*IDENTIFICATION OF 5′ LINKED ADENOSINE AS END GROUP OF TMV-RNA**

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The biological activities of nucleic acids seem largely, if not entirely, due to their nucleotide sequences. An obvious experimental approach to elucidation of such sequences is the characterization of the end groups. Recent data suggest that the terminal 5′ linked nucleoside of TMV-RNA is not phosphorylated[1] and thus should appear as a free nucleoside upon decomposition of the RNA by alkali. The detection and quantitation of a single nucleoside liberated from a molecule as large as TMV-RNA (6500 nucleotides) is technically feasible if virus is employed which carries enough of a radioactive label. The presence of 50–100 mc of $C^{14}O_2$ in the atmosphere during most of the propagation period of the virus (12–14 days) in leaves of *N. tabacum* achieves this objective by yielding virus which gives 2–10 $\times$ 10^6 cpm/mg. Less than 1 mg of the RNA of such virus suffices for the estimation of its terminal residues, particularly if carrier nucleosides are added as a guide in isolation of the liberated C^{14} nucleosides.

The evaluation of end group data requires careful consideration of the homogeneity of the preparation. In the case of TMV-RNA, the bulk of the material (50–80 per cent) shows an approximate molecular weight of 2 $\times$ 10^6 and represents the infective molecular species which is under investigation.[2] The rest of the material probably consists largely of fragments of viral RNA, and fragmentation causes the appearance of new end groups. The mode of action of most nucleases, and of alkali, would make one presuppose that most fragments would carry a 3′ (or 2′) terminal phosphate, rather than an unphosphorylated glycol group, and this supposition is supported by data concerning the alkali degradation of P^{32}-RNA.[1] The probability thus appears high that a bona-fide terminal 5′ linked nucleoside can be detected if it is present in TMV-RNA.

The first experiments showed that nucleosides could arise through accidental dephosphorylation of nucleotides. Only after conditions had been established which seemed to reduce dephosphorylation to a non-detectable level could a search for the terminal nucleoside be initiated. This was the case if the alkaline hydrolysate was carefully neutralized with the buffer and the nucleotides were then separated from the nucleosides by means of paper electrophoresis at pH 7.4. The latter could subsequently be separated from one another and further purified by 2-dimensional paper chromatography[3] followed by electrophoresis at pH 3.5. In this manner reproducible results were obtained, as shown on Table 1. It is evident that adenosine is present in about the amount expected for a single terminal residue, and that the other nucleosides occur in lesser amounts varying from 0.04 to 0.3 residues per mole, all after appropriate correction based on the specific radioactivity of the nucleoside and the recovery of the corresponding marker.

It was found of crucial importance that the unlabeled marker nucleosides were added only after the alkaline digestion. If the degradation of C^{14} polynucleotides was carried out in the presence of similar amounts of such markers 0.5 to 1.3 per

TABLE 1

DETERMINATION OF NUCLEOSIDES IN ALKALINE HYDROLYSATES OF C^{14} TMV-RNA*

Condition of alkaline digestion	Amount of RNA hydrolysed ($\times 10^{-4}$ μM)	Carrier nucleoside recovery of 0.1 mg added (%)		C^{14} found in nucleoside cpm	Count corrected for recovery, cpm	Nucleoside found ($\times 10^{-4}$ μM)	Mole nucleoside per mole RNA
Standard conditions† 24 hours' digestion. Carrier nucleosides added after completion of digestion.	1.9	A	19	77	405	1.7	0.9
		G	28	10	36	0.2	0.1
		C	27	3	11	0.1	~0.05
		U	30	11	37	0.3	0.2
48 hours' digestion. Carrier nucleosides added after completion of digestion.	1.9	A	22	94	427	1.8	0.9
		G	25	10	40	0.2	0.1
		C	35	4	11	0.1	~0.05
		U	36	10	28	0.2	0.1
48 hours' digestion. Carrier nucleosides added at 24 hours.	1.9	A	19	107	563	2.4	1.2
		G	25	21	84	0.5	0.3
		C	30	6	20	0.2	0.1
		U	37	18	49	0.4	0.2
24 hours' digestion. Carrier nucleosides added at the beginning of digestion.	1.9	A	20	2458	12300	51	27
		G	12‡	586	4880	30	16‡
		C	30	325	1080	13	7
		U	43	1023	2380	20	10
24 hours' digestion. Traces of C^{12} nucleosides ($\sim 3 \times 10^{-4}$ μM), added at the beginning of digestion. Carrier nucleoside (~0.4 μM) added after the completion of digestion.	1.9	A	21	98	467	2.0	1.0
		G	33	11	33	0.2	0.1
		C	37	4	11	0.1	~0.05
		U	18	4	22	0.2	~0.1

* About 2×10^{-4} μM C^{14}-labeled TMV-RNA was hydrolyzed in 0.1 to 0.4 *N* KOH (OH^-/P = 10 to 40) at 37°C for 18 to 48 hours. Non-labeled adenosine, guanosine, cytidine, and uridine were added as carrier and marker after the hydrolysis, unless otherwise specified. The hydroylsate was chilled in an ice bath and neutralized with ice-cold 2 *M* phosphate buffer (pH 4.5). Nucleosides were separated from nucleotides by paper electrophoresis at pH 7.4 (0.05 *M* phosphate buffer, 1000 volts per 40 cm). In this electrophoretic system, the four nucleosides stay together near the origin and are well separated from the nucleotides which move as a broad band toward the anode. After the run, the nucleosides were eluted from the paper with water, lyophilized and then spotted on Whatmann 3MM paper for two-dimensional chromatography[3] (1st dimension: 71.5 ml ethanol (95%) and 28.5 ml 1 *M* NH_4-CH_3COO (pH 7.5); 2nd dimension: 80 ml saturated $(NH_4)_2SO_4$, 2 ml isopropanol and 18 ml H_2O). The four nucleosides, which were well separated from each other, were eluted from the paper with water and then desalted by adsorbing to Norite A at pH 2.5 (0.1 *N* HCl used to adjust pH), washing with water and then eluting with 0.3 *N* NH_4OH in 50% ethanol. The eluates were dried under low vacuum over $CaCl_2$ and concentrated H_2SO_4, and the nucleosides were further purified by paper electrophoresis at pH 3.5 (0.05 *M* ammonium formate, 1000 volts per 40 cm). Each nucleoside was eluted from the paper, checked for the recovery of the non-labeled carrier by plotting its UV-spectrum, and then dried on a planchet for the counting of its radioactivity. The count actually obtained for each nucleoside was corrected for the recovery of the carrier marker and the amount of C^{14}-labeled nucleoside was then calculated by using the specific count for each nucleoside given in Table 2 (RNA preparation B3).

† After techniques had been established for the isolation of nucleosides, the standard conditions were used in six separate experiments with three samples of RNA prepared from different C^{14} virus preparations. The adenosine found in these experiments was, per mole RNA: 1.0, 0.6, 1.1, 1.4, 1.1, and 0.9 mole, while the other nucleosides ranged from 0.04 to 0.3 mole.

‡ This value is estimated, correcting for accidental loss of part of the solution. A similar relationship of A > G > C, U was obtained in another similar experiment.

cent of the C^{14} appeared in nucleosides (A > G > U, C), thus simulating 20 to 60 5′ linked end groups per mole of RNA.† This phosphate transfer did not occur to a significant extent if the nucleoside concentration was several orders of magnitude lower than that of the nucleotides, as it is the case in the digests of RNA. It appeared that only in *statu nascendi*, and thus possibly at the 2′,3′ cyclic state, did nucleotides undergo such interchange with nucleosides in alkaline solution, since incubation of digests for an additional 24-hr period with added unlabeled nucleosides did not produce greater amounts of nucleosides than observed under the standard conditions. The control of this experiment, in which marker nucleosides were absent, demonstrated that no detectable traces of nucleosides arose through hydrolysis of nucleotides under the conditions of alkaline degradation of the RNA (see Table 1).

Another measure of the phosphate transfer consisted in hydrolysing unlabeled RNA in the presence of varying amounts of C^{14}-labeled nucleosides (about 1 to 120 mole per mole RNA) and determining the label appearing in the nucleotide fraction. This corresponded to only about 1 per cent of the added nucleosides.

Previous studies of alkaline digests of P^{32}-labeled RNA had shown that no detectable amounts of nucleoside diphosphates were formed, thus proving that there was no terminal 5′ phosphate on the other end of the chain.[1] This conclusion was corroborated by the present experiments with C^{14}-RNA, which also yielded less than 0.4 mole per mole of any diphosphate, after due correction for the recovery of internal markers. Figure 1 represents a schematic presentation of our present state of knowledge concerning the chemical structure of TMV-RNA.

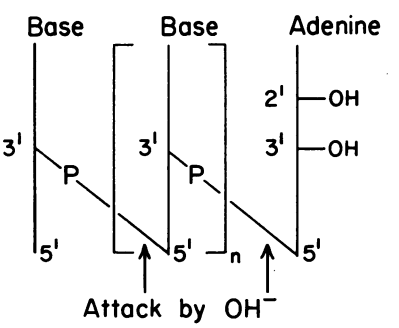

FIG. 1.—*Release of adenosine from TMV-RNA by alkaline degradation.* TMV-RNA is symbolized in conventional manner. Base stands for adenine, guanine, cytosine or uracil. n = approximately 6500.

The same amount of about one mole of adenosine per mole was obtained with the RNA derived from three separate preparations of C^{14}-TMV. In all three cases, similar marked differences were observed in the specific extent of labeling of the four nucleosides or nucleotides (see Table 2). The same difference was also observed when the bases were examined. Since the plants were held in an enclosed airspace containing $C^{14}O_2$ for most of the period of virus propagation and accumulation (12–14 days), no such differences had been anticipated. A related question is that pertaining to the uniformity of labeling throughout the length of the RNA chain. For only if this is uniform is one justified in using the average specific activity of adenosine in calculating the number of terminal adenosine residues per

TABLE 2

SPECIFIC RADIOACTIVITY OF NUCLEOTIDES, NUCLEOSIDES AND BASES IN C^{14}- LABELED TMV-RNA*

RNA preparation	Component	Specific Radioactivity ($\times 10^5$ cpm/μM) A	G	C	U
A	3′(2′)-nucleotide	12.5	6.6	3.8	5.9
	5′-nucleotide	11.3	7.2	4.3	6.8
	nucleoside	11.1	7.6	3.8	6.6
A	Base	3.6	2.0	0.7	1.3
B2	3′(2′)-nucleotide	28.3	19.8	9.9	15.4
B3	3′(2′)-nucleotide	23.9	16.4	8.1	12.2

* The 3′(2′) nucleotides were obtained by KOH hydrolysis of the RNA, the 5′-nucleotides by digestion with purified snake venom phosphodiesterase kindly supplied by M. Laskowski. The nucleotides were separated by electrophoresis at pH 3.5 (0.05 *M* ammonium formate). The nucleosides were obtained by digestion with crude snake venom, and separated by two-dimensional chromatography as described in Table 1. The bases were obtained by digestion with $HClO_4$, and separated by one-dimensional chromatography (65 ml isopropanol, 16.7 ml concentrated HCl, and 18.3 ml H_2O).

TABLE 3

LABELING IN TERMINAL AND RESIDUAL PARTS OF THE RNA CHAIN*

Portion of RNA chain	Specific Count ($\times 10^6$ cpm/μM) AMP	GMP	CMP	UMP
Terminal (18%)	2.63	2.25	1.10	2.08
Residual	2.59	1.84	0.92	1.48
Unfractionated	2.83	1.98	0.99	1.54

* 10 mg of TMV containing about 0.1 mg of C^{14}-labeled TMV was heated at 60°C for 1 min with gentle stirring in 0.04% sodium dodecyl sulfate solution at pH 8.0. The mixture was diluted 6-fold with ice-cold water and centrifuged at 30,000 rpm for 2 hr. The pellet was resuspended in water and incubated with 0.01 mg of pancreatic ribonuclease at pH 7 (overnight at 37°C). The mixture was centrifuged at 40,000 rpm for 2 hr. The supernatant showed a U.V. spectrum indicating that it was largely RNA and contained about 18% of the RNA of the whole virus. Both supernatant and pellet, resuspended in H_2O, were deproteinized by phenol extraction, and the deproteinized RNA was digested with KOH to give the 3′(2′)nucleotides, which were separated and analyzed as described in Table 2. It was found important that the unlabeled virus used as carrier in such experiments be prepared in exactly the same manner as the labeled virus.

mole. Experiments designed to test this by the partial degradation of TMV from one end through limited detergent treatment[4, 5] suggest similar labeling in terminal and residual portions of the RNA (see Table 3).

The finding of adenosine as the terminal residue must be considered in relation to the fact that adenosine is also regarded as the terminal residue of Transfer-RNA. Thus, a 1-per cent contamination with such an RNA could account for the adenosine observed in alkaline digests of TMV-RNA. However, since Transfer-RNA is reported to yield guanosine 3′5′ diphosphate in equivalent amounts to the adenosine upon alkaline degradation[6, 7] the absence of any detectable diphosphates from the digests appears to rule out the presence of as much as 0.3 per cent of Transfer-RNA in TMV-RNA. The conclusion, therefore, appears justified that TMV-RNA, at least as isolated, terminates in an adenosine residue, unphosphorylated at the 2′ and 3′ positions.

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† Preliminary data obtained by P. Whitfeld with alkali treatment of a ribonuclease digest of C^{14} TMV-RNA containing unlabeled nucleosides suggest that the phosphate exchange occurs also at the level of oligonucleotides.

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*REACTION OF CARCINOGENS WITH ACRIDINE**

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Only a very small number of the known substances is strongly carcinogenic. This indicates that carcinogenicity is linked to different qualities which all must be present to produce cancer. The various chemical reactions or qualities brought into relation with carcinogenicity reflect one or more of these factors. None of them seems to comprise all involved parameters. At the same time, none of the reactions proposed is strictly characteristic and is thus given also by substances which are not carcinogenic. This is true, also, for the "black reaction" with iodine, as described earlier by Isenberg *et al.*[1, 2]

In spite of these discrepancies the chemical behavior of carcinogens is not without interest, giving, possibly, a clue to one or the other parameter of carcinogenicity. It is in this relation that we want to report reactions with acridine.

TABLE 1

	Color with acridine	Carcinogenicity
Methylcholanthrene	+++	+++
1,2-Benzyprene	−	0
3,4-Benzpyrene	+++	+++
Pyrene	+	0
4-Dimethylaminostilbene	+++	++
Stilbene	−	0
2-Aminofluorene	++	++
4-Aminofluorene	−	0
Fluorene	−	0
Anthracene	−	0
9,10-Dimethylanthracene	+	0
Benzanthracene	+	?
10-Methyl benzanthracene	+	+
9,10-Dimethylbenzanthracene	+++	+++
Phenanthrene	−	0
1,4-Dimethylphenanthrene	++	0
1,10-Phenanthroline	+	0
Phenothiazine	+	0
Biphenyl	−	0
p-Terphenyl	−	0
Triphenylene	−	0
2-Aminobiphenyl	−	0
4-Aminobiphenyl	++	+
Picene	−	0
Chrysene	−	0
Naphthalene	−	0
1-Naphthol	−	0
2-Naphthol	−	0
α-Naphthylamine	+	?
β-Naphthylamine	++	?
Indole	+	?