[CONTRIBUTION FROM VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Peptide Chains of Tobacco Mosaic Virus

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Tobacco mosaic virus nucleoprotein was studied by two independent methods for the detection and estimation of N-terminal residues. The small number found (100–300/mole) is far less than the number of sub-units that the virus can readily be dissociated to, or than the number of C-terminal threonine residues (3000/mole). Removal of all nucleic acid, denaturation and extensive dissociation by various means does not increase the number of detectable N-terminal residues. Working hypotheses are discussed to account for these observations in terms of chemical structure of the sub-unit. Treatment of the virus with trichloroacetic acid at 100° is shown to cause the appearance of a great number of different N-terminal residues —presumably artefacts due to the hydrolytic rupture of the most labile peptide bonds.

Methods have been developed in recent years for the determination and identification of the N-terminal amino acid residues of the peptide chains of proteins. The fluorodinitrobenzene (FDNB) method[2a,b,3] which is readily applicable to all amino acids except glycine and proline is complemented by the phenyl isothiocyanate (PTC) method[4,5] for which serine, cysteine, threonine, histidine and arginine may represent special problems. Both methods have been applied by us to tobacco mosaic virus (TMV) nucleoprotein, and the preliminary result was that the total number of terminal residues detectable by either method was quite low (about 0.04 equiv. per 10^4 g., or 200/mole).[6] Since the virus can by various methods be dissociated to sub-units of the order of mol. wt. 10,000–20,000,[7] most probably without rupture of any primary chemical linkages, it would appear that the maximal number of amino ends found would be insufficient to permit the assumption that each sub-unit represents one open peptide chain. These findings suggest that the virus protein may be largely composed of cyclic peptide chains. Positive evidence for this hypothesis is being sought at present. Similar indications have recently been obtained with other complex and simple proteins (myosin, flagellae, chymotrypsinogen[8–10]).

These findings seemingly stand in contrast to the remarkable observation of Harris and Knight[11] that carboxypeptidase splits off about 3000 residues of threonine, and only threonine from TMV. While the simplest interpretation of this finding would be the presence of a similar number of peptide chains terminating in threonine, carboxypeptidase is not necessarily limited in its action to the original C-terminal amino acids as are the chemical reagents used for the amino end. Digestion of one or several polythreonine chains from the carboxyl and various other interpretations are possible to account for the observed effect. Yet, these findings suggested further intensive searching for a corresponding number of N-terminal residues. To this end we investigated TMV–protein that had been freed more or less rigorously from nucleic acid as well as TMV dissociated by the action of alkali (*p*H 10),[12] guanidine hydrochloride and sodium dodecylsulfate (SDS).[13] We also performed the end group reactions under a variety of conditions designed to favor "unmasking" of protein groups, and thus complete reaction. All these experiments gave concordant results, showing no appreciable amounts of N-terminal residues (100–300/mole), by either of the two methods.

At this time it came to our attention that Schramm believed to have evidence that TMV protein contained N-terminal proline, in amounts corresponding to the C-terminal threonine. While failure to detect this particular amino acid by the FDNB method could be envisaged, we did not see how the PTC method could possibly have failed to indicate such an amount of N-terminal proline clearly. We performed, however, additional experiments searching for proline in TMV by the latter method, while bringing definite proof for the validity of the techniques by adding a natural proline-N-terminal peptide, *i.e.*, salmine,[14] to the TMV in amounts equivalent to the end groups claimed by Schramm. We found the expected amount of proline phenylthiohydantoin (PTH) in the salmine containing sample, but none whatsoever in TMV alone.

When the paper by Schramm and Braunitzer became available to us,[15] the main difference in our experiments became immediately evident. These authors had used only one type of preparation for their study, a TMV protein sample obtained by treating the virus with 5% trichloroacetic acid for 30 min. at 100°. It appeared possible that spurious "end groups" had been generated through hydrolysis of peptide bonds under these conditions. This suspicion was confirmed when we studied preparations made according to Schramm and Braunitzer. For the first time, we obtained many end groups by both the FDNB and the PTC method. In agreement with the German authors we found evidence for much terminal proline (about

(1) Aided by a grant from The National Foundation for Infantile Paralysis.

(2) (a) F. Sanger, *Biochem. J.*, **39**, 507 (1945); **45**, 563 (1949); (b) R. R. Porter and F. Sanger, *ibid.*, **42**, 287 (1948).

(3) R. R. Porter, *Methods in Medical Research*, **3**, 256 (1950).

(4) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(5) H. Fraenkel-Conrat and J. Fraenkel-Conrat, *ibid.*, **5**, 1409 (1951).

(6) H. Fraenkel-Conrat, Ciba Conference, Chemical Structure of Proteins, London, Dec., 1952.

(7) Preliminary results of H. K. Schachman and R. T. Hersh indicate values such as S^{w}_{20} = 1.2 for 0.64% TMV in 1% sodium dodecyl sulfate (0.01 *M* cacodylate, *p*H 6.4). See also Protein Preparation III.

(8) K. Bailey, *Biochem. J.*, **49**, 23 (1951).

(9) C. Weibull, *Acta Chem. Scand.*, **7**, 335 (1953).

(10) P. Desnuelle, M. Rovery and C. Fabre, *Compt. rend.*, **233**, 1496 (1951); J. A. Gladner and H. Neurath, *Biochim. Biophys. Acta*, **9**, 335 (1952).

(11) J. I. Harris and C. A. Knight, *Nature*, **170**, 613 (1952).

(12) W. F. Harrington and H. K. Schachman, in press.

(13) M. Sreenivasnya and N. W. Pirie, *Biochem. J.*, **32**, 1708 (1938).

(14) S. F. Velick and S. Udenfriend, *J. Biol. Chem.*, **191**, 233 (1951).

(15) G. Schramm and G. Braunitzer, *Z. Naturfschg.*, **8b**, 61 (1953).

2500 residues/mole), but we also found with both methods a host of other terminal amino acids, amounting to about 1500 residues/mole. Detailed analysis of this complex mixture of 'end groups' has been postponed until evidence be adduced for its representing anything but artefacts, due to hydrolysis of the more labile peptide bonds. Control experiments on the effect of boiling trichloroacetic acid on β-lactoglobulin and bovine plasma albumin showed that many new terminal amino acids appeared also in these proteins which in the intact state have only one N-terminal amino acid (leucine and aspartic acid, respectively).[16]

Experimental

A. **Protein Preparations.**—TMV was prepared in the usual manner by differential centrifugation, the last sedimentations being performed in distilled water. Nucleic acid was removed by various techniques: I, coagulation of the protein for 10 minutes at 100° in 0.3 M sodium chloride[17]; II, solution of the nucleoprotein in concentrated guanidine hydrochloride solution, followed by ammonium sulfate precipitation of the protein moiety; III, incubation of about 1% TMV in 1% sodium dodecyl sulfate at pH 8–9 for 24 hours (37°),[13] followed by precipitation of the protein with ammonium sulfate (0.3 saturated); the protein was reprecipitated 3 times at 0.3–0.4 saturation, once dispersed in saturated ammonium sulfate, and finally redissolved in water and dialyzed. Upon prolonged dialysis, the protein, absolutely free of nucleic acid, precipitated. (N, 16.2; P, 0.00; extinction coefficient (1 cm.) 1.2 at 280 mμ (*i.e.*, maximum), 1 mg./ml. in H_2O.) Ultracentrifuge analyses, kindly performed by H. K. Schachman and W. F. Harrington, indicated values of S^w_{20} 1.2 to 1.6 for the main component in 1–3% solution in water (pH 8–9.9) or 0.1 M SDS. The marked tendency of the preparation to aggregate is shown by the presence of varying amounts (always less than one-third) of a heavy component (S^w_{20} 6.5–9.8). In 0.01 M borate, the material was all aggregated (S^w_{20} 12.9 and 15.3). Sodium chloride could not be used in the sedimentation runs since it tended to cause aggregation and precipitation.

B. **Search for End Groups and Determination of Reactive Lysine ϵ-Amino Groups with FDNB.**—Reaction was generally performed in the classical manner in bicarbonate with alcoholic FDNB,[2a,b] followed by isolation of the insoluble dinitrophenyl (DNP)-protein by the usual washing with water, alcohol and ether. At times entire reaction mixtures were repeatedly washed with ether, acidified, further washed with ether and directly subjected to hydrolysis in 6 N HCl without isolation and transfer of the DNP-protein. This technique, to be referred to as "direct" gives with insulin and other proteins[18] results in agreement to those obtained with the isolated DNP derivative, while obviating the need to determine the protein content of the derivative.

The reaction with FDNB was also often performed in aqueous solution (5% bicarbonate) for 3 hours at 40°, keeping the solution saturated with FDNB by means of continuous stirring. Reaction generally proceeds to the same extent under these as under the classical conditions, but the products often remain water soluble. TMV also reacted in aqueous solution held at pH 9 or 10 by repeated additions of alkali. After decantation from excess FDNB, the reaction mixtures were usually dialyzed thoroughly. The products were isolated either by lyophilization or by precipitation with alcohol, followed by washing with alcohol and ether.

After hydrolysis of 10–25 mg. of DNP-protein by refluxing with 6 N HCl (3 times redist.) for 8 or 16 hours, the ether extract of the hydrolysate which should contain the DNP derivatives of all N-terminal amino acids except histidine, arginine and proline, showed almost no color. Upon chromatography on columns prepared from Mallinckrodt silicic acid (for Chromatographic Purposes) (dried at 110° and mixed with half its weight of water) with chloroform followed by 1% butanol–chloroform as solvents, the only definite yellow band moved more rapidly down the column than does any DNP-amino acid, and proved to behave like dinitroaniline upon chromatography on paper,[19,20] and in the ratio of its extinction coefficient at 390/350 mμ (in 1% bicarbonate: 0.57, compared to 0.66–0.71 for DNP amino acids, 0.87 for dinitrophenol).[21] When 40–60 mg. of the DNP derivative of the nucleic-acid free protein was analyzed, two slower yellow bands could be detected (R_f 0.47 in 1% butanol for the faster, 0.68 in 5% butanol in chloroform for the slower). After evaporation of the organic solvent and solution in 4 ml. of 1% bicarbonate, the yellow color was read in the Beckman spectrophotometer, using 15,800 as the molar extinction coefficient for all DNP-amino acids (except proline). The color of the 2 faint bands amounted to only 0.04 and 0.02 μmole, equivalent to a total of about 75 residues per mole TMV (mol. wt. 50 million). Also upon 2-dimensional paper chromatography,[22] 2 faint spots were apparent, apart from dinitrophenol, which resembled DNP-serine and DNP-glutamic acid and amounted to about 50 and 30 residues per mole TMV; the dinitrophenol was equivalent to approximately 200 residues (Table I). When known amounts of DNP-serine, -glutamic acid, -alanine and ϵ-DNP-lysine were refluxed for 16 hours in 6 N HCl in presence of TMV or DNP-TMV, the recoveries ranged from 63–77%.

The aqueous fraction of the hydrolyzate should contain ϵ-DNP-lysine and any DNP-arginine and di-DNP-histidine that might have arisen from these amino acids, had they been N-terminal. ϵ-DNP-lysine was the only yellow component that could be identified with a variety of chromatographic methods (paper chromatography according to Blackburn,[19] or with 92% acetone–8% 3 M acetic acid; HCl–silicic acid columns,[2a,b] as well as buffered columns[23]). In all systems, there was some diffuse yellow material, and it proved impossible to free the ϵ-DNP-lysine band of hydrolyzates of TMV from some contaminant which absorbed strongly at 300 mμ, and still appreciably at 350 mμ, so that the ratio of its extinction at 390/350 mμ was often 0.5–0.6 instead of 0.71 for pure ϵ-DNP-lysine. This contaminant was absent from nucleic-acid free preparations and from TMV nucleoprotein if it reacted with FDNB in aqueous bicarbonate, but not at pH 10.[24]

The ϵ-DNP-lysine was usually isolated by means of HCl–silicic acid columns with 66% methyl ethyl ketone–ether as solvent,[2a,b] redissolved in N HCl and read at 390 mμ, using 10,000 as the molar extinction coefficient of ϵ-DNP-lysine. The analyses indicated the presence of about 2400 residues of ϵ-DNP-lysine in DNP-TMV (Table I). Thus a considerable part, apparently about 60%, of the 1.5% lysine of TMV[25] did not yield ϵ-DNP-lysine quite in contrast to most proteins. This was the case also with TMV denatured in various ways, as well as with the nucleic acid-free protein of low molecular weight (prepn. III). The deficit in ϵ-DNP-lysine thus seemed not due to non-reactivity of the amino groups blocked by ionic fixation to the nucleic acid or complex folding of the sub-unit chains that make up the virus molecule. This problem is under further study.

C. **The Presumed Presence of N-Terminal Proline.**—DNP-Proline is known to be quite unstable during acid hydrolysis.[3] Although some yellow fraction—similar to DNP-proline in R_f, though chromatographically not homogeneous—can be found after 2 hours heating to 103° in 12 N HCl, none of this material actually represents unchanged

(16) Leucine was found N-terminal in β-lactoglobulin by R. R. Porter (*Biochim. Biophys. Acta*, **2**, 105 (1948)); aspartic acid in serum albumin by P. Desnuelle, M. Rovery and C. Fabre (*Compt. rend.*, **233**, 987 (1951)) and others. The recent claim of L. E. McClure, L. Schieler and M. S. Dunn (THIS JOURNAL, **75**, 1980 (1953)) that there are many different N-terminal residues in serum albumin could not be confirmed by us by either of the methods used.

(17) S. S. Cohen and W. M. Stanley, *J. Biol. Chem.*, **144**, 689 (1942); C. A. Knight, *ibid.*, **197**, 241 (1952).

(18) H. Fraenkel-Conrat and R. R. Porter, *Biochim. Biophys. Acta*, **9**, 557 (1952).

(19) S. Blackburn and A. G. Lowther, *Biochem. J.*, **48**, 126 (1951).

(20) G. Biserte and R. Osteux, *Bull. soc. chim. biol.*, **33**, 50 (1951).

(21) Dinitrophenol is not colored under these conditions. It precedes the dinitroaniline on the column and thus can be separated.

(22) Unpublished results of A. L. Levy.

(23) S. Blackburn, *Biochem. J.*, **45**, 579 (1949).

(24) The contaminant resembled, but was not identical with, O-DNP-tyrosine, which could be separated cleanly from DNP-lysine on columns. It appears probable that it was a decomposition product of a DNP-purine or pyrimidine.

(25) See Table I.

TABLE I

DINITROPHENOL AND ε-DNP-LYSINE FOUND IN HYDROLYZATES[a] OF VARIOUS DNP-PROTEIN PREPARATIONS

	Dinitrophenol equivalents		DNP-Lysine, equivalents/mole
	Per 10^4 g.	Per mole[b]	
DNP-Lysosyme	0.03	0.05	5.0
DNP-Lysosyme + DNP-proline	.7	1.0	5.0
DNP-Bov. serum albumin	.004	0.03	53
DNP-Bov. + DNP-proline	.07	.5	54
DNP-β-Lactoglobulin	.025	.1	22
DNP-β-Lactoglobulin + DNP-proline	.4	1.6	22
DNP-TMV[c]	.04	180	2200
	.04	200	2600
DNP-TMV-protein (SDS, 37°)	.1	470	2400
	.04	180	2500
	.05	250	2200
DNP-TMV-protein (TCA 100°)	.4	2100	2900
	.3	1700	2700
	.6	3000	3400

[a] Refluxed for 16 hours with 6 *N* HCl. About 10 mg. of DNP-protein (thoroughly washed) was used, and 0.4 μM. DNP proline). [b] The molar ratio of dinitrophenol found to DNP-proline added was about 0.8, 1 and 0.7 in the 3 experiments listed. [c] TMV reacted in saturated guanidine hydrochloride or 1% dodecyl sulfate solution gave similar results.

DNP-proline, as indicated by the marked change in its absorption spectrum (ratio of extinction coefficient 390/360 mμ-1.9 for pure DNP-proline, 0.66–0.71 for the products formed from DNP-proline upon treatment with acid, the same as for other DNP-amino acids). Thus, the DNP-method is unsuited for the detection and estimation of N-terminal proline. However, we could confirm the findings of Schramm and Braunitzer[15] that added DNP-proline yielded dinitrophenol in the course of hydrolysis of various DNP-proteins. Thus, if extreme care is taken in removing all dinitrophenol formed during the reaction with FDNB by very prolonged washing with water and alcohol, or by prolonged dialysis, followed by washing with alcohol, then it may be possible to use the presence of much dinitrophenol in the hydrolyzate as an indication of the presence of N-terminal proline.[26] However, TMV and nucleic-acid free TMV-protein yielded DNP derivatives which, after dialysis and washing, gave rise to only small amounts of dinitrophenol (Table I). This may be regarded as indirect evidence for the absence of appreciable amounts of N-terminal-proline in this protein (for direct evidence see next section).

D. Search for End Groups with Phenyl Isothiocyanate (PTC).—PTC has been proposed by Edman[4] as a reagent for the stepwise degradation of peptides from the amino end. After suitable modification the method has been found applicable to the stepwise degradation of proteins.[5] It can be employed as an end group method, and has yielded data in accord with those obtained with the FDNB method for several proteins.[6] It is of particular advantage with proteins containing terminal glycine and proline.

The technique used for most of the experiments was as follows: 5–50 mg. of protein, in 4 ml. of 50% aqueous pyridine or preferably dioxane solution[27] was stirred at 40° with 0.1 ml. of PTC for 2 hours, maintaining an approximate *p*H 9 by dropwise addition of alkali (a magnetic stirrer, *p*H meter and infrared lamp are all that is required). The solution was then repeatedly extracted with benzene. Dissolved benzene was removed with a stream of air, and the aqueous phase then evaporated to dryness in a desiccator. The residue was redissolved in water (most phenylthioureido-proteins are soluble on the alkaline side of the isoelectric point) and brought to *N* HCl by addition of one fifth volume of 6 *N* HCl. If end group analysis, and not stepwise degradation, was the object, then the treatment with acid was performed at 103° (1 hr.). The suspension was then extracted 3 times with ether, the ether washed twice with little water, and its absorption read in the spectrophotometer over the range of 260–280 mμ. The ether solution containing the phenylthiohydantoins (PTH's) of all N-terminal amino acids except arginine, histidine, serine, cysteine and possibly threonine, should show a maximum between 267 and 272 mμ.[5,6,28] Proline PTH has a maximum closer to 273 mμ. The molar extinction coefficients of all PTH's are near 16,000. Blank experiments show low absorption without a maximum over the above range—the absorption decreasing steadily over the range.

When this technique was used with TMV (20–60 mg.) the ether extracts contained small amounts of phenylthiohydantoins, which in 12 experiments averaged 320 residues/mole (range, 200–370). Analyses of preparations freed from nucleic acid by means of sodium dodecylsulfate generally yielded ether extracts showing decreasing absorption over the range 260–275 mμ, without the definite maximum near 270 mμ characteristic of phenylthiohydantoins, yet higher than obtained without protein. This relatively high background absorption, due probably to the great amounts of protein used can be expected to introduce a positive error in those experiments where a maximum was observed at about 270 mμ. Thus, all these analyses appear to set an upper limit of about 300 PTH residues per mole TMV. When known amounts of alanyldiglycine (1 μM.) were treated with PTC alone and together with TMV the amounts of alanyl-PTH formed by *N* HCl at 100° from the peptide alone were 80% of the calculated, and the PTH in excess of this value formed in presence of TMV corresponded to 130 N-terminal residues/mole.[29]

E. The Effect of Hot Trichloroacetic Acid on TMV.—Schramm and Braunitzer in their study of the N-terminal groups of TMV, employed only one type of preparation throughout.[15] This was TMV-protein freed from nucleic acid by means of heating for 30 minutes in a boiling water-bath in the presence of 5% trichloroacetic acid (TCA). To test the effect of this treatment we prepared some TMV-protein according to Schramm's technique. Upon treatment of this material with FDNB, and analysis in the usual manner, much dinitrophenol was found after 16 hours of hydrolysis (Table I) which confirms the finding of Schramm and suggests the presence of N-terminal proline in this preparation. However, we found not only dinitrophenol, but also definite though somewhat variable amounts of stable DNP-amino acids in the hydrolyzates of such preparations. At least 8 fractions were isolated either by combined column and paper chromatographic techniques, or by two dimensional paper chromatography. Some of the bigger fractions were definitely identified (glutamic acid, threonine, serine). The total amount ranged from 900–2100 residues/50 million g. The extent of reaction of the lysine with FDNB was slightly higher than in the other TMV-preparations (Table I).

Analyses of the DNP derivatives of serum albumins and β-lactoglobulin samples which had been heated with TCA to 100° for 30 minutes showed very similar pictures with much dinitrophenol and at least 7 different amino acids (serine, threonine, phenylalanine, etc.) in addition to the one characteristic of these proteins (aspartic acid and leucine, respectively).[15] The new end groups amounted to about 0.6 and 1.3 residues, respectively, per mole of the original proteins, and the dinitrophenol which may have arisen from DNP-proline to about 1.5 residues/mole. Thus it appears that treatment with hot TCA should be avoided in the preparation of proteins for end group analysis.

The findings with the FDNB-method in regard to the action of trichloroacetic acid were confirmed with PTC. The

(26) Since some dinitrophenol is formed also from other DNP-amino acids (M. B. Williamson and J. M. Passmann, *J. Biol. Chem.*, **199**, 121 (1953)), no exact quantitative relationship can be expected.

(27) M. Ottesen and A. Wollenberger, *Nature*, **170**, 801 (1952).

(28) In a modification of this technique which is being developed at this Laboratory, ring closure occurs at room temperature and is believed to yield the PTH's of all amino acids, without exception.

(29) The quantitative value of such experiments is limited, since it was found that from some peptides *e.g.*, leucylglycylalanine the PTH is split off with great ease and that this PTH is then unstable in the reaction mixture at 100°. However, in presence of protein and probably due to its acid binding capacity, the stability of the PTH is increased, and it can be isolated in good yield. This again emphasizes the need to use the gentlest possible conditions for the formation of the PTH's from proteins and peptides.[5,28]

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]

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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.

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-

(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.

(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).