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The End-Groups of Tobacco Mosaic Virus RNA. II. Nature of the 3'-Linked Chain End in TMV and of Both Ends in Four Strains*

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Exhaustive digestion of C^{14}-labeled tobacco mosaic virus (TMV) RNA by snake venom phosphodiesterase usually yields about 0.5 moles of guanosine, cytidine, and uridine and 1.5 mole of adenosine. This strongly suggests that adenosine represents the 3'-linked ("left") end-group of the intact RNA chain, as it was previously shown to represent the 5'-linked ("right") end of the chain. Analysis of four quite different strains of TMV by both phosphodiesterase and alkaline degradation indicates that adenosine probably represents the terminal residue of each of these, notwithstanding the fact that some of the strains show differences from common TMV in regard to the end-group of their protein.

The ribonucleic acid of the tobacco mosaic virus (TMV-RNA) represents a chain molecule composed of about 6400 nucleotides. Studies of the nature of its end-groups have become practical only since virus preparations of high specific radioactivity became available. It was thus demonstrated with P^{32}-labeled RNA that the 2'- and 3'-positions of the 5'-linked end, as well as the 5'-position of the 3'-linked end of the chain, were not phosphorylated (Fraenkel-Conrat and Singer, 1962). It was further shown by alkaline degradation of C^{14}-labeled RNA that the 5'-linked end (*i.e.*, the right end by the customary methods of symbolizing polynucleotide structures) was an adenosine residue (Sugiyama and Fraenkel-Conrat, 1961). Various other methods have confirmed this and supplied information concerning the nature of the neighboring residues, which appear to be pyrimidines (Whitfeld, 1962, and unpublished data).

Snake venom phosphodiesterase supplies a tool to identify the nature of the 3'-linked end of the chain, since this terminal residue, if unphosphorylated in the 5'-position, should be present as the only nucleoside upon complete digestion of the RNA by phosphodiesterase. The problems inherent in this approach, and the tentative identification of the "left" terminal nucleotide of TMV-RNA, represents the main subject of this paper. Common TMV, as well as four typical strains, were investigated in this regard, and the strains were also subjected to alkaline degradation to ascertain their 5'-linked ("right") terminal residue.

Methods and Materials

The conditions of biosynthesis of C^{14}-TMV of 10^6 to 10^7 cpm/mg have been reported (Sugiyama and Fraenkel-Conrat, 1961). The virus was isolated by conventional methods of differential centrifugation. The preparation of TMV-RNA of highest possible purity in terms of nuclease contaminants has been previously described (Fraenkel-Conrat *et al.*, 1961). The other prerequisite for this work is the availability of snake venom phosphodiesterase of highest purity in terms of the absence of 5'-nucleotidase and of any nucleases or phosphatases splitting ribose 5'-phosphate bonds. A series of highly purified samples of this diesterase was kindly placed at our disposal by Dr. M. Laskowski (Felix *et al.*, 1960; Williams *et al.*, 1961). The conditions of enzyme treatment are indicated in the tables and text.

The isolation and identification of terminal nucleosides and nucleoside diphosphates was achieved by the same methods as previously described (Sugiyama and Fraenkel-Conrat, 1961). Known amounts of the unlabeled carrier compounds were added to the enzymatic digest and then reisolated by a series of purification steps. These usually consisted of (1) paper electrophoresis at *p*H 7.4, (2) two-dimensional chromatography, (3) desalting by charcoal adsorption and elution, and (4) paper electrophoresis at *p*H 3.5. The recovery of the absorbance of the nucleoside markers supplied the necessary correction factors for losses incurred during the complicated and time-consuming procedure of reisolation of the pure compound. The radioactivity found associated with each of these markers indicated the extent to which the given compound had been derived from the labeled TMV-RNA. The specific radioactivities of each nucleoside, which in some virus preparations differed appreciably one from another, were determined in separate experiments and used in these calculations.

Alkaline degradation was performed in the manner previously described (Sugiyama and Fraenkel-Conrat, 1961).

Results and Discussion

A. The 3'-Linked End-Group of TMV-RNA.—Degradation of a monodisperse terminally unphos-

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TABLE I

NUCLEOSIDES FOUND IN SNAKE VENOM PHOSPHODIESTERASE DIGESTS OF TMV-RNA[a]

Experiment No.	RNA Preparation	Enzyme Preparation and Digestion Conditions[a]	Nucleosides A	G	U	C
			(mole per mole RNA)			
1	C	IV, once, 2 hr.	1.3	0.5	0.4	0.4
2	C	IV, twice, 4 hr.	1.5	0.5	0.4	0.4
3	A-5	IV, twice, 4 hr.	1.8	0.8	0.8	0.8
4	C	III, twice, 4 hr.	2.4	1.2	1.2	1.0
5	B-4a[b]	IV, twice, 4 hr.	1.8	0.6	0.5	0.5
6	B-4a	IV, twice, 18 hr.	4.0	1.3	2.1	1.1
7	B-4a, preincubated[c]	IV, twice, 4 hr.	2.1	1.0	0.8	1.3
Details of one experiment (No. 2) (RNA 1 × 10^{-4} μmole)		Carrier recovery (%)	21	35	38	37
		Net cpm per nucleoside	74	26	11	20
		cpm corrected for recovery	352	74	29	54
		μmole (× 10^{-4})[d]	1.5	0.5	0.4	0.4

[a] Standard conditions of digestion are 0.2–0.3 mg C^{14}-labeled TMV-RNA in 0.2 ml *p*H 8.6 0.1 M borate buffer containing 0.5 μmole $MgCl_2$ 4 hr. at 37°. The enzyme, usually 1 to 1.5 units (Felix *et al.*, 1960), added in installments (excepting the first experiment) either at zero time and 60 min. or at zero time, 30, 60, and 120 min. In several of these experiments nucleoside 3′,5′-diphosphates were approximately determined by addition of two markers (pAp and pCp). The terminal diphosphates found amounted to 1.2 to 1.4 mole/mole RNA, and similar amounts of inorganic phosphate were found in similarly treated P^{32}-labeled TMV-RNA digests when analyzed by the method of Gordon *et al.* (1960). [b] This preparation was of low infectivity (about 1% after reconstitution, compared to about 50% for the other preparations used). [c] Preincubation was performed in the *p*H 8.6 borate + Mg used for the digestion for 1 hr. [d] From data on the specific activity of each nucleoside, as separately determined (see Sugiyama and Fraenkel-Conrat, 1961).

phorylated RNA by pure snake venom phosphodiesterase should yield besides 5′-nucleotides only one terminal nucleoside. Neither inorganic phosphate nor any diphosphates should be formed. The presence of fragmented RNA molecules due to breaks of the diester bond between the phosphorus and the 5′-position of the ribose would reveal itself by the appearance in the complete phosphodiesterase digest of equal amounts of nucleosides and nucleoside diphosphates, while breaks of the ribose-3′-phosphate bond would not be detected. Apart from the nucleosides due to breaks, these could also arise through secondary dephosphorylation of 5′-nucleotides formed by the diesterase, if this enzyme or the substrate contained traces of 5′-nucleotidase or other phosphatases.

Preliminary experiments with TMV-RNA (P^{32}- or C^{14}-labeled) gave variable and frequently quite high values for nucleosides, nucleoside diphosphates, and inorganic phosphate upon degradation by phosphodiesterase. These results seemed indicative of the occurrence of both secondary dephosphorylation and the appearance of spurious end-groups due to the action of alkali or enzymes acting similarly to pancreatic ribonuclease. Since our preparations contained about 70–80% of one molecular species of approximately 30 *S*, the rest showing lower sedimentation rates and being presumably fragments, a definite background of spurious end-groups was to be expected. However, the observed variability in the preliminary data obtained upon digestion of a single preparation of TMV-RNA indicated the appearance of new ends in the course of digestion, and experiments were initiated to establish conditions which would minimize these artifacts.

P^{32}-labeled RNA was used for most of these preliminary experiments. It appeared that borate buffers minimized the dephosphorylation of 5′-nucleotides, while attempts to inhibit the residual 5′-nucleotidase action by variations in *p*H or by the addition of metals had unfavorable results on the rate or extent of the diesterase action. The coincident appearance of spurious ends was most effectively minimized by employing great amounts of the diesterase, added in two to four aliquots in the course of a relatively short total digestion period (2 or 4 hours at 37°). Of additional importance in the furthering of this research was the arrival of a new preparation of phosphodiesterase (IV) which gave lower values for spurious end-groups than the preparations tested earlier.

When these optimal conditions had been established and when the primary electrophoretic separation of nucleosides and nucleoside diphosphates from the bulk of the 5′-nucleotides was not delayed, quite consistent results were obtained with enzyme preparation IV acting on a certain RNA preparation. The adenosine was found to exceed by 1 mole per mole RNA the background of about 0.3 to 0.5 mole of each of the four nucleosides, a value which was in line with expectation, considering the sedimentation pattern of the preparation. Both nucleoside diphosphates and the inorganic phosphate found in the digest corresponded to about 1 mole per mole RNA. Similar analyses performed with a different preparation of RNA or of the diesterase gave slightly higher background values, but still gave an excess of one adenosine above the background.

The possibility had to be considered that the adenosine arose from chain breakage due to an agent acting preferentially on X—p—A— bonds, rather than being derived from the end. This was tested by preincubating the RNA in the borate buffer (0.1 M, *p*H 8.6, containing 0.005 M Mg^{++}) used for the diesterase digestion. Subsequent end-group analysis with phosphodiesterase preparation IV indicated a slightly increased total, but no preferential appearance of excess adenosine (Table I).

Attempts to study the kinetics and the mechanism of the appearance of the excess adenosine (more than one residue above the background of nonspecific ends) were complicated by the fact that the only phosphodiesterase preparation then available (V) (possibly because it was obtained from *Bothrops* rather than from *Crotalus* venom) contained a phosphatase which could not be removed (Laskowski, M., private communication). This probably accounts for the slightly higher excess of adenosine found in phosphodiesterase V digests (about 1.5 mole per mole RNA). Data obtained with this enzyme concerning the nature of breaks in the RNA resulting from various agents will be reported in a later publication.

Another possible cause for the spurious appearance of adenosine could be the preferential dephosphorylation of 5′-adenylic acid by the action of 5′-nucleotidase or another contaminating phosphatase. In that case the amount of adenosine in the digest should be a function of the duration of the incubation period. As the data of Table I show, this did not seem to be the case, even though somewhat greater adenosine liberation was observed after 18 hours of incubation. Thus, dephosphorylation does not appear to account for the rapid formation of 1 1.5 adenosine residues per mole of TMV-RNA observed in all experiments with phosphodiesterase IV, although it may contribute to the somewhat higher adenosine values obtained with phosphodiesterase V.

One aspect which remains to be discussed is the completeness of digestion of the RNA, since the true end-group could escape detection if it formed part of an enzyme-resistant core. Snake venom diesterase is known to attack polynucleotides preferentially from the unphosphorylated 5′-linked end, and the enzyme is being used in this manner in a separate study in this laboratory designed to obtain sequential data (Singer, B., in preparation). Obviously, quite different conditions are employed for this limited attack and for complete digestion. To ascertain how closely the latter conditions approach their aim of complete digestion, the amount of P^{32}-containing material moving more slowly than the mononucleotides upon electrophoresis at *p*H 7.4 has been determined. This was found to amount to only about 0.3% under the best conditions of digestion, and control experiments indicated that about 0.2% of the radioactive material trailed in the same area of the paper when the nucleotide fraction was eluted and again subjected to electrophoresis. It thus appears that in typical experiments less than 0.2% of the material remains in the form of oligonucleotides. Since terminal oligonucleotides would lack one phosphate (XpY, XpYpZ, etc.), these would be more probably separated and detected behind the nucleotide area upon electrophoresis than would typical di- and trinucleotides (XpYp, XpYpZp). Yet, even under less rigorous digestion conditions, when the trailing and nonmoving material was found to amount to 2 to 4%, the usual amount of nucleosides with about 1 excess mole of adenosine was obtained. The fact that one terminal 3′-linked nucleoside was found in 96% and in > 99.7% digested RNA suggests that the "left" end of the chain does not represent a particularly resistant part of the molecule.

Earlier data led us to expect one terminal 3′-linked nucleoside in TMV-RNA, and only one, namely, adenosine, was found regularly in sufficient amount to fulfill this expectation. Since various control experiments failed to reveal clear contrary evidence it is tentatively concluded that adenosine represents the 3′-linked end of the TMV-RNA chain.

B. Analysis for Both Terminal Residues in Four Strains.—Four strains of TMV were grown in the customary manner in a C^{14} atmosphere, and their RNA was isolated. Separate aliquots were subjected to KOH and phosphodiesterase digestion. The strains were selected to be representative of the wide variation in the composition of coat protein observed for TMV strains (Tsugita and Fraenkel-Conrat, 1962; Tsugita, 1962a,b). Strains 171 and 223 were evoked by nitrous acid. The former, while differing from common TMV by only three amino acid replacements, shows one difference near the C-terminus (proline No. 156) (Tsugita and Fraenkel-Conrat, 1960). Strain 223 shows sixteen net replacements and belongs to group C, characterized by two methionines and an as yet unresolved difference in the nature of the N-terminal part of the peptide chain. Strain Y-TAMV, a natural isolate, is a representative of group B (eight exchanges, one methionine, and C-terminal serine instead of threonine). The HR strain, finally, belongs to group D, characterized by three methionines and one histidine.

TABLE II

DETERMINATION OF END-GROUPS OF RNA FROM STRAINS OF TMV[a]

	5′-Linked Terminal Nucleosides in Alkaline Digest (Mole/Mole)				3′-Linked Terminal Nucleosides in Phosphodiesterase Digests (Mole/Mole)			
	A	G	U	C	A	G	U	C
HR	1.0	0.2	0.1	0.2	1.2	0.5	0.4	0.4
Y-TAMV	0.9	0.2	0.1	0.2	1.2	0.5	0.3	0.4
171	0.8	0.2	0.2	0.1	0.9	0.4	0.4	0.4
223	0.8	0.1	0.0	0.1	1.6	0.4	0.7	0.6
(Common TMV)	1.0	0.1	0.1	0.2	1.5	0.5	0.4	0.4

[a] For methodological details see Sugiyama and Fraenkel-Conrat, 1961, and Sugiyama, 1962.

As shown in Table II, all four strains resemble the common TMV in having both 3′-linked and 5′-linked terminal adenosine. Some of the data are not as clear cut as those obtained with common TMV, and the limited amounts of material available have prevented repetition of the analyses. The specific radioactivity of each nucleotide was determined for only two of the strains (HR and No. 223), and these relatively close values were averaged and used in the calculation of the data for the other two strains. The symptoms given by strain 171 showed evidence of slight contamination with another strain, but the other three were biologically typical and uniform, a pleasant surprise, since all were grown simultaneously in the same crowded C^{14} atmosphere box.

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