Heat Inactivation of Tobacco Mosaic Virus Ribonucleic Acid as Studied by End-Group Analysis*

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Received January 10, 1962

The action of the alkaline phosphatase from *Escherichia coli* on tobacco mosaic virus ribonucleic acid labeled with P^{32} resulted in the release of less than one phosphate group per molecule of nucleic acid. These results indicate that the nucleic acid does not contain any monoesterified phosphate groups. Tobacco mosaic virus ribonucleic acid which had been heated to destroy about 99% of the biological activity contained approximately one additional monoesterified phosphate group per molecule. It is concluded that the heat inactivation of infectious tobacco mosaic virus does not occur by the random breakage of molecules with the resultant formation of monoesterified phosphate groups. The explanation that appears most in accord with the experimental observations is the breakage of the polynucleotide chain with the formation of cyclic 2′,3′-phosphate end-groups.

In the course of studies of the heat inactivation of tobacco mosaic virus ribonucleic acid (TMV-RNA) in the presence of ions of the first transition series (Wacker *et al.*, 1961; Huff, Gordon, and Wacker, unpublished), determinations were made of the number of monoesterified phosphate groups in samples of TMV-RNA which had been subjected to various treatments. Previous studies had made use of a highly purified human prostatic phosphomonoesterase for the detection of monoesterified phosphate groups, and it was concluded that each intact TMV-RNA molecule of some 6500 nucleotides carries at most one terminal phosphate (Gordon *et al.*, 1960). In the present investigation the readily available phosphomonoesterase from *Escherichia coli* was used for the detection of monoesterified phosphate end-groups. The detection of the small amount of inorganic phosphate liberated by the enzyme was facilitated by the use of RNA labeled with P^{32}.

The results of the present study indicate that less than one phosphate group per intact molecule of 6500 nucleotides was liberated from TMV-RNA with no concomitant loss of infectivity. The finding that TMV-RNA does not terminate in monoesterified phosphate groups is in agreement with the results of Fraenkel-Conrat and Singer (1962), who also treated P^{32}-labeled TMV-RNA with *E. coli* phosphomonoesterase. The present studies also show that, when samples of P^{32}-labeled TMV-RNA were heated to destroy about 99% of the biological activity, only about one additional phosphate group per molecule could be detected.

EXPERIMENTAL

Preparation of P^{32}-labeled TMV-RNA.—The common strain of TMV used in these studies was obtained from Dr. C. A. Knight, Virus Laboratory, University of California, Berkeley 4. The P^{32}-labeled virus was produced by incubating detached infected leaves of Turkish tobacco plants in the presence of P^{32}-labeled sodium phosphate as previously described (Gordon *et al.*, 1960). The virus prepared from the infected leaves was at least 85% homogeneous, with an $s_{20,w}$ = 193*s*. The nucleic acid was prepared by phenol extraction, and when first prepared the RNA usually had a specific activity of about 2–3 × 10^6 cpm/mg.

Nucleosides and Derivatives.—The various nucleosides, 2′- and 3′-nucleoside monophosphates, and 2′,3′-nucleoside cyclic phosphates were obtained from Schwarz Bioresearch, Inc., Orangeburg, New York.

Heating Conditions.—The solutions of RNA or nucleoside 2′,3′-cyclic phosphates were heated at 65° at concentrations of 1–4 mg per ml in 0.025 M NaCl–0.025 M NH_4OOCCH_3, *p*H 6.8 (Fuwa *et al.*, 1960).

Sonication.—An MSE Ultrasonic Disintegrator distributed by Instrumentation Associates, 17 W. 60th St., New York 23, was used in these experiments. This instrument is stated to emit 60 watts at 18,000–20,000 cycles per second. The RNA was subjected to sonication for 5 minutes in the above-mentioned buffer at a concentration of 3.3 mg per ml. The solution was kept in a test tube surrounded by ice during the sonication.

Assays of Infectivity.—The infectivity of samples of TMV-RNA was determined by the standard method of half-leaf assay (Fraenkel-Conrat, 1959) with *Nicotiana tabacum* var. Xanthi, n.c. used as a local lesion host. The results of these assays are expressed as percentages of the zero time controls. The error is estimated to be about ±10% of the stated values.

Enzymatic Release of Terminal Phosphate Groups.—The *E. coli* alkaline phosphomonoesterase used in initial experiments was obtained from the Worthington Biochemical Corporation, lot

* This work was aided by grants from the United States Public Health Service and by funds from Initiative 171 of the State of Washington.

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#6009. In later experiments highly purified enzyme was obtained from Father Donald J. Plocke, Biophysics Research Laboratory of the Department of Medicine, Harvard Medical School, Boston. The latter sample was stated to have a turnover number of 3000 moles of *p*-nitrophenylphosphate per mole of enzyme (assumed m.w. 80,000) in 1 M Tris buffer, *p*H 8.0, at 10^{-3} M substrate concentration. Solutions containing 1.3 to 3.3 mg per ml of RNA in the NaCl–NH_4OOCCH_3 buffer, *p*H 6.8, were adjusted to contain 10^{-3} M Mg^{++} and incubated at 23° for the various times indicated in Table I. In experiments 1, 2, 3, and 4 a ratio of 1 part Worthington enzyme to 150 parts by weight of RNA was used. In experiments 5 and 6, a ratio of 1 part of Father Plocke's enzyme to 600 parts of RNA was used. At the completion of the incubation periods, the reaction mixtures were stored in stoppered containers at −80°, and aliquots were analyzed for inorganic phosphate and residual infectivity within 48 hours.

Separation of Inorganic Phosphate.—The separation of inorganic phosphate from the RNA was accomplished by paper electrophoresis at *p*H 9.2 (Gordon *et al.*, 1960). This procedure gave clean separation of inorganic phosphate from the nucleic acid; however, this separation was verified in each case by radioautography, which also served to locate the inorganic phosphate. The rate of migration of the P^{32}-labeled inorganic phosphate in all cases coincided with that of known inorganic phosphate.

Separation of Nucleoside Phosphates.—The separation of nucleoside phosphates was carried out by electrophoresis at *p*H 7.0 (Markham and Smith, 1952). A 0.025 M sodium phosphate buffer, *p*H 7.0, was used in conjunction with a 50-cm long strip of Whatman No. 3MM paper. The current was 1,500 v at about 45 mamp. In a 2-hour run under these conditions the 2′- and 3′-phosphates of uridine migrated 15 cm, uridine 2′,3′-cyclic phosphate migrated 9.0 cm, and uridine migrated −1.0 cm. Similarly, the corresponding derivatives of adenine and cytidine were clearly separated. After electrophoretic separation, the compounds in question were eluted from the paper with water and analyzed by spectrophotometry.

Radioactivity Determinations.—A probable error of ±5% was obtained in all determinations except the 0 time value in experiment 3 (Table I), where the probable error was about ±10%. The radioactivity of samples of P^{32}-labeled nucleic acids was determined along with the radioactivity of isolated inorganic phosphate in order to avoid decay corrections.

Results and Discussion

Trial experiments with the two enzyme preparations indicated that a 20-minute incubation

Table I

Effect of Heating on the Phosphomonoesterase-Detectable End-Groups of TMV-RNA

Expt. No.	Nucleic Acid Preparation	Time of Heating at 65° (min.)	Time of Enzyme Treatment (min.)[a]	Av. Length of Polynucleotide Chain[b]	Ratio of Av. Length of Control Chain to Av. Length of Heated Chain	No. of Detected Chain Breaks	No. of Chain Breaks Calculated from Actual Infectivity[c]	% Actual Infectivity[d]	% Infectivity Calculated from Detected Chain Breaks[c]
1	A	0	20	7,500	2.0	1.0	—	—	37
		80	20	3,850					
2	A	0	60	6,200	2.0	1.0	—	—	37
		80	60	3,100					
3	B	0	20	14,500	1.9	0.9	4.3	1.4[e]	41
		80	20	7,700					
4	C	0	20	5,000	1.6	0.6	3.9	2.0[f]	55
		80	20	3,200					
5	D	0	20	6,500	2.6	1.6	3.7	2.6[f]	20
		80	20	2,500					
6	D	Sonicated 5 min.	20	570	11.4	10.4	6.7	0.12[f]	0.003

[a] For conditions of enzymic action see text. In experiments 1, 2, 3, and 4 the Worthington enzyme was used. In experiments 5 and 6 the *E. coli* phosphomonoesterase obtained from Father Plocke was used. [b] Average length of the polynucleotide chain = cpm in the nucleic acid sample/cpm in the inorganic phosphate released from the sample by the phosphomonoesterase. [c] See text. [d] The actual infectivity was measured by the standard half-leaf assay system. [e] In this experiment the infectivities of the control solutions and the heated samples decreased 10-fold during incubation with the phosphomonoesterase. [f] The infectivities of these samples were not significantly decreased by treatment with the phosphomonoesterase.

period at room temperature released most of the phosphomonoester groups from TMV-RNA. In experiments 1 and 2 the same preparation of TMV-RNA was used, and it may be seen that a rapid release of inorganic phosphate occurred during the first 20 minutes, followed by a more gradual release. The 20-minute period was therefore used in subsequent experiments. No inorganic phosphate could be detected in control experiments in which no enzyme was used. The average chain lengths of nucleic acid preparations from four different P^{32}-labeled virus samples are given in Table I. The virus preparations used in these experiments were at most 85–90% homogeneous, and the preparation of RNA by the phenol extraction probably introduces an additional amount of polynucleotide chain breakage (Gordon *et al.*, 1960). In a previous study the finding of the release of one phosphate per 3000 to 5000 nucleotides was interpreted as signifying that the intact TMV-RNA molecule has at most one terminal phosphate. In the present experiment an average of one phosphate per 8400 nucleotides (range 5000–14,500) was released by the enzyme. The release of no more than one phosphate group per RNA molecule of some 6500 nucleotides in length indicates that the intact molecule of TMV-RNA does not bear any monoesterified phosphate groups. The retention of infectivity under these conditions is also worthy of note. This result is in agreement with the recent findings of Fraenkel-Conrat and Singer (1962), who also concluded that intact TMV–RNA does not contain phosphomonoesterase labile groups. These workers imply that the phosphate groups labile to alkaline *E. coli* phosphomonoesterase are present in trace amounts of materials that sediment more slowly during centrifugation in a sucrose gradient than the infectious component of TMV-RNA preparations. The higher values for phosphate end-groups previously reported (Gordon *et al.*, 1960) were probably occasioned by a slightly higher content of these impurities. The very large scatter of values obtained in the present experiments is probably due to the contribution of a vanishingly small but highly variable amount of these impurities.

Samples of TMV-RNA which were heated at 65° for 80 minutes lost about 98–99% of biological activity (Table I). When these heated samples of RNA were analyzed for phosphomonoesterase-labile end-groups, it was found that approximately one additional end-group per molecule was introduced by the heat treatment. It is to be particularly noted in experiment 3 of Table I that less than one phosphomonoester end-group per 6500 nucleotides was found after heating. It has been demonstrated clearly that the loss of biological activity on heating of TMV-RNA follows first-order kinetics (Ginoza, 1958; Wacker *et al.*, 1961), and, thus, it is very likely that one inactivating event per molecule ("hit") is sufficient to destroy biological activity. Under these conditions, and assuming a random distribution of inactivating events, the Poisson distribution would indicate that $I/I_0 = e^{-h}$, where I_0 is the initial biological activity, I is the final biological activity, h is the average number of hits per molecule of biologically active RNA, and e is the basis of natural logarithms. This equation was used to calculate the infectivity which should have remained in the heated TMV-RNA sample after the observed number of new phosphomonoester groups had been formed (Infectivity Calculated from Detected Chain Breaks, Table I). Conversely, the number of chain breaks which should have been found were calculated from the observed residual infectivities of the various samples (Number of Chain Breaks Calculated from Actual Infectivity, Table I). Upon inspection of Table I it is apparent that the observed number of phosphomonoester groups is much smaller than the number which should have been found if the heat inactivation of TMV-RNA proceeds by a mechanism that ultimately results in the formation of either 2′-, 3′-, or 5′-phosphomonoester groups. The discrepancy between the observed and the calculated number of phosphomonoester end-groups is even larger when it is noted that additional end-groups should have been contributed by further cleavage of biologically inactive, fragmented molecules of TMV-RNA. The inactivation of TMV-RNA by the cleavage of a single exceptionally labile bond per polynucleotide chain with the formation of one additional phosphomonoester group per molecule appears to be ruled out by (*a*) the results of experiment 3, in which, even after heating less than one phosphate end per 6500 nucleotides was found, and (*b*) the fact that the heat of activation, ΔH^*, has been found to be the same for the degradation of molecular weight (Eigner *et al.*, 1961) and loss of biological activity (Ginoza, 1958) of TMV-RNA upon heating (see below).

As a control experiment TMV-RNA was inactivated by sonication until 0.12% of the original infectivity remained. In this case a larger number of phosphomonoester groups was found than was calculated from the remaining biological activity. The additional end-groups are probably due to the additional fragmentation of previously broken molecules. This behavior is in agreement with a mechanism of inactivation of biologically active TMV-RNA by sonication whereby chain breakage occurs with the concomitant formation of phosphomonoester end-groups.

The present study does not provide a definite mechanism for the loss of biological activity of TMV-RNA upon heating. Any explanation must take into account the fact that the heat of activation, ΔH^*, has been found to be the same in studies based upon the decrease of molecular weight (Eigner *et al.*, 1961) or from studies based upon the loss of biological activity (Ginoza, 1958). It is, thus, highly probable that the rate-limiting step in each of these reactions is identical. It is unlikely that the loss of biological activity is due to the loss of purines or to deamination of bases

under the mild conditions of the present experiment. Furthermore, the infectivity of TMV-RNA is not affected by a variety of conditions that would be expected to destroy any type of tertiary structure in the molecule (Haschemeyer *et al.*, 1959). In view of these facts the explanation that appears most in accord with the experimental observations is the breakage of the polynucleotide chain with the formation of cyclic 2′,3′ end-groups. In model experiments using the 2′,3′-cyclic phosphates of adenosine, uridine, and cytidine, no breakdown (less than 5%) of these compounds could be detected after heating at 65° for 80 minutes or by incubation of these compounds with 0.5% by weight of Plocke's alkaline *E. coli* phosphatase in the presence of 10^{-3} M Mg^{++} at 23° for 20 minutes. Nucleoside 2′,3′-cyclic phosphates have also been produced by boiling RNA with suspensions of $BaCO_3$ (Markham and Smith, 1952). In view of these results it appears that cyclic end-groups would be stable under the conditions of the present experiment and would not be released as inorganic phosphate by the alkaline *E. coli* phosphatase. Each such break in the polynucleotide chain would cause a concomitant loss of biological activity. Ginoza has advanced a similar explanation for the heat degradation of TMV-RNA (Ginoza, 1958).

An alternative explanation has been proposed by Boedtker (1960), who noted that thermal treatment of TMV-RNA produces a sharp drop in biological activity accompanied by the formation of a more compact molecule without an apparent decrease in molecular weight. It is unlikely, however, in view of a molecular weight of 2,000,000, that the methods used would have detected biologically inactivating breaks which would cause experimentally insignificant decreases in the observed molecular weight.

No unequivocal choice between these two proposed mechanisms for the inactivation of TMV-RNA may be made on the basis of presently available data. Measurement of the number of additional cyclic phosphate or nucleoside end-groups possibly produced upon heating TMV-RNA may help to solve this problem, and such attempts are under way.

The results of the present investigation rule out the possibility of chain cleavage with the resultant formation of monoesterified phosphate end-groups as the basis of the mechanism of the heat inactivation of TMV–RNA.

Acknowledgments

The authors wish to thank Mr. Roger D. Wade for help with the ultracentrifuge experiments and C. Smith for technical assistance.

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