A single salt-like link between protein and exchanger is not enough to explain the strength of this union. The bond between protein and exchanger is not hydrolyzed by water as one would expect from the salt of a weak acid and weak base. Multiple bonds (2) or a constellation of charges in each bond would overcome this weakness. However, one cannot overlook the fact that there are many groups on both the exchanger and the protein which could participate in hydrogen bonding. As the derivative is dried, these bonds may become even more important to the strength of this union and may increase the stability of the protein.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

Selective Cleavage of C-Tryptophyl Peptide Bonds in Proteins and Peptides

BY L. K. RAMACHANDRAN[1] AND B. WITKOP

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The application of the N-bromosuccinimide (NBS) cleavage to proteins under specified conditions releases new N-terminal residues. Bond cleavages generally average 20–40% and the number of new N-terminals formed corresponds to the number of tryptophans in the molecule. The results indicate the presence of Try–Lys and Try–Ala bonds in tobacco mosaic virus (TMV) protein, of a Try–Ala bond in the I-peptide from TMV protein, of a Try–Ala bond in human serum albumin, and of Try–Gly and Try–Ser bonds in bovine serum albumin. Lysozyme which contains seven tryptophans is cleaved by the reagent with much lower yields.

The traditional agents, such as acids and bases which are used for the cleavage of proteins show a moderate degree of specificity only when functional groups labilize adjacent peptide bonds. Thus, the hydroxyls of threonine and serine participate in, and facilitate, acid-catalyzed hydrolysis.[2]

The carboxyls of aspartic and glutamic acids (or the ω-carbonyls of asparagine and glutamine) exercise an accelerating influence on the breakage of neighboring peptide bonds[3] by 1,5- or 1,6-interaction.[4,5] A novel and different concept of peptide cleavage has been introduced recently[6,7]: Selective activation of inert peptide groups has been achieved by making them participants in intramolecular displacement reactions. The cleavage of the polypeptide glucagon, containing 29 amino acids, to yield the C-terminal tetra-peptide LEU–MET–ASP(NH_2)–THR served as a demonstration of the usefulness of the new method. The application to more complex peptides and proteins is reported herein.

The protein of tobacco mosaic virus (TMV) with a mol. wt. 18,270 probably contains two tryptophan residues per mole.[8] Because of analytical difficulties the possibility of three tryptophan residues has been discussed.[9] It has now been found that in 66% acetic acid the addition of six moles of N-bromoacetamide (NBA) per TMV protein sub-unit releases 0.2–0.3 mole each of alanine and lysine as new N-terminals, whereas the original protein contains no free α-amino group but is acetylated at the N-terminal.[10] The I-peptide which has the approximate composition indicated in Chart I,[11] and another fifteen-residue peptide (K 0.7-peptide)[12] constitute the only two characterized tryptophan-containing peptides isolated from tryptic hydrolysates of TMV protein. The I-peptide with 3 moles of NBA per mole of tryptophan in 66% acetic acid or with 3 moles of NBS per mole of tryptophan in aqueous 0.2 M sodium acetate–acetic acid buffer at pH 4.0 containing 0.2% sodium dodecyl sulfate (SDS) led to the cleavage of a peptide bond and the detection of 0.2 moles of *alanine* as new N-terminal. The pentadecapeptide (K 0.7 peptide) with 3 moles of NBS per mole of tryptophan was found to yield 0.4 mole of *lysine* as N-terminal. As shown in Chart I, these data indicate the presence of Try–Ala and Try–Lys bonds in TMV protein. The absence of any other major N-terminal group after cleavage with excess NBS supports the assumption that there are only two tryptophan residues in this protein.[12] From the absence of N-terminal arginine and aspartic acid one may conclude that peptide bonds attached to tyrosine carboxyls are not split[7] under the conditions used, since two of the four tyrosine sequences in TMV protein are known to be Val–Tyr–Arg- and Gly–Tyr–Asp·NH_2.[13]

Bovine and human albumins have a molecular weight in the vicinity of 70,000[14] and contain ap-

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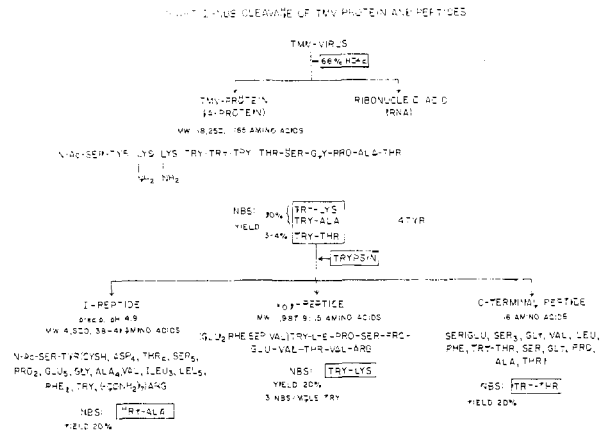

proximately two and one mole, respectively, of tryptophan residues per mole of protein.[15] Bovine serum albumin in 5.0–10.0 *M* urea solution consumed approximately 5 moles of NBS before showing any decrease in the extinction of optical density at 280 mμ, the absorption peak which changes with the oxidation of tryptophan. Since there is one titratable SH group in the protein, the initial amount of oxidizing reagent is probably utilized in the oxidation of this group. The addition of a total of 10–20 moles of NBS per mole of protein led to the release of 0.3–0.5 mole of N-terminal *glycine* and *serine* fragments. This indicates the presence of Try–Gly and Try–Ser sequences in bovine serum albumin. The absence of any further new terminal amino acids indicates again the non-participation of tyrosyl bonds in the oxidative cleavage under the chosen conditions, since eighteen tyrosyl residues are present in bovine serum albumin.[15] By the same procedure, cleavage of human serum albumin, which contains but one tryptophan residue per mole, yielded 0.3–0.4 mole of *alanine* as a new N-terminal indicating the presence of the sequence *Try–Ala* in this protein. Bovine and human serum albumins have approximately the same molecular weight and are known to contain the same N-terminal and C-terminal amino acids.[15c] A difference in the C-terminal sequence in the two has been demonstrated previously.[16] During these investigations the only N-terminal amino acid of native, performic acid-oxidized bovine serum albumin was found to be aspartic acid. This is in agreement with a recent investigation[17] which failed to confirm the reported presence of an N-terminal half-cystine.[18]

Lysozyme (mol. wt. 15,000) undergoes the cleavage reaction best in aqueous acetate–formate buffer at *p*H 4.15. The protein contains approximately seven residues of tryptophan per mole. With 3 moles of NBS per mole of tryptophan the new N-terminal amino acids appearing after the reaction indicate cleavage of a Try–Val bond in 20–30% yield, a Try-"Leu" bond in 10–20% yield, a Try–Phe bond in 5–10% yield, a Try–Arg bond in 5–10% yield. In addition, small amounts of N-terminal amino acids are found non-reproducibly and in yields <5%. It must be left open whether Try–Ser and Try-Gly sequences are present in the protein.[19,20] In preliminary experiments neither reduction of the disulfide bonds and subsequent alkylation of the SH groups with iodoacetamide[21] nor the conversion of the free amino groups to dinitrophenylamino groups improved the yields of N-terminal amino acids in the cleavage reaction. The recovery of the original N-terminal lysine as di-DNP-Lys was in many cases higher with the NBS oxidized lysozyme (0.4 mole) than with native protein (0.2 mole). The cleavage of a Try–Lys bond, possibly present, might account for this observation. To test this possibility, acetylated lysozyme was used for the cleavage reaction, but only approximately a tenth of a mole each of ε-DNP- and α-DNP-Lys were estimated (solvent system *t*-amyl alcohol–phthalate, *p*H 6.0). The identification of "α-DNP-Lys" is not certain since its R_F in a second solvent system was identical with that of ε-DNP-lys and the material could be a minor amount of a ε-DNP-Lysyl-peptide. The higher yield of di-DNP-Lys in the oxidized samples of lysozyme may be due to less decomposition during acid hydrolysis. This would be in line with the suggestion[22] that the low recoveries of N-terminal lysine as Di-DNP-Lys from lysozyme result from decomposition and side reactions with the tryptophan present in this protein.

The extent of side reactions in the NBS cleavage will depend on the nature, number and proximity of additional free or potential functional groups. The oxidation of free amino groups may assume appreciable proportions (*cf.* yields of ε-DNP-Lys from oxidized lysozyme, Table I). Thus, a fraction of the amino groups formed in the oxidative cleavage may be lost by further oxidation. The amount of oxidant in excess of that needed for complete elimination of the tryptophan absorption will determine the extent of other side reactions. With bovine albumin and hemoglobin A which take up approximately 5 moles and 20–30 moles of reagent, respectively, before a decrease in optical density at 280 mμ is detected, the oxidation of the SH groups (one[23] and eight[24] molecules per mole,

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TABLE I

CONDITIONS AND YIELDS FOR THE CLEAVAGE OF VARIOUS PROTEINS AND PEPTIDES BY N-BROMOSUCCINIMIDE (NBS) OR N-BROMOACETAMIDE (NBA) AND THE NEW N-TERMINAL AMINO ACIDS LIBERATED IN THE PROCESS

Protein	No. of tryptophan residues present in 1 mole of protein	Original N-terminal group	Medium	Moles reagent per mole tryptophan	New N-terminal groups formed moles per mole	Remarks
TMV-Protein	3[12]	N-Acetylserine	0.05 *M* sodium acetate-acetic acid *p*H 4.0 containing 0.15% SDS	4.5 NBS	Ala 0.11; traces Asp-Glu, Ser, Thr	Reaction time 20 min.
TMV-Protein	3	N-Acetylserine	12 *M* lithium acetate-acetic acid *p*H 4.15		Ala 0.04; Lys 0.03	Reaction time 20 min.
TMV-Protein	3	N-Acetylserine	70% acetic acid	6.0 NBA	Ala 0.35; Lys 0.29	Reaction time 15 min
TMV-Protein	3	N-Acetylserine	70% acetic acid	3.0 NBA	Ala 0.28; Lys 0.23 Thr 0.04	Reaction time 45 min.
I-Peptide	1	N-Acetylserine	0.02 *M* sodium acetate-acetic acid *p*H 4.0 contg. 0.15% SDS	3.3 NBS	Ala 0.18; traces Asp-Glu, Thr, Val, Ser, Phe, Leu	Reaction time 15 min.
I-Peptide	1	N-Acetylserine	70% acetic acid	3.0 NBA	Ala 0.10; Asp-Glu 0.01	Reaction time 15 min.
I-Peptide	1	N-Acetylserine	70% acetic acid	6.0 NBA	Ala 0.19; Asp-Glu 0.02	Reaction time 15 min.
Bovine serum albumin	2	Aspartic acid	7 *M* urea *p*H 4.15	10.0 NBS	Ser 0.22; Gly 0.38	Reaction time 15 min.
Bovine serum albumin	2	Aspartic acid	70% acetic acid	3.0 and 6.0 NBA	Traces Gly, Ser, Thr, Ala, Val, Phe, Ala	Reaction time 15 min.
Bovine serum albumin	2	Aspartic acid	6 *M* urea *p*H 4.15	6.0 NBA	Gly 0.05	Reaction time 15 min.
Bovine serum albumin	2	Aspartic acid	8 *M* urea *p*H 4.15	5.0 NBS	Ser 0.29; Gly 0.50	Reaction time 15 min. Protein sample was Hg dimer from Pentex Inc.
Bovine serum albumin	2	Aspartic acid	6 *M* urea *p*H 4.15	10.0 NBS	Ser 0.45; Gly 0.68	Reaction time 15 min. Protein was Hg dimer from Pentex Inc.
Bovine serum albumin	2	Aspartic acid	6 *M* urea *p*H 4.15	10.0 NBS	Ser 0.41; Gly 1.10	Reaction time 15 min. Sample from Armour
Human serum albumin	1	Aspartic acid	6 *M* urea *p*H 4.15	10.0 NBS	Ala 0.40	Reaction time 15 min. Hg dimer from H. A. Saroff
Human serum albumin	1	Aspartic acid	8 *M* urea *p*H 4.15	15.0 NBS	Ala 0.16	Reaction time 15 min. Same as above
Human serum albumin	1	Aspartic acid	8 *M* urea *p*H 4.15	20.0 NBS	Ala 0.35	Reaction time 15 min. Hg dimer from H. A. Saroff, prepared by W. L. Hughes
Human serum albumin	1	Aspartic acid	6 *M* urea *p*H 4.15	10.0 NBS	Ala 0.05	Reaction time 15 min. Same as above
Lysozyme	7	Lysine	70% acetic acid	3.0 NBA	Val 0.06; "Leu" 0.02	Reaction time 15 min.
Lysozyme	7	Lysine	70% acetic acid	6.0 NBA	Val 0.03; Phe 0.03 Ala 0.01; Ser 0.01	Reaction time 15 min.
Lysozyme	7	Lysine	6.0 *M* urea *p*H 4.15	6.0 NBA	Gly 0.04; Ser 0.03	Reaction time 15 min.
Lysozyme	7	Lysine	Formate–acetate 0.2 *M* *p*H 4.15	1.6 NBS	Val 0.01	Reaction time 15 min.
Lysozyme	7	Lysine	Formate–acetate 0.2 *M* *p*H 4.15	2.5 NBS	Val 0.06	Reaction time 15 min.
Lysozyme	7	Lysine	Formate–acetate 0.2 *M* *p*H 4.15	5.0 NBS	Val 0.05; Ser 0.02 Gly 0.15; Phe 0.01	Reaction time 15 min.
Lysozyme	7	Lysine	8 *M* lithium acetate *p*H 4.15	1.6 NBS	Val 0.02	Reaction time 15 min.
Lysozyme	7	Lysine	8 *M* lithium acetate *p*H 4.15	2.5 NBS	Val 0.03	Reaction time 15 min.
Lysozyme	7	Lysine	8 *M* lithium acetate *p*H 4.15	5.0 NBS	Val 0.02	Reaction time 15 min.
Lysozyme	7	Lysine	12 *M* lithium acetate *p*H 4.15	3.0 NBA	Val 0.02	Reaction time 15 min.
Lysozyme	7	Lysine	Formate–acetate *p*H 4.15[a]	3.0 NBA	Val 0.33; Phe 0.08 "Leu" 0.18; Arg 0.06	Reaction time 15 min.

[a] In the formate–acetate buffer, with increasing amounts of NBS, the yields of ϵ-DNP-lysine were 3.88, 3.69 and 2.73 moles per mole of protein, respectively. In 8 *M* lithium acetate the corresponding yields were 2.68, 2.46 and 1.38 moles. The untreated protein yields 4.6 moles ϵ-DNP-lysine, the amount expected from the amino acid composition being 5 moles.

respectively) may be the most rapid reaction. The oxidation of SH to $-SO_3H$ would require three moles of either NBA or NBS. With a large excess of NBA present (Table II) the oxidized hemoglobin A on hydrolysis yielded nearly 7.3 moles of cysteic acid per mole of protein. This shows that a large excess of oxidant quantitatively oxidizes SH-groups in some proteins. In preliminary experiments with other proteins the oxidation of –S–S– bonds varies and does not approach the value expected from composition. In such cases more drastic conditions of oxidation may be necessary to obtain quantitative results. Therefore, proteins with reactive SH or free amino groups pose special problems when side reactions in the oxidative peptide cleavages are to be avoided.

To summarize, under the conditions used in this investigation peptide bonds attached to the tryptophyl carboxyl in peptides and proteins are split in yields averaging 20–40%. The use of N-bromosuccinimide or N-bromoacetamide offers a specific and rapid method for fragmenting high molecular weight peptides and proteins to smaller ones. Applied in conjunction with one of the several methods available for fractionation of polypeptides and proteins the reaction should prove of value in the arduous task of determining overlapping sequences in proteins. The use of a

TABLE II

CONTENT OF CYSTEIC ACID IN HYDROLYSATES OF PROTEINS OXIDIZED WITH NBA

Protein	Ribo-nuclease	Chymo-trypsin	Insulin	Lysozyme	Bovine serum albumin	Hemo-globin A	TMV protein	I-Peptide
No. of $CyHSO_3$ estimated in hydrolyzate	2.0	4.9	1.2	4.2	17.8	7.2	0.17	0.11
No. of $CyHSO_3$ expected from reported cystine and cysteine content	8.0	10	6	10	30	6–8	1	1
Moles NBA used per mole protein	33	42	27	32	124	81	16	18

largely selective chemical agent for peptide cleavage will be a welcome supplement to the enzymatic methods.

The availability of a method capable of selective modification or cleavage of a protein suggests applications to problems such as: rapid detection of species differences in the amino acid residue next to tryptophan; modification and simplification of tryptophan-containing peptide hormones; dependence of antigenic and enzymatic properties of proteins on the extent of intact tryptophan molecules present.

Experimental

Proteins.—The sources of the proteins used in this work are as indicated: lysozyme and gramicidin: Nutritional Biochemicals Corporation; chymotrypsin: Worthington CD498–501; bovine plasma albumin: Armour, Lot No. 67009; Pentex, Lot 8052; and another sample kindly supplied by Dr. H. A. Saroff; human serum albumin (Hg dimer): sample prepared by Dr. H. A. Saroff and another by Dr. W. L. Hughes, Jr., both kindly supplied by Dr. Saroff; Zn-insulin: Eli Lilly, 535664; hemoglobin A (as a 5% solution) kindly supplied by Dr. A. Murayama. TMV protein was prepared by dissociation of TMV with 66% acetic acid,[25] and the I-peptide was isolated from tryptic digests of TMV protein.[9]

Identification and Determination of N-Terminal Groups.—1-Fluoro-2,4-dinitrobenzene[26] was used for this purpose. The two dimensional system[27] and the tertiary amyl alcohol–*p*H 6 phthalate system[28] were used for the identification of DNP amino acids. The hydrolysis of DNP-proteins was done at 104–106° in 6.0 *N* HCl, using about 1 ml. of acid for 10 mg. of protein. Standard corrections for hydrolytic and chromatographic losses have been applied.

The cleavages of proteins and peptides with NBS or NBA were done in various media (see Table I for details and results). In general, after cleavage in 66% acetic acid the solvent was removed by evaporation *in vacuo*, the dry residue suspended in water at *p*H 8 and the necessary amount of reagents added. In experiments where lithium acetate–acetic acid, urea–acetic acid or sodium formate–acetic acid buffers were used the reaction mixtures were brought to *p*H 8 with addition of dilute sodium hydroxide, and then the necessary amounts of $NaHCO_3$ and fluorodinitrobenzene were added, alcohol was removed, excess reagent extracted with ether, and the DNP proteins and peptides precipitated by adjustment of the *p*H to about 2.

Tryptophan Content of Proteins.—TMV-Protein, by the decrease of O.D. at 280[6] on addition of NBS, was found to contain 1.9 moles of tryptophan per mole of protein of mol. wt. 18,270. The literature values are about 2–3 residues per mole.[8,9] The I-peptide by this method was found to contain 0.8 mole of tryptophan per mole of peptide of molecular weight 4,500, values obtained by spectrophotometry in 0.1 *N* NaOH being of the order 0.8–1.0. Bovine serum albumin has been reported to contain two residues of tryptophan per mole assuming a molecular weight of 69,000, and human serum albumin of the same molecular weight approximately 0.6 mole per mole of protein.[15] The use of NBS indicated 1.7 mole/mole tryptophan for bovine serum albumin and 0.7 mole/mole for human serum albumin. From spectrophotometry of lysozyme in 0.1 *N* NaOH the protein is found to contain 2.9 residues of tyrosine and 7.4 residues of tryptophan per mole of protein in agreement with the literature values.[29] The OD_{280} (optical density) decrease in the presence of NBS leads to a value for tryptophan of approximately 6.5 residues per mole.[30] Gramicidin is reported to contain 45% tryptophan[31] and the OD_{280} decrease with NBS indicates the presence of 42.8% of tryptophan in this material. Hemoglobin A is found to contain 5 residues per mole of protein (mol.wt. 68,000) in agreement with literature values.[15b] In these estimations the

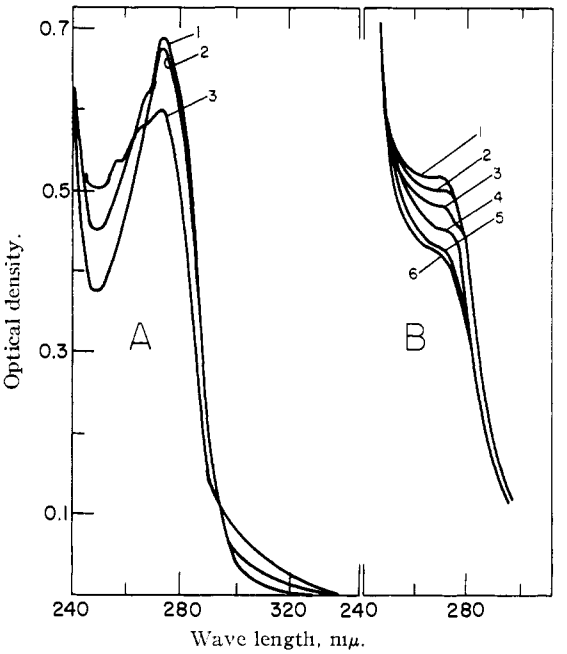

Fig. 1.—(A) Bovine serum albumin (1.65 mg.) in 1.55 ml. of 10.0 *M* urea solution at *p*H 4.15 (curve 1); after addition of 0.25 μ*M* of NBS in 25 λ of water (curve 2); after addition of 50 λ (0.5 μ*M*) of NBS (curve 3). No further decrease in OD_{280} with addition of NBS was observed. (B) human serum albumin (Hg dimer, 2.5 mg.) in 3.03 ml. 10 *M* urea solution at *p*H 4.15 (curve 1); after addition of 0.2 μ*M* of NBS in a 1 μ*M*/ml. solution in water (curve 2); 0.3 μ*M* of NBS (curve 3); 0.45 μ*M* of NBS (curve 4); 0.65 μ*M* of NBS (curve 5); 0.75 μ*M* of NBS (curve 6). The original spectrum was practically unchanged after the addition of only 0.1 μ*M* of NBS.

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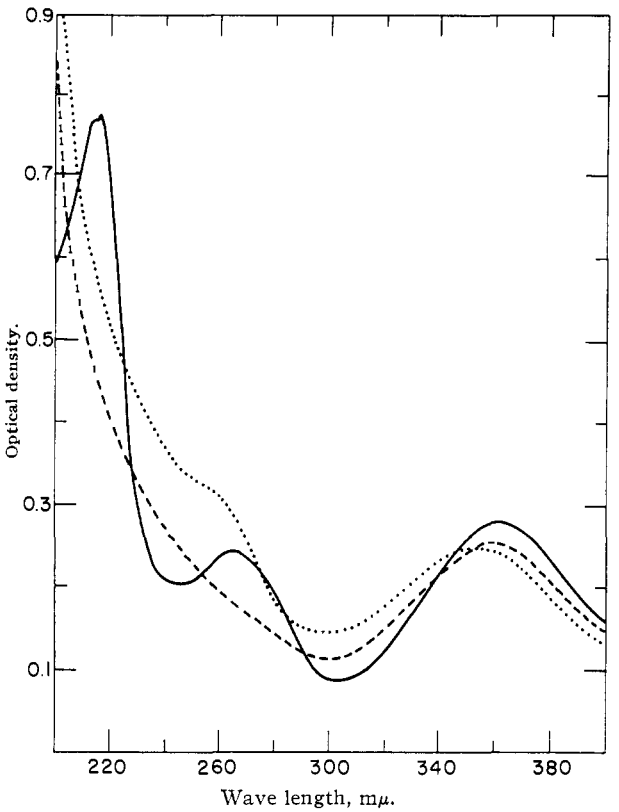
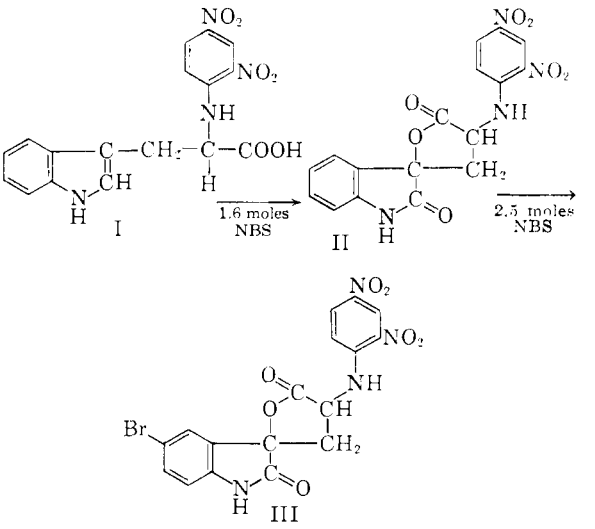


Fig. 2.—0.05 μM DNP-tryptophan in 3.03 ml. water ———; spectrum after addition of 0.08 μM NBS – – – –; spectrum after addition of 0.20 μM NBS ..... Further addition of NBS causes no change in the spectrum.

amount of tryptophan in a sample solution is calculated from the decrease in O.D. 280 mμ obtained and the use of the factor 1.31[6] and an extinction coefficient for tryptophan at 280 mμ of 5,500.

The amount of oxidizing agent taken up to obtain minimum O.D. at 280 mμ varies with the nature of the protein and the medium used. A few examples are as given: Bovine serum albumin takes about 10 moles of NBS per mole of tryptophan present in 10 M urea and human serum albumin (Hg-dimer) in 8.0 M urea 20 moles. The I-peptide in 8.0 M lithium acetate solution at pH 4.15 needs 1.95 moles of NBS, whereas in an aqueous acetate-formate buffer (0.4 M) at pH 4.15 and in the presence of 0.2% SDS 3.3 moles of NBA are required. Lysozyme in aqueous acetate–formate buffer needs 2.3 moles of NBS per mole of tryptophan, 3.0 moles of NBA in 12.0 M lithium acetate and 2.7 moles of NBA in 66% acetic acid. Typical spectrophotometric changes with bovine and human serum albumin (Hg-dimer) are depicted in Fig. 1. Figure 2 shows the changes in the spectrum of DNP-tryptophan on addition of NBS and shows that in such a model system at least two well-defined products result from the use of 1.6 and 4 moles of oxidizing agent per mole of DNP-tryptophan.[32]



Oxidation of -SH and -S-S- Groups by NBA.—Table II presents data on the number of sulfur atoms, cysteine or cystine, which are oxidized to cysteic acid when a large excess of oxidant is used under one set of experimental conditions. The oxidations were done in 0.4 M acetate-formate buffer at pH 4.15 for 10 minutes. The oxidized proteins were hydrolyzed for 24 hr. with 6.0 N HCl and the cysteic acid present in the hydrolyzates was estimated as DNP-cysteic acid. The only correction used in the calculations was for a 10% loss in paper chromatography. It is possible that with further study NBA or NBS could be used instead of performic acid[33] for the estimation of cysteine and cystine in proteins as cysteic acid.

(32) Preliminary results (elementary analyses and infrared spectra) on the isolated products (m.p. 196–198° and 248° for II; m.p. 260° (III)) would lead to tentative formulations II and III. The bromine in III is probably present in the five (or six) position.

(33) E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

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The Reversible Transformation of β-Lactoglobulin at pH 7.5[1]

BY CHARLES TANFORD, LYLE G. BUNVILLE AND YASUHIKO NOZAKI[2]

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The specific optical rotation and the titration curve of β-lactoglobulin both indicate that a reversible change in the configuration of this protein occurs at 25° near pH 7.5. A similar conclusion is reached from sedimentation data obtained by Pedersen in 1936. The configurational change parallels the titration of imidazole groups and is accompanied by the reversible release of two carboxyl groups from the interior of the molecule. The transition is a novel one in that no general unfolding of the molecule occurs: it must be regarded instead as a refolding of part of the polypeptide chains.

Pedersen,[3] in 1936, performed a thorough ultracentrifugal investigation of β-lactoglobulin in aqueous solution. He showed that there is a decrease in the sedimentation coefficient between pH 7 and 8, which, though small, lies outside the limits of

(1) Abstracted, in part, from the Ph.D. thesis of Lyle G. Bunville, State University of Iowa, 1958.

(2) On leave of absence from the National Hygienic Laboratory, Tokyo, Japan.

(3) K. O. Pedersen, *Biochem. J.*, **30**, 961 (1936).