the conversion of the enol of pyruvate to the keto acid.[20]

The rate of the enzymatic decarboxylation greatly exceeds that for the non-enzymatic process. The rate for the reaction of the enzyme–substrate complex is probably about 50,000 times as great as that observed by Graham[21] for the ferric ion–dimethyloxalacetic acid complex and exceeds the rate for the cupric ion-catalyzed decarboxylation of oxalacetic acid by a comparable factor. But the ion which activates the enzyme is Mn^{++}, and in the non-enzymatic process this ion is only $^1/_{250}$ as effective as Cu^{++}. Further, the decarboxylation is not the rate-controlling step in the enzymatic reaction and probably is faster than the rate-controlling step by a factor of at least 10. This suggests that the enzyme may promote the decarboxylation by a factor of the order of 10^8.

(20) No account has been taken of a possible role for biotin in this decarboxylation process; see H. C. Lichstein, *Federation Proc.*, **16**, 211 (1957). G. A. Hamilton and F. H. Westheimer, THIS JOURNAL, in press.

(21) R. Graham, Thesis, University of Chicago, 1953.

Acknowledgment. The authors wish to thank the National Science Foundation for financial assistance in this investigation.

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE CENTRAL RESEARCH LABORATORIES, ARMOUR AND COMPANY]

Isolation of Proteolytic Enzymes from Solution as Dry Stable Derivatives of Cellulosic Ion Exchangers

BY M. A. MITZ AND R. J. SCHLUETER

RECEIVED FEBRUARY 2, 1959

A method of isolating proteins from solution is afforded by the use of cellulosic ion exchangers. The basis of this method is a stable derivative obtained by drying the combination of protein and cellulosic ion exchanger. In this report a general method of preparation which yields an active product with a very small amount of extraneous ions is described. To demonstrate the fundamental process, six proteins and four cellulose ion exchangers are used. The acidic proteins pepsin and acylase, as well as the neutral kidney and spleen tissue proteases, combine with the anion exchanger diethylaminoethylcellulose, whereas the basic proteins, chymotrypsin and trypsin, combine with acidic forms of modified cellulose such as carboxymethylcellulose, cellulose phosphate and cellulose citrate. These cellulosic ion exchangers combined with from 1 to 50% protein by weight retain the physical characteristics of the cellulose and can be freeze dried or air dried. The protein in the dried exchanger derivatives can be solubilized under suitable acidic or basic conditions. Moreover, the desorbed enzyme compares favorably in activity with the starting enzyme.

Introduction

Since 1954, when cellulosic ion exchangers were first applied to protein chromatography,[1] many proteins have been fractionated by this type of exchanger.[2] These same exchangers should be useful in the storage, preservation and utilization of proteins, provided a protein sorbed from solution can be dried without loss in biological activity. In this report, the process of isolating proteins from solution as derivatives of cellulosic ion exchangers is described.

In most studies of protein chromatography on modified celluloses, the proteins have been sorbed from dilute buffers of low ionic strengths. Preliminary studies here have indicated that complete elimination of the buffer increases the amount of protein sorbed; the less buffer and salt in the protein solution, the more sites available for protein sorption. It is possible to form protein–exchanger combinations free of inorganic ions which might be a source of trouble on drying as well as a source of contamination. In this study, therefore, the concentration of inorganic salts has been kept as low as possible.

For several reasons, proteolytic enzymes were chosen as representative proteins for this study. They are readily available in reasonably pure form, are well characterized and can be assayed both by enzyme activity and by ultraviolet absorption. There are also wide variations in the isoelectric points and *p*H requirements for stability among this group of enzymes.

Which cellulosic ion exchanger to use in isolating a specific enzyme depends in part on the acidity or basicity of the enzyme. Most enzymes, except very basic ones like lysozyme, will react with the weakly basic cellulosic anion exchangers,[3] and basic enzymes will also react with cellulosic cation exchangers. For enzymes sorbed by either type, the choice can be based on facilitating characteristics of the enzyme or the product.

The preparations of the following combinations of enzyme and cellulosic ion exchanger were studied; **pepsin** with DEAE-cellulose[4] hydrochloride; **acylase** with DEAE-cellulose; **bovine kidney and spleen proteases** with DEAE-cellulose; **trypsin and chymotrypsin** with CM-cellulose, cellulose citrate and cellulose phosphate.

Experimental

Cellulosic Ion Exchangers.—DEAE-cellulose was prepared by the method of Peterson and Sober[5] from 2-chlorotriethylamine hydrochloride and α-cellulose. It had an exchange capacity of 0.8 meq./g.

Five cellulosic cation exchangers were used: cellulose phosphate (1.15 meq./g.) was prepared by method No. 11 of Jurgens, *et al.*[6]; cellulose citrate (2.01 meq./g.) was a

(1) H. A. Sober and E. A. Peterson, THIS JOURNAL, **76**, 1711 (1954).

(2) H. A. Sober and E. A. Peterson, "Ion Exchangers in Organic and Biochemistry," Interscience Publishing Co., Inc., New York, N. Y., 1957, Chap. 16, p. 327.

(3) M. A. Mitz and S. S. Yanari, THIS JOURNAL, **78**, 2649 (1956).

(4) The abbreviations used are DEAE-cellulose for diethylaminoethylcellulose and CM-cellulose for carboxymethylcellulose.

(5) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(6) J. F. Jurgens, J. D. Reid and J. D. Guthrie, *Textile Research J.*, **18**, 42 (1948).

research sample (Eastman Lot No. X-1987-35); CM-cellulose Lot A (0.62 meq./g.) was prepared by the method of Ellis and Simpson[7]; CM-cellulose Lot B (0.31 meq./g.) was made from Johnson and Johnson 1/2 inch bandage by the same procedure as CM-cellulose Lot A; and CM-cellulose Lot C (0.18 meq./g.) was made by the procedure of Peterson and Sober, using a low ratio of chloroacetic acid to cellulose to yield this low order of substitution.

Proteins.—The proteins used were crystalline pepsin, crystallized trypsin, crystallized chymotrypsin, porcine renal acylase I[8] and bovine kidney and spleen proteases. All except the tissue proteases were commercial preparations. The proteases were prepared by ammonium sulfate fractionation[9] and were dialyzed free of salt.

Methods.—Protein concentrations were determined from the specific extinction coefficients at 280 mμ using an extinction coefficient of 1.4/cm./mg./ml. for pepsin, of 1.0 for tissue proteases, of 2.11 for chymotrypsin, of 1.42 for trypsin and of 0.974 for acylase.

The specific activity of each enzyme after treatment with a cellulosic ion exchanger was compared with the initial specific activity. Anson's hemoglobin protease assay (D_{280}/mg. protein/10 min.) was used for pepsin (at *p*H 1.8) and for the tissue proteases (at 3.5). A spectrophotometric method was developed and used for acylase.[10] Acylase specific activity was calculated in terms of micromoles of acetyl-L-methionine hydrolyzed per hour per milligram of protein. The esterase activities of chymotrypsin and trypsin were determined on synthetic substrates, chymotrypsin on acetyl-L-tyrosine ethyl ester and trypsin on *p*-toluenesulfonyl-L-arginine methyl ester. Chymotrypsin and trypsin specific activities were defined as millimoles of ester hydrolyzed per minute per milligram of protein.

Procedure.—The following general method was used for the formation, washing, drying and dissolution of the protein cellulose derivatives: At 1°, the cellulosic ion exchanger was stirred with the protein solution for 15 minutes and then centrifuged. After centrifugation, the supernatant solution was removed leaving the wet derivative which was immediately re-suspended in 10 volumes of distilled water. After 5 minutes the insoluble derivative was centrifuged and separated from the supernatant solution. The process of washing was repeated until no protein was detected in the wash solution. Once free of unreacted materials the derivatives were suspended in a small volume of water, shell frozen and freeze dried. In order to solubilize the protein, the dried derivative was suspended in the appropriate eluting solution for 15 minutes and, after centrifugation, the protein solution was separated from the exchanger by decantation. Several minor variations in the general procedure are described later.

Results

DEAE-cellulose Derivatives.—In Table I are listed the results of typical over-all experiments with pepsin, acylase and kidney and spleen proteases on DEAE-cellulose, using the best conditions found for sorption and desorption. A single treatment with an equal weight of exchanger removed most of the protein from solution (column I). In the next column (II) are the results of desorption after drying the combination. If these results are considered with the initial and final specific activities (column III and V), it can be seen that most of the total activity was recovered from each of the four derivatives. Furthermore, the protease DEAE-cellulose combinations were active as such (column IV). Although the pepsin derivative showed only one-third of the activity present, the acylase and the tissue proteases demonstrated almost full activity.

(7) H. Ellis and M. E. Simpson, *J. Biol. Chem.*, **220**, 939 (1956).

(8) S. M. Birnbaum, L. Levintow, T. B. Kingsley and J. P. Greenstein, *ibid.*, **194**, 455 (1952).

(9) J. S. Fruton and M. Bergmann, *ibid.*, **130**, 19 (1939).

(10) M. A. Mitz and R. Schlueter, *Biochem. Biophys. Acta*, **27**, 168 (1958).

TABLE I

SORPTION AND DESORPTION OF PROTEIN–DEAE-CELLULOSE DERIVATIVES

Protein	I % Protein[a] sorbed	II % Protein[d] desorbed	III Specific activity of protein: Initial	IV As Derivative	V Final
Pepsin	88[b]	97	28.9	8.3	29.2
Acylase	69	62.6	846	1700	2035
Kidney protease	70[c]	96	0.076	0.07	0.065
Spleen protease	91[c]	89	0.127	0.11	0.114

[a] To a solution of 10 mg. protein/ml. was added a weight of exchanger equal to the weight of the protein. The percentage of protein sorbed was determined from the differences in initial and final protein concentration. [b] Sorbed by the hydrochloride form of the exchanger. It was prepared by first treating 100 mg. of the free-base form of DEAE-cellulose with 10 ml. of 0.01 *N* hydrochloric acid and then washing with two 10-ml. portions of distilled water. [c] After dialysis of the protein solution–DEAE-cellulose admixture. This was accomplished as follows: the cellulosic ion exchanger suspended in the protein solution was placed in a Visking casing and dialyzed against 3 changes of ten volumes each of distilled water over a period of 3 hr. [d] Eluting solutions used were as follows: for pepsin 0.03 *N* hydrochloric acid; for acylase 0.5 *M* phosphate, *p*H 7; for kidney and spleen proteases 0.015 *M* phosphate, *p*H 7. Values represent percentages of protein originally sorbed.

In the preparation of the pepsin derivative and its dissolution, acid was important. As seen in Fig. 1, without acid the *p*H of the mixture of pepsin and DEAE-cellulose (free-base form) was 6 and

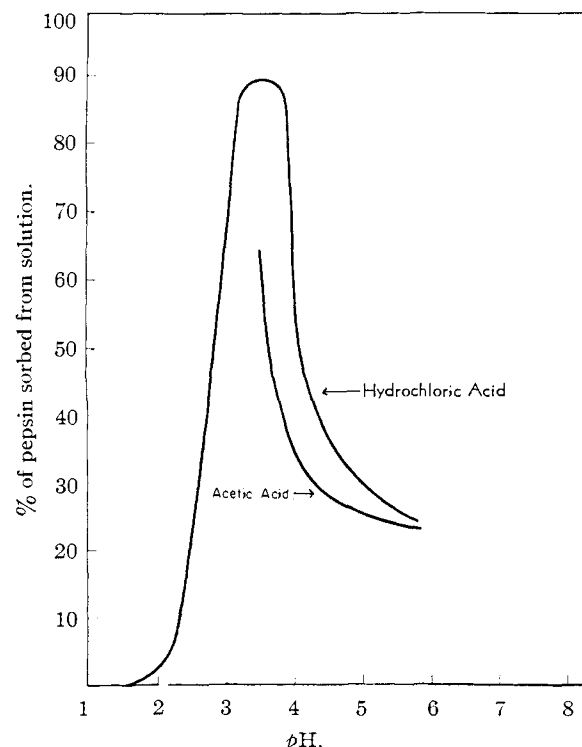

Fig. 1.—One hundred mg. DEAE-cellulose/100 mg. pepsin/10 ml. solution.

only one-fourth of the pepsin was sorbed. Increased amounts of pepsin were sorbed as the *p*H of the mixture was shifted from 6 to *p*H 3.5 where maximum sorption was reached. As the *p*H was

decreased further, the amount of pepsin sorbed on DEAE-cellulose decreased until at *p*H 2 no pepsin was sorbed. At *p*H 3.5 hydrochloric acid was more effective than acetic acid in causing pepsin to react with the exchanger. Since the use of the hydrochloride form of the exchanger was as effective as the use of acid in the reaction solutions and much more convenient, this form of the exchanger was used to prepare the pepsin derivatives.

A variation of the general method was useful for the sorption of some of the other proteins. It consisted of dialyzing the exchanger–protein solution admixture against distilled water. Under these conditions (Table II) the amount of kidney protease reacting with 100 mg. of exchanger increased from 47 to 70 mg. Even the high reaction ratio of spleen protease to exchanger was increased slightly by dialysis. On the other hand, the acylase reaction was unaffected by the dialysis treatment.

Table II

Sorption of Proteolytic Enzymes by DEAE-Cellulose

Enzyme	% Protein sorbed[a] Without dialysis	On dialysis
Kidney protease	47	70
Spleen protease	88	91
Acylase	69	69

[a] In all cases 100 mg. of protein in 10 ml. of distilled water was mixed with 100 mg. of DEAE-cellulose. Unreacted protein was determined in the solution and the percentage sorbed was calculated by difference.

Examination of the acylase reaction showed evidence of non-homogeneity in the protein. In Table I, a twofold increase in specific activity of the acylase recovered from the DEAE-cellulose over the initial acylase suggested a purification of the enzyme somewhere in the over-all process. So a separate experiment was performed to determine the nature of this enrichment. As indicated in Table III, the sorbed protein gained specific activity at the expense of the unsorbed protein. Another increase in specific activity was found on desorption. Some of the protein was not recovered from the exchanger.

Table III

Sorption and Desorption of Acylase on DEAE-Cellulose

Protein	Mg. protein	Specific activity	Total units	% Protein	% Activity
Initial	88.2	1480	130,500	100	100
Unsorbed[a]	44.0	1010	44,410	50	34
Sorbed[b]	44.2	1977	86,090	50	66
Desorbed[c]	15.4	3665	56,420	18	42.5
Recovered[d]	59.4		100,830	68	76.5

[a] Eighty mg. of DEAE-cellulose was reacted with 88.2 mg. of protein in 10 ml. of distilled water. [b] Calculated by difference between "Initial" protein and "Unsorbed" protein. [c] Desorbed with 10 ml. of 0.1 *M* phosphate buffer at *p*H 7. [d] Calculated by addition of the "Unsorbed" protein and "Desorbed" protein. The protein not "Recovered" (28.8 mg.) was still on the exchanger.

Analysis of the solution in the sorption step also showed the stability of the protein derivative to distilled water. Advantage is taken of this property to remove unreacted protein. Although the protein-exchanger combination separated from the bulk of the reaction solution on centrifugation, it nevertheless retained ten times its weight of water, which of course contained unreacted protein. Washing with distilled water diluted out the unsorbed material without eluting the protein from the derivative. A good example of this effect was one in which only half the acylase protein in solution reacted with the exchanger (Table IV). Since the precipitate occupied a little over 1 ml. and the final volume of reaction and subsequent washing solutions was kept at 10 ml., a one-to-ten dilution of the unreacted protein was expected between the reaction solution and the first wash. A second and third wash should each yield a concentration of protein $^1/_{10}$ as high as the previous solution. The expected results were obtained.

Table IV

Washing the Acylase–DEAE-Cellulose Derivative[a]

	Vol., ml.	Mg. protein/ml. soln.
Initial protein soln.	10	8.76
Supernatant of DEAE-cellulose reaction	8.9	4.40
Acylase–DEAE-cellulose (wet)	1.1	..
1st distilled water wash	8.9	0.35
2nd distilled water wash	8.9	0.04
3rd distilled water wash	8.9	<0.005

[a] Eighty-eight mg. of exchanger was added to 10 ml. of the acylase solution. After 15 minutes, the mixture was centrifuged and the clear supernatant replaced by an equal amount of distilled water. The wash solution was separated from the derivative and the process of washing repeated.

After washing, the precipitates were readily shell frozen and freeze dried. Most of the dry materials were as white as the cellulosic ion exchanger used. Some of the derivatives, such as of acylase and certain pepsin preparations, were colored; the shade and intensity of this color was related to the color of the initial protein preparation. All the protein derivatives retained the fibrous nature of the cellulose. None of the preparations showed any signs of being hygroscopic. On the contrary, several pepsin derivatives were air dried in the cold room. Although this process was slow, requiring several days, it compared favorably with the faster freeze drying process in most respects. For example, the specific activities of air-dried and freeze-dried derivatives from the same pepsin preparation were 30.2 and 29.6, respectively.

Solutions which removed the protein from the exchanger were not difficult to find. As was expected from the sorption study in Fig. 1, pepsin was solubilized by dilute hydrochloric acid at *p*H 1 to 2. At *p*H 7, the tissue proteases were removed from the exchanger with as dilute as 0.005 *M* phosphate buffer, the acylase 0.05 *M* phosphate. Salt could also be used to remove tissue proteases or acylase from their derivatives, but its use resulted in a drastic shift in *p*H to 9.5. Combinations of salt and buffer avoided this shift but offered no advantage over the use of buffers alone.

Several pepsin–DEAE-cellulose derivatives were prepared and studied using anion exchangers with a capacity of 0.4 meq./g. instead 0.8 meq./g. The over-all results were the same as with the more highly substituted cellulose except for the fact that only half as much pepsin reacted with the exchanger.

Cation Exchanger Derivatives.—In Table V are listed the results of typical over-all experiments with trypsin and chymotrypsin with several cation exchangers using small protein-to-exchanger ratios. In the sorption step 5 mg. of trypsin or 5 mg. of chymotrypsin dissolved in 10 ml. of distilled water was interacted with 100 mg. of the free-acid form of the CM-cellulose, the cellulose citrate or the cellulose phosphate. The amounts of trypsin and chymotrypsin sorbed vary with the type of modified cellulose used: cellulose citrate shows the lowest degree of sorption; carboxymethylcellulose gauze (CM-cellulose Lot B) is next in order of increasing amounts sorbed; and Lot A of the carboxymethylcellulose preparation has the highest degree of sorption. In all cases the final *p*H of the reaction was 4 to 5 without adjustment. As in the case of the DEAE-cellulose derivatives, sorbed protein was not eluted with distilled water and the precipitates could be either freeze dried or air dried. Even after three months in the refrigerator, these dried enzyme derivatives yielded most of their protein to a solution containing 0.05 *M* phosphate and 0.1 *M* sodium chloride at *p*H 7.9. In the amount of protein sorbed as well as in the specific activity of the recovered enzyme, CM-cellulose Lot A performed best.

TABLE V

SORPTION AND DESORPTION OF CHYMOTRYPSIN- AND TRYPSIN-MODIFIED-CELLULOSE DERIVATIVES[a]

Exchanger	Trypsin: % Protein Sorbed	Trypsin: % Protein Recovered	Trypsin: Final specific activity	Chymotrypsin: % Protein Sorbed	Chymotrypsin: % Protein Recovered	Chymotrypsin: Final specific activity
Cellulose phosphate	90.5	83	0.190	91.0	96	0.306
Cellulose citrate	21.5	80	.192	16.3	97.7	.238
CM-Cellulose Lot A	97	93	.268	98.5	89.6	.310
CM-Cellulose Lot B	61.7	97	.207	59.1	87.5	.267
CM-Cellulose Lot C	90.5	99	.212	91.6	91.0	.280

[a] One hundred mg. of modified cellulose (free-acid form) was mixed with 10 ml. of solution containing 5 mg. of protein. After 15 minutes the derivative was separated and washed with distilled water. All the derivatives were freeze dried except that of CM-cellulose Lot B which was air dried. After three months the dried derivatives were eluted at 0° with a solution containing 0.1 *M* sodium chloride and 0.05 *M* phosphate at *p*H 7.9. The average elution *p*H became 7.5. The supernatants from these elutions were assayed for specific activity at 25°. The specific activity value of the initial chymotrypsin was 0.311 μM per minute per mg. of protein; of the initial trypsin, 0.250 μM per minute per mg. of protein.

Slightly larger amounts of chymotrypsin could be sorbed than are shown in the comparative study in Table V, if the reaction mixture was maintained at *p*H 5. At this *p*H, 100 mg. of CM-cellulose Lot A sorbed 7 out of 8.4 mg. of protein in 10 ml. of solution. However, when still larger amounts of chymotrypsin (or trypsin) were reacted with the CM-cellulose, the amount of protein sorbed was not significantly increased.

Discussion

These stable protein-cellulosic-ion-exchanger combinations provide a general means of isolating proteins from solution which is both mild and efficient. Widely different proteins, including crude enzyme preparations, form these derivatives, and despite great differences in *p*H stability these proteins retain biological activity. In part, this is due to the fact that the *p*H at which these derivatives form coincides with the *p*H at which the proteins are most stable.

The characteristics of the product are determined mainly by the modified cellulose used to make the derivative. Although the protein content of these preparations varies from less than 1% to 50% by weight (see compilation in Table VI), the insoluble fibrous nature of the cellulose is dominant in the product. In this regard, the fact that solutions of low salt concentration favor the formation of the derivative is important. Proteins isolated in this way have few contaminating inorganic ions. Furthermore, washing with distilled water removes unreacted proteins from the derivatives without eluting the reacted protein. The result is a "clean" derivative which not only is readily dehydrated but also is not hygroscopic.

Although these enzyme derivatives can be used *per se*, the preferable procedure is to displace the protein from the exchanger. Over-all recovery is high from preparations with either small or large amounts of protein. The fact that the protein can be desorbed under conditions favorable to the enzyme is responsible for this high yield. For example pepsin, which is unstable at *p*H's above 5,[11] is desorbed by acid, whereas acylase, which is unstable at *p*H's below 6,[8] is eluted at neutral *p*H. Another factor which favors the usefulness of the method in enzyme work is the rapidity at which the reaction is completed even at near-freezing temperatures.

TABLE VI

PROTEIN CONTENT OF CELLULOSIC DERIVATIVES

TYPICAL VALUES

Derivative	% Protein by wt.
Pepsin–DEAE-cellulose	47
Acylase–DEAE-cellulose	41
Kidney protease–DEAE-cellulose	41
Spleen protease–DEAE-cellulose	47
Trypsin-CM-cellulose	5
Trypsin–cellulose citrate	1.5
Chymotrypsin-CM-cellulose	6-7
Chymotrypsin–cellulose citrate	0.8

The rapidity of sorption and desorption along with other results indicates that the primary but not necessarily the only forces in the derivatives are salt-like bonds. This must be true to account for the fact that the amount of protein bound is roughly proportional to the number of charges on protein and exchanger. A simple calculation shows that 1 g. of DEAE-cellulose, exchange capacity 0.8 meq./g. could react with as much as 0.8 g. of pepsin of equivalent weight 1000.[11] Then twice as much pepsin should react with the exchanger if half the pepsin carboxyl groups were uncharged or if the exchanger capacity were doubled. Both were found to be true.

(11) J. H. Northrop, M. Kunitz and Harriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948, p. 964.

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

RECEIVED JUNE 1, 1959

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-

(1) Visiting Scientist of the USPHS on leave of absence from the Virus Laboratory, University of California, Berkeley.

(2) (a) P. Desnuelle and A. Casal, *Biochim. Biophys. Acta*, **2**, 64 (1948); (b) D. F. Elliott, in "Ciba Foundation Symposium on the Chemical Structure of Proteins" (Eds., G. E. W. Wolstenholme and M. P. Cameron), J. and A. Churchill Ltd., London, 1953, p. 129; (c) L. K. Ramachandran and W. B. McConnell, *Can. J. Chem.*, **33**, 1638 (1955); F. Lucas, J. T. B. Shaw and S. G. Smith, *Biochem. J.*, **66**, 468 (1957); (d) K. Narita, THIS JOURNAL, **81**, 1751 (1959).

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(6) A. Patchornik, W. B. Lawson and B. Witkop, *ibid.*, **80**, 4747, 4748 (1958).

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