and adenine groups are equatorially and quasi-equatorially oriented, respectively, and the CH_2OH group is bisectionally oriented. (This conformation has been previously suggested for the purine nucleosides.)

Based on the observation that the $J_{1'-2'}$ values for 2′:3′-UMP and 2′:3′-CMP are the same within experimental error it is reasonable to suggest that their sugar rings are characterized by the same conformation. In the case of 3′-AMP, 5′-AMP and 2′:3′-AMP the coupling constant of about 4.5 c.p.s. implies a dihedral angle of about 135° between H_1' and H_2'. This could be achieved by having a C_2'-*endo* conformation in which C_2' is displaced by somewhat less than 0.4 Å. However, analysis of the 60 mc. spectra due to the other ribose protons is essential before suggesting a specific puckered form for these compounds.

Experimental

The various nucleotides were dissolved in 0.5 ml. of D_2O and the *p*H was adjusted with NaOD to the desired value. The *p*H was measured with *p*H paper accurate to 0.1 *p*H units. The nucleotide solution was transferred quantitatively to small lyophilizing flasks of 25–35 ml. capacity with an additional 1.5 ml. of D_2O and was lyophilized in a Vis-tis freeze-dry apparatus. The lyophilization was repeated 2 to 3 times and finally the sediment was dissolved in 0.5 ml. of D_2O. An aliquot was pipetted into an n.m.r. tube (5 mm. o.d.). Approximately 0.005 ml. of acetone (internal standard) was then added and the tubes were stoppered with plastic caps and kept at −20°.

High resolution spectra at 40 and 60 megacycles were obtained with standard spectrometers from Varian Associates. The shifts were obtained by superimposing the acetone side-band on the sharp peaks of the spectra while the shifts for broader lines were calculated from at least four spectra by interpolation. However, the spacings for H_1' were obtained by superposition of an equal intensity acetone side-band or by interpolation from the spectrum calibrated in the following way; an acetone side band is placed on the low field side near the doublet. The field is slowly swept through the doublet and then the frequency of the audio-oscillator is quickly changed so that the side band appears on the high field side of the doublet. The two side band frequencies are read off from a Hewlett–Packard counter. In all cases the shift of the acetone peak was measured from an external benzene standard introduced in the sample in a small capillary and all shifts were then expressed relative to the benzene peak.

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Bovine Serum Albumin in Water–Dioxane Mixtures

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In order to investigate the influence of solvent environment upon the configuration of globular proteins, the behavior of bovine serum albumin (BSA) in mixtures of *p*-dioxane and dilute salt solutions has been studied. Experimental methods included sedimentation, optical rotation, viscometry and the measurement of apparent partial specific volume. In the neighborhood of the isoelectric point the configuration of the protein molecule is only slightly affected by dioxane in concentrations up to 35% (vol.). On the other hand, the expansion of the BSA molecule in acid solution is profoundly influenced by the presence of dioxane. The expansion is greater at a given *p*H, and the onset of expansion occurs at a higher *p*H when dioxane is present. The behavior can be semi-quantitatively explained in terms of a simple electrostatic model. The influence of dioxane upon the configuration of BSA appears to result mainly from the effect of the non-polar solvent upon electrostatic interactions within the molecule, rather than from direct interaction of dioxane with the protein.

Introduction

The nature of the forces responsible for maintaining the conformations of globular protein molecules in solution has been the subject of much recent inquiry. It appears that a complex and delicate balance is involved which is sensitive to small changes in the composition of the protein molecule or its environment. Examples of the latter effect are afforded by the recent studies by Tanford and co-workers of the result of the addition of organic solvents to aqueous solutions of ribonuclease[2] and β-lactoglobulin[3] and by Bresler, *et al.*,[4,5] of human and horse serum albumin in dioxane–water mixtures. This paper describes somewhat similar experiments with bovine serum albumin (BSA), in water–1,4-dioxane mixtures. Earlier studies of the intrinsic viscosity and optical rotation of BSA in dioxane–water mixtures have been reported by Foster and Yang,[6] who suggested that the increase in intrinsic viscosity observed upon addition of dioxane was a consequence of the decrease in the dielectric constant of the solution.

Serum albumin provides an interesting material for such study, because of the large number of intrachain disulfide bonds which serve to limit the number and kind of conformations available to the molecule and because of the large body of experimental data which has been accumulated concerning the behavior of this protein.

The choice of a solvent pair was dictated in part by the desirability of minimizing flow interaction in sedimentation. This can be accomplished by using

(1) Northland College, Ashland, Wisconsin.

(2) R. E. Weber and C. Tanford, *J. Am. Chem. Soc.*, **81**, 3255 (1959).

(3) C. Tanford, P. K. De and V. G. Taggart, *ibid.*, **82**, 6028 (1960).

(4) S. E. Bresler, *Discussions Faraday Soc.*, **25**, 158 (1958).

(5) S. E. Bresler, V. P. Kushner, S. Ya Frenkel, *Biokhimiya* (consultants' Bureau English translation), **24**, 630 (1959).

(6) J. F. Foster and J. T. Yang, "Abstracts 129th Meeting, American Chemical Society," p. 4-C.

as a solvent a mixture of two liquids of nearly equal density. Water and 1,4-dioxane satisfy this requirement quite well, since their densities at 25° are 0.997 and 1.027 g./cm.^{3}, respectively. There is the additional advantage that considerable data concerning the behavior of both weak and strong electrolytes in these mixtures has been amassed.

Experimental

A. Materials.—The bovine serum albumin was obtained from Armour. Portions of three lots (P67908, V68308 and V68802) were used. Most of the experiments were performed with lot V68802. The moisture content was determined by microkjeldahl analysis, assuming 16.07% nitrogen in dry protein. Sample V68802 was dried at 105–110°, which led to an apparent protein content about 2% higher than the microkjeldahl value. The result from nitrogen analysis was used in all calculations. The 1,4-dioxane was Merck reagent grade, further purified by redistillation from sodium and stored frozen. All other chemicals were reagent grade, used without further purification.

The mixtures containing dioxane were prepared by placing the required volume of dioxane in a volumetric flask and making the solution up to volume with buffer or dilute salt solution as required. Thus a "30% dioxane" solution was prepared by diluting 30 ml. of dioxane to 100 ml. of solution. Protein solutions were prepared by dissolving the protein in these mixtures. Unless otherwise specified, all experiments were carried out with solutions containing approximately 0.75 g. protein/100 ml.

B. Sedimentation Velocity Measurements.—A Spinco Model E ultracentrifuge, equipped with phase plate and rotor temperature indicator and control unit (r.t.i.c.) was employed. During the course of this investigation, after installation of a new r.t.i.c. unit, the calibration was checked with five times recrystallized diphenyl ether in the manner described by Biancheria and Kegeles[7]; the temperature determined in this way agreed within 0.02° with that given by the r.t.i.c. reading. More confidence can be placed in the experiments carried out after the calibration. All experiments were carried out in the neighborhood of room temperature, using rotor speed settings of 56,100 or 59,780 r.p.m. Photographic plates were measured with either a Bausch and Lomb toolmaker's microscope reading to 0.0001 in. in two directions or a Mann Comparator reading to 0.0001 cm. in one direction.

C. Density Measurements.—The density of solutions and the dioxane–buffer solvents were measured at 25.00 ± 0.01°. The protein solutions were prepared by weighing both solutes and solution, and the solvent composition was corrected for water carried by the moist protein. The pycnometers were fashioned by fastening capillary necks of about 1 mm. internal diameter to 25 ml. erlenmeyer flasks. The relationship between the volume contained and the height of the meniscus above a reference mark (measured to ±0.001 cm.) was determined by calibration with distilled deionized water. In all measurements a period of at least 30 min. equilibration in the constant temperature bath plus about 20 min. in the balance case was allowed. Weights were corrected for air buoyancy. In order to prevent the formation of air bubbles within the pycnometer, the water and dioxane were either boiled or aspirated before preparation of solutions.

D. Optical Rotation Measurements.—The optical rotation was determined at a wave length of 546 $\mu\mu$, using a Schmidt and Haensch visual polarimeter, with a two decimeter tube. Ten or more readings were taken with each solution and solvent mixture; the average deviation from the mean was about ±0.007°. The temperature was 25 ± 2°.

E. Viscosity Measurements.—Relative viscosities were determined at 25.00 ± 0.02° (constant in any one determination to ±0.005° or better), using a Cannon-Ubbelohde No. 50 viscometer. The viscometer was mounted on a three-point suspension, so that when once made vertical it could be removed from the bath and replaced with precision. The flow time for this viscometer was about 228 sec. for water. The kinetic energy correction was determined by measuring flow times for both acetone and water at 25.01°. The correction was very small, amounting to only 0.2% in the largest relative viscosity measured. In most cases, solutions were pressure filtered through sintered glass before use. Flow times generally were reproducible to ±0.1 sec.

(7) A. Biancheria and G. Kegeles, *J. Am. Chem. Soc.*, **76**, 3737 (1954).

Results and Discussion

A. The Isoelectric Region.—The results obtained from experiments carried out with dioxane–water mixtures buffered in the neighborhood of the isoelectric point of BSA will be discussed first. The buffer solution contained 0.01 M acetic acid, 0.01 M sodium acetate and 0.1 M KCl. Measurement of the pH of these solutions showed a consistent variation between 4.75 (0% dioxane) and 5.45 (30% dioxane). This presumably arises from the variation in pK of acetic acid with increasing dioxane concentration. Since the pK of the acidic groups on the protein should vary in a similar fashion, it was felt that this technique yields more nearly constant conditions than adjusting the pH of each solution to a constant value. While glass electrode readings in the more concentrated dioxane solutions are subject to correction, as discussed in Section B, the exact value of the pH does not appear to be critical in this region. The solutions each contained about 0.75 g. BSA/100 ml. It was found possible to prepare such solutions containing up to about 35% dioxane; however, the solutions containing more than about 20% dioxane formed some precipitate after a few days. In general, experiments were performed on the same day that the solution was prepared.

Sedimentation coefficients were calculated from the positions of the maxima in the concentration gradient curves. This method does not include the correction relating the position of the maximum to the true boundary position, but this has been shown by Baldwin[8] to be small for BSA. Furthermore, the variation of the apparent sedimentation coefficient resulting from radial dilution has been neglected, but this effect is also small. The sedimentation coefficients thus obtained decrease rapidly with increasing dioxane concentration. The results must be corrected to a standard temperature, and for the variation in viscosity, solution density and solute partial specific volume resulting from the presence of the dioxane. It is assumed that these corrections may be expressed as

$$S_{25,w} = S_{t,m}\frac{\eta_{t,m}\eta_{25,m}(1-\bar{v}\rho)_{25,w}(1-\bar{v}\rho)_{25,m}}{\eta_{25,m}\eta_{25,w}(1-\bar{v}\rho)_{25,m}(1-\bar{v}\rho)_{t,m}} \quad (1)$$

where subscripts t and 25 refer to the temperature of measurement and 25°, respectively, and the subscripts m and w refer to the solvent mixture and water, respectively. It has been demonstrated that, in general equation 1 is not sufficient to describe results for such a multi-component system. Flow interaction should give rise to additional correction terms. For a three-component system [water (1), protein (2), dioxane (3)] for example, the thermodynamics of irreversible processes yields the expression[9]

$$S_2 = [L_{22}(1-\bar{v}_2\rho) + (1-\bar{v}_3\rho)]/C_2 \quad (2a)$$

or

$$S_2 = \frac{L_{22}(1-\bar{v}_2\rho)}{C_2}\left[1 + \frac{L_{23}(1-\bar{v}_3\rho)}{L_{22}(1-\bar{v}_2\rho)}\right] \quad (2b)$$

(8) R. L. Baldwin, *Biochem. J.*, **65**, 503 (1957).

(9) J. W. Williams, K. E. Van Holde, R. L. Baldwin and H. Fujita, *Chem. Revs.*, **58**, 715 (1958).

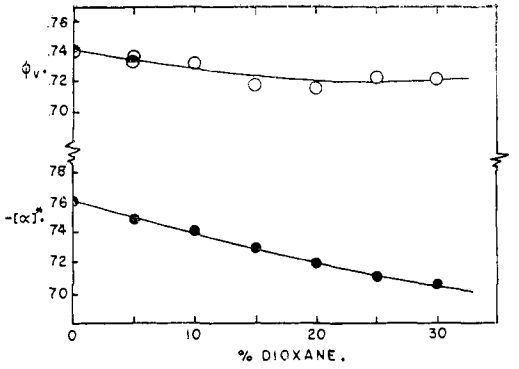

Fig. 1.—Variation of apparent partial specific volume (ϕ_v) and corrected specific optical rotation $[\alpha]^*$ with dioxane concentration. The two kinds of symbols in the ϕ_v graph indicate results obtained with two lots of BSA (O, lot T68308 and ⊖, lot V68802).

where L_{22} and L_{23} are the phenomenological coefficients relating flows to driving forces, and ρ is the solution density. It is presumed that variation of the term $(L_{22}(1-\bar{v}_2\rho)/C_2)$ with solvent environment and temperature is accounted for by equation 1, and specific effects of flow interaction are given by the second term. However, this additional correction term depends upon the magnitude of the quantity $(1-\bar{v}_3\rho)/(1-\bar{v}_2\rho)$. Dioxane–water mixtures provide an exceptionally advantageous case, since the partial specific volume of dioxane in water is nearly unity, and the above quantity is of the order of 0.1. Hence, it should be possible (unless $L_{23}/L_2 >> 1$) as a first approximation to ignore the problem of flow interaction and treat these mixtures as two-component systems. In this event, equation 1 should be sufficient, and variations in values of $S_{25,w}$ so obtained should reflect changes in the structure or hydrodynamic properties of the molecule.

The factors in equation 1 were obtained as follows:

1. The value of the ratio $\eta_{t,m}/\eta_{25,m}$ was approximated by interpolation in the data of Geddes,[10] for the temperature dependence of the viscosity of dioxane–water mixtures. This factor was quite close to unity in all cases.

2. The ratio $\eta_{25,m}/\eta_{25,w}$ was obtained from measurement of viscometer flow times and densities of the solvent mixtures, as described in the experimental section.

3. It was assumed that

$$\frac{(1-\bar{v}\rho)_{25,m}}{(1-\bar{v}\rho)_{t,m}} = \frac{(1-\bar{v}\rho)_{25,w}}{(1-\bar{v}\rho)_{t,w}} \qquad (3)$$

The temperature dependence of $\bar{v}$ for serum albumin was estimated to be 0.0004 ml./g.-deg. While no direct experimental confirmation seems available, the above assumption probably is safe, since the correction never exceeded 0.5%.

4. Measured values of the densities of the mixed solvents and solutions allowed calculation of the apparent partial specific volume of bovine serum albumin in these dioxane–buffer mixtures. Solutions containing approximately 0.7 g./100 ml. of protein in mixtures containing 5, 10, 15, 20, 25, and 30% dioxane were employed. The apparent partial specific volumes were calculated from

$$\phi_v = \frac{V - w_1/\rho_1}{w_2} \qquad (4)$$

where V is the volume of the solution in the pycnometer, ρ_1 is the density of the mixed solvent and w_1 and w_2 are the weights of solvent and protein, respectively, contained in the pycnometer. Since the apparent partial specific volume of BSA has been shown to be independent of protein concentration in water,[11] it was assumed that ϕ_v could be replaced by $\bar{v}$. The results of these determinations are shown in Fig. 1. The value of ϕ_v in buffer (0.740 ml./g.) is slightly higher than the average of the results obtained by Charlwood[12] (0.734 ml./g.). The effect of dioxane upon the apparent partial specific volume of BSA is slight, the data indicating a decrease of about 3% in ϕ_v at 30% dioxane. This decrease is of the same order of magnitude as the "excluded volume" estimated by Charlwood from comparison of values of $\bar{v}$ with values calculated, taking electrostriction into account, from the amino acid composition.

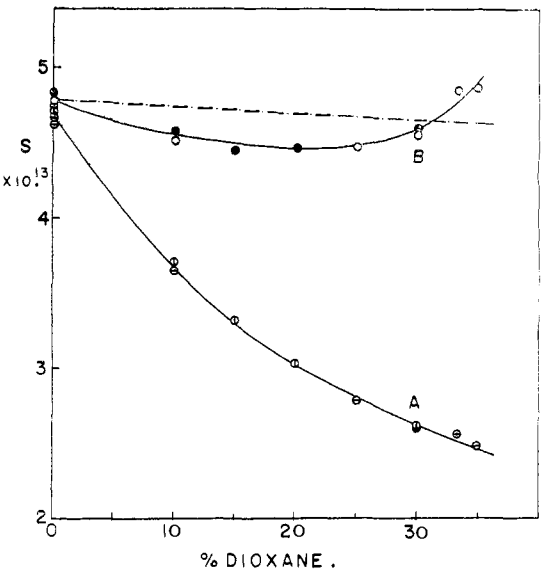

Fig. 2.—Sedimentation coefficients of BSA in dioxane–buffer mixtures. Curve A represents data corrected to 25° at various dioxane concentrations. In curve B, corrections have been applied for the variation of $\bar{v}$, ρ and η with solvent composition, according to equation 1. The symbols ⊖ and O refer to the earlier experiments (lot P67908) and the symbols ⊙ and ● to the later experiments (lot V68802).

In Fig. 2, curve A, are shown the results of the two sets of sedimentation experiments, corrected to 25°, but not corrected for the effect of dioxane upon solution density or viscosity. While concentrations were very nearly identical for separate runs in each of the two sets of experiments, there was a small difference between the average concentrations in the two sets. In order to make the results more comparable, the results from the earlier set have been multiplied by the factor (1.003) predicted by Baldwin's[8] equation for the concentration

(10) J. A. Geddes, *J. Am. Chem. Soc.*, **55**, 4832 (1933).

(11) M. O. Dayhoff, G. E. Perlmann and D. A. MacInnes, *ibid.*, **74**, 2515 (1952).

(12) P. A. Charlwood, *ibid.*, **79**, 776 (1957).

dependence of the sedimentation coefficient of BSA. The results for the later set of experiments, for which more reliable temperature data are available, are to be considered more accurate. After the data have been corrected for the relative viscosity, density and partial specific volume variation with dioxane concentration, curve B, Fig. 2, is obtained. The application of the corrections makes the variation of $S_{25,w}$ with dioxane concentration very slight. The values obtained in 0% dioxane (4.84×10^{-13} for the later data, 4.78×10^{-13} and 4.71×10^{-13} for the earlier) are in good agreement with the value predicted from the results of Baldwin[8] (4.82×10^{-13}) at the average protein concentration used.

In these experiments, a small amount of the more rapidly sedimenting component which has been frequently reported in serum albumin was observed. For two experiments, one in 0% dioxane, one in 30% dioxane, the refractive index gradient curves were analyzed in detail. Representative refractive index gradient curves are shown in Fig. 3. The broken line in each diagram represents the gradient curve for the more rapidly sedimenting component, which has been calculated in the following manner: the gradient for the main (trailing) component was assumed to be symmetrical about the maximum, and values of Z at various distances from the maximum on the trailing side of the boundary were subtracted from values of Z at the corresponding points in the leading side of the boundary. The areas under these two curves were corrected for the radial dilution effect by multiplying by the ratio $(r_h/r_0)^2$ where r_h is the position of the appropriate maximum in each photograph and r_0 the meniscus position. Average areas thus corrected, as well as values of the relative sedimentation coefficient of the fast component are shown in Table I.

TABLE I

ANALYSIS OF GRADIENT CURVES

	A_0[a] (total)	A_0[a] (fast)	% fast[b]	S_{fast}/S_{slow}
0% dioxane	4.58 ± 0.04	0.45 ± 0.03	9.8	1.50 ± 0.05
30% dioxane	3.90 ± .03	0.32 ± 0.02	8.3	1.55 ± 0.03

[a] Arbitrary units. [b] Based on areas under gradient curves, corrected for radial dilution.

The percentage of fast component is comparable to that observed by Baldwin.[8] The difference between the 30% dioxane and dioxane-free solutions may not be of significance, since the apparent amount of fast component is rather sensitive to the accuracy of the base line. The ratio of sedimentation coefficients of the two components is nearly the same in both cases. It has been suggested that the more rapidly sedimenting component in BSA preparations is a dimer; if so, these results indicate that there is no increase in dimerization in dioxane. The change, if any, is in the opposite direction.

The molecular weight has been determined by calculation of the diffusion coefficient of the main component. Values were corrected for the boundary sharpening resulting from concentration dependence of S by the procedure described in a recent note.[13] The diffusion coefficients of BSA, corrected to water at 25° by applying the appropriate viscosity factors, are: in 0% dioxane, 6.94×10^{-7}, and in 30% dioxane, 7.10×10^{-7}. These results yield for the apparent molecular weight the values 65,900 and 61,200. Since these data apply to one concentration only and have not been corrected for flow interaction, the 7% difference probably is not significant.

(13) K. E. Van Holde, *J. Phys. Chem.*, **64**, 1582 (1960).

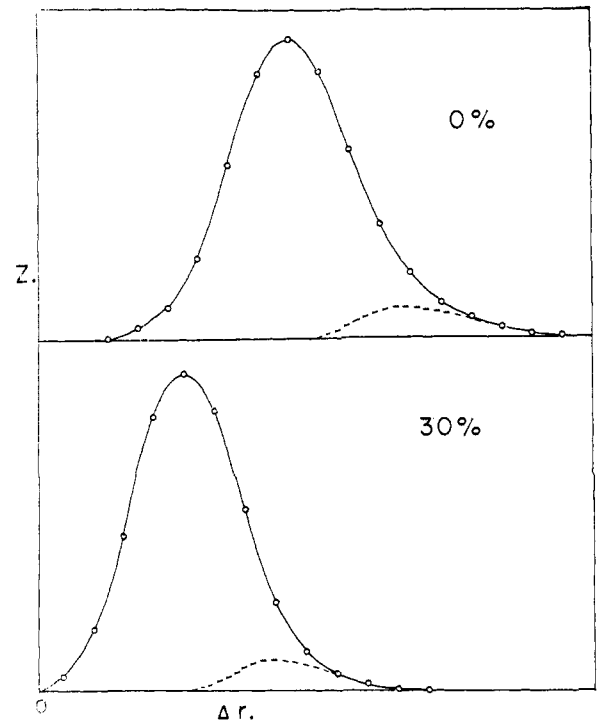

Fig. 3.—Refractive index gradient curves from sedimentation experiments in 0% and 30% dioxane. The left edge of the graph indicates the meniscus position, and the time of sedimentation is approximately the same in both. The dotted curves correspond to the rapidly sedimenting component and were calculated as described in the text.

The difference in total area under the schlieren patterns is well beyond experimental error. A difference is to be expected, since the specific refractive index increment of BSA should be smaller in buffer–dioxane mixtures than in buffer alone; furthermore, any selective solvation of the protein by one component will lead to a change in the composition of the medium across the boundary over and above that resulting from the simple presence of the protein component. If additivity of molar refractions and no volume change on mixing are assumed, it is easy to show that

$$n - n' = \frac{(n')^2 + 2}{n' + 1}(A_2\Delta\phi_2 + A_3\Delta\phi_3) \qquad (5)$$

where n and n' are the refractive indices on the solution and solvent side of the boundary, respectively, $\Delta\phi_2$ and $\Delta\phi_3$ are the changes in volume fractions of protein and dioxane, respectively, across the boundary, and A_2 and A_3 are constants given by the general equation

$$A_i = \frac{n_i^2 - 1}{n_i^2 + 2} + \frac{n_1^2 - 1}{n_1^2 + 2} \qquad (6)$$

n_1 being the refractive index of water. The constant A_3 can be calculated from known refractive indices, and the constant A_2 from the specific refractive index increment of BSA in the absence of dioxane ($dn/dc = 0.193 \times 10^{-2}$ when c is in g./100

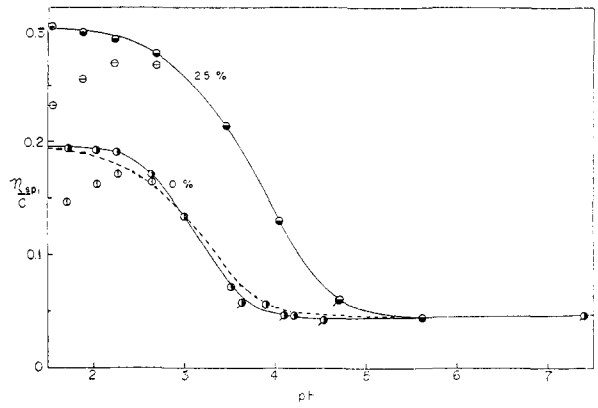

Fig. 4.—Reduced viscosity *versus* *p*H for BSA in 0.03 *M* KCl (lower curve) and in 0.03 *M* KCl containing 25% dioxane (upper curve). The unfilled circles in each case represent the original data; the half-filled circles represent data corrected for the increase in ionic strength at low *p*H. Data indicated by ◑ or ◒ were obtained upon reversal of the expansion.

ml.).[14] The quantity $\Delta\phi_2$ should not be zero even in the absence of specific solvation since addition of protein will decrease the volume fractions of all other components. The $\Delta\phi_3$ expected on this basis can be shown to be

$$\Delta\phi_3^0 = \frac{-\phi_2\phi_3}{1-\phi_2} \qquad (7)$$

where ϕ_2,ϕ_3 refer to concentrations in the protein solution. On this basis, assuming $\bar{v}_2 = 0.72$ ml./g.

$$\Delta\phi_3^0 = -0.160 \times 10^{-2}$$

From the area under the boundary in the experiment, in 30% dioxane, as compared to the area under the boundary in the experiment without dioxane, the result obtained is

$$\Delta\phi_3 = -0.223 \times 10^{-2}$$

The difference can be interpreted in terms of hydration of BSA, if it is assumed that there is no solvation by dioxane. A hydration of 0.30 g. water per g. protein would account for the difference. This is in the same range as values previously estimated for globular proteins. It should be noted, however, that this result is obtained in the presence of 30% dioxane in the surrounding medium and implies that the water is rather tenaciously held by the protein. One might ask whether the variation of the sedimentation coefficient of BSA with dioxane concentration may be attributed to such hydration. The equation of Schachman and Lauffer[15] predicts for the sedimentation coefficient in a ternary system where solvation occurs

$$S = \frac{M(1-\bar{v}\rho)}{Nf}\left(1 + \frac{h}{m}\frac{(d-\rho)}{(1-\bar{v}\rho)}\right) \qquad (8)$$

where h/m represents the volume of solvent of density d held per unit mass of solute. If it is assumed that the solvation is independent of dioxane concentration and that variation of the frictional coefficient f is accounted for by the change of solution viscosity, equation 8 may be used to estimate the variation of S with dioxane concentration. The result is shown by the dotted line in Fig. 2. Obviously, the above assumptions are insufficient to account for the observed change in S.

The conclusion to be drawn is that the observed variation in S results either from flow interaction of a type not adequately described by equation 8, and (immense) variation in solvation with dioxane concentration, or as a result of a change in the conformation of the BSA molecule produced by the dioxane. Some further information is provided by optical rotation measurements. In Fig. 1 are shown values of the quantity $\frac{n_w^2+2}{n_m^2+2}[\alpha]_{546} = [\alpha]^*$ as a function of dioxane concentration. This quantity represents specific rotation values $[\alpha]$, at 546 $\mu\mu$ corrected for the effect of the varying refractive index of the solvent (n_m).[16] The refractive indices of dioxane–water mixtures were interpolated from the data of Gillis and Delaunois[17]; n_w is the refractive index of water. The factor $(n_w^2+2)/(n_m^2+2)$ ranges between 1.000 and 0.979 for these solutions. The corrected specific rotation varies only slightly; the change with increasing dioxane concentration is in the direction usually associated with an *increase* in helix content of the protein molecule.

The changes observed in the sedimentation velocity and specific rotation of BSA in dioxane–water mixtures near the isoelectric point are consistent with those observed by Bresler, *et al.*,[4,5] with the similar human and horse serum albumin. They stand in marked contrast to the results obtained by Tanford, *et al.*,[3] with β-lactoglobulin in dioxane–water mixtures. In the latter work a very pronounced configurational change was observed as dioxane was added. The serum albumins seem to be much more resistant to the effect of an organic solvent than either β-lactoglobulin or ribonuclease.[2]

B. Acidic Solutions.—In marked contrast to the relative insensitivity to dioxane shown by BSA near the isoelectric point are the results obtained in acidic solutions. It is well known, of course, that addition of acid to aqueous BSA solutions produces a reversible "swelling" of the molecule.[18,19] Such behavior is illustrated by the lower curve in Fig. 4, which shows the variation in the reduced viscosity (specific viscosity divided by concentration) as HCl is added to a solution initially containing 1.26 g. BSA per 100 ml. of 0.03 *M* KCl. The increase of η_{sp}/C between *p*H = 4.4 and 2.2 is similar to that which has been observed by Yang and Foster[18] and Tanford, *et al.*[19] The decrease at still lower *p*H is presumably an ionic strength effect; it will be discussed later. The half-filled circles represent data "corrected" for this effect, to a constant ionic strength of 0.03.

When the same process is carried out in a solution containing 1.40 g. of BSA/100 ml. in a mixture 25% in dioxane and 0.03 *M* in KCl (adding an HCl solution containing 25% dioxane), the curve represented by the upper set of half-filled circles in Fig. 4 is obtained. In this case it is necessary to

(14) Calculated from the data of G. E. Perlmann and L. G. Longsworth, *J. Am. Chem. Soc.*, **70**, 2719 (1948).

(15) H. K. Schachman and M. A. Lauffer, *ibid.*, **72**, 4266 (1950).

(16) See, for example, J. A. Schellman, *Compt. rend. trav. lab. Carlsberg*, **30**, 363 (1958).

(17) J. Gillis and A. Delaunois, *Rec. trav. chim.*, **53**, 186 (1954).

(18) J. T. Yang and J. F. Foster, *J. Am. Chem. Soc.*, **76**, 1588 (1954).

(19) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

correct the pH values as read from the glass electrode pH meter for the presence of dioxane. This has been done by using the data obtained by Van Uitert and Fernelius.[20] In no case did the corrections amount to more than 0.14 pH unit.

The curve obtained in 25% dioxane differs from that obtained in the absence of dioxane in two respects: The maximum reduced viscosity attained is much higher, and the change commences at a higher pH. The latter effect may be semi-quantitatively understood if the swelling of the protein molecule is made possible only as carboxyl groups become protonated. Studies of the dissociation of a number of carboxylic acids in 1,4-doxane[21] have indicated that pK values will be shifted upward by about 0.6 pH unit in solutions containing 25% dioxane. The broken curve in Fig. 4 has been obtained by arbitrarily reducing the curve in 25% dioxane and then shifting this to the left on the pH scale by 0.6 unit. The curve thus obtained agrees fairly well with the experimental data.

The increase in expansion at a given pH upon addition of dioxane had been observed previously and qualitatively explained by Foster and Yang.[6] In order to understand this effect it is necessary to consider the forces involved in maintaining a particular expanded configuration of the molecule. It is most convenient to estimate the free energy change corresponding to a change in dimensions of the swollen molecule. As a rough first approximation, we may regard the molecule as a spherical swollen particle of a poly-electrolyte microgel. Because of the large number of disulfide cross links, this picture should be better suited to BSA than to most other globular proteins. The free energy change may be thought of as consisting of three parts:

A. A free energy of mixing of solvent molecules in the protein network, given approximately by Flory's[22] expression where n_1 is the number of sol-

$$\Delta F_{\text{mixing}} = kT(n_1 \ln (1 - v_2) + \chi_1 n_1 v_2) \quad (9)$$

vent molecules in the gel, v_2 the volume fraction of protein in the swollen molecule and χ_1 an interaction parameter. In the case of a mixed solvent, it is assumed that the composition of the solvent within and without the protein is the same.

B. A term resulting from the entropic elastic response of the gel network. Again from Flory[23] is obtained

$$\Delta F_{\text{elastic}} = \frac{kT\nu_e}{2}(3q^{2/3} - 3 - \ln q) \quad (10)$$

where ν_e is the number of residues in the network which are points of cross-linking and q is the swelling ratio

$$q = \frac{\text{volume of swollen gel}}{\text{volume of unswollen gel}} \quad (11)$$

C. The electrostatic free energy of a swollen sphere with a uniformly distributed charge Ze, as given by Tanford[24]

(20) L. G. Van Uitert and W. C. Fernelius, *J. Am. Chem. Soc.*, **76**, 5887 (1954).

(21) See, for example, the data given by R. A. Robinson and R. H. Stokes in "Electrolyte Solutions," 2nd Ed., Butterworth's, London, 1959.

(22) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 577.

(23) *Ibid.*, p. 578.

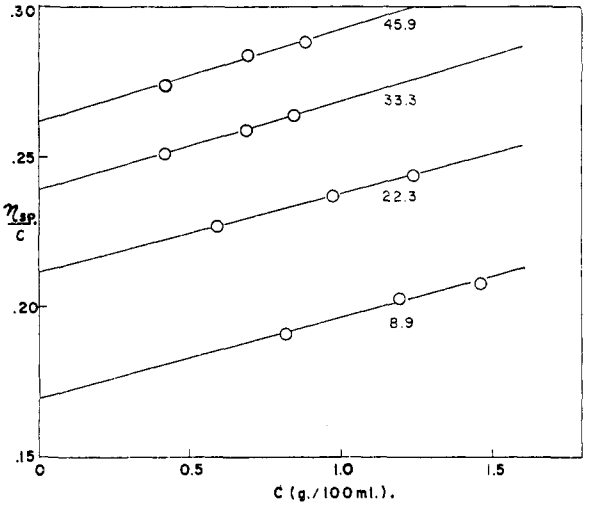

Fig. 5.—Reduced viscosity *versus* concentration for BSA in aqueous 0.03 M KCl solutions containing varying amounts of dioxane. The percentage of dioxane is indicated by the number below each line.

$$\Delta F_{\text{elast}} = \frac{3Z^2\epsilon^2}{2K^2DR^3}\left(1 - \frac{3}{2K^3R^3}[K^2R^2 - 1 + (1 + KR)^2\exp(-2KR)]\right) - F_E' \quad (12)$$

Here D is the dielectric constant of the medium within the sphere (assumed to be the same as the dielectric constant of the surrounding medium), ϵ is the electronic charge, K is the familiar Debye–Hückel parameter and R is the radius of the swollen sphere. F_E' is the electrostatic free energy of the unswollen sphere. All of the above quantities can be written in terms of the swelling factor q and summed to give the total free energy change in swelling

$$\Delta F = \frac{kTV_0}{V_1}(q - 1)(\ln (1 - 1/q) + \chi_1/q) + \frac{kT\nu_e}{2}(3q^{2/3} - 3 - \ln q) + \frac{3Z^2\epsilon^2}{2K^2DR_0^3q}\left(1 - \frac{3}{2K^2R_0^3q}[K^2R_0^2q^{2/3} - 1 + (1 + KR_0q^{1/3})^2\exp(-2KR_0q^{1/3})]\right) - F_E' \quad (13)$$

where V_0 and V_1 are molar volumes of unswollen protein and solvent, respectively, and R_0 is the radius of the unswollen sphere. The equilibrium value of q can be found by setting $\partial\Delta F/\partial q)_{T,P} = 0$. Since $KR_0 > 1$ in most cases of interest, exponential terms in the result may be neglected for moderate or large degrees of swelling.

$$-\frac{kTV_0}{V_1q^2}(1/2 - \chi_1 + 1/3q + \ldots) + \frac{kT\nu_e}{q^{1/3}}(1 - 1/2q^{2/3}) - \frac{3Z^2\epsilon^2}{2K^2DR_0^3q^2}(1 - 2/KR_0q^{1/3} + 3/K^3R_0^3q) = 0 \quad (14)$$

For highly expanded BSA molecules, it would seem reasonable that the term $1/3q$ and higher terms could be dropped. If this can be done, the approxi-

(24) C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955). Of the two models considered by Tanford, the one employed here would seem more reasonable for highly swollen protein molecules.

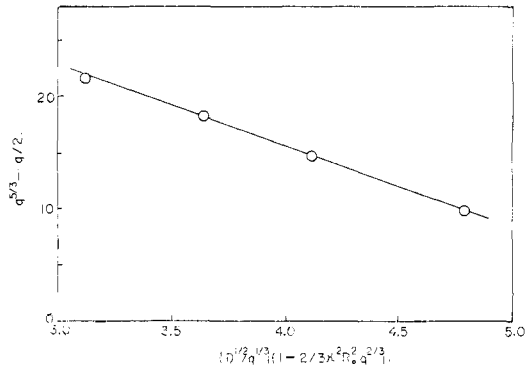

Fig. 6.—A graph of the swelling factor calculated from intrinsic viscosity plotted according to equation 15.

mate result can be put in a usable form

$$q^{5/3} - q/2 = \frac{V_0}{\nu_e V_1}(1/2 - \chi_1) + \frac{3000Z^2}{8\pi R_0^3 \nu_e N} - \frac{3000Z^2}{4\pi R_0^4 \nu_e}\left(\frac{1000kT}{4\pi\epsilon^2 N\Gamma}\right)^{1/2}\frac{D^{1/2}}{q^{1/2}}(1 - 3/2K^2R_0^2q^{2/3}) \quad (15)$$

In this equation substitution for K has been made

$$K = \left(\frac{4\pi\epsilon^2 N\Gamma}{1000DkT}\right)^{1/2} \quad (16)$$

where Γ is twice the ionic strength. Equation 15 predicts a linear relationship between $(q^{5/3} - q/2)$ and the quantity $(D^{1/2}/q^{1/2})(1 - 3/2K^2R_0^2q^{2/3})$.

Since the swelling factor q should be approximately equal to the ratio of the intrinsic viscosity of the swollen molecule to that of the unswollen molecule, an experimental test of equation 15 is available. Measurements of the intrinsic viscosity of BSA in 0.03 M KCl, at pH = 2.0–2.1, were made at a number of dioxane concentrations. The extrapolations to infinite dilution are shown in Fig. 5. The results are graphed according to equation 15 in Fig. 6, using 0.038 for $[\eta]$ of unswollen BSA. The data seem to lie on a straight line, over the rather narrow range covered. On the assumption that the mixing term is negligible, the slope and intercept of the line yield 21 Å. for R_0 and ν_e = 28. The value for R_0 is definitely too small; a value in the range 25–30 Å. would be expected.[19] The result for ν_e is reasonable, for 17 (S–S) bonds would give ν_e = 34, and it may well be expected that a number of these bonds span such short distances as to be relatively ineffective. The assumption that the mixing term is negligible is of dubious validity, but there appears to be no readily available check on this point. The charge on the protein under these conditions has been estimated as +68, from the data of Tanford.[19]

The above theory is somewhat similar to, but much simpler than, that advanced by Rice, Wada and Geiduschek.[25] It should be emphasized that it does not attempt to explain the entire process of expansion of the BSA molecule but rather only the region of relatively high expansion, in which short-range segmental interactions have become unimportant.

The theory also has been applied to the correction of the specific viscosity data in Fig. 4 for variation of ionic strength with addition of acid. From the way in which the ionic strength enters into equation 15, it is apparent that the dimensions of the molecule should decrease, and hence η_{sp}/c should decrease, with increasing ionic strength at pH values sufficiently low that the charge remains essentially constant. The half-filled circles in Fig. 4 represent data corrected to an ionic strength of 0.03 on the basis of equation 15 and the values of the parameter ν_e = 28, R_0 = 21 Å. and Z = +68. The effect of chloride ion binding upon the ionic strength was not taken into account. Examination of Fig. 4 shows that the application of these corrections yields values of η_{sp}/C which very nearly "level off" at pH values below 2, a result in accord with expectation, since the charge of the protein remains essentially constant below this point.

Yang and Foster[18] have studied the variation of $[\eta]$ with ionic strength in aqueous solutions of BSA and proposed the equation

$$[\eta] = D + \frac{1}{1/[\eta]_{00} + A\sqrt{\Gamma/2}} \quad (17)$$

where D is the value of $[\eta]$ at infinite ionic strength and $[\eta]_{00}$ the value at zero ionic strength. It is difficult to compare our theory with this equation since equation 15 should apply to neither limit. At very low ionic strength dropping the exponential terms in equation 13 is not justifiable, and at high ionic strength the value of q is not sufficiently great to justify other approximations which we have made.

Summary.—At the risk of oversimplification, the effect of dioxane upon the conformation of BSA in solution can be described in terms of effects of the altered dielectric constant of the medium upon electrostatic interactions. Near the isoelectric point the addition of dioxane seems to produce only minor changes in the protein conformation. Dioxane does, however, change the pH at which carboxyl groups are protonated, and hence the pH at which the reversible swelling of the molecule commences. At the same time, the decreased dielectric constant enhances electrostatic repulsion between the positively charged groups, resulting in greater expansion of the molecule. This conclusion agrees with the experimental results and the explanation put forward by Foster and Yang in their studies of the effect of dioxane upon the BSA molecule. It is of interest that they found that in acidic solution, a large change in intrinsic viscosity produced by dioxane was accompanied by little change in optical rotation.

While the process is likely be be more complex than the above statements would imply, this point of view explains the data available at the present time. A further test of these ideas would be the measurement of the intrinsic viscosity of BSA in acid mixtures containing various organic solvents, adjusted to the same dielectric constant.

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(25) S. L. Rice, A. Wada and E. P. Geiduschek, *Discussions Faraday Soc.*, **25**, 130 (1958).