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

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

BY EDWARD G. SAMSA AND JOSEPH F. FOSTER

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.

by heating at 100° for 15 minutes. Measurements were made over a range in gradient of 610 sec.$^{-1}$ to 3000 sec.$^{-1}$. The lengths shown are calculated from the data obtained at the highest gradient ($G\eta/T$ = 8.4). The numbers near the circles represent the function Δ/fc and Δ (in parentheses). In the *p*H range 2 to 3, Δ/fc values were not considered reliable and so are not shown. It should be mentioned that isoelectric, native ovalbumin (0.8% in 91.5% glycerol solution) showed no detectable birefringence of flow.[2]

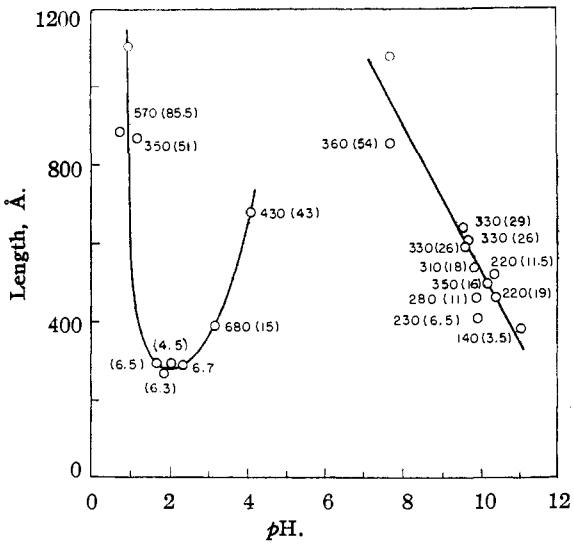

Fig. 1.—Denaturation at 100° for 15 minutes. Ovalbumin concentration: 0.39 g./100 ml.; measurements at $G\eta/T$ = 8.4.

The lengths shown can be interpreted to be those of solute particles composed of ovalbumin molecules which are partially unfolded but aggregated to a varying extent. As *p*H values increase or decrease from the isoelectric point a lesser amount of aggregation, and correspondingly shorter lengths, would be anticipated because of increasing charge on the protein molecule.

The increased lengths at the low values of *p*H are probably due to aggregation promoted by the higher ionic strength of these solutions. In support of this idea are the results of heating a solution of *p*H 2 similar in composition to that of Fig. 1, but containing NaCl so that the ionic strength equalled that of a solution having a *p*H 0.73. This solution after heating for 15 minutes at 100° was highly aggregated, as evidenced by its gelatinous character, and was too viscous for flow orientation measurements.

Effect of the Protein Concentration in the Acid Range.—It is important to know whether the denatured protein molecules act independently of each other during flow orientation measurements. Figure 2 shows the results of denaturation at *p*H 1.1 for 15 minutes in a test for interaction during flow orientation measurements. Denaturation was effected in a 0.4% solution and flow orientation measurements made on this solution, as well as on 0.2 and 0.1% solutions made by dilution with 85% glycerol adjusted to *p*H 1.1. The excellent agreement in lengths at various concentrations shows that interaction is practically absent at the concentrations used.

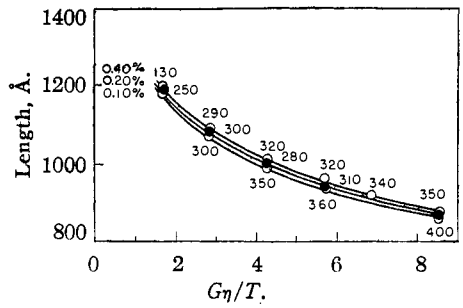

Fig. 2.—Denaturation at 100° and *p*H 1.1 for 15 minutes. Ovalbumin concentration during denaturation: 0.40; final: 0.40, 0.20, 0.10 g./100 ml.

The concentration of protein present during denaturation, however, is important in determining the length of solute particles preduced. For Fig. 3, ovalbumin was denatured by heating 15 minutes at 100° at *p*H 0.93 and at various protein concentrations. The pronounced increase in length with increased protein concentration is good evidence that aggregation is present. Even at the lowest concentration employed, 0.05%, the solution appears polydisperse.

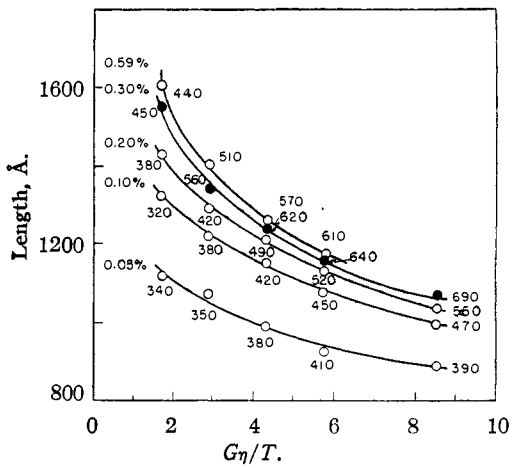

Fig. 3.—Denaturation at 100° and *p*H 0.93 for 15 minutes.

Effect of Varying Time and Temperature in the Acid Range.—Figure 4 is a summary of a time–temperature study at *p*H 0.93. Only the sample which was heated 5 minutes at 50°, and that heated 60 minutes at 25°, appeared homogeneous with respect to length; the others appeared polydisperse. The curves illustrate the rapid increase in length which occurs in the first few minutes of heating and the relatively slower increase with further heating.

The samples of Fig. 4 which were heated at 50° are shown in Fig. 5. The sample heated for 5 minutes appears homogeneous, with solute particles around 500 Å. in length.

Denaturation at *p*H 2.55 leads to decidedly shorter lengths than those obtained at *p*H 0.93, as may be seen in Fig. 6. Here the solute particles appear homogeneous in length at about 350 Å., and polydisperse at lengths around 425 Å. To facilitate measurements in this *p*H range, it was

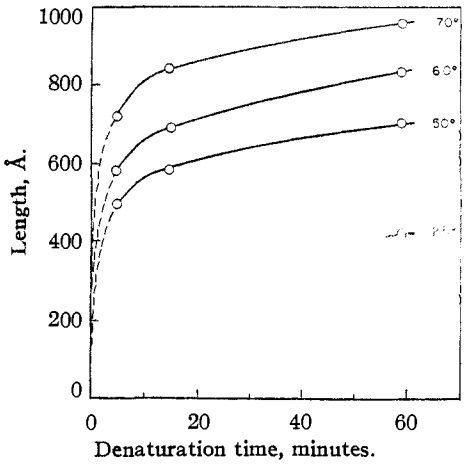

Fig. 4.—Denaturation at pH 0.93. Ovalbumin concentration 0.39 g./100 ml.; measurements at $G\eta/T$ = 8.4.

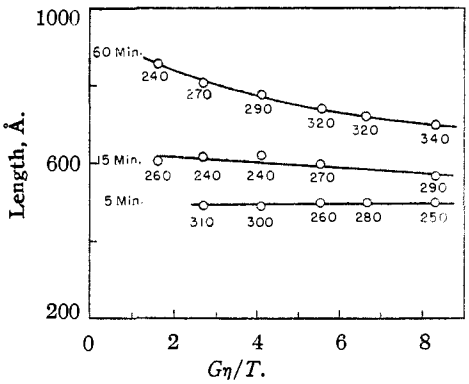

Fig. 5.—Denaturation at 50° and pH 0.93. Ovalbumin concentration: 0.39 g./100 ml.

necessary to use a higher concentration of ovalbumin, 0.79%, during denaturation.

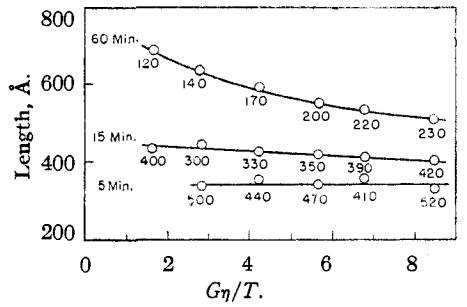

Fig. 6.—Denaturation at 100° and pH 2.55. Ovalbumin concentration: 0.79 g./100 ml.

Effect of Varying Time and Temperature in the Alkaline Range.—Throughout the entire pH range studied lengths generally were found to increase as the protein concentration, during heating, increased. At pH values around 10, however, for solutions containing 0.2 to 0.6% ovalbumin, the lengths were found to be much less dependent upon concentration than they are at pH values around 1.0. Aggregation is less prominent at these higher values of pH.

The dependence of length on time of heating and temperature at pH 10.25 is summarized in Fig. 7. Comparing these data with those of Fig. 4, obtained

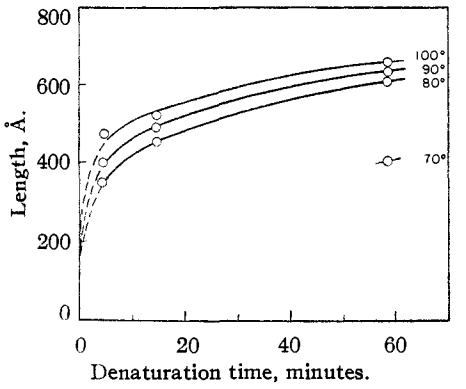

Fig. 7.—Denaturation at pH 10.25. Ovalbumin concentration 0.39 g./100 ml.; measurements at $G\eta/T$ = 5.6.

at pH 0.93, shows a striking difference in the dependence of denaturation upon temperature. The better constancy of length in Figs. 8 and 9, where the solute particles appear homogeneous to lengths of about 450 Å., suggests less aggregation to be present at the higher pH. Less emphasis was placed on the first three experimental points (and particularly the first) in drawing the curve for the 5 minute sample of Fig. 8. They were known to be too low, as the result of reflection errors which are troublesome in solutions of low birefringence.

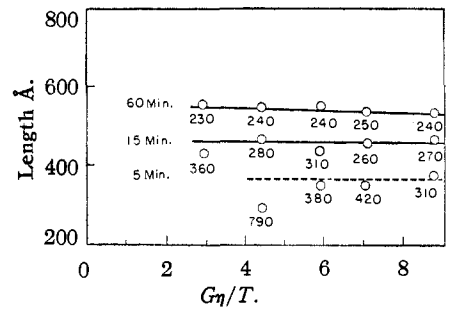

Fig. 8.—Denaturation at 80° and pH 10.25. Ovalbumin concentration: 0.39 g./100 ml.

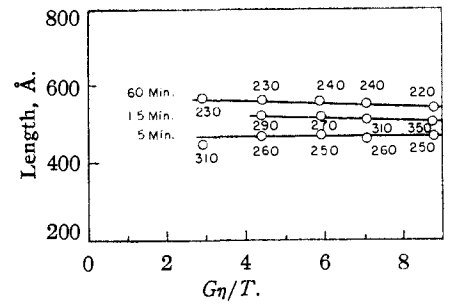

Fig. 9.—Denaturation at 100° and pH 10.25. Ovalbumin concentration: 0.39 g./100 ml.

Effect of Protein Concentration in the Alkaline Range.—In Fig. 10 results of time studies at three protein concentrations are presented. The solutions appeared to be heterogeneous except for the 5-minute samples and the lengths shown correspond to the apparent values at a single gradient. It is of extreme interest that the concentration effect is small, and actually in the inverse direction

to that which would be expected if aggregation were playing a significant role. It seems probable that under these conditions the lengths observed are those of unfolded, unaggregated molecules.

General Discussion

Some idea of the sensitivity of the measurements to polydispersity can be formed by considering the sample which was heated 60 minutes at 50° (Fig. 5). This sample showed an apparent length which varied from about 850 to 700 Å. over the gradient range studied, a variation which perhaps would not seem extreme considering the complexity of the material studied. Nevertheless, these data could be fitted rather well by assuming, for example, a mixture consisting of 80% 500 Å. and 20% 1000 Å. particles. If one disregarded polydispersity, the conclusions in such a case would be seriously in error. For this reason emphasis has been placed, in this series of investigations, on systems which appear to be homogeneous.

A further important reason for searching for conditions yielding apparently homogeneous solutions rests on the argument that unfolding might be expected to yield homogeneous solutions whereas aggregation, in general, would result in solute particles heterogeneous in length. It is recognized that the starting material, crystalline ovalbumin, is not homogeneous as judged by its electrophoretic behavior. The possibility that this heterogeneity may reside in relatively small differences in composition, however, and not in gross size and shape is indicated by the ease of crystallization of ovalbumin. In any event, the argument would be unaffected if homogeneous systems of readily measurable length were attained.

As a first approximation it might be expected that an aggregation process would lead to a random distribution in particle lengths. While the flow birefringence technique is highly sensitive to a discrete inhomogeneity such as was discussed above there remains the question as to whether it would be capable of demonstrating inhomogeneity in a random system. Therefore, calculations have been made assuming a system arising from a purely random end-to-end aggregation of molecules 100 A. long.[4] A calculation has been carried out for the case of a number average degree of polymerization of two, considering the contributions of each component from monomer up to aggregates of twenty molecules. The resultant orientation angles would lead to apparent lengths of 770 Å. at $G\eta/T$ of 1.0 dropping to 630 at 10.0. This is a heterogeneity far greater than is found in many of the solutions having lengths in this range.

The effect of *p*H is in accord with an aggregation hypothesis involving either unfolded or not-unfolded ovalbumin molecules. Whether aggregation is entirely eliminated in the region of the minimum around *p*H 2 seems doubtful. On the other hand the results of the time–temperature studies at *p*H around 10 are not easily explainable on the basis of aggregation and these data strongly suggest that unfolding occurs during denaturation.

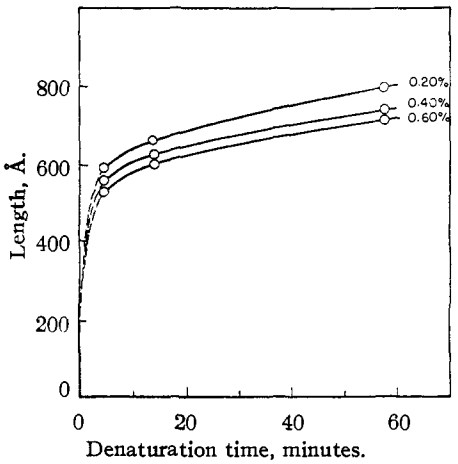

Fig. 10.—Denaturation at 100° and *p*H 9.5-10.0; measurements at $G\eta/T = 8.4$.

The length is seen to decrease with increasing *p*H and no minimum was found at very high values of *p*H, for example, around 11. However, it is possible that this may be explained either on the basis of hydrolysis or of dissociation. Some independent, though by no means convincing, evidence in this direction has been obtained in this laboratory by light-scattering. A combination of this technique with flow birefringence should be capable of providing an unambiguous answer as to the extent of unfolding in denaturation.

The quantity c in the function Δ/fc was arbitrarily taken as being equal to the total protein concentration in grams per 100 ml. of solution. It is recognized that not all of the protein present is necessarily contributing significantly to the birefringence. However, it is not possible to determine exactly what portion of the total concentration of protein is contributing to the birefringence. For this reason, the total protein concentration was employed in these calculations.

For solute particles which are homogeneous in length Δ/fc, as well as the length, should be independent of gradient. In general, this has been found to be the case, Δ/fc being constant to about $\pm 20\%$ in solutions showing constant length over the range in gradient employed in this investigation. The greatest discrepancies in Δ/fc, in solutions apparently homogeneous in length, appear when χ is near 45° and when Δ is low (at low values of the gradient, for example). Under these conditions reflection errors often are prominent. In the apparatus used this results in χ values which are too high and this, in turn, leads to f values which are too low and to Δ/fc values which are too high. It is difficult to interpret changes in Δ/fc for solutions denatured under various conditions, and such interpretations are reserved for a future communication in this series.

(4) This is essentially the mechanism proposed by M. Joly and E. Barbu, *Bull. soc. chim. biol.*, **31**, 1642 (1949), although they did not assume that the aggregation is precisely end-to-end nor that it is random. In the more general case the heterogeneity should be at least as pronounced.

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