though of markedly different average molecular weight, have practically identical spectrograms, which are appreciably lower than that of 1-ethyl-2-pyrrolidone.

It is clear then that building up a long chain polymer from this particular dialkylamide does result in diminished average absorption per amide link on the long wave length shoulder of the amide band. It also appears that beyond a certain point there is no further decrease in low wave length absorption with increase in molecular size.[17] Unfortunately, the spectra of the simpler intermediate polymers are not available.

However, extension of the neutral glycine peptide chain (up to hexapeptide) results in a similar change in the average absorption per peptide link.[3] And because all of the intermediates are available, it was possible there to offer a reasonable explanation of such results on the basis of an unequal distribution of absorption among the different peptide links in a given chain. The unequal distribution was attributed to the different structural environment surrounding each peptide link. The two systems are not entirely analogous, but it is possible that a similar explanation could be offered here.

(17) It is possible that there is actually a progressive decrease in chromophoric absorption; but beyond a certain molecular size, this decrease is exactly compensated by an increase in Tyndall scattering. On the other hand, it may be more reasonable to conclude that Tyndall scattering by molecules of this size does not add significantly to the intense chromophoric absorption found at low wave lengths.

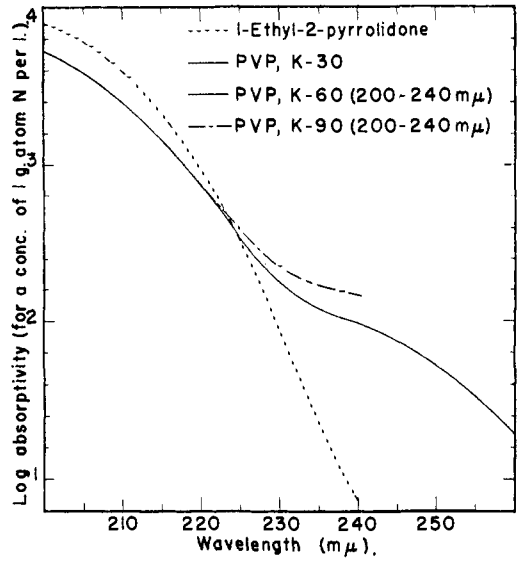

Fig. 3.—Spectrograms of PVP compared with that of 1-ethyl-2-pyrrolidone.

Acknowledgment.—The author is indebted to Miss Mildred Mosby for the N determinations.

CHICAGO 12, ILLINOIS

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

Effect of Temperature on the Intrinsic Viscosity and Optical Rotation of Bovine Plasma Albumin in Acid Media[1]

BY JOSEPH F. FOSTER[2a] AND JEN TSI YANG[2b]

RECEIVED OCTOBER 11, 1954

It has been shown previously that bovine serum albumin undergoes a rapid reversible structural alteration, at room temperature, upon reducing the *p*H of the aqueous solution below approximately 4.0. This alteration, which has been tentatively interpreted by the authors as an all-or-none transition from a compact to an expanded form, is accompanied by a marked increase in intrinsic viscosity and in levorotation. In an attempt to evaluate the thermodynamic parameters studies have now been carried out over the temperature range 2 to 80°. The expansion equilibrium is favored by elevated temperature but the effect is not marked and interpretation of the results is complicated by two general features. In the first place the temperature dependence, which is fairly normal above 20°, disappears below that temperature. Secondly, above about 40° a slow, irreversible alteration takes place. This denaturation is characterized by a marked *decrease* in intrinsic viscosity from that of the reversibly swollen form, but the optical rotation remains substantially constant. That this change is not due primarily to hydrolysis or to aggregation is indicated by preliminary light scattering studies. The kinetics of the process are not simple first-order. It is suggested that the irreversible alteration proceeds primarily through the compact form, so that the low *p*H expanded form may be regarded as a reversibly "protected" structure.

There is now considerable evidence to indicate that bovine plasma albumin (BPA) is subject to drastic reversible alterations in configuration upon change of *p*H, particularly in acid solution. The authors have shown[3] that there is marked increase in intrinsic viscosity and a parallel increase in optical rotation upon lowering the *p*H from the isoelectric point to approximately 2.0. The failure of such solutions to yield flow birefringence was taken as evidence that the alteration is essentially an isotropic expansion and the magnitude of the viscosity increase is such as to indicate an increase of some twenty-fold in effective hydrodynamic volume of the protein molecule.

The present study was undertaken in the hope of clarifying further the mechanism of this reversible alteration. In particular it was desired to ascertain the temperature dependence and attempt to evaluate the thermodynamic parameters characterizing the equilibrium which was postulated to be an all-or-none transition from one to another discrete form of the protein. Experimental measurements have been made of the effect of temperature, at various *p*H values acid to the isoelectric point, on the

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

intrinsic viscosity and optical rotation of BPA. Since a slow, irreversible alteration was observed at elevated temperature preliminary studies of the kinetics of this process also were made.

Materials and Methods

The crystalline bovine plasma albumin was purchased from Armour and Company. Stock aqueous solutions were adjusted to the desired *p*H with dilute HCl, kept in the refrigerator overnight, and filtered through fine sintered-glass filters under pressure.[4] Solutions remained clear after filtration. The concentration of protein in solution was determined by micro-Kjeldahl, using a nitrogen factor of 16.0%.

Viscosity measurements were made in two Ostwald-Fenske type viscosimeters contained in a constant temperature bath controlled to ±0.02°. The specific viscosity of protein solutions was calculated from the ratio of the flow times of the solution to that of solvent at the same temperature, without correction for kinetic energy or density effects (both of which were considered negligible for present purposes).

Optical rotation measurements were made in 2.0-dm. nickel-jacketed polarimeter tubes with a Rudolph High-precision Model 80 polarimeter, using sodium light. The polarimeter tubes were connected through a circulating pump to a constant temperature bath. Temperature control in the protein solutions is estimated at ±0.5°.

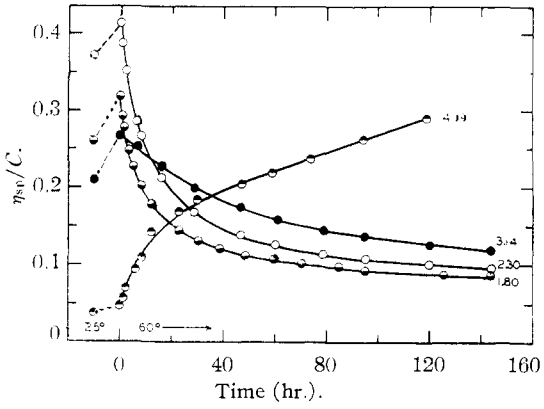

Fig. 1.—Reduced viscosity of BPA as a function of time of heating at 60° at *p*H values as indicated. The original viscosities at 25° are also shown for comparison; protein concentrations: *p*H 1.80, 2.03%; 2.30, 1.63%; 3.14, 1.60%; 4.19, 2.05%.

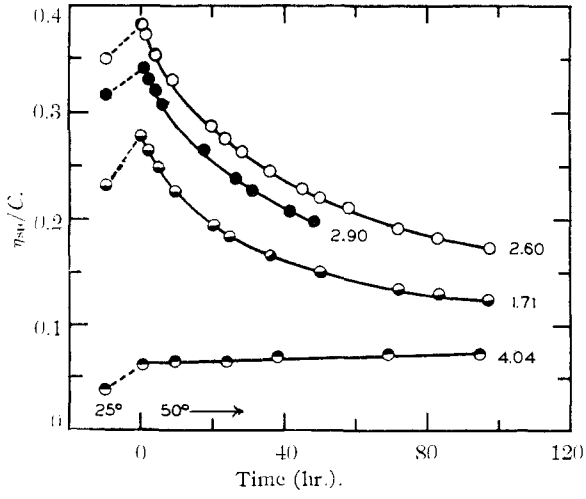

Fig. 2.—Reduced viscosity of BPA as a function of time of heating at 50° at *p*H values as indicated. The original viscosities at 25° are also shown for comparison; protein concentrations: *p*H 1.71, 2.06%; 2.60, 2.06%; 2.90, 1.04%; 4.04, 2.05%.

(4) This procedure was adopted to remove traces of insoluble material liberated by the protein on acidification. This contaminant is evidently fatty acid or other lipid like material.

Experimental Results

In Figs. 1 and 2 are shown the changes, with time, in the reduced viscosity of BPA at several values of *p*H and at two temperatures, 60 and 50°, respectively. At *p*H values below 4 there was a very rapid, possibly instantaneous, increase in viscosity, upon elevating the temperature, followed by a continous decrease with time. Above *p*H 4 a time-dependent increase was observed and fiber-like particles characteristically appeared after prolonged heating.

In Fig. 3 are shown comparable results on the effect of time on optical rotation (expressed as $[-\alpha]$) at various *p*H values and 60°. There is an essentially instantaneous increase in this property, as in the case of the viscosity, upon raising the temperature. However, in this case no time dependent decrease is seen, the rotations being substantially time independent at lower *p*H values or slowly increasing at *p*H near 4.0.

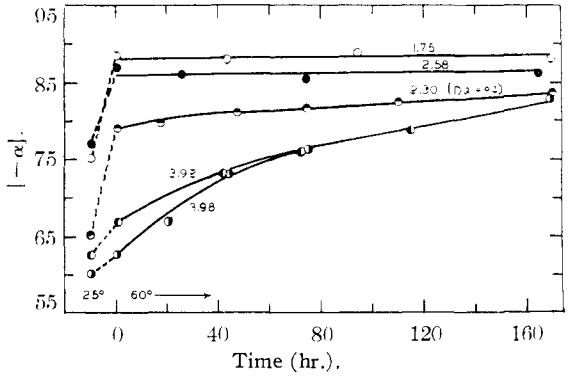

Fig. 3.—Specific rotation of BPA as a function of time of heating at 60° at *p*H values as indicated. The original rotations at 25° are also shown for comparison. Protein concentrations: *p*H 2.30, 1.86%; 2.58, 1.68%; 3.92, 1.79%; 3.98, 1.91%. The experiment at *p*H 2.30 was conducted in the presence of 0.1 molar added NaCl.

In Fig. 4 there are plotted optical rotations as a function of temperature at several *p*H values. In order to minimize irreversible changes in the protein, these experiments were carried out by connecting the polarimeter tubes to the circulating system only long enough to bring them to temperature and make the optical measurement. Between readings, while the bath temperature was being raised, the tubes were disconnected and maintained at room temperature. In light of the results in Fig. 3 this procedure is justified at the lower *p*H values since the rotation is substantially time-independent. The results at the highest *p*H (4.47) are more complicated and do not represent equilibrium values, but approximately initial values with perhaps some error due to irreversible changes.

In Figs. 5 and 6 are given reduced viscosity *vs.*

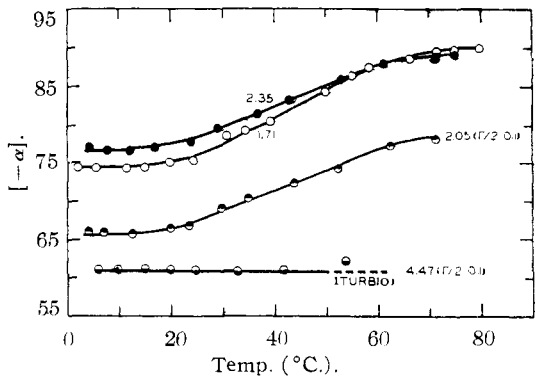

Fig. 4.—Effect of temperature on the initial specific rotation of BPA solutions at the *p*H values indicated; protein concentrations: *p*H 1.71, 2.07%; 2.05, 0.63%; 2.35, 1.92%; 4.47, 2.10%. The experiment at *p*H 4.47 was conducted in presence 0.1 ionic strength acetate buffer; at 2.05 in presence of glycine–HCl buffer ($\Gamma/2 = 0.1$).

concentration plots and optical rotation *vs.* concentration plots for BPA at various *p*H values after prolonged heating (10 days) at 60°. These data are taken as representing, approximately, the behavior after complete (irreversible) denaturation under the given conditions. Table I summarizes the derived intrinsic viscosity values and extrapolated (to zero concentration) optical rotations, both measured at 25°, for BPA solutions which had been denatured by heating at 60° for ten days. Also given here are some data, without extrapolation, in which denaturation was carried out at one *p*H, 3.73, followed by neutralization to a lower *p*H, 2.40.

TABLE I

VISCOSITY AND SPECIFIC OPTICAL ROTATION OF BPA SOLUTIONS AFTER TEN DAYS HEATING AT 60° AT VARIOUS *p*H VALUES AS INDICATED

*p*H after heating	Intrinsic viscosity	Extrapolated specific rotation
2.03	0.051	−84
3.06	.086	−86
3.73	.107	−76
2.83 ($\Gamma/2 = 0.1$)	.048	−80
	Reduced viscosity	Specific rotation
3.73 (concn. 1.7%)	0.093	−73
2.40 (concn. 1.3%) (adjusted)	.110	−84

As was mentioned above, a fibrillar precipitate was obtained in heating experiments above *p*H 4. Since further information was desirable as to the rate of this insolubilization, experiments were carried out in which the amount of insoluble protein formed was determined as a function of time at the isoelectric point. Insoluble protein was removed by centrifugation at 20,000 *g* and the residual protein in solution determined by micro Kjeldahl analysis. Data of this type obtained at 60° are given in Table II. The data before and after dialysis are considered to be in good agreement and indicate no low molecular weight split products to be formed.

TABLE II

RESULTS OF HEAT DENATURATION OF ISOELECTRIC BPA AT 60°

Heating time (hr.)	% protein in the supernatant Before dialysis	After dialysis	Supernatant after dialysis and filtration (25°) Concn., %	η_{sp}/c	(α)
0.50	71.9	73.0	0.720	0.038	−59.9
4	46.4	47.5	.612	.043	−58.9
10	36.0	37.1	.506	.050	−60.1
25	31.6	27.3	.334	.061	−63.0

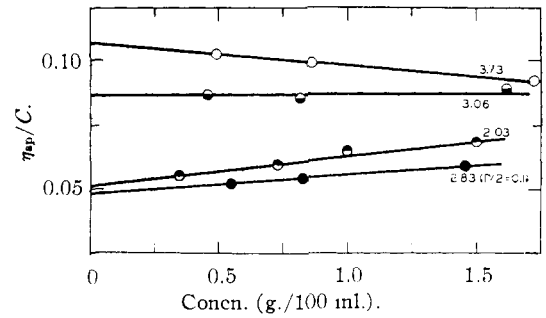

Fig. 5.—Reduced viscosity *vs.* concentration plots for BPA after 10 days' heating at 60° and at *p*H values indicated. The experiment at *p*H 2.83 was in presence of 0.1 molar added NaCl. Samples were heated at the highest concentration and dilution series performed.

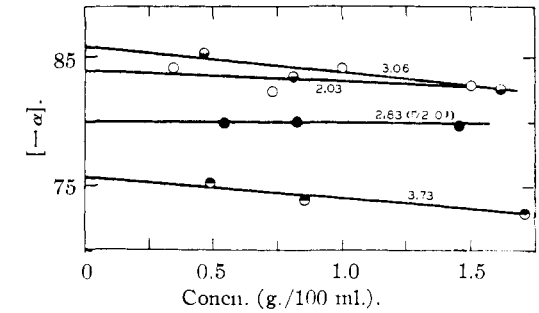

Fig. 6.—Specific rotation *vs.* concentration plots for BPA after 10 days' heating at 60° at *p*H values indicated; experimental details as in Fig. 5.

Discussion

Effect of Temperature on Expansion Equilibrium.—As Figs. 1 and 2 show, there is an instantaneous, or at least extremely rapid, increase in intrinsic viscosity of BPA solutions at low *p*H upon raising the temperature. This immediately suggests a positive enthalpy effect associated with the previously postulated expansion equilibrium. Further evidence for the positive temperature coefficient is seen in Fig. 3 where it is seen that the (negative) optical rotation also undergoes an increase under these conditions. This thermal effect is evidently reversible. If solutions were cooled after only brief exposure to 50° or 60° they regained their original room temperature viscosity and rotation values. This positive temperature coefficient might be anticipated on the basis of a recent report by Gutfreund and Sturtevant[5] who observed a delayed endothermic process in the acid titration of BPA (half-time 2.5 minutes).

Near *p*H 4 the increase in both properties, viscosity and optical rotation, is small. This is not surprising in view of the fact that at this *p*H the

(5) H. Gutfreund and J. Sturtevant, THIS JOURNAL, **75**, 5447 (1953).

equilibrium constant, at room temperature, is very unfavorable toward the expanded form.

The effect of temperature is perhaps shown more clearly in Fig. 4 where the optical rotation has been plotted as a function of temperature under four different sets of conditions. The rotation values at *p*H 1.71 and at 2.35 appear to be approaching a common limiting value of about 90° at elevated temperature which value is tentatively taken as the specific rotation corresponding to complete expansion. This is in satisfactory agreement with the limiting value of −90° obtained at room temperature by extrapolation to low *p*H and zero ionic strength.[3,6] The curve at *p*H 2.05 in presence of glycine buffer appears to be leveling off at a somewhat lower value but the data are not sufficient to be sure of this point. The insensitivity of rotation to temperature at *p*H values near the isoelectric point is clearly shown by the lowest curve in this figure. This result is in agreement with the findings of Jirgensons[7] and of Golub and Pickett.[8]

The most puzzling feature of the results depicted in Fig. 4 is the failure of the curves to approach a common value at low temperature. Thus if one is dealing with a simple endothermic equilibrium the system should shift completely to the unexpanded form in all cases at some low temperature. Instead, the rotation appears to level off below about 20°. This immediately suggests that the heat of reaction goes to zero below this temperature. It is significant in this connection that Zaiser and Steinhardt[9] have recently observed the apparent enthalpy of the acid denaturation of horse ferrihemoglobin to be zero between 15 and 25° although it is positive above this temperature range.

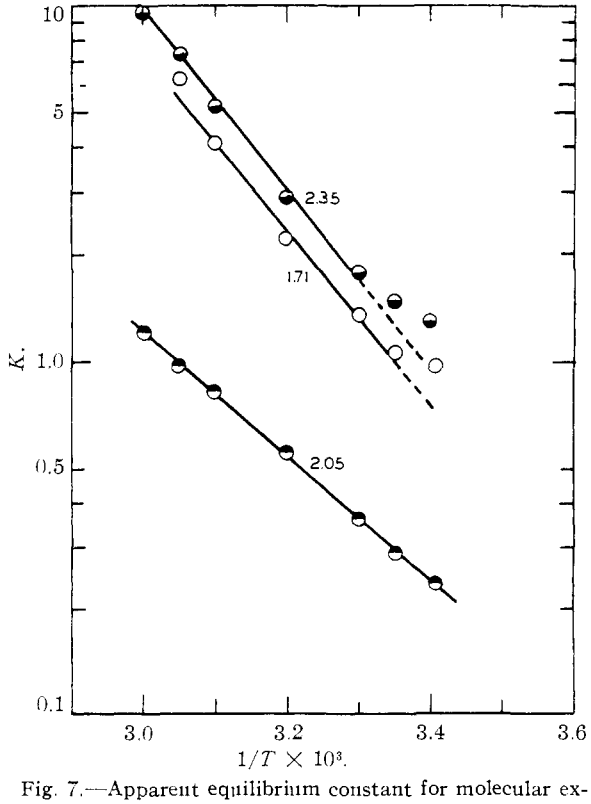

Fig. 7.—Apparent equilibrium constant for molecular expansion reaction *versus* reciprocal of absolute temperature at three *p*H values as indicated; protein concentrations: *p*H 1.71, 2.07%; 2.05, 0.63%; 2.35, 1.92%. Experiment at *p*H 2.05 in the presence of glycine buffer ($\Gamma/2 = 0.1$).

Thermodynamic Considerations.—In spite of these uncertainties, it is of interest to determine the apparent thermodynamic parameters associated with the reversible expansion process, at least as to their order of magnitude. Assuming the optical rotation to be an additive property, the equilibrium constant for the expansion is given by

$$K = (\alpha - \alpha_0)/(\alpha_\infty - \alpha) \qquad (I)$$

where α_0 is the rotation of the native (unexpanded) form and α_∞ that of the expanded form, taken to be −61 and −90°, respectively.[10] The data in Fig. 4, between the temperatures 20 and 60° where the temperature effect appears to be normal, have been utilized by means of eq. I, to estimate the temperature coefficient of the equilibrium constant. Results are shown in Fig. 7 plotted in the usual fashion. The slopes of these plots near 25° have been utilized in calculation of apparent $\Delta H°$ values which are summarized in Table III along with the other thermodynamic parameters.

The apparent entropies are clearly very large and positive. It is tempting to associate the positive entropy with the configurational change (expansion) postulated. However, as has been emphasized by Zaiser and Steinhardt,[9] no such assignment can be made without further information as to the entropies involved in the changes of ionization which are clearly a part of the over-all process.

The Irreversible Phase.—As shown in Figs. 1 and 2 there is a slow but very pronounced decrease with time in the viscosity of acid BPA solutions at 50 and 60°. Actually, this process was found to take place at an appreciable rate at temperatures as low as 35°. This change is irreversible as shown by the fact that solutions returned to room temperature were found to have suffered a permanent reduction in viscosity, the greater the longer they had been exposed to elevated temperature. The ultimate viscosity attained at high temperature was very much lower than that of the expanded protein, approximately 0.1 deciliter per g., or only two to three times that of the native isoelectric protein. In contrast, a maximal increase of ten- to twenty-fold takes place in the case of the fully expanded protein at room temperature.[3] The rate of the viscosity decrease is not greatly influenced by *p*H, nor is it very markedly temperature dependent over the range 50 to 60°.[11]

Several possibilities should be considered as possible causes of the drastic decreases in viscosity observed. Bacterial action does not seem a likely

(6) In our previous paper this value was given as −93°. It has since been found that, due to a systematic error in our Kjeldahl analyses at that time, the values given should be reduced by about 3%.

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

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

(9) E. Zaiser and J. Steinhardt, THIS JOURNAL, **76**, 2866 (1954).

(10) Advantage has been taken of the fact that the rotation is not appreciably influenced by the secondary irreversible process. Similar calculations could clearly be based on the viscosity data but would be subject to more uncertainty.

(11) In one experiment at 100°, the viscosity of a 5% BPA solution was found to decrease by about 50% in one hour at *p*H 2, a rate perhaps ten times as great as that at 60°.

explanation, in view of the high temperatures used together with the absence of any lag-period. No obvious evidence of gross microbiological growth was seen in any of the solutions. A more serious possibility is that of acid hydrolysis. The very slight dependence of rate on *p*H strongly argues against acid hydrolysis as an important factor. Preliminary light scattering studies revealed no detectable decrease in turbidity even after one week of heating at 60° and *p*H 2.5.[12]

The most logical explanation of the results would seem to be that there is another, and much more stable, configuration open to the protein, which configuration is characterized by an intrinsic viscosity (hydrodynamic volume) intermediate between that of the native form and of the reversibly expanded form. Preliminary flow birefringence studies on solutions after 1 hour heating at 100° failed to reveal any appreciable orientation, again indicating that no drastic unfolding has taken place.

The slow increase in viscosity above *p*H 4 is probably partially due to the transition from native (compact) form to the irreversibly denatured form. However, a part or perhaps most of the increase is doubtless due to the obvious aggregation which takes place in this region.

The behavior of the optical rotation during the irreversible phase is in strong contrast to that of the viscosity. Thus at low *p*H where the viscosity decreases, the rotation remains fairly constant. At *p*H around 4 where the viscosity increases, the rotation also increases. This suggests that the stable ultimate configuration has the same optical rotation, and hence possibly the same basic polypeptide configuration, as the reversibly expanded form. Thus, at low *p*H no shift in rotation could be expected since the protein is largely in the expanded configuration initially. The denatured structure is thus tentatively pictured as a collapsed one, not quite so compact as the native configuration, and possessing the same basic polypeptide configuration as the acid-expanded form.

While it is not the purpose of this paper to make a detailed kinetic analysis of the irreversible process, a few preliminary observations regarding the kinetics and possible mechanism of the heat denaturation of BPA appear in order.[13] It is generally assumed that protein denaturation is first order with respect to protein concentration, although in general rigorous first-order kinetics are not obeyed. The present case is no exception. The first-order law has been tested in Fig. 8 by plotting log $(\eta - \eta_\infty)$ *versus* time. It can be shown readily that, assuming the observed viscosity to be an additive function of the composition with respect to the two presumed components, this plot should be linear for a first-order process. The deviation observed is in the same direction as frequently observed in other cases, for example in the urea denaturation of ovalbumin[14] and of diphtheria antitoxin.[15]

Several possible explanations for such deviations already have been offered, among them heterogeneity of the protein and postulated series of two or more first-order steps. Another explanation, offered by Wright and Schomaker,[15] based on an assumed side-equilibrium with a "reversibly-protected" form, is of particular interest in the present case because of the independent evidence for an equilibrium process.

The kinetics of the irreversible denaturation of BPA have been determined over this same *p*H range by Levy and Warner[16] using isoelectric precipitation as the criterion of denaturation. They found the first-order rate law to be obeyed below *p*H 4. Gibbs[17] has studied the kinetics of denaturation of human plasma albumin by the same method. His rate curves demonstrated the same deviation from first-order behavior as is seen in Fig. 8.

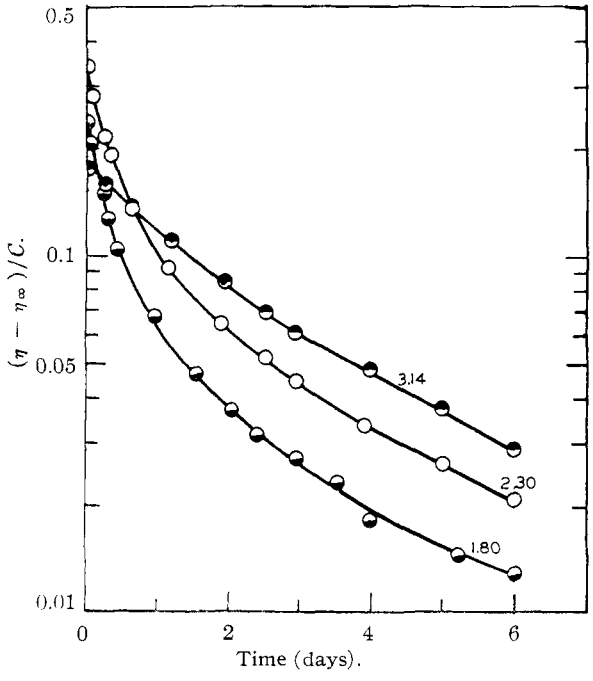

Fig. 8.—First-order rate plot for the irreversible stage of denaturation utilizing viscosity data as criterion of degree of reaction. All studies at 60° and at *p*H values as indicated; protein concentrations: *p*H 1.80, 2.03%; 2.30, 1.63%; 3.14, 1.60%.

(12) In another experiment containing 0.1 *M* NaCl the turbidity of the solution was found to increase, evidently due to aggregation.

(13) It should be pointed out that the experiments were designed primarily to study the equilibrium step. Thus buffers were not employed since the equilibrium is so strongly shifted toward the side of the native (compact) protein by presence of ions. As a consequence, significant *p*H shifts were observed during the course of prolonged heating experiments, introducing some uncertainty into the data in spite of the relatively small apparent *p*H dependence of the irreversible process. In addition, there is the uncertainty as to the effects of slight hydrolysis or aggregation, and of concentration dependence, on interpretation of the viscosity results.

Mention should also be made of certain difficulties encountered in the kinetic studies by rotation. Since the polarimeter tubes were not completely air-tight, some loss of solvent due to evaporation was unavoidable in prolonged experiments. Furthermore, protein films tended to form around the gaskets at the ends of the polarimeter tubes after prolonged heating. Both effects altered the protein concentration, but in opposite directions. To minimize such errors, stock solutions were heated in sealed test-tubes in a separate constant-temperature bath and transferred to the jacketed polarimeter tubes only intermittently for reading. Even so, some concentration change took place over several days so that the data in Fig. 3 are not as precise as would be desired.

(14) R. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5139 (1953).

(15) G. Wright and V. Schomaker, *ibid.*, **70**, 356 (1948).

(16) M. Levy and R. Warner, *J. Phys. Chem.*, **58**, 106 (1954).

(17) R. Gibbs, *Arch. Biochem. Biophys.*, **51**, 277 (1954).

In Table IV are given pseudo first-order rate constants as determined from the half-times for the viscosity drop at three *p*H values, and from the rate of the decrease in rotation at *p*H 3.98. Also given is the rate constant, determined in the same manner, from the insolubilization study at the isoelectric point. For comparison there are also given rate constants taken from the papers of Levy and Warner and of Gibbs. It is of interest that the rate constant at *p*H 3.14 determined from the viscosity drop agrees rather closely with that of Gibbs at the same temperature and *p*H. The value at the isoelectric point is also in fair agreement with his. The values at lower *p*H fall well above Gibbs' values but close to those of Levy and Warner. Quantitative comparisons should not be made because of the differing conditions employed, particularly in that our studies were conducted in absence of buffer and at very low ionic strength.[18] Furthermore the mode of calculation of the rate constants differs in that we have used the half-time whereas Gibbs employed the limiting initial slope. However, the mere fact that the rate of the viscosity drop is of the same order of magnitude as the rate of formation of insoluble (at the isoelectric point) protein is of considerable interest and suggests the irreversible alteration responsible for the viscosity drop to be the rate determining step in insolubilization (denaturation).

It is thus concluded that the irreversible alterations occurring at elevated temperature may properly be associated with the phenomenon of "heat denaturation." It is of interest, then, to consider the possible relationship between the reversible "expansion" at low *p*H and such denaturation. An intermediate, reversibly-denatured state in the irreversible denaturation of proteins was suggested as early as 1939 by Lundgren and Williams.[20] Such a mechanism has gained strong support in the beautiful studies conducted in the Carlsberg laboratories by Linderstrom-Lang and co-workers, most notably the studies of Christensen on β-lactoglobulin.[21] At first thought the present results might appear to fit into such a simple picture since the evidence for a reversible expansion equilibrium at low *p*H seems almost unequivocal. Such a mechanism might be indicated as

$$N_\alpha \rightleftarrows N_\beta \longrightarrow D \qquad \text{(Mech. I)}$$

where N_α and N_β represent the native and expanded configurations, respectively. (The implication that they are in configurations analogous to the α- and β-peptide configurations must, of course, be taken as highly speculative at present.) Kauzmann has proposed such a mechanism for the denaturation of plasma albumin.[22]

Such a simple mechanism would imply that the rate of formation of D should be highly *p*H dependent in light of the strong *p*H dependence of the prior equilibrium.[3] In particular, the rate of denaturation should be substantially nil above *p*H 4 where the equilibrium is far over on the side of N_α. That such is not the case is shown by the sharp increase in viscosity at *p*H 4.19 (Fig. 1) and of rotation at *p*H 3.92 and 3.98 (Fig. 3) (see also Table IV).

TABLE III

APPARENT VALUES OF THERMODYNAMIC PARAMETERS (Expansion of BPA at 25°.)

*p*H	*K*	$\Delta F°$(cal.)	$\Delta H°$(cal.)	$\Delta S°$(e.u.)
2.35	1.5	−230	+12,000	+39
1.71	1.1	− 40	+13,000	+43
2.05 (Γ/2–0.1)	0.29	+730	+ 8,100	+25

TABLE IV

ESTIMATION OF FIRST-ORDER RATE CONSTANTS FOR THE IRREVERSIBLE DENATURATION OF BPA AT 60°

*p*H	½ Time, *t* (sec.)	*k* (=0.69/*t*)	*k* (L and W)[a]	*k* (G)[b]
1.80	3.2×10^4	2.2×10^{-5}	2×10^{-5}	1×10^{-5}
2.30	4.1×10^4	1.7×10^{-5}	2×10^{-5}	1×10^{-5}
3.14	16×10^4	4.3×10^{-6}	2×10^{-4}	1.5×10^{-5}
3.98	25×10^4	2.7×10^{-6}	2×10^{-2}	3×10^{-4}
4.8	1.4×10^4	4.8×10^{-5}		3×10^{-5}

[a] Taken from Fig. 2 of the paper by Levy and Warner, *J. Phys. Chem.*, **58**, 106 (1954), by interpolation between the two temperatures 56.1 and 65.7. [b] Taken from Fig. 3 of the paper by Gibbs, *Arch. Biochem. Biophys.*, **51**, 277 (1954), except values at *p*H 1.80 and 2.30 which are unpublished data of Dr. Gibbs.

The data of Levy and Warner[16] and Gibbs[17] show clearly that in both bovine and human plasma albumin there is a maximal rate of heat denaturation near *p*H 4 and that the rate decreases very markedly upon reducing the *p*H below this value. One is forced to conclude that the results are almost precisely the reverse of what is to be expected on the basis of mechanism I.

Another interesting possibility stems from the studies of Wright and Schomaker on the urea inactivation of diphtheria antitoxin, previously cited.[11] This mechanism, using the symbolism of the present paper, could be given as

$$D \longleftarrow N_\alpha \rightleftarrows N_\beta \qquad \text{(Mech. II)}$$

The N_β form in this case is a reversibly "protected" species. Since the equilibrium shifts to the right with decreasing *p*H below 4 the rapid decrease in rate of denaturation with decreasing *p*H would follow naturally. The relative stability of plasma albumins toward (irreversible) denaturation, as compared to for example ovalbumin, would thus be due to the fact that they are capable of reversible intramolecular rearrangements (isomerization). This mechanism becomes even more attractive upon making closer quantitative calculations. Thus, the previously published data on intrinsic viscosity and optical rotation at low *p*H[3] suggest

(18) The extreme ionic strength dependence of the rate of denaturation of human plasma albumin under these conditions has been pointed up recently by Gibbs.[19] In particular, he finds a pronounced decrease in rate in the *p*H range 3 to 4 with decreasing ionic strength. It seems probable that this is the explanation of the lack of agreement between our kinetic constants in this range and those of Levy and Warner since our ionic strength is very much lower than theirs and also somewhat variable. It should be pointed out further that this ionic strength dependence is precisely what would be expected on the basis of Mech. II (below).

(19) R. Gibbs and A. Chanutin, Abstr. 126th Meeting A.C.S., p. 77-C (1954).

(20) H. Lundgren and J. Williams, *J. Phys. Chem.*, **43**, 989 (1939).

(21) L. Christensen, *Compt. rend. Lab. Carlsberg*, **28**, 37 (1952).

(22) W. Kauzmann, in McElroy and Glass, "The Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Maryland, 1954, p. 70.

that two protons are bound in the expansion,[23] *i.e.*

$$N_\alpha + 2H^+ \rightleftharpoons N_\beta \cdot 2H^+$$

Gibbs[17] found the slope of his plot of log rate *versus* *p*H to be two in this *p*H range and postulated that loss of two protons from the low *p*H form must precede denaturation.

As attractive as this mechanism is, it leaves unanswered several important questions, notably the apparent increase in rate below *p*H 2, seen both in our data and in that of Levy and Warner[16] and of Gibbs.[17] Further study of the process by combined use of precipitation, viscosity and optical rotation should lead to clarification of such points.

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(23) This is readily demonstrated by plotting the logarithm of the apparent equilibrium constant *versus* *p*H, the experimental slope being −1.8. We are here comparing results on bovine plasma albumin with results on the human protein. The data of Levy and Warner[16] on the bovine protein show a *p*H dependence in better accord with the binding of three hydrogen ions.

It should be pointed out that this substantial agreement of the *p*H dependence on kinetics and equilibrium could be fortuitous. Strictly speaking, it only shows that the transition complex in the irreversible process has a charge similar to or identical with that of the unexpanded form. On the other hand, the fact that the plasma albumins are rather unusual in showing the low *p*H expansion, and are also unusual in that they denature less rapidly in acid solution than at the isoelectric point, seems significant.

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The Amino Acid Composition of Certain Morphologically Distinct Parts of White Turkey Feathers, and of Goose Feather Barbs and Goose Down

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Chromatographic analyses have established that differences exist in the amino acid composition of morphologically distinct parts of white turkey feathers. Furthermore, it is probable that different types of feathers from a given species of bird are identical in amino acid composition but that feathers from different species of birds are dissimilar. The high content of proline in feather proteins makes it unlikely that the polypeptide chains of these proteins assume simple pleated sheet or helical configurations.

Feather rachis keratin has long been known to produce a complicated and well-defined X-ray diffraction pattern[1] and hence to possess a highly oriented structure. Rudall[2] has found that essentially the same X-ray pattern which is given by the rachis is also shown by the barbules, barbs, calamus and medulla of the feather (Fig. 1 of the present paper may be consulted for a definition of the various feather parts). However, if one examines the individual feather parts under a polarizing microscope, the rachis appears to be highly oriented but the other parts are much less so or virtually unoriented. Furthermore, although these parts of a feather arise from the *stratum intermedium*, one need not conclude that they must be identical in composition. Thus, the work of Lillie[3] and collaborators proves that the various portions of the papilla from which the *stratum intermedium* arises are highly specific in their ability to produce the individual parts of a feather: the barbs cannot be considered to be branches of the rachis nor the calamus an elongation of it. This information, therefore, lends credence to the possibility which arises on mere visual inspection that, perhaps, the morphologically distinct parts of a feather may not be identical in composition.

As part of an investigation of the structure of feather keratin, we have made an exact quantitative determination of the amino acid content of four distinct parts of a feather in order to determine whether or not the composition is uniform. White turkey feathers have been used for analysis because the absence of color simplified the determinations and because the size of the feathers facilitated their sectioning. In addition, less exact analyses have been made of goose feather barbs and of goose down in order to ascertain (1) whether species differences exist and (2) whether various types of feathers from the same kind of bird are identical in composition.

(1) W. T. Astbury and T. C. Marwick, *Nature*, **130**, 309 (1932).

(2) K. M. Rudall, *Biochim. Biophys. Acta*, **1**, 549 (1947).

(3) F. R. Lillie, *Biol. Rev.*, **17**, 247 (1942). This review of Lillie as well as the papers of R. M. Strong (*Bull. Mus. Comp. Zool. Harvard*, **40**, 147 (1902)) and H. R. Davies (*Morph. Jahrb.*, **15**, 560 (1889)) may be consulted for a description of entire developmental history of a feather

Experimental

Materials.—Figure 1 presents a drawing of a typical white turkey feather and a definition of terms. In this figure, the feather is viewed from the *dorsal* side, that is, the side which the feather presents to the outside world; the opposite side toward the bird itself is termed the *ventral* side. The portion of the feather from R to A is above the skin level of the bird and that from C to A is below. The *rachis* bears the *barbs* which in themselves are complex structures and support *barbules*. The distal barbules of each barb possess hooklets which interlock with the proximal barbules of the adjoining distal barb. This interlocking of the barbules maintains the form of the feather. If the hooklets are absent, the feather is fluffy as is often the case near the junction of rachis and calamus. Throughout its length, the rachis is filled with a pithy cellular substance of low apparent density which is termed the *medulla*. The barbs also contain medullary material which does not join that of the rachis. The interior dorsal side of the rachis contains grooves which extend from R approximately to A; like the rest of the interior, these grooves are packed tightly with the medulla. The rachis is thickest on the dorsal side and very thin at the junction with the barbs and at the bottom of the ventral groove. The *calamus* is essentially cylindrical but slightly thickened on the dorsal side. The interior of the calamus contains pulp caps, the origin of