STUDIES ON OVALBUMIN

I. DENATURATION BY HEAT, AND THE HETEROGENEITY OF OVALBUMIN

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Summary

The denaturation of ovalbumin by heat at pH 2–3 has been studied by following the changes in optical rotation, viscosity, solubility at the isoelectric point, and amount of aggregated protein observed in the ultracentrifuge. The laevorotation and reduced viscosity of heated solutions of ovalbumin increase with ionic strength and protein concentration. This effect is related to the state of aggregation of the denatured protein and not to the extent of denaturation as measured by changes in solubility. The initial denaturation step is irreversible but does not involve the extensive unfolding observed in urea solutions.

The presence is demonstrated, in a number of ovalbumin preparations, of a fraction which is more resistant to heat denaturation. This is shown to be a more stable form of ovalbumin ("S-ovalbumin") and its occurrence is shown to be connected with the storage history of the eggs used for the ovalbumin preparations.

I. INTRODUCTION

Crystalline ovalbumin has served as a standard protein for many investigations since its isolation from egg white by F. Hofmeister in 1890. It is easily prepared, readily purified by repeated crystallizations, and is stable on storage. Although electrophoretically heterogeneous (Longsworth 1939; Cann 1949) the three components have been shown by Perlmann (1952) to differ only in the number of phosphate groups but are otherwise identical in composition. No other evidence of heterogeneity has been reported.

The coagulation of ovalbumin solutions by heat was one of the earliest observed examples of protein denaturation, and subsequently loss of solubility in salt solutions at or near the isoelectric point has been used frequently as an index of denaturation (e.g. Gibbs, Bier, and Nord 1952). Other workers have demonstrated the irreversibility of heat denaturation and have shown that even in the absence of coagulation there is pronounced aggregation, indicated by changes in such hydrodynamic properties as viscosity (Loughlin and Lewis 1932; Bull 1940), sedimentation–diffusion (MacPherson, Heidelberger, and Moore 1945), light scattering (Bier and Nord 1949), and flow birefringence (Fredericq 1947). The influence of pH and ionic strength on the aggregation has been studied by Foster and Samsa (1951) and by Foster and Rhees (1952).

In studies on the denaturation of proteins by urea carried out in this Laboratory by McKenzie, Smith, and Wake (1963), the usefulness of following a particular denaturation process by means of several different techniques was demonstrated.

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In the work reported in this paper a similar approach has been made to the heat denaturation of ovalbumin in the acid pH region, in order to assess the relative importance of the aggregation reaction during denaturation and to enable the effects of heat to be compared with those previously reported for urea.

During the course of this work it became evident that the ovalbumin contained a minor component which had a much slower rate of denaturation. On isolating this fraction by selective heat denaturation, it was found to be distinguishable from "whole" ovalbumin only by its relative resistance to denaturation. The experiments which indicated the nature and occurrence of this form of ovalbumin are reported here.

II. Methods

(*a*) *Preparation of Ovalbumin*

Ovalbumin was prepared from commercially "fresh" hen eggs by the method of Kekwick and Cannan (1936) and recrystallized twice from a sodium sulphate solution at pH 4·7. Stock solutions containing 10–15% ovalbumin were prepared by exhaustive dialysis against distilled water, followed by centrifugation to remove traces of surface-denatured protein. The concentration was determined from semi-microKjeldahl nitrogen estimations (McKenzie and Wallace 1954) assuming 15·7% nitrogen in ovalbumin (Steven and Tristram 1958).

(*b*) *Optical Rotation*

A Schmidt and Haensch visual polarimeter reading directly to 0·01° was used to measure rotations. Unless otherwise stated, readings were made at 50°C, at the wavelength of the sodium D line. Water from a constant-temperature bath was pumped through the jackets of all-glass, 2-dm cells, controlling the temperature of the sample to 0·1 degC. A blank reading was taken for each cell filled with water at 50°C. The results are expressed as specific rotation.

(*c*) *Viscosity*

U-tube viscometers having a flow time of approximately 45 sec for water at 50°C and a working volume of 12 ml were used in a water-bath controlled to 0·1 degC. Flow times were measured to 0·1 sec with a stop-watch. The results are expressed as reduced viscosity, $(\eta - 1)/c$, where η = relative viscosity, and c is protein concentration in g/dl. The relative viscosity was taken as the ratio of the flow times of solution and solvent, no correction being made for differences in density.

(*d*) *Solubility*

A method similar to that of Gibbs (1954) was used, but with some modifications. Heated ovalbumin solution (1 ml) was run into 10 ml of cold "stopping solution" (0·5M sodium chloride, 0·1M sodium acetate–acetic acid buffer, pH 4·7). After mixing and standing for 15 min, the insoluble protein was centrifuged off and the relative concentration of soluble protein determined by ultraviolet absorption at 278 mμ in a 10-mm cell, correcting for scattering by subtracting the absorption at

320 mμ. The corrected absorption was expressed as a percentage of the absorption of an unheated portion of the solution treated in the same way, giving "percentage soluble protein", and, by difference, "percentage insoluble protein".

(e) *Sedimentation Measurements*

Measurements of sedimentation coefficients and amounts of slow and fast components were made with a Spinco model E ultracentrifuge fitted with a phase-plate and automatic temperature control. Runs were made near 20°C in 12-mm cells at a speed of 59,780 r.p.m. Sedimentation coefficients were determined as described by Schachman (1957) and corrected to the value in water at 20°C. The area of the slow peak was measured with a planimeter on a tracing of the enlarged pattern and

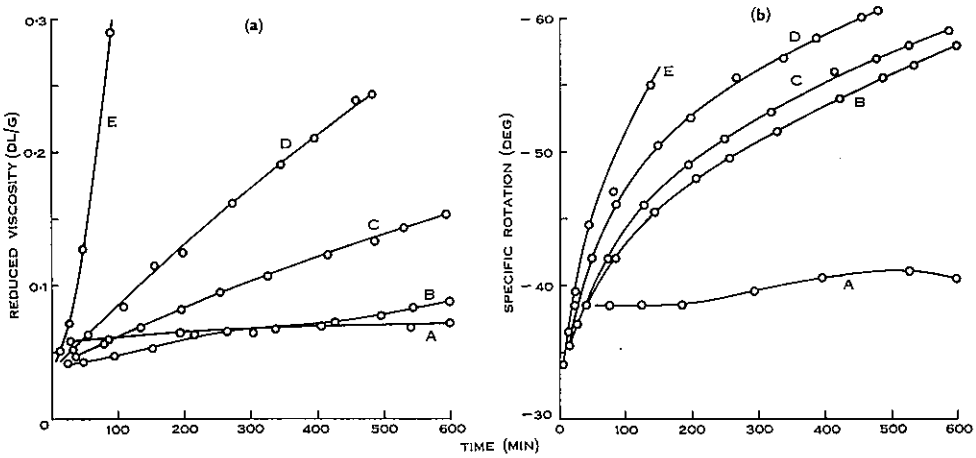

Fig. 1.—(*a*) Reduced viscosity and (*b*) specific rotation of ovalbumin solutions heated for various periods at 50°C, pH 3·0. *A*, 1% protein, no salt; *B*, 1% protein, 0·02M NaCl; *C*, 2% protein, 0·02M NaCl; *D*, 1% protein, 0·05M NaCl; *E*, 1% protein, 0·1M NaCl.

expressed as a percentage of the area of the peak given by an unheated portion of the solution. No correction was made for the relative enhancement of the area of the slow component (Johnston and Ogston 1946) or for change in concentration due to sectorial dilution. The first correction was found to be small in this system and the second was avoided by measuring the area of the peak at the same relative position in the cell for each run. The "percentage fast component" was obtained by difference.

III. Results

(a) *Effects of Ionic Strength on Optical Rotation and Viscosity*

Figures 1(*a*) and 1(*b*) show the changes in viscosity and optical rotation of 1 and 2% ovalbumin solutions at pH 3·0 while heating at 50°C in the presence of different concentrations of sodium chloride. There is a marked increase in both reduced viscosity and specific laevorotation as the ionic strength is increased. At concentrations over 0·1M sodium chloride the viscosity rises very steeply on heating, the solutions become opalescent and, at 2% protein concentration, finally gel.

Increasing the ionic strength apparently caused an increase in the degree of aggregation of the heated protein. Thus after heating a 1% solution of ovalbumin in 0·02M NaCl for 360 min at 50°C, cooling, and then adjusting to 0·1M NaCl, the reduced viscosity of the heated solution rapidly rose from 0·04 to 0·28. The sedimentation patterns [Figs. 2(*a*) and 2(*b*)] show a change in the sedimentation rate and heterogeneity of the fast (aggregated) component which also indicates an increase in the degree of aggregation. In a similar experiment, a 1% ovalbumin solution

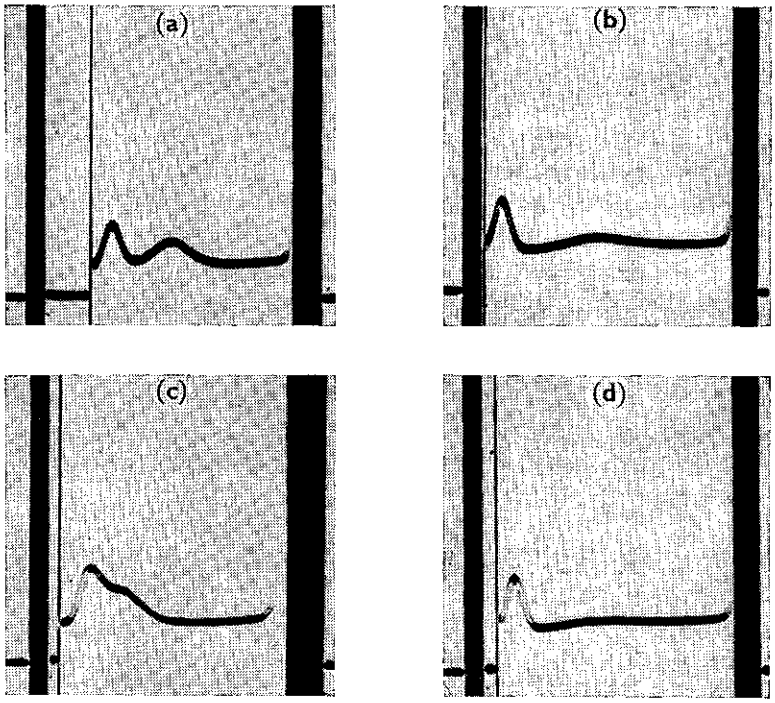

Fig. 2.—(*a*)–(*d*) Sedimentation patterns after 21, 17, 50, and 17 min at speed (59,780 r.p.m.), respectively, of 1% ovalbumin solutions which had been heated at 50°C, pH 3·0, (*a*) for 360 min in presence of 0·02M NaCl, (*b*) for 360 min in 0·02M NaCl, then adjusted to 0·1M NaCl, (*c*) for 600 min in the absence of NaCl, (*d*) for 600 min in absence of NaCl, then adjusted to 0·1M NaCl. Sedimentation is from left to right.

was heated in the absence of salt for 600 min at 50°C and after cooling was adjusted to 0·1M salt. The specific rotation, measured at 20°C, then changed rapidly from −40° to −52° and continued to decrease slowly. The sedimentation patterns are shown in Figures 2(*c*) and 2(*d*). [Due to the charge effects caused by the absence of salt there was incomplete resolution in Fig. 2(*c*).] In both experiments the slow component had a sedimentation rate near that of the native protein.

The changes in reduced viscosity and specific rotation which occur on heating at pH 2 and 45°C [Figs. 3(*a*) and 3(*b*)], and the effects of increasing salt concentration, are similar to those at pH 3 and 50°C. That these effects are due to differences in a

secondary aggregation process and not to differences in the amount of denaturation is shown in Figure 4,*A*, where the percentage insoluble protein at pH 4·7, ionic strength 0·5, is plotted against the time of heating at 45°C, pH 2·0. Here there is no significant

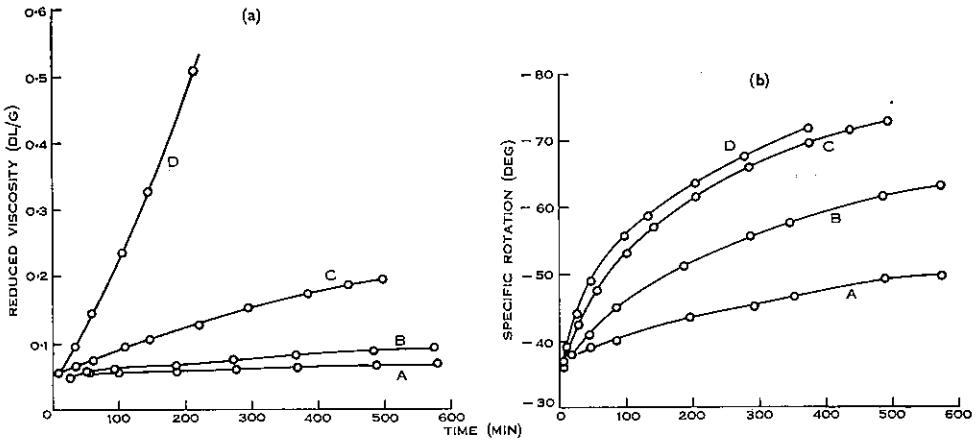

Fig. 3.—(*a*) Reduced viscosity and (*b*) specific rotation of 1% ovalbumin solutions heated at 45°C, pH 2·0. *A*, no salt; *B*, 0·02M NaCl; *C*, 0·05M NaCl; *D*, 0·1M NaCl.

difference between a solution containing no salt and one with 0·05M salt. The effect of protein concentration is also small when solubility change is measured, as shown in Figure 4, *B*, for 1 and 2% ovalbumin solutions in 0·02M NaCl at pH 3·0, heated at 50°C.

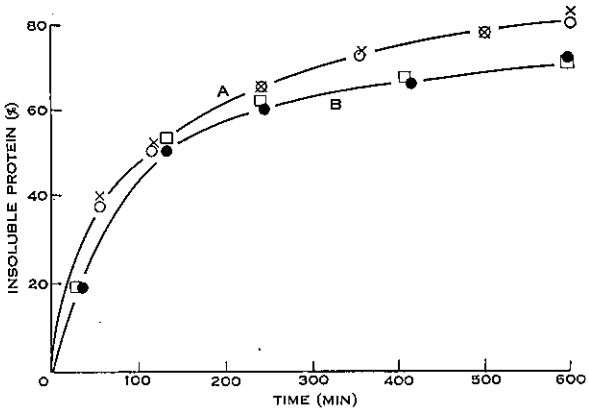

Fig. 4.—Solubility change with time of heated ovalbumin solutions: *A*, 1% protein at 45°C, pH 2·0 (×, salt absent; ○, 0·05M NaCl). *B*, 1% protein (●) or 2% protein (□) in 0·02M NaCl at 50°C, pH 3·0.

(*b*) *Primary Denaturation Process at pH 3·0 and 50°C*

Since neither reduced viscosity nor specific rotation showed any indication of reaching a final steady value after heating for 600 min (and even after 1300 min in two cases), neither of these techniques could be used for measuring reaction rates.

In any case, being dependent on the state of aggregation of the denatured protein, as shown above, they could not validly be used to follow the primary denaturation process.

Solubility change at the isoelectric point has been used by Gibbs, Bier, and Nord (1952) to measure the kinetics of the heat denaturation of ovalbumin, and this measurement, as shown in Figure 4, is independent of ionic strength and protein concentration, and therefore probably does indicate the true extent of denaturation.

It was found that the amount of insoluble protein was the same whether the sample was removed and immediately added to the stopping solution or whether it was kept in the cold for 24 hr before the solubility was determined, i.e. the denaturation is not reversible. This meant that it was reasonable also to measure the extent of denaturation by the change in the relative amounts of slow- and fast-moving components on ultracentrifugation. The effect of heating 1 and 2% solutions

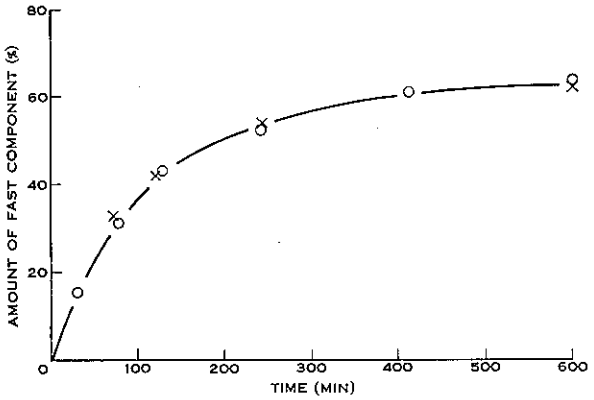

Fig. 5.—Amount of fast component on ultracentrifugation of ovalbumin (○, 1% protein; ×, 2% protein) solutions after heating in 0·02M NaCl at 50°C, pH 3·0, for the periods indicated.

of ovalbumin in 0·02M NaCl at pH 3 and 50°C for 10 hr on the amount of fast component present is shown in Figure 5. There is no significant difference between the results obtained for the two solutions.

The sedimentation coefficients of the fast component were in the range 6·8–8·0 at this salt concentration, and increased to 18–21 as the concentration was raised to 0·1M. The rates for the slow component were generally lower than the corresponding values for native ovalbumin (in 0·02M NaCl a time-extrapolated value of $S_{20,w} = 2·75$ at 1% concentration was obtained) but showed some variation in different experiments.

(c) *Stable Fraction of Ovalbumin*

It is apparent from Figures 4 and 5 that the changes in solubility and amount of fast component do not approach completion but that the curves tend to level off at a value less than 80%. This effect appeared to have two possible explanations: either the products of the reaction were inhibitory or there was a second component present which either did not denature or denatured more slowly.

A 2% ovalbumin solution in 0·02M NaCl at pH 3 was heated at 50°C for 7 hr and the denatured protein removed by precipitation at pH 4·7. The soluble protein (about 25% of the original) was salted-out with sodium sulphate, dissolved, and dialysed until free from salts. This fraction, adjusted to 1% protein concentration in 0·02M NaCl at pH 3, showed a decrease in specific rotation of only 9° on heating for 180 min at 50°C compared with a decrease of 26° for the original ovalbumin, and the sedimentation pattern of the heated solution showed only a trace of fast component. The rise in viscosity on heating this fraction was also small compared with the viscosity change of a similar solution of unfractionated ovalbumin. This showed that in a heated solution at pH 3 there was a fraction of the ovalbumin which was relatively resistant to denaturation; but there was no evidence to show whether this material was originally present in the native ovalbumin or whether it was formed by a side reaction on heating.

TABLE 1

EFFECTS OF HEATING 1% SOLUTIONS OF DIFFERENT OVALBUMIN PREPARATIONS FOR 300 MIN AT 50°C AND pH 3·0

Protein solutions in 0·05M NaCl except where indicated

Preparation No.	Specific Rotation (deg)	Reduced Viscosity (dl/g)	Insoluble Protein (%)	Fast Component (%)
1	−53*		75*	
2	−51*	0·065*		
2	−56	0·17		
3	−52		64*	57*
5	−74	0·44	69	73
6	−78	1·20	79	72
8	−51	0·21	45	37

* In 0·02M NaCl.

Several large preparations of the stable fraction were made, the conditions of denaturation being heating for 16 hr at 50°C, pH 3. The protein soluble at pH 4·7 was completely recovered by lyophilization of the dialysed solution. Preliminary characterization by sedimentation, optical rotation, paper electrophoresis, and ultraviolet absorption showed only slight differences from native ovalbumin. This fraction is therefore regarded as a distinct type of ovalbumin and called S-ovalbumin (stable ovalbumin).*

(*d*) *Occurrence of S-Ovalbumin*

When the measurements of the extent of denaturation by heat at pH 3 were repeated with different preparations of ovalbumin, an unexpected variability was disclosed. Table 1 summarizes the results obtained with six different preparations, giving the values for specific rotation, reduced viscosity, solubility, and amount of fast component after heating for 300 min at 50°C.

* In an earlier communication (Smith and Back 1962) the stable fraction was referred to as "ovalbumin-X".

Since repeated measurements on the same preparation were consistent and showed only small variations, the assumption was made that different preparations contained different amounts of either S-ovalbumin or its precursor (if S-ovalbumin is formed by heating). These differences could, in turn, be due either to variations in the eggs or to some unknown factor affecting the preparation. To examine these possibilities, four lots of eggs were obtained from different sources, ovalbumin was prepared from each lot by a strictly uniform procedure, and the reactivity of each lot of ovalbumin determined by measurements of viscosity, optical rotation, and solubility on heating at 50°C, pH 3. The results are shown in Table 2. The percentage of S-ovalbumin was calculated on the assumption that the amount of insoluble protein produced after 300 min was 97% and 17% for ovalbumin and S-ovalbumin respectively.

TABLE 2

COMPARISON OF OVALBUMINS FROM EGGS FROM DIFFERENT SOURCES

1% solutions heated for 300 min at 50°C and pH 3·0 in 0·05M NaCl

Source of Eggs	Specific Rotation (deg)	Reduced Viscosity (dl/g)	Insoluble Protein (%)	Estimated S-Ovalbumin (%)
Laying boxes	73	1·75	93	5
Poultry farm, after packing	70	0·56	79	23
Retail store	60	0·41	65	40
After 6 months cold storage	44	0·15	32	81

IV. DISCUSSION

The ovalbumin preparations which were used for this study of heat denaturation have been shown to be mixtures of two components which differ in their rates of denaturation. Because of this a quantitative evaluation of the results is not justified, but it is possible to make some general observations on the process of heat denaturation.

As other workers have shown, aggregation plays an important part in the overall reaction. Even at pH 3, where aggregation is minimal and no visible precipitation occurs, most of the denatured protein (as measured by the change in solubility) appears as a fast-moving component on ultracentrifugation.

It has generally been considered that aggregation occurs as a secondary process during denaturation. In the denaturation of ovalbumin by urea the two stages of unfolding and aggregation may be distinguished relatively easily in the changes in optical rotation and viscosity. Thus the rapid change in these properties which occurs in 7M urea at pH 3 has been shown by sedimentation and diffusion measurements to be due to a rapid unfolding, and there is no subsequent aggregation (McKenzie, Smith, and Wake 1955, 1963). At pH 7 in urea the initial unfolding is followed by a slow increase in viscosity and the appearance of a fast component in

the ultracentrifuge pattern, indicating aggregation. In all cases the sedimentation rate of the slowest component indicated that a considerable increase in asymmetry had occurred.

In the present measurements of heat denaturation there is little evidence for the presence of an unfolded monomer. The sedimentation rates of the slow component were generally less than that of the native protein at pH 3 but the results were erratic. The cause of this variation is unknown.

Charlwood and Ens (1957) found that there was a slight decrease in sedimentation rate for ovalbumin when the pH was brought from 6 to below 3·5. This has been confirmed, but since the change is reversible and there is no tendency for aggregation at pH 3 at temperatures below 30°C it does not represent the initial denaturation step. The primary reaction may be assisted by this slight expansion occurring at low pH, but it does not involve the extensive disorganization of the molecule which occurs in urea solutions. The effect of ionic strength on the specific rotation suggests that a secondary unfolding occurs during or after aggregation and that the extent of this unfolding depends on the size of the aggregate. This could be regarded as a type of micelle formation, with a reduction in the stabilizing energy of the hydrophobic bonds due to the changed environment of part of the protein molecule (Kauzmann 1959).

Although in this work heat denaturation has been studied for only one protein under a limited range of conditions, it is reasonable to point out that there are obvious differences from denaturation by urea.

The discovery of a less reactive form of ovalbumin has been an unexpected outcome of this work, and appears to be an unusual, if not unique, example of the detection of heterogeneity through measurement of denaturation rates.

It was considered important to find the reason for the variability of different ovalbumin preparations, before examining the properties of the two ovalbumins in detail. The measurements given in Table 2 suggest a relation between the age of the egg and the amount of S-ovalbumin in the preparation.

V. Acknowledgments

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