[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

Conformation Changes in Bovine Plasma Albumin Associated with Hydrogen Ion and Urea Binding. I. Intrinsic Viscosity and Optical Rotation[1,2]

BY MELVIN D. STERMAN AND JOSEPH F. FOSTER

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The behavior of plasma albumin in 2 *M* urea solution has been investigated by measurement of intrinsic viscosities and of specific rotations (extrapolated to zero concentration) over the *p*H range 2.0 to 6.0 both with and without added NaCl. The results of these studies parallel the results obtained by Yang and Foster in solutions lacking urea. However, the increase in intrinsic viscosity and levorotation begins at higher *p*H values and is larger in the presence of urea than in its absence. Extrapolation of the data to zero ionic strength yields a monotonic sigmoidal curve when either set of data is plotted against *p*H. The viscosity data are consistent with a twenty-fold increase in volume at *p*H 3 and zero ionic strength. The optical rotation data are considered to be influenced by two factors, the number of asymmetric centers in the molecule and the conformation of the molecule and are interpreted as involving the transition from a highly ordered conformation at the isoionic point to a more random conformation at low *p*H or in urea solution. The data are consistent with a reversible expansion or swelling of the molecule in which the expanded molecule maintains some element of its molecular integrity.

Introduction

The fact that profound changes in the physical characteristics of plasma albumin are observed in the presence of urea and at low *p*H has been known for some time. Early studies by Burk[3] and Neurath and co-workers[4,5] showed that the diffusion constant progressively decreased and the reduced viscosity increased with increasing urea concentration, although the molecular weight of serum albumin remained constant. Almquist and Greenberg[6] demonstrated that serum albumin undergoes a marked increase in levorotation below *p*H 4. Similar results have been reported by Jirgensons[7] and by Golub and Pickett.[8] From a study of the sedimentation, diffusion and viscosity behavior of serum albumin in acid solution Pedersen[9] concluded that a pronounced structural change in the protein molecule takes place below *p*H 3. Foster and Yang[10] observed that the combination of serum albumin with a cationic detergent at low *p*H and low ionic strength resulted in a large increase in specific rotation and specific viscosity. A detailed study of the changes in intrinsic viscosity and specific rotation of plasma albumin over the *p*H range 1.3 to 7.0 and as a function of ionic strength was conducted by these investigators.[11] The data were interpreted on the basis of a reversible expansion of the albumin molecule at low *p*H due to coulombic repulsions. It was postulated that an "all-or-none" type equilibrium existed between the native molecule and the expanded molecule.

Scatchard[12] has examined osmotic pressure data and small anion binding data for serum albumin and concluded that the molecule must swell. Light scattering and sedimentation studies of plasma albumin at low *p*H by Reichmann and Charlwood[13] yield results which are in accord with the conclusions of Yang and Foster.[11] Furthermore, these results rule out a disaggregation of the albumin molecule as postulated by Weber[14] and also disprove the concept of a gross aggregation of this protein above and below its isoelectric point as proposed by Macheboeuf and co-workers.[15,16] Tanford[17] has also been led to suggest expansion of the plasma albumin molecule in acid solution as a means of accounting for the trend in the electrostatic contribution to the free energy of binding of protons with increasing charge.

A thorough study of the urea denaturation of serum albumin has been reported recently by Kauzmann and co-workers.[18] The results of these studies show clearly that plasma albumin can undergo pronounced structural changes in presence of urea in nearly neutral solution.

The present investigation of bovine plasma albumin was designed primarily to examine the combined effect of urea and hydrogen ions on the conformation of bovine plasma albumin.

Experimental

Materials.—Crystalline bovine plasma albumin was obtained from Armour and Company and used without further recrystallization. The urea for all the experimental work was recrystallized from absolute ethanol. All other chemicals employed in this study were either C.P. or Reagent grade material.

Viscosity Measurements.—Flow time measurements were made in Cannon–Fenske type modified Ostwald Viscometers.[19] The kinetic energy corrections for the viscometers were omitted. In the calculation of relative viscosity the ratio of the solution density to the solvent density was neglected. Density experiments showed that the omission of this factor introduced no significant error in the calculated

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(2) This work was supported in part by a grant from the Office of Naval Research and in part by a grant from the Public Health Service, National Institutes of Health.

(3) N. F. Burk, *J. Biol. Chem.*, **98**, 353 (1932).

(4) H. Neurath and A. Saum, *ibid.*, **128**, 347 (1939).

(5) H. Neurath, G. R. Cooper and J. O. Erickson, *ibid.*, **142**, 249 (1942).

(6) H. J. Almquist and D. M. Greenberg, *ibid.*, **105**, 519 (1934).

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

(8) M. A. Golub and E. E. Pickett, *J. Polymer Sci.*, **13**, 427 (1954).

(9) K. O. Pedersen, *Faraday Soc. Disc.*, **13**, 49 (1953).

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(11) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954).

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(15) E. Gavrilesco, E. Barbu and M. Macheboeuf, *Bull. soc. chim. biol.*, **32**, 924 (1950).

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viscosity values. The viscosity measurements were carried out at 25.00 ± 0.02° using 10-ml. volumes of solution or solvent.

The protein dilution series utilized for these measurements was prepared from a stock plasma albumin solution. The stock albumin solution was first dialyzed against distilled water at 1–3° to yield an isoionic protein solution. The albumin solution was acidified to remove all fatty acid insoluble material by pressure filtration. An aliquot of this albumin solution was then utilized for the preparation of the dilution series by the addition of appropriate quantities of urea, salt and doubly distilled water. Protein concentration determinations were made on the acidified albumin stock solution by the micro-Kjeldahl method of nitrogen analysis using a value of 16.07 as the percentage of nitrogen in plasma albumin. Prior to making the flow time measurements, the protein solutions were exposed to two separate clarification procedures, pressure filtration through fine sintered glass filters and centrifugation in a Sorvall High Speed Angle Centrifuge type SS-1 at 20,000 *g* for about 1 hr.

Optical Rotation Measurements.—Optical rotation measurements were made with a Rudolph High Precision Model 80 Polarimeter at room temperature (25 ± 3°) using a sodium vapor source. All measurements were made in two or four decimeter center-well fill type polarimeter tubes. The results of ten readings were averaged. Optical rotation measurements were made on the same solutions utilized for the flow time measurements.

A Leeds and Northrup glass electrode *p*H meter was utilized for all *p*H measurements.

Results

The viscosity and optical rotation behavior of plasma albumin solutions in 2 *M* urea was studied as a function of protein concentration, salt concentration and *p*H. The variation in protein concentration of each dilution series was normally in the range from 0.2% to 1.8% plasma albumin. These studies were conducted exclusively at *p*H values acid to the isoionic point of the protein and covered the *p*H range from 2 to 6, at three concentrations of added NaCl, 0.00, 0.02 and 0.10 *M*.

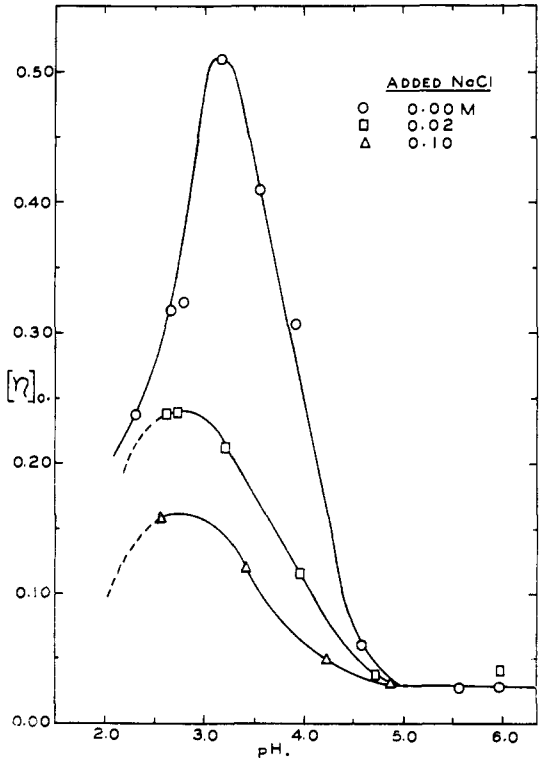

Fig. 1.—Dependence of intrinsic viscosity on *p*H in presence of 2.0 *M* urea and various levels of added salt.

Both the reduced viscosity (η_{sp}/c) and the specific rotation showed a linear dependence upon protein concentration. The slope of such plots was normally small or only slightly positive. However, in experiments with no added salt and at *p*H values where viscosity was very sensitive to *p*H, negative slopes were obtained as in the previous study.[11] Intrinsic viscosities $[\eta]_0$ and limiting specific rotations $[-\alpha]_0$ were readily obtainable by a direct linear extrapolation of the reduced viscosity or the specific rotation data to zero protein concentration. Figures 1 and 2 summarize the values for the intrinsic viscosities and limiting specific rotations determined by the method described. The downward curvature at low *p*H in curves at 0.02 and 0.10 *M* added salt, while not justified by these data, seem almost certain by analogy to the upper curve and to the curves in absence of urea, Figs. 2 and 4 of reference 11.

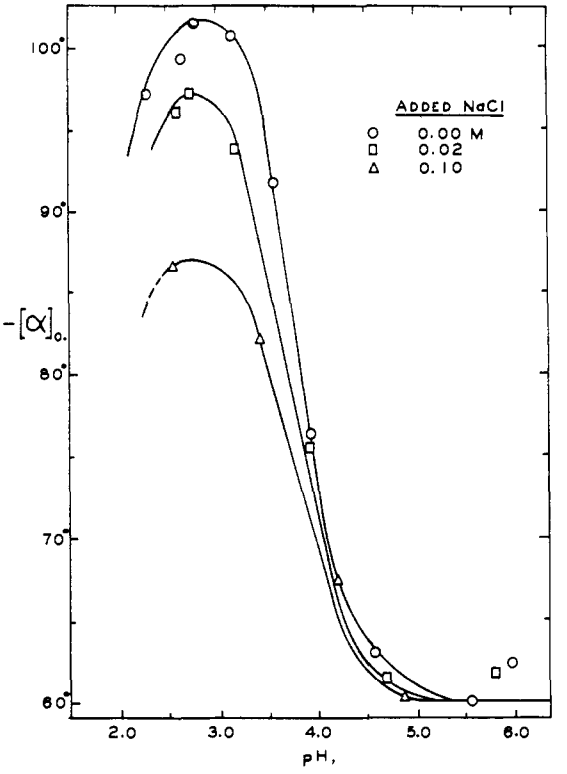

Fig. 2.—Dependence of specific rotation (extrapolated to zero protein concentration) on *p*H in presence of 2.0 *M* urea and various levels of added salt.

The data indicate very clearly the dependence of the intrinsic viscosity and limiting specific rotation upon *p*H at each of the three NaCl concentrations studied. Whereas the addition of even a small quantity of salt causes a marked depression in the values for the intrinsic viscosities, only a slight depression of the specific rotation is observed. Yang and Foster[11] have shown that extrapolation of such data to zero ionic strength can be performed through equations of the form

$$\frac{1}{[\eta]_0} = \frac{1}{[\eta]_\infty} + A\sqrt{\Gamma/2} \qquad (1)$$

and

$$\frac{1}{[-\alpha]_0} = \frac{1}{[-\alpha]_{00}} + B\sqrt{\Gamma/2} \qquad (2)$$

where $[\eta]_{00}$ and $[-\alpha]_{00}$ are the values for the intrinsic viscosity and limiting specific rotation extrapolated to zero ionic strength. These equations are similar in nature to equations which have been successfully applied to polyelectrolyte solutions.[20,21] Using equations 1 and 2 it has been possible to extrapolate both the viscosity and optical rotation data to zero ionic strength (Figs. 3 and 4). If the extrapolated values $[\eta]_{00}$ and $[-\alpha]_{00}$ are plotted as a function of pH, it is possible by an appropriate choice of ordinate values to normalize the data in such a manner whereby both sets of values fit a common sigmoidal curve (Fig. 5).

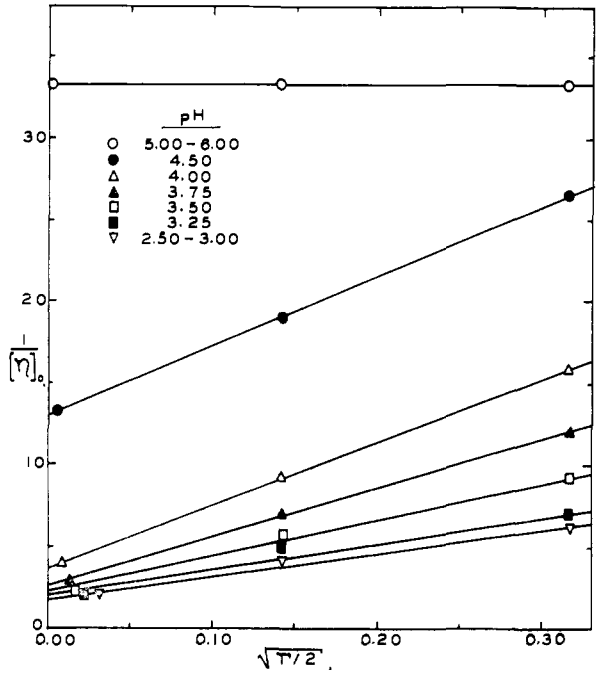

Fig. 3.—Extrapolation of intrinsic viscosity to zero ionic strength.

Studies were concurrently conducted to determine whether the observed changes in specific rotation and intrinsic viscosity were reversible. Several of the solutions from experiments in the pH range 2.5 to 3.5, with and without added salt, were dialyzed exhaustively against distilled water in the cold room. Nitrogen analysis was employed to demonstrate quantitative removal of urea. These solutions were then studied viscometrically and polarimetrically, dilution series being performed in the usual manner. The optical rotation returned in all cases to the normal value for the isoelectric protein, *i.e.*, -61 to $-65°$. The viscosities were also nearly reversible but were in all cases slightly higher than the native isoelectric protein, *i.e.*, 0.04 to 0.06. It was observed that addition of 0.2 M NaCl reduced the viscosity, in the one case where tried, to the normal value of 0.03. These results indicate

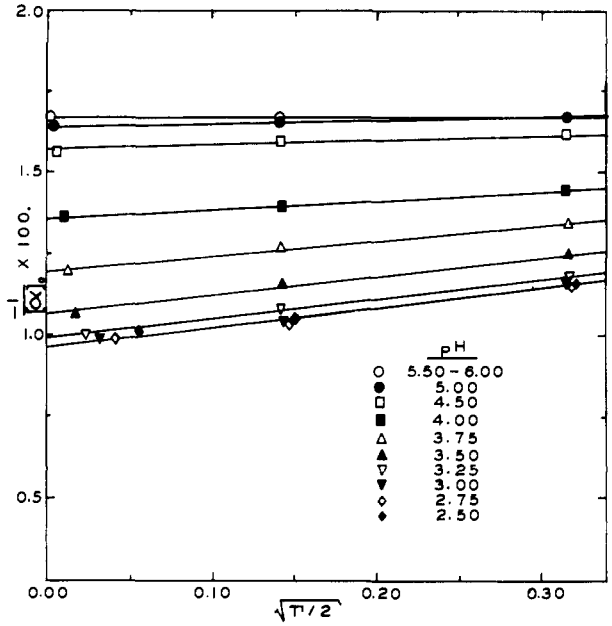

Fig. 4.—Extrapolation of limiting specific rotation to zero ionic strength.

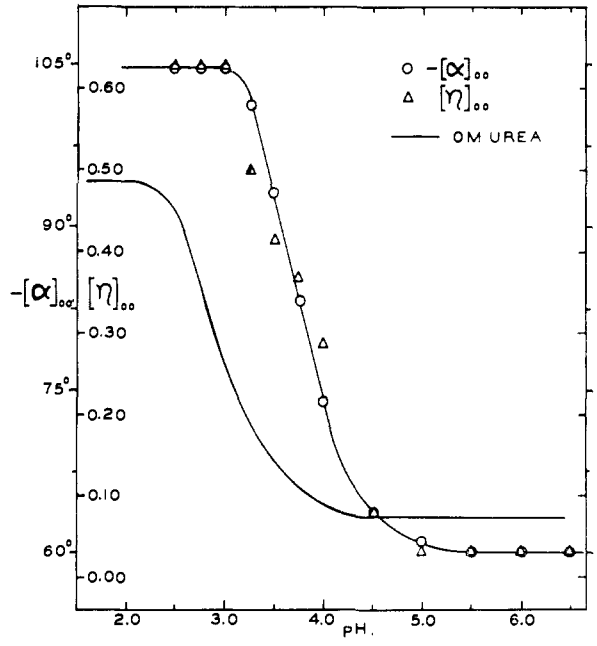

Fig. 5.—Dependence of limiting optical rotation and intrinsic viscosity, at zero ionic strength, on pH in presence of 2.0 M urea and various levels of added salt. The curve in absence of urea is for the optical rotation taken from reference 11.

the essential reversibility, at least in so far as these properties are concerned, of the low pH urea treatment. The slight irreversibility of the intrinsic viscosity undoubtedly is related to the appearance of traces of insolubilized protein in the dialyzed solutions. Interestingly, this effect was most prevalent in the most dilute solutions, a fact for which we have no explanation. One would expect greater aggregation in the more concentrated solutions.

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Discussion

A comparison of the viscosity and optical rotation data in 2 *M* urea with the results reported by Yang and Foster[11] in the absence of urea shows many similarities and some interesting differences. The presence of 2.0 *M* urea results in a characteristic shift of the curves toward higher *p*H values, the shift being of the order of 0.60 to 0.70 *p*H unit. The increase in intrinsic viscosity and levorotation is also somewhat larger in urea solution. Furthermore, the effect of salt concentration appears to be more marked in the absence of urea than in its presence and this is especially true of the optical rotation data. The viscosity data can only be interpreted qualitatively in terms of the hydrodynamic volume of the molecule in solution. At *p*H 3 approximately a twenty-fold increase in intrinsic viscosity is calculated at zero ionic strength which should correspond to a change in hydrodynamic volume of the molecule of this order of magnitude.

The specific rotation of a protein molecule must be considered to be the resultant of two additive effects: (1) the number of asymmetric centers in the molecule and (2) the spatial orientation or conformation of the polypeptide chain. Cohen[22] has recently examined specific rotation data for a number of proteins and concluded that the existence of randomized chain configurations in denatured proteins causes them to exhibit specific rotations which are the sums of effectively independent contributions of the L-amino acid residues in the molecule. It would seem reasonable to expect an increase in the degree of randomness of the molecular conformation to be associated with the low *p*H expansion. Applying these ideas to the specific rotation data for plasma albumin in 2 *M* urea solution, it should be possible to attribute the major portion of the specific rotation value observed at *p*H 3 to the independent contributions of the asymmetric centers in the protein molecules with but a small contribution from the molecular conformation superimposed upon the former primary effect. The value for the specific rotation observed at the isoionic point might be considered to be the sum of two effects, a contribution from the number of asymmetric centers in the molecule, which is identical to its contribution at *p*H 3, plus a contribution from the molecular conformation which exists at the isoionic point. Thus the change in conformation which occurs in the molecule in going from *p*H 3 to the isoionic point is characterized by a positive change in specific rotation of 44°.

At the isoionic point the native albumin molecule can be presumed to be a fairly compact and well ordered structure, having a definite intramolecular configuration which results from the specific folding of the polypeptide chain. Associated with this ordered structure is the positive contribution to the rotation which has been noted above. Similar observations have been reported by Robinson and Bott[23] and Becker and Stahman[24] as well as by Kauzmann, *et al.*[18] The suggestion of Cohen[22] that this positive contribution is the result of partial compensation of the contributions by separate asymmetric centers due to presence of helical coils of opposite sense is very interesting. However, many other reasonable possible explanations exist, notably changes in vicinal contributions[25] due to changes in configuration of the polypeptide chains.

In a very interesting recent communication Doty and Yang[26] have demonstrated a temperature dependent transition in a synthetic polypeptide, poly-γ-benzyl-L-glutamate, with a change in rotation from about −15 to +13°. They conclude the latter value to correspond to the helical form and conclude that an increment in specific rotation of +25–30° is associated with helical coiling in this polymer. Correcting for the difference in mean residue weight, approximately 120 for bovine plasma albumin as compared to 219 for the synthetic polymer, their conclusions would predict a contribution of +45–55° due to coiling in the isoelectric form if it is such a helix. The agreement with our observations is highly suggestive.

It is suggested that the transition in conformation which is observed at low *p*H and in urea solution is the result of an expansion or swelling of the albumin molecule in which there is a rearrangement in the spatial orientation of the folding of the peptide chain such that a more random configuration is obtained. Urea probably functions through its high hydrogen bonding potential causing the rupture of the extensive network of intramolecular hydrogen bonds which maintains the molecule in its ordered conformation.

Support for this hypothesis comes from the ready reversibility of the conformation change by removal of urea, acid and salt. This fact makes it almost mandatory that the less ordered conformation maintain a definite vestige of its molecular integrity so that the polypeptide chain under the proper conditions can revert to the very specific folding characteristic of the native molecule. If the conformation change were postulated to be an unfolding of the peptide chain, it seems highly unlikely that the drastic rearrangement which would be involved could be reversed to yield the very specific folding required in the native molecule.

Additional support for the expansion of the molecule can be had from the slopes of the reduced viscosity against concentration plots. The negative slopes obtained are in agreement with the results yielded by polyelectrolytes. The absence of birefringence of flow in albumin solutions at low *p*H and in urea solution[11,27] is indicative of a high degree of symmetry of shape within the molecule. This observation is also more in accord with an expansion of the molecule rather than an unfolding of the peptide chain, as the latter conformation should lead to definite molecular asymmetry.

Yang and Foster[11] suggested that the expansion process is of the nature of an all-or-none transition between two extreme forms. This suggestion was made as an explanation for the strikingly parallel course of the alterations in the two entirely different properties measured, optical rotation and in-

(22) C. Cohen, *Nature*, **175**, 129 (1955).
(23) C. Robinson and M. Bott, *ibid.*, **168**, 325 (1951).
(24) R. R. Becker and M. Stahman, THIS JOURNAL, **74**, 38 (1952).
(25) W. Kauzmann and H. Eyring, *J. Chem. Phys.*, **9**, 41 (1941).
(26) P. Doty and J. Yang, THIS JOURNAL, **78**, 498 (1956).
(27) J. F. Foster, E. G. Samsa and G. F. Hanna, *ibid.*, **76**, 6044 (1954).

trinsic viscosity. Utilizing this postulate here, and assuming further complete additivity of rotations and intrinsic viscosities in the equilibrium mixtures, it is possible to compute the *p*H dependence of the equilibrium constant. It is found that the logarithm of the apparent equilibrium constant is linear in *p*H with a slope of −1.5. Within experimental error it appears to be identical with the slope obtained in absence of urea and indicates an equilibrium in which 1.5 hydrogen ions combine with the reacting species. The non-integral value is puzzling and may represent an average value for a large collection of molecules.

On the other hand, it may be necessary to abandon the concept of an all-or-none transition. Tanford, *et al.*,[28] have very recently given rather convincing evidence that there is an intermediate form between the isoelectric and expanded forms. This intermediate they term the "expandable" form. They visualize the formation of this intermediate to be an all-or-none change, but the subsequent expansion to be a gradual stepwise process. Their arguments for a gradual expansion appear sound; indeed, we had previously made similar calculations and been led to the same conclusion. The fact that the optical rotation is much less sensitive to ionic strength than the viscosity, pointed out above, shows that complete parallelism does not exist in the two properties. Further evidence that these two changes are due to distinct structural modifications has been obtained through studies of the effect of dielectric constant.[29]

(28) C. Tanford, J. Buzzell, D. Rands and S. Swanson, THIS JOURNAL, **77**, 6421 (1955).

(29) J. F. Foster and J. T. Yang, in preparation.

The striking parallelism between rotation and intrinsic viscosity after extrapolation to zero ionic strength scarcely seems coincidental. This parallelism is now demonstrated in 2*M* urea as well as in absence of urea. The possibility of an explanation arises in the mechanism of Tanford, *et al.*,[28] if it is assumed that the rotational change is associated with formation of the expandable species and viscosity increase with the subsequent expansion. They have indicated that decreasing ionic strength would shift the two equilibria in opposite directions, toward coincidence. Thus extrapolation to zero ionic strength might represent a situation in which the expandable form would no longer exist because complete expansion of this form would take place. This possibility is under further investigation.

Previous results have shown that either urea (or guanidinium salts) or hydrogen ions can in some way dissolve the folded structure of plasma albumin leading to expansion of the molecule. The present studies show that these two factors can act in a supplementary manner. Several possible explanations are possible: (1) The presence of a limited number of key hydrogen bonds which can be ruptured by either urea or hydrogen ion; (2) a competition between attractive forces (hydrogen bonds) and electrostatic repulsion; (3) a specific effect of urea on the basicity of carboxylate anionic sites. A further elucidation of the situation requires additional information such as knowledge of the titration curve in presence of urea. This subject will be considered in the following publication.

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Conformation Changes in Bovine Plasma Albumin Associated with Hydrogen Ion and Urea Binding. II. Hydrogen Ion Titration Curves[1,2]

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Studies were conducted to discern the effect of urea (0, 2, 5 and 8 *M*) and ionic strength (0.01, 0.02 and 0.10) upon the acid titration curve of bovine plasma albumin. In absence of urea titration curves are anomalous as previously reported by others. It is concluded, however, that the deviation from ideality is due more to an increase in the intrinsic binding constant of the carboxylate anions with reduction in *p*H rather than to the molecular expansion as was previously concluded. In presence of urea hydrogen ion binding at a given *p*H is enhanced, and there is a marked elevation in the binding maximum (*n*). It is concluded that the primary effect of urea is an increase in the basicity of carboxylate anions, probably through destruction or weakening of the intramolecular protein structure, rather than a reduction in the electrostatic free energy term which is associated with hydrogen ion binding. The increase in molecular volume in presence of urea at low *p*H is thus in a sense a consequence, rather than cause, of the increased binding of hydrogen ions.

Introduction

The first paper in this series[3] presented results of an investigation of the intrinsic viscosity and specific rotation of bovine plasma albumin in 2 *M* urea solution as a function of *p*H and salt concentration. The data were consistent with a reversible expansion of the molecule in which the molecule maintains some element of its molecular integrity. It was further postulated that urea functions through its high hydrogen bonding potential causing the rupture of the extensive network of intramolecular hydrogen bonds which maintains the molecule in its ordered native conformation.

Tanford[4] has reported a very interesting investigation of the titration behavior of human plasma

(1) Based upon a thesis submitted to the Graduate Faculty of Purdue University by Melvin D. Sterman in partial fulfillment of the requirements for the degree Doctor of Philosophy. Presented before the Division of Biological Chemistry, A.C.S., Minneapolis, September, 1955.

(2) This work was supported by a grant from the Public Health Service, National Institutes of Health.

(3) M. D. Sterman and J. F. Foster, THIS JOURNAL, **78**, 3652 (1956).

(4) C. Tanford, *ibid.*, **72**, 441 (1950).