THE ACTION OF CARBOXYPEPTIDASE ON STRAINS OF TOBACCO MOSAIC VIRUS*

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In studies on the chemistry of virus mutation, thirteen strains of tobacco mosaic virus (TMV) have been characterized with respect to their protein and nucleic acid constituents (1–4). A possible chemical basis for virus mutation was provided by the finding that mutant strains of TMV can differ in amino acid content, whereas they seem not to differ in the composition of their nucleic acids. However, no chemical differences were found between some strains, and, while this may be attributable to a need for refining the analytical tools, it is also possible that these strains differ only in certain structural features not reflected in their compositions.

In this connection, it was recently found (5, 6) that the enzyme, carboxypeptidase, releases specifically from TMV about 2900 residues of threonine per mole of virus, from which it was concluded that threonine is the primary carboxyl terminal (C-terminal) residue of the native virus and, by combination with other data, that the virus is made up of about 2900 peptide chains representing subunits of the virus having a molecular weight of about 17,000. As indicated above, it was noted that carboxypeptidase does not digest along the peptide chains of TMV, but stops sharply after removal of threonine. Since these results appear to distinguish TMV from most proteins tested thus far (7), it seemed desirable to examine for such structural features all of the strains of TMV which had previously been analyzed for protein and nucleic acid components. The results of this investigation, together with preliminary data obtained on other plant viruses, are the subject of this report.

EXPERIMENTAL

Virus Preparations—Highly purified preparations of the various strains of TMV and other plant viruses were obtained from appropriate plants by the customary procedure of differential centrifugation (1, 4, 8, 9). Aqueous solutions of such preparations were used in the reaction with carboxypeptidase.

Consistent results were obtained only with strictly fresh virus prep-

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arations, for older preparations tended to develop bacterial growth, which, even though slight, frequently led upon treatment with carboxypeptidase to release of amino acids which were not observed with freshly prepared virus. This effect could not always be abolished by repurifying the virus, e.g. by differential centrifugation. This suggests the possibility that some bacteria may possess enzymes capable of altering the structure of TMV and other viruses, although it is also possible that autolysis of bacteria could release protein material which had sedimentation characteristics similar to those of the virus and which was susceptible to the action of carboxypeptidase. In any case, erratic results were frequently obtained when old preparations of the viruses were treated with carboxypeptidase; hence fresh, highly purified preparations were employed in the major part of these studies, although, in a few instances, older preparations which had been preserved with toluene were used.

Reaction with Carboxypeptidase—The conditions for the enzyme reaction were in most cases similar to those previously described (5, 6); i.e., the reaction was carried out at about pH 7.5 and approximately 25° for 2 to 8 hours with about 1.6 mg. of virus N and 0.004 mg. of enzyme N per ml. The carboxypeptidase used was an aqueous suspension, crystallized three times, obtained from the Worthington Biochemical Sales Company, Freehold, New Jersey. In some instances the reactions were run after incubation of the enzyme with an inhibitor such as diisopropyl fluorophosphate or tetraethyl pyrophosphate (10) to preclude activity of traces of endopeptidases. With strains of TMV and most of the plant viruses except potato virus X, this precaution was unnecessary, owing to the well known resistance of these viruses to trypsin and chymotrypsin. At the end of the reaction period, the viruses were removed from the reaction mixtures by centrifuging at 40,000 r.p.m. for 1 hour in the Spinco model L ultracentrifuge. The supernatant fluids were carefully drawn off down to the level of the virus pellets and analyzed.

Analyses of Digestion Products—Qualitative analyses of the reaction mixtures after removal of substrate were made by means of paper chromatography following an approximate 60-fold concentration of aliquots of the supernatant fluids. Concentration was achieved either by lyophilization or by evaporation to dryness in a stream of nitrogen. In either case the residue was dissolved in a small volume of water and 1 to 20 μl. aliquots were applied to Whatman No. 1 paper for chromatography in phenol-water or in butanol-acetic acid as previously described (5, 11).

On the basis of the results of the qualitative tests for amino acids, quantitative analyses were made either by microbiological assay (4) or by application of the photometric ninhydrin method of Moore and Stein (12).

Tests for Biological Activity of Carboxypeptidase-Treated Viruses—The
carboxypeptidase-treated viruses were removed from the enzymatic digestion mixtures by high speed centrifugation and after resuspension were tested on appropriate plant hosts by rubbing a leaf or two with dilutions containing approximately $10^{-5}$ gm. of virus per ml. The symptoms developing in the inoculated plants were noted.

**Table I**

Maximal Yields of Threonine Obtained from Strains of Tobacco Mosaic Virus by Reaction with Carboxypeptidase at 25° (Approximately 1 Part of Enzyme N Per 400 Parts of Virus N)

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Threonine, γ per 10 mg. virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMV</td>
<td>65*</td>
</tr>
<tr>
<td>M</td>
<td>66</td>
</tr>
<tr>
<td>J14D1</td>
<td>60</td>
</tr>
<tr>
<td>GA</td>
<td>64</td>
</tr>
<tr>
<td>YA</td>
<td>70</td>
</tr>
<tr>
<td>HR</td>
<td>70</td>
</tr>
<tr>
<td>B2</td>
<td>65</td>
</tr>
<tr>
<td>B2A</td>
<td>63</td>
</tr>
<tr>
<td>B3</td>
<td>72</td>
</tr>
<tr>
<td>B4</td>
<td>67</td>
</tr>
<tr>
<td>S1</td>
<td>61</td>
</tr>
<tr>
<td>S2</td>
<td>67</td>
</tr>
<tr>
<td>S3</td>
<td>66</td>
</tr>
</tbody>
</table>

* The values are averages of two or more determinations made in most cases by the quantitative ninhydrin method, although for TMV approximately equal numbers of analyses were made by this means and by microbiological assay. In the case of TMV twenty-three determinations were made on nine different preparations of virus and the standard deviation calculated was 5.

**Results**

Qualitatively and quantitatively, tobacco mosaic virus and twelve of its strains reacted with carboxypeptidase in essentially the same manner. Threonine and only threonine was detected in the enzymatic digests in all cases. An average of 65 γ of threonine per 10 mg. of virus was released from TMV, and the average values obtained for twelve strains of TMV, as indicated in Table I, were very similar in magnitude.

As illustrated in Table II, the behavior of other plant viruses when treated with carboxypeptidase differed in two major respects from TMV and its strains: (1) several different amino acids were evidently released as a consequence of treatment with the enzyme (it was not determined which of these represented primary C-terminal groups and which adjacent residues) and (2) the sum of all the amino acids released for a given weight of
virus was considerably smaller in all cases than the quantity of threonine obtained from a comparable weight of TMV.

The differences between cucumber viruses 3 and 4 and the tobacco mosaic group were especially striking in view of the common consideration that these cucumber viruses are strains of TMV. The primary C-terminal group of the cucumber viruses appears to be alanine rather than threonine, for about 80 per cent of the amino acid released in a 6 hour treatment with carboxypeptidase was found to be alanine. This point was established from a combination of the paper chromatography results, the data from

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acid released, γ per 10 mg. virus</th>
<th>Amino acids identified as reaction products by paper chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco mosaic</td>
<td>65</td>
<td>Threonine†</td>
</tr>
<tr>
<td>Potato X</td>
<td>9</td>
<td>Alanine, aspartic acid, glutamic acid, leucine, threonine, valine</td>
</tr>
<tr>
<td>Southern bean mosaic</td>
<td>3</td>
<td>Alanine, leucine, valine</td>
</tr>
<tr>
<td>Tomato bushy stunt</td>
<td>7</td>
<td>Valine, leucine, alanine</td>
</tr>
<tr>
<td>Tobacco ringspot</td>
<td>3</td>
<td>Serine and possibly arginine</td>
</tr>
<tr>
<td>Cucumber 3 and 4</td>
<td>5</td>
<td>Alanine, leucine, phenylalanine, valine</td>
</tr>
</tbody>
</table>

* Determined as amino nitrogen by the quantitative ninhydrin reaction and expressed in terms of threonine. The reactions were run at about pH 7.5 and at 25° for 6 hours with about 1.6 mg. of virus N and 0.004 mg. of enzyme N per ml., the enzyme having been previously treated with tetraethyl pyrophosphate.

† The amino acids identified as major spots are italicized.

‡ Or isoleucine in all cases in which leucine is listed.

DISCUSSION

It might have been supposed from the diverse protein compositions of some of the strains of TMV (1, 4) that these viruses would differ in their peptide structures, and particularly in the nature of their C-terminal residues. However, this does not seem to be the case, for all thirteen strains examined appear to have only threonine as the C-terminal residue and,
furthermore, probably have similar structures adjacent to the threonine in view of the fact that carboxypeptidase action on these strains stops in every case after release of the threonine.

While it would not be justified to conclude that the occurrence of C-terminal threonine is a unique feature of TMV and its strains, it is clear that this structural feature distinguishes the TMV family from the few other plant viruses tested thus far. In fact, generalizing from the present data it appears that it can be considered a chemical indication of strain relationship when viruses are found to contain the same numbers and type of C-terminal residues. (Another chemical marker of strain relationship recently proposed is that strains of a virus will have the same quantity of nucleic acid and that their nucleic acid compositions will be identical (13).)

In addition to the close similarity of TMV strains with respect to C-terminal groups, it should be pointed out that other fundamental structural inferences can be drawn. In the case of TMV, evidence has been adduced to support the idea of a fundamental subunit in the virus structure (6, 13). The existence of this unit and its size have been deduced from x-ray studies, content of the limiting amino acid, cysteine, and the number of C-terminal residues. Calculations from these data indicate the presence of a subunit with a molecular weight of approximately 17,000.¹ Actual chemical subunits obtained by treating the virus with hydrogen bond-breaking reagents are of a size consistent with the calculated unit (14). Admittedly, the present data do not permit the conclusion that all strains of TMV contain precisely the same number of C-terminal residues; nevertheless, it is clear that the strains examined (Table I) contain very similar if not identical numbers of such residues. Therefore, it can be postulated that strains of TMV contain the same number of chemical subunits of approximately the same size, and that this is a characteristic structural feature of virus strains. Support for this hypothesis is provided by the observation that all strains of TMV examined contain the same quantity of cysteine, which, on the assumption of 1 cysteine residue per subunit, leads to a calculated subunit which is close in size and number to that computed from the C-terminal data. These structural similarities seem especially significant when the strain, HR, is compared with TMV, for these two strains are grossly different in proportions of most of their constituent amino acids.

The C-terminal analyses reported for plant viruses other than the TMV

¹ This value was calculated from the maximal amount of threonine released by carboxypeptidase from a standard amount of virus in a carefully conducted kinetic experiment following which the threonine was determined by three independent types of analysis (6). If the average value given for TMV in Table I is used, the calculated subunit comes out about 18,000, but, since the data in Table I represent a heterogeneous set of conditions and less rigorously controlled experiments, the value given above is considered a better estimate.
group must be considered only indicative of what may be found in more
detailed studies with varied conditions, because the present experiments on
these viruses were conducted solely on a comparative basis with TMV and
its strains. If higher enzyme concentrations, higher temperatures, and
longer reaction times are employed, the picture may change for the other
plant viruses, although the situation for TMV is virtually unaffected by
such variations in conditions (6). In any case, under the present moderate
conditions, evaluation of the carboxypeptidase reaction for the non-
TMV viruses was rendered difficult by the multiplicity of the products
of the enzymatic action and their low concentrations.

Incidentally, the character of the carboxypeptidase results indicates
that the C-terminal groups of plant viruses are not vitally concerned per se
in their capacity to infect, since infectious quality was unimpaired by
treatment of the strains with the enzyme. Furthermore, it might be in-
ferred that the nature of the C-terminal groups has little or no effect on the
type of symptoms induced in the host, since removal of the primary
C-terminal groups from the TMV strains and from other plant viruses has
no apparent effect on the symptoms caused in the usual host. However,
so little virus gets into the host cells to initiate infection that the pathologi-
cal effects are likely due directly or indirectly to the progeny, and, in the
case of TMV, it will be recalled that the progeny of carboxypeptidase-
treated virus were found to have the C-terminal characteristics of the un-
treated virus (5). Another way of summarizing these results is to say that
the hereditary centers of these viruses are apparently unaffected by treat-
ment with carboxypeptidase.

SUMMARY

Each of thirteen distinctive strains of tobacco mosaic virus was treated
with carboxypeptidase and it was found that essentially the same quantity
of threonine, and nothing but threonine, was split from each as a result of
the enzymatic treatment. In contrast, smaller quantities of several amino
acids were split from potato X, southern bean mosaic, tomato bushy stunt,
and tobacco ringspot viruses and from cucumber viruses 3 and 4. From
the results obtained it appeared that the number and type of carboxyl-
terminal residues were characteristic of a given virus and hence were the
same for all strains of that virus. Since strains of tobacco mosaic virus
appear to contain the same number of carboxyl-terminal residues, namely
about 2900, it was concluded that they are also probably composed of the
same number of chemical subunits.

BIBLIOGRAPHY

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