Inactivation and Mutagenesis of Tobacco Rattle Virus by Nitrous Acid

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SUMMARY

The rate of inactivation of tobacco rattle virus RNA by 0.2 M-nitrous acid at pH 4.0 was comparable to that of tobacco mosaic virus RNA. In 0.0025 M-nitrous acid at pH 6.0 this rate was greatly diminished, and was slower than that of intact virus. Possible reasons for this are discussed. Methods are described for the induction of mutants of tobacco rattle virus by nitrous acid, and for the selection of temperature-sensitive mutants that are not characterized by changes in the coat protein gene.

INTRODUCTION

Treatment of tobacco mosaic virus (TMV) RNA with nitrous acid leads to deamination of base residues and inactivation of infectivity (Schuster & Schramm, 1958). Furthermore, Mundry & Gierer (1958) found that among the surviving RNA were mutants characterized by their ability to form necrotic local lesions on Java tobacco. Both inactivation and mutation reactions proceeded with single-hit kinetics. Similar reactions occur when intact TMV is treated with nitrous acid, although the relative extents to which the various bases are deaminated are quite different (Schuster & Wilhelm, 1963). Isolated RNA is inactivated more rapidly than intact virus, but at the same level of survival more mutants are obtained from intact virus (Sehgal & Krause, 1968). Nitrous acid has also been used to produce mutants of other plant viruses (e.g. cowpea chlorotic mottle virus, Bancroft et al. 1971), although all the mutations studied in detail so far have involved changes in the coat protein amino acid sequence.

Tobacco rattle virus (TRV) would seem to lend itself to the production of mutants in specific parts of its genetic material. TRV preparations contain a mixture of long and short rod-shaped particles, each particle containing a single RNA molecule of a size proportional to the particle length. Only the long particles are infective and therefore presumably carry the information for an RNA replicase system (Frost, Harrison & Woods, 1967). Such infections produce only uncoated long RNA. Short particles, although not infective alone, when mixed with long particles, give rise to infections producing coated particles of both lengths. Moreover, when a mixture of short and long particles from different strains are used as inoculum, both lengths of particle in the progeny virus have the serological specificity of the short particle parent (Sänger, 1968). It is inferred that the short particle carries the information for the coat protein sequence.

In the CAM isolate of TRV, the mol. wt. of the RNA molecules contained in the long and short particles are $2.5 \times 10^6$ and $0.7 \times 10^6$, respectively (Cooper & Mayo, 1972). Assuming that the whole of the RNA codes for protein, this represents enough information for about 5 to 10 average-sized polypeptides in the long particles, and only one or two in addition to
METHODS

Virus. The CAM isolate of TRV was propagated in Nicotiana clevelandii Gray plants grown in a glasshouse at about 20 °C. The systemically infected leaves were minced, and the sap expressed through muslin and stored at −15 °C. Virus was purified from frozen sap by differential centrifugation, as described by Cooper & Mayo (1972). Preparations of separated short particles were obtained by two successive centrifugations in sucrose density gradients (Brakke, 1960).

The CAM/DF isolate of TRV is a defective variant of CAM derived by inoculation at high dilution (Harrison, Stefanac & Roberts, 1970), and was propagated in N. clevelandii. Inocula were prepared from systemically infected leaves by cold phenol extraction.

RNA extraction. RNA was extracted from purified virus by a method based on that of Kirby (1965). Virus at about 1 mg/ml in 0.01 M-tris buffer, pH 7.6, containing 0.05 M-sodium chloride and 0.5% sodium naphthalene-1,5-disulphonate was mixed with an equal volume of phenol-cresol reagent and final concentrations of 0.5% (w/v) triisopropylphosphoryl-naphthalene sulphonate and 3% (w/v) sodium 4-aminosalicylate were added. The mixture was shaken vigorously and centrifuged to separate the phases. The aqueous phase was made 3% (w/v) in sodium chloride and re-extracted with an equal volume of phenol-cresol reagent. RNA was precipitated from the final aqueous phase with 2.5 volume ethanol at −20 °C. It was re-precipitated once from 0.15 M-acetate buffer, pH 6.0, containing 0.5% SDS, and was used as a solution in water.

After reaction with nitrous acid, RNA was extracted from dilute suspensions of virus by treatment with pronase (Calbiochem). Pronase at 2 mg/ml was dissolved in 0.015 M-sodium citrate containing 0.15 M-sodium chloride and 5 mg/ml SDS, and was incubated for 30 min at 37 °C to destroy traces of ribonuclease. An equal volume of this mixture was added to each virus sample, and kept at 37 °C overnight. After pronase treatment, the mixtures were chilled on ice and the precipitated SDS removed by centrifuging.

Assay of virus infectivity. Infectivity of virus preparations was assessed by inoculation to Chenopodium amaranticolor Coste & Reyn. Three or four inocula were compared in a randomized block design. Infectivity is expressed as the total number of local lesions on six or eight leaves.

Cloning of isolates. Suitably diluted infective sap was inoculated to Chenopodium amaranticolor to produce about 30 lesions/leaf. Well-spaced individual lesions were cut out with a No. 4 cork borer (7.5 mm) and ground in 3 drops of 0.02 M-phosphate buffer, pH 7.3. The extracts were inoculated to single leaves of C. amaranticolor, and if desired were cloned again by the same procedure.

Isolates obtained in Chenopodium amaranticolor were propagated by inoculation of sap to Chenopodium quinoa Willd. After 4 days under normal glasshouse conditions, isolates were transferred from this intermediate host to Nicotiana clevelandii, systemically infected leaves of which served as a stock inoculum of the virus. Use of C. quinoa as an intermediate host avoided problems due to the presence of virus inhibitors in C. amaranticolor sap.

Controlled environment. Type SH (Sherer-Gillett, Model CEL 25-7) controlled-environment chambers, in which the air temperature was held within 1 °C of the mean, were used. Lighting by a combination of fluorescent tubes and tungsten filament lamps, at an intensity of 4300 lx, was provided for 16 h each day.
**Mutagenesis of tobacco rattle virus**

Reaction with nitrous acid. Two vol. purified virus or extracted RNA were mixed with 1 vol. of the appropriate buffer and 1 vol. 4 M-sodium nitrite, and incubated at room temperature. Samples were withdrawn at various times and diluted at least tenfold into ice-cold 0.07 M-phosphate buffer, pH 8.0. They were kept at 4 °C until the final sample had been obtained, and all were then assayed for infectivity.

Spectrophotometry. U.v. extinction spectra were measured in a Pye-Unicam SP 1800 spectrophotometer, equipped with an SP 877 electrically heated cell holder and an SP 876 temperature programme controller. Extinction readings at elevated temperatures were corrected for thermal expansion of the solvent.

**RESULTS**

Kinetics of inactivation of TRV and its RNA by nitrous acid

Fig. 1 shows the inactivation of infectivity of TRV–RNA by 1.0 M-sodium nitrate in 0.25 M-acetate buffer, pH 4.0. The concentration of HNO₃ in this mixture is 0.20 M (Dussault, Bourgault & Verly, 1970). The log of the surviving fraction was inversely proportional to the time of treatment, indicating that inactivation follows single-hit kinetics. The rate of inactivation varied somewhat from one experiment to another, but 1% survival was reached after 20 to 30 min.

The inactivation of intact virus by nitrous acid at this concentration could not be followed because the infectivity and particle structure of TRV are unstable at pH 4.0 (Harrison & Nixon, 1959). Instead, a comparison was made between the inactivation of infectivity of TRV and of TRV–RNA by 1.0 M-sodium nitrite in 0.06 M-phosphate buffer at pH 6.0,
where the concentration of HNO$_2$ is 0.0025 M (Dussault et al. 1970) (Fig. 2). The inactivation of TRV–RNA under these conditions was much slower than at pH 4.0, as expected from the much lower concentration of active reagent. However, intact TRV was inactivated much more rapidly than extracted TRV–RNA under the same conditions, although the rate was still very slow compared with that of TRV–RNA at pH 4.0. This inactivation of virus appears to be specific to nitrous acid, because, when sodium chloride was substituted for sodium nitrite, 62% of the infectivity survived after 48 h under otherwise identical conditions.

A possible explanation for the more rapid inactivation of intact virus is that nitrous acid could be reacting with the coat protein in such a way as to prevent uncoating. To test this, RNA was extracted by the pronase method from samples of nitrous acid-treated virus and its infectivity assessed (Fig. 2). It is clear that the inactivation rate of RNA within virus particles is similar to that of the virus itself rather than that of isolated RNA. Thus it seems that the inactivation of intact virus involves a reaction between nitrous acid and RNA.

**Ultraviolet spectrometry of TRV–RNA**

Another possible reason for the difference in inactivation rates between TRV–RNA and intact TRV could be secondary structure of the free RNA in solution. Intramolecular hydrogen bonding could make amino groups unavailable for reaction with nitrous acid. In the intact virus particle, RNA is held in an extended configuration where this type of interaction is prevented (Offord, 1966). To test whether there is indeed extensive secondary structure in TRV–RNA in solution, its hyperchromicity was measured in 0.1 M-phosphate buffer, pH 7.0. For a typical RNA preparation, the $E_{258}$ was 0.532 at 27 °C and 0.679 at 91 °C, an increase of 28%. Taking the increase for completely base-paired polynucleotide
Table I. Lesion formation by CAM and CAM/DF strains and by ts mutants in Chenopodium amaranticolor at 20 °C and 30 °C

<table>
<thead>
<tr>
<th>Relative dilution . . .</th>
<th>20 °C</th>
<th>30 °C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1:1</td>
<td>1:10</td>
</tr>
<tr>
<td>CAM</td>
<td>*</td>
<td>543†</td>
</tr>
<tr>
<td>CAM/DF</td>
<td>*</td>
<td>534</td>
</tr>
<tr>
<td>N8</td>
<td>*</td>
<td>1181</td>
</tr>
<tr>
<td>N10</td>
<td>*</td>
<td>804</td>
</tr>
<tr>
<td>S36</td>
<td>*</td>
<td>737</td>
</tr>
</tbody>
</table>

* Too many lesions to count. † Total lesions in six leaves.

as 50 % (Doty et al. 1959), these measurements suggest that 56 % of the bases of TRV-RNA are involved in hydrogen bonding under these conditions.

Choice of conditions for mutagenesis of TRV

In the light of the inactivation reactions described above, the conditions chosen for mutagenesis of TRV were as follows: intact purified virus was treated with 1.0 M-sodium nitrite in 0.06 M-phosphate buffer, pH 6.0, for 40 h at room temperature.

Selection of temperature-sensitive (ts) mutants from nitrous acid-treated TRV

The effect of temperatures of 20 and 30 °C on lesion formation in C. amaranticolor by the CAM and CAM/DF strains of TRV is shown in Table I. Although the CAM strain produced almost as many lesions at 30 as at 20 °C, the CAM/DF strain was incapable of forming lesions at 30 °C. Even massive inocula of CAM/DF, sufficient to produce confluent necrotic spots at 20 °C, gave rise to no lesions at 30 °C. Clearly, many lesions picked from plants inoculated with nitrous acid-treated complete virus would be of the NM (defective) type (Frost et al. 1967), due to the high dilution of the inoculum, and could be selected as putative temperature-sensitive mutants. To avoid this complication, purified untreated short particles (final concentration 17 μg/ml) were added to inoculum containing nitrous acid-treated virus. Taking the mol. wt. of the CAM short particle as 12 × 10^6 (Harrison, 1970), this is equivalent to 8.5 × 10^11 short particles/ml, a concentration which according to Frost et al. (1967) is sufficient to ensure that all lesions are V-type (contain whole virus).

About 7 days after inoculation single lesions were picked as described in Methods. Extracts of these lesions were inoculated to two Chenopodium amaranticolor plants, which were kept in controlled-environment cabinets, one at 20 °C and the other at 30 °C. Isolates which produced at least 50 lesions/leaf at 20 °C and less than 5 lesions/leaf at 30 °C were tested further.

Isolates tentatively identified as ts mutants by this preliminary test were transferred from inoculated leaves of the Chenopodium amaranticolor at 20 °C to C. quinoa, and then to Nicotiana clevelandii as described in Methods. The mutant character of each isolate was confirmed by testing the infectivity of serial dilutions of infective N. clevelandii sap on two matched sets of C. amaranticolor, one kept at 20 °C and the other at 30 °C. Isolates, where the dilutions giving equal numbers of lesions at the two temperatures were less than one tenfold step apart, were rejected. At the same time the infectivity of the same dilution series was tested after freezing and thawing. Any remaining defective strains, whose infectivity was destroyed by this treatment, were also eliminated.
Table 2. Scheme of selection procedure for ts mutants

Purified virus in 0.06 M phosphate buffer pH 6.0 + 1.0 M-nitrite, incubated 40 h at room temperature.
Mixture diluted. Excess untreated short particles added.
Inoculated to C. amaranticolour.
Single lesions picked. Inoculated to C. amaranticolour at 20 and 30 °C.
Suspected ts mutants propagated in C. quinoa and then in N. clevelandii.
Infected sap assayed at 20 °C, 30 °C and after freezing and thawing.
ts mutants clonally purified.

The ts mutants were clonally purified by two successive single lesion isolations from C. amaranticolour, as described in Methods. The final scheme for selection of ts mutants is summarized in Table 2.

Mutant character of selected isolates

Of 131 single lesions from nitrous acid-treated virus tested, three proved to contain temperature-sensitive mutants. These are designated N 8, N 10 and S 36. Their mutant character is defined by their relative inability to form lesions in Chenopodium amaranticolour at 30 °C. Table 1 shows comparative lesion numbers at 20 and 30 °C for these three mutants at various dilutions, together with those for untreated TRV (CAM strain). Using the figures for the 1:10 dilution of sap, lesion production at 30 °C relative to that at 20 °C was 49 % for wild-type, 0 % for N 8, 3 % for N 10 and 8 % for S 36.

DISCUSSION

Sehgal & Krause (1968) found that in 1.0 M-sodium nitrite, pH 4.0, isolated TMV–RNA was inactivated about six times as rapidly as intact virus, and 99 % of TMV–RNA infectivity was inactivated in about 30 min. Fig. 1 shows that under the same conditions TRV–RNA also loses 99 % of its infectivity in a similar time. Increase of the pH to 6.0 (Fig. 2) with a consequent 80-fold reduction in concentration of the active species HNO₂, leads to survival of 30 % of RNA infectivity after 48 h. This represents a decrease in rate of inactivation of about 400 times, suggesting that some factor other than the concentration of HNO₂ could be influencing the rate of the inactivation reaction.

Under the same conditions, intact virus is inactivated about seven times as rapidly as isolated RNA. Two explanations for this surprising behaviour may be suggested.

(i) Nitrous acid may react with virus coat protein in such a way as to render intact virus non-infective. Evidence against this possibility comes from experiments in which it was shown (Fig. 2) that RNA extracted from nitrous acid-treated virus by the pronase method was inactivated to a similar extent to the virus from which it was obtained. However, nitrous acid treatment may give rise to changes in the coat protein or protein-RNA interactions such that pronase cannot liberate intact RNA from its coat.

(ii) The conformation of free RNA in solution may be such that amino groups are protected from reaction with nitrous acid, e.g. by hydrogen-bond interactions in base pairing. It has been shown (Doty et al. 1959) that such interactions can retard the reaction of TMV–RNA with formaldehyde. Such interactions would tend to be stabilized by the high ionic strength of the nitrous acid reaction mixture. However, hyperchromicity measure-
ments suggest that only a moderate proportion of bases in TRV–RNA are involved in base pairing. Moreover it does not explain why TRV is so different from TMV in the relative rates of inactivation of RNA and whole virus.

Sehgal & Krause (1968) also confirmed the prediction of Schuster & Wilhelm (1963) that at the same level of survival more mutants were induced in intact TMV than in TMV–RNA. No attempt was made to test this for TRV, but this possibility, together with the relative ease of handling of intact virus, led us to treat intact virus with nitrous acid to produce mutants.

The capacity of the long particle of TRV for independent replication, giving rise to defective strains, caused considerable confusion in preliminary attempts to isolate ts mutants, because these defective isolates are themselves temperature-sensitive in their ability to produce lesions on Chenopodium amaranticolor (Table 1). This phenomenon could merely be caused by the rate of degradation of uncoated virus RNA in plant tissue at 30 °C exceeding the rate of synthesis. A second possibility is that a protein coded for by the short-particle RNA contributes temperature stability to some function involved in virus replication. Another alternative is that the short particle may exert some form of control. For example, the distribution of RNA molecules between replication and translation could depend upon the total number of pieces of virus RNA in the infected cell, and in the absence of short RNA at 30 °C such a balance of functions may break down. Further investigation of this phenomenon is in progress.

To avoid selecting a high proportion of defective strains, an excess of untreated short particles was added to the nitrous acid reaction mixtures. This would be expected to have the additional effect of swamping any mutations induced on the short particles by nitrous acid. It is therefore predicted that the mutants found will prove to be on the long particle, and by implication that they are not coat protein mutants.

Three mutants were obtained, showing the feasibility of producing temperature-sensitive mutants of TRV. The properties of these mutants will be described in a subsequent paper. Physiological studies, and possibly complementation experiments with these mutants, should throw some light on the replicative process and genetic structure of TRV.

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REFERENCES


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