STUDIES ON OVALBUMIN

III.* DENATURATION OF OVALBUMIN AND S-OVALBUMIN

By M. B. SMITH† and JOAN F. BACK†

[*Manuscript received November* 10, 1967]

Summary

Ovalbumin and S-ovalbumin have been compared by measuring their viscosity and optical rotatory dispersion in aqueous formamide solutions at pH 7 and in urea solutions at pH 3·5; also by measuring the optical rotatory dispersion of heated solutions at different salt concentrations, and by a study of ultraviolet difference spectra in alkylamine hydrochloride solutions. The different aggregation behaviour of the two proteins after denaturation suggests a difference in covalent bond structure.

I. INTRODUCTION

S-ovalbumin is formed from ovalbumin during the normal storage of shell eggs and can also be produced from isolated ovalbumin by holding the latter in solution at pH 9–10 (Smith and Back 1962). In Part II of this series (Smith and Back 1965), the *in vitro* conversion was found to be irreversible and to follow first-order kinetics. Although no difference in any other property of native ovalbumin and S-ovalbumin was detected S-ovalbumin was more resistant to denaturing agents such as heat, urea, or guanidine. The increase in stability on converting ovalbumin to S-ovalbumin suggested that an intramolecular change in structure of the ovalbumin molecule is involved, and it was deduced (Smith and Back 1965) that a more thermodynamically stable arrangement of secondary bonds may follow the opening or displacement of some restraining bond. Such changes might involve, for example, a shift in the position of the disulphide cross-link by disulphide–sulphydryl interchange.

The results of a more detailed study of the denaturation of ovalbumin and S-ovalbumin are given in the present paper. The object was to determine whether there exist differences in the properties of the fully denatured proteins that could be interpreted as evidence of differences in covalent bond structure. Part IV (Smith and Back 1968) will be concerned with the denatured, enzymically degraded proteins in which only covalent bond differences might still exist and be detectable.

II. MATERIALS AND METHODS

Ovalbumin and S-ovalbumin, prepared as described previously (Smith and Back 1965), were stored dry at 4°C. Stock solutions (usually 5% w/v) were prepared freshly for each set of measurements by dissolving the protein in water, centrifuging to remove some insoluble protein, and adjusting to the required pH. Protein concentrations were estimated from the light absorption at 280 mμ of a diluted solution, using the value $A = 0{\cdot}712$ for a 0·1% solution in a 1-cm cell

* Part II, *Aust. J. biol. Sci.*, 1965, **18**, 365–77.

† Division of Food Preservation, CSIRO, Ryde, N.S.W. 2112.

(Glazer, McKenzie, and Wake 1963). In some instances the concentrations were checked by semi-microKjeldahl nitrogen estimations (McKenzie and Wallace 1954).

Formamide was purified by the method of Verhoek (1936). Urea was recrystallized from ethanol–water (70% v/v) and the crystals were washed with absolute ethanol and dried at 50°C. A stock 10M urea solution was made up and de-ionized as required by stirring 100 ml of the solution with 10 g of mixed-bed resin (Amberlite MB-1). Alkylamine hydrochloride solutions were prepared by dissolving Fluka (purum grade) alkylamines in the calculated amount of 1·0M hydrochloric acid.

Viscosity measurements were made with U-tube viscometers (B.S. type B) having a flow time at 20°C of *c.* 75 sec for water and 120 sec for 70% (w/v) aqueous formamide. The reduced viscosity was calculated from $(\eta-1)/C$, where η denotes relative viscosity and C the 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 the small density difference.

Optical rotation was measured in a Perkin–Elmer model 141 photoelectric polarimeter at wavelengths of 589 mμ (sodium lamp) and 578, 546, 436, and 365 mμ (mercury lamp). A 1-dm water-jacketed cell was used, all measurements being made at 25°C. The Drude dispersion parameter λ_c was obtained from the specific rotation $[\alpha]_\lambda$ at each wavelength by plotting $[\alpha]_\lambda.\lambda^2$ against $[\alpha]_\lambda$ (Yang and Doty 1957). Mean residue rotations $[m']_\lambda$ were calculated from $[\alpha]_\lambda$ and corrected for the refractive index, n, of the solvent by using the expression:

$$[m']_\lambda = [3/(n_\lambda^2+2)].(W/100).[\alpha]_\lambda,$$

the mean residue weight, W, being taken as 115. Values of n_λ for water, urea, and formamide solutions were obtained from published tables (Fasman 1963). The parameters a_0 and b_0 of the Moffitt equation:

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2}+\frac{b_0\lambda_0^2}{(\lambda^2-\lambda_0^2)^2},$$

were derived from the intercept and slope of the line obtained by plotting $[m']_\lambda(\lambda^2-\lambda_0^2)/\lambda_0^2$ against $\lambda_0^2/(\lambda^2-\lambda_0^2)$. A value of 212 mμ was assumed for λ_0 (Urnes and Doty 1961).

Ultraviolet difference spectra of 0·5% protein solutions in 10-mm cells were measured in a Beckman DK2A recording spectrophotometer. Sedimentation coefficients were determined as described in Part I (Smith 1964).

III. Results and Discussion

(*a*) *Denaturation in Formamide–Water Mixtures*

Because S-ovalbumin was denatured very slowly in 8M urea at pH 7, a mixture of urea and guanidine hydrochloride was previously used to compare its denaturation rate with that of ovalbumin (see Part II). Guanidine hydrochloride is an effective denaturant on its own but does not hold denatured ovalbumin in solution at the relatively low concentrations required. Since it appeared from the work of De Deurwaerder and Harrap (1964) that formamide–water solvents might have some advantages with respect to the ease of preparing mixtures, the avoidance of reactive hydrolysis products, and the facility of using low ionic strengths, this solvent system was examined.

It was apparent in preliminary studies that aqueous formamide, even at 70% concentration, was not as good a solvent for the denatured protein as 6–8M urea, and solutions of protein concentration higher than 0·5% were apt to form gels or precipitates. In the measurements described below, protein concentrations were 0·4% or less and the phosphate buffer was 0·1 ionic strength, pH 7·0. In making up the solutions, the formamide was weighed out and diluted with the strong buffer solution

and water (an amount previously determined to give the correct final volume) before a measured volume of stock protein solution was added.

Figures 1(*a*) and 1(*b*) show the effects of formamide concentration (from 45 to 70%) on the rate of change of reduced viscosity of 0·4% ovalbumin and S-ovalbumin. It is apparent that ovalbumin shows a much greater viscosity change than S-ovalbumin at low formamide concentrations, but in 70% formamide the difference is small. The changes in viscosity are probably caused by a combination of unfolding and aggregation similar to that observed in urea denaturation (McKenzie, Smith, and Wake 1963).

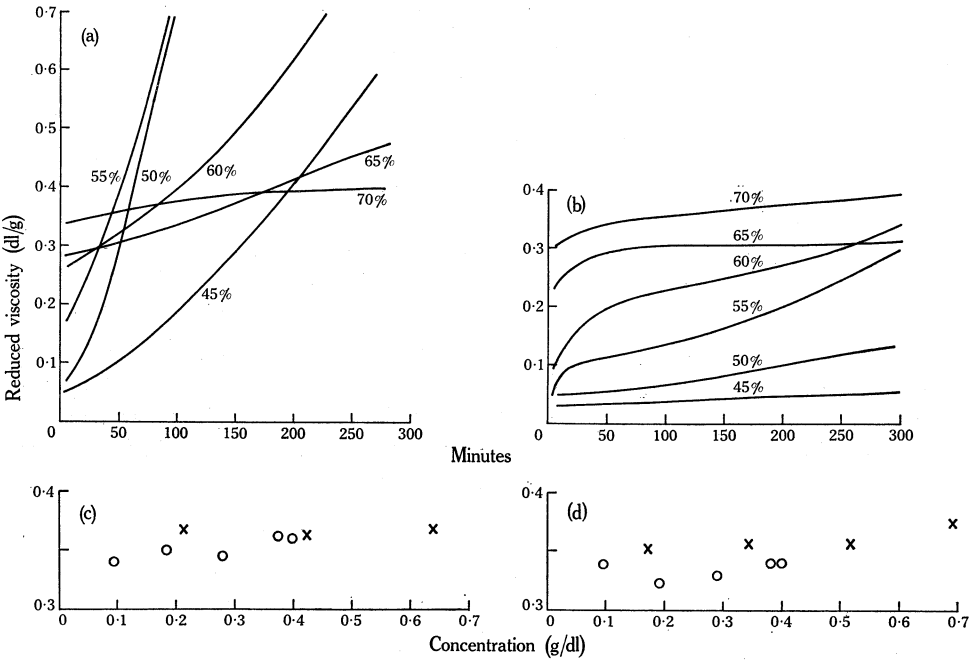

Fig. 1.—(*a*), (*b*) Effect of different percentages of formamide on the rate of change in reduced viscosity of ovalbumin (*a*) and S-ovalbumin (*b*). Protein concentration 0·4% (0·4 g/dl), pH 7·0. (*c*), (*d*) Reduced viscosity of different concentrations of ovalbumin (*c*) and S-ovalbumin (*d*), after 60 min in 70% formamide. × In presence of 0·005M *p*-chloromercuribenzoate.

Since aggregation of the unfolded protein is more extensive at low formamide concentrations, the relatively less stable ovalbumin exhibits higher viscosities under these conditions.

Figures 1(*c*) and 1(*d*) show the reduced viscosity after 60 min for different concentrations of ovalbumin and S-ovalbumin in 70% formamide. In this solvent there is little aggregation, as shown by the small positive slope. No decrease in reduced viscosity was found when measurements were made in the presence of *p*-chloromercuribenzoate, indicating that no intermolecular disulphide bonds were formed under these conditions. The intrinsic viscosity (reduced viscosity extrapolated to zero protein concentration) is $0 \cdot 33 \pm 0 \cdot 02$ dl/g for both ovalbumin and S-ovalbumin, a value representative of the "random coil" configuration (McKenzie, Smith, and Wake 1963).

The specific rotations of ovalbumin and S-ovalbumin in formamide–water mixtures did not change after the first 10 min for formamide concentrations of 60–80%. Table 1 shows the specific rotation at 578 mμ for three different protein concentrations, calculated from the mean readings taken 10–60 min after mixing. There is no dependence of [α] on protein concentration, but the rotations for S-ovalbumin are about 4° higher than for ovalbumin.

TABLE 1

SPECIFIC ROTATION AT 578 mμ OF OVALBUMIN AND S-OVALBUMIN AT DIFFERENT PROTEIN AND FORMAMIDE CONCENTRATIONS

Protein	Concentration (% w/v)	Formamide Concentration (% w/v) 60	65	70	80
Ovalbumin	0·4		−90·6	−87·3	
	0·2	−90·5	−90·4	−87·3	
	0·1	−91	−90	−88·5	−86
S-ovalbumin	0·4		−86·4	−83·9	
	0·2	−85·3	−85·9	−84·5	
	0·1	−87	−85	−84	−81

The change in $[\alpha]_{578}$ from −31·5° for the native proteins to the values in Table 1 indicates an extensive loss of native structure, and this is supported by the optical rotatory dispersion data given in Table 2. The Drude and Moffitt parameters are calculated from the specific rotations of 0·4% protein solutions in 70% formamide at pH 7 after 30 min reaction at 25°C.

TABLE 2

DRUDE AND MOFFITT PARAMETERS FOR OPTICAL ROTATORY DISPERSION OF 0·4% OVALBUMIN AND S-OVALBUMIN IN 70% FORMAMIDE, AFTER 30 MIN AT pH 7, 30°C

Protein	Mercaptoethanol	λ_c(mμ)	a_0	b_0
Ovalbumin	0	220	−482	−13
	0·1M	219	−479	−7
S-ovalbumin	0	220	−462	−8
	0·1M	220	−468	−9

Reported values for the optical rotatory dispersion parameters of ovalbumin are in general agreement with our results. Native ovalbumin has a_0 near −175 and b_0 in the range −150 to −200, changing on denaturation in urea to an a_0 value of −500 or less, with b_0 close to zero (Urnes and Doty 1961; Meyer and Kauzmann 1962; Tomimatsu and Gaffield 1965). Schellman (1958) found that λ_c changed from 266 mμ for the native protein to 226 mμ in 9M urea at pH 7.

The intrinsic viscosity and optical rotatory data for ovalbumin and S-ovalbumin in 70% formamide indicate complete denaturation, i.e. the complete loss of organized structure. If the difference in stability between ovalbumin and S-ovalbumin were

caused only by a difference in non-covalent interactions the two proteins should be identical after denaturation. However, the following experiment suggests that there is still a difference.

Solutions of ovalbumin and S-ovalbumin were denatured in 70% formamide for 30 min in the presence of 0·005M *p*-chloromercuribenzoate and then diluted to a concentration of 55% formamide by the slow addition of water, with constant stirring. The subsequent changes in viscosity are shown in Figure 2(*a*). The curves have been drawn to average the readings, which were erratic, presumably owing to the presence of some precipitated protein. Three experiments, in which two different initial concentrations of protein were used, showed the same tendency for ovalbumin to aggregate more rapidly than S-ovalbumin. The curves in Figure 2(*b*) were obtained when 0·1M mercaptoethanol was added to the solutions in 70% formamide (no *p*-chloromercuribenzoate) before dilution. The effect of this reagent is to increase the aggregation rate but some difference between ovalbumin and S-ovalbumin still persists.

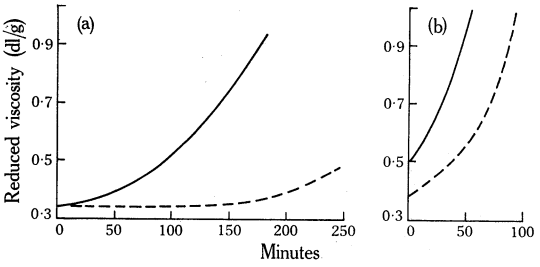

Fig. 2.—Changes in reduced viscosity on diluting from 70% to 55% formamide, (*a*) in presence of 0·005M *p*-chloromercuribenzoate, (*b*) in presence of 0·1M mercaptoethanol. —— Ovalbumin. – – – S-ovalbumin.

(*b*) *Denaturation in Urea at pH 3*

McKenzie, Smith, and Wake (1963) observed that in 7M urea at pH 3·3 ovalbumin showed a very rapid change in viscosity and optical rotation; also that, as judged by viscosity and sedimentation patterns, no aggregation occurred after unfolding, even at protein concentrations of 1%.

S-ovalbumin is also denatured rapidly in 7M or 8M urea at pH 3–3·5. Solutions of 0·2–0·8% ovalbumin and S-ovalbumin in 8M urea, buffered with 0·2M formate buffer at pH 3·5, had reduced viscosities of 0·40–0·42 and Moffitt dispersion parameters of −600 for a_0 and 0–10 for b_0. There was no significant difference between the two proteins. Thus they are both fully unfolded at pH 3·5 and are probably in a more extended configuration than at pH 7, owing to the increased electrostatic repulsion between charged groups.

On diluting the 0·8% protein solutions in 8M urea to a final urea concentration of 5M, no sign of aggregation was visible in the ultracentrifuge. However, dialysis of the 8M solutions against 20 volumes of 0·2M formate buffer at pH 3 for 48 hr at 5°C gave the ultracentrifuge pattern shown in Figure 3(*a*). The approximate sedimentation coefficients of the two components are 3·5 and 7. There is a difference in the proportions of the two components, ovalbumin again aggregating more than S-ovalbumin (as shown by the relatively larger amount of the faster moving component).

(c) *Heat Denaturation at pH 3*

At pH 3·0, ovalbumin is 95% denatured after 90 min at 55°C, whereas S-ovalbumin is 12% denatured under the same conditions (Smith 1964). This earlier work also showed that aggregation of ovalbumin depended on the ionic strength and protein concentration. Large changes in specific rotation occurred that were associated with the aggregation stage. To see whether the behaviour of S-ovalbumin was similar to that of ovalbumin it was necessary to denature both proteins to comparable extents.

It was found that heating S-ovalbumin for 300 min at 65°C produced a loss of solubility of over 95%. Accordingly, 1% salt-free solutions of ovalbumin and S-ovalbumin were adjusted to pH 3 with 1M hydrochloric acid, heated at 65°C for 300 min, cooled, and diluted to 0·5% protein with the addition of water and 1M NaCl to give 0, 0·02, and 0·05M NaCl. The solutions were left at 5°C overnight (it was shown previously that denaturation is not reversible under these conditions) and the specific rotations were measured at five wavelengths.

TABLE 3

SPECIFIC ROTATION AT 578 mμ AND MOFFITT DISPERSION PARAMETERS OF 0·5% HEAT-DENATURED OVALBUMIN AND S-OVALBUMIN AT pH 3

Protein	Salt Concn.	$[\alpha]_{578}$	a_0	b_0
Ovalbumin	0	−43·4	−250	−34
	0·02M	−62·4	−361	−57
	0·05M	−81·2	−470	−46
S-ovalbumin	0	−43·6	−248	−44
	0·02M	−53·4	−309	−51
	0·05M	−71·6	−419	−37

Table 3 shows the specific rotation at 578 mμ and the Moffitt dispersion parameters at the different salt concentrations. The b_0 values suggest that about one-quarter of the initial helical structure is present. Although b_0 is only slightly affected by salt concentration the values for $[\alpha]_{578}$ and a_0 indicate an increasing exposure of internal residues to the new environment as the salt concentration (and the degree of aggregation) increases (Chignell and Gratzer 1966), the effect being more pronounced with ovalbumin than with S-ovalbumin.

(d) *Effect of Alkylamines at pH 3*

The relative stability to detergents of ovalbumin and S-ovalbumin was studied with alkylammonium cations at acid pH, in order to avoid aggregation through disulphide interchange and the oxidation of sulphydryls which could occur at neutral or alkaline pH. Measurement of the ultraviolet difference spectra was the most sensitive method of detecting slight changes in internal structure (Wetlaufer 1962).

Initially, 0·5% solutions of ovalbumin and S-ovalbumin at pH 7 were compared with solutions of the same concentration at pH 3 containing 0·1M alkylamine

hydrochlorides of chain lengths C_4, C_5, C_7, C_8, C_{10}, and C_{12}. All of the solutions at pH 3 had a lower absorbance in the region of 293 mμ than the pH 7 solutions. The difference is shown in Figure 4*A* as a difference peak, the usual convention being adopted of expressing the difference as:

$$\Delta D = D_{\text{reference solution}} - D_{\text{sample solution}}.$$

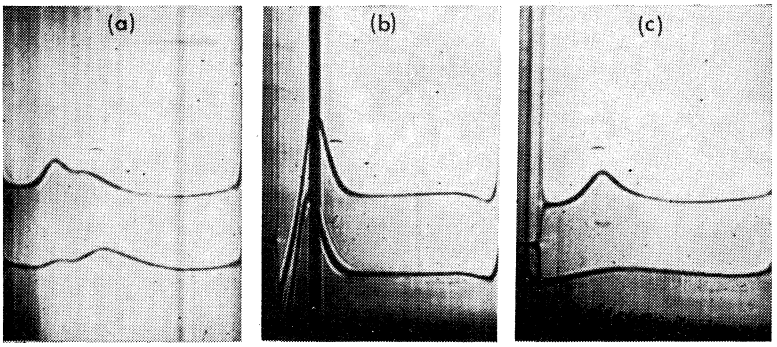

Fig. 3.—Sedimentation of ovalbumin (lower patterns) and S-ovalbumin (upper patterns) at 59,780 r.p.m., 20°C. (*a*) 0·8% protein solutions dialysed against 0·2M formate at pH 3 after denaturation in 8M urea at pH 3·5; (*b*), (*c*) 0·5% protein in 0·2M formate at pH 3 with 0·1M C_{10} alkylamine (*b*) before and (*c*) after dialysis.

The difference peak at 293 mμ was observed by Glazer, MacKenzie, and Wake (1963) when comparing solutions of ovalbumin at pH 7 and pH 3, and is apparently not caused by the detergent; but with the C_{10} and C_{12} alkylamines an additional peak appeared at 287·5 mμ and the higher-wavelength peak became broader (Fig. 4*B*).

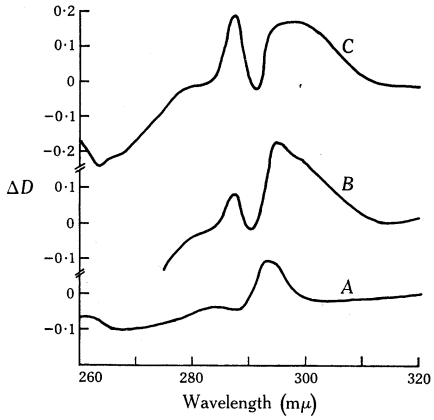

Fig. 4.—Ultraviolet difference spectra of 0·5% ovalbumin in 0·1M alkylamine hydrochoride. Reference solution at pH 7 *v.* solutions at pH 3 with (*A*) C_8 and (*B*) C_{10} alkylamine. (*C*) Both solutions at pH 3, C_{10} alkylamine. S-ovalbumin gave similar spectra.

Further measurements were made with the reference solution at pH 3. No difference peaks were evident with the C_4, C_5, C_7, and C_8 alkylamines but the C_{10} and C_{12} solutions gave the difference spectrum shown in Figure 4*C*.

For both ovalbumin and S-ovalbumin the difference peak reached a maximum height within 10 min of mixing, and remained constant for a further 20 min. The ovalbumin solution in C_8 then started to develop opalescence, but the C_{10} solutions remained clear. There was a marked effect of the concentration of the C_{10} alkylamine on the appearance of the different peaks: at 0·02M no peaks were evident, between 0·04 and 0·06M a rapid increase in peak height occurred, and at 0·1M this reached a maximum. No difference between ovalbumin and S-ovalbumin was observed.

The difference peaks indicate changes in the environment of the tyrosine and tryptophan chromophores, similar to those brought about by urea; however, the specific rotations of ovalbumin and S-ovalbumin solutions in 0·1M C_{10} had changed only to −48° and −47°. Figure 3(*b*) shows the sedimentation patterns for 0·5% protein in this solvent. Both proteins gave a single peak with a sedimentation coefficient of 1·2 (uncorrected). It is probable that this peak is not unfolded monomer but a monodisperse complex or micelle of both protein and detergent. Molecular weight determinations in this solvent require information on the partial specific volume and composition of the complex (Smith and Alexander 1957), and have not been attempted.

Attempts were made to reverse the effect of the detergent by fivefold dilution with 0·2M formate buffer, and by dialysis against water followed by buffer; but in all cases the proteins had lost solubility at pH 4·8. The solutions dialysed at pH 3 showed extensive aggregation in the ultracentrifuge [Fig. 3(*c*)].

IV. Conclusion

Denaturation of ovalbumin involves the two distinct processes of molecular disorganization (unfolding) and aggregation. The sequence of these processes is usually considered to be native protein → unfolded → aggregated. It is apparent from previous work on urea and heat denaturation (Holme 1963; McKenzie, Smith, and Wake 1963; Gagen and Holme 1964; Smith 1964) that there are multiple forms of both the unfolded and aggregated states and that the final product depends on the composition of the solvent (pH, ionic strength, and concentration of other solutes). At low pH and in high concentrations of urea the tendency to form aggregates is slight, so the unfolded form predominates. At neutral pH in urea or formamide aggregation occurs more readily, but molecular unfolding may still be distinguished by extrapolating relevant data to zero protein concentration. In the absence of an organic solubilizing agent, denaturation by heat apparently leads directly to aggregation, and the presence of an intermediate state can only be inferred (Smith 1964). That an intermediate form can undergo further internal disorganization after the initial aggregation, is shown by changes in specific rotation and a_0 as the salt concentration is increased. Detergents, also, may cause proteins to convert to this intermediate form, represented by the sedimenting peak in Figure 3(*b*).

The primary object in making the present measurements was to determine whether the difference in stability of ovalbumin and S-ovalbumin is due to a difference in covalent structure or whether it is due only to a difference in the disposition and relative strength of weak internal bonds (hydrogen, hydrophobic bonds, etc.).

Since the difference in denaturation behaviour has been observed only under conditions favouring aggregation, there may be no real difference in internal stability but only in the aggregation properties of the intermediate "unfolded" state. This does not necessarily eliminate the concept of "stability", since those interactions which stabilize the internal structure (e.g. the association of non-polar side-chains) may also control intermolecular association.

If all non-covalent bonds are destroyed or randomized in 70% formamide at pH 7 and in 8M urea at pH 3·5, the differences in aggregation properties of ovalbumin and S-ovalbumin observed on subsequent dilution of the denaturing agent must be caused by a difference in covalent structure. The evidence for complete destruction of secondary bonds is based on the values for intrinsic viscosity and the optical rotatory dispersion parameters, which have no absolute significance. The effect on aggregation properties of a known covalent cross-link is shown in Table 2 and Figure 2. The dispersion parameters are not affected by the addition of 0·1M mercaptoethanol to the 70% formamide solution but the subsequent viscosity changes on dilution to 55% formamide are more rapid than in its absence. It is not known to what extent the disulphide is reduced by this concentration of mercaptoethanol nor whether the same reduction is obtained for both ovalbumin and S-ovalbumin. The results are not inconsistent with the theory that ovalbumin and S-ovalbumin differ in the position of the disulphide and the experiment demonstrates that a difference in covalent bonding can affect aggregation.

Differences in aggregation properties have been observed with pepsin and trypsin digests of the urea-denatured proteins [see Part IV (Smith and Back 1968)]. These differences and the irreversible nature of the conversion process indicate a covalent bond change. Such a change might be detected by comparison of enzymically produced fragments of ovalbumin and S-ovalbumin, and in Part IV this approach is used.

V. Acknowledgments

The expert technical assistance of Mr. R. W. Sleigh during the course of this work is gratefully acknowledged. Financial assistance from the United States Department of Agriculture, under Public Law 480, is also acknowledged.

VI. References

Chignell, D. A., and Gratzer, W. B. (1966).—*Nature, Lond.* **210**, 262.
De Deurwaerder, R., and Harrap, B. S. (1964).—*Makromolek. Chem.* **72**, 1.
Fasman, G. D. (1963).—In "Methods in Enzymology". Vol. 6. p. 928. (Eds. S. P. Colowick and N. O. Kaplan.) (Academic Press, Inc.: New York.)
Gagen, W. L., and Holme, J. (1964).—*J. phys. Chem., Wash.* **68**, 723.
Glazer, A. N., McKenzie, H. A., and Wake, R. G. (1963).—*Biochim. biophys. Acta* **69**, 240.
Holme, J. (1963).—*J. phys. Chem., Wash.* **67**, 782.
McKenzie, H. A., Smith, M. B., and Wake, R. G. (1963).—*Biochim. biophys. Acta* **69**, 222.
McKenzie, H. A., and Wallace, H. S. (1954).—*Aust. J. Chem.* **7**, 55.
Meyer, M. L., and Kauzmann, W. (1962).—*Archs Biochem. Biophys.* **99**, 348.
Schellman, J. A. (1958).—*C.r. Trav. Lab. Carlsberg (Ser. chim.)* **30**, 429.
Smith, M. B. (1964).—*Aust. J. biol. Sci.* **17**, 261.

SMITH, M. B., and ALEXANDER, A. E. (1957).—Proc. 2nd Int. Congr. Surf. Activ., London. p. 349.
SMITH, M. B., and BACK, J. F. (1962).—*Nature, Lond.* **193**, 878.
SMITH, M. B., and BACK, J. F. (1965).—*Aust. J. biol. Sci.* **18**, 365.
SMITH, M. B., and BACK, J. F. (1968).—*Aust. J. biol. Sci.* **21**, 549.
TOMIMATSU, Y., and GAFFIELD, W. (1965).—*J. Polym. Sci.* D **3**, 509.
URNES, P., and DOTY, P. (1961).—*Adv. Protein Chem.* **16**, 485.
VERHOEK, F. H. (1936).—*J. Am. chem. Soc.* **58**, 2577.
WETLAUFER, D. B. (1962).—*Adv. Protein Chem.* **17**, 346.
YANG, J. T., and DOTY, P. (1957).—*J. Am. chem. Soc.* **79**, 761.