Studies of Nucleotide Sequences in Tobacco Mosaic Virus Ribonucleic Acid. III. Periodate Oxidation and Semicarbazone Formation*

A. Steinschneider† and H. Fraenkel-Conrat

ABSTRACT: Treatment of tobacco mosaic virus ribonucleic acid (TMV–RNA) with a 100-fold molar excess of periodate oxidizes the terminal glycol group. Subsequent treatment with [^{14}C]semicarbazide causes the binding of about 2 equiv of this reagent/mole of the RNA in excess of the level of binding by nonoxidized RNA (0.5 equiv). The oxidation causes loss of about 80% of the infectivity. Neither semicarbazone formation nor repeated periodate treatment causes further losses of infectivity. Overoxidation of TMV–RNA with much greater amounts of periodate gradually increases the semicarbazide binding capability up to 4.2 equiv. Under these circumstances, much further loss of infectivity results from semicarbazide treatment.

Selective chemical modification of the 5′-linked end groups as a preliminary to nucleotide sequence analysis of tobacco mosaic virus ribonucleic acid (TMV–RNA) has been attempted by Dulbecco and Smith (1960), Gordon *et al.* (1960), and Whitfeld (1965). In no case, however, has a clear case been made that the reaction was specific for the end group. The present study is concerned with periodate oxidation of the 5′-linked terminal glycol group followed by coupling of the resulting two aldehyde groups by means of ^{14}C-labeled semicarbazide. Evidence will be presented that both of these reactions show a high degree of specificity.

Materials and Methods

Sodium metaperiodate was purchased from G. Frederick Smith Chemical Co. Stock solutions (10^{-3}–10^{-1} M) in water were stored in dark bottles in the refrigerator. Semicarbazide hydrochloride was purchased from Eastman Organic Chemicals and [^{14}C]semicarbazide hydrochloride (sp act. 0.9 mc/mM) from Orlando Research Inc. Semicarbazide was freshly dissolved to 1 mg/ml in water. ^{14}C-Labeled adenosine was isolated from generally ^{14}C-labeled TMV–RNA, as described by Sugiyama and Fraenkel-Conrat (1961). Snake venom phosphodiesterase (Williams *et al.*, 1961) was kindly supplied by Dr. M. Laskowski.

Radioactivity was determined in the Packard 314E Tricarb liquid scintillation spectrometer. Samples were usually 25–50 μl and no more than 0.5 ml/10 ml of scintillation liquid containing naphthalene, PPO, and POPOP (80, 5.0 and 0.5 g/l. respectively), dissolved in toluene, dioxane, and absolute alcohol (385/385/230, v/v/v).

Periodate Oxidation. TMV–RNA (5 mg/ml of reaction mixture) was treated with $NaIO_4$ (sodium periodate) in 0.15 M sodium acetate of pH 5.3 usually for 30 min at 0° in the dark. Selective oxidation of the end groups was performed by addition of a 10^{-3} M solution to a 100-fold molar excess over the TMV–RNA (0.25 ml/5 mg of RNA). The RNA was precipitated by the addition of 3 volumes of ice-cold ethanol and was centrifuged after 40 min for 20 min. Some kinetic experiments were terminated by addition of ethylene glycol (10% in ethanol).

Semicarbazide Reaction. To 1 mg of RNA or oxidized RNA in 0.2 ml of 0.15 M pH 5.3 sodium acetate 60 μl of a 0.1% semicarbazide hydrochloride solution (about 1000-fold molar excess) was added and the solution held at room temperature for 1 hr. The reaction was terminated by the addition of 3 volumes of ice-cold ethanol and sedimented as above.

The washing of the RNA to achieve removal of all unbound [^{14}C]semicarbazide consisted of four precipitations from 70% ethanol, usually interspersing precipitation in the presence of 0.05 M acetate (pH 5.3) with precipitation from 0.005 M EDTA (pH 7.0). Usually two additional precipitations were performed after holding the RNA for 30 min in a solution of 0.15 M pH 5.3 sodium acetate to which 0.1 volume of 0.1 M [^{12}C]semicarbazide hydrochloride was also added. All manipulations were performed at 0°. All experiments were carried out in duplicate. The preparation of TMV–RNA and its reconstitution and assay were per-

* From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received April 17, 1966.* This investigation was supported by Grant GB 3107 from the National Science Foundation.

† Taken in part from a thesis by A. Steinschneider in partial satisfaction of the requirements for the degree of Doctor of Philosophy, Department of Molecular Biology, University of California, Berkeley, Calif., 1965.

[1] Abbreviations used: SC, semicarbazide hydrochloride; SVPDE, snake venom phosphodiesterase; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

TABLE I: Effect of Periodate Concentration on the Infectivity of TMV–RNA and Its Ability to Bind Semicarbazide.[a]

Periodate				Subsequent [^{14}C]Semicarbazide				
				SC Bound (Mole/Mole)		Infectivity[b] (%)		
Molar Excess	Infectivity (%)[b]							
0	100		100[c]	0[d]	0.5	100[d]	100	100[c,d]
10	57			1.1		64		
50	28			2.1		45		
100	17[e]	16	44[c]	2.7	2.7	35[e]	45	64[c]
500		12	70[c]		2.9		18	19[c]
1,000		12	39[c]		3.2		17	6[c]
10,000		10	29[c]		4.4		0.4	0.03[c]

[a] Reaction mixtures contained 1 mg of TMV–RNA in 0.2 ml of sodium acetate, pH 5.3. Sodium periodate (5–50 μl), and [^{14}C]semicarbazide (SC) (1000-fold molar excess) were added and allowed to react as described in Materials and Methods. The RNA was isolated by one reprecipitation from water, two from EDTA, two treatments with non-radioactive semicarbazide, and again two precipitations from water, followed by radioactivity counting. Infectivities were measured prior to addition of the [^{14}C]semicarbazide and at the end of the experiment. The data represent two typical experiments and are entered accordingly in the various columns of the table. [b] Per cent of control, all assayed on 24–72 half-leaves of *N. Tab. var.-Xanthi nc* after reconstitution except where indicated. [c] Unreconstituted RNA assayed. The difference between these data and those obtained after reconstitution are not regarded as significant since they are not observed in other experiments. [d] No [^{14}C]semicarbazide added. [e] This seeming increase in infectivity upon semicarbazide treatment was not borne out in other experiments (*e.g.*, 31% after periodate and 25% following semicarbazide).

formed according to the standard procedures of this laboratory (Fraenkel-Conrat *et al.*, 1961).

Sedimentation in the Model E ultracentrifuge equipped with ultraviolet optics was performed in 0.05 M pH 7 phosphate (4°, 59,000 rpm). The RNA used for the experiments showed a main component of approximately 30 S amounting to 40–80% of the total absorbance.

Results

Periodate Oxidation. As a preliminary study to the oxidation of the terminal nucleoside of TMV–RNA, ^{14}C-labeled adenosine was oxidized at a similar molarity to that practical for TMV–RNA (10^{-6} M) in the presence of a similar amount of the RNA (2 mg/ml). It appeared that more periodate or more time was required in the presence of the RNA than in its absence. A 10-fold molar excess of periodate sufficed for complete oxidation in 30 min in the absence of RNA, but less than half of the adenosine was oxidized in the presence of RNA (pH 5.3, 0.06 M acetate at 0°). A 50-fold excess achieved complete oxidation in 15 min even in the presence of RNA. These data were obtained (after reducing excess periodate by brief gassing with SO_2 followed by air) by electrophoretic isolation (0.1 M sodium bisulfite, pH 4.7) of the unoxidized adenosine from the supernatant after alcohol precipitation of the RNA, if any.

On the basis of these model experiments it was concluded that treatment of TMV–RNA with a 50- to 100-fold excess of periodate might cause complete terminal oxidation with minimal side reactions of the type observed in previous studies. The chemical criteria used to ascertain the effect of periodate on TMV–RNA were (a) the resulting ability of the molecule to bind semicarbazide (which will be discussed in the following section), and (b) the resulting tendency of the molecule to release the terminal modified nucleoside [which will be the subject matter of an accompanying paper (Steinschneider and Fraenkel-Conrat, 1966)].

When the effect of the oxidation reaction on the infectivity of TMV–RNA was studied, it appeared that a loss of about 80% of the infectivity occurred rapidly and under minimal condition for complete oxidation, but that no further appreciable inactivation resulted when considerably more time or reagent was used. This 80% figure represented an average of many experiments which actually ranged from 30 to 96% (Table I). Repeated periodate treatment did not cause additional loss of infectivity.

The possibility that this inactivation was due to diester bond breakage was ruled out by sedimentation analyses which showed that the amount of 30S component in TMV–RNA (about 50% in the preparations used) was not affected by minimal or excessive periodate treatment under our reaction conditions.

Semicarbazide Binding. As shown on Table I, treatment of TMV–RNA with 1000 equiv of semicarbazide at pH 5.3 and washing under our standard conditions (including the use of [^{12}C]semicarbazide) led to a "binding" of about 0.5 mole/mole, which was not appreciably reduced by further washing.

When TMV–RNA was oxidized with a 100-fold

excess of periodate, it became capable of binding about 2.2 moles of [^{14}C]semicarbazide over the control. The same amount of semicarbazide remained bound to oxidized or control RNA also when the reaction mixtures were passed through a Sephadex G-75 (medium) column at pH 6.8. Rechromatography did not lead to any decrease in the amount bound.

Employing a 1000-fold molar [^{14}C]semicarbazide excess, the reaction with oxidized RNA appeared complete after 60 min at 25° and little additional labeling was observed during the second hour of treatment (Figure 1). When the molar excess was increased to the 1000-fold level, the total amount of [^{14}C]semicarbazide bound to oxidized and control RNA was higher and continued to increase slowly with time (Table II). On the other hand, the use of less semicarbazide (100-fold excess) or a higher pH (pH 7, 1000-fold semicarbazide), while causing less semicarbazide to be bound by unoxidized RNA, failed to achieve quantitative semicarbazone formation with oxidized RNA in 2 hr (Table II). Consequently, semicarbazide treatment with a 1000-fold molar excess at pH 5.3 for 1 hr at 25° was adopted as standard procedure.

TABLE II: Effect of Reaction Conditions on Semicarbazide (SC) Binding by TMV-RNA.

RNA	SC Added (Moles/ Mole of RNA)	pH	Moles of SC Bound/ Mole of RNA (min) 30	60	120
Oxidized[a]	100	5.3	0.8	0.9	1.9
Control	100	5.3	0.2	0.2	0.2
Oxidized[a]	1000	5.3	2.1	2.7	2.4
Control	1000	5.3	0.3	0.3	0.4
Oxidized[a]	10000	5.3	3.1	3.4	3.8
Control	10000	5.3	1.0	1.1	2.0
Oxidized[a]	1000	7.0		1.8	2.0
Control	1000	7.0		0.2	0.2

[a] With 100-fold excess of periodate (see Methods).

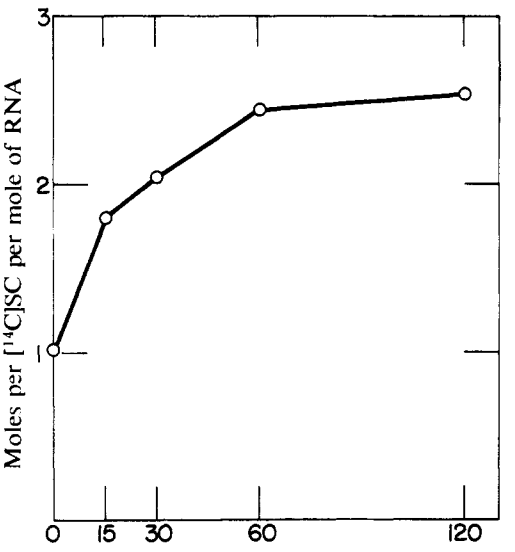

FIGURE 1: Semicarbazide binding to oxidized TMV-RNA as a function of time. About 3.5 mg of TMV-RNA was oxidized with a 100-fold molar excess of periodate, precipitated, and treated with a 1000-fold molar excess of [^{14}C]semicarbazide as described in Materials and Methods. Aliquots were removed at specified time periods (0-min time aliquots were secured within 30 sec of [^{14}C]semicarbazide addition) and frozen, and the excess counts were removed as described.

Employing a 100-fold molar periodate excess for oxidation and terminating the reaction by precipitation of the RNA with ethanol, the oxidative reaction appeared complete within 15 min and probably much earlier (Table III). No additional semicarbazide was bound even after 2 hr of oxidation. Decreasing the periodate excess (Table I) resulted in an approximately proportional decrease in the number of semicarbazide binding sites created. Yet, when periodate concentrations were progressively increased to the 10,000-fold molar excess level, only an additional 1.7 moles of semicarbazide was bound (Table I).

The interpretation that periodate oxidation and semicarbazone formation occurred predominantly at the end of the RNA molecule when a 100-fold molar excess of periodate was employed was at this stage based only on the stoichiometry and kinetics of the two reactions. This conclusion was strongly supported by studying the effect of repeated treatment with these reagents, as illustrated by one such experiment described in Table IV. As expected, TMV-RNA which had previously been oxidized with periodate and converted into the [^{12}C]semicarbazone was no longer able to bind 2 moles of [^{14}C]semicarbazide with or without an additional periodate treatment. The location of the labeled semicarbazide at the end group as opposed to random nucleotides was also suggested by the finding that most of the counts seemed to be associated with the sugar rather than the base moieties when the products of complete digestion of the RNA with snake venom phosphodiesterase were examined (see next section).

In terms of infectivity, semicarbazide treatment showed no appreciable effect on TMV-RNA. The semicarbazone formation of oxidized RNA also did not cause further loss of its infectivity. Only after very excessive periodate treatment which, by itself was no more inactivating, did semicarbazide cause a marked loss of infectivity (Table I). Similar results were obtained whether the RNA was assayed directly or after reconstitution with TMV protein, suggesting

TABLE III: Effect of Periodate Excess on Subsequent Binding of Semicarbazide.

Periodate Reaction Time (min)	Moles of Semicarbazide Bound/Mole of RNA: Periodate Excess 100-Fold[a,b]	10,000-Fold[c]
0	(2.1)	0.3
1		2.6
15	2.6	2.8
30	2.6	2.6
60	2.7	3.4
120	2.6	4.1

[a] TMV-RNA (5 mg) in 1.4 ml of 0.15 M acetate, pH 5.3, was held in the dark at 0° after addition of 0.25 ml of 10^{-3} N $NaIO_4$. Aliquots of 0.250 ml were withdrawn after about 30 sec (0 min), and later times as indicated, and immediately frozen on Dry Ice. All samples were thawed simultaneously and precipitated with ice-cold ethanol. The precipitates were taken up in 0.2 ml of water and further treated with [^{14}C]semicarbazide as described in Materials and Methods. [b] In another experiment [^{14}C]semicarbazide binding under similar conditions was 0.3, 1.8, and 3.0 moles/mole of RNA after 0, 2, and 60 min of oxidation, respectively. Infectivities were 100, 59, and 32% for these samples. Similar results were obtained whether the RNA was treated at 25° for 10 min with a 100-fold molar excess of ethylene glycol prior to alcohol precipitation, or precipitated directly with three volumes of ethanol, or ethanol containing 0.03 μl/ml of ethylene glycol (10-fold molar excess). [c] TMV-RNA (5.4 mg) in 1 ml of 0.15 M acetate, pH 5.3, was held in the dark at 0°. After removal of 0.16-ml aliquots at zero time, 0.720 ml of 0.1 N $NaIO_4$ was added and 0.22-ml aliquots were removed as indicated. All samples were immediately frozen on Dry Ice and thawed simultaneously before precipitation with 0.6-ml of ice-cold ethanol containing 1.5% (v/v) ethylene glycol. The RNA was once reprecipitated from water and then treated with [^{14}C]-semicarbazide. (We have frequently observed that when ethylene glycol was used to consume high excesses of periodate (about 10^{-2} M) subsequent [^{14}C]semicarbazide binding was erratic. No such adverse effects were observed when similar amounts of ethylene glycol were used to destroy periodate at the 100-fold excess level (10^{-4} M).

TABLE IV: Effect of Repeated Periodate and Semicarbazide Treatment on Semicarbazide (SC) Binding.[a]

	First Treatment: IO_4	First Treatment: [^{12}C]SC	Second Treatment: IO_4	Second Treatment: [^{14}C]SC	Equiv [^{14}C]SC Bound/Mole of RNA
1	+	0	0	+	2.4
2	+	+	0	+	0.6
3	+	+	+	+	1.1[b]
4	+	0	+	+	2.9[b]
5	0	+	0	+	0.5
6	0	0	+	+	2.3

[a] TMV-RNA (1 mg) was oxidized with a 100-fold molar excess of periodate and treated with [^{12}C]-semicarbazide as described in Materials and Methods. The excess semicarbazide was removed by five alcohol precipitations, the first and third from EDTA, the others from water, but no treatment with excess unlabeled semicarbazide was included. The RNA was then oxidized with periodate, treated with [^{14}C]-semicarbazide, and purified as described in Materials and Methods. + = treated, 0 = treatment omitted. [b] We have frequently observed (see also Steinschneider and Fraenkel-Conrat, 1966) that a second treatment of oxidized TMV-RNA with 100 equiv of periodate led to an increase of 0.3–0.5 mole in [^{14}C]semicarbazide binding. This may in part be due to oxidation of molecules which have escaped oxidation during the first periodate treatment (see Steinschneider and Fraenkel-Conrat, 1966).

that modification of the end group had no effect on the reconstitutability of TMV-RNA.

As far as the integrity of the RNA molecules is concerned, even the inactivated preparation resulting from maximal periodate followed by semicarbazide treatment remained intact, as indicated by the fact that a similar fraction (46%) of the treated material was of approximately 30 S.

Properties of Oxidized TMV-RNA Semicarbazide. It was hoped that the labeled end group would supply a useful handle for the characterization of terminal nucleotide sequences. Prior to testing this possibility the stability of the label was investigated by exposing the treated RNA to conditions of pH which it would encounter during the standard methods of isolating and characterizing oligonucleotides. It appeared that much of the label was lost, the semicarbazone being only moderately stable even at pH 7–8 (Figure 2). Attempts to stabilize the semicarbazone by reduction with $NaBH_4$ and other reducing agents were not successful, as judged from similar pH-stability tests. It must be noted, however, that no proof is actually available that the reaction product is a semicarbazone, and not an addition product. The term semicarbazone is used throughout only for brevity's sake.

Enzymatic degradation of the ^{14}C-labeled TMV-RNA dialdehyde disemicarbazone was performed with snake venom phosphodiesterase and the digest was electrophoretically separated in a 0.2 M pH 8.4 sodium borate buffer (Figure 3). It appeared that about 60% of the counts moved in a manner to be

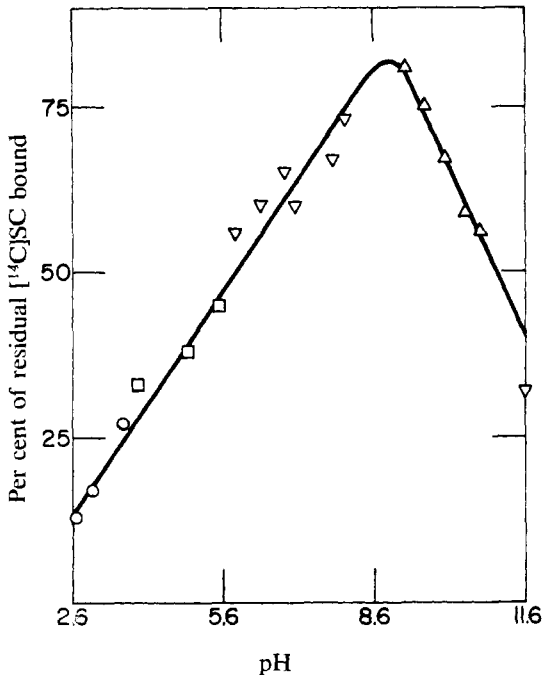

FIGURE 2: Stability of semicarbazide binding by TMV-RNA: 130 μg of oxidized and [^{14}C]semicarbazide-treated TMV-RNA (2.5 moles of semicarbazide/mole of RNA) in 75 μl of water was mixed with 75 μl of 0.1 M buffer, 2×10^{-4} M in Versene, and adjusted to a final $\Gamma/2 = 0.15$ with KCl. The actual pH was determined potentiometrically in solutions lacking RNA. The samples were held in sealed tubes for 12 hr at 30° and then made 0.1 M in sodium acetate, pH 5.3, before precipitation with 3 volumes of ice-cold ethanol. Following one reprecipitation from water the amount of residual [^{14}C]semicarbazide bound was determined. Buffers: sodium formate, (○), sodium acetate, (□), sodium phosphate, (▽), and sodium bicarbonate, (Δ).

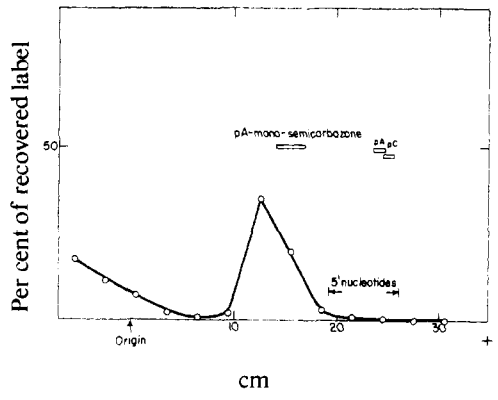

FIGURE 3: Electrophoresis of snake venom digest of oxidized, semicarbazide-treated TMV-RNA. The oxidized [^{14}C]semicarbazide-treated RNA (790 μg, 2.5 moles of semicarbazide bound/mole of RNA, in 0.15 ml of H_2O) was treated with SVPDE (2.5 units in 0.5 ml of H_2O) for 3 hr at 37°, after making the solution 0.015 M with respect to pH 8.6 Tris–HCl and 0.0015 M with respect to $MgCl_2$. Another 2.5 units of SVPDE was added and incubation was continued for another 4 hr. The digest was then freeze dried, taken up in 0.12 ml of water, applied to Whatman's 3MM filter paper, and subjected to electrophoresis in 0.2 M sodium borate, pH 8.4, for 3 hr at 20 v/cm, with 5′-adenylic acid (pA), 5′-cytidylic acid (pC), and the dialdehyde monosemicarbazone of 5′-adenylic acid (Dulbecco and Smith, 1960) as markers. The ultraviolet absorbing spots were located, and paper strips 4.5-cm wide and 3 cm long were submerged in 15 ml of scintillation fluid and counted directly. The radioactivity of a paper blank cut from a different alley was 21 cpm. Applying a correction factor of about 75% for the efficiency of counting on paper (as determined separately), the total recovery of counts was 77%. The losses are probably due mainly to failure to include one additional strip further toward the cathode as well as to handling of the sample. The recovered counts (58%) were located where adenylic dialdehyde disemicarbazone is expected to move. Virtually no counts were associated with 5′-nucleotides.

expected for 5′-nucleotide dialdehyde semicarbazones, *i.e.*, less far than the free 5′-nucleotides which are able to form borate complexes and then move faster in the electric field. The monosemicarbazone of oxidized 5′-adenylic acid, prepared according to Dulbecco and Smith (1960), which also is unable to bind borate, moved similarly to the bulk of the ^{14}C-containing products in the digest. The rest of the counts stayed at the origin, as does free semicarbazide, and less of this material was present if the enzyme treatment was for a shorter time period. Thus the instability of the semicarbazone presents a definite problem even within the range of pH 8–9 which was not exceeded in this experiment.

Discussion and Conclusions

A method is described to selectively convert to a dialdehyde the glycol end of a nucleic acid as big as TMV-RNA, which is believed to have only one such group per 6400 nucleotides. This consists of oxidation of the end group with a 100-fold molar excess of periodate. The dialdehyde end is then condensed with ^{14}C-labeled semicarbazide. From 2.5 to 3 moles of that reagent/mole of RNA is bound as a consequence of the oxidation. Since 0.4–0.5 mole is bound by unoxidized RNA, the binding of 2.7 moles after oxidation approaches the expected value for two aldehyde groups created by oxidation of the terminal glycol. The semicarbazide bound by unoxidized TMV-RNA may be due to an incipient reaction of cytosine residues as described by Hayatsu and Ukita (1964). The small excess over the theoretical binding observed with oxidized RNA (about 0.2 mole/mole) may be attributed

to fragments of TMV–RNA terminating in glycol groups which would be expected to be of that small magnitude (Singer and Fraenkel-Conrat, 1963). However, there occurs also a slow, progressive, and seemingly nonterminal binding of semicarbazide after oxidation with a great excess of periodate, the nature of which is not yet understood. It appears most likely that it is indicative of the destruction of base rings.

The conclusion that the predominant reaction is with the end group is based on (a) the stoichiometry and kinetics of periodate oxidation and semicarbazide binding, (b) the inability to effect additional [^{14}C]semicarbazide binding upon repeated oxidation of TMV–RNA or its semicarbazone, and (c) the detection after complete digestion of the modified RNA with SVPDE of ^{14}C-labeled fragments which have the electrophoretic mobility in a pH 8.4 borate buffer expected of a 5′-nucleotide dialdehyde disemicarbazone.

It is noteworthy that oxidized TMV–RNA binds 2 moles of semicarbazide while on the basis of experiments with thiosemicarbazide (Dulbecco and Smith, 1960) and isonicotinoyl hydrazide (Hunt, 1965), the binding of only one molecule to form a cyclic monosemicarbazone was expected. Since all our data are consistent with the notion that both semicarbazides are equivalent and bound terminally, the difference in behavior could be regarded as characteristics of the various aldehyde reagents used. However, the nature of the RNA also seems to play a role since preliminary data obtained by T. Sugiyama (private communication) and confirmed by us indicate that the RNA of the bacteriophage, MS2, binds only 1 equiv of semicarbazide under the conditions of oxidation and coupling used by us with TMV–RNA.

The drop of about 80% in the infectivity of TMV–RNA upon periodate treatment, which was also observed by Whitfeld (1965), is attributed to oxidation of the end group since no appreciable further loss of infectivity is observed upon longer treatment, or repeated treatment, or the use of higher periodate concentration, while a decrease in the extent of terminal oxidation results in lesser inactivation. The infectivity of terminally oxidized TMV–RNA is not affected by semicarbazone formation. However, overoxidation by an increasing excess of periodate gradually renders the RNA susceptible to subsequent inactivation by semicarbazide. As the overoxidation reaction presumably involves destruction of base rings by periodate (see Tomasz *et al.*, 1965) one would expect a loss of biological activity, but we are unable to account for the observation that this occurs only upon further reaction with semicarbazide. The techniques worked out in the course of this study are being applied in monitoring stepwise degradation of TMV–RNA as reported in the following paper (Steinschneider and Fraenkel-Conrat, 1966).

References

Dulbecco, R., and Smith, J. D. (1960), *Biochim. Biophys. Acta 39*, 358.

Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.

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

Hayatsu, H., and Ukita, T. (1964), *Biochem. Biophys. Res. Commun. 14*, 198.

Hunt, J. A. (1965), *Biochem. J. 95*, 541.

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

Steinschneider, A., and Fraenkel-Conrat, H. (1966), *Biochemistry 5*, 2735 (this issue; following paper).

Sugiyama, T., and Fraenkel-Conrat, H. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1393.

Tomasz, M., Sanno, X., and Chambers, R. W. (1965), *Biochemistry 4*, 1710.

Whitfeld, P. R. (1965), *Biochim. Biophys. Acta 108*, 202.

William, E. J., Sung, S., and Laskowski, M. (1961), *J. Biol. Chem. 236*, 1130.