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 pH dependence of the equilibrium constant. It is found that the logarithm of the apparent equilibrium constant is linear in pH 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 $2M$ 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.

LAFAYETTE, INDIANA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

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

BY JOSEPH F. FOSTER AND MELVIN D. STERMAN

RECEIVED DECEMBER 29, 1955

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 pH rather than to the molecular expansion as was previously concluded. In presence of urea hydrogen ion binding at a given pH 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 pH 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 pH 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).

albumin. The curve in the carboxyl titration region could be described adequately on the basis of a single pK for the carboxyl groups (4.00) and an electrostatic repulsion term of the usual form $-2RTwZ$ (where w is a constant independent of charge Z) only if binding of a very large number of chloride ions was assumed. In a later paper Tanford concluded[5] that w could not be considered constant but rather decreases with decreasing pH. These results are in contrast to the results which have been reported for β-lactoglobulin[6] and ovalbumin[7] by Cannan and co-workers. For these proteins w is found to be constant and independent of pH. Since w is related to the dimensions of the molecule, Tanford[5] concluded that plasma albumin undergoes a structural modification in acid solution which he postulated to be either an expansion or partial unfolding of the molecule.

The primary objective of the present investigation was to examine the effect of urea on the titration behavior of plasma albumin in the acid region. It was felt that such information might contribute considerably to our understanding of the curious low pH behavior of this protein. Considering the large number of investigations into the action of urea solution on proteins, the literature is almost devoid of data on the effect of urea on protein titration curves, doubtless due to difficulties of interpretation brought out in this paper. Mihalyi[8] has conducted limited studies of the titration behavior of fibrinogen and fibrin in the alkaline region in 3.33 M urea.

Experimental

Materials.—Crystallized bovine plasma albumin was obtained from Armour and Company and used without further recrystallization. The urea employed in all experimental work was recrystallized from absolute ethanol. All other chemicals were either C.P. grade or Reagent grade material. The HCl for all titration studies was prepared from concentrated C.P. grade HCl, which was diluted with doubly distilled water and standardized gravimetrically by the precipitation of AgCl.

Measurement of pH.—A Beckman Model G pH meter was employed for all pH measurements herein reported. The internal electrode system supplied with the pH meter was not used in making these measurements. In its place a specially designed external electrode cell assembly was utilized. The cell assembly is a modified version of a design by Tanford[9] and uses a Beckman #1190-80 General Purpose glass electrode with a thirty inch shielded lead and a Beckman #1070-71 sleeve type calomel reference electrode, also with a thirty inch lead. This type of cell assembly offers a number of distinct advantages. It permits the formation of a free diffusion liquid junction between the solution whose pH is to be measured and the saturated KCl solution resulting in more accurate and more reproducible pH measurements. With the denser solution—saturated KCl—at the bottom of the liquid junction, contamination by mixing of the solutions is minimized. New liquid junctions can be formed easily. All temperature sensitive parts of the electrode cell assembly are maintained at constant temperature, eliminating experimental variations in the measured pH due to temperature fluctuations or the lack of complete thermal equilibrium of the glass electrode with the solution being measured. This latter effect will produce significant errors in the measured pH.[10]

The electrode cell assembly was thermostated at 25.00 ± 0.02°. The solution whose pH was to be measured was permitted to attain thermal equilibrium in the compartment for about five minutes before the glass electrode was introduced. The electrode and solution were kept in contact for about five minutes to allow complete thermal equilibrium to be reached before the pH measurement was made. The pH meter was standardized daily by employing two standard buffer solutions of pH 4.01 (potassium acid phthalate) and pH 7.00 (phosphate) and it could be read with a precision of ±0.01 pH unit.

The following procedure was employed in determining each protein titration curve. A series of protein solutions was prepared which had the same protein concentration, urea concentration and ionic strength, but which contained varying amounts of HCl. In each case the pH was measured within 10 to 15 minutes after preparation of the solution. Each member of the series was prepared from the same stock albumin solution in double distilled water.

The stock albumin solutions were passed through a mixed bed ion-exchange column of a type similar to that described by Oncley and co-workers.[11] An aliquot volume of the stock albumin solution was utilized for the determination of protein concentration. Optical densities were measured with a Beckman DU spectrophotometer at a wave length of 279 mμ from which the protein concentration was calculated using an extinction coefficient $E^{1\%}_{1\,cm.}$ of 6.67 for isoionic plasma albumin.

For the titration studies it was necessary to have calibration curves which yielded an exact relationship between the hydrogen ion concentration and the pH of a solution. Thus blank titration curves were measured, in a manner identical to that employed for the measurement of the protein titration curves, at each urea concentration and ionic strength studied. In the absence of urea these calibration curves were determined over the pH range 1–4. Above pH 4 the hydrogen ion concentration was considered to be sufficiently dilute that it could safely be assumed that ideal behavior was obeyed. In the presence of urea calibrating data were measured over the same pH range as the protein curve. No assumptions about ideal behavior were made even at pH 6.

In view of the high molarities of urea employed, it is clear that pH in urea containing systems does not necessarily have the same significance as in purely aqueous systems. An attempt was made to partially establish a pH scale in aqueous urea by conducting titration curves on acetate at ionic strength 0.1 in 0, 2.0 and 5.0 M urea. The curves obtained were of ideal shape but showed a progressive shift in the alkaline direction with increasing molarity of urea. The pK' values deduced from the inflection points were respectively 4.62, 4.80 and 5.01.

Results

The titration behavior of plasma albumin on the acid side of the isoionic point has been investigated under a variety of conditions. These studies were designed to discern the effect of urea concentration and ionic strength upon the titration curves of this protein. Thus studies were conducted at four urea concentrations—0, 2, 5 and 8 M—and at three ionic strengths—0.01, 0.02 and 0.10. A protein concentration of approximately 0.5% was utilized in most of the studies.

Plots of equivalents of hydrogen ion bound per mole of plasma albumin of molecular weight 70,000, as function of pH (Figs. 1, 2 and 3) show that in the absence of urea normal sigmoidal titration curves are obtained. In the presence of urea the curves at an ionic strength of 0.10 are indicative of two distinct binding regions. This effect is not observed at lower ionic strengths as the acid concentration is not high enough to yield maximum binding. The

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

(6) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(7) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(8) E. Mihalyi, *J. Biol. Chem.*, **209**, 723, 733 (1955).

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

(10) R. G. Bates, "Electrometric pH Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954.

(11) J. L. Oncley, H. M. Dintzis and N. R. S. Hollis, Abstracts, 22nd Meeting, Am. Chem. Soc., 1952, p. 12-P.

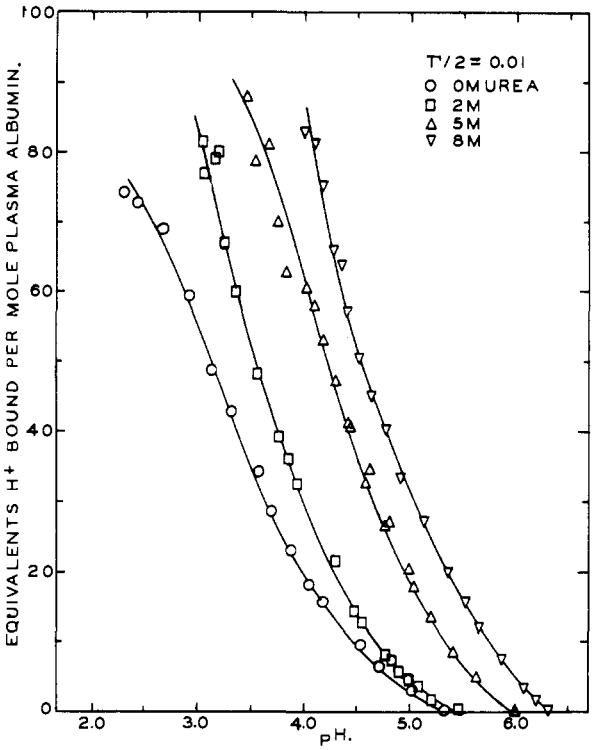


Fig. 1.—Titration curves at 0.01 ionic strength in absence of urea and at urea molarities as indicated.

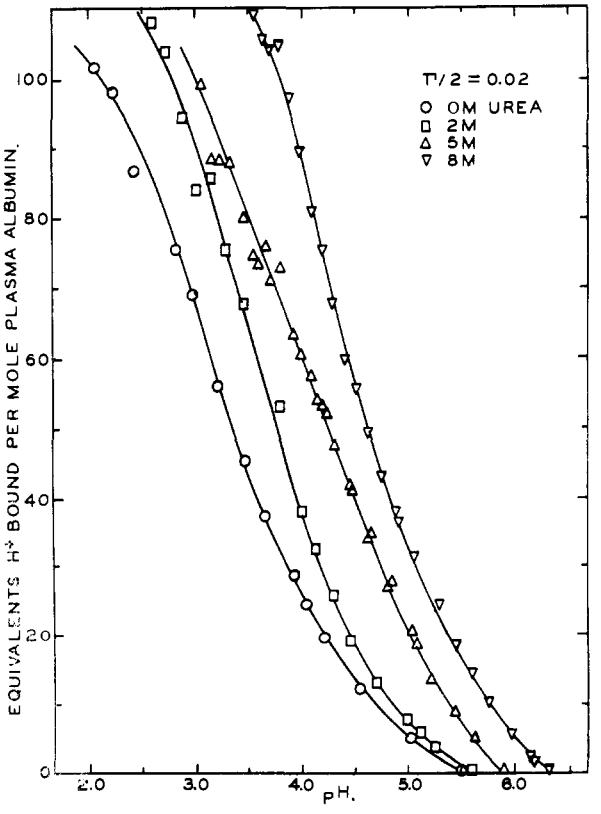


Fig. 2.—Titration curves at 0.02 ionic strength in absence of urea and at urea molarities as indicated.

curves suggest, however, that the binding limit

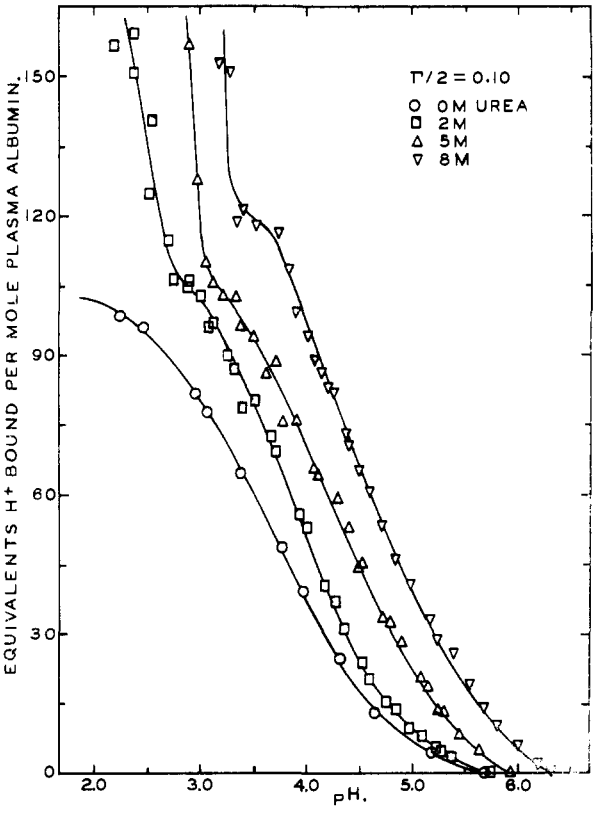


Fig. 3.—Titration curves at 0.10 ionic strength in absence of urea and at urea molarities as indicated.

must be much higher in the presence of urea than in its absence.

The effect of ionic strength is most marked in the absence of urea, the titration curves being shifted toward lower *p*H values with decreasing ionic strength. In 2 *M* urea solution the effect of ionic strength is still very evident but is not quite as pronounced as in the absence of urea. In 5 and 8 *M* urea the effect of ionic strength upon the titration curve has diminished greatly.

Discussion

One of the earliest theoretical treatments for the interpretation of protein titration curves was proposed by Linderstrøm-Lang.[12] This approach has been elaborated upon by Tanford[4,13] and by Scatchard.[14] Beginning with the fundamental definition of the dissociation of a proton from a carboxyl group on the protein molecule, Tanford derived an equation which predicts the shape of a protein titration curve. This equation is of the form

$$\log \frac{n - r}{r} = p\mathrm{H} - pK_0' + 0.868Zw \qquad (1)$$

where n is the total number of basic groups of a particular species in the molecule, r is the number of protons that are bound to that species, pK_0' is the intrinsic dissociation constant for the particular species considered, Z is the net charge on the

(12) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, 7 (1924).

(13) C. Tanford, *Proc. Iowa Acad. Sci.*, **57**, 225 (1950).

(14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

molecule and w is a parameter which depends upon the dimensions of the molecule.

The titration results clearly show that the binding of hydrogen ions by bovine plasma albumin, on the acid side of the isoelectric point, is markedly enhanced at any given pH by the presence of urea. In the previous paper[3] it was shown that the expansion of the protein molecule is also enhanced by urea under these conditions. Two obvious possibilities present themselves. (1) Urea weakens the protein structure, presumably by destruction of intramolecular hydrogen bonds, thereby favoring expansion at any given protein charge and thus reducing the magnitude of the electrostatic free energy term. From this point of view increased hydrogen ion binding is a consequence of increased expansion. (2) Urea produces an alteration of the intramolecular structure of the protein which enhances the tendency toward combination with hydrogen ions, either by rendering available additional binding sites (increasing n) or increasing the basicity of the sites (increasing pK_0'). In this case increased hydrogen ion binding is the cause of increased expansion. An attempt will now be made to decide between these extreme possibilities, although it must be recognized from the outset that actually a combination of the two effects may operate.

Calculations were first made adopting the first point of view, *i.e.*, assuming n and pK_0' to be constant. This is the treatment employed by Tanford and co-workers.[4,5,13,15] In the calculations n was assumed equal to 105 in all cases based on a protein molecular weight of 70,000. This conforms closely to the value found by Tanford, *et al.*,[15] namely 99 based on a 65,000 molecular weight. In the presence of urea the actual binding exceeds this value at low pH, a fact which is discussed further below. This enhanced binding would obviously invalidate the calculations at low pH, perhaps below 3, but would have little effect closer to the isoelectric point.[16]

A major problem arises in the choice of charge Z at a given pH and ionic strength. Reasonable estimates of the number of chloride ions bound in each case were made employing the chloride binding data of Scatchard, *et al.*,[19] and of Carr.[20] Since no such data are available in the presence of urea, it was necessary to make the assumption that urea is without effect on the binding of chloride ions at a given net charge Z.

On the basis of these assumptions, plots were made of the function $p\mathrm{H} - \log (n-r)/r$ *versus* corrected charge Z. The curves in absence of urea showed a strong negative slope close to the isoelectric point, with considerable curvature, both curvature and slope decreasing with increasing charge. On the basis of the assumptions made we would reach the same conclusion as Tanford, *et al.*, namely, that w is large at the isoelectric point but decreases rapidly between that point (*ca.* pH 4.5) and pH 4.0. Estimates of pK_0' at the three ionic strengths are given in Table I. Such estimates are most unreliable because of the curvature of the plots in the region of zero charge and further depend strongly on the correction for chloride binding.[21] It should be pointed out that our estimated values show even more ionic strength dependence than those of Tanford, *et al.*,[15] who commented on the unusually large ionic strength dependence of this parameter.

While admitting the uncertainties of these calculations, we feel that the trends cannot be ignored and conclude that either pK_0' is not a constant[23] or that the assumption of a constant number of binding sites n is invalid. The possibility of "masked" sites[24] should perhaps be considered.

TABLE I

THE VALUES FOR pK_0' AS A FUNCTION OF IONIC STRENGTH AND UREA CONCENTRATION

Ionic strength	pK_0' 0 *M* urea	2 *M* urea	5 *M* urea	8 *M* urea
0.10	3.97	4.02	4.48	4.89
.02	3.86	3.85	4.38	4.77
.01	3.74	3.68	4.35	4.77

Another strong argument against the assumption of constancy of pK_0' and n is the fact that the largest apparent decrease in w, both in Tanford's[15] data and ours, occurs in a pH range, namely, 4.5 to 4.0, where both his data[25] and those of Yang and Foster[26] show only a trivial increase in intrinsic viscosity. At lower pH values where obvious molecular expansion occurs, the decrease in w is relatively small. We conclude that in the main deviations from the ideal equation 1 arise in the transition to the "expandable" form rather than in the expansion *per se*. *The origin of the deviations must lie more in changes in either pK_0' or n, or both, than in w.*

Similar analysis of the $p\mathrm{H} - \log (n-r)/r$ *vs.* Z plots in the presence of urea raised even more formidable difficulties. The most obvious characteristic of the curves is the fact that they are very much

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

(16) Estimation of the limiting binding capacity (n) is very difficult utilizing the titration curves as given in the figures. However, we find reasonably linear graphs of such binding data when the binding equations of either Scatchard[17] or Klotz[18] are employed. Extrapolation of such plots yielded 103 to 105 sites in absence of urea and at ionic strength either 0.1 or 0.02. At ionic strength 0.01 the results are much less precise, since titration cannot be continued to pH below about 2.3, and yielded 84 to 91 sites. In the presence of urea the Scatchard plot yielded n values of 135–140, 130–150 and 150–162 at 2, 5 and 8 *M* urea, respectively, and at the two higher ionic strengths.

(17) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(18) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946); I. Klotz, F. Walker and R. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(19) G. Scatchard, I. Scheinberg and S. Armstrong, *ibid.*, **72**, 535 (1950).

(20) C. Carr, *Arch. Biochem. Biophys.*, **40**, 286 (1952).

(21) Corrections for chloride binding were made using the binding data of Coleman,[22] which data were also used by Tanford, *et al.*

(22) J. Coleman, Ph.D. Thesis, Massachusetts Institute of Technology, 1953.

(23) Tanford, *et al.*,[15] suggested strong electrostatic interactions involving carboxylate anionic groups as an explanation for both the anomalously low pK_0', values and the ionic strength dependence. We utilize their model in the discussion below. There is thus no essential qualitative difference between their point of view and ours. However, while they have suggested the carboxyl groups in the isoelectric form to be "anomalous" they have not utilized this fact in the quantitative interpretation of the titration data where a constant pK_0', was assumed. It is our suggestion that the w terms they calculate, as summarized in their Fig. 6, are thus purely fictitious. The actual decrease in w must be very much less than indicated by their calculations.

(24) J. Steinhardt, *Adv. Protein Chem.*, **10**, 152 (1955).

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

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

flatter in the region of the isoelectric point than when urea is absent. This is true even in 2 *M* urea (Fig. 4) where viscosity studies[3] show almost no enhancement of hydrodynamic volume. Again we conclude that the effect of urea is not primarily on the electrostatic free energy term, but rather one of either exposing (un-masking) basic sites or enhancing the basicity of the sites. Attempts were made to evaluate pK_0' at each level of urea, the results being included in Table I. It should be emphasized that little if any credence can be attached to these numbers because of the uncertainty in chloride binding, the downward trend of the points near the isoelectric point (as indicated in Fig. 4) and the added uncertainty of the *p*H scale in presence of urea. With respect to the last point, it would appear that the values at 2 and 5 *M* urea should be reduced by about 0.2 and 0.4 unit, respectively, as judged by the results on acetic acid given above. The correction in 8 *M* urea would presumably be even larger.

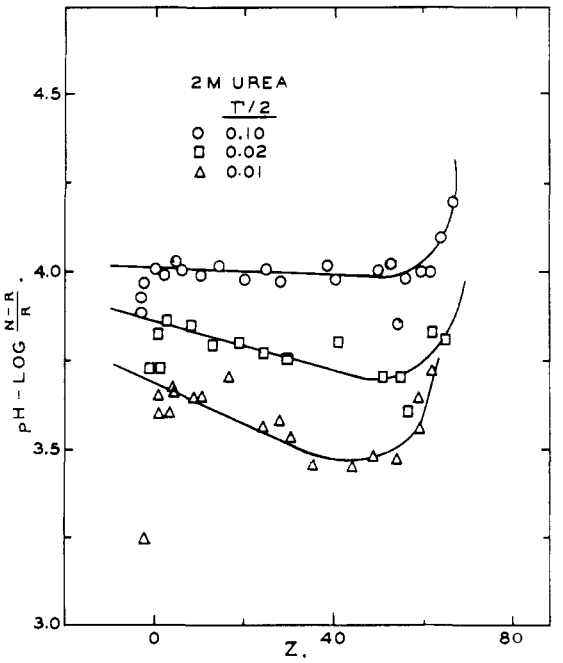


Fig. 4.—Plots according to equation 1 of data in 2 *M* urea. Net charge *Z* corrected for chloride binding.

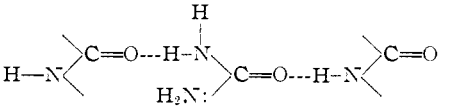
The assumption has been made throughout that the only groups being titrated in these studies are the (approximately 105) carboxyl groups. This assumption is probably reasonably valid in absence of urea. At most it seems probable that not over one or at most two of the imidazole groups remain untitrated at the isoelectric point. The situation in urea may, however, be quite different. Information on the effect of urea on the entire titration curve of this protein would be most helpful.

Tanford[9] has commented on the unusually low pK_0' values deduced for the plasma albumins as contrasted to the more normal value of about 4.6 expected and exhibited by such proteins as β-lactoglobulin. To account for this fact, plus the fact that expansion takes place on the acid side in the region of the titration of carboxyl groups and on the alkaline side in the amino titration region, Tanford and co-workers have suggested[25] strong ionic interactions of the type

$$\begin{vmatrix} -R & & R- \\ -COO^- & & {}^+H_3N- \\ -R & & R- \end{vmatrix}$$

Whatever the nature of the interaction responsible for the anomalously low pK_0' values, it is worthy of note that *they must involve essentially all of the carboxyl groups*. If an appreciable proportion had normal basicity, they would tend to bind hydrogen ions preferentially just below the isoelectric point, weighting the calculated pK_0' toward 4.6. It is our conclusion that it is the rupture of such carboxyl interactions in the *p*H range 4.5 to 4.0 which is responsible for the anomalous titration curve in this region and which is associated with the conversion to the "expandable" form. We further conclude that the predominant effect of urea is on this equilibrium, either through attack of urea on the carboxyl interactions directly or, more likely, through a general weakening of the protein structure through hydrogen-bond rupture.

In conclusion, the enhanced hydrogen ion binding observed in presence of urea merits consideration. That the enhanced binding at low *p*H is real and not due to decomposition of urea in the presence of the protein was established rather conclusively by the observation that there was no time dependence in the titration curves. It is important to emphasize that corrections were made for binding by urea itself through determination of blank titration curves. However, this correction is valid only if the basicity of urea is unaffected by combination with protein. It seems possible to us that the formation of hydrogen bridges with the protein peptide groupings might enhance the basicity of urea appreciably. Since a very large number of urea molecules is doubtless so involved and since the pK_B of urea is approximately 13, only a slight enhancement of basicity would suffice. A possible formulation is



In this structure one of the amino groups is essentially frozen out of the normal resonance existing in urea so that its free electron pair might approach that of an amine in so far as affinity for hydrogen ions is concerned. An alternative possibility exists, namely, that the enhancement is due to increased basicity of the peptide or amide groupings. Steinhardt[27] concluded that the amide group is more basic than the peptide linkage. He further concluded combination of hydrogen ions by amide groupings to take place in the presence of strongly bound anions such as dodecylsulfate. From the work of Hall and Conant,[28] urea would appear to be 10 to 100-fold more basic than the amide grouping. The most likely explanation of the enhanced binding thus appears to be combination with bound urea.

LAFAYETTE, INDIANA

(27) J. Steinhardt, *J. Biol. Chem.*, **141**, 999 (1941).
(28) N. Hall and J. Conant, THIS JOURNAL, **49**, 3047 (1927).