References

Berger, A., Loewenstein, A., and Meiboom, S. (1959), *J. Am. Chem. Soc. 81*, 62.

Bradbury, J. H., Fenn, M. D., and Gusney, I. (1965), *J. Mol. Biol. 11*, 137.

Cassim, J. Y., and Taylor, E. W. (1965), *Biophys. J. 5*, 553.

Doty, P. (1957), *Proc. Intern. Symp. Macromol. Chem.* 5; *Tetrahedron Suppl. 2*, 1951.

Doty, P., and Yang, J. T. (1956), *J. Am. Chem. Soc. 78*, 498.

Hanlon, S., and Klotz, I. M. (1965), *Biochem. 4*, 37.

Hanlon, S., Russo, S. F., and Klotz, I. M. (1963), *J. Am. Chem. Soc. 85*, 2024.

Karasz, F. E., O'Reilly, J. M., and Bair, H. E. (1964), *Nature 202*, 693.

Klotz, I. M., and Franzen, J. S. (1962), *J. Am. Chem. Soc. 84*, 3461.

Klotz, I. M., Russo, S. F., Hanlon, S., and Stake, M. A. (1964), *J. Am. Chem. Soc. 86*, 4774.

Kolthoff, I. M., and Chantooni, M. K., Jr. (1963), *J. Am. Chem. Soc. 85*, 426.

Moffitt, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.

Perlmann, G. E., and Katchalski, E. (1962), *J. Am. Chem. Soc. 84*, 452.

Ruttenberg, M. A., King, T. P., and Craig, L. C. (1965), *J. Am. Chem. Soc. 87*, 4196.

Shechter, E., and Blout, E. R. (1964a), *Proc. Natl. Acad. Sci. U. S. 51*, 695.

Shechter, E., and Blout, E. R. (1964b), *Proc. Natl. Acad. Sci. U. S. 51*, 794.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.

Watanabe, H., Yoshioka, K., and Wada, A. (1964), *Biopolymers 2*, 91.

Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.

The Preparation and Tryptic Hydrolysis of *S*-Aminoethylated Tobacco Mosaic Virus Protein*

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ABSTRACT: A method of preparative aminoethylation of tobacco mosaic virus (TMV) protein with ethylenimine under reducing and denaturing conditions has been described. The reaction proved to be essentially quantitative and specific for the sulfhydryl group. The aminoethylcysteinyl bond has been shown to be completely digested with trypsin though more slowly than lysyl and arginyl bonds.

A single phenylalanine residue (position 10) in TMV protein is also attacked upon prolonged digestion with trypsin, and this action is inhibited by the chymotrypsin inhibitor, 1-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK). The combined introduction of one additional tryptic site by aminoethylation of the cysteine residue and liberation of aspartic acid by dilute acid have permitted the facile allocation of an amino acid exchange in a nitrous acid mutant (No. 273) of TMV. A serine residue was thus found to replace one of four aspartic acid residues (33) in the core peptide containing 41 residues.

Trypsin continues to be the most specific reagent available for the cleavage of polypeptide chains. Alkylation of cysteine residues with $CH_2CH_2NH_2$ groups has, therefore, been proposed as a means of transforming these residues into trypsin-susceptible sites (Lindley, 1956). Raftery and Cole (1963) have demonstrated that ethylenimine may be used advantageously in place of haloethylamines. This reaction would be of particular advantage for the tobacco mosaic virus (TMV) protein where the single cysteine residue is located near the middle of the large N-terminal tryptic peptide consisting of 41 residues. Although the difficult task of determining the sequence of this peptide has now been completed (Funatsu *et al.*, 1964), the location of the frequent exchanges of amino acid residues in various virus strains in this part of the molecule makes methods for its clear-cut fragmentation most valuable. This appears to be the case if ethylenimine is used to alkylate the cysteine residues of the protein or peptide. In addi-

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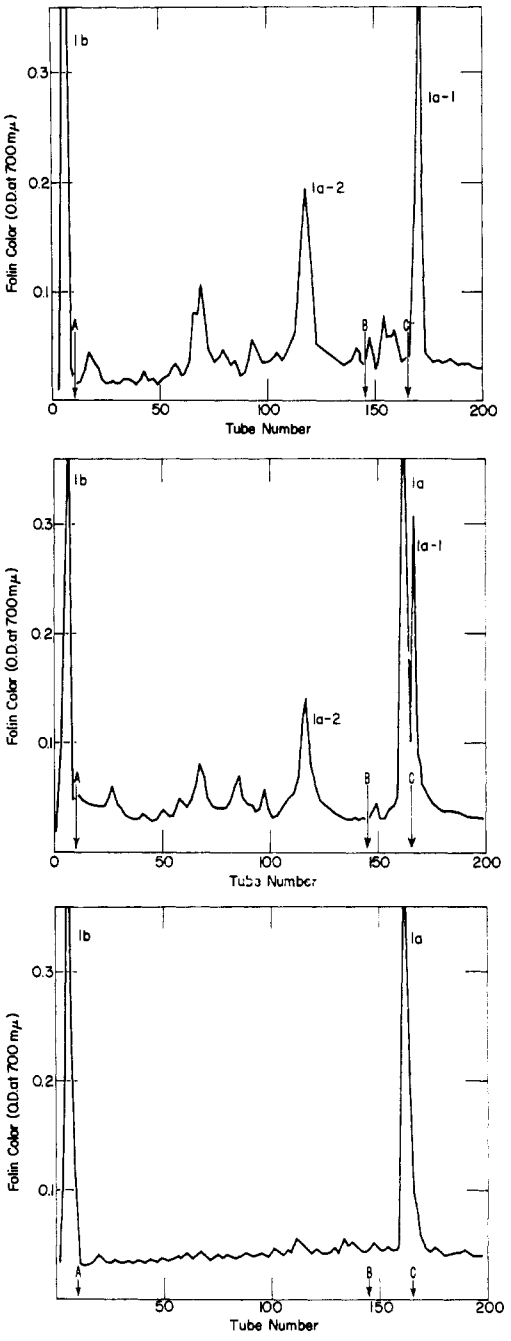

FIGURE 1: Chromatographic separation of fractions obtained from tryptic hydrolysis of aminoethylated peptide 1. Column: 0.9 × 60 cm Doxew 1×2 (200–400 mesh); four buffer systems were employed and the arrows indicate buffer changes (see Methods for details). Temperature 37°; flow rate 30 ml/hr by Minipump (Milton Roy Corp.); fractions, 3 ml. Sample: Peptides derived from 25 mg (5 μmoles) of aminoethylated peptide 1 using twice-crystallized (1a), thrice-crystallized (1b), and TPCK trypsin (1c) (see Experimental Results). The numbers identify the main component of each peak with the sequential peptide number on peptide 1.

tion, the combined use of this method with dilute acid treatment which causes the liberation of aspartic acid from peptides (Tsung and Fraenkel-Conrat, 1965, and earlier papers there referred to) has proved useful for rapid allocation of amino acid exchanges in the large trypsin-resistant peptide in TMV mutants.

Methods and Materials

The virus protein was prepared from TMV and strain 273 (Tsugita and Fraenkel-Conrat, 1962) with cold 67% acetic acid (Fraenkel-Conrat, 1957). Peptide 1 was precipitated from tryptic digests of TMV protein by adjustment to pH 4.6, and purified by three cycles of solution at pH 8 and precipitation at pH 4.6.

Trypsin (twice crystallized, and thrice crystallized, sterile) was obtained from Worthington Biochemical Corp. Occasionally trypsin was used which had been treated with 1-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) (Schoellmann and Shaw, 1963) to inhibit chymotryptic activity, and which was kindly supplied by Drs. S. S. Wong and F. H. Carpenter (Kostka and Carpenter, 1964). Ethylenimine was supplied by Matheson Coleman and Bell Co.

Amino Acid Analyses. Amino acids were determined by ion-exchange chromatography with recording equipment (Spackman *et al.*, 1958) using the Beckman/Spinco Model 120. Samples were hydrolyzed in constant boiling HCl at 108° for 24 hr in evacuated sealed tubes. β-Aminoethylcysteine (AEC) was eluted between lysine and histidine on the 15-cm column (Raftery and Cole, 1963). The integration constant for lysine was used to determine the quantity of β-aminoethylcysteine (AEC). No correction was made for destruction of serine and threonine or incomplete release of leucine and valine.

Titration of the Sulfhydryl Group. To 1 ml of the aqueous solution of protein or peptide (0.2–0.5 μmole) containing 0.2 M Tris–HCl buffer (pH 8.6) and 4.5 M guanidine hydrochloride (pH 8.6) was added 0.05 ml of 10% sodium nitroprusside solution. The solution was titrated with 10^{-3} M methylmercuric nitrate to the point of disappearance of the pink color.

Reaction with Ethylenimine. The complete reaction mixture contained 0.5–1 μmole of protein or peptide, 2 ml of 8 M urea, 0.025 M mercaptoethanol, 0.01 M EDTA, and 0.4 M Tris–HCl buffer of pH 8.6. The reaction mixture in screw-cap vials was held at about 25° under nitrogen for usually 2 hr. Ethylenimine (0.05 ml) was then added five times at 5-min intervals while maintaining the pH in the range from 8.6 to 9.0 by the dropwise addition of 2 N acetic acid in 8 M urea. The reaction was allowed to proceed for 1 hr after the final addition of ethylenimine.

The reaction mixture was diluted with an approximately equal volume of water and dialyzed *vs.* distilled water overnight with two changes. The aminoethylated products precipitated during dialysis and the precipitate

[1] Abbreviations used: TPCK, 7-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; AEC, β-aminoethylcysteine; MET, mercaptoethanol.

TABLE I: Aminoethylation of TMV Protein and Peptide 1 Under Various Conditions.

	Condition[a]						
	Tris	Urea	EDTA	N_2	MET	SH[b]	AEC[c]
TMV protein	+	−	−	−	−	0.9	0.06
	+	+	−	−	−	1.0	0.63
	+	+	+	−	−	1.0	0.79
	+	+	+	+	−	1.0	0.85
	+	+	+	+	+	1.0	0.93
TMV peptide 1	+	−	−	−	−	0.5	0
	+	+	−	+	−	0.47	0.13
	+	+	−	+	+	0.5	0.49
	+	+	+	+	+	0.5	0.52

[a] The concentrations of the reagents used are: Tris, 0.4 M Tris–HCl buffer, pH 8.6; urea, 8 M; EDTA, 0.1 M ethylenediaminetetraacetate, pH 8.6; MET, 0.025 M mercaptoethanol. The molarity indicates the final concentration, with 0.2–0.5 μmole of the material in a final volume of 1 ml. In each case the total amount of ethylenimine (0.25 ml) was added in five steps (see Methods). [b] SH was determined on each sample by the titration procedure of sulfhydryl group as described in the Methods, referred to the molecular weight of protein or peptide. Both the data for the yield of AEC and for SH titer represents the average of three experiments. [c] AEC represents the ratio of aminoethylcysteine to two lysines found by amino acid analysis for aminoethylated TMV protein, and the ratio of AEC/arginine for peptide 1.

could be purified through two cycles of dissolving at pH 10 and precipitating at pH 4.6.

Tryptic Digestion. Tryptic digestion was carried out in a pH-Stat. The aminoethylated protein was not soluble at pH 8 but gradually dissolved almost completely in the course of the tryptic digestion which was performed at 37° for 3 hr using an enzyme–substrate ratio of 1:50 and protein concentration of 1%. The aminoethylated peptide 1 was soluble at pH 8, but 4–6 hr was nevertheless required for complete tryptic digestion.

Chromatographic Separation of Tryptic Peptides. Ion-exchange chromatography of the peptides was performed on Dowex 1×2, 0.9 × 60, or 0.9 × 150 cm columns which were water jacketed at 37°. The column was equilibrated with pH 8.8 buffer, 1% collidine–1% pyridine–1.09 × 10^{-3} N acetic acid. The sample was dissolved in 2 ml of pH 8.8 buffer and the pH was adjusted to 9.2 with 0.1 N NH_4OH. After the application of the sample, the column was first developed with pH 8.3 buffer, 1% collidine–1% pyridine–4.36 × 10^{-3} N acetic acid, then with (A) polygradient buffer: Autograd (Technicon Chromatography Corp.) chambers 1–3 were charged with pH 7.2 buffer: 1% collidine–1% pyridine–0.033 N acetic acid; chambers 4–9 were acetic acid with normalities of 0.02, 0.02, 0.2, 0.35, 0.5, and 0.65, respectively; and (B) acid gradient; chambers 1–3 were charged with 1 N, 15% and 30% acetic acid, respectively. (C) The column was finally washed with glacial acetic acid. Each chamber was filled with 120 ml for the 0.9 × 150 cm column and with 60 ml for the 0.9 × 60 cm column. The flow rate was *ca.* 30 ml/hr, and 3 ml of eluate was collected/tube; 0.1 ml of each fraction was dried at *ca.* 50° by an air stream and analyzed by the Folin–Lowry method (Lowry *et al.*, 1951).

Results

Reaction of TMV Protein and Peptide 1 with Ethylenimine. The extent of aminoethylation of TMV protein or peptide 1 was examined under the conditions listed in Table I. The results indicate that the single cysteine residue reacted at pH 8.6 only after denaturation with 8 M urea, more extensively in the presence of EDTA and under nitrogen, and almost quantitatively by the further addition of mercaptoethanol to protect the SH group from autoxidation. The aminoethylated TMV protein was brought into solution by means of a renaturation procedure (Anderer, 1959) by dissolving it in 8 M urea containing 0.2% mercaptoethanol and dialyzing it *vs.* 0.01 M phosphate buffer (pH 6.8). The protein remained in solution upon dialysis *vs.* water. However, the aminoethyl protein was insoluble in 0.1 M pyrophosphate buffer and thus failed to reconstitute with TMV–ribonucleic acid. Further attempts to characterize the physicochemical properties of the aminoethylated protein are now in progress.

Peptide 1 generally contained no more than 0.5 mole of a titratable cysteine residue which was quantitatively aminoethylated under the optimal conditions described above. The rest of the sulfur seemed to be present in higher states of oxidation, since it was not reducible to SH by mercaptoethanol. It is, therefore, advantageous to aminoethylate TMV protein rather than peptide 1.

Tryptic Hydrolysis of Aminoethylated Peptide 1. For preparative purposes, peptide 1 (5 μmoles) was aminoethylated with ethylenimine and then digested with twice-crystallized trypsin for 6 hr at which time

TABLE II: Amino Acid Compostion of Tryptic Peptides Derived from Aminoethylated Peptide 1.

	Peptide 1b		Peptide 1a − 1		Peptide 1a − 2	
	Residues[a] Found	Theoretical Compn	Residues[a] Found	Theoretical Compn	Residues[a] Found	Theoretical Compn
Asp	2.10	2	...	0	2.01	2
Thr	1.87	2	1.62	2	...	0
Ser	...	0	2.33	3	1.57	2
Glu	4.00	4	1.00	1	1.00	1
Pro	...	0	1.00	1	0.89	1
Gly	1.03	1	...	0	...	0
Ala	1.94	2	...	0	1.98	2
Val	...	0	...	0	0.63	1
Ileu	...	0	0.81	1	1.37	2
Leu	1.04	1	...	0	2.13	3
Tyr	...	0	0.88	1	...	0
Phe	0.94	1	0.93	1	0.67	1
Arg	1.00	1	...	0	...	0
AEC[b]	...	0	...	0	0.92	1
Try[c]	...	0	...	0	1	1

[a] Molar ratios based upon glutamic acid; ... indicates values <0.01 residue. [b] *S*-(β-Aminoethyl)cysteine. [c] Separate determination was made spectrophotometrically for tryptophan (Beavan and Holiday, 1952).

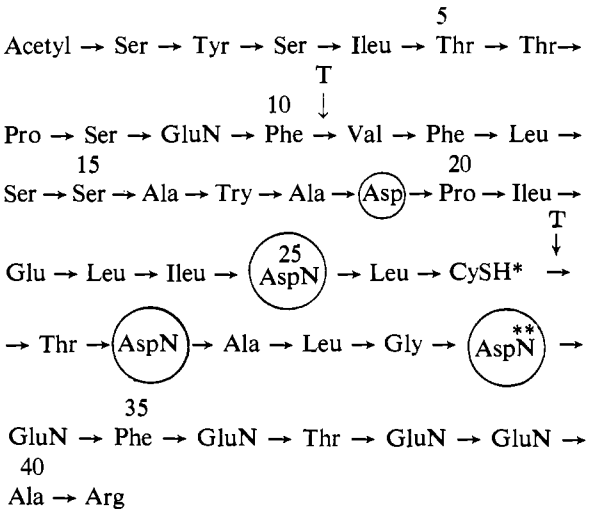

FIGURE 2: Amino acid sequence of peptide 1 of TMV protein. The selective cleavages of peptide 1 by the tryptic hydrolysis of aminoethylated peptide 1, indicated by T, followed by the preferential release of aspartic acid by dilute acid treatment (circled residues). Peptide 1a: residues 1–27; peptide 1a − 1: residues 1–10; peptide 1a − 2: residues 11–27; peptide 1b: residues 28–41. * Aminoethylcysteine after treatment with ethylenimine. ** Ser in strain 273.

the alkali uptake corresponded to *ca.* 10 μmoles. The chromatographic separation of the resulting tryptic peptides on a Dowex 1×2 column of 0.9 × 60 cm is shown in Figure 1a. In contrast to the expected result three major peaks were obtained rather than two. The fractions under these peaks were pooled and analyzed for amino acid composition (Table II). The results show that tryptic cleavage occurred, as expected, at the peptide bond next to the AEC residue at position 27 yielding peptide 1b (residues no. 28–41). However, an additional cleavage had occurred next to phenylalanine at position 10, thus splitting peptide 1a (residues no. 1–27) to peptide 1a − 1 (residues no. 1–10) and peptide 1a − 2 (residues no. 11–27) (see Figure 2).

In another experiment, 5 μmoles of aminoethylated peptide 1 was hydrolyzed with thrice-crystallized trypsin for 6 hr. The chromatographic resolution of the resulting tryptic peptides into four main peaks is shown in Figure 1b. This result suggests that in this experiment peptide 1a was partially split into peptide 1a − 1 and peptide 1a − 2. These data are compatible with the theoretical composition of the corresponding peptides.

Finally, another 5 μmoles of aminoethylated peptide 1 was hydrolyzed with TPCK trypsin which is free from chymotryptic activity. The chromatographic pattern of the peptides on Dowex 1×2, as shown in Figure 1c, reveals only two major fractions. The amino acid compositions of the material in the peak tubes from these two fractions approach the theoretical, as indicated in parenthesis: peptide 1b: Asp 1.87 (2), Thr 1.75 (2), Glu 4.00 (4), Ala 2.07 (2), Leu 0.95 (1), Phe 0.89 (1); peptide 1a: Asp 1.97 (2), Thr 1.56 (2), Ser 3.21 (5), Glu 2.00 (2), Pro 1.47 (2), Gly 0.10 (0), Ala 1.73 (2), Val 0.75 (1), Ileu 2.05 (3), Leu 2.32 (3), Tyr 0.86 (1), Phe 1.78 (2).

The amino acid composition of peptide 1b is always very close to the theoretical composition. However, that of peptide 1a, or 1a − 1 when it is present, deviates slightly from the theoretical composition. The original

TABLE III: Amino Acid Composition of Peptides 1a and 1b of TMV Wild Strain and Mutant Strain 273.

	Peptide 1a				Peptide 1b			
	Wild Strain		Strain 273		Wild Strain		Strain 273	
	Residues[a] Found	Theoretical Compn	Residues[a] Found	Whole No.	Residues[a] Found	Theoretical Compn	Residues[a] Found	Whole No.
Asp	2.02	2	1.94	2	1.87	2	0.96	1
Thr	1.63	2	1.87	2	1.75	2	1.87	2
Ser	3.84	5	3.73	5	...	0	0.73	1
Glu	2.00	2	2.00	2	4.00	4	4.00	4
Pro	1.55	2	1.60	2	...	0	...	0
Gly	0.03	0	0.02	0	1.22	1	0.84	1
Ala	2.05	2	1.74	2	2.07	2	1.92	2
Val	1.24	1	0.81	1	...	0	...	0
Ileu	2.61	3	2.22	3	...	0	...	0
Leu	3.05	3	2.48	3	0.95	1	0.95	1
Tyr	0.81	1	0.76	1	...	0	...	0
Phe	1.75	2	1.60	2	0.89	1	0.83	1
Try[b]	1	1	1	1	...	0	...	0
Arg	0.05	0	0.05	0	1.00	1	1.00	1
AEC[c]	0.97	1	0.88	1	...	0	...	0

[a] Molar ratios based upon glutamic acid; ... indicates values <0.01 residue. [b] Separate determination was made spectrophotometrically for tryptophan (Beavan and Holiday, 1952). [c] *S*-(β-Aminoethyl)cysteine.

peptide 1 always contained only 0.5 mole of aminoethylcysteine and thus the digest of aminoethylated peptide 1 was always contaminated with an equal amount of the unmodified peptide 1. It is apparent, therefore, that peptide 1a and peptide 1a − 1, though chromatographically separable on Dowex 1×2, were contaminated with the unmodified peptide 1 which was slowly eluted by the concentrated acetic acid.

Tryptic Hydrolysis of Aminoethylated TMV Protein. TMV protein (53 mg) (3 μmoles) was aminoethylated and digested with thrice-crystallized trypsin. The tryptic digest was separated by isoelectric precipitation at pH 4.6 into a precipitate and a supernatant fraction. The precipitate was purified by three cycles of isoelectric precipitation at pH 4.6. The combined supernatant fraction was lyophilized and subjected to chromatographic separation on Dowex 1×2 (0.9 × 150 cm) as shown in Figure 3. The chromatographic pattern was essentially the same as the standard one for unmodified TMV protein (Tsung *et al.*, 1964), except that the first peak had a much higher Folin color. The material under the first peak was, therefore, further purified by paper chromatography using 1-butanol–acetic acid–water–pyridine (30:6:24:20, v/v) (Waley and Watson, 1953) as solvent. In addition to two peptides which usually elute as the first peak (*i.e.*, peptides 2 and 7 with R_F values of 0.32 and 0.20, respectively), a new peptide was found to remain at the origin of the paper chromatogram which gave positive tests with both the ninhydrin and Sakaguchi reagent (Block *et al.*, 1958). The amino acid composition of this peptide, as shown in Table III, was that of peptide 1b.

The pH 4.6 insoluble fraction of this digest, in contrast to that of nonalkylated TMV protein, was not found to give a positive Sakaguchi test for arginine which indicated that peptide I was absent from this fraction. The amino acid composition of this fraction, shown in Table III, resembled that of peptide 1a, although it could well be in part composed of the two peptide fragments, peptide 1a − 1 and 1a − 2.

Allocation of Amino Acid Exchange in the Nitrous Acid Mutant of TMV, Strain 273, by the Method of Chemical Cleavage. Strain 273 is a nitrous acid mutant of TMV (Tsugita and Fraenkel-Conrat, 1962). The amino acid composition of the protein of this particular strain had indicated an amino acid exchange of Asp → Ser. This exchange had been located in peptide 1 which contains four aspartic residues. Thus this strain appeared particularly suitable to test the applicability of both the method of selective cleavage at the cysteine residue and preferential release of aspartic acid residues (Tsung and Fraenkel-Conrat, 1965) for the exact location of amino acid replacements.

The protein of strain 273 was reduced, aminoethylated, and digested with thrice-crystallized trypsin according to the standard procedure as described in the Methods. The chromatographic separation of the soluble tryptic peptides was as shown in Figure 3. The amino acid composition of peptide 1b, as well as of peptide 1a as obtained by three cycles of isoelectric precipitation at pH 4.6, are summarized in Table III and they indicate that the Asp → Ser exchange occurred in peptide 1b.

Peptide 1b from both the wild strain and strain 273

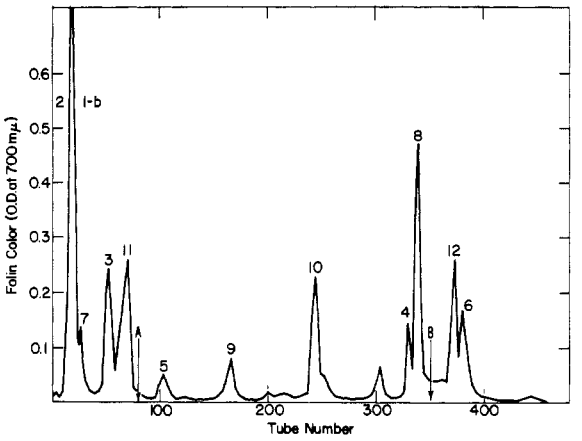

FIGURE 3: Chromatographic separation of tryptic peptides from aminoethylated protein of TMV wild strain and of strain 273. Column: 0.9 × 150 cm. Dowex 1 × 2 (200–400 mesh); three buffer systems were employed and the arrows indicate buffer changes (see Methods for details). Temperature 37°; flow rate 30 ml/hr by Minipump (Milton Roy Corp.); fractions, 3 ml. Sample: soluble tryptic peptides (pH 4.6) derived from 50 mg each of protein. Wild strain and mutant showed the same pattern. The numbers identify the main component of each peak with the sequential peptide number on the polypeptide chain. (Peptide 1a is insoluble at pH 4.6 and was removed by centrifugation prior to chromatography.)

was treated with 0.03 N HCl at 105° for 48 hr. The analyses for free amino acids in the partial acid hydrolysates obtained from both strains are summarized and compared as follows: wild strain: $Asp_{2.11}$, $Thr_{0.98}$; strain no. 273 $Asp_{1.00}$, $Thr_{1.06}$. This result indicates that the aspartic acid is released from the asparagine residue in position 29 but not from position 33, because in that case the N-terminal threonine (position 28) would not be released (see Figure 2). It is thus evident from this experiment that a rapid allocation of the Asp → Ser exchange at position 33 in peptide 1 is made possible by the combined use of the two cleavage methods studied.

Discussion

The aminoethylation of TMV protein clearly indicates the specificity of the ethylenimine reaction with the single cysteine residue in the protein. The introduction of one additional trypsin-susceptible site in the core peptide (peptide 1) represents a useful technique for structural analysis and for rapid allocation of amino acid replacements in strains of TMV. The same technique is now being applied successfully to the protein of the coliphage MS-2 and related proteins. The reduction and aminoethylation of SH-containing proteins may at times be preferable to the more customary techniques of reduction followed by carboxymethylation as a means of protecting SH group against oxidation during enzymatic digestion.

A further advantage of aminoethylation is that it may lead to a decrease in the size of big trypsin-resistant peptides, thus making them much easier to purify by chromatographic methods. However, the peptide bond of the aminoethylcysteine residue is digested more slowly than that of arginine and lysine residues. Therefore, the complete hydrolysis of aminoethylated proteins or peptides requires much longer time during which chymotryptic activity may become appreciable. The aminoethylated peptide 1 is split at the phenylalanine residue no. 10, yet the phenylalanine residues at positions 12 and 35, the tryptophan at position 17, and the tyrosine residue at position 3 are not attacked (Figure 2). This selectivity suggests an enzymatic action resembling and yet differing from chymotrypsin. Whether this activity is an inherent property of trypsin or due to a contaminating enzyme remains to be solved. This activity appears to be inhibited by the chymotrypsin inhibitor, TPCK. Whatever its nature, this peculiar enzyme action has proved to be a useful bonus in providing an additional selective cleavage at a strategic location in the trypsin-resistant core peptide which contains more than one-fourth of the total amino acid residues of the TMV protein.

Summary

A method of preparative aminoethylation of TMV protein with ethylenimine under reducing and denaturing conditions has been described. The reaction proved to be essentially quantitative and specific for the sulfhydryl group. The aminoethylcysteinyl bond has been shown to be completely cleaved by digestion with trypsin though more slowly than lysyl and arginyl bonds. A single phenylalanine residue (position 10) in TMV protein is also attacked upon prolonged digestion with trypsin, and this action is inhibited by the chymotrypsin inhibitor, TPCK.

The combined introduction of one additional tryptic site by aminoethylation of the cysteine residue and liberation of aspartic acid by dilute acid has permitted the facile allocation of an amino acid exchange in a nitrous acid mutant (273) of TMV. A serine residue was thus found to replace one of four aspartic acid residues (no. 33) in the core peptide containing 41 residues.

Acknowledgments

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References

Anderer, F. A. (1959), *Z. Naturforsch. 14b*, 642.

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Block, R. J., Durrum, E. L., and Zweig, G. (1958), A

Manual of Paper Chromatography and Paper Electrophoresis, 2nd ed, New York, N. Y., Academic, p 128.
Fraenkel-Conrat, H. (1957), *Virology 4*, 1.
Fraenkel-Conrat, H. (1959), *in* Symposium on Sulfur in Proteins, Benesch, R., *et al.*, Ed., New York, N. Y., Academic, p 339.
Funatsu, G., Tsugita, A., and Fraenkel-Conrat, H. (1964), *Arch. Biochem. Biophys. 105*, 25.
Kostka, V., and Carpenter, F. H. (1964), *J. Biol. Chem. 239*, 1799.
Lindley, H. (1956), *Nature 178*, 647.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10*, 467.
Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Tsugita, A., and Fraenkel-Conrat, H. (1962), *J. Mol. Biol. 4*, 73.
Tsung, C. M., and Fraenkel-Conrat, H. (1965), *Biochemistry 4*, 793.
Tsung, C. M., Funatsu, G., and Young, J. D. (1964), *Arch. Biochem. Biophys. 105*, 42.
Waley, S. G., and Watson, J. (1953), *Biochem. J. 55*, 328.

Nucleohistone Composition in Stationary and Division Synchronized *Tetrahymena* Cultures*

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ABSTRACT: The nonionic detergent Triton X-100 was used for the isolation of the macronuclei which were found by electron microscopy to be free from cytoplasmic contamination. Nuclei of stationary phase cells contain in weight per cent of substances analyzed 2.3% ribonucleic acid (RNA), 22.4% deoxyribonucleic acid (DNA), and 75.2% protein. The nuclear proteins (44%) are histones. The lysine/arginine ratio varies from 3.4 to 5.3. The amino acid composition of *Tetrahymena* histone is comparable to published results on histones of chicken 5rythrocytes. By electrophoresis on polyacrylamide gel the histones could be separated into nineteen bands. Similar electrophoresis patterns were found for log and stationary cells, as well as for cells at the end of the heat treatment (EHT) and 1 hr later (EHT + 1). Minor but distinct differences were observed between these two groups of samples. Histones extracted from log and stationary phase cells contain slow-moving components which are absent in the patterns of histones prepared at EHT and EHT + 1. The latter show fast-moving components which are absent in the patterns of histones prepared from log and stationary phase cells. The specific activity of L-[U-^{14}C]lysine was determined in 17 histone fractions. Although the nuclear histone content decreases prior to synchronized cell division (EHT + 1 hr), the rate of lysine incorporation is twice the rate found in log phase cells or in the division-blocked cells at the end of the heat treatment (EHT).

Studies of nucleohistone composition and metabolism in animal and plant tissues provide evidence for the current view that histones, among other suggested functions, are involved in regulation of gene activity (Stedman and Stedman, 1950; Huang and Bonner, 1962; Allfrey *et al.*, 1963; Irvin *et al.*, 1963; Moore, 1963; Dulbecco, 1964). Consequently, histones could serve as gene modulators in the differentiation of higher organisms and in the life cycles of single cell organisms lacking differentiation but exhibiting highly ordered development. The infraciliature of ciliates is a useful biological structure for studies of cellular development (Frankel, 1962). During the intermittent heat treatment for induction of synchronized division in *Tetrahymena*, synthesis of total protein and nucleic acids does occur, but cells are blocked at the same stage of their development in oral morphogenesis (Holz *et al.*, 1957). In preparation for synchronized division additional protein synthesis has to occur (Zeuthen, 1964).

In this investigation we have determined the amino acid composition, electrophoresis pattern, and incorporation of L-[U-^{14}C]lysine into *Tetrahymena* histones during exponential multiplication, stationary phase, and synchronized cell division. The results are compared with published data on the amino acid composition (Vande Woude, 1964, 1965; Iwai *et al.*, 1965) and electrophoresis patterns (Hardin and Lindsay, 1965) of *Tetrahymena* histone.

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