Location of Amino Acid Exchanges in Chemically Evoked Mutants of Tobacco Mosaic Virus*

G. FUNATSU† AND H. FRAENKEL-CONRAT

From the Virus Laboratory, University of California, Berkeley

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Through the use of limited digestion by Nagarse the big N-terminal tryptic peptide of tobacco mosaic virus protein was fragmented and the amino acid exchanges occurring in this peptide in several mutants were localized. Enzymatic methods and partial acid degradation made it possible to localize exchanges also in other parts of the molecule. Of about forty-four exchanges observed and localized in our laboratory twenty-four were in the N-terminal and C-terminal 25-residue segments. No mutant or natural strain was found by us or elsewhere which differed from the wild type in the segment comprised by residues 108–122, and very few exchanges have been observed in the preceding 8-residue segment. The total number of different exchanges observed by us were thirteen, four of which recurred frequently and usually involved the same amino acid residues in the peptide chain. The mechanism leading to exchanges in terms of known codons was discussed.

Studies on the protein structure of chemically evoked mutants of tobacco mosaic virus (TMV)[1] have been carried out over the past years in our laboratory and at the Max Planck Institut für Biologie at Tübingen (Tsugita and Fraenkel-Conrat, 1962; Tsugita, 1962a; Wittmann, 1962), in the hope that these would supply definite information about the genetic code. In both laboratories many of the mutants studied showed replacements of one or two and more rarely three amino acid residues, while many others showed no exchanges. Though these exchanges were scattered over the entire sequence, they occurred frequently in peptide 1[2] (Tsugita, 1962a). The localization of these amino acid exchanges in peptide 1 was not attempted because of technical difficulties related to its size (forty-one amino acids) and to the lability of some of its peptide bonds.

In this paper we present data on the location of these amino acid replacements in ten strains previously reported and in nine new strains.

Methods and Methods

The method of isolation of mutants has been described in the previous papers (Tsugita and Fraenkel-Conrat, 1962; Tsugita, 1962a). Data obtained since make it appear possible that some but not all of the identical mutants studied by us resulted from contamination owing to the remarkable heat resistance of TMV, in that only boiling in water seems to assure sterilization of equipment. The virus proteins were prepared by the acetic acid method (Fraenkel-Conrat, 1957). Twice and thrice crystallized trypsin, crystallized pepsin, chymotrypsin, and DFP thrice crystallized carboxypeptidase were obtained from Worthington Biochemical Corp. Nagarse (protease from *Bacillus subtilis*) was supplied by the Nagase Co. (Osaka, Japan).

After tryptic digestion, the fractionation of tryptic peptides was performed by three cycles of isoelectric precipitation of peptide 1 followed by Dowex 1 × 2 column (0.9 × 150 cm) chromatographic separation of the soluble peptides as described in the preceding paper (Funatsu, 1964). Enzymatic digestions of these tryptic peptides were performed as before (Funatsu *et al.*, 1964; Tsung *et al.*, 1964). The peptides 1, 6, and 8 were digested with 0.5% of Nagarse for 20–40 minutes at room temperature using an autotitrator to maintain *p*H 8.0, and peptide 10 was digested with 4% of pepsin at *p*H 2.0 for 5 hours at 37°. Peptide 12 was digested with 2% of chymotrypsin at *p*H 8.0 for 1 hour at 37° in an autotitrator.

Partial acid hydrolysis of peptides with 0.03 N HCl was carried out at 108° in evacuated sealed tubes for 24 hours (Tsung and Fraenkel-Conrat, 1963). Under these conditions most of the aspartic acid was split off. Since peptide 6 was insoluble in 0.03 N HCl, longer hydrolysis in the presence of 50% ethanol was used (Tsung *et al.*, 1964).

Initially, the resulting peptides were fractionated after dinitrophenylation (Funatsu *et al.*, 1964), using paper chromatography with *n*-BuOH–1 N NH_3 (1:1), *n*-BuOH–AcOH–H_2O–pyridine (30:6:24:20), and *tert*-amyl alcohol–1 N NH_3 (1:1) as solvents. In later experiments, however, the peptides were chromatographed on a Dowex 1 × 2 column (0.9 × 60 cm) by means of a polygradient apparatus (Autograd, Technicon Chromatography Corp.) charged with *p*H 7.3 pyridine-colllidine-AcOH as used for the tryptic peptides (Fig. 1) (Funatsu, 1964). The Nagarse digest of peptide 1 was developed only with three chambers of the polygradient, containing 200 ml of 0.5 N, 1.7 N, and 5 N acetic acid, respectively.

When necessary, the peptides eluted from the column were further purified by paper chromatography using *n*-BuOH–AcOH–H_2O–pyridine (30:6:24:20) as solvent. The amino acid compositions of the peptides were analyzed by the Spinco automatic amino acid analyzer (Spackman *et al.*, 1958), after hydrolysis for 24 hours with 6 N HCl at 109° in evacuated sealed tubes.

Carboxypeptidase digestion was performed at 37° either at *p*H 8 using the autotitrator or at *p*H 8.2 in 0.1 M Tris buffer. The liberated amino acids were analyzed by the amino acid analyzer. In order to distinguish serine from asparagine and glutamine, the liberated amino acids were identified after dinitrophenylation (in 67% ethanol at *p*H 8–9 for 1–2 hours at room temperature) by paper chromatography using the upper layer of *n*-BuOH–N NH_3 (1:1). The modified hydrazinolysis method (Funatsu *et al.*, 1964) was employed for determination of the C-terminal amino acids. The peptides were hydrazinolyzed with 95% hydrazine

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† Present address: Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.

[1] Abbreviations used in this work: TMV, tobacco mosaic virus; DNP, dinitrophenyl-.

[2] All peptides will be referred to by their sequential numbers, starting with the acetyl N-terminal peptide (peptide 1).

TABLE I
LOCALIZATION OF AMINO ACID REPLACEMENTS IN CHEMICALLY EVOKED MUTANTS

Mutant[a]	Mutagenic Reaction	Amino Acid Changes	Peptide	Location Methods[b]	Location Residue
218	Bromination	Pro → Leu	1	Nag.	20
235 (p)	Bromination	Pro → Leu	1	Nag.	20
342 (p, 330)	Bromination	Ileu → Thr	1	Nag. Cp.	21
344 (p)	Methylation	Thr → Ala	6	Nag.	81
		Ser → Phe	12	Chy. Cp.	148
346	Deamination	Pro → Leu	1	Nag.	20
357	Deamination	Asp(NH$_2$) → Ser	1	Nag. Ac.	25
359 (p, 284)	Deamination	Arg → Lys	2		46
		Glu → Gly	8	Nag. Cp.	97
369 (p)	Deamination	Arg → Gly	2		46
		Ileu → Val	10	Pep.	129
371 (p, 329)	Deamination	Ser → Phe	11		138
		Ser → Phe	12	Chy. Hyd.	148
378 (p, 326)	Bromination	Asp(NH$_2$) → Ser	1	Nag. Cp. Ac	25
		Asp(NH$_2$) → Ser	1	Nag.	33
		Asp(NH$_2$) → Ser	10	Pep. Ac.	126
383 (p, 171)	Deamination	Asp(NH$_2$) → Ser	1	Nag. Ac.	25
		Thr → Ala	6	Ac.	81
		Pro → Leu	12	Cp.	156
384 (p, 282)	Deamination	Asp(NH$_2$) → Ser	1	Nag. Cp. Ac.	25
		Thr → Ala	6	Nag.	81
403 (p, 178)	Methylation	Arg → Gly	2		46
414	Bromination	Ser → Gly	4	Ac.	65
415	Deamination	Ileu → Val	1	Nag. Cp.	21
419	Deamination	Ser → Phe	12	Chy. Hyd.	148
421	Deamination	Val → Met	1		11
427	Deamination	Glu(NH$_2$) → Arg	8		99[c]
430	Spontaneous	Pro → Thr	1	Nag. Cp. Ac.	20

[a] (p) indicates that the analyzed sample represented progeny of an earlier preparation; (p) followed by a number means that data on the earlier preparation had been reported in the literature under that strain number (Tsugita and Fraenkel-Conrat, 1962; Tsugita, 1962a). [b] Tryptic digestion was the primary step (except for residue 156), followed by Nag., Nagarse; Cp., carboxypeptidase; Chy., chymotrypsin; Pep., pepsin; Ac., partial acid hydrolysis; Hyd., hydrazinolysis. [c] Not established with certainty.

in evacuated sealed tubes at 108° for 8–15 hours. After removal of hydrazine in an evacuated desiccator containing sulfuric acid the hydrazinolysate was applied directly to the amino acid analyzer. The subtractive method modified by Konigsberg and Hill (1962) was used in connection with the Edman degradation of the peptide.

RESULTS

The strains analyzed for exchange location are listed in Table I. Eight additional strains which were analyzed are not included because they showed identical changes with others tested at the same time and may therefore be suspected of having arisen through contamination.

The Dowex 1 × 2 column (0.9 × 150 cm) chromatography of Nagarse digests of peptide 1 from common TMV, performed by the method used for fractionation of tryptic peptides of TMV protein (Funatsu, 1964), is shown in Figure 1. Each peak was further purified on paper without or after dinitrophenylation. One of the Nagarse peptides obtained and easily isolated from peptide 1, and comprising residues 15–26 (peak i in Fig. 1), contained several of the amino acids which were exchanged frequently (proline, aspartic acid, and isoleucine). This peptide was also easy to purify after dinitrophenylation, because its DNP derivative was insoluble at *p*H 2–3 (Funatsu *et al.*, 1964). The amino acid exchanges from proline to leucine at residue 20 (mutants 218, 235, and 346) were established by amino acid analysis of the DNP peptide (DNP-Ser; $Asp_{2.0}$, $Ser_{0.3}$, $Glu_{1.2}$, $Ala_{1.7}$, $Ileu_{1.7}$, $Leu_{2.9}$ instead of DNP-Ser; Asp_2, $Ser_{0.1}$, Glu_1, Pro_1, Ala_2, $Ileu_2$, Leu_2) (Fig. 2).

The exchanges from asparagine to serine in mutants 357, 378, 383, and 384 were identified by direct poly-gradient chromatography of the Nagarse digest of peptide 1 using a slower gradient-elution system than that in Figure 1. The amino acid composition of the peptide fraction corresponding to peak i in Figure 1 (i in Fig. 3) was $Asp_{1.1}$, $Ser_{1.3}$, $Glu_{1.0}$, $Pro_{1.0}$, $Ala_{1.8}$, $Ileu_{1.9}$, $Leu_{2.0}$. This peptide has one aspartic acid and one asparagine at residues 19 and 25. In order to determine which aspartic residue was changed to serine, carboxypeptidase digestion and partial acid hydrolysis were used. In the case of mutant 378, for instance, Ser [or Asp(NH$_2$)]$_{0.4}$, $Ileu_{0.4}$, and $Leu_{0.95}$ were liberated from this peptide by carboxypeptidase digestion, but the serine was differentiated from asparagine after dinitrophenylation. By partial acid hydrolysis of this peptide, $Asp_{1.0}$, $Ser_{0.1}$, $Ala_{0.3}$, $Leu_{0.2}$ were liberated as free amino acids from this mutant instead of $Asp_{1.9}$, $Ala_{0.2}$, $Leu_{1.0}$ from normal peptide i. These results confirmed that asparagine at residue 25 was replaced by serine (Fig. 2).

As shown in Figure 3, the peak b (glycine-asparagine) of Figure 1 appears to be absent from the digest of strain 378. The fractions from tube 173 to 191 were pooled and lyophilized. After dinitrophenylation, a peptide which was composed of DNP–Ala, $Ser_{0.94}$, $Glu_{1.02}$, $Gly_{0.94}$, $Leu_{10.0}$, and $Phe_{0.83}$ was isolated by paper chromatography. Since there is only one glycine in peptide 1, this peptide is DNP-Ala-Leu-Gly-Ser-Glu(NH$_2$)-Phe

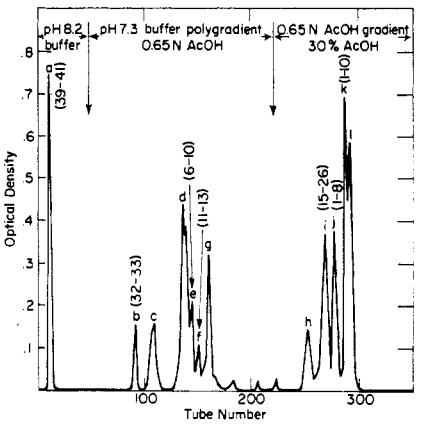

FIG. 1.—Chromatography of Nagarse digest of peptide 1 (common TMV) on Dowex 1 × 2 column (0.9 × 150 cm). Nagarse digest of peptide 1 was applied to the Dowex 1 × 2 column which was equilibrated with pyridine-collidine-AcOH buffer (*p*H 8.8) and then eluted with the same buffer system as that used for the tryptic peptides of TMV (Funatsu, 1964). A 250-ml quantity of *p*H 8.2 collidine–pyridine-acetic acid (1%, 1%, 6.25 × 10^{-3} M), then nine-chamber polygradient containing in two chambers a *p*H 7.3 collidine–pyridine-acetic acid (1%, 1%, 0.03 N), followed by 0.02 N acetic acid (two chambers) 0.1, 0.2, 0.35, 0.5, 0.65 N acetic acid (135 ml/chamber), and then by three chambers with 0.65 N, 1.7 N, and 5 N acetic acid (10, 30%). Flow rate, 40 ml/hr; temperature, 35°. Most of the peptide fractions were purified by paper chromatography using *n*-BuOH-AcOH-H_2O-pyridine (30:6:24:20) and then analyzed for amino acid composition. Numbers in parentheses show the amino acid residue sequential numbers of the main components (see Fig. 2 for complete sequence). The coordinates represent absorbance at 700 mμ of the Folin-Lowry test (optical density), performed on 0.2-ml aliquots unless otherwise stated, and tube number, each tube containing about 6.3 ml.

and the exchange from asparagine to serine occurred at residue 33.

The isolation of the peptide comprising residues 15–26 by a simplified elution scheme is illustrated in Figure 4 (peak b). The same elution pattern of a Nargase digest of peptide 1 from mutant 430 is shown in Figure 5. In this chromatogram, a new peptide peak appeared preceding peak b. This new peak had the amino acid composition $Asp_{1.1}$, $Ser_{1.5}$, $Ala_{2.0}$, [Try][3] corresponding to residues from 14 to 19, while the peptide corresponding to peak b consisted of $Asp_{2.0}$, $Thr_{0.9}$, $Ser_{0.5}$, $Glu_{1.0}$, $Ala_{2.0}$, $Ileu_{1.9}$, $Leu_{2.0}$, [Try]. The new peptide was produced by splitting the peptide bond between aspartic acid (residue 19) and the threonine (residue 20) which replaced the proline.

In the mutants 342 and 415 which showed exchanges from isoleucine to threonine and from isoleucine to valine, respectively, the amino acid compositions of this same peptide fraction (residue 15–26) were $Asp_{2.0}$, $Thr_{0.9}$, $Ser_{1.4}$, $Glu_{1.2}$, $Pro_{1.0}$, $Ala_{2.0}$, $Ileu_{1.1}$, $Leu_{2.1}$; and $Asp_{2.0}$, $Ser_{1.0}$, $Glu_{1.0}$, $Pro_{1.2}$, $Ala_{2.0}$, $Val_{1.0}$, $Ileu_{1.0}$, $Leu_{1.9}$, respectively. Since there are isoleucines at both residues 21 and 24 in this peptide in common TMV, the mutant peptides were digested further with carboxypeptidase. From both mutants asparagine, glutamic acid, isoleucine, and leucine (about 1:1:1:2) were liberated, but not threonine nor valine. These results indicated that isoleucine at residue 24 was present while isoleucine at residue 21 was changed to threonine or valine.

Dowex 1 × 2 column chromatographic patterns of certain enzymatic digests of other tryptic peptides of the TMV protein have already been published (Funatsu *et al.*, 1964; Tsung *et al.*, 1964). The points of enzymatic cleavage of these peptides are summarized in Figure 6. These methods were used to locate mutational exchanges in the peptides.

In mutants 344, 383, and 384, one less threonine and one more alanine were found in peptide 6. Therefore peptide 6 from mutants 344 and 384 was digested with Nagarse and fractionated by paper chromatography using *n*-BuOH–AcOH–H_2O–pyridine (30:6:24:20) after dinitrophenylation. A peptide with the composition

[3] Amino acid residues given in brackets are presumed to be present but were not determined by analysis.

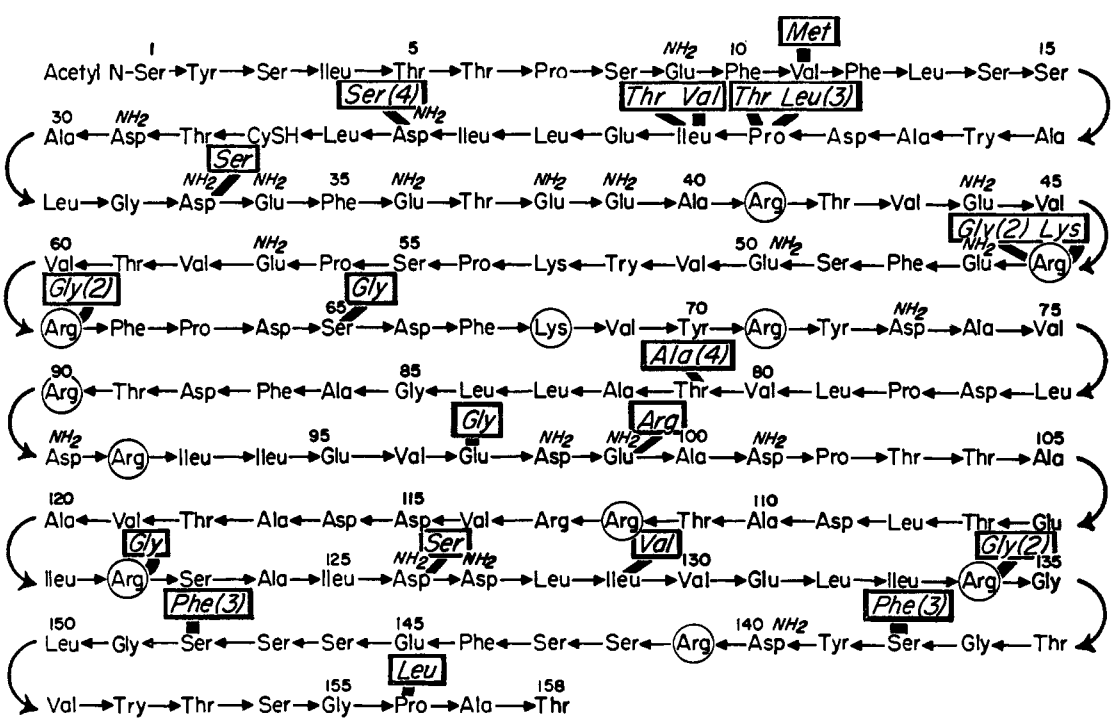

FIG. 2.—Distribution on the peptide chain of TMV protein, and frequency, of all amino acid exchanges observed in this laboratory. Number in parentheses shows the frequency of the exchange, omitting questionable mutants.

DNP-Val, $Ala_{2.0}$, and $Leu_{2.0}$ was found to replace that of common TMV, composed of DNP-Val, Thr_1, Ala_1, and Leu_2. The peptide 6 of mutant 383 was hydrolyzed with 0.03 N HCl in 50% ethanol and fractionated by Dowex 1 × 2 (0.9 × 60 cm) column chromatography (Fig. 7). The amino acid composition of peak c was $Pro_{1.0}$, $Gly_{2.3}$, $Ala_{4.5}$, $Val_{1.0}$, $Leu_{3.0}$, $Phe_{2.3}$, and lacked Thr. This peak apparently represented a mixture of the two peptides Pro_1, Gly_1, Ala_3, Val_1, Leu_3, Phe_1, and Gly_1, Ala_1, Phe_1. The amino acid analysis of peak a gave Thr, [Arg]. These results indirectly indicated that it was the threonine at residue 81 which was replaced by alanine. The exchange from glutamic acid to glycine in peptide 8 of mutant 359 was easily detected by the change of peak position on Dowex 1 × 2 column chromatography (Funatsu, 1964). After digestion with Nagarse, the digest was chromatographed on a 60-cm Dowex 1 × 2 column. As shown in Figure 8, the amino acid composition of peak c was $Asp_{1.0}$, $Glu_{1.8}$, $Gly_{1.0}$, $Val_{0.9}$, $Ileu_{0.7}$. This peptide liberated $Asp(NH_2)$ and/or $Glu(NH_2)_{1.8}$, $Gly_{1.0}$, $Val_{0.5}$ upon carboxypeptidase digestion. The corresponding peptide from common TMV was usually eluted after peak d (Thr-Ala-Glu-Thr-Leu) as reported in a previous paper (Funatsu *et al.*, 1964).

The exchanges from isoleucine to valine and from asparagine to serine in peptide 10 of mutants 369 and 378, respectively, were located after peptic digestion or partial acid hydrolysis (Figs. 9 and 10). The amino acid compositions of peak c in Figure 9 and peak d in Figure 10 were $Val_{2.0}$, $Glu_{1.0}$, and $Ser_{1.8}$, $Ala_{1.3}$, $Ileu_{1.0}$ instead of Val_1, Glu_1, $Ileu_1$ and Ser_1, Ala_1, $Ileu_1$, $Asp(NH_2)$, respectively. It is noteworthy that the erroneous sequence previously reported (Tsugita *et al.*, 1960) for residues 125 and 126 (aspartyl-isoleucine) of common TMV was first suspected on the basis of the data obtained with this mutant.

The exchanges from serine to phenylalanine in peptide 12 of mutants 344, 371, and 419 were located at residue 148. As shown in Figure 11, the chymotryptic digest of peptide 12 was cleanly separated by Dowex 1 × 2 column chromatography. Hydrazinolysis of peak c, which has the amino acid composition $Ser_{3.4}$, $Glu_{1.0}$, $Phe_{1.8}$, gave only phenylalanine, and carboxypeptidase digestion gave also phenylalanine and a small amount of serine.

In cases where an amino acid exchange involved a residue occurring only once in a tryptic peptide, sequential analyses were not always performed. The exchanges from arginine to lysine or to glycine (mutants 359, 369, and 403) were easily located by disappearance of the peak of the tryptic peptide 2 upon Dowex 1 × 2 chromatography. The exchange from serine to phenylalanine in peptide 11 did not cause a noticeable change of the peak position for this peptide, but was detected by amino acid analysis of all serine-containing peptides. When the proline at residue 156 was changed to leucine in mutant 383, the digestion of virus with carboxypeptidase released threonine, alanine, and leucine instead of threonine only (Tsugita and Fraenkel-Conrat, 1960). The partial acid hydrolysis of peptide 4 of mutant 414 gave $Asp_{2.0}$, $Gly_{0.96}$ instead of Asp_2, Ser_1. In the mutant 421, one methionine was found in peptide 1 instead of one valine.

The location of the exchange from glutamine to arginine in strain 427 was attempted using only 6 mg of protein. After tryptic digestion, the entire digest was applied to a 60-cm Dowex 1 × 2 column and developed with almost the same elution schedule as usual, but with only half the volume of each solvent. As shown in Figure 12, peak i was eluted earlier than peptide 8 is customarily, although it corresponded in amino acid

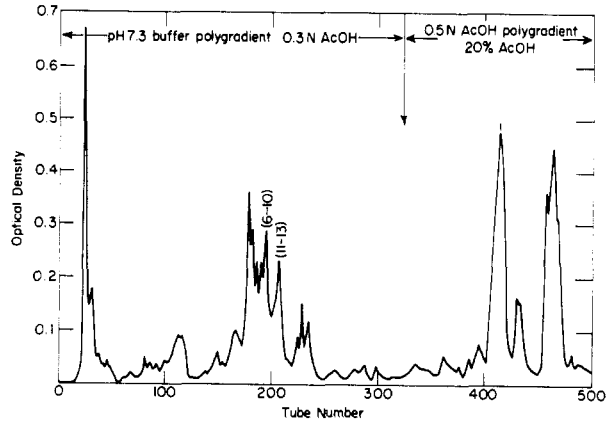

FIG. 3.—Chromatography of Nagarse digest of peptide 1 (mutant 378, 2 aspartic acid → 2 serine) on Dowex 1 × 2 column (0.9 × 150 cm). Nagarse digest of peptide 1 was applied to the Dowex 1 × 2 column which was equilibrated with pyridine-collidine-AcOH buffer (*p*H 8.8) and then eluted by means of the polygradient-elution system having each 135 ml of pyridine-collidine-AcOH buffer (*p*H 7.3; three chambers), 0.02 N AcOH (2 chambers), 0.1 N AcOH, 0.2 N AcOH, and 0.3 N AcOH, followed by a gradient having each 200 ml of 0.5 N AcOH, 1.7 N AcOH, and 3.4 N AcOH. Flow rate, 40 ml/hr; temperature, 35°. Peptide corresponded to residues 15–26 with an aspartic residue (25) being replaced by serine. In this and all subsequent elution patterns each tube contained about 3.3 ml.

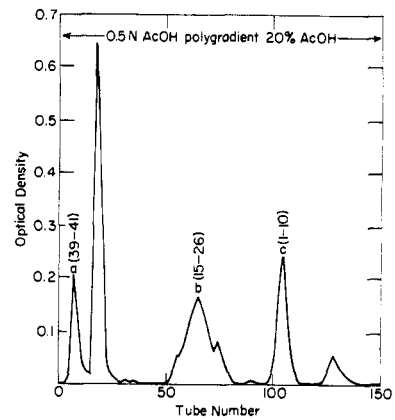

FIG. 4.—Chromatography of Nagarse digest of peptide 1 from common TMV on Dowex 1 × 2 column (0.9 × 60 cm). Nargase digest of peptide 1 was applied to the Dowex 1 × 2 column (0.9 × 60 cm) which was equilibrated with pyridine-collidine-AcOH buffer (*p*H 8.8) and then eluted by means of a polygradient-elution system having each 200 ml 0.5 N AcOH, 1.7 N AcOH, and 3.4 N AcOH at room temperature. Flow rate, 10 tubes (33 ml)/hr. Numbers in parentheses show the residue numbers.

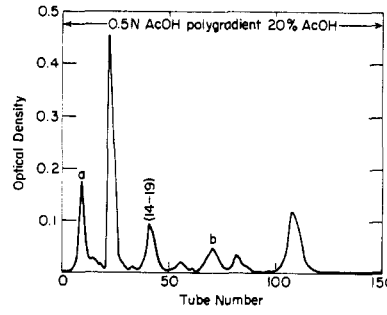

FIG. 5.—Chromatography of Nagarse digest of peptide 1 (mutant 430, proline → threonine) on Dowex 1 × 2 column (0.9 × 60 cm). Chromatographic conditions were the same as described in Fig. 4. Peptide b had the composition corresponding to residues 15–26, with the proline (20) replaced by threonine.

Peptide 1. Nagarse

5 10 15
acetyl-N-Ser-Tyr-Ser-Ileu-Thr-Thr-Pro-Ser-Glu(NH_2)-Phe-Val-Phe-Leu-Ser-Ser-
20 30
Ala-Try-Ala-Asp-Pro-Ileu-Glu-Leu-Ileu-Asp(NH_2)-Leu-CySH-Thr-Asp(NH_2)-Ala-Leu-Gly-
35 40
Asp(NH_2)-Glu(NH_2)-Phe-Glu(NH_2)-Thr-Glu(NH_2)-Glu(NH_2)-Ala-Arg

Peptide 6. Nagarse
Tyr-Asp(NH_2)-Ala-Val-Leu-Asp-Pro-Leu-Val-Thr-Ala-Leu-Leu-Gly-Ala-Phe-Asp-Thr-Arg

Peptide 8. Nagarse
Ileu-Ileu-Glu-Val-Glu-Asp(NH_2)-Glu(NH_2)-Ala-Asp-Asp(NH_2)-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg

Peptide 10. Pepsin
Ser-Ala-Ileu-Asp(NH_2)-Asp(NH_2)-Leu-Ileu-Val-Glu-Leu-Ileu-Arg

FIG. 6.—Enzymatic digestion of tryptic peptides of TMV protein. Points of enzymatic cleavage of peptides 1, 6, 8, and 10. These are deduced from information already reported (Funatsu *et al.*, 1964; Tsung *et al.*, 1964). Solid arrows indicate main points of enzymatic attack, and dotted arrows secondary points of cleavage.

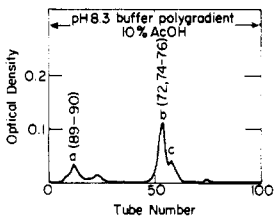

FIG. 7.—Chromatography of partial acid hydrolysate of peptide 6 (mutant 383, threonine → alanine) on Dowex 1 × 2 column (0.9 × 60 cm). The elution system of the column was a gradient having per chamber 135 ml of pyridine-collidine-AcOH buffer (*p*H 7.3), 0.2 N AcOH, 0.65 N AcOH, and 1.7 N AcOH. Elution was done at room temperature at the same flow rate as others. Peptide c analyzed as if it were a mixture of residues 85–87 and 78–87, with the replacement of the threonine by an alanine.

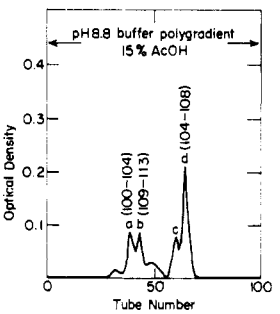

FIG. 8.—Chromatography of Nagarse digest of peptide 8 (mutant 359, glutamic acid → glycine) on Dowex 1 × 2 column (0.9 × 60 cm). (Elution schedule of Fig. 1.) Peak c analyzed for residues 93–99, with a glutamic acid replaced by a glycine residue.

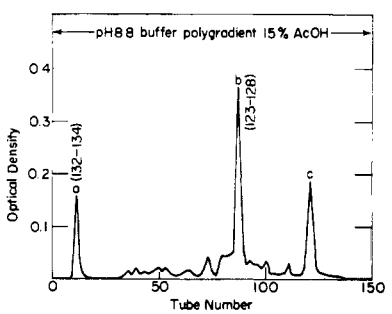

FIG. 9.—Chromatography of peptic digest of peptide 10 on the Dowex 1 × 2 column (0.9 × 60 cm). (Elution schedule of Fig. 1.) Peak c analyzed for residues 129–131, with isoleucine replaced by valine.

(neutral and acidic) composition, but for one less glutamic acid, to peptide 8. After the first step of Edman degradation, one isoleucine and one alanine were missing in the residual peptide (Funatsu, 1964). These findings are interpreted as owing to the replacement of glutamine residue 99 by arginine. It appears probable that the two peptides produced by tryptic digestion of the modified peptide 8 (glutamine replaced by arginine), would not separate on the Dowex 1 × 2 column since they would have the same net charge and lack of aromatic residues.

DISCUSSION

A survey of the location of all amino acid exchanges observed in this laboratory (Fig. 2) shows that the majority occur among the C-terminal and N-terminal 25 residues. Other frequently replaced residues are 46 and 81. No exchanges were observed from residue 100 to 121, a part of the molecule which also seems similar and in good part identical in all natural strains investigated (Tsugita, 1962b; Wittmann, 1962; Funatsu *et al.*, 1964). It appears probable that this part of the molecule plays a particular role in the folding of the chain which assures the proper shape of the molecule and enables it to coaggregate with RNA to form stable virus particles. On the other hand, the function of other segments, and particularly the C-terminal, appears to be less critically dependent upon a singular sequence of amino acids, and at the same time more susceptible to enzymatic and chemical alteration (Harris and Knight, 1952; Fraenkel-Conrat, 1962).

In previous papers from this and other laboratories, the amino acid exchanges observed in TMV mutants have been compared to the codons as deduced from amino acid incorporation data in the cell-free *Escherichia coli* system (Tsugita and Fraenkel-Conrat, 1962; Tsugita, 1962a). The first codons to be reported by Nirenberg and Matthaei (1961) were in excellent agreement with some of the TMV data: proline (=CCC) was frequently lost by deamination, and phenylalanine (=UUU) was never lost but frequently appeared in deamination mutants. However, not all the exchanges could be rationalized, even if one assumed that the hypoxanthine resulting from deamination of A would code like G. In the past two years the number of recognized codons has been greatly increased through the study of the action of U-free polynucleotides in the Nirenberg system (Nirenberg and Jones, 1963; Ochoa, 1963). When the present data are compared with this

TABLE II
AMINO ACID REPLACEMENTS OBSERVED IN CHEMICALLY INDUCED TMV-MUTANTS

Nature of Exchanges	Frequency of Observation of: Deamination	Bromination	Methylation	Possible Condon Exchanges[a]
Gly	(2)[b]			AGC → GGC
Asp ↗ ↘ Ala	(4)			
Asp(NH$_2$) → Ser	4 (2)	3		ACA → GCA
Ala	2			ACA → GCA
Thr → Ileu	(9)			CAA → UAA
Met	(3)			
Phe	4 (4)	2	2	CUU → UUU
Ser → Gly		1		ACG → GCG
Leu	(2)			
Glu → Gly	1 (1)			AUG → GUG
Arg ↗ Glu(NH$_2$) ↘ Val	1 (2)			
Leu	3 (3)	4	4	CUC → UUC
Pro ↗ ↘ Ser	(3)			CCU → UCU
Val → Met	1			
Val	2 (3)			AUU → GUU
Ileu → Thr		1		
Met	(1)			AUA → GUA
Leu → Phe	(1)			CUU → UUU
Gly	3		1	AGA → GGA
Arg ↗ ↘ Lys	1			

[a] On the basis of codons reported by Ochoa (1963) and Nirenberg and Jones (1963); sequences arbitary. [b] Figures in parentheses: exchanges reported by Wittmann (1962) and Wittmann and Wittmann-Liebold (1963).

broadened spectrum of codons (Table II), it becomes evident that all six nitrous acid mutant exchanges obtained by us more than once (total about eighteen), four of them also repeatedly obtained by Wittmann, can be accounted for by C-to-U and A-to-G transformations, although this may no longer be true if the order of the coding nucleotides becomes definitely established and is taken into consideration. It should be noted that two exchanges previously reported from this laboratory (Tsugita, 1962a) were erroneous. Strains 171 and 282 have asparagine → serine, and threonine → alanine exchanges, rather than aspartic acid → alanine, and threonine → serine.

In contrast to our exchanges several of the recurring exchanges observed by Wittmann in nitrous acid mutants do not accord with the codon data (e.g., aspartic acid → alanine, threonine → methionine, glutamine or glutamic acid → valine, four, three, and two times, respectively) (Wittmann, 1962; Wittmann and Wittmann-Liebold, 1963). Besides this fact, that the exchanges observed most frequently by us are not the same as those observed in Tübingen, one is also disturbed by the observation that the frequently recurring exchanges observed here are the same regardless of the

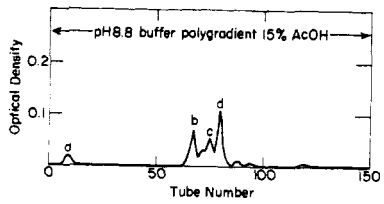

FIG. 10.—Chromatography of partial acid hydrolysate of peptide 10 (mutant 378, asparagine → serine) on Dowex 1 × 2 column (0.9 × 60 cm). (Elution schedule of Fig. 1.) Peak d analyzed for residues 123–126, with the asparagine replaced by serine.

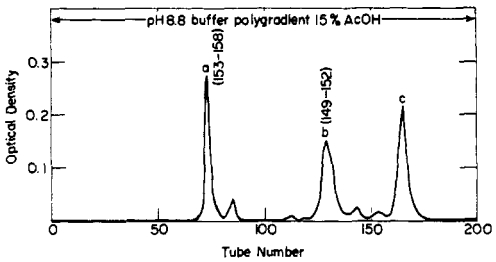

FIG. 11.—Chromatography of chymotryptic digest of peptide 12 (mutant 419, serine → phenylalanine) on Dowex 1 × 2 column (0.9 × 60 cm). (Elution schedule of Fig. 1.) Peak c analyzed for residues 142–148, with the last serine replaced by phenylalanine.

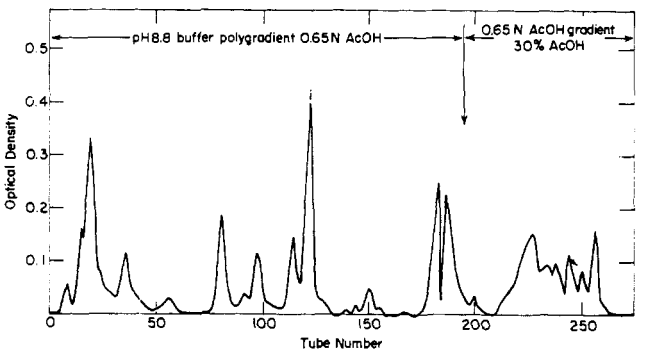

FIG. 12.—Chromatography of the tryptic digest of mutant 427 (glutamine → arginine) on Dowex 1 × 2 column. The tryptic digest of mutant 427 (6 mg) was applied to a Dowex 1 × 2 column (0.9 × 60 cm) which was equilibrated with pyridine-collidine-AcOH buffer (*p*H 8.8) and eluted by means of a polygradient-elution system having per chamber 70 ml of pyridine-collidine-AcOH buffer (*p*H 8.8), pyridine-collidine-AcOH buffer (*p*H 8.2), pyridine-collidine-AcOH buffer (*p*H 7.3), 0.02 N AcOH, 0.02 N AcOH, 0.2 N AcOH, 0.35 N AcOH, 0.5 N AcOH, and 0.65 N AcOH, and finally a gradient having each 50 ml of 0.65 N AcOH, 1.7 N AcOH and 5 N AcOH at room temperature. Flow rate, 36 ml/hr. For peak i analysis see text.

mutagen used. If one postulated that 5-bromocytosine might code like uracil, this could account for the proline → leucine exchange which was observed in four bromination mutants, but it could not account for the asparagine → serine exchange observed with the same frequency. On the other hand, if one wanted to postulate that methylation of A might make it code like G, then this could account for the single arginine → glycine exchange, but not for the more frequent serine → phenylalanine and proline → leucine exchanges observed in methylation mutants.

In support of previous conclusions, neighboring amino acids were never found to be exchanged, an observation which rules out all overlapping codes. Further, all exchanges were unidirectional (proline → leucine, serine → phenylalanine, etc., but never leucine → pro-

line, phenylalanine → serine), as would be expected for those exchanges caused by definite chemical reactions (e.g., deamination), but not for spontaneous and/or random changes in the coding behavior of bases, which are held to be responsible for some of our and Wittmann's data.

However, as stated, the testing of our virus preparations has been plagued by a considerably higher level of spontaneous mutation or contamination in the past 2 years than at earlier stages of this study. Thus we cannot safely assume that all mutants isolated are actually the consequence of the chemical agent to which the RNA (or the virus) was exposed. It is for this reason that we have not isolated new mutants produced by the low-efficiency mutagenic reactions (bromination or methylation) but have concentrated our efforts on nitrous acid mutants which with high probability are the actual result of deamination. The localization of exchanges was in good part performed on mutants isolated several years ago. The preparation of progeny of such mutants showing the original amino acid exchanges has not been affected by the problems encountered with the isolation of new mutants.

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Further Attempts to Characterize Products of TMV-RNA-directed Protein Synthesis*

H. G. AACH,† G. FUNATSU,‡ M. W. NIRENBERG, AND H. FRAENKEL-CONRAT

From the Virus Laboratory, University of California, Berkeley, and the National Institutes of Health, National Heart Institute, U. S. Public Health Service, Bethesda, Md.

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Radioactive peptides or proteins synthesized in a cell-free *E. coli* amino acid–incorporating system under the direction of TMV-RNA were characterized in order to assess possible similarities to TMV-specific products. Chromatographic separations of proteins and tryptic peptides as well as serological methods were used to determine whether TMV-coat protein or other unknown TMV-specific protein products were formed. No clear evidence was obtained that a significant fraction of the amino acids incorporated in soluble proteins or peptides could be identified with such products.

In some preceding experiments the thesis was tested whether purified TMV-RNA could direct the cell-free synthesis of TMV protein in *Escherichia coli* extracts under conditions optimal for amino acids incorporation (Tsugita *et al.*, 1962). Different methods used in those experiments for the identification of the products which were synthesized by the *E. coli* system gave somewhat conflicting results. While one serological experiment as well as certain chemical tests seemed to support that thesis, reconstitution experiments with the *in vitro*–synthesized proteins failed to do so. Therefore the possibility was considered that under our experimental conditions protein might be synthesized *in vitro* in such a manner that it was unable to form its proper 3-dimensional structure. This would be expected if the product of cell-free protein synthesis represented either denatured or incomplete TMV protein.

Additional experiments employing both chemical and serological methods have now been performed to attempt substantiation of the conclusion that soluble products synthesized by the *E. coli* system were related to TMV-coat protein, or to other proteins synthesized in TMV-infected tobacco leaf cells. The results of these experiments were essentially negative, so that the nature of the proteins synthesized in the cell-free *E. coli* system under the influence of TMV-RNA must at present be regarded as uncertain.

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† Present address: Botanischen Institut, Technische Hochschule, Aachen, Germany.

‡ Present address: Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.