Artificial Histidine-Containing Mutants of Tobacco Mosaic Virus*

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ABSTRACT: The amino acid replacements in several artifically evoked histidine-containing mutants of the coat protein of tobacco mosaic virus have been allocated. These are the first artificial histidine mutants to be reported for this virus. They could be of interest in studies concerned with tobacco mosaic virus coat protein structure and synthesis as well as virus assembly when carried out by X-ray analysis or immunochemical techniques. In all mutants, the histidine residue appears near the N-terminal end of the protein chain. In mutant 483, the amino acid replacement is $GluNH_2 \rightarrow$ His at residue 9. Mutant 470 represents the first instance of a double-compensatory replacement being detected, involving a Ser → His shift at residue 8, and a Gly → Ser shift at residue 149. Of the three replacements, only Ser → His is not compatible with a presumed mechanism of mutation involving a single base change in the codon.

The classification of these new histidine mutants among the other known mutants is discussed.

Histidine does not appear in the coat protein of the wild-type strain of TMV (Tsugita *et al.*, 1960). Of all the naturally occurring mutant strains of TMV, only one, Holmes ribgrass virus (HR) (Holmes, 1941), was shown to contain a single histidine residue (Tsugita, 1962a). None of the artifically evoked mutants isolated by the Berkeley group (Funatsu and Fraenkel-Conrat, 1964) or by the Tübingen group (Wittmann-Liebold and Wittmann, 1965) showed so far any histidine replacements.

Tsugita (1962b) proposed a classification system of four classes for the natural and artificial mutant strains of TMV in which the methionine content of the coat protein was used as one of the distinguishing criteria. The class A group of strains of an amino acid composition either identical with common TMV, or quite similar to it, show up to three net amino acid replacements and always lack methionine and histidine. The classes B and C include strains which show greater numbers of exchanges, and in addition contain 1 and 2 methionine residues, respectively. The total number of residues in the coat protein, however, always remains 158. HR[1] with 17 net exchanges, 3 methionines, and 1 histidine is so far the only member of class D.

Recent data published on the amino acid composition of CV_4 (Van Regenmortel, 1967) and ORSV (Paul *et al.*, 1965) show that their coat proteins are also 158 amino acid residues long. ORSV possesses 17 net exchanges as compared with common TMV and 3 methionines. It resembles HR but lacks a histidine. On the other hand, CV4 has 18 net exchanges, but has neither methionine nor histidine and in over-all amino acid composition is closer to the strains of class A. These strains could be assigned to two additional classes (E and F) in the system of Tsugita (1962b) (Fraenkel-Conrat, 1968). Even so, D remains the class to harbor the sole histidine mutant known until this work. We now describe the allocation of histidine replacements in several artificial mutants obtained in this laboratory. The classification of these mutants in Tsugita's system will be discussed.

Materials and Methods

Strain 470 was a nitrous acid mutant obtained by the customary method of treating TMV-RNA with nitrite in pH 4.8 acetate. Strain 483 was obtained by the concerted action of thiopyronin and light (Singer and Fraenkel-Conrat, 1966).

Strain 487 was obtained by treating TMV-RNA with ferrous sulfate in the presence of light. After reconstitution of the virus, the reaction mixture was put on Sylvestris tobacco plants (*Nicotiana sylvestris* Spegaz and Comes). A single local lesion was isolated and purified in the usual way.

Strain 498 was isolated by floating a leaf disk of Turkish tobacco (*Nicotiana tabacum* L. var. Turkish), infected 1 day before with wild TMV, on a solution containing proflavin. Whole virus was then extracted and put on Sylvestris tobacco for isolation of local lesion mutants.

Strain 539 originated from a TMV-RNA treated with periodate and aniline to eliminate stepwise the nucleotide residue from the 5′-linked end group (Steinschneider and Fraenkel-Conrat, 1966b).

The virus coat proteins were separated from the ribonucleic acid by the cold 67% acetic acid procedure of Fraenkel-Conrat (1957). Amino acid analyses were made on samples hydrolyzed for periods of 24 and 72 hr (Tsugita, 1962b) by the automatic procedure of Spackman *et al.* (1958). Tryptic digestions were made accord-

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[1] Abbreviations used that are not given in *Biochemistry 5*, 1445 (1966), are: HR, Holmes ribgrass virus; CV4, cucumber virus 4; ORSV, Odontoglossum ringspot virus.

ing to Tsugita (1962a), and the large N-terminal peptide was isolated and purified by repeated isoelectric precipitation at pH 4.5. The soluble tryptic peptides were separated on a 150 × 0.9 cm Dowex 1-X2 column with the system of Funatsu (1964). Tryptic peptide 1[2] of mutant 470 and 483 and 12[2] of mutant 470 were further digested with chymotrypsin according to Funatsu and Fraenkel-Conrat (1964), or with thermolysin, following Matsubara *et al.* (1965), and separated on a 60 × 0.9 cm Dowex 1-X2 column (Funatsu *et al.*, 1964). When necessary, peptides were further purified by descending chromatography in the 1-butanol–acetic acid–pyridine–water (30:6:24:20) system of Waley and Watson (1953). Histidine-containing peptides in the chromatographic eluates were located by spotting an aliquot of each peptide peak as determined by the Folin–Lowry method (Lowry *et al.*, 1951) on paper and spraying with the Pauly reagent for histidine (Baldridge and Lewis, 1953.

The sequential degradation of the N- and C-terminal ends of peptides was done by the phenyl isothiocyanate method and carboxypeptidase A, respectively, as described in an earlier paper (Rombauts, 1966).

Results

Mutant 470. Total amino acid analysis showed one less glycine and one more histidine than in wild-type TMV (Table I). This single net exchange proved to be a double one when the amino acid composition of the insoluble tryptic peptide 1 and of the soluble peptides was determined. As shown in Table I, peptide 1 has one less serine and contains one extra histidine, and peptide 12 has one less glycine and one more serine as compared with the wild strain. The other tryptic peptides showed no exchanges on the basis of either amino acid composition or position of elution upon chromatography, as compared with the standard pattern, and these are therefore not listed on Table I.

Peptide 1 was further hydrolyzed with thermolysine which under the conditions used (37°, 5 hr, enzyme:substrate ratio 1:50) is expected to split mainly at the N-terminal side of leucine, isoleucine, alanine, and phenylalanine (Matsubara *et al.*, 1965). The resulting peptides were separated on a 60 × 0.9 cm Dowex 1-X2 column and the elution pattern is presented in Figure 1. Peak fraction 1 was strongly positive in the Pauly test. Amino acid analysis of this fraction after 24-hr hydrolysis gave the composition: $Thr_{1.8}$,Ser_{none},$Glu_{1.0}$,$Pro_{1.0}$,$Ile_{1.0}$,-$His_{1.0}$. This hexapeptide can only correspond to the sequence in wild-type TMV from residues 4 to 9: -Ile^{4}-Thr-Thr-Pro-Ser-Glu^{9}NH$_2$-, with the exception that the serine is missing and one histidine is present (Figure 2).

The hexapeptide was found to have the following structure: H_2N-Ile-Thr-Thr-Pro-His-GluNH$_2$-OH. The sequence of the first five residues was determined by Edman degradation. In the fifth step, no serine was found and histidine was isolated in 20% over-all yield, which corresponds to an average yield per step of about 75%. Carboxypeptidase A liberated glutamine (determined as serine on the amino acid analyzer) as the only C-terminal amino acid in 80% yield, and no histidine. This is to be expected because of the presence of the proline residue penultimate to the glutamine and corresponds to the known effect and yield of carboxypeptidase A on the C-terminal end, -Pro-Ala-Thr-OH, of wild-type TMV where 80% of the threonine only is liberated

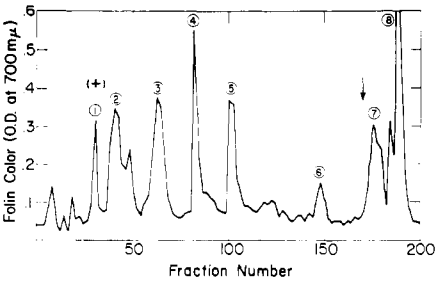

FIGURE 1: Chromatography of a thermolytic digest of peptide 1 of mutant 470 on a Dowex 1-X2 column (0.9 × 60 cm) equilibrated with pyridine–collidine–acetic acid buffer of pH 8.6. Elution was done with a pyridine–collidine–acetic acid gradient starting with pH 7.3 and ending with 0.65 N acetic acid (Funatsu *et al.*, 1964). At the completion of the gradient (indicated by an arrow) the column was washed with 50% acetic acid. The flow rate was 12 fractions of 3.3 ml/hr. Two-tenths of every second fraction were analyzed with the Folin reagent. The (+) mark indicates the peak which gave a positive reaction for histidine with the Pauly reagent. For the composition of the numbered peaks, see Results.

Because of the importance of specific splitting methods for the allocation of amino acid exchanges in the 41-residue-long tryptic peptide 1, we also report here some results based on the analysis of the other peptide fractions of the thermolytic digest. Peak 2 in Figure 1 had a composition corresponding to residues 31–33 (Leu-Gly-AspNH$_2$); peak 4 contained peptide 23–30 (Leu-Ile-AspNH$_2$-Leu-CysSH-Thr-AspNH$_2$-Ala), peak 5 was peptide 10–12 (Phe-Val-Phe), and peak 7 was peptide 16–20 (Ala-Trp-Ala-Asp-Pro). Peaks 3, 6, and 8 contained not readily identifiable mixtures.

Peptide 12 of wild-type TMV has two glycines, one at position 149 and the other at position 155 (Funatsu and Fraenkel-Conrat, 1964). Gish (1959) reported the isolation in good yield of the C-terminal peptide H_2N-Thr^{153}-Ser-Gly-Pro-Ala-Thr^{158}-(OH) from a chymotryptic digest of peptide 12. After such treatment of peptide 12 of mutant 470, the corresponding C-terminal peptide was isolated (the first eluted peak in Figure 3) and had the expected composition (24-hr hydrolysis): $Thr_{1.5}$,$Ser_{0.8}$,-$Pro_{0.9}$,$Gly_{1.0}$,$Ala_{1.0}$.

From the total composition of mutant peptide 12 and the unchanged composition of its C-terminal peptide it may reasonably be assumed that a Gly → Ser replacement must be located at position 149.

We conclude that mutant 470 has a Ser → His replacement at position 8 and a Gly → Ser one at posi-

[2] The numbering of the tryptic peptides correspond to their sequential position in the peptide chain.

TABLE I: Amino Acid Composition of the Coat Protein and of Peptides 1 and 12 of TMV and Mutants 470 and 483.[a]

Amino Acid	Coat Protein					Tryptic Peptide 1					Tryptic Peptide 12		
		Mutant 470		Mutant 483			Mutant 470		Mutant 483			Mutant 470	
	TMV	Found	Nearest Integer	Found	Nearest Integer	TMV	Found	Nearest Integer	Found	Nearest Integer	TMV	Found	Nearest Integer
Asp	18	18.1	18	18.2	18	4	4.4	4	4.2	4			
Thr	16	15.9	16	16.1	16	4	4.0	4	3.8	4	2	2.0	2
Ser	16	16.3	16	16.3	16	*5*	4.1	*4*	4.6	5	*6*	6.6	*7*
Glu	*16*	16.0	16	15.0	*15*	*6*	6.2	6	5.0	*5*	1	1.1	1
Pro	8	7.8	8	8.1	8	2	2.1	2	1.9	2	1	0.8	1
Gly	*6*	5.0	*5*	6.0	6	1	1.2	1	1.2	1	*2*	1.0	*1*
Ala	14	14.0	14	13.9	14	4	4.3	4	4.0	4	1	0.9	1
Cys (1/2)	1	n.d.	(1)	n.d.	(1)	1	n.d.	n.d.	n.d.	n.d.			
Val	14	13.8	14	13.6	14	1	1.1	1	1.1	1	1	0.9	1
Met	0	0	0	0	0	0	0	0	0	0			
Ile	9	8.9	9	8.6	9	3	3.0	3	2.9	3			
Leu	12	11.8	12	11.9	12	4	4.1	4	4.0	4	1	0.9	1
Tyr	4	3.8	4	3.9	4	1	1.0	1	0.9	1			
Phe	8	7.9	8	7.9	8	3	2.9	3	2.8	3	1	0.9	1
Trp	3	n.d.	(3)	n.d.	(3)	1	(+)	(+)	(+)	(+)	1	(+)	(+)
Lys	2	2.0	2	2.0	2	0	0	0	0	0			
His	*0*	1.0	*1*	1.0	*1*	0	1.0	*1*	1.0	*1*			
Arg	11	10.9	11	10.7	11	1	1.2	1	1.0	1			

[a] The amino acid composition of the mutants and the mutant peptides was determined after 24- and 72-hr acid hydrolysis and the values for serine and threonine were corrected for destruction. When tryptophan and cysteine were not separately determined, they are listed as n.d. Tryptophan was detected spectrophotometrically in the mutant peptides and listed as (+) when found.

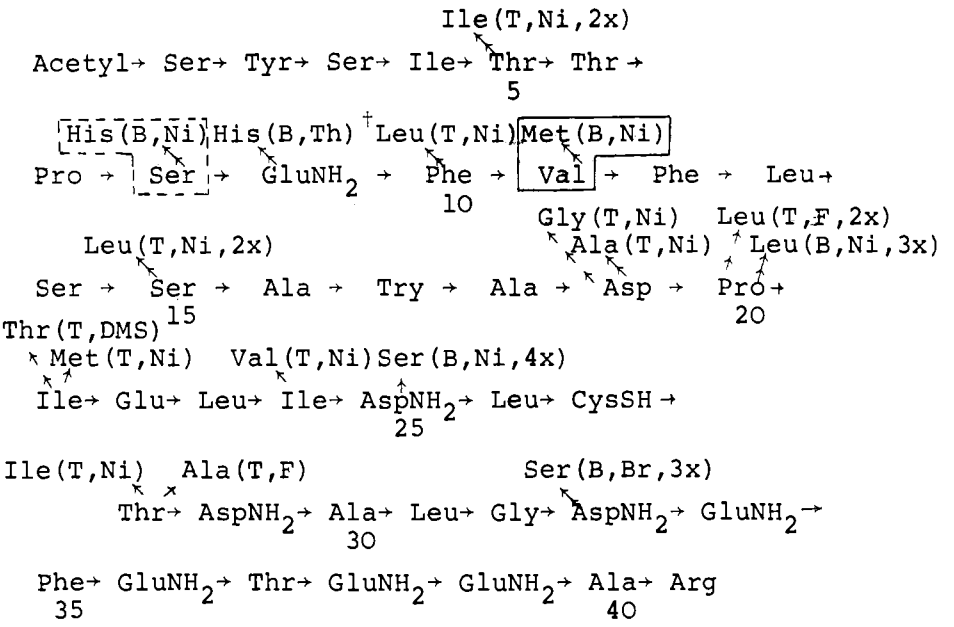

FIGURE 2: The figure lists the sequence and amino acid exchanges in peptide 1 of TMVP located in Berkeley (B) and Tübingen (T), in artificially evoked mutants by treatment with nitrous acid (Ni), fluorouracil (F), bromination (Br), thiopyronin and light (Th), and dimethyl sulfate (DMS). The frequency of the observed exchanges is also given. The amino acid exchanges boxed in with a solid line do not follow the presumed deamination action of nitrous acid, and the one boxed in with a dashed line in addition does not fit the single base-exchange hypothesis (see text). The data were taken from Funatsu and Fraenkel-Conrat (1964), Wittmann-Liebold and Wittmann (1965), Jockusch (1966), and this work. † The same exchange was also observed, once in each case, in a proflavin mutant, a mutant caused by the action of ferrous sulfate and light, and in a mutant obtained from RNA previously treated with periodate followed by aniline.

tion 149. It therefore has one change in composition and two in sequence.

Mutant 483. Total amino acid analysis showed one less glutamic acid and one more histidine than in wild-type TMV (Table I). This replacement was found to be located in the N-terminal peptide 1, as is evident from its analysis (Table I). The whole peptide was digested with chymotrypsin and the resulting peptides separated on a 60 × 0.9 cm Dowex 1-X2 column according to Funatsu *et al.* (1964). The resulting elution pattern is presented in Figure 4. Only one peak was strongly positive for histidine, as indicated in Figure 4. The peaks immediately preceding and following it gave a weak reaction, probably through incomplete separation from the main histidine peptide. This strongly positive fraction was further purified by paper chromatography and gave the following amino acid composition: $Thr_{1.9}$,-$Ser_{1.0}$,$Glu_{<0.1}$,$Pro_{1.0}$,$Ile_{0.9}$,$Phe_{1.0}$,$His_{1.0}$. In addition about half a residue of alanine and leucine was present, due to incomplete purification. This composition can only correspond to the sequence 4–10 in wild-type TMV, -Ile-Thr-Thr-Pro-Ser-$GluNH_2$-Phe-, except that the glutamine at position 9 has been replaced by a histidine. We conclude therefore that mutant 483 has a glutamine → histidine replacement at position 9. Further possible compensatory exchanges in the soluble tryptic peptides have not been investigated.

Recently, three additional histidine mutants, 487, 498, and 539, were isolated. Upon amino acid analysis, they all showed a GluX → His net replacement. By techniques similar to those used for mutant 483, this replacement was located in tryptic peptide 1 in all three strains. After digestion of this peptide with chymotrypsin, a peptide with an amino acid composition corresponding to

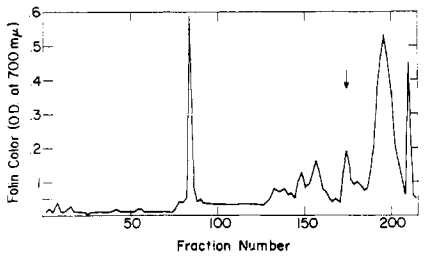

FIGURE 3: Chromatography of a chymotryptic digest of peptide 12 of mutant 470. The chromatographic conditions were identical with those in Figure 1.

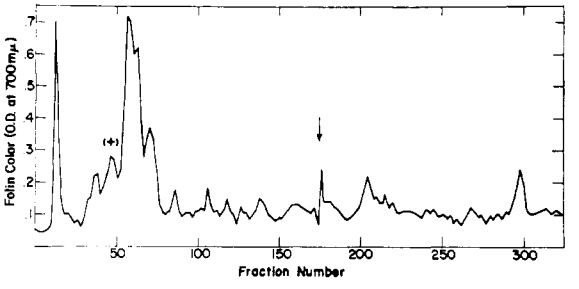

FIGURE 4: Chromatography of a chymotryptic digest of peptide 1 of mutant 483. The chromatographic conditions were identical with those in Figure 1. The (+) mark indicates the peak giving a positive reaction for histidine with the Pauly reagent.

the sequence -Ile-Thr-Thr-Pro-Ser-His-Phe was isolated in the three cases. These three mutant strains thus show the same replacement as mutant 483.

Discussion

The artificially evoked mutants of TMV reported in this paper are the first mutants among the many already described to show a histidine exchange in the coat protein. These exchanges are in position 9 in mutants 483, 487, 498, and 539, and in position 8 in mutant 470.

In this region of the chain around position 10 rather few amino acid replacements have so far been located (Figure 2). Funatsu and Fraenkel-Conrat (1964) reported a Val → Met shift at position 11 and Wittmann-Liebold and Wittman (1965) described a Phe → Leu shift at position 10. The now reported histidine replacements at positions 8 and 9 together with these earlier results indicate that the sequence around position 10 is more "mutable" than it appeared so far, and consequently that it is less critical for maintaining a viable conformation of the coat protein in the virus. HR is the only other histidine-containing TMV strain known. Its amino acid sequence has been reported (Tokyo, 1967) at the International Meeting on Plant Viruses by both Funatsu and by Wittmann and its histidine residue seems to be located near position 135. Our histidine mutants completely differ from HR, not only in the position of the His exchange, but also in that they have only 1 or 2 over-all net exchange(s) as compared to TMV, while HR has 17 and two deletions. Therefore they do not belong with HR in class D of Tsugita's (1962b) classification system. They are clearly related to the mutants of Tsugita's class A, differing from TMV with a maximum of three exchanges. Consequently the limiting requirement of no histidine for this class (Tsugita, 1962b) should now be dropped.

These His strains could provide a very promising tool for studies of the three-dimensional structure of TMV. The His at position 8 or 9 could form a potential handle to attach a heavy atom substituent in a specified position along the chain, useful for X-ray diffraction studies.

The His replacement might also change the antigenic character of TMV and TMV protein, thus indicating whether His replacements in this portion of the chain influence their three-dimensional conformation. Van Regenmortel (1967) has suggested that such serological differences are only detectable with exchanges occurring in certain critical regions along the chain. Exchanges in the area of our histidine replacements at positions 8 and 9 have not been studied immunologically, but exchanges at positions 20 and 21, the nearest locations studied, could not be serologically distinguished from the wild type by Van Regenmortel (1967) and von Sengbusch (1965). On the contrary, both authors reported serological differences with exchanges involving the seryl residues at position 148, next to the location of our observed Gly → Ser shift in mutant 470.

A further point of interest with these mutants is the relation of their observed amino acid exchanges to the mechanism of mutagenesis. There are two major aspects to this problem: one, whether or not the exchange can be explained by a single base change in the codon; the other, whether this base change corresponds with the presumed mutagenetic reaction.

Mutant 470 was isolated after treatment with nitrous acid. Its Gly → Ser shift in peptide 12 is compatible with a single base exchange in the codon: GG(U,C) → AG(U,C), when related to the codon dictionary (Söll *et al.*, 1965). On the other hand, the Ser → His replacement cannot be accounted for by a single nucleotide exchange: AG(U,C) or UC(U,C,G,A) → CA(U,C). The single-base-exchange hypothesis did seem to hold for all artificial mutants so far isolated in which specific amino acid exchanges were identified (Wittmann-Liebold, 1966; for a review, see Fraenkel-Conrat, 1968). However the data supporting the observed Ser → His shift are unambiguous and leave no alternative but to conclude that although very rare, such exchanges do occur in artificially evoked mutants.

As for the mechanism of mutagenesis, the two observed exchanges in nitrous acid mutant 470 do not seem to follow a base change in TMV-RNA involving C → U or A → G. Such is, however, the mechanism of action of nitrous acid on RNA *in vitro* (Tsugita, 1961; Fraenkel-Conrat, 1961). The same mechanism is operative for the intact virus. Almost all exchanges so far allocated in the protein of nitrous acid mutants can be explained by a deamination mechanism (Fraenkel-Conrat, 1968; von Sengbusch, 1967).

There are only a few exceptions. Wittmann-Liebold and Wittmann (1965) mention a once-observed Glu → Asp exchange at position 95. Two other such potential exchanges were repeatedly observed (Asp or $AspNH_2$ → Ala, four times, and Glu or $GluNH_2$ → Val, twice) but only located as being in peptide 1 (Wittmann, 1964). Their exact allocation would be of interest. Funatsu and Fraenkel-Conrat (1964) mention two other such exchanges, Val → Met at position 11 and Arg → Lys at position 46. Both exchanges were observed only once.

It is possible that these single net exchanges are actually double compensatory ones for each of which the deamination mechanism could apply. Only when all tryptic peptides of a TMV mutant are checked for amino acid composition and sequence can this possibility be ruled out. This is usually not done, as statistically such compensatory exchanges occur very rarely, probably because most chemical RNA mutations are monodirectional (C → U, not U → C).

However the very fact that we report in this paper the first allocation of such a compensatory exchange in mutant 470 points to this danger. Both exchanges, one in peptide 1, the other in peptide 12, make exception to the deamination mechanism. The amino acid composition and elution position of all other tryptic peptides were checked and found to be as in the wild type. The possibility of further concealed exchanges is therefore in this particular mutant remote and, on statistical grounds, very improbable. A mechanism other than deamination must apply.

Tessman *et al.* (1964) have suggested on the basis of purely genetic evidence that in phage S 13 nitrous acid induces G → A type mutations. This mechanism, if

valid for TMV, could explain the Val → Met exchange (GU(U,C,GA) → AUG) reported by Funatsu and Fraenkel-Conrat (1964), but it offers no complete explanation for the other "abnormal" nitrous acid mutants cited here.

A more probable explanation for their aberrant behavior is that any rare or singly observed exchange can always be due to spontaneous mutation and need not necessarily be attributable to the chemical used.

The $GluNH_2$ → His exchange in mutants 483 and 498, isolated after treatment with thiopyronin and proflavin, respectively, and light, fits the single-base change CA(A,G) → CA(U,C). This change in the codon involving a purine replacement by a pyrimidine could be accounted for by the observation of Singer and Fraenkel-Conrat (1966) that these dye-catalyzed photoinactivations of TMV-RNA are correlated with destruction of guanine residues. Perhaps a similar mechanism applies to the mutant induced by ferrous sulfate and light, yielding strain 487, which also is characterized by the same $GluNH_2$ → His exchange. However, mutant 539, obtained after treatment with periodate and aniline for removal of nucleotides at the 5′-linked end of TMV-RNA (Steinschneider and Fraenkel-Conrat, 1966a,b), also had this exchange. As these authors have demonstrated that such treatment under normal conditions quite preferentially affects the glycol end of TMV-RNA, the mutation in strain 539 is most likely the result of a spontaneous event.

The types of our observed exchanges do not completely correspond with the general replacement types that were found to occur most frequently by von Sengbusch (1967) in a statistical analysis of TMV mutants. Polar residues are mostly replaced by Ser or Gly, while in our case a strongly polar $GluNH_2$ is replaced by a similarly polar His. On the other hand, the Ser → His exchange in mutant 470 fits in this author's generalization that Ser and Thr are most frequently replaced, but very rarely by Ala or Gly.

In this work, thermolysin has been used to hydrolyze the large insoluble peptide in TMV, and the splits observed are in general agreement with the proposed specificity of this enzyme (Matsubara *et al.*, 1965). Thermolysin seems to be much more specific in its action on peptide 1 than chymotrypsin, as is apparent from a comparison of the peptide patterns obtained after chromatographic separation shown in Figures 1 and 4. Thermolysin treatment therefore is a useful addition to the other specific cleavage methods available for peptide 1, *e.g.*, tryptic digestion after aminoethylation (Tsung and Fraenkel-Conrat, 1966), and should facilitate the allocation of amino acid exchanges in this large peptide.

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