STUDIES ON OVALBUMIN

II. THE FORMATION AND PROPERTIES OF S-OVALBUMIN, A MORE STABLE FORM OF OVALBUMIN

By M. B. SMITH* and JOAN F. BACK*

[*Manuscript received July* 2, 1964]

Summary

Ovalbumin is changed to a more stable form (S-ovalbumin) during the storage of shell eggs. Conversion also occurs in an isolated ovalbumin solution, the rate increasing with pH and temperature. First order rate constants for the reaction have been measured at pH 9–10 and at temperatures between 20 and 50°C. The reaction is apparently irreversible and does not appear to involve loss of amino acids or small peptides.

Ovalbumin and S-ovalbumin have been compared by measurements of sedimentation rates, molecular weights, electrophoretic and serological properties, sulphydryl and disulphide groups, ultraviolet absorption spectra, specific rotation, crystal form, and solubility. No difference which would explain their difference in stability was observed in the properties of the native proteins. Measurements of changes in specific rotation on denaturation in urea–guanidine hydrochloride solutions indicate more complete unfolding in ovalbumin than in S-ovalbumin.

I. INTRODUCTION

In Part I of this series (Smith 1964) it was shown that a less reactive form of ovalbumin was present in variable amounts in a number of ovalbumin preparations made from commercially fresh eggs. This form of ovalbumin (S-ovalbumin) was detected by its slower rate of heat denaturation at pH 3. After isolation by selective heat denaturation, preliminary examination showed that it resembled ovalbumin very closely in all properties except its denaturation behaviour.

The amount of S-ovalbumin in shell eggs stored at 20°C was found to increase with time of storage at a rate which increased with pH (Smith and Back 1962). The pH of the egg white was controlled by oiling the eggs at various times between 4 and 36 hr after laying, thus preventing natural loss of carbon dioxide and tending to hold the pH constant from the time of oiling.

In the present paper, the results of a limited study of the formation of S-ovalbumin *in vitro* are presented, a method for preparing S-ovalbumin from ovalbumin is given, and a comparison is made of the properties of ovalbumin and S-ovalbumin.

II. MATERIALS AND METHODS

(*a*) *Preparation of Ovalbumin and S-ovalbumin*

Ovalbumin was prepared from eggs less than 6 hr old at the time of starting the preparation by the method of Kekwick and Cannan (1936) and recrystallized two or three times. For one preparation eggs were collected within 15 min of laying and

* Division of Food Preservation, CSIRO, North Ryde, N.S.W.

immediately cooled in ice-water, but there was no significant difference in heat-denaturation behaviour between this preparation and the others. Ovalbumin so prepared is regarded as "pure" ovalbumin for the present measurements.

S-ovalbumin has been prepared by two methods. The first method consisted of selectively heat-denaturing the ovalbumin in a mixture prepared from eggs that had been cold-stored for 6 months and which appeared to contain about 80% S-ovalbumin. A 2% solution of the mixed ovalbumins in 0·02M NaCl was adjusted to pH 3·0 and heated at 50°C for 16 hr. After cooling, sodium acetate, acetic acid, and NaCl were added to give concentrations of 0·1M acetate, 0·5M NaCl, and a pH of 4·7. The precipitate of denatured ovalbumin was filtered off, and the filtrate was dialysed against water until free of salts and then lyophilized. This material was somewhat variable in its properties, possibly because of contamination with some denatured protein.

A more satisfactory product was obtained by the direct conversion of ovalbumin to S-ovalbumin. A 5% solution of ovalbumin in water was adjusted to pH 9·9 at room temperature and then heated for 16 hr at 55°C. After cooling, a small amount of denatured protein (less than 1% of the total) was precipitated at pH 4·7 and the solution dialysed and lyophilized. This material has been taken as 100% S-ovalbumin for these measurements.

Solutions were prepared freshly as required by dissolving the lyophilized protein in water, centrifuging to remove a small amount of surface-denatured protein, and then adjusting to the required ionic strength and pH. For electrophoretic measurements the protein solutions were dialysed for 24 hr at 2°C against a large volume of the required buffer.

(*b*) *Estimation of S-ovalbumin and Measurement of Conversion Rates*

Since ovalbumin and S-ovalbumin could not be distinguished by a number of the usual physical and chemical methods (Smith and Back 1962) it was necessary to use the difference in rates of denaturation as a basis for an analytical method. It was shown in Part I that the change in solubility at the isoelectric point is the most convenient and accurate index of the extent of denaturation, and this was used together with a standard set of conditions for denaturation.

Two different sets of conditions have been used. In the egg-storage experiments (Smith and Back 1962) the change in solubility on heating for 90 min at 55°C and pH 3·0 was measured. In the experiments on the kinetics of the conversion of isolated ovalbumin, it was more convenient to carry out the heating at pH 7·0 for 60 min at 73·5°C. To avoid gelling, it was found necessary to use protein concentrations below 1·5% and an ionic strength below 0·1, although, as shown in Part I, the solubility change is independent of protein concentration and ionic strength. The amount of protein soluble at pH 4·7 was determined as described previously (Smith 1964). The percentage of S-ovalbumin was calculated from $100(A_n-A_0)/(S-A_0)$, where A_0, S, and A_n are the percentages of soluble protein in pure ovalbumin, S-ovalbumin, and the mixture, respectively, after the standard heating.

The procedure for measuring the rates of conversion at different pH values and temperatures was as follows:

A 2·5% solution of ovalbumin (or S-ovalbumin for a control) in 0·025M $NaHCO_3$ was adjusted with 1M NaOH to that pH at room temperature that would give the required pH at the reaction temperature. The pH–temperature relationship for this protein–buffer system had previously been determined, as a guide in making the adjustment. The solution, in a stoppered tube, was placed in a water-bath controlled to 0·05°C and reached bath temperature within 1 min. After 5–10 min the pH was checked with a Leeds and Northrop type 7666 pH-meter and Metrohm type EA125 miniature glass electrodes, standardized with 0·01M sodium borate at the temperature of the bath. Samples were removed at intervals and 2 ml pipetted into 8 ml of phosphate buffer, ionic strength 0·06, pH 7·0 (2·13 g Na_2HPO_4 and 2·34 g $NaH_2PO_4.2H_2O$ per litre). The percentage soluble protein after heating for 60 min at 73·5°C was determined as described previously (Smith 1964).

To prevent bacterial growth in the solutions kept at 20 and 30°C, the ovalbumin solution was sterilized by filtration through a bacterial filter into a sterile tube, and samples were removed aseptically.

(*c*) *Sedimentation and Molecular Weight*

Sedimentation coefficients were determined as described in Part I (Smith 1964). Molecular weight determinations were made by the approach-to-equilibrium method (Archibald 1947) in which the general procedure described by Schachman (1957) was used. Runs were made at 20°C with 1% solutions of ovalbumin and S-ovalbumin in 0·05 ionic strength phosphate buffer, pH 6·9, containing 0·1M NaCl. A layer of Kel-F polymer oil was used to provide a bottom meniscus in the cell. A speed of 6565 r.p.m. was used. Values of $\delta = (1/xc)(dc/dx)$, where x is the radial distance and c the concentration at a point in the cell, were determined from the photographed schlieren patterns by the integration method of Klainer and Kegeles (1955). A plot of δ against x was extrapolated to give values of δ_m and δ_b at the top and bottom menisci. Apparent molecular weight ($M_{app.}$) was obtained from the relation:

$$M_{app.} = \delta.\mathbf{R}T/(1-\bar{V}\rho)\omega^2$$

where **R** is the gas constant, T the absolute temperature, $\bar{V}$ the partial specific volume (taken as 0·745), ρ the solution density, and ω the speed (radians/sec).

(*d*) *Electrophoresis*

Moving boundary electrophoresis was carried out in the apparatus described by McKenzie and Wake (1959). Protein solutions (concn. 1%) in the following buffers were used:

Glycine: pH 3·0 (0·2M glycine, 0·05M HCl);

Phosphate: pH 6·8 (0·0125M NaH_2PO_4, 0·0125M Na_2HPO_4);

Veronal: pH 8·7 (10·3 g sodium diethylbarbiturate plus 1·85 g diethylbarbituric acid per litre).

Paper electrophoresis, in which the same buffers were used, was carried out in the L.K.B. apparatus at 20°C for 16 hr at 150–220 V.

(*e*) *Immunology*

Immunological analyses were performed by the following method: Antisera were prepared against ovalbumin and S-ovalbumin in rabbits. Antigen (20 mg made up in Freund's adjuvant) was injected at eight sites, four subcutaneous and four intramuscular. Four weeks later a booster injection of 20 mg was given intravenously and the rabbits were bled 1 week later. Standard Ouchterlony gel diffusion tests (Ouchterlony 1948) and Björklund inhibition tests (Björklund 1952) were run in agar plates made up according to the method described by Reisner and Sobey (1962).

(*f*) *Sulphydryl and Disulphide Groups*

Sulphydryl groups were determined by amperometric titration with methyl mercuric iodide (MeHgI), with a dropping mercury electrode (Leach 1960). For measuring the reactive groups in the native proteins, 2 ml of 2% ovalbumin or S-ovalbumin were added to 5 ml of Tris buffer, pH 7·3, and titrated with 0·01M MeHgI in dimethylformamide. The current at a fixed potential of −0·7 V (*v.* saturated calomel electrode) was measured after each addition, and the end-point obtained from the break in the titration curve. Measurements were also made after allowing the protein to stand overnight with excess MeHgI and then determining the excess by continuing the titration. Total thiol was determined by titration in a solution of 8M urea, 0·5M KCl, 0·1M NH_4OH, at pH 9·5 and a potential of −0·8 V, and thiol plus disulphide by adding 0·2M sodium sulphite as described by Leach (1960).

(*g*) *Ultraviolet Absorption Spectra*

Ultraviolet absorption spectra were measured with 0·2% solutions of ovalbumin and S-ovalbumin in 0·1 ionic strength glycine buffer, pH 9·5, in 0·1M acetate at pH 4·6, and at pH 2·0 (dilute HCl). A Zeiss model PMQ11 spectrophotometer was used at wavelengths between 240 and 320 mμ. Difference curves were also obtained by directly measuring in 10-mm cells the difference in absorption between 1% solutions of both proteins in 0·05 ionic strength phosphate, pH 6·8. The concentrations of the two solutions were adjusted for these measurements to give zero difference at 278 mμ; for the other measurements the actual concentrations of the solutions were determined.

(*h*) *Other Methods*

Protein concentrations were determined from semi-microKjeldahl nitrogen estimations (McKenzie and Wallace 1954) on the basis of 15·7% nitrogen in ovalbumin (Steven and Tristram 1958).

Specific rotation was measured as described in Part I (Smith 1964).

A comparison of the solubilities of the two proteins was made by titration with 95% saturated ammonium sulphate, as described by Ottesen (1958).

Methods used for comparing denaturation by heat or by urea and guanidine hydrochloride were the same as those described in Part I and by McKenzie, Smith, and Wake (1963) respectively.

III. Results

(*a*) *Conversion of Ovalbumin to S-ovalbumin* in vitro

The effects of pH and temperature on the rate of conversion are shown in Figure 1. The rate constants k are given in Table 1. These were obtained from the slopes of straight lines drawn through the points obtained on plotting log $(S-A_n)$ against time, as shown in Figure 1. The value for S (the percentage of S-ovalbumin soluble at pH 4·7 after heating at 73·5°C at pH 7·0 for 60 min) was found to increase slowly (up to 5%) in control solutions kept under the same conditions of

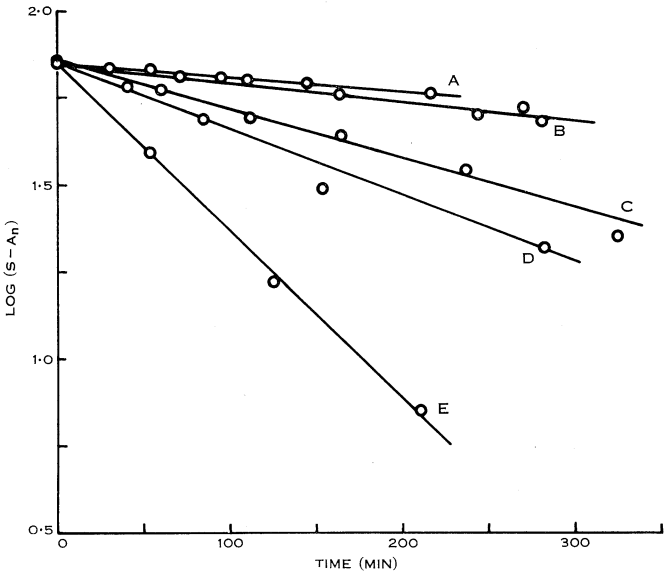

Fig. 1.—Rates of conversion of ovalbumin to S-ovalbumin: *A*, pH 9·5, 40°C; *B*, pH 9·0, 50°C; *C*, pH 10·0, 40°C; *D*, pH 9·5, 50°C; *E*, pH 10·0, 50°C.

pH and temperature as the ovalbumin solution. The reason for this is not known; it may be due to a slight increase in stability, caused possibly by slow oxidation of the sulphydryl groups. It is not possible to correct for this change since it is not known whether both ovalbumin and S-ovalbumin are affected or only the latter. Accordingly S has been assumed constant and a mean value of 83·5% taken; similarly A_0, the percentage ovalbumin initially soluble, has been assumed constant. The method of plotting used assumes a first-order reaction.

On plotting log k against $1/T$ for each pH, similar slopes are obtained, giving a value for the activation energy of 25 ± 5 kcal/mole. Because of the inherent inaccuracy of the method used for the determination of S-ovalbumin in the mixture, a more rigorous treatment could not be applied.

To investigate the possibility of small peptides or amino acids being split off during the conversion, a 5% ovalbumin solution was adjusted to pH 9·9 at room temperature and divided into halves. One part was heated at 55°C for 16 hr to

bring about conversion to S-ovalbumin, and then cooled. The protein was precipitated from both the heated and unheated solutions by treatment with 6% trichloroacetic acid and soluble nitrogen was then determined by Kjeldahl analysis. The difference was equivalent to less than 0·05% of the original protein nitrogen, i.e. equivalent to less than 0·25 mole of glycine per mole of ovalbumin.

TABLE 1

FIRST ORDER RATE CONSTANTS, k, FOR THE CONVERSION OF OVALBUMIN TO S-OVALBUMIN

Temperature (°C)	$10^5k(\text{sec}^{-1})$		
	pH 9·0	pH 9·5	pH 10·0
20			0·6
30		0·4	1·6
40	0·7	1·7	6·0
50	2·2	7·4	19

(b) Comparison of the Properties of Native Ovalbumin and S-ovalbumin

(i) *Sedimentation.*—Sedimentation coefficients ($S_{20,w}$) in phosphate–NaCl at pH 6·9 were determined for four concentrations of ovalbumin and S-ovalbumin.

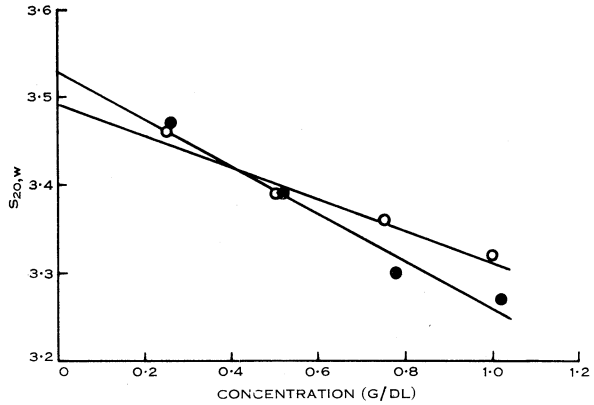

Fig. 2.—Sedimentation coefficient ($S_{20,w}$) *v.* concentration for ovalbumin (●) and S-ovalbumin (○) in 0·15 ionic strength phosphate–NaCl buffer at pH 6·9.

The results are plotted in Figure 2 together with the calculated regression lines,

$$S_{20,w} = 3{\cdot}53 - 0{\cdot}27C \text{ for ovalbumin,}$$

and

$$S_{20,w} = 3{\cdot}49 - 0{\cdot}18C \text{ for S-ovalbumin,}$$

where C is expressed in g/dl. The extrapolated values of $S_{20,w}$ at zero concentration are in reasonable agreement with the value of 3·67 obtained by Kegeles and Gutter

(1951). The slight difference in concentration dependence may represent a slightly more compact configuration for S-ovalbumin.

A decrease in sedimentation rate at pH 3 was noticed for both ovalbumin and S-ovalbumin and has been previously reported (for ovalbumin) by Charlwood and Ens (1957). For 1% solutions at pH 3·0, ionic strength 0·1, values of $S_{20,w}$ of $2{\cdot}8_7$ and $3{\cdot}0_1$ were obtained for ovalbumin and S-ovalbumin respectively. The lower value for ovalbumin may be an indication of partial unfolding. As mentioned in Part I, the sedimentation of ovalbumin at pH 3 appeared to be affected by some unknown variation.

Sedimentation of ovalbumin at pH 4·7 in 0·15 ionic strength acetate–NaCl buffer showed the presence of aggregates as noted by Creeth, Nichol, and Winzor (1958) and as previously reported (Smith and Back 1962) with one batch of ovalbumin, but not when repeated at a later date with two other batches. The formation of aggregates at pH 4·7 has now been shown by Gordon and Ottesen (1963) to be due to a complex of ovalbumin with aluminium, the aluminium in some cases apparently being derived from the aluminium alloy centrepiece of the ultracentrifuge cell. When ovalbumin and S-ovalbumin were run in 0·15M acetate at pH 5·0 in the presence of 0·001M aluminium chloride both proteins showed the same fast-moving component as reported by Gordon and Ottesen.

TABLE 2

APPARENT MOLECULAR WEIGHTS OF OVALBUMIN AND S-OVALBUMIN

	δ_m	δ_b	M_m	M_b
Ovalbumin	0·201	0·224	41,900	46,700
S-ovalbumin	0·198	0·218	41,300	45,500

(ii) *Molecular Weight.*—Mean values of δ_m and δ_b, obtained by extrapolation of the graphs $\delta = (1/xc)(dc/dx)$ against x, are given in Table 2. No consistent change in δ_m or δ_b with time of centrifuging was found. The apparent molecular weights, also given in Table 2, show no significant difference between ovalbumin and S-ovalbumin and together with the sedimentation measurements indicate that any difference in size or shape is small.

(iii) *Electrophoretic Measurements.*—No difference was found between ovalbumin and S-ovalbumin with moving boundary and paper electrophoresis at pH 3·0 in glycine buffer, at pH 6·8 in phosphate buffer, and at pH 8·7 in veronal buffer. The moving boundary electrophoresis patterns in veronal, pH 8·7, are shown in Figure 3. At pH 6·8 and pH 8·7 resolution into three components, corresponding to the A_1, A_2, and A_3 components observed by Longsworth (1939), was observed for both proteins. Since these components correspond to ovalbumin molecules having two, one, and no phosphate groups respectively (Perlmann 1952), it appears that conversion of ovalbumin to S-ovalbumin does not involve the phosphate groups (e.g. by production of a di-ester). Removal of one phosphate by treatment of ovalbumin with acid phosphatase at pH 5·4 (Perlmann 1952) was found not to affect

the conversion, and no phosphate soluble in trichloroacetic acid could be detected after heating ovalbumin to convert it to S-ovalbumin.

(iv) *Immunological Analysis.*—No serological difference between ovalbumin and S-ovalbumin could be detected. On carrying out standard Ouchterlony gel-diffusion tests no spur formation was detectable when the two antigens were run in adjacent wells against either antiserum. Similarly, Björklund inhibition plates did not demonstrate any heterologous antibody after cross-absorption.

(v) *Sulphydryl and Disulphide Groups.*—The results of the titrations of ovalbumin and S-ovalbumin with MeHgI are given in Table 3. In Tris buffer at pH 7·3, three equivalents of MeHgI per mole (45,000 g) of ovalbumin react rapidly. After

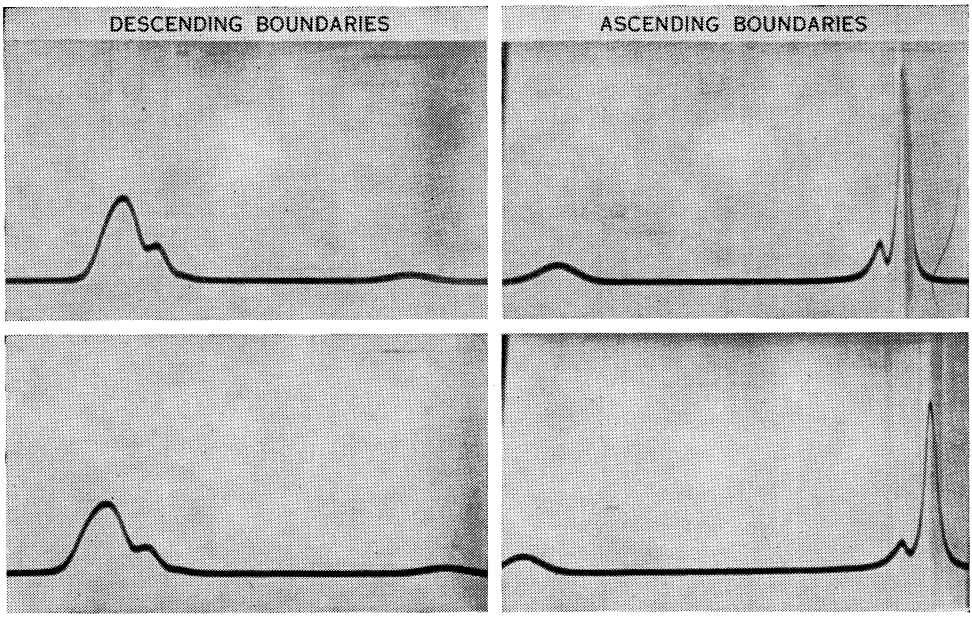

Fig. 3.—Moving boundary electrophoresis in veronal buffer, pH 8·7, voltage gradient 6 V/cm. Top, ovalbumin after 11,800 sec. Bottom, S-ovalbumin after 13,700 sec.

standing for 24 hr with excess MeHgI a fourth group partly reacts. In 8M urea, pH 9·5, the four groups may be titrated directly and no further uptake occurs on standing. The addition of 0·2M sulphite results in a fifth group becoming reactive, but this is only immediately apparent with ovalbumin, S-ovalbumin showing a significantly slower reaction rate.

The additional group reacting with MeHgI in the presence of sulphite is formed from a disulphide by the reaction:

$$R_1\text{–}S\text{–}S\text{–}R_2 + \text{–}SO_3^{2-} \rightleftharpoons R_1\text{–}SSO_3^- + R_2S^-.$$

Thus there appears to be a difference in the reactivity of the disulphide group in S-ovalbumin, compared with ovalbumin, although there is little difference in the total number of reactive sulphur groups. Leach (1960) found that the disulphide group

in ovalbumin was unusually labile, reacting with MeHgI even in the absence of sulphite. This may account for the high value (5·3) reached after standing, the disulphide group reacting with more than one equivalent of MeHgI.

(vi) *Ultraviolet Absorption Spectra.*—No differences between the ultraviolet spectra of ovalbumin and S-ovalbumin were found when comparisons were made at pH 2·0, 4·6, or 9·5. The difference spectrum of 1% solutions at pH 6·8 did not show any peaks, indicating close correspondence of the wavelengths of absorption maxima.

(vii) *Specific Rotation.*—Early measurements on S-ovalbumin indicated a specific rotation of *c.*—40°, compared with —30° for ovalbumin (Smith and Back 1962). For these measurements S-ovalbumin prepared by selective heat denaturation of a mixture was used. Later measurements on S-ovalbumin prepared by direct conversion have given —30° for the specific rotation. It is thought likely that the lower values for early preparations were caused by contamination with denatured ovalbumin or S-ovalbumin not completely removed at pH 4·7 after the selective heat denaturation.

TABLE 3

REACTION OF OVALBUMIN AND S-OVALBUMIN WITH METHYL MERCURIC IODIDE

Values given are equivalents of MeHgI reacting with 45,000 g protein when titrated immediately and after standing 24 hr with excess MeHgI

Solvent	Reaction Time (hr)	Ovalbumin	S-ovalbumin
Tris buffer, pH 7·3	0	3·1	3·2
Tris buffer, pH 7·3	24	3·6	3·6
8M urea, pH 9·5	24	4·1	4·0
8M urea, 0·2M sulphite, pH 9·5	0	4·8	3·8
8M urea, 0·2M sulphite, pH 9·5	24	5·3	4·8

(viii) *Solubility and Crystal Form.*—Although it has been observed that S-ovalbumin is more difficult to crystallize than ovalbumin, no difference in solubility was found on titrating 1% protein solutions at pH 6·1 with 95% saturated ammonium sulphate and determining the end point by the turbidimetric method described by Ottesen (1958). The crystals formed on allowing these mixtures to stand also appeared to be indistinguishable on examination at low magnification.

(*c*) *Denaturation Measurements*

The distinction between ovalbumin and S-ovalbumin has been made on the basis of their different stability to heat denaturation. At pH 3·0 and 55°C, ovalbumin was 95% denatured after 90 min, S-ovalbumin was 12% denatured. At pH 7·0 and 73·5°C, heating for 60 min caused 88% and 16% denaturation, respectively.

S-ovalbumin is also more resistant to denaturation by urea and guanidine hydrochloride. Since the changes in specific rotation and viscosity were very slow in 7M or 8M urea, mixtures of urea and guanidine hydrochloride were used for a comparison of ovalbumin and S-ovalbumin. Guanidine hydrochloride is about three times as effective a denaturant as urea, but does not hold the denatured protein

in solution at concentrations around 1M; hence 6M urea was used together with the guanidine hydrochloride (Schellman, Simpson, and Kauzmann 1953).

Figure 4 shows the changes in specific rotation at 25°C of 1% solutions of ovalbumin and S-ovalbumin at pH 7·5 in 6M urea and various concentrations of guanidine hydrochloride. It can be seen that comparable initial rates of change occur with ovalbumin in 0·4M guanidine–6M urea, and S-ovalbumin in 1·75M guanidine–6M urea. Expressed in terms of equivalent urea concentrations, this corresponds to 7·2M for ovalbumin and 11·2M for S-ovalbumin, the latter being actually above the solubility limit for urea at 25°C.

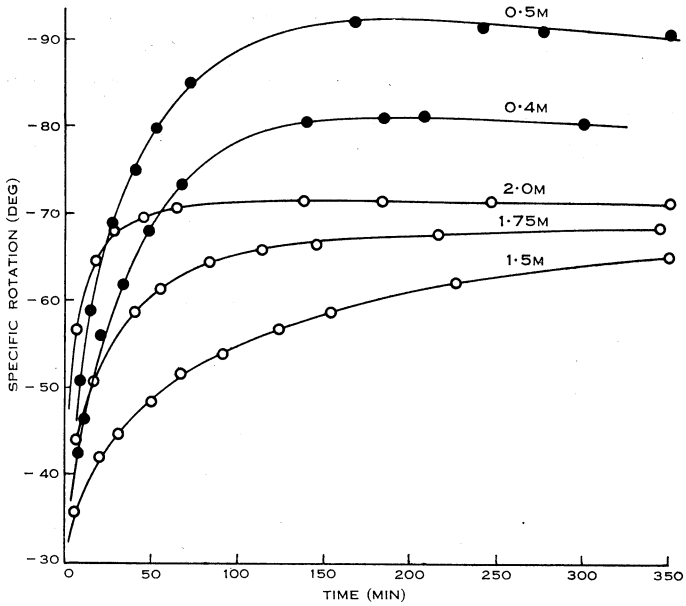

Fig. 4.—Specific rotation of 1% ovalbumin (●) and S-ovalbumin (○) at 25°C in 6M urea, pH 7·5, with added guanidine hydrochloride at concentrations indicated on the curves.

The specific rotation of ovalbumin in urea or guanidine salts appears to approach a limiting value of *c.*—95° as the concentration of denaturant is increased (Simpson and Kauzmann 1953; Schellmann, Simpson, and Kauzmann 1953). This value has been considered to be a characteristic of the fully unfolded molecule. It appears, from the limited range of concentrations studied, that S-ovalbumin may have an appreciably lower value for its limiting rotation, perhaps *c.*—75° (see Fig. 4).

IV. Discussion

The earlier egg-storage experiments (Smith and Back 1962) and the *in vitro* conversion studies reported here provide strong evidence that S-ovalbumin is a distinct form of ovalbumin produced on storage in alkaline solutions. Its physical and chemical properties indicate that it is a native protein and not a partially

denatured or degraded form of ovalbumin. Since it is likely that partial conversion of ovalbumin to S-ovalbumin occurs during the first days of incubation of fertile eggs, it can be regarded as a naturally occurring protein.

The kinetics of the *in vitro* conversion show that the process is first order with respect to protein, but this does not necessarily imply a monomolecular reaction since water, which is present in large excess, may be a reactant. The pH-dependence of the reaction may indicate an initial ionization step involving a sulphydryl or amino group, or a dependence on hydroxyl ion concentration (e.g. as in alkaline hydrolysis). No change in pH has been detected during conversion in unbuffered solution at pH 9·5, but the buffering capacity of the protein itself is high at this pH. Titration curves have not shown any significant difference between ovalbumin and S-ovalbumin but it is likely that the measurements would not detect a difference of one ionizing group at pH 9–10.

No evidence for the reverse reaction has been found. S-ovalbumin solutions stored at 20 or 5°C and at pH 4·7 or 3·0 have not shown any change in behaviour on heating. The slight *increase* in stability which occurs at pH 9–10 may be due to oxidation, as mentioned earlier.

Attempts were made to study the effect on the conversion rate of reagents that might be expected to modify the reactivity of certain side-chain groups of the protein, e.g. methyl mercuric iodide to block sulphydryl groups, hydroxylamine to combine with ester-like linkages, and ethylenediamine as a competitive inhibitor of reactive lysyl ε-amino groups. The results were negative or inconclusive owing to the effect of the added reagent on the heat denaturation stage and the resulting uncertainty as to the amount of ovalbumin converted. This approach requires a method of analysing the mixed ovalbumins which is not based on heat denaturation.

The significance of the conversion of ovalbumin to S-ovalbumin in stored eggs in relation to the well-known changes in the physical properties of egg white cannot at present be judged. The formation of S-ovalbumin during storage of untreated and oiled eggs at 20°C appears to be roughly paralleled by the breakdown of the thick white (Shenstone and Vickery 1958). The latter, however, has been considered to involve a change in the ovomucin fraction of the white (Brooks and Hale 1961) although it is possible that other proteins, including the ovalbumin, may take part in the change.

A recent report by Meehan, Sugihara, and Kline (1962) suggests a direct connection between S-ovalbumin formation and quality changes in stored eggs. They were able to show that the inferior baking properties of egg white from stored eggs were related to an increase in the temperature of heat coagulation of the white and could be improved by the addition of ovalbumin (from fresh eggs) to the "damaged" whites.

The results of the comparison of ovalbumin and S-ovalbumin given here generally confirm our earlier view that S-ovalbumin is indistinguishable from ovalbumin except under denaturing conditions. Although the results do not throw any light on the actual change in the ovalbumin molecule which confers greater stability, certain changes in structure may be discounted:

(1) Dissociation into subunits and association to oligomers are ruled out by the molecular weight measurements.

(2) Change in shape should be indicated by a change in sedimentation coefficient. The slight difference observed in the concentration dependence of $S_{20,w}$ may indicate a slightly more compact shape for S-ovalbumin, but any gross change such as that occurring on denaturation can be discounted.

(3) An intramolecular rearrangement in structure could be of only limited extent in view of the serological identity and agreement in specific rotation and absorption spectra.

(4) The loss of a peptide fragment during the conversion of ovalbumin to S-ovalbumin is unlikely, as shown by the absence of nitrogen in a trichloroacetic acid filtrate. Thus the conversion does not appear to be similar to the plakalbumin transformation (Ottesen 1958).

(5) Change in the number of ionizable side-chain groups is unlikely to occur without altering the electrophoretic properties.

Since little is known of the relative importance of the different non-covalent interactions in maintaining the compact, folded structure of the native protein (Kauzmann 1959), it is not possible to assess the degree of structural change which might be responsible for the increase in stability on converting ovalbumin to S-ovalbumin. However, since the conversion is irreversible, it is possible that the original structure of ovalbumin is not the most thermodynamically stable arrangement and that opening or displacing some restraining bond allows immediate rearrangement to a more stable configuration. The pH dependence of the conversion (in the range of ionization of –SH groups), the activation energy of conversion, the labile nature of the disulphide group of ovalbumin, and the known interchange properties of sulphydryl and disulphide groups (Cecil and McPhee 1959) suggest that the ovalbumin disulphide group is involved in the conversion. Displacement of the disulphide, as well as allowing a more favourable disposition of groups participating in non-covalent interactions, could in itself add to the stability of the molecule by enclosing a greater length of the polypeptide chain in cyclic form; the limiting rotations in urea–guanidine solutions offer some support for this idea.

Evidence to support this hypothesis is unfortunately all negative, and since the disulphide and at least one sulphydryl group are inaccessible to most modifying or blocking reagents without denaturing the protein, it is probable that direct evidence of covalent bond alteration will only be obtained by methods of sequence analysis.

V. Acknowledgments

The authors are grateful to Dr. A. H. Reisner and Dr. W. R. Sobey, both of the Division of Animal Genetics, CSIRO, for immunological analyses, and to Dr. S. J. Leach, Division of Protein Chemistry, CSIRO, for help in measuring sulphydryl groups.

VI. References

Archibald, W. J. (1947).—*J. Phys. Colloid Chem.* **51**: 1204.

Björklund, B. (1952).—*Proc. Soc. Exp. Biol., N.Y.* **79**: 319.

Brooks, J., and Hale, H. P. (1961).—*Biochim. Biophys. Acta* **46**: 289.

Cecil, R., and McPhee, J. R. (1959).—In "Advances in Protein Chemistry." Vol. 14. (Eds. C. B. Anfinsen Jr., M. L. Anson, K. Bailey, and J. T. Edsall.) (Academic Press, Inc.: New York.)

Charlwood, P. A., and Ens, A. (1957).—*Canad. J. Chem.* **35**: 99.

Creeth, J. M., Nichol, L. W., and Winzor, D. J. (1958).—*J. Phys. Chem.* **62**: 1546.

Gordon, J. A., and Ottesen, M. (1963).—*Biochim. Biophys. Acta* **75**: 453.

Kauzmann, W. (1959).—In "Advances in Protein Chemistry". Vol. 14. (Eds. C. B. Anfinsen Jr., M. L. Anson, K. Bailey, and J. T. Edsall.) (Academic Press, Inc.: New York.)

Kegeles, G., and Gutter, F. J. (1951).—*J. Amer. Chem. Soc.* **73**: 3770.

Kekwick, R. A., and Cannan, R. K. (1936).—*Biochem. J.* **30**: 227.

Klainer, S. M., and Kegeles, G. (1955).—*J. Phys. Chem.* **59**: 952.

Leach, S. J. (1960).—*Aust. J. Chem.* **13**: 547.

Longsworth, L. G. (1939).—*J. Amer. Chem. Soc.* **61**: 529.

McKenzie, H. A., Smith, M. B., and Wake, R. G. (1963).—*Biochim. Biophys. Acta* **69**: 222.

McKenzie, H. A., and Wake, R. G. (1959).—*Aust. J. Chem.* **12**: 723.

McKenzie, H. A., and Wallace, H. S. (1954).—*Aust. J. Chem.* **7**: 55.

Meehan, J. J., Sugihara, T. F., and Kline, L. (1962).—*Poultry Sci.* **41**: 892.

Ottesen, M. (1958).—*C. R. Lab. Carlsberg* (Ser. chim.) **30**: 211.

Ouchterlony, O. (1948).—*Acta Path. Microbiol. Scand.* **25**: 186.

Perlmann, G. E. (1952).—*J. Gen. Physiol.* **35**: 711.

Reisner, A. H., and Sobey, W. R. (1962).—*Aust. J. Sci.* **25**: 26.

Schachman, H. K. (1957).—In "Methods in Enzymology". Vol. 4. (Eds. S. P. Colowick and N. O. Kaplan.) (Academic Press, Inc.: New York.)

Schellman, J., Simpson, R. B., and Kauzmann, W. (1953).—*J. Amer. Chem. Soc.* **75**: 5152.

Shenstone, F. S., and Vickery, J. R. (1958).—CSIRO Div. Food Pres. Tech. Pap. No. 7.

Simpson, R. B., and Kauzmann, W. (1953).—*J. Amer. Chem. Soc.* **75**: 5139.

Smith, M. B. (1964).—*Aust. J. Biol. Sci.* **17**: 261.

Smith, M. B., and Back, J. F. (1962).—*Nature* **193**: 878.

Steven, F. S., and Tristram, G. R. (1958).—*Biochem. J.* **70**: 179.