# Diffusion Studies of Bovine Serum Albumin by Quasielastic Light Scattering†

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ABSTRACT: The translational diffusion coefficient, $D$, of bovine serum albumin (BSA) is measured in a pH range from 7.0 to 2.0 as independent functions of ionic strength and BSA concentration. At relatively high ionic strength the diffusion coefficients are BSA concentration independent at relatively low concentrations and the molecule appears to expand (smaller diffusion constant) as the pH is lowered below the isoelectric point near pH 5. At low ionic strengths $D$ increases as the pH is lowered and $D$ is strongly dependent on the solution concentration, increasing linearly with concentration. However, the infinite dilution diffusion coefficient is ionic strength independent and indicates the same expansion as the pH is lowered from 5.0 to 2.0 as observed at high ionic strengths. These results are interpreted in terms of solution electrostatic effects.

Bovine serum albumin (BSA)[1] exists as a compact molecule at and near its isoelectric point (pH $\simeq$5). Although the molecule shows no change in configuration or conformation in the pH range 4.5–10.5, all past studies reveal that BSA solutions change very markedly, though reversibly, certain of their physicochemical properties over the pH range 4.5–2.0. The sedimentation constant (Harrington *et al.*, 1956; Kronman and Foster, 1957; Charlwood and Ens, 1957), translational diffusion coefficient (Champagne, 1957), specific viscosity (Yang and Foster, 1954; Tanford *et al.*, 1955), specific optical rotation (Yang and Foster, 1954; Sogami and Foster, 1968), and low-angle X-ray scattering studies (Luzzatti *et al.*, 1961) all suggest expansion of the BSA molecule at low pH. This expansion is reversible and apparently electrostatic in nature, as it is highly dependent on the ionic strength of the BSA solution; the expansion increases as the ionic strength is lowered. However, an overall swelling of the molecule with no change in shape is ruled out because of the decrease in the rotational relaxation time, $\rho_h$, as the pH is lowered (Weber, 1952; Weber and Young, 1964; Harrington *et al.*, 1956). A model has been proposed by Foster (1960) which accounts for the above changes where the protein is composed of globular parts linked by flexible peptide chain segments. The compact molecule (at and above pH 5) undergoes a transformation and expansion from pH 5 to about 3.5 and at lower pH the molecule expands further.

BSA is a highly charged molecule at low pH. Most polyelectrolytes are studied in the presence of an excess of a low molecular weight salt to partially screen the charges on the macroion. In the studies mentioned above, the molecular charges are considered to cause intramolecular expansion because of similar charges on different segments of the macromolecule, but the intermolecular interactions due to increased ionic atmosphere are usually ignored. For example, Yang and Foster (1954) found that the intrinsic viscosity of BSA increased inversely with the ionic strength and they related it directly to an increase in the effective (hydrodynamic) volume of the macromolecule. This will lead to an increased frictional coefficient. However, Alexandrowicz and Daniel (1963) and Daniel and Alexandrowicz (1963) assumed the frictional coefficient of the macroion to be independent of the ionic strength and were able to explain the sedimentation and the diffusion data of poly(L-lysine) in terms of the direct effect of the intermolecular electrostatic fields on the macroion.

Since the diffusion coefficient, $D$, is inversely proportional to the hydrodynamic radius of the macromolecule, the measurement of $D$ as a function of pH and ionic strength should show whether there is an actual expansion (hydrodynamic expansion) of the molecule at low pH and low ionic strength.

In this paper we report the measurement of the translational diffusion coefficients of monomer BSA in the pH range from 7.0 to 2.0 at several ionic strengths. There were two objectives in this work: (i) to study the hydrodynamic changes in the BSA molecule as the pH is lowered below the isoelectric point, and (ii) to study the effect of low ionic strength on the diffusion coefficient and hence on the frictional coefficient of BSA at low pH.

Diffusion coefficients were measured by the relatively new technique of laser beat frequency spectroscopy, also called inelastic or quasielastic light scattering (Dubin *et al.*, 1967; Cummins *et al.*, 1969). The physical basis of this technique is that laser light scattered from solutions of macromolecules is Doppler broadened by Brownian motion of the macromolecules. In the case of spherical particles, the spectrum of the scattered light is Lorentzian with angular frequency half-width at half-height equal to $DK^2$, where $D$ is the translational diffusion coefficient of the macromolecule, and $K$ is the magnitude of the scattering vector, which is given by

$$K = (4\pi n/\lambda_0) \sin(\theta/2)$$

$n$ is the refractive index of solution, $\lambda_0$ is the incident vacuum wavelength, and $\theta$ is the scattering angle. Measurement of the half-width at half-height of the spectrum leads to a direct determination of $D$. This technique can be used for particles of virtually any size [barring complications due to the effects of rotational diffusion (Cummins *et al.*, 1969)]. Details of the theory and instrumentation are available in several reviews of this technique (French *et al.*, 1969; Cummins and Swinney, 1969; Chu, 1970; Ford, 1972).

Our results show that in the ionic strength range $I$ = 0.07–0.50, the diffusion coefficient is independent of the ionic strength and there is no dependence on BSA concentration up to 2% BSA. Thus, the pH-dependent changes in the diffusion coefficient of 1% BSA in solutions of an ionic strength $\geq$0.07 can only represent changes in the hydrodynamic volume. The

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[1] Abbreviation used is: BSA, bovine serum albumin.

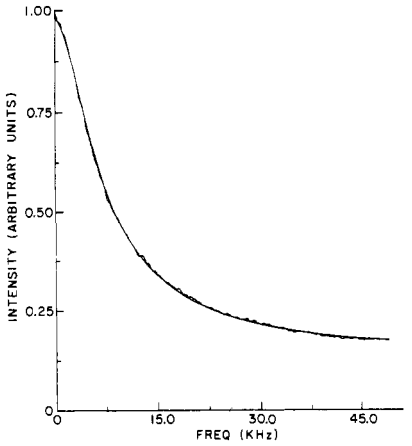


FIGURE 1: Spectrum of the photocurrent for light scattered at $\theta$ = 58.2° by 2% BSA in 0.1 M Tris + 0.2 M NaCl buffer. The smooth curve represents a calculated Lorentzian line of 5.00-kHz half-width and the rough curve is the experimental curve.

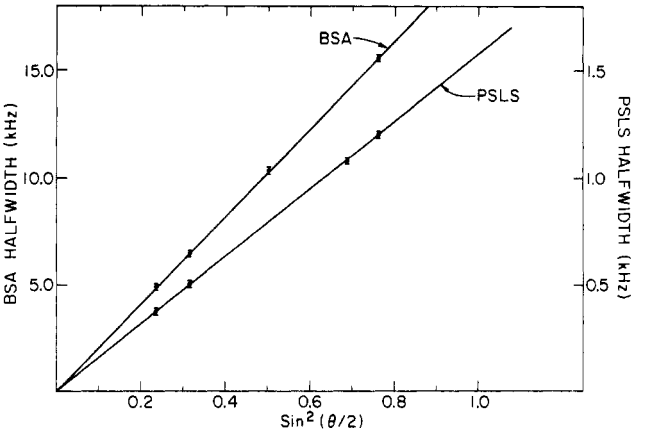


FIGURE 2: Dependence of the observed half-width at half-height on $\sin^2(\theta/2)$ for BSA and polystyrene latex spheres (PSLS).

plot of diffusion coefficient *vs.* pH given in this work at any ionic strength ≥0.07 follows the "expansion" plots of previous studies (Tanford *et al.*, 1955; Sogami and Foster, 1968). However, the infinite dilution diffusion coefficient, $D^0$, was found to be independent of ionic strength in contrast to the increased expansion with lower ionic strength observed by Yang and Foster (1954). At the lower ionic strengths, the observed diffusion coefficient was very sensitive to the BSA concentration and the observed $D$ values for a fixed BSA concentration (about 1%) do not represent hydrodynamic changes which have apparently been observed by other techniques. These results are explained in terms of screened interparticle electrostatic interactions.

## Experimental Section

*Preparation of Sample.* Crystallized BSA (lot J72104) was obtained from the Reheis Chemical Co., Chicago, Ill. Monomer BSA was separated from its oligomers by gel filtration on Sephadex under the conditions described by Pedersen (1962). The column was 5.2 cm in diameter and 150 cm long, with the bed volume of 3000 ml. The column was packed with Sephadex G-150, bead form (lot no. 860), with particle size 40–120 $\mu$. The buffer was 0.200 M in NaCl and 0.100 M in Tris buffer, adjusted to pH 7.3.

Following gel filtration, the monomer was collected. Solutions at various pH's were obtained by dialyzing 10 ml of monomer solution for at least 2 days against 6 l. of buffer solutions. HCl–NaCl solutions were used for pH 2.00–4.25 and phosphate buffer for pH values above 4.5. Citrate–phosphate buffers were also employed over the entire pH range. All buffer solutions contained 0.0005 M EDTA to suppress dimerization. Deionized water was used for making all solutions and analytical reagents were used without further purification. BSA concentrations were determined with a Zeiss Model PMQ II spectrophotometer using a value for the extinction coefficient, $\epsilon_{1cm}^{1\%}$, of 6.67 at 278 m$\mu$. pH measurements were made with a Sargent-Welch Model DR at 20°.

*Measurement of D.* We performed light-scattering experiments by the homodyne (self-beat) method (Dubin *et al.*, 1967; Cummins and Swinney, 1969; Chu, 1970; Ford, 1972) using an $Ar^+$ laser as a source of incident monochromatic light. The photocurrent was analyzed by a Federal Scientific Model UA-15A spectrum analyzer, the output of which is averaged by a Federal Scientific Model 1015 averager. The resulting spectrum is then analyzed by fitting to a single Lorentzian by a nonlinear least-squares procedure to obtain the half-width, from which the diffusion coefficient can be directly calculated.

The scattering cells were cleaned by closed circulation of distilled water through a 0.22-$\mu$ Millipore filter and dried by forcing filtered $N_2$ through the cells. The BSA solution was filtered three to four times through a 0.22-$\mu$ filter before transferring into the scattering cell. This procedure was quite satisfactory for removing dust from the samples. The scattering cell was placed in the spectrometer and maintained at a constant temperature of 20.0 ± 0.1°. A typical light-scattering spectrum taken at a scattering angle of 58.2° is shown in Figure 1.

All measurements were taken within a week of preparation of the BSA monomer solution. Polyacrylamide gel electrophoresis (Davis, 1964) was used to check the extent of dimerization of BSA. No significant dimerization was detected in low pH solutions for more than 1 week after the solutions were prepared. The pH as well as the optical density was measured before and after the scattering experiment and no significant changes were observed.

The accuracy of our instrument was examined by measuring the homodyne spectrum from a sample of polystyrene latex spheres (Dow Chemicals, run number LS-1132-B) known to be 910 ± 60Å in diameter. A plot of the half-width at half-height *vs.* $K^2$ for polystyrene spheres gives the predicted straight line shown in Figure 2. Our results for the diffusion coefficient were $D = (4.68 \pm 0.04) \times 10^{-8}$ cm$^2$ sec$^{-1}$ as compared to $D = 4.72 \times 10^{-8}$ cm$^2$ sec$^{-1}$ given by Stokes–Einstein equation ($D = kT/6\pi\eta r$ where $\eta$ is the medium viscosity and $r$ is the radius of the particle). The other curve in Figure 2 is for 1.35% by weight of monomer BSA at pH 6.78 and gives $D = (6.01 \pm 0.04) \times 10^{-7}$ cm$^2$ sec$^{-1}$, in agreement with numbers reported earlier (Champagne, 1957; Dubin *et al.*, 1967). We estimate the accuracy of our measurement of $D$ to be better than 3%.

## Results

$D$ was studied as a function of pH, BSA concentration, and the ionic strength of the solution by varying one of these properties while the other two were held constant. For example, to study $D$ as a function of pH, the BSA concentration and the ionic strength were both held constant by dialyzing equal volumes of the same monomer solution of BSA and then diluting the samples after dialysis to the same concentration of BSA using the appropriate buffer solution.

The diffusion coefficient is found to be independent of the ionic strength, BSA concentration, and the type of buffer used for pH values ~6–7 as shown in Table I, but at pH's lower than the isoelectric point (pH 4.8), $D$ depends upon all the above factors.

TABLE I: Diffusion Coefficients for BSA at Several Values of pH above the Isoelectric Point.[a]

| pH | Buffer | Ionic Strength | BSA Concn (%) | $D \times 10^{+7}$ cm² sec⁻¹ |
|---|---|---|---|---|
| 6.78 | Citrate–phosphate | 0.02 | 1.35 | 6.0 ± 0.1 |
| 6.79 | Citrate–phosphate | 0.02 | 1.77 | 6.1 ± 0.1 |
| 7.27 | Tris + NaCl | 0.30 | 1.96 | 6.1 ± 0.1 |
| 6.91 | Phosphate + NaCl | 0.03 | 0.85 | 6.1 ± 0.1 |
| 6.32 | Phosphate + NaCl | 0.03 | 0.71 | 5.9 ± 0.1 |
| 5.94 | Phosphate + NaCl | 0.03 | 0.90 | 5.9 ± 0.1 |

[a] Various BSA concentrations and ionic strengths are also listed.

*Dependence of D on Ionic Strength.* Figure 3 shows the values of $D$ for a 1% BSA solution as a function of ionic strength for pH values of 5, 4.2, 3.6, and 3.1. At and near the isoelectric point (pH ≃5 in Figure 3), the ionic strength in the range 0.01–0.5 has no effect on the diffusion coefficient of BSA. This result is expected as a polyelectrolyte near its isoelectric point behaves similar to neutral macromolecules. The solutions for this set of data (at the isoelectric point) were prepared by dialyzing the neutral sodium chloride solutions of appropriate ionic strength and the pH ranged from 4.9 to 5.1.

At pH's lower than the isoelectric point (4.2, 3.6, and 3.1 in Figure 3), $D$ for 1% BSA is not dependent on the ionic strength in the range 0.5–0.07, but $D$ increases as the ionic strength is lowered to 0.01. Thus, an ionic strength ≥0.07 is sufficient to screen the charge on BSA in a 1% solution.

*Dependence of D on pH.* Figure 4 shows the values of $D$ for a 1% solution of BSA as a function of pH for ionic strength values of 0.10 and 0.03. As the pH is lowered below the isoelectric point at an ionic strength ≥0.07, $D$ decreases (to about pH 3) and then levels off as the pH is further lowered to 2 as shown in Figures 4 and 5. The plot at the ionic strength of 0.10 in Figure 4 is similar to the "expansion" curve of Tanford *et al.* (1955) and Sogami and Foster (1968) in the pH range 5–2 where our results are for nondefatted BSA and Sogami and Foster's plot is for charcoal-defatted deionized BSA. According to Sogami and Foster, the first transition (from pH 5 to 3.6) corresponds to a conformation change (N–F transition) and the second step (from pH 3.6 to 2.0) corresponds to a further expansion of BSA (F form).

At the low ionic strength of 0.03, $D$ for 1% BSA solution increases as the pH is lowered from 5 to 4 and then virtually

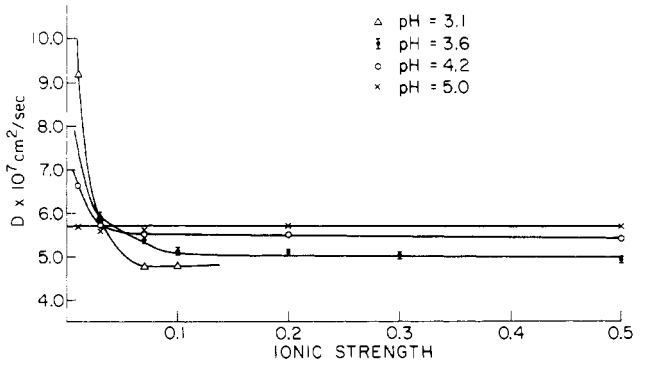


FIGURE 3: Dependence of $D$ on the ionic strength. The BSA concentration is 1% by weight in a HCl–NaCl solution. The data are taken at a scattering angle of 90° at the various values of pH as shown.

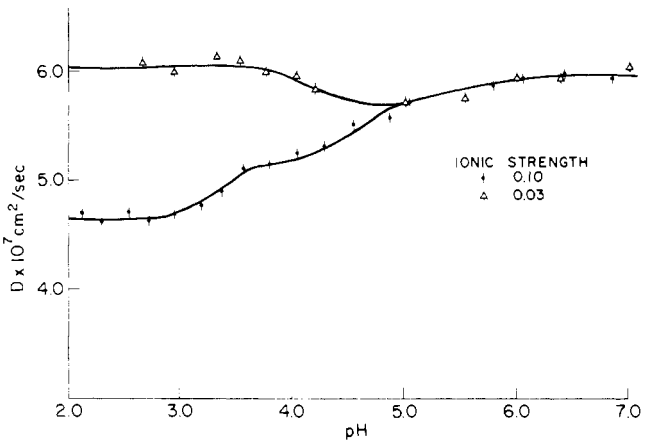


FIGURE 4: The diffusion coefficient, $D$, *vs.* pH at ionic strengths 0.10 and 0.03. The other conditions are the same as in Figure 3.

stays constant as shown in Figures 4 and 5. At the lower ionic strength of 0.01, $D$ increases sharply as the pH is lowered below the isoelectric point as shown in Figure 5.

*Dependence of D on Concentration.* The dependence of $D$ on BSA concentration was studied at ionic strengths of 0.20, 0.03, and 0.01 and at pH values of 3.65 and 3.10. Figure 6 shows $D$ as a function of BSA concentration at various ionic strengths at pH 3.65. The data extrapolate to the infinite dilution value of $D = 4.8 \times 10^{-7}$ cm² sec⁻¹. Similar data were recorded at pH 3.10 giving the infinite dilution value of $D = 4.2 \times 10^{-7}$ cm² sec⁻¹. Thus, our diffusion measurements for 1% monomer BSA at high ionic strength (≥0.1) are the same as the infinite dilution result, $D_{20,w}{}^0$.

At lower ionic strengths of 0.03 and 0.01, $D$ increases linearly with BSA concentration and at any finite concentration, $D$ changes inversely with the ionic strength. $D_{20,w}{}^0$ obtained by extrapolation to zero concentration is independent of the ionic strength within the accuracy of our results.

## Discussion

*Results at High Ionic Strength.* BSA exists in a compact form (N form) in the high pH range. The molecule has a relatively small negative charge from pH 7 to 5 (Tanford *et al.*, 1955), and only a small ionic strength is required to shield the

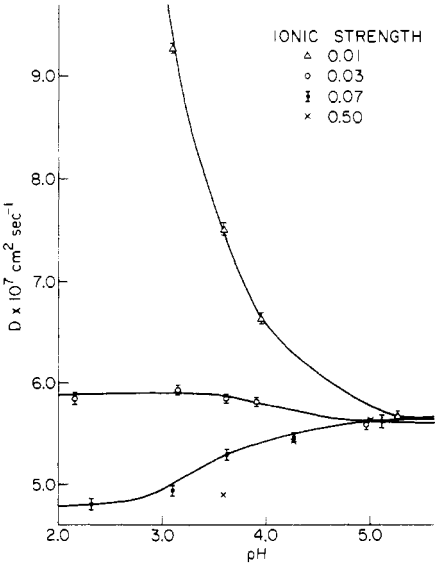


FIGURE 5: The diffusion coefficient, $D$, *vs.* pH at ionic strengths 0.01, 0.03, 0.07, and 0.50. The other conditions are the same as in Figure 3.

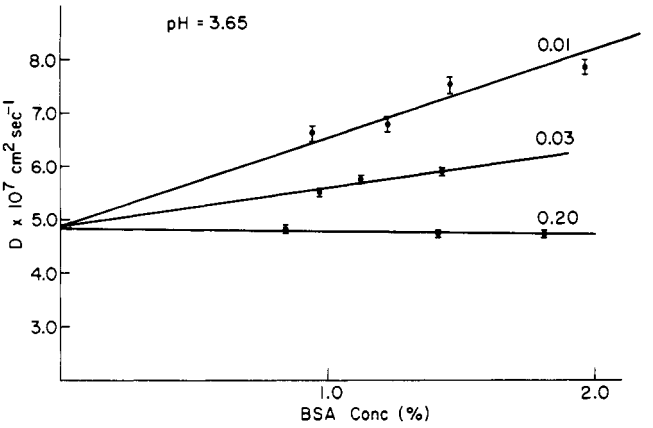


FIGURE 6: Dependence of $D$ on BSA concentration at pH 3.65 and at ionic strengths equal to 0.01, 0.03, and 0.20.

TABLE II: Typical Values of Our Effective Hard Sphere Radius in BSA, $r_{HS}$, As Obtained from Equations 6 and 7.[a]

| pH | Ionic Strength | $(B_2 - B')$, cm³/g | $B_2$, cm³/g | $r_{HS}$, Å | $r$(Tanford), Å |
|---|---|---|---|---|---|
| 3.6 | 0.03 | 12.5 | 17.4 | 38.1 | 44 |
| 3.6 | 0.01 | 44.2 | 49.1 | 54.7 | 49 |
| 3.1 | 0.03 | 20.2 | 25.5 | 44.0 | 48 |
| 3.1 | 0.01 | 87.1 | 92.4 | 67.5 | 55 |

[a] $B_2 - B'$ values are obtained from the measured values of $D_{20,w}^{low}$ and $D_{20,w}^{high}$ at low and high ionic strengths, respectively (see eq 7). $B'$ is obtained from the data of Harrington *et al.* (1956). $r_{HS}$ is calculated from $B_2$ by using eq 6. $r$(Tanford) is from viscosity data.

intraionic repulsions between different parts of the BSA molecule in this pH range. On the other hand, BSA acquires a large positive charge as the pH is lowered below the isoelectric point, a part of which is neutralized by the strong binding of $Cl^-$ ions. Hence, a significantly higher ionic strength of the solution ($\geq 0.1$) is required to provide ionic shielding in these highly charged BSA molecules. In the presence of excess of salt, the concentration dependence of $D$ can then be explained in terms of the second virial coefficients (or excluded volume).

At relatively low, but nonzero, concentrations of a polyelectrolyte, the diffusion coefficient can be described by the expression (Tanford, 1961)

$$D = \frac{kT}{f}\left[1 + C\left(\frac{\partial \ln y}{\partial C}\right)_{T,p}\right] \quad (1)$$

$$= \frac{kT}{f}[1 + B_2C + \ldots] \quad (2)$$

where $y$ is the molar activity coefficient, $C$ is the concentration in grams per cubic centimeter, and $B_2$ is the second virial coefficient, the same quantity which characterizes the first-order departure from linearity in the concentration dependence of the osmotic pressure and in the concentration dependence in the scattered light intensity as obtained by conventional light scattering. The frictional coefficient, $f$, also depends upon the concentration of the macromolecule and can be described by (Tanford, 1961)

$$1/f = (1/f_{20,w}^0)(1 - B'C) \quad (3)$$

where $f_{20,w}^0 = 6\pi\eta_{20,w}r$ for a spherical molecule with radius $r$ and $B'$ is an empirical factor. Then

$$D \simeq (kT/f_{20,w}^0)[1 + (B_2 - B')C] \quad (4)$$

or

$$D_{20,w} \simeq D_{20,w}^0[1 + (B_2 - B')C] \quad (5)$$

to a first order of approximation. For uncharged macromolecules, $B_2$ depends only on the excluded volme and is given by (Tanford, 1961)

$$B_2 = (N/M)(32/3)\pi r^3 \text{ cm}^3/\text{g} \quad (6)$$

where $N$ is Avogadro's number and $M$ is the gram molecular weight. $B'$ at a given pH was obtained from the data of Harrington *et al.* (1956) on the concentration dependence of the sedimentation coefficient and was assumed to be independent of ionic strength (Daniel and Alexandrowicz, 1963). At and near the isoelectric point, $B' \simeq 4.5$ cm³/g (with a large uncertainty of ~2 cm³/g). For BSA at or near the isoelectric point, this correction turns out to be less than 2% for 1% BSA. The high ionic strength data at pH's 3.65 (Figure 6) and 3.10 show that there is no observed dependence of $D$ on BSA concentration even up to 2%. Thus, the diffusion coefficients for 1% BSA at all pH's studied at high ionic strength ($\geq 0.1$) are very close to the corresponding $D_{20,w}^0$ values and the changes in these $D$ values reflect true changes in the hydrodynamic volume.

*Low Ionic Strength Data.* At low pH, a BSA molecule can carry a charge up to 100 electronic units and the electrostatic (repulsive) energy between two unscreened charged molecules plays an important role in the diffusive motion. Though the scattering molecule may still be small, its large charge can give rise to an increased effective diameter. We can explain the low ionic strength data by replacing $r$ (hydrodynamic radius) in eq 6 by an effective hard sphere radius, $r_{HS}$. $r_{HS}$ will depend on the degree of screening produced by the salt solution, as well as on the total charge on the protein molecules. Doty and Steiner (1952) used a similar approach to explain the effects found in a conventional light-scattering study of BSA. Pusey *et al.* (1972) also explained the diffusion results of R17 virus in terms of $r_{HS}$. The interaction of two partly shielded macroions, of course, cannot be strictly described by a hard sphere potential, but this simplifying approximation provides a qualitative explanation of the data at low ionic strength. Since, at high ionic strength, the diffusion coefficient of 1% BSA at low pH was found to be independent of the ionic strength (Figure 3) as well as the concentration of BSA (Figure 6), we replace $D_{20,w}^0$ in eq 5 by $D_{20,w}^{high}$ at the corresponding pH.

$$D_{20,w}^{low} \simeq D_{20,w}^{high}[1 + (B_2 - B')C] \quad (7)$$

The experimental values of $B_2$, $B_2 - B'$, and $r_{HS}$ calculated from eq 6 and 7 and radii values from the viscosity data of Tanford *et al.* (1955) are listed in Table II. The value of $r_{HS}$ increases with increasing BSA charge as the pH is lowered. The value of $r_{HS}$ also increases with decreasing ionic strength due to the decreasing screening effect of the salt.

It should be pointed out that the values of $r_{HS}$ at the low ionic strengths are only of qualitative significance, since the net charge on the protein molecule is expected to vary with both concentration of BSA as well as the ionic strength (especially because of strong binding of the negative ions to the molecule). Moreover, $B'$ should be known accurately as a function of the ionic strength and the pH.

Figure 6 shows that $D_{20,w}^0$ is independent of the ionic strength. Thus, the apparent increase in the effective diameter is due to unscreened charges on the macromolecules and is not

due to an increased physical expansion of the molecule at low ionic strength over the values observed at high ionic strength.

In summary, at ionic strengths in excess of 0.07 with a 1% BSA solution, BSA expands as the pH is lowered below the isoelectric point. No further physical expansion occurs when the ionic strength is lowered.

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# Fluorine Nuclear Magnetic Resonance Studies of Trifluoroacetyl-insulin Derivatives. Effects of pH on Conformation and Aggregation†

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ABSTRACT: $^{19}F$ nuclear magnetic resonance techniques have been used to study the effects of solvent and pH on the conformation and aggregation properties of several trifluoroacetyl derivatives of insulin. Sedimentation velocity studies were also performed in an effort to obtain independent data on the aggregation state of these derivatives at pH 2.0 and 6.8. At pH 2.0, insulin and the trifluoroacetyl-insulin derivatives exist largely as dimers, as suggested by the $s_{20,w}$ values and the three narrow $^{19}F$ resonance peaks derived from the trifluoroacetyl moiety bound to the $\alpha$-amino groups of the glycine$^{A-1}$ and phenylalanine$^{B-1}$ and the $\epsilon$-amino group of lysine. At pH 6.8, high molecular weight aggregates were indicated by the $s_{20,w}$ values and the considerable broadening of the $^{19}F$ resonance spectra. Spectral analysis at this pH suggested that the trifluoroacetyl group on glycine$^{A-1}$ possessed significantly more motional freedom than on phenylalanine$^{B-1}$ or lysine$^{B-29}$. Further elevation of the pH to 8.7 resulted in disaggregation of the trifluoroacetyl-insulin derivatives, as indicated by the line widths of the $^{19}F$ nuclear magnetic resonance spectra. Spectral analysis of the chemical shifts at pH 8.7 also suggested conformational alterations in the regions of phenylalanine$^{B-1}$ and lysine$^{B-29}$

The use of nuclear magnetic resonance (nmr) techniques to study structural and conformational properties of protein molecules has afforded a wealth of information. Proton magnetic resonance spectroscopy affords extremely complex spectra due to the large number of equivalent protons and the broadening of the various peaks resulting from the incorporation of the absorbing species into a macromolecule. Nevertheless, considerable information has been obtained from the examination of those resonance peaks which are resolved from the main absorption envelope (Jardetsky and Wade-Jardetsky, 1971).

In an effort to simplify these complex spectra, several studies have been concerned with the chemical introduction of fluorine as the trifluoroacetyl group to serve as an environmental probe at defined sites in a macromolecule. This procedure allows for the identification of individual resonance peaks and the analysis of observed spectral alterations resulting from differences in

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