from those of BSA. The variation of w with pH is also essentially the same, as seen on the acid side by the fact that the curve for HSA is as steep as that for BSA, and as best shown on the alkaline side by the similarity between the titration curves for the phenolic groups.[10]

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IOWA CITY, IOWA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Reversible Expansion of Bovine Serum Albumin in Acid Solutions[1]

BY CHARLES TANFORD, JOHN G. BUZZELL, DAVID G. RANDS AND SIGURD A. SWANSON

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Bovine serum albumin exists in a compact form between pH 4.3 and 10.5. Below pH 4.3 and above pH 10.5 it undergoes expansion. This paper shows that the compact form is held together by a network of bonds involving the side-chain groups of the protein. Near pH 4.2 or 10.5 the titration of some of these side chains results in transition to an *expandable* form. This form undergoes continuous expansion, increasing with charge and decreasing with ionic strength, so as to reduce the electrostatic free energy. A further slow molecular change occurs on standing. The entire process, including the final slow change, is reversible.

Numerous investigators during the last five years have discovered, independently, that the behavior of serum albumin in acid solutions is considerably different from that of other well-known proteins. The first indication of this difference came from the titration curve of human albumin which, in the acid region, is much steeper than theory predicts.[2] Both Scatchard and one of the present authors have interpreted this as the result of an *expansion* of the protein molecule with increasing acidity.[3,4] About the same time, Macheboeuf, Barbu and co-workers[5] observed an anomalous viscosity increase, which they interpreted as an *association*, and shortly thereafter Weber[6] observed an anomalous increase in the apparent rotational diffusion constant (from studies of the depolarization of fluorescence) which he interpreted as a *dissociation*. Pedersen[7] has stated, however, that unpublished diffusion–sedimentation studies made by him preclude the possibility of a change in molecular weight, and the same conclusion was reached from light scattering by Yang and Foster.[8]

Yang and Foster's is the most detailed study of the problem to date: they studied viscosity, optical rotation and light scattering, showing that all of them point to expansion as the probable cause of the anomalous behavior. Moreover, they found an absence of flow birefringence, so that the expansion appears to be essentially isotropic. Yang and Foster have also pointed out that the decrease in fluorescence polarization observed in acid BSA[6] is compatible with expansion if, as seems reasonable, the expansion is accompanied by increase in freedom for internal rotation.

Jirgensons[9] has also observed the same changes in optical rotation as Yang and Foster, and Gutfreund and Sturtevant[10] have observed an anomalous uptake of heat in acid solutions, which they have also ascribed to expansion.

The present investigation of bovine serum albumin (BSA) was intended primarily to confirm Yang and Foster's work, and to compare the progress of the expansion as reflected by viscosity measurements with its progress as reflected by changes in the empirical electrostatic interaction factor derived from the titration curves of BSA.[11,12] During the course of the investigation it soon appeared, however, that the expansion process is more complicated than was originally supposed. Yang and Foster[8] had visualized it as a single "all-or-none" equilibrium between an expanded and a compact form of BSA; our own initial idea[4,11] had been that it was a continuous change in configuration. It is shown in the present paper that there are actually at least three stages to the reaction: an initial "all-or-none" conversion from a compact to an *expandable* form, followed by continuous expansion of the expandable form, followed by a slow third stage, possibly an aggregation.

As a result of the discovery of this greater complexity of the reaction, this paper actually represents only a preliminary study, for each stage of the reaction now requires more detailed investigation, not only by the methods here given, but by some of the other techniques which have been used to provide evidence for the existence of the expansion.

Experimental

Crystalline BSA was purchased from Armour and Co. (lot P67502). The protein was dissolved in water and the solutions were passed through a mixed bed ion-exchange column as described by Dintzis.[13] The resulting stock

(1) A preliminary report on this work was presented at the 126th National Meeting of the American Chemical Society, New York, N. Y., September, 1954.

(2) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(3) G. Scatchard, *American Scientist*, **40**, 61 (1952).

(4) C. Tanford, *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).

(5) E. Gavrilesco, E. Barbu and M. Macheboeuf, *Bull. soc. chim. biol.*, **32**, 924 (1950); S. Bjornholm, E. Barbu and M. Macheboeuf, *ibid.*, **34**, 1083 (1952).

(6) G. Weber, *Biochem. J.*, **51**, 155 (1952).

(7) K. O. Pedersen, *Disc. Faraday Soc.*, No. **13**, 49 (1953).

(8) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(9) B. Jirgensons, *Arch. Biochem. Biophys.*, **41**, 333 (1952).

(10) H. Gutfreund and J. M. Sturtevant, THIS JOURNAL, **76**, 1595 (1954).

(11) C. Tanford in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

(12) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

(13) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

solutions were assumed salt-free and isoionic. Their concentrations were determined by drying to constant weight at 107°.

Solutions for measurement were prepared from such stock solutions by addition of appropriate amounts of standard HCl, KCl and conductivity water. All solutions were filtered through fine fritted Pyrex glass funnels immediately preceding their introduction into a viscometer.

Viscosity Measurements.—A study of the viscosity of serum albumin solutions in the region of *p*H 4.3 to 10.5, where no expansion takes place, has been reported elsewhere.[14] The method in the present paper differs from that previously used only in so far as changes were necessitated by the time dependence of viscosity below *p*H 4.

The viscometers used in this study were capillary viscometers of the Ubbelohde "suspended level" type.[15] The relation between viscosity and flow time for such viscometers is $\eta = \rho(At - B/t)$, where η is the viscosity, t the flow time, ρ the density[16] and A and B are constants determined by calibration with conductivity water.[17]

A complication arose in the present study because the flow times for many of the solutions changed with time (*cf.* Fig. 2). Accordingly, measurements of flow time were made over a period of time up to 20 hours, the first being made about 10 minutes after the solutions were mixed. The results were extrapolated to zero time. No significant change in density or *p*H with time was observed, so that no extrapolation of these quantities was necessary.

Intrinsic viscosities were obtained as previously described[14] by linear extrapolation, by the method of least squares, of reduced viscosity, $\eta_{red} \equiv \eta_{sp}/c \equiv (\eta - \eta_0)/\eta_0 c$, to zero protein concentration. Here η_{red} is reduced viscosity, η_{sp} is specific viscosity, η is the viscosity of a protein solution containing c grams of BSA per 100 ml., and η_0 is the viscosity of the solvent. Intrinsic viscosities were computed from the viscosities obtained from flow times extrapolated to zero elapsed time, and for some of the solutions also from those taken at 20 hours after mixing.

All viscosity measurements were made at 25.0 ± 0.002°.

Light Scattering Measurements.—Measurements of light scattering were made with a Brice–Phoenix light scattering photometer. Only a crude calibration of the instrument was made since the purpose of the measurements was to detect only changes in molecular weight. Solutions used for light scattering were filtered through Millipore filters[18] before measurement. Light scattering was measured at 25.0 ± 1.0°.

Results

All previous work indicates that the major portion of the expansion process is essentially complete within five minutes or less. Yang and Foster[8] report that their changes in optical rotation and in viscosity were "immeasurably fast." The titration curve data of the preceding paper[12] were often determined within less than 10 minutes, and no changes in *p*H were ever observed thereafter. Only Gutfreund and Sturtevant[10] report a "slow" reaction: the anomalous heat uptake observed by them took place, however, over a period of only 3.5 minutes. In the present study therefore, where changes in viscosity with time occurred, the values extrapolated to zero time are of principal interest. Since the earliest flow time measurements were made not less than 10 minutes after a solution was prepared, the Gutfreund–Sturtevant "slow" reaction is complete before our first measurements.

Table I lists the intrinsic viscosities obtained in this way, together with the average molecular charge Z at the *p*H and ionic strength where the measurements were made. The value of Z was computed from the data of the preceding paper.[12] For the sake of comparison, Table I lists also some intrinsic viscosities determined in the range of *p*H 4.3 to 10.5, intrinsic viscosities determined from 20-hour flow times, and corresponding data of Yang and Foster.[8,19] There is good agreement between our data and Yang and Foster's at $\mu = 0.15$, but the rise in viscosity observed by Yang and Foster at $\mu = 0.03$ occurs at a somewhat higher *p*H than ours.[20]

TABLE I

INTRINSIC VISCOSITY AT 25°

Ionic strength, μ	*p*H	Z	$[\eta]$ This paper, $t = 0$	$[\eta]$ This paper, 20 hr.	$[\eta]$ Yang and Foster
0.03	10.5	−37	0.041		
	Isoionic	− 5	.037		0.038
	4.3	+ 8	.038		.038
	4.0	+15	.043		.044
	3.5	+31	0.049	0.050	.076
	3.0	+47	.100	.105	.133
	2.5	+65	.153	.150	.165
	2.0	+69	.175	.176	.173
0.15	10.5	−45	0.040		
	Isoionic	−10	.0371		0.038
	4.50	+ 3	.0373		
	4.40	+ 6	.0371		
	4.30	+ 8	.0375		.038
	4.22	+10	.0379		
	4.10	+12	.0407		
	4.00	+13	.044		.040
	3.92	+15	.044		
	3.72	+21	.046		
	3.63	+24	.044		
	3.5	+29	0.046	0.050	.050
	3.0	+41	.065	.083	.071
	2.5	+57	.083	.084	.086
	2.0	+61	.083	.084	.086

The data at $\mu = 0.15$ are shown graphically in Fig. 1. They show clearly that the expansion process (quite apart from the subsequent changes with time) occurs in two distinct stages. There is a sharp increase in $[\eta]$ between *p*H 4.3 and 4.0, from the value of 0.037, characteristic of the compact protein in the neutral *p*H region, to about 0.045. Little further change then occurs until *p*H 3.5, where the major portion of the increase in $[\eta]$ begins. It should be noted that the titration curve of BSA is at its steepest between *p*H 3.5 and 4.0, so that the plateau of Fig. 1 is not the result of a diminished effect of *p*H on charge. In fact, the opposite is true: if $[\eta]$ is plotted against Z instead of *p*H the plateau becomes a little more pronounced.

(14) C. Tanford and J. G. Buzzell, *J. Phys. Chem.*, in press.

(15) L. Ubbelohde, *Ind. Eng. Chem., Anal. Ed.*, **9**, 85 (1937).

(16) Determined in the present study with pycnometers of the type described by M. R. Lipkin, *et al.*, *ibid.*, **16**, 55 (1944).

(17) *Am. Soc. Testing Materials, Standards*, Pt. V., 899 (1949).

(18) Millipore Filter Corp., Watertown 72, Mass.

(19) Values at the appropriate ionic strengths were obtained from the linear plots of Yang and Foster's Fig. 5. A personal communication from Dr. Foster indicates that the interest in their work lay in relative rather than absolute viscosity data. They therefore made no kinetic energy correction to their flow times, and also ignored the density difference between their solutions and the solvent. We have estimated (ref. 14, footnote 25) that the error so introduced is about 0.005 in $[\eta]$, and have corrected all of Yang and Foster's data by this amount. Yang and Foster's data were obtained after a lapse of several hours and should thus agree more closely with our twenty-hour results than with those at zero time.

(20) This may be due at least in part to the fact that we used the linear plots of Yang and Foster (their Fig. 5) to obtain the data. There is some question whether their experimental data justify a linear plot in the region of *p*H 3.2 to 3.8.

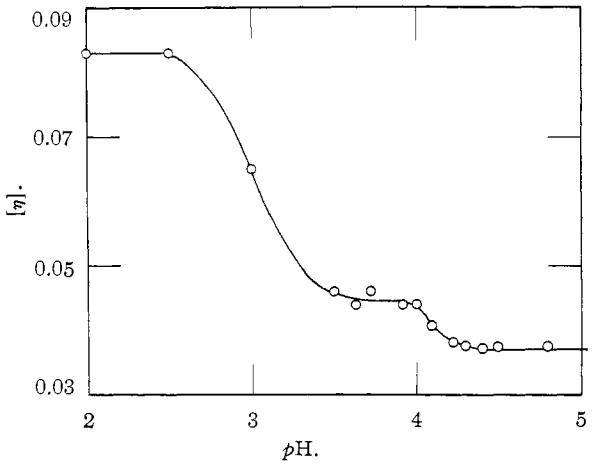


Fig. 1.—The intrinsic viscosity of BSA as a function of *p*H at $\mu = 0.15$, $T = 25°$.

A similar plateau is observed in the *p*H-dependence of the electrostatic interaction factor, shown in Fig. 6 of the preceding paper.[12] A large decrease in w occurs between *p*H 4.5 and 4.0, only a small change between *p*H 4.0 and 3.5, and then a further large decrease below *p*H 3.5.

There are not sufficient data at $\mu = 0.03$, either from our viscosity studies or from the titration curve, to decide unequivocally whether the two-stage feature of the expansion is observable at this ionic strength. It is certainly not seen in the dependence of w on *p*H at $\mu = 0.01$. As will appear in the discussion below this is to be expected: the two-stage nature of the process should be most evident at relatively high ionic strength.

Time Dependence.—Yang and Foster[8] have reported that there is a slow development of turbidity in low *p*H albumin solutions, but that it can be removed without apparent loss of protein in the solution. This observation has been confirmed by us. At the same time, however, we have observed small changes in viscosity (mostly increases), which Yang and Foster apparently did not observe. These changes in flow time are illustrated by some examples shown in Fig. 2. Removal of the precipitated material by filtration did not affect the rate of change of viscosity, so that we must conclude that a further change in the albumin structure occurs, beyond the two-stage expansion process.

The change which occurs is much greater at $\mu = 0.15$ than at $\mu = 0.03$, where the 20-hour intrinsic viscosities (Table I) are essentially the same as those at zero time. The change is also much greater at high than at low concentration. Both these properties suggest that an aggregation reaction producing greater asymmetry may be involved. The following section would indicate that only a small fraction of the BSA molecules could participate in such a reaction.

Changes in Molecular Weight.—Yang and Foster[8] have reported preliminary light scattering data indicating that no increase in molecular weight occurs in acid solutions of BSA. Our measurements confirm this. In one experiment, for example, the turbidity of a freshly-prepared 1% isoionic BSA solution was measured. Relatively

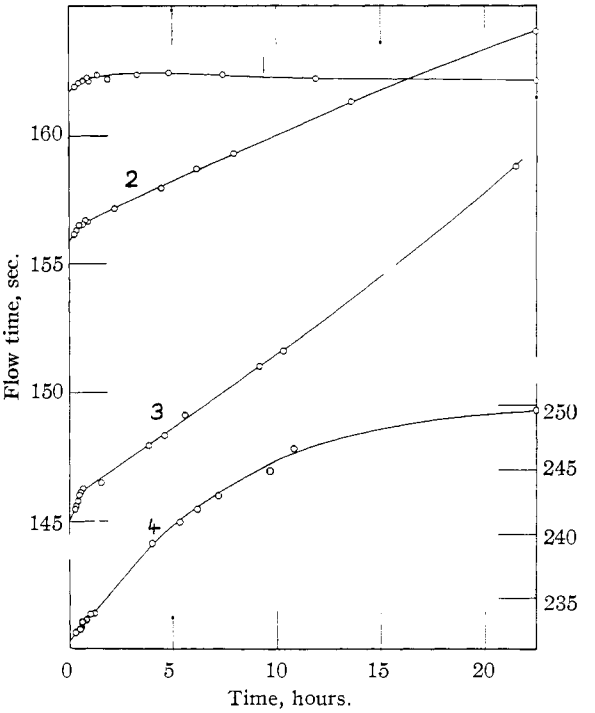


Fig. 2.—Time dependence of flow times of acid BSA solutions, at $\mu = 0.15$, 25°, and c approximately 4 g./100 ml. at *p*H 2.0 (curve 1), *p*H 2.5 (curve 2), *p*H 3.0 (curve 3) and *p*H 3.5 (curve 4). The curve at *p*H 3.5 was obtained in an early experiment using a Fenske viscometer rather than the Ubbelohde type used elsewhere in this paper. It should be noted that the changes in flow time here depicted are the maximal changes: less change is observed at lower ionic strength and at lower protein concentrations. It also should be noted that the initial steepness in these curves, lasting usually one or more hours is a characteristic of most of the time studies that were made.

concentrated, filtered HCl was added to bring the *p*H to 2, and the turbidity was followed as a function of time. A rapid increase with time was observed, with an initial induction period of 3 to 4 minutes (the same as the time required for the "slow" reaction of Gutfreund and Sturtevant[10]). The resulting turbidity, however, was greatly reduced by subsequent filtration, suggesting that the major portion of the observed increase is due to the precipitated material, in contrast to the slow increase in viscosity, which is unaffected by filtration. In any event, essentially no increase in molecular weight occurs during the few minutes at the end of which the two principal stages of the expansion are complete.

Figure 3 shows light scattering data obtained after lengthy exposure to *p*H 2, and filtration to remove the precipitated material. A small increase in molecular weight is suggested by the data on solutions which stood at *p*H 2 at or above 1% concentration, but none for the solution which stood at 0.35% concentration.

It is of interest in this connection that sedimentation studies of Saroff, Loeb and Scheraga[21] show the *reversible* transformation of a fraction of the BSA to a faster sedimenting, and therefore presumably a

(21) H. A. Saroff, G. I. Loeb and H. A. Scheraga, THIS JOURNAL, **77**, 2908 (1955).

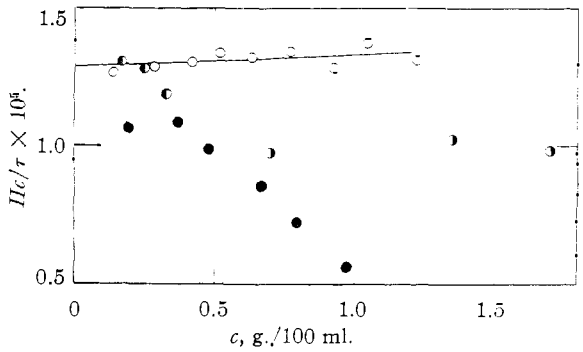


Fig. 3.—Light scattering measured at pH 2: ●, after 4 days standing at room temperature at 1% concentration; ◐, after 4 days standing at room temperature at 0.35% concentration; ◑, after 27 days standing (mostly in the refrigerator) at 2.6% concentration. The open circles and the line drawn through them represent measurements on fresh isoionic BSA. All determinations were made at 25° and $\mu = 0.15$.

heavier form on standing for two days at pH 3.5 and $\mu = 0.3$. No such change was observed after one day at the same pH and $\mu = 0.1$.

Reversibility.—When a solution exposed to pH 2 is returned to the isoionic region the viscosities obtained are essentially the same as for fresh isoionic solutions. This is true even for solutions exposed to pH 2 for several days, although several hours standing at the isoionic pH is then required before the viscosity returns to normal. (This, incidentally, is further indication that a molecular change occurs on standing at low pH.)

Figure 4 is an especially rigorous test of reversibility, on solutions exposed to pH 2 for 17 days. One of these was returned to the isoionic point, the other to pH 8.5. In both cases the viscosity data are not appreciably different from those obtained for fresh BSA not exposed to low pH. This shows that not only the isoionic viscosity, but also the resistance to expansion on the alkaline side, are unaffected even by lengthy exposure to pH 2.

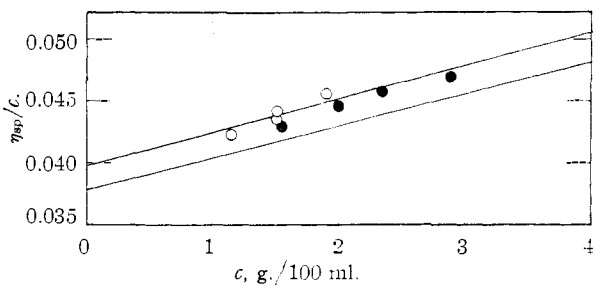


Fig. 4.—Reversibility study: the experimental points are for solutions reversed, after 17 days standing at pH 2, to pH 5 (●) and to pH 8.5 (○), respectively. The lines drawn represent similar data for solutions freshly brought to pH's indicated. All determinations are at 25° and $\mu = 0.15$.

Comparison of Viscosities with Electrostatic Interaction Factor.—We have mentioned above the similarity in the effect of pH and ionic strength on viscosity and on the electrostatic interaction factor (w) given by Fig. 6 of the preceding paper.[12] In order to compare the two types of measurement quantitatively, we have calculated from both the radii, R, of spheres equivalent to the average BSA molecule at the various pH's and ionic strengths.

For the relation between intrinsic viscosity and R we have used the relation derivable from Einstein's viscosity equation[22]

$$100[\eta] = 10\pi NR^3/3M \qquad (1)$$

where N is Avogadro's number, and M the protein molecular weight.

To relate w to R we have used the equation recently derived[23] for expanded spheres into which salt ions from the solvent may penetrate

$$w = \frac{\epsilon^2}{4DkT\kappa R^2}\left[1 - \frac{1 - \kappa R_0}{1 + \kappa R_0}\, e^{-2\kappa(R - R_0)}\right] \qquad (2)$$

where D is the dielectric constant of water, k is Boltzmann's constant, T the absolute temperature, κ the Debye–Hückel constant proportional to the square root of the ionic strength, ϵ the protonic charge, and R_0 the radius which the protein ion would have had if no solvent had penetrated it other than the tightly-bound water associated with the charged and polar groups. In the present calculation R_0 was placed equal to 28.8 Å. (allowing for about 20% hydration). The choice of R_0 is not critical, and any value between 26 and 31 Å. would have given essentially the same result.[24]

The values of R so calculated between pH 2 and 4 are shown in Fig. 5, those obtained from values of w having an uncertainty of about 2 Å. There is seen to be a discrepancy between R calculated from $[\eta]$ and that calculated from w at the lower ionic strengths near pH 3.5, but the over-all similarities are much more striking than the inconsistencies, and there seems no reason to doubt that the changes in $[\eta]$ and in w are a reflection of the same phenomenon.[25]

To relate w to R between pH 4.3 and 10.5 requires that one use the equation for an impenetrable sphere, equation 2 of the preceding paper,[12] rather than equation 2 of this paper. This yields $R = 33$

(22) A. Einstein, *Ann. Physik*, **19**, 289 (1906); **34**, 591 (1911).

(23) C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).

(24) Equation 2 assumes that salt ions penetrate some distance into the interior of the protein ion, but that the protein fixed charges remain on the surface. An alternative model was considered in ref. 23, in which the fixed protein charges are allowed to penetrate into the interior of the protein ion along with the solvent salt ions. The equation corresponding to this model does not give as good agreement with viscosity data as equation 2. The R values calculated by equation 2 tend to be higher at low ionic strength than those obtained from viscosity; those calculated by the equation permitting the fixed charges to be in the interior would be even higher, by about 4 Å. at $\mu = 0.03$ and 6 Å. at $\mu = 0.01$. This suggests that the protein charges must still be close to the surface in the expanded form of BSA, just as they are in the compact isoionic molecule. It is of interest in this connection that the intrinsic pK of the carboxyl groups of BSA does not appear to change during the expansion process,[12] which indicates that the environment of these groups remains unchanged.

(25) Since the completion of this paper we have seen the light scattering and sedimentation study of M. E. Reichmann and P. A. Charlwood, *Can. J. Chem.*, **32**, 1092 (1954). They, too, report no change in molecular weight with pH, except on standing, but they do find a change in sedimentation constant with both pH and ionic strength, which they interpret as a configurational change. We have calculated radii of equivalent spheres from their values of $s_{20,w}$, assuming a molecular weight of 65,000. Near neutrality we obtain $R = 36$ Å.; at pH 1.9 in 0.1 M KCl, $R = 48$ Å.; and at pH 1.9 in 0.5 M KCl, $R = 42$ Å. These values agree remarkably well with those of Fig. 5.

± 2 Å., compared with the value of 33.7 Å. obtained from the intrinsic viscosity. The reason for the large drop in w at $\mu = 0.15$ during the first stage of the expansion, as compared to the small drop in $[\eta]$ lies in the fact that even a small amount of expansion, resulting in some penetration of salt ions into the protein molecule, gives rise at least at an ionic strength as high as 0.15, to an entirely disproportionate change in w.[26]

Electroviscous Effect.—It has been shown previously[14] that intrinsic viscosity changes of the magnitude here observed cannot be ascribed to the electroviscous effect, *i.e.*, to the interaction between a protein ion and its ionic atmosphere. If one computes by Booth's equation[27] the contribution of the electroviscous effect to be expected for the expanded protein, one obtains a *maximum* effect, at *p*H 2 to 2.5, of about 0.001 at $\mu = 0.15$, and about 0.005 at $\mu = 0.01$. In calculating R from $[\eta]$, this contribution should, strictly speaking, be subtracted. This would result in a decrease in R at *p*H 2.5 of 0.2 Å. at $\mu = 0.15$ and of about 0.5 Å at $\mu = 0.01$, which is clearly negligible.

Discussion

Except at the isoelectric point a protein ion must always have a positive electrostatic free energy, which can always be reduced by configurational changes leading to an increased separation between the charges. When such configurational changes do not take place with increasing charge it necessarily implies the presence of internal bonds which require more free energy for rupture than can be gained by the change in configuration.

In BSA no configurational change occurs between *p*H 4.3 and 10.5. At *p*H 4.3 a two-stage expansion sets in, and a similar process appears to begin at *p*H 10.5 (see below). Two possible explanations may be proposed for this kind of *p*H effect.

(1) Acidic or basic side chain groups are involved in the internal bonds. As these groups are titrated with changing *p*H the corresponding internal bonds are broken or weakened, so that expansion previously characterized by a *net* positive free energy change is now accompanied by a *net* negative free energy change and thus occurs spontaneously. If this is the mechanism of the configurational change, it will not be primarily a function of charge or ionic strength, but rather of the number of groups titrated.

(2) Alternatively, no change in the strength of internal bonds with *p*H may occur. Instead the internal bonds required for maintenance of a compact structure may be weak, or easily adaptable to new, more extended configurations. In this case changes in configuration will occur wherever the electrostatic free energy becomes sufficiently great. The changes will take place most readily at high charge and at low ionic strength, regardless of the number of groups titrated.

(26) If one were to assume that the albumin molecule is still penetrable to salt ions above *p*H 4.3 and were to use equation 2 to evaluate R from w, one would get a value of R less than 30 Å. at $\mu = 0.15$, and, especially, an entirely erroneous dependence of w on ionic strength. If one assumes that the expanded albumin molecule is impenetrable to salt ions, one would obtain from w at $\mu = 0.15$ values of R as high as 65 Å.

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

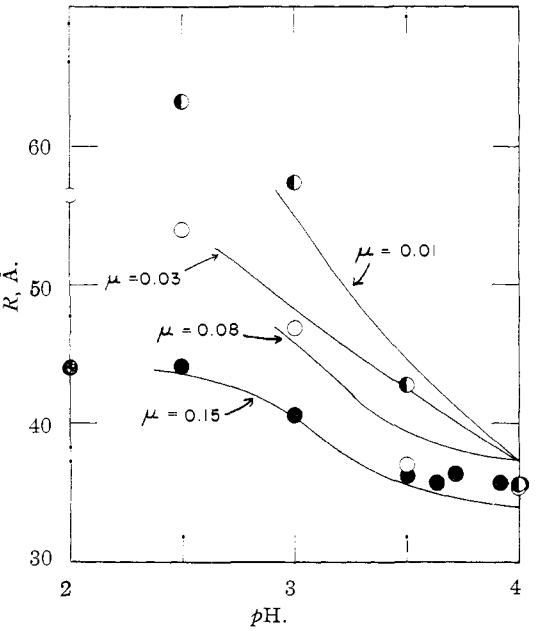


Fig. 5.—The radius of an equivalent sphere. The four curves represent the variation of R with *p*H as calculated from the variation of w with *p*H. Viscosity data are shown by the points: ●, $\mu = 0.15$; ○, $\mu = 0.03$; ◐, $\mu = 0.01$. The values at $\mu = 0.01$ are from Yang and Foster (ref. 8).

Our results indicate that the first of these explanations must apply to the initial change in the configuration of BSA. This initial change is not primarily caused by charge, for it occurs on the acid side where Z is approximately +10, but on the alkaline side not till Z is about −50. It occurs on the acid side slightly more readily at $\mu = 0.15$ than at $\mu = 0.03$, although the potential reduction in electrostatic free energy is greater at $\mu = 0.03$. This result is to be expected if the critical factor is the number of groups titrated, for the titration curve of BSA, like that of all proteins, is steeper at $\mu = 0.15$ than at $\mu = 0.03$, so that more groups have been titrated at a given *p*H at the higher ionic strength.

On the other hand, the second mechanism must apply to the events which occur between *p*H 4 and *p*H 2, for here the greatest change is observed at the lowest ionic strength and highest charge. The number of groups titrated can have but little specific effect on the second stage of the expansion.

This difference in mechanism between the two stages of the expansion enables one to understand why the separation between the two stages should be more clearly exhibited at the higher ionic strengths used. If the completion of the first stage depends primarily on the number of groups titrated, it will take place at higher ionic strength at a lower *net* charge than at low ionic strength, because of greater chloride ion binding at the higher salt concentration. This lower charge, combined with the fact that the electrostatic free energy is in any event lower at high ionic strength, even with the same *net* charge, means that the BSA molecule at the completion of the first stage of the expansion has a considerably lower electrostatic free energy at higher ionic strength. If, then, the second stage of

the expansion depends on the ability of the electrostatic free energy to overcome the binding energy of the remaining weak or adaptable internal bonds, the second stage may begin at once at lower ionic strength, *i.e.*, it will be more or less continuous with the first stage. At higher ionic strength, on the other hand, it may well be necessary to reduce the *p*H further, so as to increase the charge, and, hence, the electrostatic free energy, before the second stage can begin. In this way a plateau such as that of Fig. 1 can arise.[28]

"All-or-None" or Continuous Change.—The first stage of the expansion of BSA occurs over a narrow region of *p*H, and depends on the number of groups titrated. The curves of $[\eta]$ or R *vs.* *p*H between *p*H 4.3 and 4.0 probably represent an equilibrium between two forms (an "all-or-none" process).

Yang and Foster[8] have suggested that close resemblance between the *p*H-dependence of optical rotation and of viscosity implies the existence of an "all-or-none" process. If this argument is applied to the present data, one would conclude that the second stage of the expansion, like the first, represents an "all-or-none" process, *i.e.*, an equilibrium between the form existing near *p*H 4.0 and a single, fully-expanded form. The values of $[\eta]$, w, R and other properties in the region of *p*H 2 to 4 would then be *average* values reflecting this equilibrium. The wide range of *p*H over which the second stage of the expansion occurs, however, argues against this conclusion, and this can be shown quantitatively, as follows.

If the process occurring is an "all-or-none" process involving only two forms, then all curves of any physical property *vs.* *p*H must reach the *same* limiting values at each ionic strength.[29] Since the limiting values actually observed at *p*H 2 are strongly ionic-strength dependent, an "all-or-none" process would require that the conversion to the fully-expanded form is incomplete, *i.e.*, that the limiting values of $[\eta]$, $[\alpha]$, w or R have not been reached.

To illustrate the inadequacy of the "all-or-none" concept, keeping in mind the fact that the driving force for the process is primarily electrostatic, we shall examine Yang and Foster's curve of specific rotation, $[\alpha]$, *vs.* *p*H at $\mu = 0.02$. If the process is an all-or-none process, then $[\alpha]$ at any *p*H is given by

$$[\alpha] = x[\alpha]_1 + (1 - x)[\alpha]_0 \qquad (3)$$

where x is the mole fraction of the fully expanded form at that *p*H, $[\alpha]_1$, is the specific rotation of the fully expanded form, and $[\alpha]_0$ that of the form existing at *p*H 4. From equation 3 one may compute experimental values of x

$$x = ([\alpha] - [\alpha]_0)/\Delta\alpha \qquad (4)$$

where $\Delta\alpha = [\alpha]_1 - [\alpha]_0$. The value of $[\alpha]_0$ must be close to 63°, the value of $[\alpha]_1$, is unknown. The value of $[\alpha]_1$ cannot, however, be less than the maximum value of $[\alpha]$ observed in any experiment, *i.e.*, it cannot be less than 88°, the highest value observed by Yang and Foster in the absence of added salt. The smallest possible value of $\Delta\alpha$ is therefore 25°. Using this value[30] we may then calculate the apparent equilibrium constant, $K = x/(1 - x)$, as a function of *p*H.

For an expanded protein ion of constant dimensions, at constant ionic strength, one may write for the standard free energy

$$F^0 = F^0_{\text{int}} + F^0_{\text{elec}} \qquad (5)$$

where F^0_{int} is the intrinsic standard free energy, *i.e.*, the value of F^0 which the protein molecule would possess at $Z = 0$; while F^0_{elec} is the electrostatic contribution to the free energy, given, using the same model as for equation 2, by

$$F^0_{\text{elec}} = \frac{N\epsilon^2 Z^2}{4D\kappa R^2}\left[1 - \frac{1 - \kappa R_0}{1 + \kappa R_0} e^{-2\kappa(R - R_0)}\right] \qquad (6)$$

The radius of the form of BSA at *p*H 4 (Fig. 5) must be about 36 Å., that of the fully-expanded form cannot be less than the maximum radius observed in any experiment. The highest value of $[\eta]$ observed for any acid solution of BSA is 0.4 (Yang and Foster[8]), which corresponds to a radius of 74 Å. Using this value[31] and placing $\mu = 0.02$, one obtains with the aid of equations 5 and 6

$$\log K = -\Delta F^0_{\text{int}}/2.303RT + 0.0107Z^2 \qquad (7)$$

where ΔF^0_{int} is the intrinsic free energy change of the transition between the form of BSA at *p*H 4 and the fully-expanded form. (Our previous conclusion about the nature of the expansion process does not allow any important effect of the state of titration of the protein on ΔF^0_{int}.)

Figure 6 shows an experimental plot of $\log K$ *vs.* Z^2, and, for comparison, the slope predicted by equation 7. The experimental plots are seen to be non-linear, and much too flat. The difference in slopes is far outside any conceivable error in these calculations.

A similar result would be obtained if one were to use $[\eta]$ or w as a measure of K. The discrepancy would be worse at $\mu = 0.15$ than at $\mu = 0.02$; it would be worse if one were to assume that the two-stage nature of the expansion is somehow an artifact, and that the "all-or-none" equilibrium exists between the fully-expanded form and the compact, isoionic form, as Yang and Foster had originally supposed.[8]

One must conclude, therefore, that there is only a single *expandable* form of BSA below *p*H 4, which is able to expand to minimize its electrostatic free energy within the limits imposed by internal bonds such as disulfide bonds. The situation is very similar to that observed when BSA is "denatured" by urea,[32] where a continuous expansion with increasing urea concentration appears to take place.

Returning to equation 5, and applying it to a

(28) If we suppose for purpose of calculation that the BSA molecule at the end of the first stage of the expansion is a sphere of radius 36 Å., then, by equation 6, F^0_{elec} is 3600 cal./mole at *p*H 4.0 and $\mu = 0.01$, with 20 protons bound. At $\mu = 0.15$, although 36 protons are bound at *p*H 4.0, F^0_{elec} is only 1000 cal./mole. It attains a value of 3600 cal./mole at *p*H 3.6.

(29) A fully-expanded form having a different radius at different ionic strengths but not at different values of Z is improbable because the effect of Z on electrostatic free energy is very much greater than that of ionic strength (ref. 14).

(30) A larger value of $\Delta\alpha$ would produce a result even less favorable to the "all-or-none" hypothesis.

(31) Once again a higher value would produce a result less favorable to the "all-or-none" hypothesis.

(32) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5167 (1953).

continuous expansion rather than an "all-or-none" process, one can see that the equilibrium degree of expansion of the BSA molecule at a given concentration, charge and ionic strength, will be given by the relation $(\partial F^0_{int}/\partial R)_{\mu,z} + (\partial F^0_{elec}/\partial R)_{\mu,z} = 0$. The second term in this equation can be evaluated by equation 6. If now, F^0_{int} is a function of R only, and independent of the number of groups titrated (a quantity depending on μ and Z), then, at equilibrium $-(\partial F^0_{elec}/\partial R)_{\mu,z} = dF^0_{int}/dR$ = function of R only, independent of ionic strength. A plot of $-(\partial F^0_{elec}/\partial R)_{\mu,z}$ *vs.* R actually shows systematic, though not very great, variation with ionic strength. This suggests that F^0_{int}, and therefore the intrinsic ability to expand, depends a little, though not much, on the number of groups titrated. It is of interest to mention that the experimental values of dF^0_{int}/dR are about 250 cal./mole/Å. near the beginning of the expansion and rise to about 1 kcal./mole/Å. when $R = 60$ Å.

The Structure of the Compact and Expandable Forms of BSA.—The principal bonds which have been suggested for the maintenance of a compact structure in proteins are hydrogen bonds between peptide carbonyl and imine groups, disulfide bonds and "hydrophobic" bonds, *i.e.*, bonds arising from the minimization of the area of contact between non-polar portions of the molecule and water.[33] These particular bonds cannot be the critical bonds in the maintenance of the compact structure of BSA, for they are not directly affected by *p*H, whereas it was demonstrated above that the transition from the compact to the expandable form occurs as a result of the titration of critical bonds with changing *p*H. The *critical* bonds in the maintenance of the compact structure between *p*H 4.3 and 10.5 must therefore be the intrinsically weaker hydrogen bonds between side chain groups[34] or salt bridges, possibly acting in concert with weak "hydrophobic" links.

There is good evidence that such weak bonds do indeed exist in BSA to an extent not found in other proteins, as shown by the abnormal intrinsic dissociation constants of the carboxyl, amino and phenolic groups,[12] and by the configurational adaptability of the molecule, which permits it to bind a greater variety of small molecules than do other known proteins.[35] The fact that the side chain groups are so readily accessible, and that the adaptability toward binding of small molecules occurs in the range of *p*H 4.3 to 10.5, where the compact structure is normally maintained, suggests that these weak bonds are near the surface of the BSA molecule. They may be thought of, perhaps, as a net holding tightly together what would otherwise be the expandable form of BSA.

Near *p*H 4.2 and near *p*H 10.5, one of two things happens: *either* a few particular bonds in the network are broken,[36] *or* a sufficiently large fraction of all the weak bonds are broken.[37] In any event, the internal bonds, taken all together, are no longer capable of maintaining a rigid structure below *p*H 4 and above *p*H (about) 10.5, and the molecule expands at will at the behest of the electrostatic forces. The remaining bonds must be weak or adaptable to allow the more or less continuous configuration changes during the second stage of the expansion to occur rapidly and reversibly.

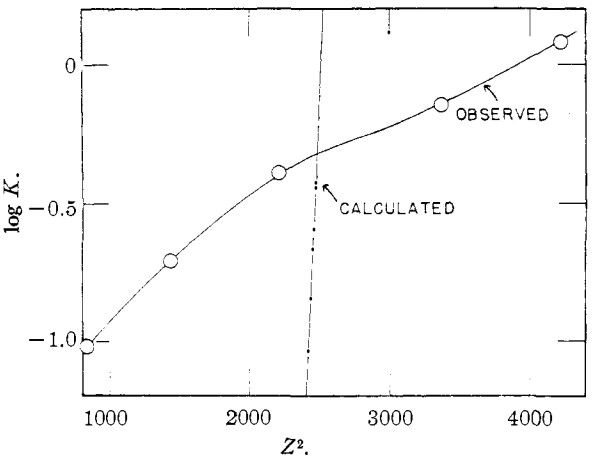


Fig. 6.—The apparent equilibrium constant for the second stage of the expansion, based on the assumption of an "all-or-none" equilibrium. The derivation of the calculated slope is given in the text. The experimental curve is based on Yang and Foster's optical rotations at $\mu = 0.02$.

Of interest in this connection is the finding of Levy and Warner,[36] that the irreversible heat denaturation of BSA occurs much more rapidly in the compact isoionic form than in the range of *p*H 2 to 4. This suggests that the expandable form of BSA has a greater capacity to absorb thermal energy in a reversible manner, making it less likely that such energy is concentrated in a particular bond or bonds leading to an activated complex for irreversible denaturation. Conversely, one may conclude that an increase in temperature in the isoionic region does not lead to a greater likelihood of transition to the expendable form, a conclusion not unexpected if the critical bonds are salt bridges, which are held together by entropy rather than bond energy (*i.e.*, their formation constants would have little temperature dependence.)

It should be mentioned, finally, that the intrinsic viscosity of BSA never attains values which would be characteristic of randomly-coiled polyelectrolytes, so that there must be a limit to the ability to expand. This limit is also seen in the increasingly high values of dF^0_{int}/dR observed with increasing R

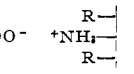
(33) W. Kauzmann, in W. D. McElroy and B. Glass, ed., "The Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1954.

(34) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(35) F. Karush, *ibid.*, **72**, 2705 (1950).

(36) The existence of a few critical bonds has been suggested to account for the *p*H-dependence of the heat denaturation of BSA: M. Levy and R. C. Warner, *J. Phys. Chem.*, **58**, 106 (1954).

(37) Near *p*H 4 about 25 carboxyl groups have been converted from the anionic to the acid form, near *p*H 10.5 about 20 amino groups have been converted from the cationic to the free form. Thus one could account for the observed result if bonds of the type



where R represents any non-polar side chain, were responsible for the compactness of the protein in the neutral region. That thermodynamic evidence for salt bridges exists is shown in the preceding paper.[12] It is difficult to conceive of such salt bridges as existing without the aid of "hydrophobic" bonding between non-polar groups (ref. 33).

(see above). As Kauzmann[32,33] has already pointed out in connection with the urea denaturation of BSA, the numerous disulfide bonds are probably at least in part responsible for this limit, and perhaps also for the reversibility of the expansion.

Expansion in Alkaline Solution.—Reference has been made a number of times in this paper to the fact that expansion of BSA occurs above *p*H 10.5 as well as below *p*H 4.3. This conclusion is based on the decrease in w occurring above *p*H 10.5, and on the behavior of optical rotation[9] and fluorescence polarization[6] above that *p*H, which parallels that below *p*H 4.3. We have made only a few exploratory viscosity measurements in this region, and have observed both an increase in viscosity and time dependence. It is probable that the expansion in alkaline solution will not show the plateau observed in Fig. 1, for the higher charge at which the first stage occurs should result in a high enough electrostatic free energy even at the higher ionic strengths so that there is immediate expansion of the expandable form. It is possible that the entire process in alkaline solution will be more difficult to study, because of the readiness with which aggregation initiated by the ionized sulfhydryl group is likely to take place.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Studies of Free Diffusion in Liquids with the Rayleigh Method. I. The Determination of Differential Diffusion Coefficients in Concentration-dependent Systems of Two Components

BY J. M. CREETH[1,2]

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The work described gives expressions relating the deviations between ideal and observed fringe positions in Rayleigh diffusion interferograms to the various coefficients describing the concentration-dependence of both the diffusion coefficient and the refraction increment; rigorous methods are given for the calculation of differential diffusion coefficients in such systems. The expressions have been tested by using them to predict the deviations to be expected in the cases of sucrose, glycine and butanol, where adequate concentration-dependence data are already available. Reasonable success has been achieved in this prediction. Some of the likely sources of error in Rayleigh diffusion work are examined, and two examples are given of a comparison between Gouy and Rayleigh results obtained in the same experiment.

In diffusion experiments conducted between solutions of two different concentrations C_1 and C_2, of the same solute, it has frequently been found[3,4] that for systems where the concentration-dependence of the diffusion coefficient is relatively small, the *mean concentration* $\bar{C} = (C_1 + C_2)/2$ of the experiment is the concentration corresponding to the measured diffusion coefficient, the latter being independent of the actual magnitude of the concentration increment $\Delta C = C_2 - C_1$. Such experiments are therefore taken to yield the differential diffusion coefficient, $D_{\bar{C}}$, corresponding to the mean concentration. Recently, however, results obtained on the markedly non-ideal butanol–water system by Lyons and Sandquist[5] indicated a dependence, albeit small, of the diffusion coefficient upon ΔC at constant $\bar{C}$, so that unambiguous values of $D_{\bar{C}}$ may not be obtained directly in this way.

Furthermore much interest has arisen recently[6,7] in the question whether the Gouy and Rayleigh optical interference methods now in widespread use do in fact yield identical results when applied to similar systems; in attempting to answer this question, it is clearly important that experimental data be analyzed to give results in terms of well-defined quantities and that any approximation introduced be closely scrutinized and estimated. The work of Fujita and Gosting[8] has shown how diffusion coefficients measured by the "height–area" method may be corrected for concentration-dependence of diffusion coefficient and of specific refraction increment to yield the true differential diffusion coefficient; the purpose of this paper is to show how parts of Fujita and Gosting's[8] theoretical development may be used to provide a basis whereby the results of Rayleigh diffusion experiments upon non-ideal systems may be similarly corrected. The work has shown, in addition, how the results may be treated to give an estimate of the concentration-dependence of the diffusion coefficient.

Theory

(a) Ideal Systems.—For the purposes of this analysis, ideal systems are defined as those in which both the diffusion coefficient and the refraction increment are independent of the concentration. For experimental systems where concentration is measured as a function of height in the diffusion cell, the relevant solution of Fick's[9] law is, in the ideal case, an expression of the form

(1) Rockefeller Foundation Fellow, 1952–1954.

(2) Department of Physical and Inorganic Chemistry, University of Adelaide, South Australia.

(3) *E.g.*, L. J. Gosting and M. S. Morris, THIS JOURNAL, **71**, 1998 (1949).

(4) M. S. Lyons and J. V. Thomas, *ibid.*, **72**, 4506 (1950).

(5) P. A. Lyons and C. L. Sandquist, *ibid.*, **75**, 3896 (1953).

(6) L. G. Longsworth, private communications to L. J. Gosting.

(7) F. J. Gutter and G. Kegeles, THIS JOURNAL, **75**, 3893 (1953).

(8) H. Fujita and L. J. Gosting, *ibid.*, in press.

(9) A. Fick, *Pogg. Ann.*, **94**, 59 (1855).