# Studies of Nucleotide Sequences in Tobacco Mosaic Virus Ribonucleic Acid. IV. Use of Aniline in Stepwise Degradation*

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ABSTRACT: Aniline was found to be an effective catalyst for the release of phosphate from periodate-oxidized 5′-nucleotides at pH 5 and 25°. Aniline also eliminated the terminal residue of periodate-treated tobacco mosaic virus ribonucleic acid (TMV-RNA), without, in contrast to more basic amines, causing appreciable internal phosphodiester bond breakage. By the combination of periodate oxidation, aniline-catalyzed cleavage, and removal of the terminal phosphate with alkaline phosphatase, nucleotides have been split off $^{14}$C-labeled TMV-RNA in five cycles and a sequence, (-Gp)-Cp-Cp-Cp-A, is tentatively proposed on the basis of these data. The elimination of the terminal base by means of aniline caused a marked loss in infectivity. Subsequent phosphomonoesterase treatment had no effect on the infectivity.

A procedure was proposed for stepwise degradation of oligoribonucleotides consisting of the oxidation of the terminal glycol group with periodate, followed by hydroxide ion catalyzed $\beta$ elimination of the terminal 5′-linked nucleoside dialdehyde derivative and removal of the new 3′-phosphate by a phosphomonoesterase (Whitfeld, 1954; Brown *et al.*, 1955). This method has not been applied to larger polynucleotides until recently because of the uncertainty about the specificity of these reagents for the end group, and because the conditions advocated for $\beta$ elimination endangered the internal phosphodiester bonds in the molecule, as did endonuclease contaminants of the monoesterase preparations available at that time. In recent years, the use of certain primary amines was proposed in lieu of hydroxide ions, and elimination was reported to proceed at pH 7–9 (Ogur and Small, 1960; Yu and Zamecnik, 1960; Khym and Cohn, 1961; Neu and Heppel, 1964). The purification of the alkaline phosphatase from *Escherichia coli*, in turn, has supplied a tool for the dephosphorylation of macromolecular RNA without appreciable chain breakage (Fraenkel-Conrat and Singer, 1962). Thus, together with the development of methods for selective oxidation of terminal nucleosides and for monitoring terminal dialdehyde groupings with [$^{14}$C]semicarbazide (see Steinschneider and Fraenkel-Conrat, 1966), the stage appeared set for application of these methods to high molecular weight ribonucleic acids. However, the advocated conditions for $\beta$ elimination were still too rough to permit repeated use of these methods with an RNA of the size of tobacco mosaic virus ribonucleic acid (TMV-RNA), which was found to be largely degraded by methylamine or lysine under conditions required to achieve elimination of the terminal nucleoside.

Aniline has been employed as catalyst for the degradation, presumably *via* a $\beta$-elimination mechanism, of apurinic acid in 66% formic acid (Burton and Petersen, 1960), and more recently near neutrality (Livingston, 1964). Our independent finding that aniline is able to catalyze the elimination of the terminal nucleoside dialdehyde at pH 5 without phosphodiester bond breakage appears to render this method applicable to the analysis of several terminal nucleotides in TMV-RNA. The effect of the various steps in the removal of single nucleotides on the infectivity of the viral RNA was also studied.

## Materials and Methods

Aniline was purchased from Eastman Organic Chemicals, redistilled, and stored in a dark bottle in the cold. It was diluted 1:33 (v/v) with water and the solution adjusted to the desired pH by means of concentrated HCl. This solution was prepared fresh for use.

Preparations of phosphomonoesterase,[1] the alkaline phosphatase from *E. coli*, were kindly given us by Drs. M. Gordon and W. Wacker.

TMV-RNA, [$^{32}$P]TMV-RNA, [$^{14}$C]TMV-RNA, and [$^{32}$P]5′-nucleotides were isolated by the standard methods of this laboratory (Fraenkel-Conrat and Singer, 1962; Sugiyama and Fraenkel-Conrat, 1961).

Periodate oxidation, [$^{14}$C]semicarbazide treatment, and sedimentation analysis were performed as in the preceding paper (Steinschneider and Fraenkel-Conrat,

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[1] Abbreviations used: PME, phosphomonoesterase; A, adenine; G, guanine; C, cytosine; U, uracil.

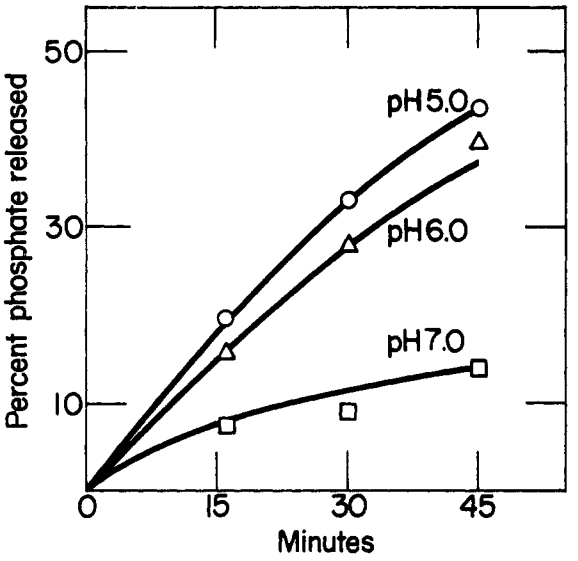


FIGURE 1: Initial rate of aniline-catalyzed phosphate release from adenosine 5′-phosphate dialdehyde. 5′-Adenylic acid (22 μmoles, 0.8 ml of 10 mg/ml in $H_2O$) was treated with 40 μmoles of $NaIO_4$ (0.4 ml, 0.1 M) for 30 min at 24° in the dark. To terminate the reaction, ethylene glycol (144 μmoles, 8 μl) was added and allowed to react for 15 min at room temperature. Of this solution 0.25 ml (4.6 μmoles of pA dialdehyde) was added to solutions made up of 0.6 ml of buffer $\Gamma/2 = 0.1$ (sodium cacodylate at pH 7.0, 6.0, sodium acetate at pH 5.0) and 0.3 ml of 0.33 M aniline adjusted to the same pH. Aliquots (75 μl) (0.3 μmole of pA derivatives) were withdrawn at indicated times, and immediately reduced with 1–2 mg (25–50 μmoles) of crystalline $NaBH_4$ (Khym and Cohn, 1961) for at least 30 min at 24°. Inorganic and total phosphate was determined according to Chen *et al.* (1956). Complete elimination corresponds to liberation of the total phosphate as inorganic phosphate. Because of interference by cacodylate, total phosphate was determined in pH 5.0 acetate solutions only. The inorganic phosphate released at zero time was 3.2, 2.9, and 2.5, at pH 7.0, 6.0, and 5.0, respectively, and was subtracted accordingly. All reactions were in duplicate.

1966). Details of the procedures used for the present experiments are given in footnotes to the tables. Infectivity tests, with and without reconstitution, were performed as usual.

*The Degradative Cycle.* OXIDATION, ELIMINATION, AND PHOSPHATASE. The conditions for complete oxidation of the glycol group of 5′-nucleotides and of TMV-RNA, and the monitoring of this reaction by semicarbazone formation, have been reported in the preceding paper (Steinschneider and Fraenkel-Conrat, 1966). A preliminary survey of the action of various amines in splitting the terminal dialdehyde off the macromolecule indicated that the use of lysine according to Neu and Heppel (1964), as well as of 1.5 M methylamine at pH 7 (2 hr, 24°), caused complete elimination, while methylamine used according to Whitfeld (1965), led to elimination of only 50% of the terminal [$^{14}$C]semicarbazide binding sites. However, after each of these treatments, most of the RNA was fragmented. In contrast, aniline (0.3 M) appeared able to achieve extensive elimination without causing concomitant phosphodiester bond breakage.

The conditions most favorable for elimination with aniline were at first studied with 5′-adenylic acid dialdehyde as model. As shown on Figure 1, the rate of phosphate elimination was greater at pH 5 than at 6. At pH 7 it was yet slower. All four 5′-nucleotide

TABLE I: Phosphate Released from 5′-Nucleotides upon Oxidation and Elimination.[a]

| Nucleotide | Periodate Treatment | Aniline Treatment | % $P_i$ |
|---|---|---|---|
| 5′-Adenylic acid | + | + | 100 |
| | + | − | 4 |
| | − | + | 0 |
| | − | − | 7 |
| 5′-Uridylic acid | + | + | 86 |
| | + | − | 9 |
| | − | + | 11 |
| | − | − | 16 |
| 5′-Cytidylic acid | + | + | 90 |
| | + | − | 18 |
| | − | + | 10 |
| | − | − | 23 |

[a] The $^{32}$P-labeled 5′-nucleotides (5′-adenylic acid $4.6 \times 10^{-4}$ mM, 5 μl; 5′-uridylic acid $5.8 \times 10^{-4}$ mM, 10 μl; or 5′-cytidylic acid, $5.2 \times 10^{-4}$ mM, 10 μl) were added to 0.1 N pH 5.3 sodium acetate (15 μl) and treated with periodate ($5 \times 10^{-3}$ mM, 5 μl) for 20 min at 24° in the dark. After addition of ethylene glycol to terminate the reaction ($1.8 \times 10^{-2}$ mM, 1 μl) the solutions were kept for 10 min at 24°, when 0.135 ml of 0.33 M aniline, pH 5.0 (or water), was added. Addition of the periodate and aniline is indicated by +, while omission of the reagent is indicated by −. At the end of 3 hr at 24° the mixtures were reduced with $NaBH_4$ (10 μl of a freshly prepared 0.1 N solution) for 30 min at 24°. The solutions were dried under an air stream, the residue was taken up in 0.1 ml of $H_2O$, and the inorganic phosphate ($P_i$) was separated by electrophoresis in 0.05 M pH 7.4 phosphate, with $^{32}P_i$ and picric acid as markers in a separate alley. $^{32}$P was detected and determined directly on paper strips in a gas-flow counter as described by Gordon *et al.* (1960). The total counts recovered (100–290 cpm) ranged from 42 to 94%. A similar experiment with 5′-guanylic acid in which no controls were run resulted in elimination of 99% of the phosphate, with 66% of the counts recovered.

dialdehydes reacted at similar rates at pH 5. The reaction was also studied at the low concentrations of aldehyde encountered in oxidized TMV-RNA reaction mixtures ($10^{-6}$ M) by means of $^{32}$P-labeled 5′-adenylic acid. As the data on Table I show, the elimination reaction proceeded satisfactorily also under these conditions.

The results of our studies on the pH and time dependence of the aniline-catalyzed elimination reaction, as applied to oxidized TMV-RNA and monitored by its ability to bind semicarbazide, are given in Table II. Apparently the reaction approaches completion in 3 hr at pH 5 and at 25°, and requires longer time at pH 6. Lower pH values were not investigated since these *per se* would tend to increase the rates of diester bond breakage and depurination.

TABLE II: Aniline-Catalyzed Elimination of the Terminal Nucleoside Dialdehyde from TMV-RNA.[a]

| | Aniline Treatment at pH 5.0 | | Aniline Treatment at pH 6.0 | |
|---|---|---|---|---|
| Time (min) | Moles of Semicarbazide Bound/Mole of RNA | Elimination (%) | Moles of Semicarbazide Bound/Mole of RNA | Elimination (%) |
| 0 | 2.4 | 0 | 2.55 | 0 |
| 30 | 1.65 | 38 | 2.05 | 25 |
| 60 | 1.35 | 53 | 1.85 | 35 |
| 120 | 0.70 | 85 | 1.30 | 63 |
| 180 | 0.45 | 98 | 1.20 | 68 |

[a] TMV-RNA (24 mg) was oxidized with a 100-fold $NaIO_4$ excess as described in the previous paper and sedimented in four equal portions. Two were treated with aniline at 25° and pH 6.0 (pellet dissolved in 2.16 ml of 0.33 M aniline, pH 6.0, and 0.240 ml of sodium cacodylate, $\Gamma/2 = 0.1$, pH 6.0), and two at pH 5.0 (same volumes of 0.33 M aniline, pH 5.0, and 0.1 N sodium acetate, pH 5.0, respectively). Aliquots of 0.4 ml were removed after indicated time intervals and frozen on Dry Ice. All samples were thawed simultaneously and precipitated by addition of 2 M NaCl (50 $\mu$l) and 1.35 ml of ice-cold ethanol. After one additional reprecipitation from water the remaining aldehyde groups were determined with [$^{14}$C]semicarbazide as described in the preceding paper (Steinschneider and Fraenkel-Conrat, 1966). The extent of terminal $\beta$ elimination was calculated using the average of the duplicates and assuming that the excess over 2 moles of [$^{14}$C]semicarbazide bound/mole of RNA at 0 min was bound nonterminally.

To obtain definitive evidence that the loss of the aldehyde groups was due to the expected cleavage of the terminal 5′-phosphoester bond the effect of PME on $^{32}$P-labeled TMV-RNA at various stages of the reaction was investigated. As shown in Table III, no appreciable amounts of phosphate were released from a sucrose gradient purified sample of the RNA (Fraenkel-Conrat and Singer, 1962) after oxidation with periodate or after direct treatment with aniline. But after treatment of oxidized RNA with aniline, about one phosphate per mole of RNA was released by the enzyme. Thus the complete cycle was accomplished.

TABLE III: Release of Phosphate from $^{32}$P-TMV-RNA by Phosphomonoesterase after Various Treatments.[a]

| Periodate Treatment | Aniline Treatment | Moles of Phosphate Released/Mole of RNA |
|---|---|---|
| + | + | 1.1 |
| + | − | 0.1 |
| − | + | 0.2 |
| − | − | 0.1 |

[a] [$^{32}$P]TMV-RNA was fractionated on a sucrose gradient (2–20%, pH 5.1, 0.1 N NaCl–0.01 N sodium acetate), 14 hr at 23,000 rpm, SW 25, and the half of the RNA nearer the bottom of the tube was used. This (0.625 mg) was oxidized with a 100-fold molar excess of $NaIO_4$, as described in the preceding paper (Steinschneider and Fraenkel-Conrat, 1966). An equal amount served as unoxidized control. The RNA in each tube was alcohol precipitated in two equal portions. One portion of each precipitate was dissolved in 0.135 ml of 0.33 M aniline, pH 5.0, and 15 $\mu$l of 0.1 N sodium acetate, pH 5.0, the other served as control (0.135 ml of $H_2O$, 15 $\mu$l of buffer). After 3 hr at 25° the RNA was precipitated by addition of 2 M NaCl (25 $\mu$l) and 0.5 ml of ethanol. The precipitates were reprecipitated from water twice and then redissolved in 0.15 ml of $H_2O$. After addition of 0.2 M pH 7.4 ammonium acetate (20 $\mu$l) and 0.1 M $MgCl_2$ (15 $\mu$l), the RNA was treated with alkaline PME (10 $\mu$g in 10 $\mu$l) for 30 min at 37°. It was then precipitated by addition of 3 M pH 5.3 sodium acetate (20 $\mu$l) and 3 volumes of ice-cold ethanol. The supernatants (after sedimentation) were collected and dried under an air stream. The residue was taken up in water and the inorganic phosphate separated by electrophoresis and determined as described previously. The extent of $^{32}$P released was determined by comparison to a known amount of $^{32}$P-RNA spotted on a strip from the electropherogram and counted simultaneously.

No noticeable loss of 30S material occurred during these treatments (Table IV). The sample after dephosphorylation was ready to be again subjected to periodate, aniline, and PME, in turn. Each of these steps, including the new availability of the RNA to periodate after completion of the cycle, was also monitored by studying the amount of [$^{14}$C]semicarbazide bound at each stage (Table V).

TABLE IV: Sedimentation Behavior of TMV-RNA after Successive Periodate, Aniline, and PME Treatments.[a]

| $IO_4$ Excess | Aniline | 30 S[b] |
|---|---|---|
| No periodate | None | 35, 39 |
| No periodate | Standard | 30 |
| $10^2$ | Standard | 29 |
| $10^4$ | Standard | 37 |
| $10^2$ | Standard | 49[c] |

[a] Sedimentation analysis was performed in 0.05 M pH 7 phosphate with 0.04–0.05 mg of RNA, after two reprecipitations from water subsequent to treatment with aniline or directly after treatment with PME. [b] Percentage of total ultraviolet absorbance sedimenting with a sharp boundary of approximately 30 S, most of the rest sedimenting more slowly. [c] Different RNA preparation, more typical, since most preparations were 50–70% homogeneous. The same sample after subsequent treatment with phosphomonoesterase (see Table III) showed 44% of 30S material. In isolated cases, the RNA appeared degraded after removal of one nucleotide.

*The Identification of the Five Terminal Nucleotides.* The 75% alcohol supernatant obtained upon precipitation of the RNA after aniline treatment contained the released nucleoside fragments. Identification of the base present in the single nucleoside fragment released from a polynucleotide as large as TMV-RNA required the use of $^{14}$C-labeled RNA. Although nucleoside dialdehydes were generally found to be unstable under the conditions previously employed for amine-catalyzed $\beta$ elimination, the liberation of the free base was often incomplete (Khym and Cohn, 1961; Neu and Heppel, 1964). This fact causes complications when the eliminated fragment is to be not only identified but quantitatively determined, which is essential when the reactions are to be carried through several cycles, and particularly if this has to be done by isotope dilution methodology. On the one hand, one would expect to find the released products only partly associated with the bases added as carriers. On the other hand, if any released base carrying a sugar fragment behaved chromatographically like the free base, this would lead to over estimation of the amount of base present, since in our [$^{14}$C]TMV-RNA the ribose moiety is three to five times as radioactive as the bases.

The chromatographic purification schedule described in Table VI was intended to remove most of such fragments as well as any residual incompletely precipitated [$^{14}$]CRNA. The amounts of each base released were established, as usual, from the total radioactivity that remained associated with each marker, the re-

TABLE V: Effect of Various Treatments on Semicarbazide Binding of TMV-RNA.[a]

| Periodate Treatment | Aniline Treatment | PME Treatment | Second Periodate Treatment | Moles of Semicarbazide Bound per Mole of RNA |
|---|---|---|---|---|
| + | + | + | + | 1.8,[b] 2.9 |
| + | + | + | − | 0.4 |
| + | + | − | − | 0.5 |
| + | − | − | − | 2.0[c] |
| + | − | − | + | 2.3,[d] 2.8[d] |
| + | + | − | + | 0.75,[d] 1.1[d] |
| + | − | + | + | 2.4, 2.8 |
| + | − | + | − | 1.9 |
| − | + | + | +[e] | 2.3 |
| − | + | + | − | 0.4 |
| − | + | − | − | 0.3 |
| − | − | + | − | 0.4 |

[a] All treatments performed in duplicate as described previously. $NaIO_4$ (100-fold molar excess) and PME (1 $\mu$g/50 $\mu$g of RNA) were added on the basis of spectrophotometrically determined amounts of treated RNA. Water was substituted for aniline in the controls. The RNA was reprecipitated twice from water prior to treatment with PME. [b] Too little $Mg^{2+}$ present for complete activation of PME. This experiment was in part repeated with sufficient amounts (second column). [c] This slightly low value is attributed to loss of some of the labile dialdehyde group during the many manipulations. [d] The additional binding caused by a second periodate treatment is discussed in a footnote to Table V of the preceding paper (Steinschneider and Fraenkel-Conrat, 1966). [e] First periodate treatment.

covery in terms of absorbance of the marker, and the specific activity of the base in question (as ascertained in separate experiments) (Table VII). However, until the exact nature of the released material is known, both at the time that the carrier bases are added, and as they are finally counted, the quantitative aspects of the data must be regarded as approximations. The results for two experiments in which the RNA was passed five times through the cycle of reactions are listed in Table VI. From these data a sequence ... G-C-C-C-A is tentatively proposed, although the lack of stoichiometry and the considerations given above create some uncertainty. This is particularly true in regard to the identification of the G and C in fifth and fourth positions, since these are less clearly above the background of radioactivity found in the other three bases.

*Loss of Infectivity in the Course of Stepwise Degradation.* It has previously been reported that periodate oxidation of TMV-RNA causes a partial loss of its infectivity (usually 50–90%). The standard aniline treatment decreased the infectivity of unoxidized TMV-RNA to about 50%. As stated, aniline caused no decrease in 30S material. Thus the loss of infectivity cannot be attributed to internal phosphodiester bond breakage. By means of $^{3}H$-labeled aniline it was possible to show that a small amount (about 0.5 mole/mole of RNA) of the reagent remained bound to the RNA and was not displaceable by unlabeled aniline. It appears not impossible that this interaction somewhere along the chain leads to inactivation.

The combined effect of aniline treatment after periodate oxidation, which causes elimination, is definitely more inactivating than the sum of the two reactions, since only 1–2% of the infectivity remains, rather than about 10%. No greater inactivation was observed whether the aniline treatment was preceded by a total of three consecutive treatments with a 100-fold molar excess of periodate or by a single treatment with a 300-fold excess of periodate. Thus the elimination reaction appears to cause loss of infectivity beyond, and independent of, that due to side reactions.

PME treatment after elimination of the terminal nucleoside dialdehyde generally caused no significant change of infectivity. These results, summarized in Table VIII, show also that a second periodate treatment again caused marked loss of infectivity. This was so whether PME had been used and a new glycol group exposed or not, and could presumably be attributed to the action of aniline on oxidized RNA, since attempts to sensitize RNA to periodate inactivation by pretreatment with aniline or various dispersing solvents (dimethyl sulfoxide, formamide, and dimethylformamide) were unsuccessful.

## Discussion

Preliminary experiments had shown that the amines which had recently been advocated for the stepwise degradation of soluble RNA (s-RNA) (Neu and Heppel, 1964) and TMV-RNA (Whitfeld, 1965) caused appreciable phosphodiester bond breakage, as indicated by the sedimentation pattern of the TMV-RNA. To eliminate this hazard a search for more selective amines as catalysts was initiated. Aniline, which readily catalyzed the elimination of phosphate from 5′-nucleotide dialdehydes in model systems at pH 5 and 25°, caused no chain breakage when acting under similar conditions on TMV-RNA. Employing [$^{14}C$]semicarbazide (as described by Steinschneider and Fraenkel-Conrat, 1966), the disappearance of dialdehyde groups upon treatment of periodate-oxidized TMV-RNA with aniline, and their regeneration upon enzymatic dephosphorylation followed by a further oxidation with periodate, indicated that degradation proceeded in the expected manner. Additional evidence for this was provided by the detection of about 1 mole of a newly created phosphomonoester group. Finally, the release of 0.8 mole of adenine upon elimination, and the detection of a new end group (cytosine) in alkaline digests of RNA after one cycle of degradation (B. Singer, private communication), completed the evidence.

Two trial experiments, in which two different RNA preparations were passed through five degradation cycles, yielded predominantly one base in three of the cycles and less clear results in the fourth and fifth. This led to the tentative proposal of a sequence reading (-Gp)CpCpCpA. For the technical reasons discussed above the amounts of bases as reported are only approximations. They probably represent the lower limit of those actually liberated. In view of these uncertainties and the progressively more uniform base pattern, indicative of increasing heterogeneity of the RNA preparation after several cycles, no definitive conclusions are justified beyond the third step.

Contrary to our general experience after one cycle, sedimentation analysis after five cycles revealed no 30S material. In addition to possible endonuclease contamination, data available from the literature on the rate of phosphodiester bond hydrolysis in RNA would indicate that TMV-RNA should have suffered about 0.75 break/molecule in five cycles. Preliminary investigations with a cyclic phosphodiesterase from *E. coli* (Anraku, 1964) suggest that the majority of these newly created breaks terminate in cyclic diesterified phosphate ends. This observation could account for the fact that we did not find increasing amounts of bases released, even though chain ends were presumably generated through progressive degradation. Also, the semicarbazide binding ability of the RNA after periodate treatment of deadenylated RNA indicated that phosphomonoesterase generated few if any glycol groups at the ends of RNA fragments. If cyclic ends predominated in such fragments, as has previously been suggested after other means of degradation (Singer and Fraenkel-Conrat, 1963b), these would be expected to be resistant to phosphomonoesterase and thus would account for the resistance of fragments to the cycle of reactions presently studied.

The finding that adenine is the first base to be liberated upon terminal attack on TMV-RNA is in line

TABLE VI: Identification of the Bases Released upon Stepwise Degradation of [$^{14}$C]TMV-RNA.[a,b]

| Degradation Step | [$^{14}$C]RNA Used: Prepn | [$^{14}$C]RNA Used: Amt ($\times 10^{-4}$ $\mu$M) | Carrier Base | % Recovered | [$^{14}$C]Base Found: Counts Found (cpm) | [$^{14}$C]Base Found: Counts Corrected for Recovery (cpm) | [$^{14}$C]Base Found: Total Amt ($\times 10^{-4}$ $\mu$M) | [$^{14}$C]Base Found: Moles/Mole of RNA |
|---|---|---|---|---|---|---|---|---|
| I[b] | 77b | 1.7 | Adenine | 67 | 198 | 296 | 1.2 | 0.71 |
| | | | Guanine | 61 | 6 | 9 | 0.06 | 0.04 |
| | | | Uracil | 73 | 10 | 14 | 0.05 | 0.03 |
| | | | Cytosine | 62 | 2 | 4 | 0.05 | 0 03 |
| | | | | | | | | Total 0.81 |
| II | 77b | 1.5 | Adenine | 60 | 31 | 52 | 0.2 | 0.14 |
| | | | Guanine | 52 | 3 | 6 | 0.04 | 0.03 |
| | | | Uracil | 73 | 11 | 15 | 0.1 | 0.07 |
| | | | Cytosine | 84 | 35 | 42 | 0.5 | 0.33 |
| | | | | | | | | Total 0.57 |
| III | 77b | 1.2 | Adenine | 64 | 11 | 17 | 0.22 | 0.18 |
| | | | Guanine | 38 | 5 | 12 | 0.09 | 0.08 |
| | | | Uracil | 35 | 10 | 28 | 0.19 | 0.16 |
| | | | Cytosine | 35 | 30 | 85 | 1.1 | 0 92 |
| | | | | | | | | Total 1.34 |
| IV | 77b | 0.70 | Adenine | 62 | 14 | 23 | 0.09 | 0.13 |
| | | | Guanine | 34 | 6 | 18 | 0.13 | 0.19 |
| | | | Uracil | 67 | 7 | 10 | 0.07 | 0.10 |
| | | | Cytosine | 59 | 17 | 24 | 0.3 | 0.43 |
| | | | | | | | | Total 0.85 |
| V | 77b | 0.07 | Adenine | 33 | 12 | 35 | 0.14 | 0.20 |
| | | | Guanine | 49 | 19 | 38 | 0.27 | 0.39 |
| | | | Uracil | 51 | 7 | 14 | 0.09 | 0.13 |
| | | | Cytosine | 55 | 7 | 14 | 0.16 | 0.11 |
| | | | | | | | | Total 0.83 |

[a] About 3.4 and 4.4 mg of [$^{14}$C]TMV-RNA preparations 77b and 48, respectively, were successively treated with periodate, aniline, and PME as previously described. The alcohol supernatant after precipitation of the RNA from the aniline reaction mixture was evaporated under an air stream after addition of known amounts (about 2 absorbancy units) of each of the four bases as markers. The residues were taken up in 150 $\mu$l of water and applied to Whatman 3MM filter paper for two-dimensional chromatography (70% isopropyl alcohol with ammonia in the vapor phase, 24 hr, descending; followed by 86% *n*-butyl alcohol for 32–38 hr, also descending). The bases (adenine and uracil did not separate occasionally) were eluted with 0.01 N HCl and separated by rechromatography on Whatman No. 1 filter.

with previous conclusions based on alkaline degradation and pancreatic ribonuclease digestion (Sugiyama and Fraenkel-Conrat, 1961; Whitfeld, 1962). The latter data indicated an adjacent pyrimidine, and alkaline digestion, as well as amine-catalyzed cleavage when applied to TMV-RNA deadenylated by one complete cycle, indicated that this pyrimidine was cytosine (Whitfeld, 1965, and our findings).

That the third nucleotide was not a purine was deduced by Whitfeld (1962) from the absence of UpA or CpA from TMV-RNA digests obtained with the purine specific *Bacillus subtilis* ribonuclease. However, the further sequence of two or more C residues, possibly followed by a G, as indicated by the present data, is in conflict with the sequence suggested on the basis of limited snake venom phosphodiesterase digestion (Singer and Fraenkel-Conrat, 1963a) since the latter experiments showed that more 5′-adenylic and 5′-uridylic acid were liberated than 5′-cytidylic and particularly 5′-guanylic acid if the total number of nucleotides released was about 2–10. The possibility that some of the material liberated by the enzyme came from small molecular weight fragments is not completely ruled out. Yet, it was shown that the

TABLE VI (*continued*)

| Degradation Step | [$^{14}$C]RNA used: Prepn | [$^{14}$C]RNA used: Amt ($\times 10^{-4}$ $\mu$M) | Carrier Base | % Recovered | [$^{14}$C]Base Found: Counts Found (cpm) | [$^{14}$C]Base Found: Counts Corrected for Recovery (cpm) | [$^{14}$C]Base Found: Total Amt ($\times 10^{-4}$ $\mu$M) | [$^{14}$C]Base Found: Moles/Mole of RNA |
|---|---|---|---|---|---|---|---|---|
| I[c] | 48 | 2.2 | Adenine | 69 | 80 | 116 | 1.9 | 0.85 |
| | | | Guanine | 55 | 3 | 5 | 0.12 | 0.05 |
| | | | Uracil | 51 | 2 | 4 | 0.1 | 0.05 |
| | | | Cytosine | 90 | 1 | 1 | 0.04 | 0.02 |
| | | | | | | | | Total 0.97 |
| II | 48 | 2.2 | Adenine | 64 | 11 | 17 | 0.27 | 0.12 |
| | | | Guanine | 55 | 4 | 8 | 0.2 | 0.09 |
| | | | Uracil | 72 | 3 | 4 | 0.15 | 0.07 |
| | | | Cytosine | 63 | 19 | 30 | 1.3 | 0.59 |
| | | | | | | | | Total 0.87 |
| III | 48 | 2.1 | Adenine | 77 | 13 | 17 | 0.27 | 0.13 |
| | | | Guanine | 72 | 4 | 5 | 0.12 | 0.06 |
| | | | Uracil | 83 | 7 | 8 | 0.30 | 0.14 |
| | | | Cytosine | 66 | 24 | 35 | 1.5 | 0.72 |
| | | | | | | | | Total 1.05 |
| IV | 48 | 2.1 | Adenine[d] | ... | ... | ... | ... | ... |
| | | | Guanine | 39 | 6 | 15 | 0.4 | 0.19 |
| | | | Uracil | 80 | 12 | 15 | 0.6 | 0.29 |
| | | | Cytosine | 54 | 17 | 31 | 1.3 | 0.62 |
| | | | | | | | | Total 1.10 |
| V | 48 | 2.1 | Adenine | 62 | 22 | 35 | 0.56 | 0.27 |
| | | | Guanine | 47 | 18 | 39 | 0.93 | 0.44 |
| | | | Uracil | 54 | 10 | 19 | 0.70 | 0.33 |
| | | | Cytosine | 62 | 15 | 24 | 1.0 | 0.48 |
| | | | | | | | | Total 1.52 |

paper in isopropyl alcohol–concentrated HCl–$H_2O$ (170:41:39, v/v/v/) for 24 hr. They were then eluted from the paper with 0.01 N HCl, dried under an air stream, and redissolved in 0.01 N HCl. Finally, the recovery of the unlabeled carrier base was determined spectrophotometrically and its radioactivity measured in a gas-flow counter after spreading on an aluminum planchet with a few drops of 0.01% Triton-X and drying under a heat lamp. The count actually obtained for each base was corrected for the recovery of the carrier, and the amount of $^{14}$C-labeled base then calculated using the specific radioactivity for each base given in Table VII. [b] Experiment 1. [c] Experiment 2. [d] Not determined.

snake venom diesterase (in contrast to polynucleotide phosphorylase, Singer and Fraenkel-Conrat, 1965) definitely attacked intact viral RNA, since the terminal adenosine as detected by alkaline digestion was lost after the phosphodiesterase treatment. When the RNA was deadenylated by the presented elimination procedure and then treated with the phosphodiesterase, no predominant nucleotide was found released by the enzyme although cytidylic acid should have been found to exceed the others on the basis of our present results with stepwise degradation. We are thus left with a discrepancy which will have to be resolved by additional evidence and presumably different methods.

The fact that complete periodate oxidation of the terminal glycol group of TMV–RNA causes only partial loss of infectivity has previously been reported and discussed (Whitfeld, 1965; Steinschneider and Fraenkel-Conrat, 1966). Aniline treatment of non-oxidized TMV–RNA also causes a limited loss of infectivity, a fact which is tentatively attributed to a very slow random interaction of aniline with bases in the chain, possibly analogous to the interaction of cytosine with semicarbazide (Hayatsu and Ukita,

TABLE VII: Specific Radioactivity of Bases in [$^{14}$C]TMV-RNA.[a]

| [$^{14}$C]RNA Prepn | Cpm/$\mu$mole | | | |
|---|---|---|---|---|
| | Adenine | Guanine | Uracil | Cytosine |
| 77b | $2.5 \times 10^5$ | $1.4 \times 10^5$ | $1.5 \times 10^5$ | $0.8 \times 10^5$ |
| 48 | $6.2 \times 10^4$ | $4.2 \times 10^5$ | $2.7 \times 10^4$ | $2.3 \times 10^4$ |

[a] 2′,3′-Nucleotides obtained by alkaline hydrolysis of preparation 77b and the intact RNA preparation 48 were hydrolyzed for 1 hr at 100° with 70% perchloric acid. The hydrolysate was applied directly to Whatman No. 1 filter paper for development in the isopropyl alcohol–HCl system described previously (Table VI). The bases were eluted with 0.01 N HCl, dried, and rechromatographed in the same system; after a second elution with 0.01 N HCl and drying, total base concentration was determined spectrophotometrically in 0.01 M HCl, and the radioactivity measured as described previously (Table VI) on 10- to 50-$\mu$l aliquots.

TABLE VIII: Effect of Various Treatments on Infectivity of TMV-RNA.

| Infectivity (%)[a] after | | | |
|---|---|---|---|
| Final Periodate Treatment | Aniline Treatment | PME Treatment | Final Periodate Treatment |
| 23 | 1.5 | 1.7 | 0.6 (35%)[b] |
| 23 | 1.5 | (None 1.6)[c] | 0.7 (44%) |
| None (100) | None (100)[c] | None (100) | 37 |
| 22 | 2.4 | ... | 1.7 (71%) |
| (None 94)[c] | 52[d] | ... | 11 (21%) |
| None (100) | None (100) | ... | 17 |

[a] Assayed after reconstitution. Data given as percentage of untreated control RNA, reconstituted under the same conditions. [b] Percentages in parentheses represent levels of inactivation resulting from periodate treatment, to be compared to 23, 22, 37, and 17% in control samples. [c] Samples exposed for same lengths of time to same pH and temperature as used for the respective reaction but lacking periodate, aniline, or PME, respectively. These conditions generally caused no loss of infectivity. [d] A similar sample that had been treated with aniline for 6 hr prior to oxidation showed 14% infectivity, compared to 20% for a sample that had been precipitated immediately after addition of the aniline.

1964). In contrast, aniline treatment after periodate, when it causes the terminal elimination reaction, leads to loss of about 95% of the infectivity of the periodate-treated sample. The interpretation that this additional inactivation is due to elimination of the terminal residue rather than to interactions along the polynucleotide chain is supported by our failure to cause additional inactivation by intensifying the periodate treatment.

The loss of biological activity upon elimination could be the consequence of the exposure of an unusual end group. In that case, reactivation would be expected upon removal of the 3′-phosphate from the next sugar. The fact that enzymic dephosphorylation causes no appreciable change in infectivity indicates that the state of phosphorylation of the 5′-linked chain end is not critical in determining its biological activity.

When the RNA after loss of one complete nucleotide was subjected to a second periodate oxidation, part of the residual low biological activity was again lost. The extent of inactivation was in most experiments comparable to that observed upon oxidation of intact RNA (about 70%), and occurred regardless of whether the phosphate end group was removed by PME and the second ribose residue exposed or not, thus apparently excluding oxidation of the second sugar as the inactivating event. It was thought that this observation could be due to a small fraction of the end groups being masked and escaping the first periodate treatment, but becoming oxidizable after the aniline treatment. However, attempts to test this working hypothesis by substituting dimethylformamide or dimethyl sulfoxide for the aniline have given ambiguous results.

Although the exact level of contributions by side reactions is difficult to assess, the present experiments seem to clearly indicate that most infectivity is lost when the terminal nucleotide is removed from TMV-RNA. This conclusion is in conflict with the data

obtained with limited snake venom diesterase digestion of the RNA (Singer and Fraenkel-Conrat, 1963a,b), which showed only very slow inactivation of TMV-RNA, one lethal hit (37% residual infectivity) resulting only when over four nucleotides were released. Even if one assumes that part of these nucleotides came from fragments rather than the chain terminus, the fact that preparations which had been deadenylated by the exonuclease (as ascertained by alkaline degradation) still retain most of their infectivity is in conflict with the concept emerging from the experiments discussed above, that the removal of the terminal nucleoside by chemical means (with or without enzymatic dephosphorylation of the new end group) causes loss of about 99% of the infectivity of the molecule. Again, resolution of this discrepancy will depend on the development of new approaches and on further study and evaluation of the methods already in use.

With most of the modifications described in this paper the RNA was assayed both directly and after reconstitution. Although quantitative results are more difficult to obtain by direct assay of unreconstituted nucleic acid, the response to modifications seemed to follow similar patterns, indicating that the reconstitutability of the RNA is rather indifferent to removal of the terminal nucleoside dialdehyde and subsequent dephosphorylation. A similar conclusion seemed warranted for the effect of terminal oxidation and semicarbazone formation, as described in the previous paper (Steinschneider and Fraenkel-Conrat, 1966). It would also seem that the mechanism involved in the entry of the nucleic acid into the host cell is insensitive to these modifications, and that they are expressed only at the level of the interaction of the modified viral nucleic acid, devoid of its protein coat, with the pertinent cellular mechanism.

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