PTH's split off with hot HCl amounted to 3000 residues/50 million g. (2 analyses, 2900 and 3100 found). The observed maximum at 272.5 mμ indicated the presence of proline-PTH. Upon hydrolysis a great number of amino acids (proline, alanine, glycine, aspartic acid, etc.) were identified chromatographically.

Discussion

The ease with which carboxypeptidase splits off about 3000 residues of apparently C-terminal threonine per mole[11] of TMV stands in marked contrast to the absence of a similar number of detectable N-terminal residues. The fact that no more such residues can be revealed by separation of the protein from nucleic acid, nor by dissociation or denaturation by alkali, guanidine salts or detergents, strongly suggests the absence of a corresponding number of free chain end amino groups. Such a conclusion can be reconciled with the presence of C-terminal residues by assuming that either the threonine (or peptides terminating in threonine) or the N-terminal residues of the chains are bound through the ω-carboxyl group of aspartic or glutamic acid to the chain, yielding either true α-peptide rings with one or two radial appendages, or 6-shaped chain-rings containing one ω-linkage and C-terminal threonine. Similar structures were recently proposed for tropomyosin.[30] The incidental finding that only about half of the lysine ε-amino groups of TMV appear to react with FDNB under all experimental conditions represents a further challenge to the structural organic chemist. No similar behavior has been noted with any other protein.[31]

After treatment of TMV with hot trichloroacetic acid (TCA), the entire picture changes. The number of N-terminal residues shows an approximately tenfold increase with many different amino acids being represented. The susceptibility to attack by carboxypeptidase has also been shown[32] to be altered with such preparations; a heterogeneous mixture of amino acids is formed and continues to increase with time to a fivefold of the amount of threonine released from TMV. In all these respects, the TCA-prepared sample differs markedly, not only from TMV, but also from the nucleic-acid free preparation dissociated to very low molecular weight by sodium dodecylsulfate. The latter preparation can be regarded as thoroughly denatured; thus the finding that hot TCA leads to the appearance of new amino, and probably of new carboxyl, groups strongly suggests their formation through hydrolysis of peptide bonds. The appearance of new "end groups" in simple proteins under similar conditions seems to support this conclusion.

(30) K. Bailey, *Proc. Roy. Soc.*, **B141**, 45 (1953).

(31) R. R. Porter found up to one-third of the ε-amino groups of certain native proteins unavailable to FDNB. In contrast to TMV, however, all became reactive after denaturation) *Biochim. Biophys. Acta*, **2**, 105 (1948)).

(32) H. Fraenkel-Conrat, J. I. Harris and C. A. Knight, Abstract ACS meeting Chicago, Sept. 1953, p. 9 C.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Disulfide Cross-links in Denatured Ovalbumin[2]

BY MURRAY HALWER

RECEIVED MARCH 4, 1953

Denatured ovalbumin consists of aggregates which are at least partially cross-linked by disulfide bonds, whether denaturation is brought about by heat, shaking, alcohol or urea. The evidence for this is that reagents which break disulfide bonds diminish the light scattering of a solution of denatured ovalbumin in 6 *M* urea. The cross-linking is probably due to a chain reaction between the protein sulfhydryl and disulfide groups.

An observation, made by the light scattering method, that urea has little disaggregating effect on ovalbumin which had been caused to aggregate by heat denaturation, suggested that the aggregates may be held together by primary bonds. It was postulated that sulfhydryl groups, which are exposed by denaturation, become oxidized, possibly by air, to give intermolecular disulfide bonds. If such bonds are formed, the addition to a denatured ovalbumin solution of reagents which break disulfide bonds—mercaptans, for example—should produce at least a partial dispersal of the aggregates. Experiment indicated that such reagents do, in fact, show a dispersing effect. Thus, if the observations have been correctly interpreted, it must be concluded that denatured ovalbumin is at least partially cross-linked by disulfide groups.

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Presented at the Atlantic City meeting of the American Chemical Society, Division of Biological Chemistry, September 16, 1952.

Experimental

Ovalbumin was prepared from fresh egg whites by the method of Sørensen and Høyrup.[3] It was recrystallized four times from ammonium sulfate solution, dialyzed against distilled water at 2°, and dried from the frozen state.

In most experiments, denaturation was brought about by heating in a boiling water-bath. Two hundred fifty mg. of ovalbumin was dissolved in water, the *p*H brought to 4.8 and insoluble matter filtered off. 0.2 ml. of 2 *M* phosphate buffer of *p*H 8 was added, the *p*H adjusted to 8.0 with ammonia and the solution diluted to 20 ml. This solution, contained in a test-tube, was placed in a boiling water-bath for from one to five minutes. After cooling, an additional 0.8 ml. of buffer was added, and 60 ml. of a 10 *M* solution of urea at *p*H 8. The solution was diluted to 100 ml., giving a urea concentration of 6 *M*, and filtered through a sintered glass filter of "fine" porosity into two light scattering cells. Light scattering intensity, defined here as the ratio of galvanometer readings at 90° to those at 0°, was determined, using a light scattering photometer developed in this Laboratory.[4] The extent of the aggregation produced on de-

(3) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **12**, 12 (1916).

(4) B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Am.*, **40**, 768 (1950).

naturation can be judged from the fact that the scattering of the urea solution of ovalbumin that had been kept five minutes in boiling water was 21 times as great as native ovalbumin under the same conditions.

To one cell was now added a sufficient amount of a reagent which breaks disulfide bonds to give a concentration of 0.02 *M*. The reagents used were potassium thioglycolate, added as a 4 *M* solution; sodium sulfite, added as a 1 *M* solution; mercaptoethanol, added as the pure liquid; and cysteine, added as the solid. To the other cell of the pair was added a control reagent, resembling the first as closely as possible in charge type, buffer range and other characteristics, but incapable of breaking disulfide bonds. These were, respectively, sodium acetate, potassium oxalate, ethylene glycol and serine. They were added in the same forms as the first-named reagents, and the concentration of each in the solution was 0.02 *M*. The solutions were allowed to stand at room temperature and light scattering was measured from time to time.

Results and Discussion

Figure 1, curves A and B, shows the results for ovalbumin denatured by heating five minutes in boiling water, with potassium thioglycolate and sodium sulfite as the reducing agents. The ratio of the scattering of the solution containing the reducing agent to that of its control is plotted against time. Cysteine and mercaptoethanol produced similar curves. In each case, the reducing agent brought about a diminution in scattering relative to the control. The control solutions themselves showed decreasing scattering with time due, probably, to a gradual break-up of aggregates that are not cross-linked, but the decrease was slower than for the solutions containing the reducing agents. Thus, in approximately 20 hours, the control solutions showed the following decreases in scattering: oxalate, 22%; ethylene glycol, 18%; acetate, 26%; serine, 23%. These are to be compared with 48% for sulfite, 39% for mercaptoethanol, 46% for thioglycolate and 34% for cysteine.

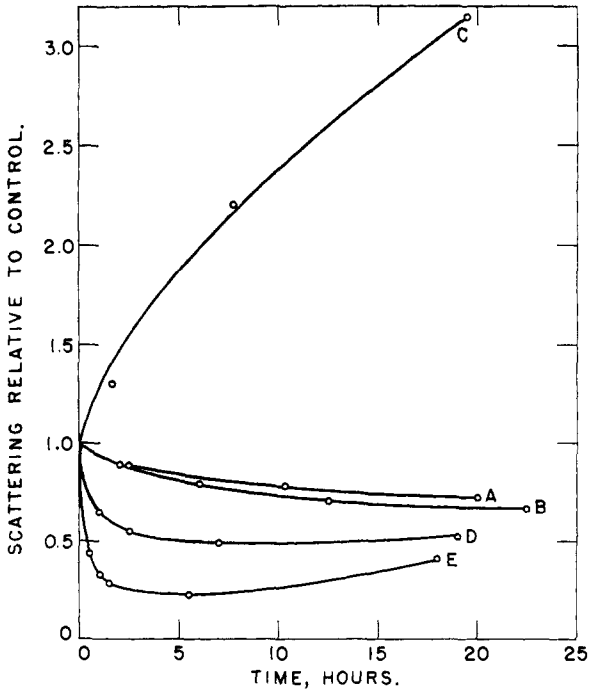

Fig. 1.—Effect of reducing agents on light scattering of ovalbumin in 6 *M* urea, *p*H 8: A and B, heat-denatured ovalbumin; C, native ovalbumin; D, surface-denatured ovalbumin; E, alcohol-denatured ovalbumin. Reducing agent is 0.02 *M* potassium thioglycolate and control reagent 0.02 *M* sodium acetate except for B, which is 0 02 *M* sodium sulfite *vs.* 0.02 *M* potassium oxalate.

These results are not, in themselves, proof of the existence of disulfide cross-links between molecules, since the same effect might be produced if the individual ovalbumin molecules themselves consisted of segments held together by disulfide bonds. We should then expect to observe that reducing agents lower the light scattering of native ovalbumin as well as denatured. Experiment shows the opposite effect, however (Fig. 1, curve C). Conditions were as described above, except that 500 mg. of protein was used, and the heating step was omitted. It is seen that 0.02 *M* potassium thioglycolate increases the scattering of native ovalbumin relative to an acetate control. Sodium sulfite and mercaptoethanol had the same effect. It is not entirely clear why these reagents increase the relative scattering. A possible explanation is that the disruption of the disulfide groups of the protein speeds up the process of denaturation and that the rising scattering is due to the aggregation of the denatured molecules.[5] Another explanation is that the reducing substances, by disrupting internal loops held together by disulfide bonds, permit a more complete extension of the denatured molecules and therefore an increased degree of lateral interaction with neighboring molecules. A combination of these two effects is also possible.

Miller and Andersson[6] concluded from ultracentrifuge studies that reduction of ovalbumin does not produce fragmentation.

Ovalbumin heated only one or two minutes at 100° before making 6 *M* in urea and 0.02 *M* in thioglycolate showed scatterings, relative to acetate controls, which decreased at first, but after about 2.5 hours began to increase, reaching values in 20 hours that were higher than the initial. Here, evidently, relatively few cross-links have formed, and the behavior is intermediate between that of native ovalbumin and denatured ovalbumin containing many cross-links. It was only after three minutes in boiling water that sufficient cross-links were formed to give a continuously decreasing scattering throughout a 20-hour period.

Formation of disulfide cross-links is not limited to heat denaturation. Surface-, alcohol- and urea-denatured ovalbumin gave evidence of the same effect. Surface-denatured ovalbumin was prepared by shaking 50 ml. of 0.4% solution at *p*H 4.8 for 20 hours at room temperature in a 250-ml. flask filled with nitrogen. The protein was completely precipitated. It was filtered off and the precipitate suspended in 20 ml. of 0.1 *M* phosphate buffer of *p*H

(5) Evidence that mercaptans increase the denaturation rate of ovalbumin in urea was supplied by the following experiment. A 0.5% solution of native ovalbumin at *p*H 8 in 6 *M* urea, containing 0.02 *M* mercaptoethanol, was allowed to stand 20 hours at room temperature. It was then diluted 5-fold and the *p*H brought to 4.8. The precipitate was centrifuged and collected in a graduated centrifuge tube. The same was done with a control solution containing 0.02 *M* ethylene glycol instead of the mercaptan. The solution containing the reducing agent produced twice as much precipitate as the control.

(6) G. L. Miller and K. J. I. Andersson, *J. Biol. Chem.*, **144**, 465 (1942).

8. The suspension was shaken three hours under nitrogen. Urea solution was added to give a final concentration of 6 *M*, the solution diluted to 100 ml. and filtered through a fine porosity sintered glass filter into two light scattering cells. To one, thioglycolate solution was added to give 0.02 *M*; the other was made 0.02 *M* with sodium acetate. The light scattering curve (Fig. 1, curve D) shows that the reducing agent decreases the scattering to a marked degree. The acetate control itself showed a 4% decrease in scattering.

Alcohol-denatured ovalbumin was prepared by adding 10 ml. of ethanol to 10 ml. of a 2% ovalbumin solution at *p*H 4.8 and centrifuging the precipitate down. Total time of contact between the precipitate and the alcoholic solution was about 12 minutes. The precipitate was washed twice with water, then treated as given above for the surface-denatured material. The urea solution was centrifuged a short time at 10,000 r.p.m. to remove haze before adding the reducing agent. Figure 1, curve E, shows that the reduction in relative scattering is very marked, in this instance.

Urea-denatured ovalbumin was prepared by letting a solution containing 0.5% protein, 8 *M* urea and 0.02 *M* phosphate buffer, at *p*H 8, stand under nitrogen. After 1.5, 3.5 and 18 hours, samples were removed, filtered and the effects of 0.02 *M* thioglycolate and 0.02 *M* acetate compared as previously described. In this system, the acetate controls changed more rapidly than did the solutions containing thioglycolate. The former showed scatterings which increased continuously with time, attributable to a combination of the crosslinking reaction and the aggregation consequent upon denaturation. In the latter, the sulfhydryl groups of the thioglycolate hinder the cross-linking process, with the result that the first sample (age 1.5 hours before adding thioglycolate) showed a nearly constant scattering until, after 8 hours, it began to increase. The two older samples showed decreases in scattering, on adding thioglycolate, of 15 and 17%, respectively, in 3 hours, after which there was little change during the total period of 20 hours. The explanation offered is that the two older samples contained enough cross-links so that addition of thioglycolate not only prevents further cross-linking but brings about a reduction in average particle size.

The initial hypothesis that the cross-links are due to air-oxidation of sulfhydryl groups had to be discarded, since heat denaturation in an atmosphere of purified nitrogen gave a product which was indistinguishable from one made in an air atmosphere. We have already seen that the surface- and urea-denatured materials gave evidence of cross-linking, although prepared in a nitrogen atmosphere.

Huggins, Tapley and Jensen[7] observed that solutions of ovalbumin, serum albumin, serum globulin and fibrinogen in concentrated urea solutions set to gels on standing. Ramsden[8] earlier noted the gelation of ovalbumin in saturated urea solution. The former authors attributed the gelation to the formation of intermolecular disulfide bonds and proposed a chain mechanism whereby a sulfhydryl group from one protein molecule reacts with a disulfide group from another molecule, forming a cross-link and leaving a new sulfhydryl group which can react with a third molecule, etc. (Fig. 2). They offered proof of this mechanism, insofar as it applies to serum albumin. For example, treating the sulfhydryl group with iodoacetamide gives a product which will no longer form a gel.

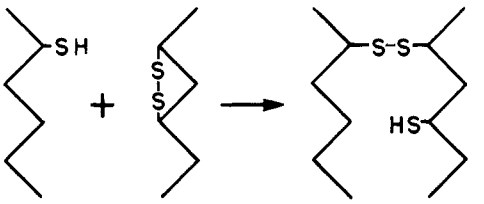

Fig. 2.—Probable mechanism for formation of disulfide cross-links.

The mechanism of the cross-linking of ovalbumin is probably the one diagrammed in Fig. 2, but it is not easy to prove, since the sulfhydryl groups of native ovalbumin are inaccessible to nearly all reagents. An exception is iodine, which reacts stoichiometrically with the sulfhydryl groups of native ovalbumin, according to Anson and Stanley.[9] Furthermore, according to Anson[10] this oxidation takes place entirely intramolecularly. The present author was able to confirm this observation by the light scattering method.

If the sulfhydryl groups in native ovalbumin can be completely destroyed by iodine oxidation, the above mechanism predicts that the resulting material should not cross-link on denaturation. Oxidized ovalbumin was prepared by the method of Anson,[11] using a 30% excess of iodine over the stoichiometric amount. After oxidation, the protein was denatured by heating for five minutes in a boiling water-bath and the effects of thioglycolate and acetate on the scattering in 6 *M* urea were compared, as previously described. Contrary to the prediction of the above mechanism, evidence of cross-linking was found, since the scattering of the thioglycolate-containing solution decreased relative to the acetate control. However, the oxidized sample was distinctly different in behavior from an unoxidized sample, prepared under the same conditions, but omitting the iodine. The latter showed a relative scattering which decreased throughout the 20-hour observation period, whereas that of the former decreased for about one hour, then increased, attaining a value after 20 hours close to the initial. The oxidized sample thus resembles a normal sample which has been heat-denatured for only one or two minutes. It is not clear how the cross-links in the oxidized ovalbumin arise. The iodine may not succeed in reaching all the SH groups, although the nitroprusside test[11] was negative for the oxidized sample. Or, a few SH groups may be formed by hydrolysis of S–S groups in the denaturation step. In this connection, Sanger[12]

(7) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).

(8) W. Ramsden, *Chemistry and Industry*, **8**, 851 (1930).

(9) M. L. Anson and W. M. Stanley, *J. Gen. Physiol.*, **24**, 679 (1941).

(10) M. L. Anson, "Advances in Protein Chemistry," Vol. II, Academic Press, Inc., New York, N. Y., 1945, p. 369.

(11) M. L. Anson, *J. Gen. Physiol.*, **24**, 399 (1941).

(12) F. Sanger, *Nature*, **171**, 1025 (1953).

has recently shown that disulfide compounds can undergo interchange reactions of the type

$$R^1—S—S—R^2 + R^3—S—S—R^4 \rightleftharpoons R^1—S—S—R^3 + R^2—S—S—R^4$$

He considers the probable mechanism to involve the formation of a few SH groups by reduction or hydrolysis, followed by a chain reaction of the type proposed by Huggins, Tapley and Jensen. He cites evidence that insulin undergoes such an interchange reaction on hydrolysis.

All the above experiments were performed in urea solution. We might expect disulfide-splitting reagents to diminish the light scattering of a denatured ovalbumin solution in the absence of urea as well as in its presence. The fact is, however, that they do not. 0.02 *M* potassium thioglycolate, for example, was substantially without effect on the scattering of a denatured ovalbumin solution which contained no urea. There are several possible explanations for the apparent failure of these reagents to act in the absence of urea. One is that urea facilitates the reduction. Another is that the cross-links actually are broken in the absence of urea, but that it requires the solubilizing action of urea to separate the fragments thus produced. Experiment indicated that both these hypotheses are correct. A 1% ovalbumin solution at *p*H 8, containing 0.02 *M* phosphate buffer, was denatured by heating five minutes in a boiling water bath. The solution was divided in two, and to one portion, thioglycolate to make 0.02 *M* was added, while acetate to make 0.02 *M* was added to the other. After 1, 2.5, 6.5 and 20 hours, 10-ml. portions were withdrawn from each and diluted to 50 ml. with urea to make 6 *M*. The solutions were filtered rapidly into light scattering cells, and scattering determined.[13] The 1-hour sample containing thioglycolate showed 3.5% less scattering than the acetate sample; at 2.5 hours, the difference was 4.8%; at 6.5 hours, it was 9.6%; at 20 hours, 10.3%. These results confirm the supposition that the cross-links are broken in the absence of urea, but that the fragments do not separate until urea is added. Comparison with the 27% decline obtained when thioglycolate is allowed to act in the presence of urea (Fig. 1) also shows that urea must facilitate the reduction.

This experiment also shows that cross-links are present in denatured ovalbumin before urea is added, rather than the alternative possibility, that the cross-links form only after urea is added. Further evidence for the prior existence of the cross-links is the fact that mercaptoethanol, added to a denatured ovalbumin solution, produced a scattering diminution which was the same in rate and over-all extent regardless of whether the mercaptoethanol was added before or after the urea. If the cross-linking takes place only upon adding urea, one would expect the sulfhydryl groups of the mercaptoethanol to hinder the crosslinking process to a marked degree, but the above experiment shows no such effect.

It was noted that potassium thiocyanate, which has a strong solubilizing effect on proteins[14] but lacks the ability to break disulfide bonds, was without effect, in 0.02 *M* concentration, on the scattering of denatured ovalbumin in 6 *M* urea. Also, 0.02 *M* mercaptoethanol was without effect on the scattering of β-casein in 6 *M* urea, while it diminished that of α-casein by not far from 50%. The latter contains one S–S group per 56,000 g. of protein, whereas the former contains no S–S groups.[15]

It should be pointed out that there is no obvious reason to assume that the cross-linking reaction would be confined to ovalbumin. It seems probable that any protein containing both sulfhydryl and disulfide groups would crosslink on denaturation. In fact, in view of Sanger's observations,[12] disulfide groups alone may suffice.

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(13) Since the scattering of both solutions diminished with time after adding urea, the scattering was extrapolated back to the moment of addition of the urea. A straight line, for extrapolation purposes, was obtained by plotting log $(S - c)$ *vs.* time, where S is the scattering ratio and c is a constant defined on p. 5 of "Empirical Equations and Nomography," by D. S. Davis, McGraw-Hill Book Co., Inc., New York, N. Y., 1943.

(14) A. R. Docking and E. Heymann, *J. Phys. Chem.*, **43**, 513 (1939).

(15) W. G. Gordon, W. F. Semmett, R. S. Cable and M. Morris, THIS JOURNAL, **71**, 3293 (1949).