the flash contacts of the recording camera. The specially designed delayline circuit is shown in Fig. 1 because it is simple and can be used in other chemical applications, *e.g.*, studies of high-field conductance, dielectric saturation and flash photolysis. With a 500 foot cable and a high voltage supply variable to 16 kv., the circuit generated rectangular voltage pulses of 1.7 μsec. duration and variable amplitude to 8000 v. The pulse appeared at a resistive element of 52 ohms, across which the Kerr cell was connected with short leads; thus, the peak power exceeded a megawatt.

The five ohm section of the load consisted of two ordinary 2 watt, 10 ohm carbon resistors in parallel. The 47 ohm resistor was a 10 watt International Resistance Corporation Type MPP high frequency resistor. Sprague non-inductive wirewound resistors introduced enough reactance into the circuit to cause noticeable ringing on the top of the pulse, and Continental Carbon 5 watt type NR-50 metal film resistors were found unsatisfactory because they increased in resistance after each pulse. During the 1.7 μsec. of the pulse, the 47 ohm resistor had to withstand a momentary 100,000-fold overload. Nevertheless, the same resistor performed satisfactorily for the total period of operation of the pulser. A cylindrical copper shield was placed around the thyratron and the coaxial shields carrying pulse current were soldered directly to this shield to keep the loop impedance low.

The optical system was similar to the ones described previously[3,4] with the following changes: (1) the entrance system was arranged so either a tungsten source with filters or a monochromator (Beckman DU) could be employed; (2) the retardation device was a new arrangement consisting of two λ/8 fused quartz prisms, which give the desired λ/4 retardation without displacement of the optic axis; (3) a longer cell (5 cm.) was designed to increase the sensitivity, but it was not required for the results described here; (4) an end-window photomultiplier was used; (5) a very fast cathode-follower stage was employed at the photomultiplier output so instrument response time could be adjusted down to about

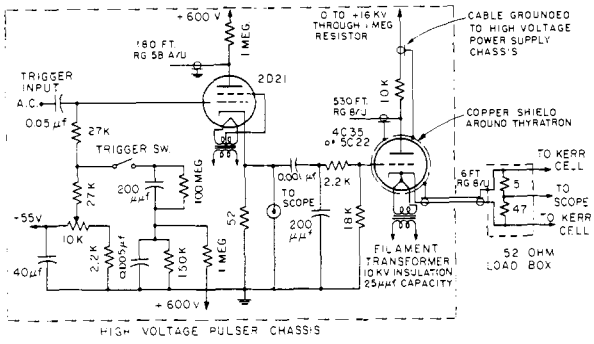
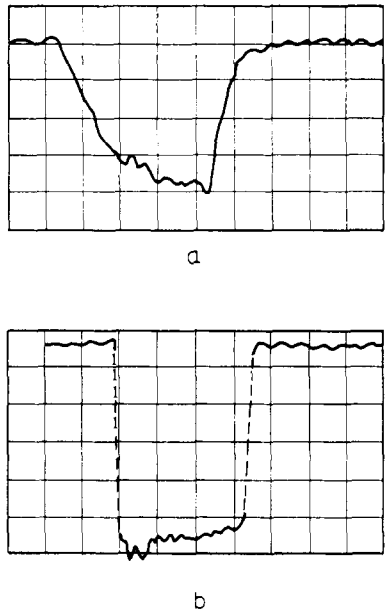


Fig. 1.—Delay-line pulser for fast birefringence studies.

3×10^{-8} sec. Extreme care was taken to minimize distributed capacitance and inductance. The multiplier was magnetically shielded with two layers of "fernetic" shielding obtained from the Perfection Mica Company, Chicago, Illinois.

The decay time of the rectangular pulses was around 0.01 μsec. to 50% and 0.1 μsec. to 25%; the 10 to 90% rise time was 0.05 μsec. Thus, birefringence relaxation times down to 0.1 microsecond could be measured. This corresponds to a rotational diffusion constant of 1.7×10^6 sec.$^{-1}$. The electrodes and Kerr cell employed for this study were those described by Pytkowicz and O'Konski.[12] The electrode separation was 0.21 cm., so fields up to about 35,000 v./cm. were achieved.

The birefringence transients were displayed on a Hewlett-Packard Model 150A oscilloscope and photographed with an Exacta reflex prism camera, with an f/1.5 lens, employing Kodak Linagraph Pan film. A typical BSA birefringence signal is shown in Fig. 2a. To show that the transient characteristics of the signal are caused by the BSA and not by time constants in the electronic circuits, a birefringence signal typical of nitrobenzene or aqueous urea solutions is shown in Fig. 2b.



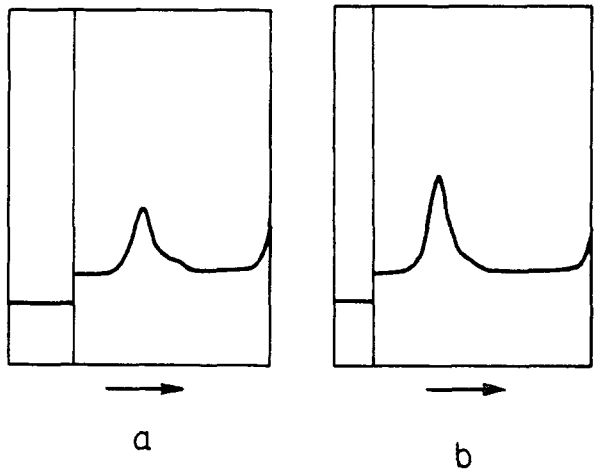
a

b

Fig. 2.—(a) Birefringence pulse from BSA solutions; (b) birefringence pulse from urea solutions.

Characterization of the BSA.—Two samples of BSA were studied. One was from Armour Laboratories bovine plasma albumin lot No. P67908; the other was Pentex Incorporated bovine serum albumin lot No. B12016P. These samples were crystalline and were used without further purification for some of this work: those preparations will be referred to as BSA, untreated. Two solutions of Pentex BSA and one of Armour BSA were deionized by passing through an ion-exchange column as first described by Dintzis.[13] These will be referred to as BSA, deionized. The concentration of the deionized BSA was determined gravimetrically by evaporating the water from a weighed amount of solution in an oven at 103–107° and then weighing the residue. In agreement with Timasheff and co-workers,[14] it was found that this method agrees with one based on the ultraviolet absorption of the molecule at 279 mμ within 1%. It was also found that the ultraviolet absorption spectrum of the BSA, deionized, from 245 to 305 mμ, was in excellent agreement with that obtained for untreated BSA by Rideal and Roberts.[15]

Figure 3a is a photograph of the ultracentrifugation pattern of BSA, untreated, after 56 minutes; Fig. 3b is a photograph of the pattern of BSA, deionized, after 56 minutes. The patterns are almost identical except for the difference in peak height caused by the concentration difference between the two samples. Both show evidence of a small amount of material with sedimentation constant higher than the value of the main component; this has been noticed in other samples of BSA by many other workers.[14,16–20] Champagne[16] estimated that this impurity comprised about 8% by weight of her sample. For the untreated BSA, $S_{20,w} = 3.89$, and $S_{20,w}$ was 3.85 for the deionized BSA. These values are comparable to those found under similar conditions by other workers.[16,17]



a b

Fig. 3.—Ultracentrifugation pattern of BSA; a, untreated; b, deionized.

Calculation Procedure.—The attenuated applied voltage pulse and the birefringence signal were always photographed across an illuminated grid on the oscilloscope face in rapid succession, keeping all experimental conditions constant. The vertical calibrations of the oscilloscope were checked against an internal calibrator before each run. Once a month this calibration was checked against batteries whose voltages had been determined potentiometrically within 1%. It remained constant to ±2%. The applied voltage was obtained from the photograph and not from the setting of the high voltage pulser because it depended somewhat on the conductance of the Kerr cell containing the solutions, which at times reached a substantial percentage of the terminating resistor conductance. The possible procedure of varying the terminating resistor to keep the output load constant was inconvenient and was not followed here.

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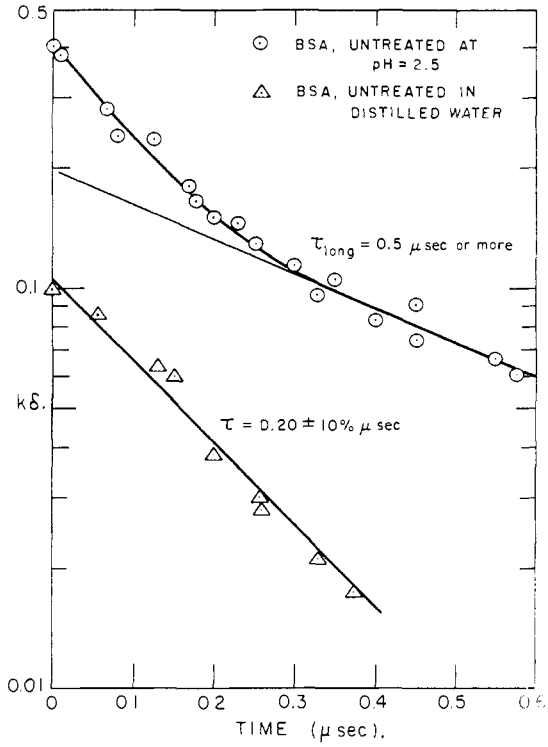

Fig. 4.—Log δ *vs.* time for untreated BSA.

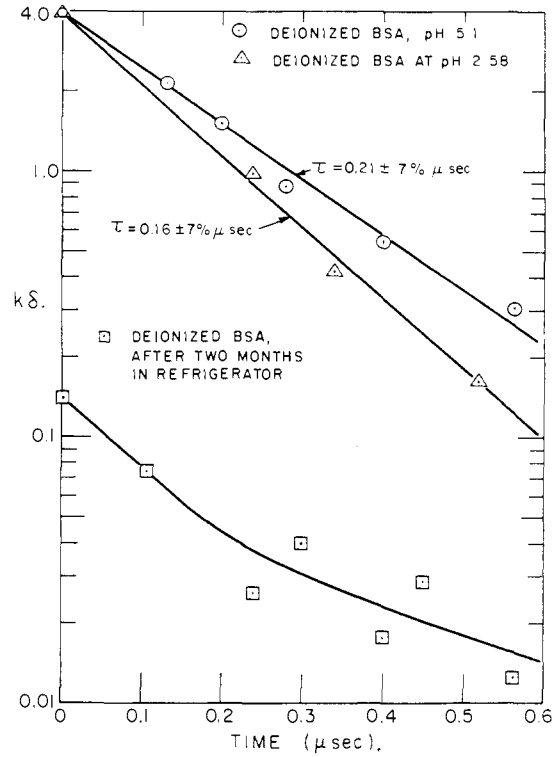

Fig. 5.—Log δ *vs.* concentration for deionized BSA.

Calculations of optical retardation were handled in the way described previously by O'Konski and Haltner.[4] The equation[21] of the optical system employing a $\lambda/4$ retardation device is

$$\Delta I_\delta / I_0 = [\sin(2\theta - \delta) - \sin 2\theta]/2 \qquad (1)$$

where the symbols have the following significance: ΔI_δ is the change of intensity of light passing through the analyzer as a result of the retardation; δ is the retardation, in radians, of the component of polarized light parallel to the applied field with respect to the perpendicular component, upon traversing the cell; I_0 is the light intensity, not including stray light, which would pass the analyzer if polarizer and analyzer were parallel and δ were zero; θ is the angle of the analyzer, all angles being measured with respect to the direction of the applied field. The equation was derived for an optical system containing a $\lambda/4$ retardation prism with slow axis at $3\pi/4$; the polarizer is at $\pi/4$ radians.

The magnitude of the applied voltage was found from the photographs of the applied voltage pulse. The birefringence relaxation times were found from plots of log δ *versus* time in the usual manner.[4] The relation between the birefringence relaxation time, τ, and the corresponding rotational diffusion constant, Θ is[3,7,9]

$$\Theta = 1/6\tau$$

Specific Kerr constants were calculated from the steady-state value of the retardation after the birefringence buildup was complete, employing the relation[5]

$$K_{sp} = \lambda\delta/2\pi lCnE^2 \qquad (2)$$

where λ is the mean wave length of the incident light *in vacuo*, l is the length of the electrodes, C is the volume concentration of the BSA, n is the refractive index of the solution and E is the value of the applied field. The cgs. system was used in all calculations. In this work, l was 1 cm. and λ was the mean wave length of the tungsten light source, 5400 Å.[5] C was calculated from the known weight concentration of the BSA and its partial specific volume, 0.734.[19]

Before inserting a value of δ in equation 2, a correction for the birefringence of the solvent was made, since the electric fields used in this work were sufficient to produce perceptible birefringence in pure water. The birefringence of pure water was subtracted from the experimental values for the solutions, as BSA concentration was low, seldom exceeding 1%. The correction was generally from 1 to 5% except at the lowest BSA concentrations where it went as high as 50%.

(21) The previously given equation,[3] as subsequently corrected for a typographical error,[4,5] is an approximation valid only for small δ. However, all data of this Laboratory were computed with the aid of curves constructed from the exact equation 1.

Results

Figures 4 and 5 show log δ *versus* time plots for BSA under various conditions. Values of τ found from the plots are given on the graphs. The points in some of the log δ *versus* time plots have been moved up or down an arbitrary distance to allow several decay curves to appear on the same graph, so the ordinates are labeled $k\delta$ instead of δ. Moving the points up or down does not affect the relaxation times as found from the graph.

Figure 6 shows the concentration dependence of the specific Kerr constant of BSA. The untreated BSA was dissolved in distilled water while the deionized BSA was dissolved in deionized water. The experimental data for BSA, deionized, were taken on two samples of Pentex and one of Armour BSA. The more dilute solutions were obtained by diluting the more concentrated solutions. It can be seen that the specific Kerr constants of untreated and deionized BSA both approach $1.3 \pm 0.2 \times 10^{-8}$ e.s.u. as the concentration of the BSA approaches zero at 30°. The specific Kerr constant of deionized BSA rises with increasing concentration, whereas that of untreated BSA decreases with increasing concentration.

Figure 7 shows the *p*H dependence of the specific Kerr constant of deionized BSA at 30°. The concentration of all solutions measured for this plot was either 6.5 mg. or 6.9 mg. BSA/ml. The added electrolyte was phosphoric acid, one of the potassium phosphates, potassium chloride or zinc chlo-

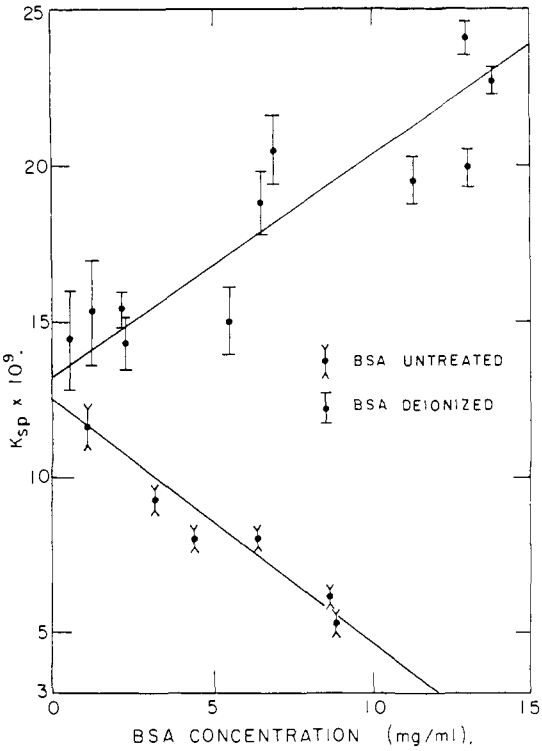

Fig. 6.—K_{sp} *vs.* concentration for BSA.

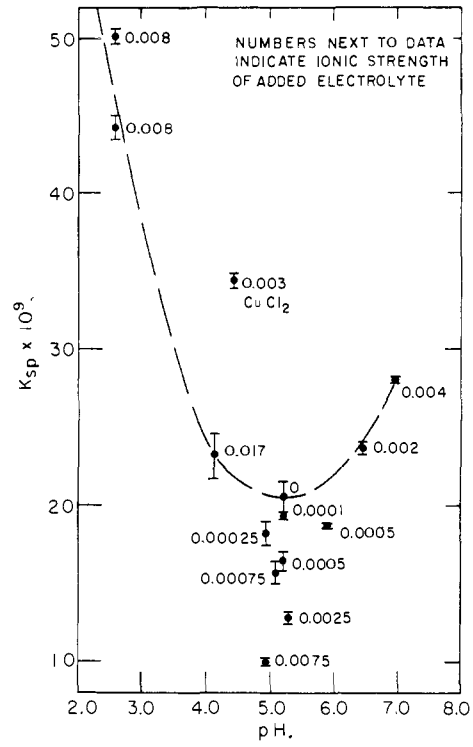

Fig. 7.—K_{sp} *vs.* *p*H for BSA, deionized.

ride in all cases except for the point labeled $CuCl_2$. The dashed curve is drawn through the maximum measured value of the specific Kerr constant at each *p*H. As the ionic strength of the solution was increased at any *p*H, the specific Kerr constant of the BSA decreased. Since it is not possible to change the *p*H of BSA solutions without adding an appreciable amount of electrolyte, the dashed curve is probably close to the maximum possible specific Kerr constant of BSA in the *p*H range studied. Probable reasons for the anomalous position of the point labeled $CuCl_2$ will be discussed below.

The ionic strength dependence of the specific Kerr constant of BSA is shown in Fig. 8. The experimental data used for this plot are the same as those in the *p*H range 4.9 to 5.3 in Fig. 7. The specific Kerr constant of BSA does not change appreciably with *p*H in this range.

Discussion

Size and Shape of the Macromolecule.—If the log δ *vs.* time plot is a straight line, one obtains only one relaxation time. This implies a single rotating macromolecular species in the solution. Most of the log δ *vs.* time plots for BSA (Figs. 4 and 5) show a single relaxation time within the experimental accuracy. The additional relaxation time expected from the small amount of faster sedimenting impurity revealed by the ultracentrifuge was not observed, possibly because the amount of birefringence produced by the heavier component was small.

When untreated BSA was acidified to *p*H 2.58, a longer relaxation time, about 0.5 μsec., was observed in addition to the usual shorter one. This longer relaxation time did not appear when deionized BSA was acidified to *p*H 2.58. These results in acid solution can be explained by aggregation of the untreated BSA aided by impurities in the sample, possibly fatty acids, which Dintzis[13] suggested are removed from BSA by the type of ion-exchange column used in this work.

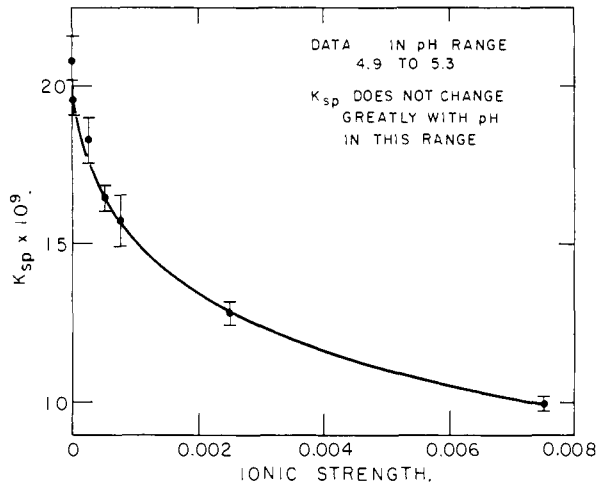

Fig. 8.—K_{sp} *vs.* ionic strength for BSA, deionized.

Previous work on the low *p*H properties of BSA corroborates this view. Foster[22] also attributed the occurrence of aggregation to the action of impurities believed to be fatty acids. Kronman, Stern and Timasheff[17] found, by a study of molecular weights in solution using light scattering, that untreated BSA aggregated at *p*H 4.5 and lower but that deionized BSA did not aggregate at *p*H 3.0 if deionized at 4°, although BSA deionized at room temperature aggregated at this *p*H. (In the

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present work, the BSA was deionized in a refrigerator below 4°.) The disappearance of the aggregation at low *p*H upon deionization of the sample also was observed by Champagne,[23] Charlwood and Ens,[24] Saroff, Loeb and Scheraga[25] and Bro, Singer and Sturtevant.[26]

The lowest curve in Fig. 5 shows that deionized BSA, after standing for two months in the refrigerator in a glass-stoppered volumetric flask, became heterogeneous as far as relaxation times were concerned. For this reason, samples were stored in a refrigerator and were never used in this research more than two weeks after deionization.

The relaxation time at 1% concentration, of BSA, untreated, in distilled water at 25° and *p*H 5 was 0.20 μsec. This is equal within experimental error to that of BSA, deionized, in deionized water at 30° and *p*H 5.1. The corresponding rotational diffusion constant is 8.3×10^5 sec.$^{-1}$.

Using this rotational diffusion constant, the dimensions of BSA were computed from Perrin's equations[27] for rigid ellipsoids of revolution. The assumed models and the results are presented in Table I.

TABLE I

DIMENSIONS OF BSA COMPUTED FROM THE BIREFRINGENCE DECAY

Model assumed	Vol. of single molecule (Å.^{3})	Calcd. dimensions $2a$(Å.)	$2b$(Å.)	$p = a/b$
Sphere	1,050,000	126	126	1
Unhydrated prolate ellipsoid	81,000	250	25	10
Unhydrated oblate ellipsoid	81,000	5.4	168	$(31)^{-1}$
Hydrated prolate ellipsoid	171,000	220	38	6
Hydrated oblate ellipsoid	171,000	12	165	$(14)^{-1}$
Hydrated prolate ellipsoid	130,000	235	33	7

The volume of the sphere follows directly from the rotational diffusion constant since this quantity depends only upon the radius of the sphere, the temperature and the viscosity. A spherical shape is clearly improbable as it would require hydration of about 10 g. H_2O/g. protein, which is excessive. The hydration is about 0.5 g. H_2O/g. protein, according to results of Ritland, Kaesberg and Beeman,[28] Champagne[23] and Buchanan and co-workers.[29]

The volume of the unhydrated molecule, 81,000 Å.^{3} was calculated from the partial specific volume of 0.734[19] and the molecular weight of 67,000.[16,30-34] Higher molecular weights have been reported for BSA,[35-38] but in those studies no attempt was made to correct for the heavier impurity. The volume of 171,000 Å.^{3} for the hydrated ellipsoid was taken from Champagne,[23] whose value is based on sedimentation constant, intrinsic viscosity and translational diffusion measurements. Recently, Champagne[39] found a volume of 130,000 Å.^{3} for the BSA molecule at *p*H 5.1 by low angle X-ray scattering. It is interesting that the essentially equal value of 137,000 Å.^{3} is computed if one employs the hydration value[28,29] of 0.5 g. of H_2O/g. protein, assuming the density of hydrated water is unity. The unhydrated models of Table I are not realistic but are included to illustrate the rather small dependence of the longer dimension upon the degree of hydration. The longer dimension is about 200 Å. whether the ellipsoid is oblate or prolate. The prolate model is strongly preferred for reasons discussed below, and the last model of Table I is our best estimate of the molecular size and shape.

Tanford and Buzzell[40-41] concluded from a comparison of their intrinsic viscosity data on BSA with other hydrodynamic data, such as translational diffusion coefficient, in the manner suggested by Scheraga and Mandelkern,[42] that BSA is either not rigid or cannot be an ellipsoid of revolution. There is a factor, β, in the Scheraga and Mandelkern treatment, which has a minimum value of 2.12×10^6 for spheres and increases for both prolate and oblate ellipsoids. For BSA, $\beta = (2.04 \pm 0.06) \times 10^6$. Loeb and Scheraga[43] also found a low value of β using some of their own measurements, and assumed that BSA was a sphere. Champagne,[23] on combining her data for intrinsic viscosity, sedimentation constant and translational diffusion coefficient, found an anomalously low value for a factor similar to β. She attributed this to experimental error, although admitting that BSA might not be an ellipsoid of revolution at all and assumed a spherical shape for BSA. Harrington and colleagues[33] have suggested that the anomalously low value of the Scheraga and Mandelkern coefficient was caused by approximations in the equation for intrinsic viscosity, rather than an odd shape of the BSA molecule.

Non-hydrodynamic methods give non-ellipsoidal shapes for BSA. Low[44] on the basis of X-ray measurements on crystalline human serum albumin, which is very similar to BSA in most physical prop-

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erties,[45] attributed an asymmetic form with a preferred length of 138 Å. to this molecule. Anderegg, Beeman, Shulman and Kaesberg,[46] in order to reconcile Low's X-ray studies with their own work on BSA solutions by low angle X-ray scattering, proposed a rectangular parallelepiped structure for BSA with dimensions 82.5 × 22.5 × 63 Å.; they suggested that the best equivalent ellipsoid was oblate with axial ratio $(3.5)^{-1}$. The former study is in better accord with our result, as it is clear that the longest dimension must be greater than 126 Å., the diameter of the rigid sphere in Table I.

If BSA has a shape less smooth than that of an ellipsoid of revolution, its longest dimension would be somewhat smaller than the 235 Å. calculated for the prolate ellipsoid, because of more resistance from the surrounding medium during rotation.

Comparison of Fluorescence Depolarization, Dielectric Dispersion and Birefringence Relaxation Studies.—The rotational diffusion constants of BSA were determined from dielectric dispersion measurements by Oncley.[47] Analysis of the dispersion data gave dielectric relaxation times of 0.36 and 0.075 μsec. Assuming these correspond to rotation of a prolate ellipsoid of revolution about the minor and the symmetry axes, respectively, Oncley found an axial ratio of 6. The agreement of this value with the one in Table I calculated from completely independent results for the hydrated prolate ellipsoid suggests that the prolate ellipsoid is better than the oblate model. It was pointed out[5,48] that there may be important contributions to the dielectric dispersion from polarization of the ion atmosphere about polyelectrolytes; this might have invalidated Oncley's analysis, in which this effect was ignored. For example, the shorter relaxation time, not observed in birefringence, might be caused by ion atmosphere relaxation. It is possible that the amplitude of the ion atmosphere contribution is not great enough in BSA to cause difficulty in computing the axial ratio.

Edsall and Foster[49] studied the streaming birefringence of BSA in 88.45% glycerol solution. From their measurements, the expected birefringence relaxation time at 25° in water solution can be estimated, assuming the solvent may be treated as a continuum and that the macromolecule in glycerol solutions has the same hydrodynamic dimensions as in water. This gives 0.17 μsec., in remarkably good agreement with the experimental value of this study.

The harmonic mean of the (dielectric) rotational relaxation times of BSA was found by the technique of fluorescence depolarization by Weber,[50,51] Steiner[52] and Harrington and colleagues.[33] The value of 0.124 μsec. at *p*H 7.3 reported by Harrington and colleagues is probably the most accurate one, since these workers used two fluorescent dyes, made direct measurements of the lifetime of the fluorescent molecules in the protein-conjugate state and employed these values in their calculations. Assuming the second hydrated prolate model of Table I for BSA, the dielectric relaxation time of the short axis can be calculated from Perrin's equations.[27] This value is 0.11 μsec. The 0.20 μsec. birefringence relaxation time of the long axis corresponds to a dielectric relaxation time of 0.60 μsec. The harmonic mean of the three dielectric relaxation times for the prolate ellipsoid is 0.15 μsec. For the oblate hydrated ellipsoid, the harmonic mean would be close to 0.60 μsec., so the prolate model is better. The discrepancy between the measured value (0.124 μsec.) and the one calculated here for the hydrated prolate model (0.15 μsec.) may be considered an indication that BSA is not completely rigid or not an ellipsoid of revolution.

Relaxation Time in Acid Solution.—It was found in this work that the birefringence relaxation time of BSA decreased, *i.e.*, the rotational diffusion constant increased, in going from *p*H 5.1 to 2.58. The relaxation time for BSA at 30° at *p*H 5 was 0.20 ± 0.01 μsec., corresponding to a rotational diffusion constant of 0.83×10^6 sec.$^{-1}$, whereas in the acid solution, it was 0.16 ± 0.01 μsec., corresponding to a rotational diffusion constant of 1.04×10^6 sec.$^{-1}$. The change is just outside experimental error. In the absence of other data, this might be interpreted as a contraction or a dissociation of the molecule or as a result of decreased hydration. However, numerous workers,[16,22-24,43,53-61] have interpreted the titration curves, the viscosity and the optical rotation properties of BSA in acid solution in terms of an expansion of the molecule. For example, Reichmann and Charlwood[59] showed that although the increase in viscosity and sedimentation constant in BSA solutions at low ionic strength and at *p*H 1.9 was accompanied by a change in molecular weight, as determined from light scattering, an expansion of the molecule was necessary to explain the data in solutions containing excess salt at the same *p*H. In the latter solutions, the light scattering of BSA indicated no aggregation. The authors suggested that the viscosity and sedimentation constant increases could not be due to aggregation of the BSA but were probably the result of an expansion or an increase in axial ratio or both.

Weber,[50,51] Steiner[52,62] and Harrington and colleagues[33] found that the mean relaxation time

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found for BSA in acid solution by fluorescence depolarization, 0.06 or 0.08 μsec. depending on the fluorescence conjugate, was less than that found in more neutral solution, *p*H 7.3, 0.124 μsec. This 40% decrease in relaxation time was considered too great to be attributed only to the electroviscous effect. Weber attributed the decrease to dissociation of the BSA in acid solution, but Harrington and colleagues, in view of the other physical properties of BSA in acid solution, ascribed the lowered relaxation time to sub-unit rotation in the BSA molecule in acid solution. The increased intrinsic viscosity was attributed to the weakening of the secondary bonds holding the BSA molecule rigid in more neutral solution, thus allowing more water to penetrate into the molecule. Tanford, *et al.*,[61] and Hill[63] suggested that the swelling of the BSA molecule in acid solution could be due to the breaking of some of the crosslinks in the molecule. Harrington, *et al.*, remarked that the breaking of secondary bonds and the onset of sub-unit rotation could account for the hydrodynamic properties in acid solutions of BSA. The decrease of the relaxation time at *p*H 2.58 found in the present work is consistent with an increase of molecular flexibility. Alternatively, it is possible that rupture of secondary bonds allows the molecule to become slightly shorter, *i.e.*, assume a more symmetric form with a shorter relaxation time. Then the increase of viscosity could be due to expansion transverse to the long dimension.

Specific Kerr Constants.—In Fig. 6, it is seen that the specific Kerr constants of untreated and deionized BSA approach each other at infinite dilution, but there is an increase with concentration for the deionized material and a decrease with concentration for the untreated material. The opposite signs of the slopes of the K_{sp} *vs.* C curves are considered to reflect qualitatively different modes of interaction between BSA molecules for the two cases. For example, the deionized material might interact so as to produce parallel alignment of permanent electric dipoles or reinforcement of principal electric polarizabilities, with the interacting molecules becoming more strongly aligned in the applied field, thus giving rise to an increase of K_{sp} with C; the reverse might be true with the untreated material. As mentioned above, previous workers have suggested that fatty acids are removed by deionization, and aggregation appears to be decreased with deionized preparations. The present results indicate significant interactions at relatively low concentrations even in deionized preparations.

It appears from the data shown in Fig. 7 that the specific Kerr constant of BSA has a minimum near the isoelectric point of BSA, that is, near *p*H 5. This minimum can be explained as follows: the increase in charge of the BSA molecules as the *p*H departs from the isoelectric *p*H could have two effects which would tend to increase the specific Kerr constant of the solution. First, if the charged groups are not situated symmetrically to the center of rotation of the molecule, the dipole moment can increase with increasing ionization. Secondly, the increasing macromolecular charge can cause an increase in specific Kerr constant as a result of the increased counterion atmosphere, which is polarizable.[3,5,48] On the basis of the present results, it is not possible to evaluate the relative importance of the two effects.

Figure 8 shows that the specific Kerr constant of BSA decreases with increasing ionic strength, that is, increasing solution conductivity, at constant *p*H. This is in accord with qualitative predictions[5] for an ion atmosphere polarization mechanism; a more quantitative treatment for large macromolecules supports this prediction.[64]

Although potassium ions are not bound by BSA[65] whereas zinc ions are bound,[66] the specific Kerr constants of their solutions were essentially the same. The only solution exhibiting anomalous behavior was one containing $CuCl_2$ (see Fig. 7). Klotz and co-workers[67] found that cupric ion caused slow changes in the spectrum of BSA which they attributed to unspecified irreversible changes in the structure of the molecule. Saroff and Choate[68] found by ultracentrifugation that concentrations of cupric ion higher than those investigated by Klotz caused reversible aggregation of BSA. The increase in the Kerr constant would be consistent with aggregation, but structural changes are not ruled out by the datum. A detailed birefringence study, including relaxation analyses, undoubtedly would shed further light on the Cu^{++}–BSA interaction.

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