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The State of Plasma Albumin in Acid pH

INTRODUCTION

Measurements on bovine and human plasma albumins in acid solutions have resulted in a lack of agreement on the state of these proteins under such conditions. It has been shown that irreversible loss of solubility at pH 5 (1-4) occurs much more slowly after treatment in the pH region of 2-3 than near the isoelectric point (pH region 4-5), while the viscosity and levorotation increase strikingly but reversibly with an increase in acidity in the same region (4-7). Similar observations have been made in urea solutions at about pH 5 (8, 9).

Although the solubility studies point to enhanced stability at low pH, the viscosity and levorotation measurements have been interpreted (8) as evidence that plasma albumin is unstable under such conditions. Thus, Kauzmann (10) proposed that reversible denaturation occurs, but that a soluble "renatured" protein forms very rapidly during the precipitation step used in solubility studies. Furthermore, it has been stated that aggregation (11, 12) or dissociation (13) occurs at low pH. Either molecular change, if it occurs, could influence the reliability of the various denaturation criteria.

Obviously, further direct measurements of the possible denaturation and of attendant changes in molecular weight should help to resolve this question. The present investigation, then, describes (a) the results ob-

¹ One of the laboratories of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

² Present address: Eastern Utilization Research and Development Division, U. S. Department of Agriculture, Beltsville, Md.

tained using a generally accepted biological criterion of denaturation, and (b) light-scattering and ultracentrifuge measurements on bovine plasma albumin under appropriate conditions of pH and ionic strength.

EXPERIMENTAL

Armour bovine plasma albumin was dissolved in redistilled water and passed through a deionizing column at 4° (14). Solutions were adjusted to the desired pH and ionic strength ($\Gamma/2$) using KCl and HCl or KOH as needed. The pH measurements were made at room temperature as previously described (2), except that no correction was made for temperature effects or the presence of urea.

In the denaturation experiments, protein mixtures were made up to the proper conditions within 5–10 sec. (2). At stated times aliquots were removed and, where necessary, cooled rapidly. For digestion a 0.5% albumin aliquot was treated for 15 min. at 25° with an equal volume of an aqueous solution of the proteolytic enzyme (pepsin at pH 2.3 or trypsin³ at pH 6.8). After addition of trichloroacetic acid and filtration, soluble split products were estimated from the absorbance (optical density/cm.) of the filtrate (18) as measured at 280 m μ , using a Beckman DU spectrophotometer. The protein mixture was considered denatured in proportion to the amount of soluble ultraviolet-absorbing product formed. A fully precipitable protein control was obtained by heating 1.0% albumin at 60°, pH 4.1, for 1 hr.⁴

For light scattering, aliquots of the deionized solution were adjusted to the desired pH and $\Gamma/2$ by adding the proper amount of HCl and KCl. The protein solution was then stored for 24 hr. in a refrigerator, to permit the last traces of the ketone⁵ impurity to precipitate. The solution was then brought to room temperature and finally filtered through a specially designed sintered-glass filter of ultrafine porosity (19). Diluting solvents of identical pH and $\Gamma/2$ to the protein solutions were prepared using redistilled water. These were also filtered through an identical filter. Measurements were performed at $25 \pm 1^\circ$ in a Brice-Phoenix instrument, using the narrow-slit optics.

³ During the assays it would have been desirable to use Ca⁺⁺ to stabilize the trypsin (15, 16). However, no Ca⁺⁺ was added since it stabilizes plasma albumin (16). In this way one possible pathway to reversal of denaturation during assay was avoided. No rate study of peptic or tryptic hydrolysis was made, but when urea was present, the trypsin proteolysis was found (Table II, Expts. 4, 5, and 6) to be the same as in the absence of urea. This indicates either that trypsin is not inactivated under this condition [see, however, Harris (17)] or that proteolysis is substantially complete before the assay period ends, owing to the presence of an excess of trypsin.

⁴ After this treatment the protein is completely insoluble in a stopping buffer at pH 5 (1), indicating that plasma albumin has been transformed into an irreversibly denatured product. Under this condition the interpretation of the results should not be compromised by the possibility [pointed out by Kauzmann (10)] that denatured protein may be "renatured" during the assay steps.

⁵ P. Bro, private communication.

RESULTS

In order to follow the alteration of plasma albumin by a biological method, the protein was exposed to the digestive action of pepsin or trypsin, both of which preferentially hydrolyze denatured proteins. To have appropriate controls, different conditions of pH, temperature, and urea concentration were selected for pretreatment of the protein, in particular conditions where the loss of solubility and increase in viscosity do not coincide. The results obtained in the digestion experiments are presented in Tables I and II.

Table I reveals a definite correlation between the pretreatment of the protein and its digestibility by pepsin. Thus, when bovine plasma albumin is treated at 25° at any of the pH's investigated, no soluble ultraviolet-absorbing products are formed. After 1 hr. at 60° at pH 4.1 (the fully precipitable control), an amount of soluble products is formed which, as would be anticipated, does not increase with further treatment at lower pH, even at 60°. Heating only at pH 3.0 results in an absorbance increment which is 30 % of that of the fully precipitable control, while heating at pH 2.3 gives only about 10 % of the control value. Heating at both pH's 3.0 and 2.3 gives about 38 % of the maximum absorbance. All these data agree well with the loss of solubility found by Levy and Warner (1) for the same system.

From Table II it can be seen that no ultraviolet-absorbing products are formed to any significant amount upon tryptic digestion either in the presence or absence of urea, except when the fully precipitable control is used.

The light-scattering data are plotted in the usual manner (20) in Fig. 1. It can be seen that there are no drastic differences in intercepts between the measurements carried out at the isoionic point or at pH 2.6-3.2, whether at $\Gamma/2 = 0.20$ or 0.01. No dissymmetry measurements were taken since it has been shown (21) that even with strong aggregation, under similar conditions, there is no significant variation in the angular dependence of the scattering of this protein.

Ultracentrifuge patterns obtained with some of the solutions used for light scattering are shown in Fig. 2. An albumin solution (pH 2.8, $\Gamma/2 = 0.01$) was kept at 25° for 5 days, then for 3 weeks in a refrigerator. After this the solution was adjusted by dialysis to pH 4.7 (acetate buffer,

TABLE I

Proteolysis of Plasma Albumin by Pepsin, pH 2.3, 25°, $\Gamma/2 = 0.2$

Bovine plasma albumin was pretreated for 1 hr. to conditions of pH and temperature as indicated. In a given experiment the protein was subjected in sequence (from left to right) only to the conditions checked (xxx). For example, in Expt. 4, plasma albumin was kept for 1 hr., first at pH 4.1, 25°; then at pH 3.0, 25°; then at pH 2.3, 60°; finally it was assayed with pepsin for 15 min. In Expt. 27, the protein was adjusted from the isoionic point directly to pH 2.3, and immediately assayed. The albumin concentration used was 1.0% at pH 4.1; 0.8% at pH 3.0; 0.6% at pH 2.3; 0.5% during the assay.

Conditions of pretreatment of protein

Experiment	$\frac{\text{pH}4.1}{25^{\circ}\text{C}.60^{\circ}\text{C.}}$	$\frac{\text{pH}3.0}{25^{\circ}\text{C}.60^{\circ}\text{C.}}$	$\frac{\text{pH}2.3}{25^{\circ}\text{C}.60^{\circ}\text{C.}}$	Absorbance increment
1	xxx	xxx	xxx	0.005
2	xxx	xxx		0.006
3	xxx			0.000
4	xxx	xxx		0.034
5	xxx		xxx	0.125
6	xxx		xxx	0.097
7	xxx	xxx		0.101
8	xxx		xxx	0.001
9	xxx			0.035
10	xxx	xxx	xxx	0.323
11	xxx	xxx		0.318
12	xxx			0.320
13	xxx	xxx		0.325
14	xxx		xxx	0.329
15	xxx		xxx	0.324
16	xxx		xxx	0.325
17	xxx		xxx	0.323
18	xxx			0.318
19		xxx		0.007
20		xxx	xxx	0.003
21		xxx		0.042
22			xxx	0.099
23			xxx	0.094
24			xxx	0.127
25			xxx	0.008
26				0.038
27				0.000

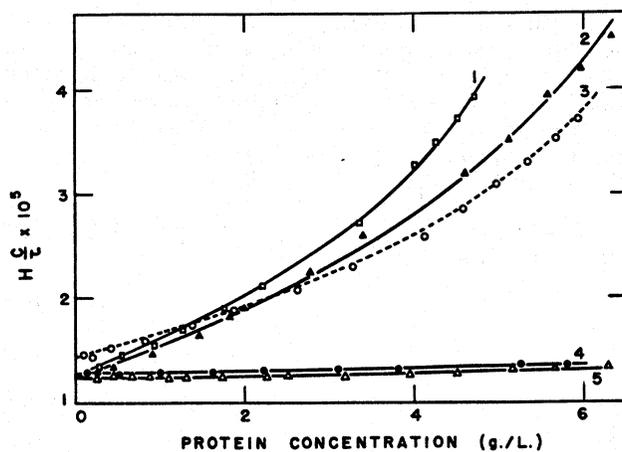


FIG. 1. Light-scattering data for Armour bovine plasma albumin in HCl-KCl. 1. pH = 2.8, $\Gamma/2 = 0.01$; 2. pH = 3.1, $\Gamma/2 = 0.01$; 3. pH = 3.2, $\Gamma/2 = 0.01$; 4. pH = 3.2, $\Gamma/2 = 0.2$; 5. isoionic, $\Gamma/2 = 0.1$.

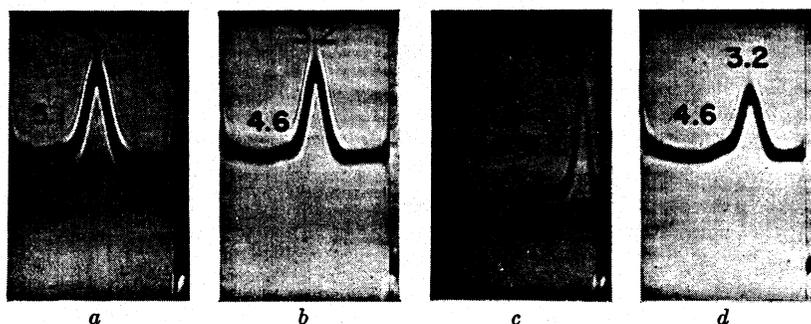


FIG. 2. Ultracentrifugal patterns of bovine plasma albumin treated at low pH. *a*. Solution kept in pH 2.8 HCl-KCl ($\Gamma/2 = 0.01$) for 5 days at 25°, then 21 days at 4° (analyzed in pH 4.7 acetate, $\Gamma/2 = 0.1$) (88 min.). *b*. Solution kept 24 hr. at pH 2.6, $\Gamma/2 = 0.2$, run immediately after light scattering (104 min.). *c*. Solution *b* after standing 4 days at 25° (16 min.). *d*. Same solution (64 min.). (In all cases, sedimentation proceeds from right to left. The time after reaching full speed is indicated in parentheses.) The sedimentation constants of the components are marked on each pattern.

$\Gamma/2 = 0.10$)⁶ The ultracentrifuge pattern (Fig. 2*a*) is similar to that normally obtained in a fresh preparation of bovine plasma albumin, the

⁶ Since at conditions of low pH and low ionic strength the ultracentrifugal analyses are complicated by electrostatic effects, all runs on the 0.01 $\Gamma/2$ solutions were made at pH 4.7. The analysis of the patterns is thus subject to the limitations of possible pH-dependent equilibria.

"dimer" (22) constituting about 7 % of the total proteins.⁷ With solutions at about pH 3.0, $\Gamma/2 = 0.20$, the patterns obtained directly after the light-scattering experiment, either in the same medium or after dialysis to pH 4.7 (as above), reveal a slight increase in the amount of "dimer" (Fig. 2b). After 4 days at 25° (pH 3.0, $\Gamma/2 = 0.20$), 17 % of the albumin was present (Fig. 2c) as an even more rapidly sedimenting component ($s_{20,w} = 8.9$). Longer standing resulted in an even greater degree of irreversible aggregation (21), while the reversible transformation (4-7) of albumin at low pH is complete after several minutes.

DISCUSSION

The Definition of Denaturation

The concept of denaturation has been defined in a number of ways over the course of years by various investigators working in the field. The general approaches used can be classified into two types: (a) molecular, attempting to describe the actual changes which take place on the molecule; (b) operational, or in terms of changes in properties measurable in the laboratory. Thus, Neurath *et al.* (24) define denaturation as "any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties," while Wu (25) states that "denaturation is a change in the natural protein molecule whereby it becomes insoluble in solvents in which it was previously soluble." The last definition requires that the solubility of every protein sample must be measured at the same condition of pH, salt environment, solvent, protein concentration, ionic strength, and temperature.

Recognizing that the most complete and ideal definition would be one describing the exact changes which occur in the protein molecule, the present investigators are inclined to accept for present purposes an operational definition of denaturation, since this permits one to state exactly what is being observed and discussed, while the molecular definition requires an extrapolation from the actual measurements to as yet badly known and poorly understood changes in the molecular structure. Eventually, as our knowledge of the changes which occur in a molecule upon denaturation increases and as experimental techniques become more sensitive, the two types of definition should extrapolate to the same asymptote and take on an identical meaning.

⁷ All sedimentation constants and compositions reported are those obtained in 0.8-1.0% protein solutions. The data were neither extrapolated to zero concentration nor corrected for the Johnston-Ogston anomaly (23).

Accepting the operational definition for the purpose of this communication, it should be pointed out that many symptoms, other than a change in solubility measured at standard conditions, are usually associated with denaturation [see, for example, Neurath *et al.* (24), Wu (25), and Putnam (26)]. Since certain of these properties can be measured very conveniently (such as viscosity, levorotation, and biological activity), the definition of the term "denaturation" as used in this paper is "a change in the natural protein molecule whereby one or more properties are altered as measured under some standard reference set of conditions."

In recent years the definition has been occasionally extended to mean a change in the structure of the protein molecule as evidenced by a change in certain properties of a protein even when these properties are not measured under a standard set of conditions. Thus, an instantaneous, reversible increase in viscosity and levorotation of plasma albumin (as compared to the isoionic protein) on lowering the pH or adding urea has been called denaturation (10). It is merely a question of semantics whether one calls such a change denaturation or a transformation⁸ which is peculiar to native protein molecules of this type. This being the case, we will refer, wherever possible, to the property which has been measured on plasma albumin, rather than to denaturation or stabilization, as has been the case previously (1-10).

Proteolytic Hydrolysis

Although a study of the rate of loss of solubility of bovine and human plasma albumins indicates that these proteins are protected at pH 2-3 as compared with pH 4-5, Kauzmann (10) has correctly pointed out that this may be only an apparent stability due to the experimental procedures used. In solubility experiments (1-4) the reacting protein mixture was cooled, adjusted to pH 5, and introduced to an environment containing large concentrations of salts. Any or all of these changes could conceivably allow a protein, which would be insoluble at pH 5 if brought immediately to that condition, to recover its solubility, and thus the denaturation of the protein, if rapidly reversible with a change of conditions, could be concealed in terms of the operational definition.⁹

⁸ Tsiperovich and Loseva (27) have found for a number of proteins that changes in viscosity and levorotation do not necessarily accompany denaturation as determined from loss of solubility.

⁹ It should be pointed out, however, that measurements of loss of solubility under appropriate conditions (1-4) give no evidence of a time-lag in the formation

Viscosity and levorotation measurements have the advantage of being made on the reacting mixture itself. They are especially valuable properties to study when changes are noted over experimentally measurable time intervals, as in the case of egg albumin (8). With plasma albumin both properties exhibit almost instantaneous reversible increases as the pH is lowered, indicating that the protein molecules are in fact very rapidly (but reversibly) altered at low pH. Subsequently, Foster and Yang have reported (6), that there is a time-dependent decrease in these properties at 60°. This decrease apparently conforms with the rate of insolubilization (1-4), especially when ionic strength effects (4) are considered.

Both pepsin and trypsin hydrolyze denatured proteins much more readily than they attack the corresponding native proteins, thereby affording a ready means of differentiation. At pH 2.3, and at pH 6.8 in the presence of urea, the viscosity and levorotation of plasma albumin are high (4-9). The experiments in Tables I and II therefore provide a direct test of whether loss of solubility or an initial increase in viscosity and levorotation signify denaturation in the same sense as proteolysis does.

It is evident from the tables that there is no direct correlation between the latter properties and proteolysis. In fact, as shown in Table III, proteolysis, which is customarily considered a biological measure of the extent of denaturation, parallels the findings obtained by the solubility method, and is consequently a measure of the same alteration in the protein molecule. Particularly where pepsin was used is this comparison a direct one.

Light-Scattering and Ultracentrifuge Measurements

The molecular weights¹⁰ obtained for bovine plasma albumin under various conditions of pH and ionic strength are summarized in Table IV. Also included are data on the amount of "dimer" (22) observed in the ultracentrifugal patterns under the same solutions.

of insoluble protein, as would be expected if reversal occurred. Since a normal kinetic treatment can be applied to solubility data when the protein has been treated at pH 3 to 5, the precipitation technique is apparently suitable for analysis of the product as it is formed.

¹⁰ The values reported for the molecular weights are the reciprocals of the intercepts of the light-scattering plots, uncorrected for the thermodynamic interaction term (28), since it has been shown that for this protein its contribution is small (29). Also, no correction for depolarization has been applied (30).

TABLE III
Observations on Plasma Albumin at $\Gamma/2 = 0.20$

pH	Conditions of treatment T °C.	Urea	Initial viscosity and levorotation ^a	Solubility denaturation rate ^b	Proteolytic hydrolysis ^c	
					Extent	Enzyme
2.3	25	—	Very high	Negligible	Negligible	Pepsin
	60	—	Very high	Very slow	Very slow	Pepsin
3.0	25	—	High	Negligible	Negligible	Pepsin
	60	—	High	Slow	Slow	Pepsin
4.1	25	—	Normal	Negligible	Negligible	Both
	25	2 M	Slightly high	—	Negligible	Trypsin
	25	8 M	Very high	—	Negligible	Trypsin
	60	—	Normal	Very rapid	Very rapid	Both
6.8	25	—	Normal	Negligible	Negligible	Trypsin
	25	2 M	Slightly high	—	Negligible	Trypsin
	25	8 M	High	—	Negligible	Trypsin
	60	—	Normal	Negligible	Negligible	Trypsin

^a Data from Refs. (4-9).

^b Data from Refs. (1-4).

^c Data from this paper.

TABLE IV
Molecular Weight Data for Plasma Albumin

pH	$\Gamma/2$	Mol. wt. ^a	Per cent "dimer" ^b
5.6	0.20	79,400	—
5.6	0.10	80,600	8
5.5	0.01	78,100	—
3.2	0.20	78,100	—
2.8	0.20	84,600	14
2.6	0.20	79,700	10
3.2	0.01	71,400	—
3.1	0.01	79,800	7
2.8	0.01	79,300	—

^a From the intercept of light-scattering plots. The value of the refractive increment used was the same as reported previously (29).

^b From relative areas in ultracentrifugal patterns on 1% albumin solutions.

From these results it becomes apparent that no aggregation whatever occurred even after long storage at pH 3.0, $\Gamma/2 = 0.01$. This is particularly striking since under this condition the viscosity attains a high value (4-7). At 0.20 ionic strength incipient aggregation is evident even after 1 day's storage at 4° or only a few hours at room temperature [see also Ref. (21)]. Association becomes much more extensive after further time at room temperature, as shown in Figs. 2c and 2d.

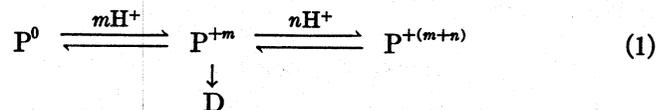
The lack of change in average molecular weight and in ultracentrifugal pattern at pH 3.0, $\Gamma/2 = 0.01$, eliminates either aggregation (11, 12) or dissociation (13) as the cause of the observed behavior of bovine plasma albumin at low pH, and especially as the reason for the high viscosity obtained with this protein at low pH, as has been recently suggested (31).

The stronger intermolecular repulsion obtained in the $\Gamma/2 = 0.01$ solution at pH 3.0 is the cause of the rapid rise and curvature (32) with concentration, of the reduced turbidity, as plotted in Fig. 1. Indeed, the curvature becomes more pronounced with a decrease in pH and an increase in net charge on the protein, reflecting the progressively increasing repulsive force. This strong repulsive force may also account in great part for the lack of aggregation at $\Gamma/2 = 0.01$, as opposed to the present observations at $\Gamma/2 = 0.20$ and the recently reported aggregations at $\Gamma/2 = 0.30$ (31). In the latter cases there is sufficient screening to permit much closer approach of the protein molecules and thus a greater chance of association.

The slow aggregation noted above quite likely is evidence of a very small rate of insolubilization at 25°, consistent with what would have been predicted from solubility studies at higher temperatures (1). It is interesting to note that these measurements denote such small extents of alteration that solubility changes and decreases in viscosity and levorotation would hardly exceed experimental error. In this case again, the sensitivity of techniques such as light scattering to detect very small amounts of alteration has been shown, in the same manner (33, 34) as was effective in demonstrating incipient denaturation with egg albumin.

The State of Plasma Albumin

Solubility studies on plasma albumin have indicated that the following mechanism (2-4) describes the insolubilization process:



where P^0 , P^{+m} , and $P^{+(m+n)}$ signify protein molecules with the corresponding numbers of ionizations suppressed on key groups in the respective states of the molecule. These key groups (35) refer to only a small portion of the total ionizable groups of the protein, and are therefore not directly referable to the titration curve. In general,¹¹ one or more protons

¹¹ With bovine plasma albumin (1), $n = 3$ at $\Gamma/2 = 0.20$. With human plasma

appear to combine at critical sites to make the protein labile (P^{+m}). On the suppression of further ionizations ($P^{+(m+n)}$), a form resistant to insolubilization is obtained.

P^0 obviously has the familiar properties of the native protein molecule. It resists insolubilization, has normal (low) viscosity and levorotation, and is not attacked readily by pepsin and trypsin. P^{+m} is labile to insolubilization, but has (probably) normal viscosity and levorotation, and is resistant to the enzymes. It yields, over a measurable time interval, a product D which is fully-precipitable and easily attacked by pepsin and trypsin. D probably [see Ref. (6)] has an intermediate viscosity and levorotation, and aggregates slowly outside the precipitation range. $P^{+(m+n)}$ is stabilized against insolubilization, resists the action of pepsin (and probably trypsin), but has considerably elevated viscosity and levorotation. Plasma albumin in this state most likely does not form an insoluble product directly, inasmuch as the conditions which favor its formation (low $\Gamma/2$ and/or low pH) are least favorable to the formation of a precipitable protein.

P^0 is native plasma albumin, while D represents the irreversibly denatured (insoluble at pH 5) protein, P^{+m} is labile toward insolubilization, and conceivably conforms to the "expandable" protein postulated by Tanford *et al.* (7). $P^{+(m+n)}$ is the "expanded" protein. Whether albumin in this state is also "reversibly denatured" is a question of semantics. While it has viscosity and levorotation as high as that found for other irreversibly denatured (capable of insolubilization) proteins (8, 10), Foster and Yang (6) report that it transforms slowly into an insolubilizable form only with an accompanying decrease in viscosity.

Sterman and Foster (9) have noted a parallel in the action of urea and of hydrogen ions toward plasma albumin. It is therefore conceivable that the protein present in high concentration of urea is similar to if not identical with $P^{+(m+n)}$.

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We would like to thank Professor Walter Kauzmann for discussing this work with us, for reviewing the manuscript, and for his very helpful comments on the definition of denaturation.

albumin at $\Gamma/2 = 0.20$ (2, 3), $m = 3$ and $n = 2$; as the ionic strength is decreased (4), m diminishes while n increases. Since bovine and human plasma albumins exhibit qualitatively (and almost quantitatively) the same behavior, the subsequent discussion applies to both proteins.

SUMMARY

Bovine plasma albumin has been subjected to attack by pepsin and trypsin after exposure to possible conditions of denaturation at 25 and 60°, in the pH range 2.3–6.8. Proteolysis parallels the extent of denaturation as indicated by loss of solubility. Increases in viscosity and levorotation previously observed at low pH or in the presence of urea have been shown to signify the transformation of plasma albumin into a form which is resistant to insolubilization.

Light-scattering and ultracentrifuge measurements have established that this protein is neither aggregated nor dissociated at low pH, but in fact exhibits considerable molecular stability at low pH, and especially so at low ionic strength.

NOTE ADDED IN PROOF

Since the preparation of this manuscript, a paper has appeared which bears directly on the work described here. In it, Loeb and Scheraga [*J. Phys. Chem.* **60**, 1633 (1956)] raise a question concerning the validity of the expansion of plasma albumin at low pH. As an alternate interpretation these authors suggest that the observed change in viscosity could be due to the aggregation of albumin molecules. It is considered that the light-scattering data at pH 3, $\Gamma/2 = 0.01$ reported in the present paper definitely eliminate the possibility of aggregation, suggested by Loeb and Scheraga, as an explanation for the observed changes in viscosity, especially since the viscosity change is greatest at low ionic strength.

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