STUDIES OF NUCLEOTIDE SEQUENCES IN TMV-RNA

II. THE ACTION OF SPLEEN DIESTERASE*

B. Singer, M. Sherwood and H. Fraenkel-Conrat

Virus Laboratory and Space Sciences Laboratory, University of California
Berkeley, California 94720

It has been reported that limited treatment of TMV-RNA with snake venom phosphodiesterase (SV-PDE) causes the release of a few nucleotides from the 5'-linked ("right") end of the chain without marked loss of infectivity (1). Spleen diesterase has been shown to attack polynucleotides from the 3'-linked ("left") end of the chain (2) and this enzyme was therefore used to obtain information on the nucleotide sequence at that end, and its essentiality for the infectivity of the RNA.

Methods and Materials.- P^{32}-labelled TMV-RNA was prepared as usual (3,4). Worthington Spleen PDE was further purified by phosphocellulose chromatography according to (5). About 0.15-0.5 mg of the RNA was treated in pH 5.2 acetate and Mg^{2+} with 0.02-0.12 units of enzyme for various time periods at temperatures ranging from 15-37°. The RNA was then precipitated with 2.5 volumes of ethanol, generally in the presence of EDTA. The supernate was concentrated and two-dimensionally chromatographed (6) after addition of known amounts of the four 3'(2')-nucleotides and the corresponding 2'-3' cyclic phosphates, as well as occasionally of the 3',5'-nucleoside diphosphates. The radioactivity associated with each of these markers represented a measure of the extent of digestion. Radioautography was used to detect other reaction products for which markers had not been added.

* This investigation was supported by GB-3107 from the National Science Foundation and grant NsG 479 from the National Aeronautics and Space Administration.

The RNA precipitate was redissolved in H_2O and tested for infectivity after reconstitution, as well as for its macromolecular integrity by ultracentrifugal analysis (Model E Spinco, equipped with U.V. optics).

Results and Discussion.- As illustrated by a typical experiment on Table I, limited digestion at 0° of TMV-RNA caused the release of a consistent pattern of nucleotides (A ≈ U > G > C). No appreciable amounts of 2',3'-cyclic nucleotides or of nucleoside 3',5'-diphosphates or other products were detected. The infectivity of such treated preparations decreased rapidly with first-order kinetics. The proportion of 30 S material remained approximately unchanged until after about 10-20 nucleotides had been released. At 0°, in contrast to SV-PDE and polynucleotide phosphorylase (7), spleen diesterase caused little digestion and erratic results.

Prolonged treatment or greater amounts of enzyme caused disappearance of the 30 S material and the appearance of appreciable amounts of the cyclic 2',3'-nucleotides and of other distinct products detected on radioautograms. These observations indicate the need for further purification and characterization of spleen phosphodiesterase. Obviously, indiscriminate use of this enzyme, even after the further step of purification, may yield data which are difficult to interpret.

The question whether the enzyme is able to attack a 5'-phosphorylated terminal nucleotide is in dispute. In the case of S-RNA, although spleen phosphodiesterase releases small molecular products, with or without prior dephosphorylation with phosphomonoesterase, these have not been identified (8,9,10). Our finding that nucleotides are released by exonuclease action without the appearance of a 3',5'-diphosphate confirms previous conclusions that TMV-RNA carries no 5'-terminal phosphate (10). The pattern of progressive nucleotide release is in accord with the results of complete digestion by SV-PDE which showed that A was the 3'-linked terminal residue (11). A sequence such as ApUpUpApGpC..could be tentatively proposed on the basis of the data presented.

The interpretation that the released nucleotides come at least in part from the infective molecule, rather than from fragments or contaminants, is justified in view of the loss of infectivity without appreciable loss of 30 S material which results from minimal spleen diesterase action. It appears that one lethal hit (37% residual infectivity) corresponds to a release of 2.3 nucleotides, up to 1.3 of which could be derived from fragments. In contrast, when snake venom diesterase acts on the other end of the chain the release of 4-5 nucleotides corresponds to one lethal hit, and these 5'-nucleotides are not likely to come from fragments whose predominant nature (ApBp....YpZp) renders them resistant to SV-PDE (12). The importance of the integrity of the 3'-linked, as contrasted to the 5'-linked, chain terminus of TMV-RNA for its infectivity may be significant in view of recent evidence that the code is read starting at the 3'-linked (i.e., 5'-OH) end of the chain (13).

REFERENCES

1. Singer, B., and Fraenkel-Conrat, H., Biochim. Biophys. Acta, 72, 534 (1963).

2. Hilmoe, R. J., J. Biol. Chem., 235, 2117 (1960).

3. Gordon, H. P., Singer, B., and Fraenkel-Conrat, H., J. Biol. Chem., 235, 1014 (1960).

4. Singer, B., and Fraenkel-Conrat, H., Virology, 14, 59 (1961).

5. Richardson, C. C., and Kornberg, A., J. Biol. Chem., 239, 242 (1964).

6. Felix, F., Potter, J. L., and Lashowski, M., J. Biol. Chem., 235 1150 (1960).

7. Singer, B., and Fraenkel-Conrat, H., Federation Proc. 24, No. 2, 602 (1965).

8. Preiss, J., Berg, P., Offengand, E. J. Bergmann, F. H., and Dieckmann, M. Proc. Natl. Acad. Sci. U.S., 45, 319 (1959).

10. Clark, J. M., Jr., Eyzaguirre, J. P., and Gunther, J. K., J. Biol. Chem., 239, 1877 (1964).

9. Harkness, D.R., and Hilmoe, R.J. Biochem. and Biophys. Research Commun. 9 393 (1962)

10. Fraenkel-Conrat, H., Singer, B., Biochemistry, 1, 120 (1962).

11. Sugiyama, T., and Fraenkel-Conrat, H., Biochemistry 2, 332 (1963).

12. Singer, B., and Fraenkel-Conrat, H., Biochim. Biophys. Acta, 76 143 (1963).

13. Tahanami, M., and Yan, Y., Biochem. Biophys. Research Commun., (in press).

TABLE I

Effect of Spleen Diesterase on TMV-RNA

Time of incubation*	Nucleotides split/mole TMV-RNA					% Infectivity**	% $S_{20,w}$ = 30**
	Ap	Gp	Up	Cp	Total	(of untreated control)	(of untreated control)
1'	0.31	0.11	0.27	0.13	0.82	65	93
5'	1.8	0.8	1.9	0.5	5.0	14	83
10'	3.1	1.6	3.7	1.1	9.5	3	64
15'	8.5	3.2	6.1	1.9	19.7	0.65	10

*The reaction mixture contained 360 µg ^{32}P-labelled TMV-RNA, 10 µl M pH 5.2 sodium acetate, 15 µl 0.1 M $MgCl_2$, 0.12 units spleen diesterase in 0.3 ml volume. Incubation was at 37°. The reaction was terminated by the addition of 50λ 0.1 M EDTA and 2.5 volumes of alcohol at 0°.

**Control infectivity was 48% of standard TMV. The 30 $S_{20,w}$ component of the control RNA was 53% of the total RNA present.