Denaturation of ovalbumin as revealed by streaming orientation

Edward G. Samsa
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DENATURATION OF OVA LUMIN AS REVEALED
BY STREAMING ORIENTATION

by

Edward G. Samsa

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
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X. ACKNOWLEDGEMENTS
I. INTRODUCTION

Proteins are substances of high molecular weight and great complexity. Ovalbumin, for example, a protein with which this dissertation is primarily concerned, has a molecular weight of 45,000 and contains about 400 amino acid residues. In the native state the molecule is known to be roughly globular in shape, rather than fibrillar, and so must possess a folded structure. This folded configuration is stable toward some chemical and physical treatments, but unstable toward others. Thus, ovalbumin, with careful manipulation, can be crystallized repeatedly without damage to the molecule as determined by electrophoretic and other techniques. Yet, when ovalbumin solutions are heated the protein becomes very insoluble at its isoelectric point, and it no longer can be crystallized. In this condition, the protein is termed "denatured".

The profound change in solubility of globular proteins on denaturation is one of the important indications of differences in structure between the native and the denatured state. Much evidence, accumulated from studies of the physical and chemical properties of native and denatured globular proteins, such as changes in viscosity, sedimentation rate, and solubility, suggests that the denatured molecule is
considerably more asymmetric than is the native molecule.

The increased asymmetry has generally been interpreted to be a consequence of an unfolding of the protein molecule. However, measurement of the length of the unfolded molecule, or of the axial ratio, is often complicated by aggregation. The unfolded molecules are particularly susceptible to aggregation, and so solutions of denatured protein usually are not composed simply of molecularly dispersed unfolded polypeptide chains. This makes the interpretation of some data difficult since, for example, the effect of aggregation on the axial ratio cannot be predicted quantitatively, or even the presence of aggregation clearly established in some cases.

Among the techniques available for the investigation of denaturation of globular proteins birefringence of flow, the one selected for this dissertation seems particularly suitable. Birefringence of flow is the double refraction produced in a liquid, optically isotropic at rest, when it is subjected to a velocity gradient. The orientation of the optic axis of the flowing solution with respect to the flow lines is measured over a range of velocity gradient. Also, the magnitude of the birefringence is measured over the same velocity gradient range. Application of the theory of flow birefringence, derived for rigid ellipsoidal particles homogeneous in length, enables a determination to be made of the length of the solute molecule, as well as of its
intrinsic birefringence. If the solute molecules are heterogeneous in length, some idea can be formed as to the amount of polydispersity.

The experimental work to be described encompasses the denaturation of ovalbumin by (1) heat, at various pH values in the acid region, (2) heat, in the presence of a cationic detergent in the acid region, (3) heat, in the presence of urea, (4) heat, at various pH values in both the acid and the alkaline region, in 85 per cent glycerol, and (5) heat, in the presence of an anionic detergent in the alkaline region, in 85 per cent glycerol. In the course of the work, flow orientation and birefringence measurements were made on more than 250 separate solutions, and the results of approximately 100 are described.
II. LITERATURE SURVEY

A. Protein Denaturation

The chemistry of protein denaturation has been thoroughly and ably reviewed by Neurath and co-workers (52). The survey presented in the following pages is not meant to be comprehensive in scope. Instead, investigations which seemed most pertinent in their relationship to the interpretation of flow birefringence studies are reviewed.

1. Heat denaturation

The kinetics of denaturation of ovalbumin by heat, and by chemical agents, has been reviewed by Eyring and Stearn (30). The extent of denaturation usually has been determined by precipitation techniques, based on the insolubility of denatured ovalbumin at its isoelectric point. Changes in other physical properties, such as optical rotation, or ultraviolet absorption, unfortunately, usually are not sufficiently large to be useful in following denaturation.

Chick and Martin (18, 19, 20) in a series of papers, have shown that heat denaturation is a first-order reaction, when the denaturation is carried out at constant pH. The denaturation reaction has an extraordinarily high temperature coefficient. The rate of denaturation increases markedly
either with increasing hydrogen ion or with increasing hydroxyl ion concentration, and this suggests that electrically charged species may be taking part in the reaction. In support of this idea, Lewis (41) found the rate to pass through a minimum at pH 6.8, at which point the hydrogen and hydroxyl ion concentrations are approximately equal. It was suggested by Bernhart (6) that water enters into the reaction as well, since dried ovalbumin is much more resistant to heat denaturation than is ovalbumin in solution, and since heat denaturation of the dried material does not follow a first-order law.

Bull (12) calculated the axial ratio of heat-denatured ovalbumin to be 7.4 to 1, and that of the native material to be 3.9 to 1. His conclusions were derived from viscosity data on ovalbumin denatured at 100° for 7 minutes at pH 8.0 in 0.02 M phosphate buffer. The extent of denaturation was 87 per cent, as estimated by isoelectric precipitation.

Such data illustrate the increase in asymmetry resulting from denaturation. It should be emphasized that solutions prepared in this manner may be aggregated, and that the aggregation may affect the calculated axial ratios in a not obvious manner. In this connection, Philippoff (60) has considered the problem of aggregation in general, and has concluded that viscosity may be increased, or decreased, as aggregation proceeds, depending upon the type of aggregation present.
Denatured ovalbumin was prepared, under closely controlled conditions, by MacPherson and Heidleberger (44) for investigations of changes in fluidity of denatured ovalbumin solutions on standing long periods of time. Their samples were prepared presumably with a minimum of degradation using acid, alkali, and heat as denaturing agents. They were purified by re-solution and re-precipitation. When these solutions were aged until fluidities became constant, they were shown by MacPherson and co-workers (45) to be aggregated, the aggregates consisting of 5 to 20 molecules. Salts in excess of 0.02 M, or exposure to 37°C, decreased the fluidity and increased the opalescence of the acid-denatured ovalbumin, which previously had been aged to constant fluidity.

These results are of general interest, insofar as flow orientation is concerned. The extent of aggregation in the above samples is relatively small, considering that they were re-precipitated and aged. Samples prepared for flow-orientation measurements, under the most favorable conditions, might be expected to be aggregated to a negligible extent, since they would not be precipitated from solution.

Precipitated, heat-denatured ovalbumin, particularly when it is stretched, was shown by Astbury and Bell (3) and by Senti and co-workers (68, 69) to give an X-ray pattern characteristic of β-keratin. The denatured ovalbumin has a side-chain spacing of about 10 Å, and a back-bone spacing of 4.5 to 4.6 Å. This X-ray pattern is indicative of an
oriented structure, composed of extended polypeptide chains oriented in an approximately parallel manner, and is good evidence that unfolding of the globular protein, ovalbumin, has occurred during denaturation.

2. Urea denaturation

The denaturation of ovalbumin, and other proteins, by urea has received rather intensive investigation. Concentrated urea solutions, for example 6 to 8 M, denature ovalbumin and at the same time act as solvents for the denatured protein. These solutions of denatured protein are suitable for study by various physical measurements such as osmotic pressure, ultracentrifugal analysis, diffusion, and viscosity.

Certain proteins, such as amandin and excelsin, when dissolved in 6.7 M urea, were found by Burk (16), using osmotic pressure methods, to be dissociated into units having about one sixth the molecular weight of the native protein. Burk and Greenberg (17) showed the molecular weight of ovalbumin to be unchanged under the same treatment. Likewise, sedimentation, diffusion, and viscosity measurements by Rothen (65) indicated the ovalbumin to be unchanged in molecular weight. Williams and Watson (72), on the other hand, believed that 50 per cent aqueous urea solution causes ovalbumin to dissociate into halves, as judged by ultracentrifugal analysis. However, this change
could also have been interpreted as having resulted from increased asymmetry due to denaturation. Clark (21) has presented evidence, from a study of the temperature coefficient of urea denaturation of ovalbumin, which indicates that some splitting may occur in concentrated urea solution. Thus, there is evidence in the literature for, and against, dissociation of ovalbumin in concentrated urea solution, the greater amount of evidence favoring the idea that dissociation does not occur.

Neurath and Saum (54), and Neurath and co-workers (51) studied horse-serum albumin denatured by urea, and by heat, and showed that the diffusion constant decreased with increasing urea concentration whereas the relative viscosity increased. These findings are indicative of increased asymmetry of the protein molecule. Similar results were obtained when heat denaturation was carried out either in the presence of 3 M urea, or at pH 3.2. The urea-denatured solutions of this protein were found to be monodisperse at urea concentrations above 0.5 M, whereas the heat-denatured solutions were polydisperse. The particles became elongated, with increasing urea concentrations. Assuming their shape to be that of an ellipsoid of revolution, their dimensions approached a major axis of 360 A and a minor axis of 20 A.

Even in fairly concentrated urea solutions of ovalbumin, however, a variable amount of aggregation, which
results in heterogeneity, may be present. Thus Mirsky (47), by an ultracentrifugal analysis, found the particle weight of ovalbumin in 9.5 M urea solution to be the same as that of native ovalbumin in aqueous solution in the absence of urea. Lowering the urea concentration, by dilution, resulted in polydispersity. At 6 M urea concentration the particle weight was doubled, and increased further as the concentration was lowered to 2 M. The solution again appeared monodisperse when the urea concentration was raised to 9 M.

Hopkins (36) found that the rate of denaturation of ovalbumin by urea decreased with increasing temperature over the range 0° to 37°. He contended that the reaction has a negative temperature coefficient, and Ramsden (64) supported him. Hopkins interpreted the unexpected negative temperature coefficient in terms of a protein-urea complex, highly dissociated except in the presence of high concentrations of urea, and of which the dissociation increased with rise in temperature sufficient to account for the observed diminution in rate of denaturation. In these experiments, the amount of denaturation was determined by dilution of the urea denatured solutions with a large volume of water containing a small amount of ammonium sulfate or acetic acid, if necessary, to facilitate precipitation. Clark (21), on the other hand, brought forth evidence, obtained by measuring opalescence
photometrically during dialysis of urea-denatured solutions, suggesting that splitting of ovalbumin may occur along with denaturation. This could account for the negative temperature coefficient, when the amount of denaturation is measured by the amount of insoluble matter formed upon dilution of the urea-denatured protein solution. Both the denaturation and the splitting reactions have positive temperature coefficients, according to Clark, and the second is more affected by increase in temperature than is the first.

Wu and Yang (74) have shown that the rate of urea denaturation of ovalbumin is dependent upon pH as well as upon the ionic strength. The rate was found to be at a minimum at pH 7.6 and to rise rather steeply on either side of this value. It was surprisingly dependent upon ionic strength. For example, a 1 per cent solution of ovalbumin in 40 per cent urea solution at pH 7.6 (unbuffered) and 31° was 62 per cent denatured after 2 hours, whereas a similar solution made up in 0.10 M phosphate buffer containing 1.0 M sodium chloride was 4 per cent denatured, and in 0.10 M phosphate buffer containing 2.0 M sodium chloride was zero per cent denatured, as measured by the amount of precipitate formed upon dilution with a large volume of buffered water.

Liu (42) also observed a pronounced effect attributable to the presence of buffer ions. On standing 20
hours an unbuffered ovalbumin solution was 80 per cent denatured, while a solution buffered at pH 6.2 with citrate buffer was only 20 per cent denatured, in 40 per cent urea solution. The viscosity increased with increasing amount of denaturation; it showed a minimum at pH 5.7.

Denaturation of ovalbumin in urea solution as well as by heat is accompanied by an increase in pH according to Wu, Liu, and Chou (73), who have interpreted this change as proof of alteration of acid or base-binding power on denaturation. Such changes might be expected, as a result of unfolding and disrupting of the bonds holding the molecule together in a folded configuration.

Klotz and co-workers (39) made a mass-law analysis for competitive binding of ions by bovine-serum albumin, using methyl orange-bovine serum albumin complexes as reference solutions. The binding ability of bovine-serum albumin was found to be slowly destroyed by heating at 55°, or by exposure to 0.01 M sodium hydroxide. Urea at high concentrations was shown to be capable of rapidly displacing methyl orange from the dye-protein complex. The displacing ability of the urea reached a maximum at about 6 M concentration.

Films of urea-denatured ovalbumin were studied by Astbury and Dickinson (5). They found the films to possess an X-ray pattern similar to that of β-keratin,
a protein having an extended structure.

3. Detergent denaturation

Many cationic, and anionic, detergents are potent denaturing agents. These compounds are, generally speaking, strongly bound to proteins. They are promising for study, since the protein-detergent combinations can be described with somewhat more definiteness than can, for example, protein-urea or protein-guanidine hydrochloride combinations. Detergent denaturation is of particular interest for flow-birefringence measurements, because it can be effected at relatively low detergent concentrations. This is advantageous, since the refractive index of the solvent is not seriously altered, and also the solubility of the denaturing agent is not likely to be exceeded. As an example, the work of Anson (1) is cited. He showed that isoelectric beef-hemoglobin is only slowly denatured by 8 M urea, whereas 0.0008 M Duponol P.C. denatures it rapidly, as judged by colorimetric methods.

Both native, and heat-denatured, ovalbumin form well-defined complexes with alkylbenzene sulfonates in solutions alkaline to the isoelectric point, according to Lundgren and co-workers (43). The native protein combines with one third its weight of detergent, in mixtures containing relatively large amounts of native protein, and the heat-denatured protein combines with larger amounts. The
complexes were studied electrophoretically. When the fraction of detergent was 0.3 or more, the protein showed the electrophoretic behavior of completely denatured protein combined with detergent.

Desreux and Fabry (22) also observed the greater combining power for detergent of denatured ovalbumin as compared to native ovalbumin. They investigated the change in specific rotation upon denaturation with Texapon (a mixture of sodium alkyl sulfates), and concluded that 0.46 gm. of detergent are fixed by 1.00 gm. of native protein, and 0.7 gm. of detergent are fixed by 1.00 gm. of denatured protein.

Putnam and Neurath (63) investigated sodium dodecyl sulfate complexes of horse-serum albumin. Complexes were formed on the acid, as well as on the alkaline side, of the isoelectric point. Complete precipitation was obtained within the limits of detergent-protein ratio of 0.2 to 1 and 0.4 to 1. They concluded that precipitation is due to electrostatic forces, since it occurs only on the acid side of the isoelectric point, and the amount of detergent required for complete precipitation corresponds closely to the total acid-binding capacity of the protein. They also showed that anionic detergents cause denaturation, as measured by rise in viscosity, on either side of the isoelectric point. The anionic detergents, are more effective as denaturants than is urea. For example, 0.17 M
sodium dodecyl sulfate increased the intrinsic viscosity of serum albumin to 25, and 8 M urea increased it to 22, from a value of 4.3 for the native protein.

Not all of the positive groups of serum albumin are equally available for binding with straight chain alkyl sulfates. This was shown by Karush and Sonenberg (38), in a study of reversible binding, by means of equilibrium dialysis.

Two discrete complexes were identified by Neurath and Putnam (53) in the horse serum albumin-sodium dodecyl sulfate system at pH 6.8 and 1°. Other complexes were observed at 20°. The first two complexes contained detergent anions equivalent to one half, and all, respectively, of the cationic groups of the protein. The higher complexes, of variable composition, were shown to be electrophoretically monodisperse, up to a detergent-protein ratio of 1, at which level free detergent appears. Putnam and Neurath (62) suggested that the first complex results from a combination between detergent anions and cationic groups of the native protein, and the second as a result of partial unfolding with liberation of additional cationic groups. Additional reagent caused further increase in viscosity possibly as a consequence of non-polar adsorption of detergent, or by non-stoichiometric association of detergent molecules with all of the available groups.
In a quantitative study of horse serum albumin-sodium dodecyl sulfate complexes, Putnam and Neurath (61) have ascertained that precipitation is governed by the protein-detergent ratio, pH, temperature, and ionic strength. The protein formed by dissociation of the protein-detergent complex was shown to be in a "regenerated" rather than the native state, as revealed by diffusion, viscosity, and electrophoretic studies.

Bull (11) found evidence of binding of sodium lauryl sulfate to ovalbumin, in surface film measurements, and concluded that the two complexes formed contain 17 and 32 molecules of detergent per molecule of ovalbumin.

Boyer and co-workers (10) investigated the effect of fatty acids and related compounds on the thermal stability of human, and bovine, serum albumin by cloud point, nephelometric, and viscometric procedures. They concluded that fatty acids stabilize the native protein, but not the denatured protein, against viscosity rise on heating, and that stabilization arises from association of both polar and non-polar portions of the fatty acid anion with certain groups of the albumin molecule in its native state. Boyer and co-workers (9) also studied the effect of fatty acid salts on the denaturation of human, and bovine, serum albumin by urea or guanidine hydrochloride, viscosity rise being used as the criterion of denaturation. Low concentrations of fatty acid salts were found to
prevent the viscosity increase which otherwise results when the albumin is dissolved in 6 M urea solution. Also, sodium caprylate, added to albumin solutions previously denatured by 6 M urea, caused a prompt pronounced viscosity decrease, which was interpreted in terms of refolding of the denatured protein molecule.

Fibers made from ovalbumin treated with detergent, and drawn under steam, were shown by Palmer and Calvin (55) to have a $\beta$-keratin configuration, and to consist of polypeptide chains arranged parallel to the fiber axis. They concluded that one of the functions of the detergent is to unfold the corpuscular protein.

4. Surface denaturation

Using surface-balance techniques, Bull (14) measured the rate of surface denaturation of ovalbumin monolayers, and found it to decrease with increasing protein concentration. Rate of surface denaturation was shown to be strongly dependent upon pH, and proceeded most rapidly at the isoelectric point. Bull (13) concluded that spread films of native, heat-denatured, and urea-denatured protein are similar in structure, and represent unfolded molecules in the form of an asymmetric polar film.

The mechanism of surface denaturation, of course, may be different from that of heat denaturation in solution. Nevertheless, Bull's observation of decreasing rate of
denaturation with increasing concentration, is of interest for flow-birefringence measurements. It hints that one condition which ought to be fulfilled is that of low protein concentration during denaturation.

The X-ray pattern of protein monolayers was determined by Astbury and co-workers (4), and found to be like that of \( \beta \)-keratin. They found that the side chains preferentially orient themselves perpendicular to the plane of the film, and that the thickness of the monolayer is about 10 Å.

5. Sulfhydryl group liberation

Sulfhydryl liberation is one of the main lines of chemical evidence supporting the concept of unfolding of the ovalbumin molecule during denaturation. Native ovalbumin possesses no detectable sulfhydryl groups. Sulfhydryl groups are exposed upon denaturation by heat, as was first shown by Arnold (2) and by alcohol, shaking, and ultraviolet radiation, as was shown by Harris (35). Other chemical agents such as urea, guanidine hydrochloride, and detergents are effective sulfhydryl liberators. They are of especial interest since they are also good solvents for the denatured protein.

Guanidine hydrochloride is a more powerful sulfhydryl group liberator than is urea, according to Greenstein (33, 34). Sulfhydryl group liberation increased, with
increasing urea concentration, without reaching a maximum value. With guanidine hydrochloride, however, maximum liberation was attained at about 5 M, and no further liberation occurred at higher concentrations. This maximum amounted to about 1.28 per cent protein sulfhydryl calculated as cysteine; with a very much higher concentration of urea, 17 M, only 0.97 per cent was obtained.

In contrast to the high concentrations of urea, and guanidine salts, needed for maximum sulfhydryl group liberation is the lower concentration of detergents required, as shown by Mirsky (48) who denatured ovalbumin with Duponol. The ionic detergents, in general, have been shown to be powerful denaturing agents.

Guanidine thiocyanate, iodide, and bromide were shown by Greenstein (33) to be more effective in liberating sulfhydryl groups than was the chloride. The carbonate, acetate, and sulfate were completely ineffective even at 6 M concentration. These data point toward antagonistic action between the guanidinium ion and the carbonate, sulfate, and acetate ions.

Burk (15) studied the effect of various compounds on urea denaturation of ovalbumin, which he followed by means of sulfhydryl liberation. Sulfates, and acetates inhibited liberation at all concentrations of urea. Magnesium and manganese chlorides inhibited in concentrated urea solution, but enhanced liberation in dilute urea solution. Hydrochloric acid enhanced
liberation in urea solutions of ovalbumin, but inhibited liberation in urea solutions of lactalbumin. The effects of magnesium chloride, likewise were opposite on ovalbumin and lactalbumin.

Such divergent results emphasize the complexity of the problem. In reality, little is known about the details of the mechanism of sulfhydryl liberation by the action of urea, guanidine salts, or of detergents. It seems certain, nevertheless, that sulfhydryl groups are present, but inaccessible, in the native protein, and are made accessible through the process of denaturation. This result seems reasonable if it be assumed that unfolding takes place during the denaturation.

B. Theory of Flow Birefringence

1. Two-dimensional orientation theory

The theory of flow birefringence has been developed principally by Boeder (7), by Peterlin (57), by Peterlin and Stuart (58, 59), and by Snellman and Bjornstahl (70).

Boeder solved the problem of orientation in two dimensions. He assumed the solute particles to be long, thin rods oriented in one plane under conditions of laminar flow by a velocity gradient, G, and disoriented, at the same time, by Brownian motion. The disorienting tendency is expressed in terms of the rotary diffusion constant, \( \Theta \). He defined the orientation distribution function,
\[ \rho(\phi), \quad \text{as} \]
\[ \rho(\phi) = \lim_{\Delta \phi \to 0} \frac{\Delta n}{\Delta \phi} \]

Here \( n \) is the number of particles per unit volume whose axes lie between \( \phi \) and \( \phi + \Delta \phi \).

The differential equation describing the steady state wherein \( \rho \) does not change with time (that is, when the orientation induced by the velocity gradient is exactly counterbalanced by the disorientation induced by the Brownian motion) is
\[ \frac{\partial \rho}{\partial \phi} + \alpha \rho \sin^2 \phi = C \]

Here \( \alpha = \frac{G}{\delta} \). Boeder gives graphical solutions for this equation in the form of graphs of \( \rho \) as a function of \( \phi \), as well as graphs of the extinction angle, \( \chi \), as a function of \( \alpha \), and of \( n_e - n_o \), the birefringence of the flowing solution, as a function of \( \alpha \). The extinction angle is defined as the angle between the optic axis of the flowing solution and the flow lines. The optic axis is assumed to coincide with the average position of the major axes of the solute particles. \( \chi \) approaches 45° at low velocity gradients, and 0° at high gradients.

2. Three-dimensional orientation theory

Boeder solved the three-dimensional case in a less complete manner, and derived a formula for \( \chi \) in terms of \( \alpha \), valid for small values of \( \alpha \),
\[
\chi = \frac{1}{2} \tan^{-1} \frac{G}{\varpi} = \frac{\pi}{4} - \frac{\varpi}{12} \left( 1 - \frac{\varpi^2}{108} + \cdots \right)
\]

Here \( \chi \) is expressed in radians. The rotary diffusion constant, \( \vartheta \), equal to \( G/\varpi \), can be obtained from this equation.

The problem of orientation in three dimensions of rigid ellipsoids of revolution was solved by Peterlin and Stuart. Their distribution function, \( \mathcal{F} \), depends on \( \varpi \) and on \( R \), where \( R = \frac{b^2 - 1}{p^2} \), and \( p = a/b \), the ratio of the major semi-axis, \( a \), to the minor semi-axis, \( b \); \( \varpi = G/\vartheta \) as in Boeder's treatment.

Their differential equation is,

\[
\Delta \mathcal{F} = \frac{1}{2} R \cos 2\varphi \frac{\partial \mathcal{F}}{\partial \varphi} + \frac{R \sin \beta \cos \beta \sin 2\varphi}{2} \frac{\partial \mathcal{F}}{\partial \varphi} - \frac{3R \sin^2 \beta \sin 2\varphi}{2} \mathcal{F}
\]

\[
\Delta = \frac{1}{\sin \beta} \frac{2}{\partial \beta} \left( \sin \beta \frac{\partial}{\partial \beta} \right) + \frac{1}{\sin^2 \beta} \frac{\partial^2}{\partial \varphi^2}
\]

\( \beta \) is the angle between the major axis of an individual particle and an axis parallel to the concentric cylinder axis; and \( \varphi \) is the angle between the projection of the major axis of the particle on a plane perpendicular to the cylinder axis and the flow lines of the solution.

A limiting form of the solution to this equation, for \( \varpi < 1.6 \), is

\[
\chi = \frac{\pi}{4} - \frac{\varpi}{12} \left[ 1 - \frac{\varpi^2}{108} \left( 1 + \frac{2 \varpi \varrho^2}{38} \right) + \cdots \right]
\]

This solution is practically identical to that of Boeder for small values of \( \varpi \).
Until lately, the theories of Peterlin and Stuart have been of limited applicability, because of the large amount of labor involved in obtaining solutions for the flow orientation equations, except for the limiting case where \( \alpha \) is small. Recently, Scheraga, Edsall, and Gadd (67) solved the complicated simultaneous equations involved, by use of the Mark I Electronic Computer at Harvard University. They give tables of the extinction angle, \( \chi \), as a function of \( \alpha \) (0 to 200) for various axial ratios (1 to infinity), and the orientation factor, \( f \), as a function of \( \alpha \) (0 to 200) for various axial ratios (1 to infinity). Graphs made from these data were used for calculations of length, and for the function \( \frac{A}{f c} \), defined later.

Applying the Ferrin (56) equation, relating \( \theta \) with the dimensions of the ellipsoid of revolution, permits a calculation of the length of the solute particles to be made from flow orientation measurements. The Ferrin equation, a limiting equation for rotary Brownian motion about the minor semi-axis, \( b \), for the case \( a > 5b \), is

\[
\theta = \frac{3 k T}{\pi \eta a^3} \left[-1 + 2 \ln \frac{2a}{b} \right]
\]

Here \( k \) is the Boltzmann constant, \( T \) the absolute temperature, \( \eta \) the viscosity of the solvent, and \( a \) the major semi-axis. The expression in brackets is relatively insensitive to variation in the axial ratio, \( a/b \). In
practice, the ratio can be estimated from viscosity measurements, or approximated arbitrarily.

3. **Magnitude of birefringence**

Peterlin and Stuart obtained, for the birefringence of the flowing solution, the relationship,

\[
\frac{n_e - n_o}{n} = \Phi \frac{2\pi}{n^2} (g_1 - g_2) \int (\alpha, \frac{a}{b})
\]

Here \( n \) is the refractive index of the solution at rest; \( \Phi \) is the volume fraction of the ellipsoids of revolution giving rise to the birefringence; \( (g_1 - g_2) \) is an optical anisotropy factor depending on the refractive index of the solvent, \( n_s \), and on the principal refractive indices, \( n_1 \) and \( n_2 \), of the ellipsoidal particles; \( f (\alpha, a/b) \) is a complicated orientation factor. For values of \( \alpha < 1.5 \),

\[
f(\alpha, \frac{a}{b}) = \frac{\alpha}{15} \left( \frac{a^2 - \frac{a}{b}^2}{a^2 + b^2} \right) \left[ 1 - \frac{a^2}{12} \left( 1 + \frac{b^2}{3} \right) \frac{a^2 - \frac{a}{b}^2}{a^2 + b^2} + \cdots \right]
\]

The optical anisotropy factor has the form,

\[
(g_1 - g_2) = \frac{1}{4\pi n_s^3} \frac{n_s^3(n_1^2 - n_s^2) + \epsilon (n_1^2 - n_s^2)(n_2^2 - n_s^2)}\left[ \frac{n_1^2 + 2n_s^2}{3n_s^2} - 2\epsilon \frac{n_1^2 - n_s^2}{3n_s^2} \right] \left[ \frac{n_2^2 + 2n_s^2}{3n_s^2} + \epsilon \frac{n_2^2 - n_s^2}{3n_s^2} \right]
\]

The factor \( \epsilon \) is a function of the axial ratio of the ellipsoids. For long rods, \( \epsilon = 0.5 \); for spheres, \( \epsilon = 0 \); for flattened discs, \( \epsilon = -1 \).

4. **Polydispersity**

Sadron (66) has investigated the effect of polydispersity of lengths on the extinction angle, and on the
birefringence. He derived the following relationships,

\[
\tan 2\chi = \frac{\sum_{i=1}^{n} (n_e - n_o)_i \sin 2\chi_i}{\sum_{i=1}^{n} (n_e - n_o)_i \cos 2\chi_i}
\]

\[
(n_e - n_o)^2 = \left[ \sum_{i=1}^{n} (n_e - n_o)_i \sin 2\chi_i \right]^2 + \left[ \sum_{i=1}^{n} (n_e - n_o)_i \cos 2\chi_i \right]^2
\]

Here \(\chi\) and \((n_e - n_o)\) are the extinction angle and the birefringence of the flowing solution, respectively, containing I solute components, each with its characteristic diffusion constant; \((n_e - n_o)_i\) is the birefringence produced by the \(i\)th component alone; \(\chi_i\) is the extinction angle produced by the \(i\)th component alone.

C. Application of Flow Birefringence to Proteins

Flow birefringence has been used to a limited extent in studying pure liquids such as benzene and octyl alcohol. Most of the applications of this technique, however, have not been with liquids of small molecular size but rather with high molecular weight substances in solvents showing negligible orientation, in comparison to that of the solute. Some representative materials which have been investigated are polymethacrylate, polystyrene, nitrocellulose, pectins, viscose, amylose, and rubber. The method has been used to determine the length of protein molecules in the undenatured state, as for example, myosin (49, 50) tobacco mosaic virus (46), fibrinogen (27), zein (31), gelatin (26), Helix hemocyanin (70), and horse-antibody...
globulin (70). The theory of flow birefringence, as well as its application to proteins has been excellently reviewed by Edsall (23, 24).

There are only a few reports in the literature dealing with flow birefringence of native albumins, or of denatured albumins. Native ovalbumin, in aqueous solution, was shown by Boehm and Signer (8) not to produce birefringence of flow, presumably because the molecule is too short. Similar results were obtained by Joly and Barbu (37) using horse-serum albumin. Edsall and Foster (25) were able to measure the length, 190-200 A, of native human, and bovine-serum albumin by making measurements in concentrated glycerol solutions.

There is a lesser amount of published work dealing with the changes in flow birefringence resulting from heating, or chemical treatment, and much of it was done using the muscle protein, myosin. Von Muralt and Edsall (49) found that the flow birefringence of myosin solutions can be made to disappear upon addition of urea, iodides, or thiocyanates, and this change was interpreted in terms of a breakdown of elongated particles into fragments too small to be oriented. Likewise, other compounds not ordinarily classed as potent denaturing agents were shown by Edsall and Mehl (29) to be effective in destroying the flow birefringence of myosin solutions.

Fredericq (32) measured the dimensions of ovalbumin
particles resulting from denaturation at 100° for 30 minutes in 0.4 to 1.2 N hydrochloric acid solution, and obtained lengths of 900 to 2300 Å. Ovalbumin denatured at room temperature with the anionic detergent, dodecyl benzene sodium sulfonate, gave lengths of 1900 to 2400 Å. The magnitude of the birefringence was lower in the solutions containing detergent, and so a rather high concentration of protein, 2.5 to 5 per cent, was used. A lower concentration, up to 1.6 per cent, was employed in the solutions denatured with hydrochloric acid. Fredericq's solutions appear to have been highly aggregated, in general, and the lengths given should be interpreted as belonging to unfolded, but aggregated, ovalbumin molecules, rather than to unfolded, molecularly dispersed polypeptide chains.

During the course of this experimental work, an investigation of the flow birefringence of thermally denatured horse-serum albumin by Joly and Barbu (37) was reported. Some of their experimental results are qualitatively similar to those obtained with ovalbumin described in this dissertation.

Joly and Barbu obtained lengths of 350-4000 Å depending upon the conditions of denaturation. Their experimental conditions did not permit measurements of particles less than 350 Å in length. For a given protein concentration, pH, and time of heating, length was found to increase with temperature of heating. For a given protein concentration,
pH, and temperature of heating, length was found to increase with time of heating. Lengths increased in the pH range 6.7 to 8.0. Hardly any birefringence was apparent in solutions which had been heated 10 minutes at pH 0.6 to 3.5, or 10 minutes in the pH range 8.4 to 10.6. Since their measurements were made in aqueous solutions, as were those of Fredericq (32), it was necessary to use rather high protein concentrations, as high as 5 per cent, in many of their experiments.

Joly and Barbu interpret their data, not in terms of unfolding of polypeptide chains, but rather in terms of sub-microscopic filaments produced by the unsymmetrical aggregation of denatured protein molecules by means of ionic and van der Waal's type bonds. They assume that the protein molecules become perturbed, as a result of thermal agitation, and groupings are thus exposed which are available for subsequent intermolecular bonding.
III. BIREFRINGENCE OF FLOW MEASUREMENTS

A. Description of Apparatus

Figure 1 is an over-all view of the apparatus used. A 100 watt low pressure mercury vapor lamp (General Electric Co., AH-4), the source of illumination, is housed in a water-cooled shield. The light beam is collimated by means of a slit and a cylindrical lens between which is a green filter serving as a monochromatizer. The light passes through a Polaroid disc, of the quality used in polarizing microscopes, and enters the solution, contained between the cylinders, by way of the bottom window and emerges through the top window. Both windows were annealed of all but a trace of birefringence before assembling, and are set into mountings which are intended to keep them free of strain. The upper optical assembly holds a removable quarter-wave plate, rotatable through approximately 90°, and a Polaroid analyzer, of the same quality as the polarizer, mounted on a circular scale graduated in degrees. A system of worm and bevel gears permits rotation of the analyzer simultaneously with the polarizer, or independently of it. After leaving the analyzer, the light beam passes to the observer by way of a telescope focussed on the upper window.
Figure 1. Birefringence of flow apparatus.
Figure 2. Concentric cylinder assembly.
Figure 2 shows the parts of the concentric cylinder assembly, consisting of an outer cylinder which is jacketed for circulation of thermostated water, an inner cylinder, and a top plate which, when in place, is separated from the outer cylinder by a paper gasket. All of the metal parts are of stainless-steel. The base-plate of the outer cylinder assembly is detachable, but ordinarily is not removed except for cleaning between runs on solutions of different character. Annular grooves and projections in the upper plate, and in the top portion of the inner cylinder, form an air lock which prevents liquid from entering the bearing.

The diameter of the inner cylinder is 6.20 cm.; length of the inner cylinder is 6.50 cm.; length of path in the liquid, from the top of the base plate to the bottom of the upper plate, is 6.75 cm. The gap between the cylinders is 0.099 cm. The velocity gradient in sec.\(^{-1}\), for the apparatus is equal to 3.40 times the speed in revolutions per minute. The critical speed, above which turbulence develops, is given by \((7000) (\eta)/\rho\), where \(\eta\) is the viscosity of the solution in poises, and \(\rho\) is its density.

A standard Ward-Leonard circuit is used to control the speed of the inner cylinder. The motor is coupled to the inner cylinder by means of a flexible drive and the speed of rotation is measured with a strobotron. Much of the construction is patterned after the equipment of Edsall and co-workers (28) and details of construction can be
found in their publication.

The gap width selected, 0.099 cm., is a compromise. A narrower width would be more suitable, from the standpoint of increasing the turbulence limit and so permitting higher gradients to be employed, and less suitable, from the standpoint of increasing the error due to reflections of the incident beam from the sides of the cylinders.

Polaroids were found to be superior to conventional Nicol prisms, in the optical assembly. They are easier to mount, have excellent extinction properties, and do not cause a shift in direction of the emergent beam, as Nicol prisms often do. The latter is advantageous in reducing reflection errors, and in otherwise producing superior images.

B. Method of Making Measurements

1. Orientation angle

The space between the concentric cylinders was filled from below with the deaerated sample with particular care being taken to avoid entrapment of air bubbles. The inner cylinder was revolved slowly to facilitate attainment of thermal equilibrium, which required about twenty minutes time, after which minor adjustments of the cylindrical lens were made, if necessary, to improve the image. All of the runs were performed at 25.0°C ± 0.3°C.
The orientation angle was found as follows: With the inner cylinder at rest, the Polaroids were accurately crossed and locked so they could be rotated simultaneously. With the inner cylinder revolving at a definite, predetermined speed, the Polaroids were rotated until a minimum in light intensity appeared. There are four such minima in a complete revolution of the locked Polaroids; they are associated with two particular directions in the flowing solution. These two directions correspond to the maximum and the minimum refractive indices in the flowing solution, which in turn are associated with the average position of the major and minor axes of the solute particles, assumed to be ellipsoids of revolution.

Similar measurements were made at the same speed of rotation, but with the cylinder revolving in the opposite sense. An average value of $\chi$ was obtained from the 8 readings of the minima (4 in each sense of rotation) in the following manner: Arithmetical differences were taken of corresponding positions of the minima for rotation in the two senses, the average halved and subtracted from 45° to get an average value of $\chi$. The process was repeated for several speeds of rotation, and thus a range in velocity gradient was covered.

This method of determining $\chi$ is of particular advantage when solutions of low birefringence are studied. With such solutions it was found, presumably because of reflections
from the side walls of the cylinders, that $\chi$ calculated using a particular minimum in the two senses of rotation may be several degrees different from that obtained using the minimum at right angles. The procedure used in this dissertation of measuring all four minima in both senses of rotation leads to more accurate values of $\chi$ than are found using only one minimum in both senses of rotation. The method of measuring all of the minima generally has not been employed, judging from the construction of the apparatus described in the literature, since the analyzer and polarizer were usually not designed to be rotatable through much more than 90° when locked together.

2. Birefringence

Birefringence measurements, using a quarter-wave plate, were made in the manner of Senarmont. Birefringence of the flowing solution is given by the expression,

$$n_e - n_o = \frac{\Delta \lambda}{180 \, \delta}.$$

Here $\Delta$ is the phase angle whose determination will be described, $\lambda$ is the wavelength of the incident light in vacuo, and $\delta$ is the length of the liquid path traversed. For the apparatus used in this dissertation,

$$n_e - n_o = 4.50 \times 10^{-8} \, \Delta$$

The phase angle, $\Delta$, was found as follows: $\chi$ values were examined over the entire range in speeds, for rotation
in a given sense. An average was taken, for a particular set of minima, if the range in \( \chi \) were not more than \( 10^\circ \). The coupled, crossed Polaroids were set at 45\(^\circ\) plus the above average, and uncoupled. A quarter-wave plate was inserted below the analyser, rotated until minimum intensity was observed, and then left in this position. A check was made by rotating the analyser independently of the polarizer and verifying the original setting of the locked Polaroids. Thereafter, in determining \( \Delta \), only the analyser was rotated, the quarter-wave plate and the polarizer remaining fixed. When the range in \( \chi \) was greater than \( 10^\circ \), more than one setting of the quarter-wave plate was necessary.

When the inner cylinder is rotated, the solution becomes anisotropic, and a measure of the anisotropy is obtained from, \( \Delta \), the number of degrees the analyser must be rotated to obtain again a minimum in light intensity. Measurements of \( \Delta \) were made over the same range in velocity gradients as were used in measuring \( \chi \). In effect, the method treats the flowing solution as though it were a uniaxial spherocystal of birefringence \( n_e - n_o \).

C. Calculations

1. Length of solute particles

Lengths of solute particles were calculated from the experimental values of \( \chi \), determined at various velocity
gradients, using the data of Scheraga, Edsall, and Gadd (67), and the Perrin (56) equation relating rotary diffusion constant to the dimensions of the particle. From the tables given by Scheraga, Edsall, and Gadd a plot was made of \( \chi \) as a function of \( \alpha \) for \( \rho \) equal to 50. Here \( \alpha = \frac{a}{b} \); \( \rho = a/b \); \( a \) is the gradient; \( \theta \) is the rotary diffusion constant; \( a \) is the semi-major axis; and \( b \) is the semi-minor axis of the ellipsoid of revolution.

This plot allows evaluation of values of \( \alpha \) to be made from experimental values of \( \chi \). These values of \( \alpha \), in turn, are required for the determination of lengths through use of the Perrin equation described on page 22. This equation, assuming \( \rho = 50 \) and with the length expressed in Angstrom units can be written as,

\[
1^{3} = 538 \times 10^{-18} \left( \frac{G \eta}{\alpha T} \right)^{-1} 
\]

or

\[
1^{3} = 538 \times 10^{-18} \left( \frac{\theta \eta}{T} \right)^{-1}
\]

Here \( \eta \) is the viscosity of the solvent in poises, and \( T \) is the absolute temperature. Accordingly, a plot was made of \( 1 \) as a function of \( (\frac{G \eta}{\alpha T}) \) from this relationship, and this plot was used in determining lengths from values of \( \alpha \) derived from the experimental values of \( \chi \).

2. The function \( \Delta / f \) c

The magnitude of the birefringence of the flowing solution, at a given value of \( \chi \), depends upon the extent
of orientation of the solute particles, as well as upon their concentration and their intrinsic birefringence.

To correct for the extent of orientation, a plot was first made of the orientation factor, $f$, as a function of $\alpha$ (for $\rho = 50$) from the tables of Scheraga, Edsall, and Gadd (67). For the experimental values of $\alpha$ corresponding values of $f$ were taken from this graph, and values of $\Delta/fc$ calculated. The latter quantities are shown on the curves of length as a function of $\varepsilon \eta$, or of pH.

In the expression $\Delta/fc$, $c$ is arbitrarily taken as equal to the total concentration of protein in gms. per 100 ml. of solution. It is recognized that not all of the protein present necessarily is contributing significantly to the birefringence. The amount of protein contributing to the birefringence can not easily be determined.

The magnitude of $\Delta/fc$, thus, is proportional to the birefringence which would be produced, at unit concentration, if the solute particles were completely oriented. Over a range in gradients, $\Delta/fc$ should be constant if the solute consists of rigid particles, homogeneous with respect to length, ellipsoidal in shape, and showing no interaction during flow birefringence measurements. Lack of constancy of the function is taken as an indication of polydispersity in a given sample. Changes in magnitude of the function would be difficult to interpret quantitatively, for different solutions, since the effects of
aggregation are not obvious, and likewise the amount of denaturation may vary from sample to sample.
IV. PREPARATION OF SAMPLES FOR FLOW BIREFRINGENCE MEASUREMENTS

Dilute aqueous solutions of ovalbumin are too fluid for adequate flow birefringence measurements in the apparatus used. Turbulence, with its quantitatively unpredictable effects on orientation angle and birefringence, sets in before a sufficiently high gradient can be attained to orient the solute particles satisfactorily. Consequently, it was necessary to augment the viscosity of the protein solution. Glycerol was chosen for this purpose since it is optically inactive, chemically neutral, of small molecular size, and compatible with ovalbumin.

Two procedures were used for preparing the samples. In the first procedure denaturation was brought about in aqueous solution. Three-times crystallized ovalbumin was dissolved in water, or in buffer solution, and the chemical agent added. The latter was, for example, a dilute solution of hydrochloric acid, sodium hydroxide, or detergent. The solution contained in a test tube was heated, with manual stirring for the first two minutes, in a constant temperature bath, after which it was diluted with 95 per cent glycerol to a final glycerol content of 70.0 per cent. It was filtered through a fritted glass filter of coarse texture to remove fragments of floating denatured ovalbumin, and centrifuged at 20,000 times gravity for several minutes.
to remove traces of suspended debris and many of the air bubbles. Finally, the sample was deaerated in a pear-shaped separatory funnel, with the aid of a water aspirator, and was then ready to be placed in the birefringence of flow apparatus. This technique was used in obtaining data for Figures 3-18 inclusive.

In the second procedure denaturation was brought about in the presence of glycerol. Three-time crystallized ovalbumin was dissolved in water, 95 per cent glycerol added and the solution mixed by inverting it in a stoppered test tube. The chemical reagent, such as dilute hydrochloric acid solution, was added carefully without mixing with the more dense glycerol solution of ovalbumin, the test tube stoppered, tilted to permit the reagent to flow over a larger area of glycerol solution, and then rapidly inverted to mix the contents. Solution compositions were arranged so that the final composition contained 85.0 per cent glycerol. The solution was heated, with manual stirring for the first three minutes, in a constant temperature bath, cooled, filtered, centrifuged, and deaerated as in the first procedure. This procedure was used in obtaining the data of Figures 19-36 inclusive. Time was measured from the instant the test tube was immersed in the constant temperature bath, in both methods. About two minutes were required for the aqueous solutions to come to bath temperature, and about three minutes for the glycerol solutions.
The pH values given throughout the dissertation were taken immediately after completion of the flow birefringence measurements.

The second procedure was developed when it became clear from the experimental work that aggregation of the denatured protein would be the greatest obstacle in obtaining correct lengths of the unfolded molecule. Denaturation was induced in ovalbumin dissolved in glycerol solution and the protein concentration, during denaturation, was thus advantageously lowered. The high viscosity of the glycerol solution, during denaturation, aided in reducing aggregation; there is some evidence which suggests that glycerol retards aggregation for other reasons also. In these solutions, a minimal ionic strength was employed, to lessen aggregation, by using sodium hydroxide or hydrochloric acid solutions to produce the desired pH, rather than by the use of standard buffer solutions.
V. EXPERIMENTAL RESULTS AND DISCUSSION

A. Heat Denaturation in Aqueous Solution

1. Denaturation in the pH range 1.0 - 4.0

Figure 3 summarizes heat denaturation in aqueous solution at various pH values from 1.0 to 4.0 (obtained with glycine-hydrochloric acid buffers). The solutions were heated, cooled, and diluted with glycerol to 70 percent glycerol content before measuring the extinction angle and the birefringence. Each length, at a given pH, was calculated from $\lambda$ measured at $G \eta / T$ equal to 2.3 (gradient of 3500 sec.$^{-1}$). This gradient is about the highest obtainable in such solutions without danger of turbulence. All of the solutions used in obtaining data for Figure 3 as well as all the others investigated throughout the dissertation were free of visible precipitation even after addition of glycerol.

The data demonstrate the dependence of apparent length upon hydrogen ion concentration during denaturation. The lengths shown are not necessarily those of unaggregated, unfolded molecules. For example, the length of around 1600 A at pH 4.0 is longer than that of a maximally extended single polypeptide chain from protein of molecular
<table>
<thead>
<tr>
<th>Component</th>
<th>Denaturation</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin (µg/100 µl)</td>
<td>0.50 %</td>
<td>3.19 %</td>
</tr>
<tr>
<td>Glyceral (weight percentage)</td>
<td>0 %</td>
<td>70 %</td>
</tr>
<tr>
<td>Temperature</td>
<td>100°C</td>
<td></td>
</tr>
<tr>
<td>G η/T</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Denaturation in the pH range 1.0-4.0.
weight 45,000. Still greater lengths can be obtained, simply by heating for longer periods of time. Aggregation, surely, must be present under such conditions.

2. Denaturation at pH 2.3; varying time, 0.6 per cent protein

Figure 4 represents a time study of denaturation at pH 2.3, which pH is in the region giving minimum lengths, (see Figure 3) and hence may possibly also be a region of minimum aggregation. The numbers below the circles are values of the function, $\Delta / f_0$, described on page 36; lack of constancy over a substantial range in velocity gradient indicates heterogeneity in lengths of solute particles.

It is evident that the greatest change in length occurs in the first 5 minutes of heating. The progressively enhanced deviation from constancy of length (over a several-fold increase in gradient), with increasing time of heating, is caused by increasing polydispersity.

The presence of polydispersity could be explained in several ways, two of which will be outlined here. (1) Rapid unfolding of the molecules to about 600 A is followed by relatively slow aggregation to give particles of various lengths. At the same time, further slow unfolding of the free, or aggregated, extended molecules could produce lengths of individual extended molecules which are longer than 600 A. (2) Rapid unfolding to about 600 A lengths occurs, with no
subsequent aggregation. Length of the unfolded molecules increases with increasing heating time. Polydispersity is the consequence of uneven unfolding of molecules, or of the presence of hydrolysis products, of diverse sizes, mixed with the unfolded molecules. Experimental results, to be described throughout the dissertation, generally support the first explanation rather than the second.

Considering Figure 4 again, all of the samples, except the 5 minute one, exhibit decreasing length with increasing gradient. This is best explained, as a first approximation, as resulting from polydispersity only.

The slight upward trend in the 5 minute sample probably is not significant. Double refraction of a flowing solution is smallest at low gradients, and under these conditions the error in $\kappa$, and therefore, in length which is calculated from $\kappa$ is greatest due to spurious birefringence introduced by reflections of incident light from the walls of the apparatus. With the apparatus used $\kappa$ is invariably raised by these reflections, and so the calculated length is lowered, and $\Delta/f c$ raised.

Mixtures of ethyl cinnamate and mineral oil were used to estimate the error in $\kappa$ due to reflections. Such solutions were weakly birefringent and gave values of 46.1° instead of the expected 45°. Thus, for weakly birefringent protein solutions, $\kappa$ values in the neighborhood of 45° could certainly be 1° too high.
Figure 4. Denaturation at pH 2.3; varying time, 0.6% protein.
Figure 5. Birefringence of samples of Figure 4.
Some idea of the $\Delta$ values involved can be seen in Figure 5, from which the $\Delta/f$ c values of Figure 4 were calculated. In most cases, the error in $\Delta$ is probably of not much consequence for $\Delta$ measurements of more than 5°.

3. Denaturation at pH 2.7: varying time, 2.4 per cent protein

The concentration of ovalbumin present during denaturation is an important variable influencing the length, as can be seen in Figure 6 in which the initial concentration is four times as great as that of Figure 4. The 5 minute sample at the higher concentration is more heterogeneous, and longer, than the corresponding one at the lower concentration. After 240 minutes of heating, the lengths found at low gradients are greater than 1600 A. These lengths are longer than would be expected from single, maximally extended ovalbumin chains (approximately 1400 A, assuming a polypeptide spacing of 7.5 A), in spite of any hydrolysis which occurred as a result of prolonged heating. Actually, there were particles even longer than 1600 A present, as evidenced by orientation which could be induced in some of the solutions simply by rotating the inner cylinder manually through several degrees.

The solutions were intensely birefringent. Comparison of Figures 7 and 5 illustrates the much greater $\Delta$ values obtained at the higher concentration.
Figure 6. Denaturation at pH 2.3; varying time, 2.4% protein.
Figure 7. Birefringence of samples of Figure 6.
The results are most realistically explained as resulting from greater aggregation in the solutions of higher protein concentration during denaturation. An alternate hypothesis, that the unfolding process is very much concentration dependent, does not seem promising. At the same time, the question of whether unfolding to greater lengths is taking place concurrently with aggregation would be very difficult, if not impossible, to answer by any experimental techniques now available.

4. Interaction study at pH 2.3

It was assumed, in deriving the theory of flow orientation, that the solute particles act entirely independently of each other during orientation. To establish the validity of this assumption for the ovalbumin solutions being investigated, an aqueous solution was denatured and then diluted with buffer solution to various concentrations of ovalbumin before addition of glycerol.

Figure 8 shows the outcome of this test. Inasmuch as the lengths found, at the various dilutions, are quite similar, interaction must be negligible. This conclusion is supported by birefringence measurements; there is an almost linear relationship between $\Delta$ and ovalbumin concentration. It seems certain that the effects of interaction during flow birefringence measurements are unimportant compared to those of concentration during denaturation.
Length, Å

1800

1600

1400

1200

1000

800

600

400


0 0.4 0.8 1.2 1.6 2.0 2.4

Figure 8. Interaction study at pH 2.3.
5. Denaturation at pH 1.2; varying protein concentration

Denaturation conditions were sought which would lead to lengths interpretable in terms of unfolding, uncomplicated by aggregation. Since low concentrations of protein, a condition for minimum aggregation, yield correspondingly low birefringence and, therefore, high reflection errors, it was hoped that high concentrations of protein could be employed.

The results of a concentration study at pH 1.2 are shown in Figure 9. The denaturation process clearly is concentration dependent, and polydispersity is prominent, even at the lowest concentration. Comparison with a corresponding sample of Figure 4, at pH 2.3, shows much greater lengths at the lower pH.

These long lengths would not have been anticipated, on the basis of net charge on the protein molecule. The protein should have its maximum charge in this pH region, and it would be expected that the molecules would repel each other and aggregate to a minimal extent as a result of intermolecular repulsion, and at the same time, unfold to a maximal extent as a result of intramolecular repulsion. Near the isoelectric point, however, lack of charge on the molecule without doubt enhances aggregation.
Figure 9. Denaturation at pH 1.2; varying protein concentration.
B. Heat and Detergent Denaturation in Aqueous Solution

1. Denaturation at pH 2.2; varying time, detergent present

Increasing the net charge on the molecules of denatured protein, particularly through binding of detergent ions, appeared promising as a possible aid in reducing aggregation. Some cationic detergents for instance are firmly bound by ovalbumin, without causing precipitation, even at pH values below the isoelectric point, and thereby increase its positive charge. Zephyran is such a detergent. It is an alkyl dimethyl benzyl ammonium chloride, with the alkyl group containing 8-14 carbon atoms.

Figures 10 and 11 represent heat denaturation of ovalbumin for varying time, with different detergent-protein ratios, at pH 2.2. These solutions were sparkling clear after heating, and this exceptional clarity suggested that the solutions might show less heterogeneity than usual. The lengths and birefringence in Figure 10, with the lower detergent-protein ratio, are about the same as those of Figure 4, with no detergent. The samples containing detergent appear slightly more homogeneous, however, than the ones without detergent. The samples containing higher detergent ratio, Figure 11, show evidence of distinctly less aggregation. The birefringence of the 5 minute sample of Figure 11 was too weak to measure, throughout most of
Figure 10. Denaturation at pH 2.2; varying time, detergent present.
Ovalbumin (gm./100 ml.)
- Denaturation Final
  2.5%  0.19%
Glycerol (weight percentage)
- Denaturation Final
  0 %  70%
Zephiran/ovalbumin 0.32
Temperature 1000 C.
pH (glycine buffer) 2.2

Figure 11. Denaturation at pH 2.2; varying time, detergent present.
the range of velocity gradient, and so the two points given may be too low, because of reflection error.

Again, in both of these samples, the length of about 600 Å seems significant. This length is about the same as that obtained in the absence of detergent, and this suggests that the over-all effect of the detergent on length is due principally to intermolecular repulsion, tending to reduce aggregation between molecules.

The function \( \Delta/f \) is decidedly of lesser magnitude in Figure 11. Since addition of the relatively small quantity of detergent would not affect the refractive index of the solvent much, it seems likely that the lowering of \( \Delta/f \) is due to changes in the principal refractive indices of the solute particles (see page 69).
2. Denaturation at pH 2.2: effect of detergent

The experiments of Figure 12 were designed to determine the effect of Zephiran on denatured ovalbumin. One sample was a 0.60 per cent solution of ovalbumin heated for 60 minutes before dilution with glycerol. The other was a 0.75 per cent solution of ovalbumin heated for 45 minutes, after which a 5 per cent solution of Zephiran was added and the heating continued for 15 minutes more before dilution with glycerol. The final protein concentration was the same in both samples. A long heating time was selected to insure thorough denaturation.

Detergent is bound by the denatured ovalbumin, as judged by the much lower birefringence and $\Delta f / c$ values. Lengths for the two samples, nevertheless, are approximately equal. The slightly higher lengths for the sample containing detergent might be due to its having been heated for 45 minutes at a higher concentration than the other.

Samples containing detergent during the entire period of heating gave distinctly lower lengths than those of Figure 12, and gave $\Delta f / c$ values lower than those of the sample of Figure 12 containing no detergent (data not shown). This could mean that detergent is bound at the start of denaturation, and lessens aggregation between molecules by augmenting their charge. Detergent added after the
Ovalbumin (g/100 ml.)

<table>
<thead>
<tr>
<th>Denaturation</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60</td>
<td>0.19</td>
</tr>
<tr>
<td>0.75</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Glycerol (weight percentage)

<table>
<thead>
<tr>
<th>Denaturation</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>70 %</td>
</tr>
</tbody>
</table>

Zephirin/Ovalbumin | 1.2 %
Temperature       | 1.00 °C
Time              | 60 minutes
40 minutes; detergent added;
Heated 15 minutes
pH (glycine buffer) | 2.2

* Contains no detergent
** Contains detergent

Figure 12. Denaturation at pH 2.2; effect of detergent.
ovalbumin has been denatured to a large extent is also bound, as is evident from Figure 12, but does not cause the already formed aggregates to disaggregate.

3. Denaturation at pH 2.3: varying protein concentration

A concentration study, at pH 2.3 using the same detergent-protein ratio, 0.45, throughout, is represented by Figure 13. A short heating time of 5½ minutes was selected. It was hoped that unfolding would be substantially completed, and aggregation not a complication, under these conditions.

The dependency of length on concentration again indicates aggregation is present. Nevertheless, the sample containing 1.20 per cent ovalbumin during denaturation has about the same length as does a 0.60 per cent sample without detergent, which implies that aggregation has been impeded noticeably by the Zephiran. The slight downward trend of lengths in the 1.20 per cent sample indicates that some polydispersity is present, and suggests that the unaggregated, and unfolded molecule is somewhat shorter than 600 Å; perhaps 500 Å would be a fair estimate of the length. A sample containing 0.60 per cent ovalbumin and having the same detergent-protein ratio as the above samples had very weak birefringence, and so reliable readings could not be made on it.
CJvalbumin (gm./100 ml.)

Denaturation Final
1.80 % 0.27 %
1.20 % 0.38 %

Glycerol (weight percentage)

Denaturation Final
0 % 70 %

Zephiran/ovalbumin 0.45

Temperature 100° C.

Time 5½ Minutes

pH (glycine buffer) 2.3

---

Figure 13. Denaturation at pH 2.3; varying protein concentration.
C. Heat and Urea Denaturation

1. Denaturation at 37°: varying time, protein concentration, and pH

Denaturation of proteins by urea has been considered quite extensively in the literature. Some of the evidence, derived from ultracentrifugal and osmotic pressure measurements, has been interpreted to mean that ovalbumin in concentrated aqueous solution unfolds to give a monodisperse solution of solute particles. Urea, accordingly, appeared to be an important chemical agent to use in studying denaturation by means of flow birefringence.

Figure 14 gives the lengths resulting from denaturation for relatively long periods of time at 37° in 6.9 M urea having a pH 7.5 (phosphate buffer). These concentrated urea solutions were diluted with glycerol, before making the orientation runs, giving a final glycerol content of 70 per cent considering only the glycerol and water.

Pronounced polydispersity is apparent in all of the samples. Likewise, a concentration study at the same pH and temperature reveals much heterogeneity, as is clear from Figure 15. Evidently, the experimental conditions selected were not conducive to the production of homogeneous solutions.
Figure 14. Urea denaturation at 37° C.; varying time.
Figure 15. Urea denaturation at 37°C; varying protein concentration.
Figure 16. Urea denaturation at 37°C; varying pH.
It was observed that solutions possessing a higher pH than those used here, as well as those made using veronal buffer instead of phosphate buffer, developed less haziness on standing. Accordingly, ovalbumin was denatured at pH 7.0, and at pH 9.3, at 37°C. Figure 16 shows that conspicuously shorter lengths are produced at the higher pH, and this most likely represents less aggregation, since extensive hydrolysis, which also could produce short lengths, is not anticipated at this pH and temperature. Again, the first experimental point of the curve at pH 9.3 was known to be too low, because of reflection error, and so emphasis was placed on the next two points. Upon which much more reliance may be placed.

2. Denaturation at pH 8.0; varying urea concentration

Figures 14, 15, and 16 are concerned with denaturation at 37°C, in urea solution, for relatively long periods of time. Figure 17, in contrast, deals with denaturation at 100°C, for 5 1/2 minutes in, (a) undiluted veronal buffer, (b) diluted veronal buffer, and (c) undiluted veronal buffer 2.0 M, 3.0 M, and 4.0 M in urea.

Clearly, the concentration of buffer ions influences the lengths. They are shortest at the lower concentration of buffer; this is in accord with the frequently observed effect of ionic strength of salts on the precipitation of protein in heated solution.
Figure 17. Urea denaturation at 37° C.; varying urea concentration.
Increasing concentrations of urea reduce the lengths, in spite of the fact that the effectiveness of urea as a denaturing agent rises with its concentration, as judged by viscosity rise and sulphydryl group liberation. This is interpreted to mean that the action of urea results principally in restraining the formation of aggregates. Unfortunately, it was not possible to employ higher concentrations of urea than 4.0 M, since the birefringence was already very weak at this concentration.

Lowering of the birefringence of ovalbumin solutions in the presence of high concentrations of urea probably has a different origin than that produced by relatively much lower concentrations of detergent. In the latter case, binding of the detergent to the ovalbumin may result in a complex whose double refraction is less than that of the denatured ovalbumin. This could be explained easily, if it were assumed that the alkyl residues of the detergent molecules interact laterally with the side-chains of the amino acid residues of the partially extended ovalbumin molecules, thereby increasing the refractive index in a direction perpendicular to the length of the polypeptide chain and, thus, reducing the birefringence. With concentrated urea solutions of ovalbumin, weak birefringence could very likely be due more to an unfavorable increase in refractive index of the solvent, rather than to complex formation.
3. **Denaturation at pH 2.6; varying protein concentration**

Heat denaturation led to minimal lengths at pH in the region 2-3 (see Figure 3), and it was of interest to know whether the presence of urea would cause further extension of the heat denatured molecule in this pH range. Figure 18 shows the results of heat denaturation at pH 2.6, with 2.0 M urea in glycine buffer, using two concentrations of ovalbumin.

A comparison of the sample of Figure 4, containing 0.60 per cent ovalbumin during denaturation, with a corresponding sample of Figure 18, containing 2.0 M urea, shows the lengths to be approximately the same. This similarity suggests once more that the length around 600 A may be that of an unfolded, unaggregated polypeptide chain. This length appears significant, in view of its frequent appearance as an apparently limiting length, under diverse conditions of denaturation in aqueous solution. An implication is that once the bonds holding the protein molecule together in its native configuration are broken, the molecule extends to about 500 or 600 A in length regardless of the charge on the molecule, and regardless of whether the denaturing agent is heat or some chemical agent such as urea.
Figure 18. Urea denaturation at pH 2.6; varying protein concentration.
D. Heat Denaturation in 85 Per Cent Glycerol Solution

1. Denaturation in the pH range 0.73-11.0

The second procedure of page 40, namely, that of denaturation in 85 per cent glycerol solution rather than in aqueous solution, was employed in preparing samples for Figures 19-36, inclusive. Advantages of this method over the first procedure in minimizing aggregation are enumerated on page 41.

Figure 19 summarizes the length data for denaturation of ovalbumin, in 85 per cent glycerol, over the pH range 0.73-11.0. Magnitude of $\Delta/f_c$ and of $\Delta$ (in parentheses) is shown, except in the neighborhood of pH around 2. The values of $\Delta/f_c$ are omitted for this region, since they are not reliable, principally because of the uncertainty in $f$, which is a function of $\chi$, which, in turn, is not reliable because of reflection errors. Inasmuch as ethyl cinnamate-mineral oil mixtures which should have given a $\chi$ of 45° gave a $\chi$ of 46.1°, the experimental values of $\chi$ around 45° in the protein solutions were arbitrarily corrected by subtracting 1° from them, before proceeding to calculate lengths. These lengths are the least reliable of any shown, and very probably still are too low.

An excellent correlation exists between the shape of the curve in Figure 19, below pH 4, and that of Figure 3 (where denaturation was brought about in aqueous solution).
Figure 19. Denaturation in the pH range 0.73-11.0.
It must be remembered that the data of Figure 19 are not to be accepted as a representation of limiting lengths of the denatured ovalbumin molecule, since the effect of several variables such as pH, time, concentration, and ionic strength on the relative rates of the unfolding, aggregation, and, in some cases, hydrolytic reaction are merged together.

The extended lengths found in the neighborhood of the isoelectric point (about 4.6 - in water) seem reasonable, as the result of intermolecular interaction by virtue of van der Waal forces. Decreasing lengths with increasing (or with decreasing) pH would be anticipated on the basis of lessened aggregation due to the presence of increasing negative (or positive) charge on the solute particles. There is no hint of increasing lengths due to intramolecular repulsion, as a result of increasing positive (or negative) charge—except at very low pH. The long lengths obtained here can be attributed, instead, to increased ionic strength of the solution. The protein may already have attained its maximum positive charge around pH 2 and additional hydrochloric acid, needed to lower the pH, would then only attenuate aggregation. In support of this idea, it was found that an ovalbumin solution at pH 2.0 and containing sodium chloride sufficient to make the ionic strength similar to that of a hydrochloric acid solution of pH 0.73 became highly aggregated when heated for 15
minutes at 100° C., and was too viscous for flow-orientation measurements.

Thuman and co-workers (71) in a study of the foaming properties of ovalbumin, interestingly enough, obtained a curve rather similar to that of Figure 19, except that they found a rise in foaming properties at very high pH. A corresponding rise in lengths was not seen in Figure 19. Even at pH values 11.5-12, very short lengths were found and only a trace of birefringence.

The possible effect of charge on aggregation is worthy of examination. Considering only the magnitude of the net charge of the denatured ovalbumin molecule, and disregarding the binding of ions other than hydrogen, there is reason to expect less aggregation at high pH than at low pH. At a pH around 1, the maximum positive charge amounts to 37 cationic residues per molecule. The net positive charge is also 37, assuming that carboxyl, hydroxyl, and sulfhydryl groups are unionized.

The total negative charge is not as certain at pH around 10, for example, principally because of the uncertainty in the number of amide groups hydrolysed during heating. There are 48 free carboxyl groups, and 31 potentially free carboxyl groups as amides. Tyrosine and cysteine residues could contribute 12 more negative.
charges. Thus, the total negative charge could be as high as 91 per molecule, and the net negative charge 77 per molecule, assuming that the guanidyl groups have not lost their charge. With none of the amide hydrolysed, the net negative charge would be 46 per molecule. This possible value of 77 net negative charges around pH 10 is considerably greater than the net positive charge of 37 around pH 1, and could explain, in part, the lesser amount of aggregation found at high values of pH.

2. Denaturation at pH 0.93: varying protein concentration

A concentration study over a large range in concentration seemed to be a necessary step in clarifying the denaturation problem at low pH. The results of such a study are presented in Figure 20. Here, denaturation was carried out at pH 0.93 with over a ten-fold variation in ovalbumin concentration.

The very pronounced dependence of length on concentration is strong evidence of polydispersity. Even at the lowest concentration, 0.049 per cent, which is very much less than has been used in most of the other experiments since at this low concentration birefringence usually is too weak to permit measurement, there is much heterogeneity as seen from the lack of constancy of length with increasing gradient. It seems very likely, then, that the lengths given for this low pH region, in
Figure 20. Denaturation at pH 0.93; varying protein concentration.
Figures 20 and 19, are not those of individual molecules.

3. Interaction study at pH 1.1

It was desirable to ascertain to what extent inter-
action of solute particles during flow orientation measure-
ments has deceptively altered the lengths. To this end,
an investigation of interaction was made at pH 1.1. An
85.0 per cent glycerol solution containing ovalbumin and
hydrochloric acid was heated. Flow birefringence measure-
ments were made on this solution, as well as on two others
made from it by dilution with an 85.0 per cent glycerol
solution containing only hydrochloric acid.

According to Figure 21, interaction is of no practical
consequence even in the most concentrated solution studied.
Interaction almost unquestionably can safely be disregarded,
in analyzing the data of this dissertation.

4. Denaturation at pH 0.93; varying time and temperature

Previous experiments have proved that denaturation is
rapid; most of the reaction occurs in the first few minutes
of heating at 100°C. It seems logical that, by varying the
experimental conditions, limiting conditions could be
found which are relatively favorable for unfolding, and
relatively unfavorable for aggregation.

Denaturation was, therefore, effected at 25°, 50°,
60°, and 70° C. as shown in Figures 22-26, inclusive. This
Figure 21. Interaction study at pH 1.1.
Figure 22. Denaturation at pH 0.93; varying time at 50° C.
Figure 23. Denaturation at pH 0.93; varying time at 60° C.
Figure 24. Denaturation at pH 0.93; varying time at 70° C.
Figure 25. Denaturation at pH 0.93; varying time at 25°C.
Figure 26. Denaturation at pH 0.93; varying time and temperature.
time-temperature data is summarized in Figure 26.

Increased time of heating, and increased temperature produce increased polydispersity. Five minutes heating at 50° C. gives what appears to be a homogeneous system, and the 500 A length is most reasonably interpreted as belonging to unfolded, essentially unaggregated, polypeptide chains.

At 25° C., denaturation proceeds much more slowly than at the higher temperatures. After 2½ hours, a limiting length of about 450 A becomes prominent. The function, $\Delta f_c$ is constant, within experimental error, for this sample, and it is problematical to decide, by comparison with the 5 minute sample at 50°C., whether the 50 A difference between them is significant. The birefringence was reasonably high in the 1.0 hour sample at 25°C., and although the left end of the curve should in reality be displaced upward somewhat, the essential constancy of length with increasing gradient could signify the presence of unfolded, unaggregated molecules of slightly shorter length than are present in solution after 2½ hours. There might, thus, be evidence here for an increase in length of an unfolded molecule, with no aggregation apparent.

5. Theoretical distribution of lengths

Superposition of the 60 minute sample at 50°, the 15 minute sample at 60°, and the 5 minute sample at 70° from
Figures 22, 27, and 24, respectively, shows the lengths to be nearly alike, and the function $\Delta f c$ as well. All of the samples are, no doubt, aggregated. The good correlation suggests that the distribution of lengths is about the same, and that the aggregation is of the same type in the various samples.

Most logically, interaction between extended polypeptide chains would be expected to lead to lateral aggregation, since many polar and non-polar groups distributed along the chain are capable of interacting. A head-to-tail aggregation involving bonding at a single point does not seem likely, and neither does aggregation to form a large three dimensional net-like structure.

Several distributions of solute particles could, of course, describe the polydispersity of the 60 minute sample of Figure 22. In order to reduce the amount of calculation involved in curve fitting, graphs were made of $\chi$ as a function of $G \eta / T$ in 20 per cent intervals of solute particles 500 A long with particles 700 A, 900 A, 1000 A, and 1100 A long. The best fit occurs for 80 and 75 per cent 500 A particles mixed with 20 and 25 per cent, respectively, of 1000 A. These data were calculated by the methods of page 24 and are shown in Figure 27. They do not claim to represent the actual distribution, but do give an idea of a specific distribution which fits the experimental data.
Figure 27. Theoretical distribution of lengths; sample of Figure 22.
Figure 28. Theoretical birefringence; sample of Figure 22.
In Figure 28 is shown the theoretical birefringence, for the same distributions, as well as the experimentally obtained birefringence for the 60 minute sample of Figure 22. The ordinate is in arbitrary units. The experimental curve was placed so as to lie near the calculated curves for easy comparison. In these calculations of theoretical birefringence, as well as in those of length distribution, it was assumed that the birefringence of completely oriented 1000 A particles is identical to that of completely oriented 500 A particles. Again, the birefringence curves are not given with the idea of proving that a specific distribution is present, but rather only to show that decreasing values of $\Delta/fc$ can be explained in terms of polydispersity.

6. Denaturation in the pH range 9.5-10; varying time and concentration

Denaturation of ovalbumin at high pH, for example around pH 10, is less complicated by aggregation than it is at a low pH, for instance around pH 1. It appeared an interesting region to study, inasmuch as it still was hoped that experimental conditions could be found whereby high concentrations of ovalbumin could be employed without danger either of aggregation or of interaction. Figure 29 summarizes the data for a concentration study at pH 9.5-10.

The individual curves (not shown) of length as a
function of $G \eta / T$, from which Figure 29 was composed, each show evidence of polydispersity, even at 5 minutes heating time, and so some aggregation presumably is present in all of the samples. The lengths for the 5 minute samples of Figure 29 are, thus, somewhat longer than would be expected for the unaggregated molecule.

There is an unexpected trend, in Figure 29, of decreasing length with increasing concentration. In all previous concentration studies, at pH values below the isoelectric point, increasing concentrations during denaturation resulted in increasing lengths. It is conceivable, of course, that unfolding might be inhibited by interaction between partially unfolded molecules. However, this explanation seems unlikely, in view of the almost negligible interaction found during flow orientation in Figure 21. As a crude approximation, Figure 29 can be interpreted to mean that the denaturation is essentially independent of concentration at these high pH values.

The data of Figure 29 were collected in a scattered fashion, and so it is not likely that some of the curves are entirely displaced due to some consistent error in measurement or in technique of preparing samples. Some unavoidable variations may account for some of the discrepancies, however, in a not obvious manner. For example, there was a marked lowering in pH, on heating these samples, which amounted to about 0.7 pH units. A faint odor of
Figure 29. Denaturation at pH 9.5-10; varying time and concentration.
ammonia noticed during heating suggests that some of the change is due to hydrolysis of amides. To keep the final pH about the same in the samples, the one containing 0.60 per cent ovalbumin contained 25 per cent more sodium hydroxide than did the other two samples, and so the ionic strength is slightly higher in this sample.

7. Denaturation at pH 10.25: varying time and temperature

The promising results of Figure 29 made it desirable to denature at a slightly higher pH to take possible further advantage of increased charge in reducing aggregation. Accordingly, a temperature study was made at pH 10.25 at temperatures of 70°, 80°, 90°, and 100° C. The results are shown in Figures 30, 31, 32, and are summarized in Figure 33.

Polydispersity appears at lengths of 500-600 A; the limiting length is around 450 A. Using constancy of length as a criterion, there is less aggregation in these samples than in most others described in this dissertation. If the 90° sample truly is unaggregated throughout the first 15 minutes of heating, then the rate of unfolding is being measured, uncomplicated by aggregation. Denaturation is slow at 70°; only the 60 minute sample had measurable birefringence.

The $\Delta/f$ c of 790 for the 5 minute sample at 80° is undoubtedly too high because of reflection errors. It
Figure 30. Denaturation at pH 10.25; varying time at 80° C.
Figure 31. Denaturation at pH 10.25; varying time at 90° C.
Figure 32. Denaturation at pH 10.25; varying time at 100°C.
Figure 53. Denaturation at pH 10.25; varying time and temperature.
It was obtained using the $f$ value calculated from the corresponding experimental $\chi$ value of 44.1°. At a lower value of $\eta / T$, 2.9, $\chi$ was 45.6°, which exceeds the theoretical limit by more than 0.6°. Assuming that the correct value for $\Delta / f c$, at $\eta / T$ equal to 4.2, is 310 instead of 790 gives an $f$ value of 0.0287, and a corresponding $\chi$ of 43.0°. The length calculated from this is 375 A, rather than the 295 A shown, and it would seem to be closer to the true length than is 295 A.

8. Denaturation at pH 2.5; varying time

Heat denaturation, in 85 per cent glycerol, in the pH region 2-3 is of special interest. Distinctly shorter lengths, than are found in any other pH region studied, are found here. Figures 34 and 35 represent heat denaturation at pH 2.45 and 2.55, and ovalbumin concentrations of 0.59 and 0.79 per cent, respectively. Since the lengths are short, the birefringence is low, because of relatively poor orientation, and it was necessary to use higher concentrations than usual.

Polydispersity becomes noticeable at lengths of 300-400 A, which implies that the unaggregated, unfolded polypeptide chain may be of even shorter length. The function $\Delta / f c$ has rather high values for these samples. Previously, as in Figure 30, certain extraordinarily high values of $\Delta / f c$ were interpreted as a
Figure 34. Denaturation at pH 2.45; varying time.
Figure 75. Denaturation at pH 2.55; varying time.
consequence of reflection errors. However, in the 5 minute sample of Figure 35, the magnitude of $\Delta$ ranged from $5^\circ$, at $G \eta /T$ of 2.8, to $15^\circ$ at $G \eta /T$ of 8.4. Even the lower value of $5^\circ$ is usually high enough to preclude significant reflection error in the apparatus used. The spread in readings of minimum intensity for the two senses of rotation likewise is very small and this, too, is indicative of negligible reflection error.

The high $\Delta/\eta c$ values can be interpreted in several ways. For example, since the molecules under consideration are only approximately 300 A long, and so are only partially extended, a high intrinsic birefringence would not have been anticipated unless possibly the glycerol in an unexplained way forms a complex, of high intrinsic birefringence, with the protein. There is some evidence in the literature of the solvent properties of polyols on ovalbumin, and so complex formation with glycerol is not entirely implausible. Another explanation for high $\Delta/\eta c$ values, invoking amount of denaturation, implies that with the 300 A lengths a much higher percentage of denaturation is present than occurs, for example, with the 500 A lengths found in some of the other pH regions studied. This explanation does not seem too likely.
E. Heat and Detergent Denaturation in 85 Per Cent Glycerol Solution

1. Denaturation at pH 10.25; varying time, detergent present

Previously, it was shown that the cationic detergent, Zephiran, influences heat denaturation of ovalbumin in aqueous solution. There are good indications that detergent cation is bound to the denatured ovalbumin thereby reducing aggregation, presumably by increasing the net positive charge on the protein. In an analogous manner, the anionic detergent, dodecylbenzene sodium sulfonate, is bound in spite of unfavorable conditions of high pH. It was desirable to learn how such detergent binding affects heat denaturation in 85 per cent glycerol solution. Of course, binding of anionic detergent would take place even more completely at low pH, but such combination usually leads to precipitation which it is imperative to avoid in flow-orientation measurements.

Figure 36 shows heat denaturation at pH about 10.5 and a detergent-protein ratio of 1.0. The lengths obtained are uniform, and perhaps slightly shorter than those found in Figure 32 for denaturation at pH 10.25 in the absence of detergent. Even after 60 minutes of heating the solution still appears homogeneous, insofar as constancy of length is a test; this is unusual. (In the 5 minute sample, the line was arbitrarily drawn through
Figure 36. Denaturation at pH 10.25; varying time, detergent present.
the final point. It was assumed to be the most accurate; the other three, certainly, are too low.

The $\Delta f_c$ values in Figure 56 are lowered as a consequence of the presence of detergent, just as they are at low pH in solutions containing Zephiran. Inasmuch as the detergents reputedly are powerful denaturing agents even at low concentrations of detergent, this is taken to mean that $\Delta$ is less for a given length rather than that denaturation is less complete in the presence of detergent.

F. Heat Denaturation in the Presence of Guanidine Salts; Thioglycollic Acid; and Formaldehyde

1. Guanidine salts

Guanidine hydrochloride, and guanidine thiocyanate were tried as denaturing agents. These compounds are of particular interest, inasmuch as they have been reported to be potent sulfhydryl liberators, and so presumably also cause extensive unfolding. Neither was found to be suitable for denaturing, preparatory to flow orientation measurements, because of the tendency of ovalbumin solutions containing them to become cloudy on standing, and particularly on heating.

2. Thioglycollic acid

Heat denaturation at pH 2.2 in aqueous solution
containing thioglycollic acid gave highly aggregated solutions containing long solute particles. This pH is in the region giving minimum lengths, as seen in Figure 3. Thioglycollic acid is of interest, since it has the ability to reduce disulfide linkages. Such reduction could possibly lead to longer lengths of the unfolded molecule if these linkages were a barrier to extension.

The aggregating action of the thioglycollic acid would seem to be most reasonably explained on the basis of decreased positive charge on the protein due to binding of the negatively charged thioglycollate anion.

3. Formaldehyde

Combination of ovalbumin with detergent, at suitable pH, was shown to lead to lessened aggregation. The flow birefringence of such complexes, however, is distinctly lower than that of the protein alone. Other compounds were sought which would increase the net charge on the protein, and still not lower the birefringence significantly.

Formaldehyde seemed to be a good choice, since as the result of its action on the amino groups of the protein the negative charge is increased by one unit for each formaldehyde molecule which reacts. At the same time the formaldehyde, after reaction, would not be expected to appreciably change the birefringence.
VI. GENERAL DISCUSSION

Some additional comment is in order, by way of comparison with the work of this dissertation, on the investigations of Fredericq (32), and of Joly and Barbu (37) which are discussed in the Literature Survey.

Fredericq calculated lengths by application of a limiting equation,

$$\lim_{\gamma \to 0} \frac{45^\circ - \kappa}{6\gamma} = \frac{1}{12\theta}$$

and the Perrin equation. Thus, for a given sample upon which measurements of \( \kappa \) were made, only the value of \( \kappa \) obtained by extrapolation to zero gradient was used in calculating the length.

This procedure suffers from the disadvantage that the presence of polydispersity can not be detected. For example, one of the samples containing ovalbumin and Nacconal was reported by Fredericq to have a length of 2400 A. However, application of the solutions of Scheraga, Edsall, and Gadd for the equations of Peterlin and Stuart, and the Perrin equation, assuming an axial ratio of 50 in each case, results in a length of 3600 A at the lowest gradient and 2300 A at the highest.

This variation of calculated length from 3600 A to 2300 A would be considered as evidence of considerable
polydispersity in this dissertation, and not much reliance would be placed even in the 2300 A value. For example, in the 60 minute sample of Figure 22, the length varies from 850 A to 700 A over the range in gradient employed. This variation, at first consideration, would not seem to be extreme in view of the complexity of the material being investigated. However, the experimental data can be rather well matched by assuming the solute consists of 80 per cent particles 500 A long and 20 per cent particles 1000 A long. Thus, the preponderance of solute particles in the solution might easily be of length 500 A rather than of length 700 A. For such reasons greater emphasis has been placed on solutions containing solute particles which appear to be homogeneous in length than on those showing evidence of polydispersity.

Joly and Harbu used the relationship of Edsall, Foster, and Scheinberg (27) and the Kuhn (40) formula. The latter is,

$$\theta = \frac{8 \kappa T}{\pi \eta \lambda^3}$$

The former relationship gives values of $\alpha$ corresponding closely to those of Scheraga and co-workers in the range $\lambda = 45^\circ$ to $25^\circ$. (It was set up assuming that Boeder's three-dimensional case is valid for values of $\alpha$ below 2, and his two-dimensional case is valid for values of $\alpha$ above 20). The Kuhn equation gives values of length about 15 per cent higher than those obtained with the Perrin
equation, assuming an axial ratio of 50.

Joly and Barbu did not include enough of their original data to permit calculation of lengths at various gradients to test for polydispersity. They assumed that the amount of polydispersity was negligible, and that the lengths measured were those of solute particles occurring with maximum frequency. These assumptions probably are not justified, but do not alter the conclusions they have drawn from their experiments.

Polydispersity, in the denatured protein solutions, may come about as the result of aggregation, or of hydrolysis. There is reason to believe that aggregation is of more importance in the solutions studied than is hydrolysis. For example, lengths invariably increased (1) with increased protein concentration, (2) with increased time of heating, (3) with increased temperature, (4) with increased ionic strength, and (5) when the pH values were near the isoelectric point during denaturation. Each of these factors would be expected to augment aggregation. The extent of hydrolysis would be expected to be increased by factors (2) and (3), and the others would not be anticipated to be important in hydrolysis. In general, hydrolysis of the ovalbumin molecule would lead to shorter molecules which would have a relatively small influence on the orientation of the unhydrolysed ovalbumin due to the nature of the weighting.
Throughout this dissertation, experimental conditions were sought which would result in solute particles homoge-
neous in length. Homogeneous solutions were produced invariably only under those conditions which logically
would result in the least amount of aggregation, and in
the least amount of hydrolysis. Thus, when using a
relatively low protein concentration, and low ionic strength
during denaturation (both of which would tend to reduce
aggregation) the most homogeneous solutions were obtained
after the shorter periods of heating and at the lower
temperatures when denaturation was carried out at a given
pH. For this reason, it is believed that aggregation, and
hydrolysis, are negligible in these apparently homogeneous
solutions. It would, of course, be very desirable to know
the particle weight of the denatured ovalbumin in solution.
At the present time there is no satisfactory experimental
method for obtaining this information, although light-
scattering techniques offer some hope for success.

Both Fredericq and Joly and Barbu made their flow
birefringence measurements in aqueous solution. Fredericq
used concentrations of 0.4 to 1.6 per cent when denaturation
was effected by heating in acid solution, and concentrations
as high as 5 per cent when denaturation was brought about
by detergents. Joly and Barbu used concentrations mostly
of 2.9 per cent in their experiments. In this dissertation,
much of the denaturation was brought about at a concen-
tration of 0.39 per cent in 85 per cent glycerol solution, and
at 0.6 per cent when denaturation was brought about in aqueous solutions. These lower concentrations are considered advantageous in reducing extent of aggregation.

Fredericq interpreted his data in terms of rather completely unfolded, partly aggregated ovalbumin molecules. Joly and Barbu, on the other hand, interpreted their data in terms of aggregation without invoking the concept of unfolding. They concluded that unfolding does not occur, but that, due to thermal agitation, groups ordinarily positioned in the interior of the molecule are exposed, and these exposed groupings are involved in forming bonds in the aggregate. The data of this dissertation would seem to support a concept involving unfolding to lengths of approximately 350 to 500 Å, followed by aggregation.
SUMMARY AND CONCLUSIONS

1. Isoelectric, native ovalbumin in 70 per cent as well as in 91.5 per cent, glycerol solution showed only a trace of birefringence and no measurable orientation angle.

2. Solutions containing 0.6 to 2.4 per cent ovalbumin in glycine-hydrochloric acid buffers at pH 1 to 4 were denatured by heating at 100°C for periods of time from 5 to 240 minutes. Measurements of orientation angle, and of birefringence, were made at 25°C, at various gradients, after addition of glycerol to a final concentration of 70 per cent. Solutions having solute particles in the range 500 to 600 Å in length appeared homogeneous, and the others polydisperse. Increased lengths were obtained with increased time of heating, and with increased concentration of ovalbumin during denaturation. Aggregation is an important cause of the polydispersity.

3. Heat denaturation at 100°C for 5 minutes at pH 2 in the presence of a cationic detergent, alkyl dimethyl benzyl ammonium chloride, produced apparently homogeneous solute particles around 500 to 600 Å in length.
Heat denaturation in the presence of the detergent resulted in a lesser amount of polydispersity.

4. The magnitude of the birefringence of heat denatured solutions containing the above cationic detergent was distinctly lower than that of ovalbumin denatured in its absence. It is suggested that this is the consequence of a preferential arrangement of the alkyl residues of the detergent molecules perpendicular to the length of the polypeptide chain. Detergent added after heating the ovalbumin solution was also bound by the protein, as judged by the reduction in birefringence, but was not effective in reducing length or polydispersity.

5. Heating for 5½ minutes at 100° in veronal buffer containing urea, 2 to 4 M in concentration, at pH 8 resulted in decreased lengths with increased urea concentration. Flow birefringence measurements are not easily made in solutions of high urea content, because of the unfavorable high refractive index of the solvent. At the limit of the measurements, in 4 M urea solution, solute particles of about 700 Å length were obtained. They were apparently homogeneous in length. At pH 2.6 in glycine-hydrochloric acid buffer, heating 5½ minutes at 100° in 2 M urea solution resulted in solute particles, apparently homogeneous
in length, about 600 A long. The over-all effect of urea in heat denaturation seems to be that of decreasing aggregation.

6. Denaturation was effected in 0.39 per cent ovalbumin solutions containing 85 per cent glycerol and HCl or NaOH to obtain the desired pH in the range 0.73 to 11.0. Lengths were measured after 15 minutes heating at 100°. In the pH range 7.5 to 11.0, lengths decreased from 1100 to 375 A. In the acid range, lengths of about 300 A were obtained at pH values around 2. The lengths rose steeply on either side of this pH value; at pH 0.73 the length was about 1100 A, and at pH 4 about 700 A.

7. Highly aggregated solutions were produced upon heating for 15 minutes at 100° and pH 0.93 in 85 per cent glycerol solution. At a lower temperature, 50°, and a shorter time of heating, 5 minutes, the solute particles appeared homogeneous and about 500 A long.

8. Heat denaturation at pH 2.5 in 85 per cent glycerol solution resulted in shorter lengths, 350 A, than are produced at pH 0.93. These samples contained a higher concentration of ovalbumin, 0.59 per cent, to facilitate measurements.

9. At pH 10.25 heating at 80° in 85 per cent glycerol
solution yielded solute particles, apparently mono-disperse, and approximately 450 Å long. Increasing the temperature to 100°, but otherwise keeping the denaturation conditions the same, gave a limiting length again around 450 Å. At this pH protein concentration during denaturation influenced the length less than at pH 0.93 or at pH 2.5. Aggregation is much less prominent at pH 10.25 than at the other pH values studied.

10. The results of this dissertation have been interpreted on the basis that unfolding of ovalbumin molecules to lengths of 350 to 500 Å occurs during denaturation; the lengths obtained depend on the experimental conditions. Aggregation of the unfolded molecules takes place readily. Interpretation of the data are complicated by aggregation.
VIII. LITERATURE CITED

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IX. APPENDIX

A. Preparation of Samples and Viscosity Measurements

1. Preparation of ovalbumin

The ovalbumin used was prepared from fresh egg white by applying essentially the ammonium sulfate procedure of Sorensen. The ovalbumin was precipitated three to four times with ammonium sulfate at the isoelectric point, dialysed until free of salts, and finally lyophilized. Almost the entire preparatory procedure was carried out at 2°C. in a cold-room; occasionally addition of reagents was made at room temperature to chilled samples, and pressure-filtration was performed, to clarify samples, under the same conditions.

2. Composition of samples for flow orientation measurements

Some compositions of representative samples prepared by the first procedure of page 39 and by the second procedure of page 40 are given in Tables 1, 2, and 3.

3. Viscosity measurements

Viscosity of the solvent at 25°C. was determined using a capillary viscometer. The solvent composition was taken to be that of the final solution excluding protein. It
was found that, in most cases, the solvent viscosity could be approximated sufficiently well by assuming the solvent consisted only of glycerol and water.
Table 1

Composition of samples for heat denaturation in aqueous solution

<table>
<thead>
<tr>
<th>Figure</th>
<th><strong>pH</strong></th>
<th>#Ovalbumin</th>
<th>0.100 M Glycine</th>
<th>0.100 M HCl</th>
<th>4% Aqueous Zephiran</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4.0</td>
<td>0.100 gm</td>
<td>14.5 ml</td>
<td>0.6 ml</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>&quot;</td>
<td>13.5 ml</td>
<td>1.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>&quot;</td>
<td>12.8 ml</td>
<td>2.2 ml</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>&quot;</td>
<td>12.0 ml</td>
<td>3.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>2.2</td>
<td>&quot;</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>&quot;</td>
<td>4.7 ml</td>
<td>11.3 ml</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>&quot;</td>
<td>0 ml</td>
<td>15.0(0.67 M)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>&quot;</td>
<td>0 ml</td>
<td>15.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>2.2</td>
<td>&quot;</td>
<td>7.32 ml</td>
<td>7.32 ml</td>
<td>0.36 ml</td>
</tr>
<tr>
<td>11</td>
<td>2.2</td>
<td>&quot;</td>
<td>7.15 ml</td>
<td>7.15 ml</td>
<td>0.70 ml</td>
</tr>
<tr>
<td>12</td>
<td>2.2</td>
<td>&quot;</td>
<td>6.00 ml</td>
<td>6.00 ml</td>
<td>3.00 ml</td>
</tr>
<tr>
<td>13</td>
<td>2.3</td>
<td>0.300 gm</td>
<td>6.00 ml</td>
<td>6.00 ml</td>
<td>3.00 ml</td>
</tr>
<tr>
<td>13</td>
<td>2.3</td>
<td>0.200 gm</td>
<td>6.50 ml</td>
<td>6.50 ml</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>13</td>
<td>2.3</td>
<td>0.100 gm</td>
<td>7.00 ml</td>
<td>7.00 ml</td>
<td>1.00 ml</td>
</tr>
</tbody>
</table>

*Contains approximately 10% moisture
**After heating and dilution with glycerol
Table 2
Composition of samples for heat and urea denaturation in aqueous solution

<table>
<thead>
<tr>
<th>Figure</th>
<th><strong>pH</strong></th>
<th><strong>Ovalbumin</strong></th>
<th><em><strong>Composition</strong></em></th>
<th>pH</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>14, 15</td>
<td>-</td>
<td>(0.200 gm)</td>
<td>2.50 ml K_2HPO_4</td>
<td>7.5</td>
<td>10.5 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.300 gm)</td>
<td>3.00 ml KH_2PO_4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.400 gm)</td>
<td>9.60 ml H_2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>0.200</td>
<td>8.10 ml Veronal-Sodium</td>
<td>7.0</td>
<td>10.5 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.90 ml HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>0.200</td>
<td>14.20 ml Veronal-913</td>
<td>10.5 gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sodium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.90 ml HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>7.9</td>
<td>0.100</td>
<td>10.70 ml Veronal-Sodium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.30 ml HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>7.7</td>
<td>0.100</td>
<td>5.41 ml Veronal-Sodium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.30 ml HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.21 ml H_2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>8.0</td>
<td>0.100</td>
<td>Veronal-Sodium/HCl = 10.70/4.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urea Conc'n. = 2.0 M, 3.0 M, 4.0 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2.6</td>
<td>{0.100 gm}</td>
<td>Glycine/HCl = 2.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>{0.200 gm}</td>
<td>3/7 Urea Conc'n = 2.0 M</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Contains approximately 10% moisture
**After heating and dilution with glycerol
***K_2HPO_4, KH_2PO_4, HCl, veronal-sodium, and glycine are 0.100 M*
### Table 3

Composition of samples for heat denaturation in 85 per cent glycerol solution

<table>
<thead>
<tr>
<th>Figure</th>
<th>**pH</th>
<th>Ovalbumin</th>
<th>H2O</th>
<th>HCl Volume</th>
<th>HCl Concentration</th>
<th>NaOH Volume</th>
<th>NaOH Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>0.73</td>
<td>0.200 gm</td>
<td>2.40 ml</td>
<td>3.50 ml</td>
<td>0.50 M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>0.93</td>
<td>0.200 gm</td>
<td>3.30 ml</td>
<td>2.60 ml</td>
<td>0.50 M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>1.1</td>
<td>0.200 gm</td>
<td>2.40 ml</td>
<td>3.50 ml</td>
<td>0.25 M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>1.65</td>
<td>0.200 gm</td>
<td>2.00 ml</td>
<td>3.90 ml</td>
<td>0.100 M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>1.75</td>
<td>0.200 gm</td>
<td>2.00 ml</td>
<td>7.90 ml</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>1.95</td>
<td>0.200 gm</td>
<td>3.00 ml</td>
<td>2.90 ml</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>2.25</td>
<td>0.200 gm</td>
<td>2.90 ml</td>
<td>3.00 ml</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>2.25</td>
<td>0.200 gm</td>
<td>7.00 ml</td>
<td>2.90 ml</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>3.1</td>
<td>0.200 gm</td>
<td>4.00 ml</td>
<td>1.90 ml</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>4.05</td>
<td>0.200 gm</td>
<td>5.00 ml</td>
<td>0.90 ml</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>7.65</td>
<td>0.200 gm</td>
<td>5.00 ml</td>
<td>-</td>
<td>-</td>
<td>0.90 ml</td>
<td>0.100 M</td>
</tr>
<tr>
<td>19</td>
<td>7.75</td>
<td>0.200 gm</td>
<td>5.00 ml</td>
<td>-</td>
<td>-</td>
<td>0.90 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>9.35</td>
<td>0.200 gm</td>
<td>4.40 ml</td>
<td>-</td>
<td>-</td>
<td>1.50 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>9.6</td>
<td>0.200 gm</td>
<td>3.90 ml</td>
<td>-</td>
<td>-</td>
<td>2.00 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>9.65</td>
<td>0.200 gm</td>
<td>4.00 ml</td>
<td>-</td>
<td>-</td>
<td>1.90 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>9.7</td>
<td>0.200 gm</td>
<td>3.90 ml</td>
<td>-</td>
<td>-</td>
<td>2.00 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>9.85</td>
<td>0.200 gm</td>
<td>3.50 ml</td>
<td>-</td>
<td>-</td>
<td>2.40 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>9.95</td>
<td>0.200 gm</td>
<td>4.50 ml</td>
<td>-</td>
<td>-</td>
<td>1.75 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>10.15</td>
<td>0.200 gm</td>
<td>2.50 ml</td>
<td>-</td>
<td>-</td>
<td>3.60 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>10.4</td>
<td>0.200 gm</td>
<td>2.00 ml</td>
<td>-</td>
<td>-</td>
<td>2.90 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>10.5</td>
<td>0.200 gm</td>
<td>1.00 ml</td>
<td>-</td>
<td>-</td>
<td>4.90 ml</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>10.95</td>
<td>0.200 gm</td>
<td>1.90 ml</td>
<td>-</td>
<td>-</td>
<td>4.00 ml</td>
<td>0.235 M</td>
</tr>
</tbody>
</table>

*Contains approximately 10% moisture

**After heating
X. ACKNOWLEDGEMENTS

It is a pleasant duty for the author to express here his genuine appreciation to Prof. J. F. Foster, under whose direction the research was conducted. Others who have given assistance in various ways are Prof. R. E. Rundle, under whom research was started on a similar problem, Prof. R. H. Forsythe, Dr. D. Zucker, Dr. J. H. Pazur, and Mr. W. L. Gatch.
At pH 8.5-10, when heat denaturation was effected in aqueous or in glycerol solution in the presence of formaldehyde, very short lengths were obtained. In some cases they were too short to measure. Heat denaturation at pH 8.5-10 with subsequent addition of formaldehyde at room temperature, however, gave lengths about the same as those obtained from heat denaturation in the absence of formaldehyde. At pH around 1, formaldehyde did not appreciably affect heat denaturation.

These data suggest that cross-links are formed by reaction of formaldehyde with protein during heat denaturation at pH 8.5-10, and these bridges inhibit unfolding. Addition of formaldehyde subsequent to heat denaturation probably results in reaction, but without any appreciable disaggregation taking place as a result of increased negative charge, and without further appreciable extension of the already unfolded molecule. At pH around 1, heat denaturation probably precedes any appreciable reaction with formaldehyde, and so unfolding and aggregation are not inhibited.