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

Streaming Orientation Studies on Denatured Proteins. I. Heat Denaturation of Ovalbumin in Acid Media[1]

BY JOSEPH F. FOSTER AND EDWARD G. SAMSA

An investigation has been made of the streaming birefringence behavior of ovalbumin solutions denatured by heating in acid media at 100°. The effects of protein concentration, time of heating and *p*H on the apparent length of the solute particles have been examined. In general the solutions are very heterogeneous with respect to length, as judged by the Peterlin-Stuart theory, and aggregation can be concluded to play an important role. Aggregation appears to be minimal in the *p*H range 2 to 3; in this range solutions have been obtained which appear to be homogeneous and of length about 600 Å.

The term denaturation, as applied to proteins, probably includes a number of more or less closely related changes in physical, and to a lesser extent chemical, properties incited by many different causative agents.[2] It is now generally believed that these changes are manifestations of configurational changes taking place in the polypeptide chains. In the native globular proteins these chains are folded, probably in a very specific manner, and the denaturation process presumably involves an unfolding, or at least an alteration in the nature, of the folded structure.

An increase in axial ratio as a consequence of heat denaturation has been indicated in some cases by increase in intrinsic viscosity. In the case of ovalbumin, Bull[3] found a moderate increase, indicating an axial ratio of 7.4:1, much less than expected for the maximally extended structure. Such results may be seriously complicated by aggregation or by changes in the degree of solvation.

The phenomenon of streaming orientation, usually manifested in the form of birefringence, provides a technique which would seem to be especially well adapted to a study of this problem. This technique affords a means of determining molecular length of sufficiently long molecules without serious complication from uncertainties with regard to molecular weight, axial ratio or degree of solvation. The theory of this method has been reviewed most adequately by Edsall.[4] In the case of native proteins excellent agreement has been attained between molecular lengths determined by this means and those calculated from other physical chemical data.[5] Recently Scheraga, Edsall and Gadd[6] have greatly extended the range of usefulness of the flow orientation theory of Peterlin and Stuart[7] by computing numerical solutions to the flow orientation differential equation which should be valid to relatively high gradients.

Fredericq[8] investigated the streaming birefringence behavior of ovalbumin denaturated by heating in acid media. He concluded unfolding to occur, the unrolled molecules possessing a strong tendency toward aggregation. Joly and Barbu,[9] on the other hand, in a study of the thermal denaturation of horse serum albumin by this technique, have concluded the effect to be a result of aggregation of slightly perturbed, but not unfolded, molecules into filaments. This latter paper was not published until after completion of the experimental work reported in the present paper.

It is proposed in this and the subsequent papers of this series to explore the streaming orientation behavior of some of the better characterized proteins subjected to various denaturation procedures in the hope that some light can be shed on the mechanism of protein denaturation and possibly on protein structure. Ovalbumin has been selected as the first subject of investigation due to its ease of preparation in crystalline form and because it has been the subject of so many denaturation studies by other techniques.

Experimental

Instrument.—The instrument used in this study consists of concentric stainless steel cylinders of mean radius 3.22 cm. with a gap width of 0.099 cm. and has been described previously.[10] In this study polaroids were substituted for the previously used nicol prisms, it having been found that they provide somewhat better extinction and freedom from annoying spurious reflections.

Preparation of Solutions.—The ovalbumin was recrystallized at least three times with $(NH_4)_2SO_4$ by the method of Sørensen, dialyzed free of salt as judged by conductivity, and lyophilized. Weighed samples were dissolved in the buffer used (mixtures of 0.1 *M* glycine and 0.1 *M* hydrochloric acid). In studies without buffer ions, weighed samples were dissolved in water and the calculated amount of dilute acid added. The total volume at this stage was in all cases 15.0 ml. This solution was then heated for the desired length of time in a boiling water-bath, immediately cooled rapidly to room temperature under the water tap and diluted with 42.0 g. of 95% glycerol. This yields a solution containing 70.0% (by weight) glycerol.

Measuring Technique.—The solutions were scrupuously clarified by filtration through a sintered-glass filter to remove any traces of floating debris followed by centrifugation at approximately 20,000 × gravity.[11] The solutions were next degassed by holding under vacuum (water aspirator) for several minutes (evaporation was negligible in this treatment as shown by suitable control) and immediately run into the cylinders.

(1) Journal Paper No. J-1876 of the Iowa Agricultural Experiment Station, Project 978. Supported in part by a grant from Swift and Company. Presented in part before the Division of Biological Chemistry at the Chicago Meeting of the A. C. S., Sept., 1950. Some of the principal results of this and following papers of this series have been summarized briefly by J. F. Foster and E. G. Samsa, *Science*, **112**, 473 (1950).

(2) An excellent review of the voluminous literature in this field has been given by H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(3) H. B. Bull, *J. Biol. Chem.*, **133**, 39 (1940).

(4) J. T. Edsall, *Advances in Colloid Sci.*, **1**, 269 (1942).

(5) (a) J. F. Foster and J. T. Edsall, THIS JOURNAL, **67**, 617 (1945); (b) J. T. Edsall, J. F. Foster and I. H. Scheinberg, *ibid.*, **69**, 2731 (1947); (c) J. T. Edsall and J. F. Foster, *ibid.*, **70**, 1860 (1948).

(6) H. A. Scheraga, J. T. Edsall and J. O. Gadd, Jr., "Double Refraction of Flow and the Dimensions of Large Asymmetrical Molecules," issued by the Computation Laboratories of Harvard University, 1949.

(7) A. Peterlin, *Z. Physik*, **111**, 232 (1938); A. Peterlin and H. A. Stuart, *ibid.*, **112**, 1 (1939).

(8) E. Fredericq, *Bull. soc. chim. Belg.*, **56**, 223 (1947).

(9) M. Joly and E. Barbu, *Bull. soc. chim. biol.*, **31**, 1642 (1949).

(10) J. F. Foster and I. H. Lepow, THIS JOURNAL, **70**, 4160 (1948).

(11) Servall Model SS1 centrifuge, Ivan Sorval Inc.

In early experiments considerable difficulty was encountered with foaming upon subjection of the solutions to the gradient, and anti-foamants such as decyl alcohol were used. It was later found that foaming was not a problem if care was exercised to avoid the presence of air bubbles and never to exceed the critical turbulence limit of the solution so that the anti-foamant was eliminated.

Measurements were made of each of the four positions of minimum intensity. Readings at a given gradient were made in both senses of rotation before proceeding to the next higher gradient. Usually five or six gradients were employed covering the range 180 to 1200 r.p.m. (gradient range 610 to 4100 sec.$^{-1}$). Occasionally checks were made at the lower gradients following the studies at high gradient but no changes were ever observed. Following the extinction angle measurements the magnitude of birefringence over the same gradient range was measured in the usual manner using the Senarmont compensator. The spread in extinction positions over the gradient range was usually small enough that a single setting of the polarizer, analyzer and $\lambda/4$ plate sufficed.

The birefringence is related to Δ, the angular rotation of the analyzer in degrees, by the equation $n_e - n_0 = 4.8 \times 10^{-8} \Delta$. All measurements were made at 25°, the outer cylinder being regulated by water circulating at high rate of flow from a thermostat. A complete set of measurements could usually be completed in less than a half-hour.

Reliability of Measurements.—Table I summarizes the data obtained on a mixture consisting of equal weights of mineral oil and ethyl cinnamate. This mixture was used as a calibrating system and should theoretically have an extinction angle of 45° at all attainable gradients. The value obtained by averaging all 72 readings (36 in each rotational sense) is 46.3°. The birefringence under these conditions corresponded to 2.3° rotation of the analyzer in the Senarmont compensator ($n_e - n_0 = 1.1 \times 10^{-7}$). The discrepancy is supposedly due to reflection errors and can be expected to diminish with increasing degree of birefringence. It should be pointed out that this error is substantially less than that observed earlier using a narrower gap (0.25 mm.).[5a]

TABLE I

OBSERVED EXTINCTION POSITIONS WITH ETHYL CINNAMATE–MINERAL OIL MIXTURE

Each value is the average of nine independent measurements

A Sense	B Sense	χ
49.4 ± 0.48	49.6 ± 0.54	45.2 ± 0.72
134.9 ± .29	140.9 ± .23	48.0 ± .37
229.3 ± .31	228.8 ± .26	44.7 ± .41
316.6 ± .25	321.6 ± .14	47.5 ± .29

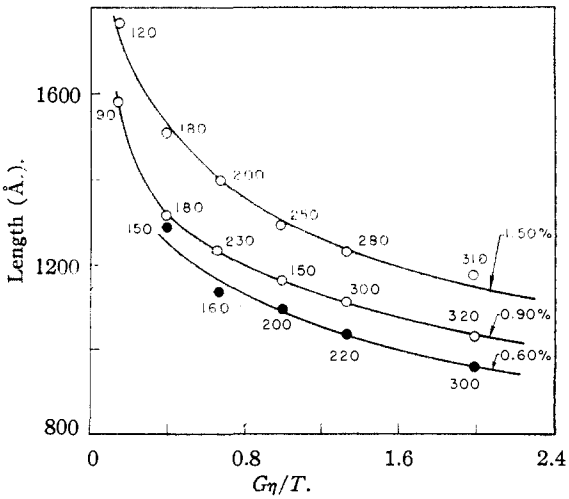

Fig. 1.—Denaturation at 100° and *p*H 1.2 for 10 minutes. Ovalbumin concentration during denaturation: 1.50, 0.90, 0.60; final concentration: 0.47, 0.28, 0.19 g./100 ml., respectively.

Another anomaly is brought out clearly by the data in Table I. It will be noted that the four minima in a given rotational sense do not lie exactly at 90° to one another although alternate minima are 180° apart within the experimental error. In other words the apparent extinction position differs depending on whether it is measured with the polarizer or analyzer in the parallel position. This effect has been regularly observed, not only with the cinnamate but with denatured ovalbumin solutions, when the amount of birefringence is low. The magnitude of the anomaly diminishes with increasing birefringence, usually becoming negligible when Δ is about 5°. It has been noticed that the effect is greatest when χ is near 45°. This effect probably has its origin in reflections from the cylinder walls but no adequate explanation can be given at present. It would appear desirable to measure all four extinction positions in both senses and average the results. This practice has been followed in the authors' laboratory for several years.[10]

No reliance was placed on χ values when the corresponding Δ values were less than 2°. In general Δ was greater than 5° in the experiments reported in this paper.

Results

Measurements on Undenatured Ovalbumin.—No measurable birefringence was observed in solutions containing 0.80% ovalbumin at glycerol concentrations of 70% and even 91.5%.

Effect of Gradient.—Apparent lengths were calculated by means of the S.E.G. tables[6] and the Perrin equation assuming a prolate ellipsoidal structure of axial ratio 50:1. In Figs. 1, 2, 3 and 4 such lengths are plotted as a function of $G\eta/T$ (the velocity gradient in sec.$^{-1}$ times viscosity in poise divided by absolute temperature). In general there is a pronounced drop in apparent length with increasing gradient, as is to be expected from the nature of the weighting in the case of heterogeneous systems.

The numbers written close to each experimental point in these figures are the experimentally measured Δ values divided by protein concentration c(g./100 ml.) and by the magnitude of the orientation factor f corresponding to the observed extinction position (f is determined from χ by means of the S.E.G. tables). This quantity, Δ/fc, may be considered as an apparent intrinsic birefringence and for a homogeneous system of rigid molecules should be independent of gradient. In general there is a pronounced increase in this quantity with increasing gradient, a result which can be accounted for, at least qualitatively, on the basis of heterogeneity.

Effect of Protein Concentration.—In Fig. 1 are given results, at three different protein concentrations, on ovalbumin solutions heated 10 minutes at 100° and *p*H 1.2. There is seen to be an appreciable increase in length with concentration. Also a comparison of Figs. 2 and 3 at comparable times indicates an appreciable concentration effect at *p*H 2.3 in glycine buffer. The results in Fig. 4 were obtained on a single solution denatured at 2.4% concentration but diluted to various concentrations for streaming birefringence measurements. The concentration effect is seen to be very small in this case showing that it is the concentration at the denaturation stage which is important.

Effect of Time of Heating.—Figures 2 and 3 show results for samples heated at 100° and *p*H 2.3 for various lengths of time at two protein concentrations. There is a pronounced increase in length with time of heating and this is true at

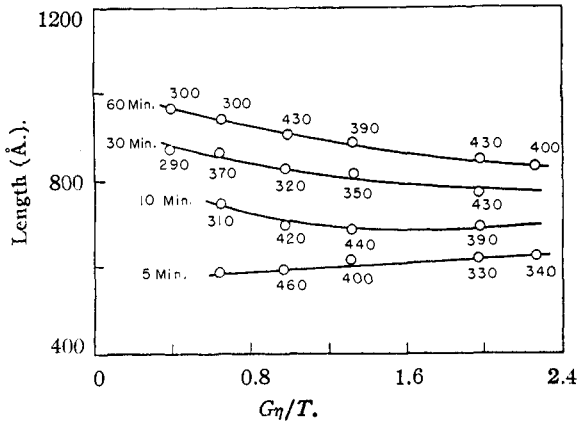

Fig. 2.—Denaturation at 100° and *p*H 2.3. Ovalbumin concentration during denaturation: 0.60; final: 0.19 g./100 ml.

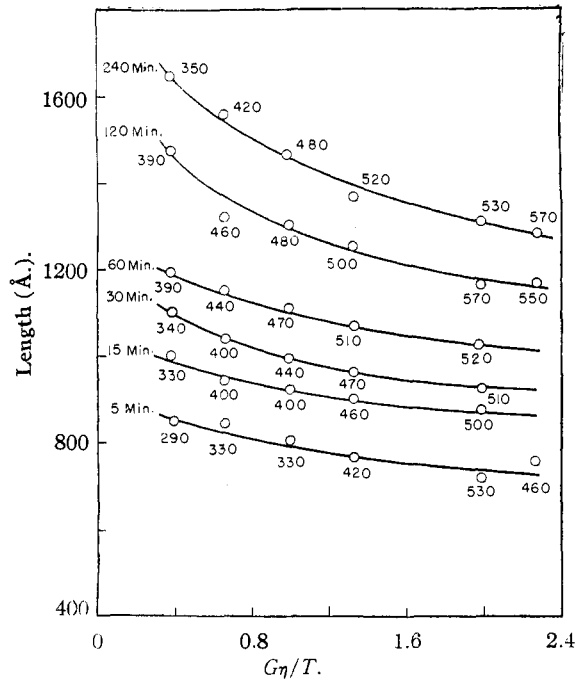

Fig. 3.—Denaturation at 100° and *p*H 2.3. Ovalbumin concentration during denaturation: 2.40; final: 0.21 g./100 ml.

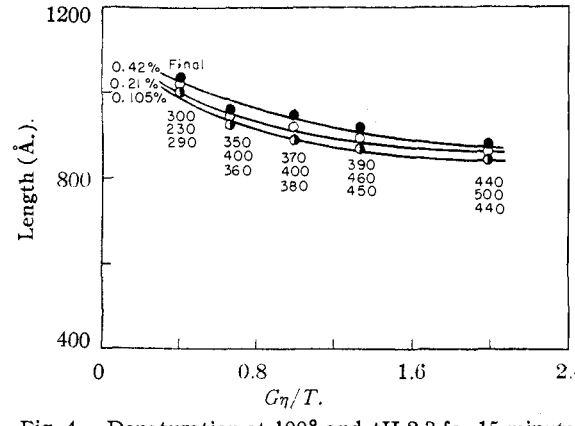

Fig. 4.—Denaturation at 100° and *p*H 2.3 for 15 minutes. Ovalbumin concentration during denaturation: 2.40; final: 0.42, 0.21, 0.105 g./100 ml.

all *p*H values studied. In general the apparent length calculated at a given gradient under a given set of conditions increases approximately linearly with the logarithm of the time of heating between 5 and 240 minutes.

Effect of *p*H.—In Fig. 5 are presented results at a single gradient on samples denatured at various values of *p*H, other conditions being held constant except for ionic strength. The *p*H was obtained by means of buffers composed of mixtures of 0.1 *M* glycine and 0.1 *M* HCl solutions above 1.3, HCl alone below this *p*H. There is a pronounced minimum in apparent length in the *p*H range 2–3.

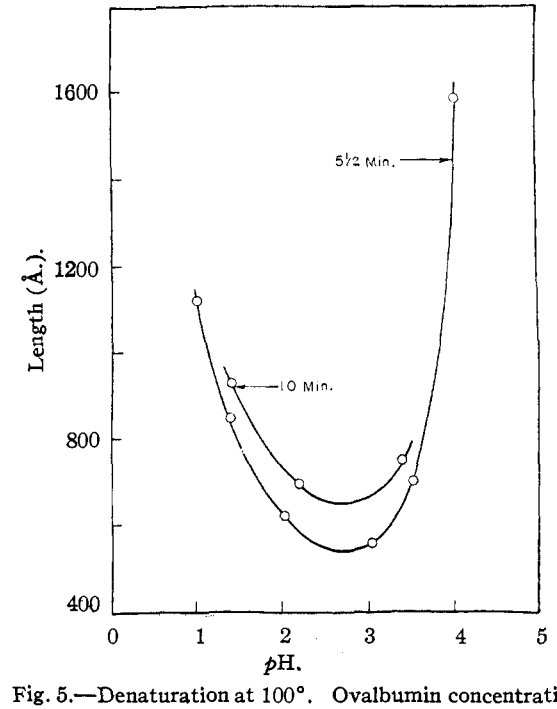

Fig. 5.—Denaturation at 100°. Ovalbumin concentration during denaturation: 0.60; final: 0.19 g./100 ml. Measurements at $G\eta/T = 2.3$.

Discussion

The early experiments at very low *p*H were carried out by way of repeating the experiments of Fredericq[8] which were conducted in 0.04, 0.08 and 1.2 *N* HCl. The results under comparable denaturing conditions were in reasonable agreement with his in spite of the fact that the present measurements were conducted on solutions diluted with glycerol to 70% while his measurements were made on the aqueous solutions. However, it seems doubtful if the lengths have any particular significance since the apparent lengths vary so widely over the gradient range studied. Fredericq reported only an average length, in any given case, obtained by extrapolation of the data to zero gradient and application of the limiting form of the orientation equation. Recalculation of some of his data using the S.E.G. table indicates that his solutions, too, were very polydisperse.

The molecular weight of ovalbumin corresponds to approximately 400 amino acid residues. Since the spacing per amino acid residue in a fully stretched polypeptide chain is about 3.5 Å. it can be concluded that lengths above 1400 Å. are not

possible on a molecular basis. It is doubtful that lengths even approaching this value would be obtained in solution in the absence of aggregation. Lengths greater than this are seen in Figs. 1 and 2, and much greater lengths can be obtained by prolonging the heating time or by increasing the concentration. From the shape of the length–gradient curves it must be concluded, too, that there are present even much longer particles since the calculated values are weighted averages. These long particles must be aggregates.

The importance of aggregation in the acid and heat denaturation of ovalbumin has been demonstrated by MacPherson, Heidelberger and Moore.[12] It should be emphasized that the conditions they used may have been conducive to aggregation since their samples were purified by isoelectric precipitation. Particular care was taken in the present investigation to avoid experimental conditions leading to precipitation or even to obvious turbidity.

(12) C. F. C. MacPherson and N. Heidelberger, THIS JOURNAL, **67**, 574 (1945); C. F. C. MacPherson, M. Heidelberger and D. H. Moore, *ibid.*, **67**, 578 (1945).

Whether aggregation is responsible for the whole effect, as was concluded by Joly and Barbu in the case of horse serum albumin,[9] or is only a secondary factor cannot be clearly answered from these studies. The increase in length as the isoelectric point is approached is undoubtedly due to aggregation. The increase below *p*H 2 can also be explained on the basis of an increase in aggregation resulting from the increased ionic strength. In the *p*H range 2–3 aggregation is evidently at a minimum but may not be absent.

Under suitable experimental conditions solutions can be obtained which appear to be homogeneous on the basis of the Peterlin–Stuart theory. This situation is approached in the 5 and 10 minute heated samples at *p*H 2.3 and the lower protein concentration (Fig. 2). It seems somewhat significant that solutions showing this ideal behavior have invariably been found to have lengths in the neighborhood of 600 Å. It appears possible that this value may have a molecular significance.

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Streaming Orientation Studies on Denatured Proteins. II. Heat Denaturation in 85% Aqueous Glycerol[1]

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An investigation has been made of the streaming birefringence of ovalbumin denatured in 85% aqueous glycerol solution by heating under a variety of conditions. Highly elongated particles are obtained in the vicinity of the isoelectric point and at *p*H values of 1 and below. A minimum is found near *p*H 2. Systems which apparently were homogeneous, as judged by independence of the calculated length and also the apparent intrinsic birefringence on the velocity gradient, were obtained after 5 minutes heating at 50° at *p*H 0.93 (500 Å.), 5 minutes heating at 100° at *p*H 2.5 (350 Å.) and 5 minutes heating at 80° at *p*H 10.3 (350–450 Å.). In general, lengths were found to increase with increasing time of heating and with increase in the concentration of protein present at the time of heating. At *p*H 10 the apparent lengths did not increase with increasing protein concentration, indicating that under these conditions aggregation plays a negligible role and unfolding actually is taking place.

The previous investigation in this series was concerned with the heat denaturation of ovalbumin.[2] Denaturation was brought about in aqueous solutions, following which glycerol was added to give a final concentration of 70% prior to flow orientation measurements. Some evidence for an unfolded ovalbumin unit of about 600 Å. length was presented but it was shown that aggregation seriously complicates interpretation of the data, particularly in samples denaturated at higher protein concentration and ionic strength.

The dilution with glycerol, which is necessary to achieve adequate orientation in these systems, makes it necessary to denature at somewhat higher protein concentrations than would be desirable. In this paper are presented results in which the following modifications of procedure have been made: (1) denaturation was carried out directly in 85% aqueous glycerol media and (2) *p*H adjustment was made entirely with HCl or NaOH to minimize the ionic strength of the systems.

(1) Journal Paper Number J-1877 of the Iowa Agricultural Experiment Station, Project 978. Supported in part by a grant from Swift and Company. Presented before the Division of Biological Chemistry at the Chicago Meeting of the American Chemical Society, Sept., 1950.

(2) J. F. Foster and E. G. Samsa, THIS JOURNAL, **73**, 2187 (1951).

Experimental

Three times crystallized ovalbumin (ammonium sulfate precipitation) was dissolved in water in a test-tube, 95% glycerol was added, and the solution mixed by inverting the stoppered tube. Dilute hydrochloric acid or sodium hydroxide solution, sufficient in amount to give the desired *p*H after heating, was carefully added so as not to mix with the glycerol solution. The test-tube was then stoppered and rapidly inverted to mix the contents. The final glycerol content was 85% in all cases.

The solutions were heated in a constant temperature bath with stirring, after which they were cooled, filtered through a coarse fritted-glass filter (to remove traces of floating debris), centrifuged at 20,000 times gravity for 10 minutes, deaerated with a water-aspirator and flow orientation measurements made at 25° using the apparatus and technique previously described.[2]

Lengths of the solute particles, as well as the function Δ/fc, were calculated as before,[1] using the data of Scheraga, Edsall and Gadd[3] relating χ and f to α (referred to as S.E.G. tables).

Results and Discussion

Effect of *p*H.—An over-all view of the dependence of apparent length on *p*H is presented in Fig. 1. In this study denaturation was effected

(3) H. A. Scherega, J. T. Edsall and J. O. Gadd, Jr., "Double Refraction of Flow and the Dimensions of Large Asymmetrical Molecules," Issued by the Computation Laboratories of Harvard University, 1949.