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*THE AMINO ACID COMPOSITION AND C-TERMINAL SEQUENCE OF A CHEMICALLY EVOKED MUTANT OF TMV**

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Most naturally occurring strains of tobacco mosaic virus (TMV) have been found to show differences in their amino acid composition.[1, 2] When it was found that mutants could be artificially produced by means of chemical modification of the virus or its RNA,[3] the question arose whether such mutants would also show detectable changes in the composition or amino acid sequence of their protein. Because of recent advances in the methodology of amino acid analysis,[4] the detection of changes as small as the replacement of one amino acid residue per peptide chain has become possible. A preliminary survey of several different chemically evoked mutants suggests that such replacements actually occur. The detailed study of the amino acid composition of one particular mutant, to be reported here, indicates the replacement of one residue each of proline, aspartic acid, and threonine by leucine, alanine, and serine. Furthermore, it is shown that at least one of these changes takes place near the C-terminus of the peptide chain, thereby greatly affecting the susceptibility of the protein to attack by carboxypeptidase.

Experimental.—The RNA was isolated from TMV by the phenol method[5] with 10^{-3} *M* versenate as buffer.[6] The nitrous acid treatment was performed according to the method of Schuster and Schramm.[7] Other reactions used to produce mutants were with small amounts of dimethyl sulfate, and with *N*-bromosuccinimide, both at 0° and pH 6–8; details of these modifications will be reported elsewhere.

After the chemical treatment, the RNA was isolated by repeated alcohol precipitations and reconstituted with native protein by the standard procedure.[8] The extent of inactivation was determined by assay on the Xanthi variety of *N. tabacum*. For the detection of mutants, the reconstituted virus was applied to *N. sylvestris* and Java Tobacco. In confirmation of Gierer and Mundry,[3] we observed frequent local lesions on these systematically-diseased test plants when nitrous acid had been employed but almost never with untreated, reconstituted virus. In addition, we obtained local lesions with somewhat lesser frequency after treatment with the methylating and brominating agents listed above but hardly ever after a variety of other chemical modifications.

The local lesions were cut out and serially transferred (2–4 times) in the same hosts until only local lesions without systemic infection resulted. Subsequent

TABLE 1

ANALYSIS OF NITROUS ACID MUTANT (171) AND TMV*

	Mutant 171			Tobacco Mosaic Virus (TMV)				
Amino acid	24-hr hydrol.	48-hr hydrol.	Nearest integer value	24-hr hydrol.	48-hr hydrol.	Nearest integer value	Ramachandran[12]	Wittmann and Braunitzer[13]
Asp	16.6	16.8	*17*	17.3	18.1	18	*19*	18
Thr	14.0	13.2	*15*†	15.0	14.4	16†	*17*	16
Ser	14.7	13.1	(16–) *17*†	14.2	12.8	(15–) 16†	*18*	16
Glu	15.5	15.4	16	15.4	15.5	16	*17*	16
Pro	7.0	6.8	*7*	8.1	7.9	8	8	8
Gly	6.0	6.0	6	6.0	6.0	6	6	6
Ala	14.8	14.8	*15*	14.0	14.0	14	*15*	14
Cys/2	0.5	0.3	1	0.6	0.4	1	1	1
Val	13.4	13.7	14	13.2	13.7	14	14	14
Ileu	8.0	8.6	9	7.8	8.5	9	*9*	*8*
Leu	12.6	12.7	*13*	11.7	11.9	12	*13*	12
Tyr	3.9	3.8	4	3.9	4.0	4	4	4
Phe	7.9	7.9	8	7.9	8.0	8	8	8
Lys	2.0	2.1	2	2.1	2.0	2	2	2
Arg	11.1	11.0	11	11.1	10.9	11	11	11
Try	..	..	(3)	..	..	(3)	(2)	3
Total			158			158	164	157

* Average of duplicate or triplicate results. Maximum error = ±0.1. Calculation is based on Kjeldahl nitrogen using Prot. N content = 16.2%. Calculated as number of residues per gram mole virus protein (i.e., = 18,000 g) as analyzed after 24 and 48 hr of hydrolysis (6 *N* HCl, 108° in high vacuum-sealed tubes). Values for 72 hr of hydrolysis of TMV were not listed since they differed significantly only in regard to serine and threonine. In italics are values which show differences from present analysis of common TMV.

† Values for threonine after extrapolation to 0-time of hydrolysis (see Fig. 1) were 14.8 and 15.5 for the mutant and TMV, respectively; for serine the corresponding values were 16.3 and 15.4, respectively.

transfer to *N. tabacum* produced a systemic disease which varied in severity for different isolates but was more often quite severe and necrotising on this host. The virus mutants were harvested from *N. tabacum* 3 weeks after inoculation, and isolated, as usual, by differential centrifugation. They were again tested on the various test plants to ascertain that they had not changed biologically.

Protein was prepared from the virus by the acetic acid method.[9] The protein (6–12 mg) was then hydrolyzed for 24, 48, and, if possible, 72 hr in 1 ml of 6 *N* twice redistilled HCl sealed under high vacuum. The acid was then evaporated; the residue was redissolved in water and again evaporated, and then redissolved in pH 3.2 buffer, freed from insoluble humin, and applied to the ion exchange column of the Spinco Amino Acid Analyzer.[4]

For the carboxypeptidase treatment of viral protein, commercial crystalline

carboxypeptidase (Worthington Biochem. Corp., #CO570) was treated with diisopropylphosphofluoridate by the procedure of Gladner *et al.*[10] The protein was treated with one two-hundredth of its weight of carboxypeptidase at room temperature for various time periods up to 47 hr. A second addition of enzyme was made to digests held longer than 24 hr. At the end of the digestion period, the samples were adjusted to pH 4.3, the insoluble protein was removed, and the supernatant brought to pH 2 and directly applied to the Amino Acid Analyzer. Control experiments with the enzyme alone showed no detectable amounts of liberated amino acids. TMV gave the expected threonine (0.9 equivalents), and a small amount of serine in one 24-hr digest.

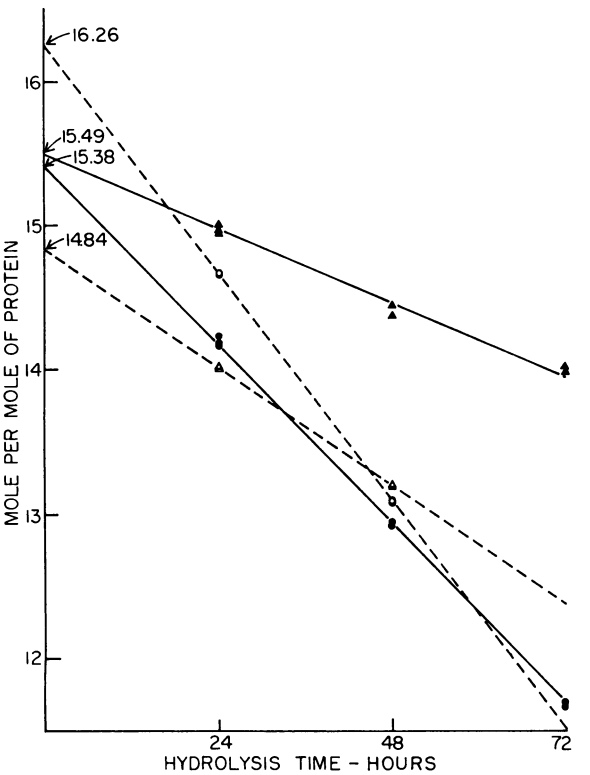

FIG. 1.—Decomposition of serine and threonine during acid hydrolysis. The results of individual analyses are plotted. Those for serine are indicated by circles, those of theonine by triangles. The open symbols are the results obtained with the mutant #171, the filled-in symbols those with common TMV. The intercept values obtained by extrapolation to zero time are given. (For experimental details, see text.)

Results and Discussion.—Several mutants obtained with nitrous acid or *N*-bromosuccinimide were analyzed in preliminary studies when the accuracy of analysis had not yet been stabilized. In each case one or several differences between the mutant and the starting material, common TMV, were detected. The strain which was selected for a thorough study was a nitrous acid mutant (#171) which had been obtained from an RNA preparation inactivated to about 99 per cent. Since mutants were observed with similar or greater frequency concomitant with much lesser inactivation, and, as stated above, only with certain inactivating agents and not with others, we conclude, in full accord with Mundry,[11] that mutagenesis and not selection is here operative. The mutant #171 gave only local lesions on *N. sylvestris* and Java Tobacco, and produced a mosaic disease on Turkish which was milder than that given by common TMV.

The results of the amino acid analysis of the mutant strain #171 and of TMV are listed on Table 1. Comparison of these analyses shows clearly a decrease by one residue in the number of aspartic acids, threonines, and prolines and an increase by one alanine, serine, and leucine residue. Also included in Table 1 is a comparison of the present analysis of common TMV with that reported by

Ramachandran,[12] and that derived from a summation of the 12 component peptides by Wittmann and Braunitzer.[13] It appears that the present analysis differs from Ramachandran's by 2 residues of serine, and one each of aspartic and glutamic acids, threonine, alanine, and leucine. With the exception of one isoleucine residue, the present analysis agrees completely with that obtained more laboriously by Wittman and Braunitzer.[13] Since isoleucine is liberated only slowly from peptide linkage, and since Wittman has recently also obtained values approaching our preferred value of 9 residues,[14] it would seem that this is more probably the correct value. The over-all outcome of this comparison of results obtained in different laboratories is astonishingly concordant. It thus appears that the tech-

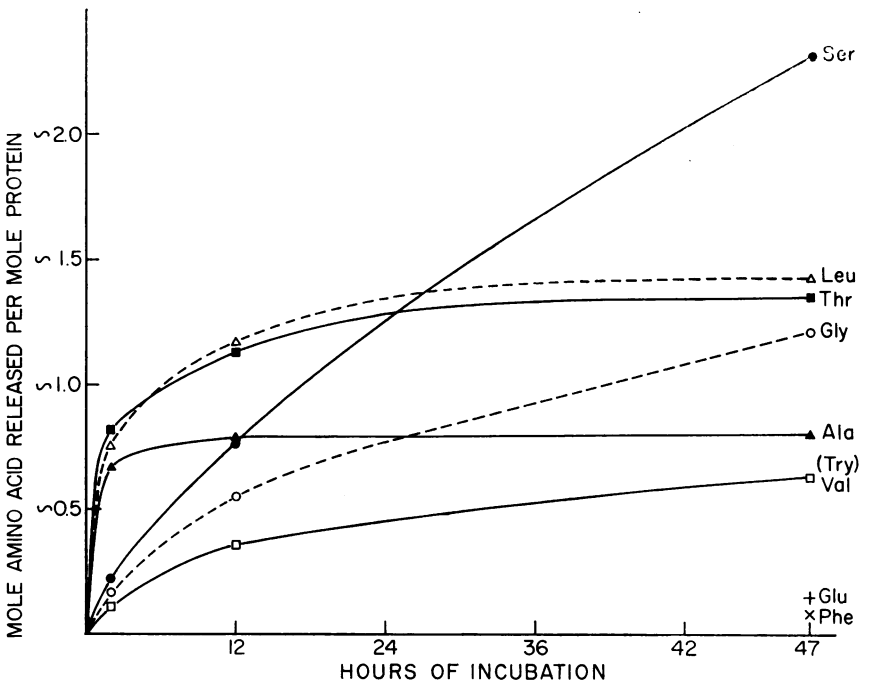

FIG. 2.—Carboxypeptidase digestion of the protein of mutant #171. The released amino acids were determined by means of the Amino Acid Analyzer after removal of the protein by isoelectric precipitation. The aliquot size after the longest (47-hr) enzyme treatment is ±10%, an error which could affect the absolute level of each amino acid, but not the relative amounts. (For experimental details, see text.)

nique of amino acid analysis has now advanced to a point where, under favorable circumstances, complete analysis of a protein containing 158 residues is possible with sufficient accuracy to establish the number of residues of almost all amino acids. One remaining slight uncertainty is the exact number of serine and threonine residues, which is due to the progressive decomposition of these amino acids during hydrolysis. Extrapolation of the resultant decay curves to zero time is the accepted procedure (see Fig. 1), but such extrapolation magnifies the observed error of each analysis and thus leads to a range of about one residue out of 16 between the highest and lowest extrapolated value. Thus, the listed serine and threonine content of TMV as well as of the mutant may possibly be in error by one residue.

The values reported by Wittmann[14] show a slightly greater range of uncertainty in this regard. The analysis and summation of the isolated peptides, each containing maximally only 6 serine residues, should be expected to give reliable values were it not for the fact that no details concerning such factors as hydrolysis times, extrapolation slope, and number of replicate analyses have as yet been reported for the isolated peptides.[13] The fact remains that the reported exchange of one threonine for a serine residue in mutant 171 is established with slightly less certainty than the other two amino acid replacements.

Treatment of TMV and all its natural strains with carboxypeptidase has been shown to lead to the release of one residue of threonine and no other amino acids from each peptide chain.[15, 16] This has been attributed to the since-established C-terminal -pro-ala-thr sequence[17, 18] since proline has been found in several other instances to present a block to carboxypeptidase action which may extend to its neighboring residue. It therefore seemed of interest to test the effect of carboxypeptidase on the protein of the mutant strain #171, which lacked one of the 8 proline residues. It became immediately evident that this strain behaved quite differently from all previously studied strains of TMV in this regard. Analysis of the released amino acids indicated, after as short a period as 2 hr, the presence of nearly one residue of threonine, leucine, and alanine and lesser amounts of serine, glycine, and valine (see Fig. 2). Analysis after varying time periods up to 47 hr showed a faster release of glycine, valine, and particularly several serine residues. Tryptophane and small amounts of glutamic acid and phenylalanine were also detected. An inspection of the known amino acid sequence of the C-terminal peptide of common TMV, as listed below,[19, 20] indicates that this would be the expected pattern of amino acid release if the proline which normally blocks the action of the enzyme were replaced by leucine. The appearance of glutamic acid and phenylalanine indicates that digestion has progressed as far as 15 residues into part of the peptide chains. The data definitely prove marked alteration of the C-terminal sequence in the mutant under study and the absence of proline from the degraded part of the molecule. Its replacement by a leucine residue represents the most probable interpretation of these data. The rest of the C-terminal sequence may be the same as in common TMV, although the presence of many serine residues in this part of the molecule renders the interpretation somewhat equivocal. Thus, according to an alternate interpretation of the relative amino acid release rates, the threonine in sixth position might be exchanged by a serine residue:

-ser-ser-phe-glu-ser-ser-ser-gly-leu-val-try-thr-ser-gly-*pro*-ala-thr-OH →
-ser-ser-phe-glu-ser-ser-ser-gly-leu-val-try-thr-ser-gly-*leu*-ala-thr-OH.

Further studies on the structure of this mutant are in progress.

The results reported clearly demonstrate that a mutant strain of TMV has been isolated which differs markedly from all previously studied mutants. It is impossible to prove unequivocally that this mutant was actually produced by the chemical treatment and did not originate spontaneously. However, the proportion of local lesions evoked on *N. sylvestris* by modified virus to those given by common TMV is certainly at least 100 to 1, and thus the probability greatly favors chemical origin for this mutant. As stated, other similarly isolated chemically induced mutants have also given indications of changes in amino acid composition.

In contrast, it has recently been reported by Wittmann[14] (a paper that became accessible to us only after completion of this study) that a nitrous acid mutant isolated by him has not shown any difference in composition from TMV. This important and well-documented finding, owing to its negative nature, is intrinsically neither definite nor final. While differences are, with luck, easily detectable, the absence of difference can be demonstrated only by exhaustive analysis. Thus the possibility that two amino acids are interchanged in position within one peptide segment can be excluded only by complete sequential analysis of each peptide. Furthermore, the problems connected with serine and threonine destruction, which were discussed above, leave some doubt as to the certainty of both the differences, and the absence of differences, in regard to these amino acids. However, it is quite possible that Wittmann's cautious and tentative conclusion will prove correct, and that he has studied a nitrous acid mutant of TMV which does not differ in its amino acid composition from the parent strain. If the complete amino acid sequence of the two were identical, this would lead one to the conclusion, which appears in no way unexpected, that certain parts of the polynucleotide chain carry the code for the amino acid sequence of the viral coat protein while other parts of the RNA chain are not concerned with the structure of this protein. It is of interest to note, in this connection, that Wittmann has studied a mutant which differs less markedly from the common strain than does the one studied by us. It has been reported[21] that so-called symptom mutants differing only in the severity of disease symptoms which they produce on *N. tabacum*, or in lesion size as noted on *N. glutinosa*, occur with very high frequencies of 50 per cent or more, quite in contrast to the much rarer differential host mutants on which we have concentrated our efforts. Thus it appears possible that the latter are more likely to show pronounced protein structural changes than the former. Such questions will become resolved only when statistically significant numbers of the various types of mutants produced by different chemical agents have been analyzed with regard to protein structure and other properties.

Summary.—The protein of a differential host mutant isolated after nitrous acid treatment and reconstitution of TMV-RNA differs from that of the parent strain in that 3 amino acid residues are replaced by three others (proline, aspartic acid, and threonine, by leucine, alanine, and serine). The composition of the parent strain appears identical with that of the *Vulgare* strain studied in Tübingen.

The action of carboxypeptidase on the mutant leads to extensive digestion, indicating that one of the changes, namely the replacement of proline by leucine, has occurred near the C-terminus of the peptide chain.

The implication of the fact that some chemically produced mutants differ in their protein composition, while others may not do so, as reported by Wittmann recently, appears of considerable interest in connection with the mechanism of coding of genetic properties by the RNA.

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*NON-VIABILITY OF STALK CELLS IN DICTYOSTELIUM**

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The Dictyosteliaceae, a family of simple amoeboid slime molds, are distinguished by their capacity to construct multicellular fruiting structures through the aggregation and orderly differentiation of large numbers of previously independent myxamoebae. The onset of the fruiting stage is marked by a dramatic shift from a free-living state to an interdependent population in which the cells become conspicuously elongated and orient themselves toward inflowing streams of myxamoebae which they join to form a cell community, or pseudoplasmodium. Subsequent development is characterized by a progressive integration of the assembled cells, the mass of myxamoebae behaving as a coordinated functioning whole wherein the differentiation of each member cell is influenced, apparently, by the remainder of the cell aggregate. The end result is the construction of a well-proportioned fructification, or sorocarp, consisting of two types of contrasting and strongly differentiated cells: spores, or propagative cells, that form an apical rounded spore head, or sorus; and polygonal, vacuolated, pith-like cells that comprise the stalk, or sorophore, which supports the sorus (or sori in branched forms).

It is this relatively simple behavioral pattern involving progressive specialization and ultimate differentiation into two cell types that has led investigators to utilize the Acrasieae, and particularly species of *Dictyostelium*, for researches on cell differentiation. Most students of these slime molds have believed the two cell types to be not only morphologically but also functionally distinguishable. Harper's statement of this concept expresses the generally accepted view: "The very cells which initiate and carry out the process of building the sorophore are sacrificed in the interests of giving the remainder a better place in the sun";[1] i.e., a substantial