2-Thiouracil Does Not Inhibit TMV Replication in Tobacco Protoplasts

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SUMMARY

2-Thiouracil (2TU) did not inhibit either the incorporation of $^{32}$P into tobacco mosaic virus (TMV) RNA or virus multiplication in tobacco protoplasts. However, the specific infectivity of TMV extracted from 2TU-treated protoplasts was 10 to 20% of that of the virus extracted from untreated protoplasts. Using a fluorescent antibody technique we have demonstrated that up to 60 to 70% fewer cells became infected in 2TU-treated as compared to untreated half leaves. Therefore, 2TU may express its inhibitory effect in leaves by producing defective TMV particles which fail to spread the infection from cell to cell rather than by causing direct inhibition of virus multiplication.

2-Thiouracil (2TU) is a nucleic acid base analogue known to inhibit virus multiplication (Commoner & Mercer, 1951; Bawden & Kassanis, 1954). 2TU has been shown to be incorporated into tobacco mosaic virus (TMV) RNA (Matthews, 1955; Francki & Matthews, 1959, 1962; Ralph et al., 1965), and a large proportion of virus particles synthesized in the presence of 2TU have been demonstrated to be non-infectious (Francki & Matthews, 1959, 1962; Francki, 1960). In addition to the inhibition of viral RNA synthesis found by Meyer et al. (1977), the total amount of extractable virus was about 50% less in leaves treated with 2TU than in untreated leaves (Francki, 1962).

Most of the studies on the mode of action of 2TU have been done with leaves. Using this system the lack of synchronous virus infection is a great disadvantage. Protoplasts permit simultaneous infection of the majority of the cell population by viruses (Zaitlin & Beachy, 1974; Farkas, 1976; Takebe, 1977) and would seem to be a good system to study the direct effect of 2TU on virus replication.

Nicotiana tabacum cv. Xanthi plants were grown in a light- and temperature-controlled greenhouse at 25 °C (day and night), 10000 lux light intensity and 16 h daily illumination. Purified TMV-OM strain (Nozu & Okada, 1968) was used in all of the experiments. Infectivity assays were performed on N. tabacum cv. Xanthi nc. plants using the half leaf method (see Otsuki et al., 1972).

Protoplasts were isolated by a modified two-step procedure of Mühlbach et al. (1977), washed three times with 0.7 M-mannitol and suspended in the TMV infection mixture (Kubo et al., 1974) containing 0.025 M-phosphate buffer pH 6.7, 0.7 M-mannitol, 1 μg/ml TMV and 1 μg/ml poly-L-ornithine. After 10 min incubation, protoplast samples were washed twice, divided and incubated with and without 10 μg/ml 2TU in the 'TMV incubation medium' described by Aoki & Takebe (1969). Protoplast samples were incubated in a temperature-controlled chamber at 27 °C and illuminated with fluorescent lamps at a light intensity of 500 lux. Six h pulse labelling was carried out from 18 h after protoplast infection by adding carrier-free $^{32}$P (potassium orthophosphate) to the incubation medium at a concentration of 1.85 mBq/ml. Each experiment was carried out using $2 \times 10^7$ protoplasts in 20 ml incubation medium.

Nucleic acids were extracted, separated by electrophoresis in polyacrylamide gels and radioactivity was measured as described by Föglein et al. (1975).
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Fig. 1. Gel electrophoresis of $^{32}$P-labelled TMV RNA and ribosomal RNAs extracted from (a) untreated and (b) 2TU-treated protoplasts.

Table 1. **Effect of 2TU on the amount of virus produced and its specific infectivity in protoplasts treated or untreated with 2TU**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Extracted virus (µg) from 2 × 10⁷ protoplasts</th>
<th>Specific* infectivity</th>
<th>Loss of infectivity in 2TU (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−2TU</td>
<td>166</td>
<td>373</td>
<td>89.8</td>
</tr>
<tr>
<td>+2TU</td>
<td>170</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>No. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−2TU</td>
<td>148</td>
<td>596</td>
<td>76.2</td>
</tr>
<tr>
<td>+2TU</td>
<td>140</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−2TU</td>
<td>151</td>
<td>462</td>
<td>88.4</td>
</tr>
<tr>
<td>+2TU</td>
<td>162</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

* Lesion no/0.01 A unit TMV in 20 µl 0.01 M-phosphate buffer.

Fluorescent antibody staining technique was used to determine the number of infected cells (Otsuki & Takebe, 1969). Only those experiments in which 80 to 90% of protoplasts became infected were analysed.

TMV was extracted from 2 × 10⁷ infected protoplasts by homogenization in 10 ml 0.1 M-sodium pyrophosphate, pH 7.25 (Fraenkel-Conrat & Williams, 1955) and purified by precipitation with polyethylene glycol (mol. wt. 6000) (Hebert, 1963), using 0.1 M-sodium pyrophosphate pH 7.25. Virus concentration was determined by spectrophotometry ($A_{260} = 2.7$ for 1 mg TMV/ml).

Electron microscopic counting of virus particles was carried out using the spray drop technique described by Backus & Williams (1950). Polystyrene latex spheres (0.3 µm diam.) were used as a reference for the drop volume.

There was no significant difference between incorporation of $^{32}$P into TMV RNA or ribosomal RNAs extracted from 2TU-treated and untreated protoplasts (Fig. 1). No differences were detected in the amount of virions produced by protoplasts treated or untreated with 2TU (Table 1). Electron microscopic counting showed no differences in the numbers of particles in 2TU-treated and control protoplasts (data not shown). However, the specific infectivity of TMV extracted from 2TU-treated protoplasts was only 10 to 20% of that of the virus extracted from untreated ones (Table 1).

To enrich the protoplasts with 2TU before infection, detached tobacco leaves were cut in halves, one half was floated on water, the other on a solution of 2TU (10 µg/ml). After 48 h incubation, protoplasts were prepared from these half leaves; in the case of 2TU-treated half leaves, both the enzyme solution for protoplast preparation and the washing medium
contained 2TU. The protoplasts were then infected with TMV, and each preparation was divided into two parts. One portion was incubated in the presence and the other in the absence of 2TU for an additional 24 h. At the end of this period TMV was extracted and measured. The results show that pretreatment of plant material with 2TU had no effect on TMV multiplication in any of the treatments.

To study the effect of 2TU on the number of infected cells in leaf tissue inoculated with TMV, protoplasts were isolated from TMV-infected leaves which had been cut in half and the halves were floated on either water or 2TU (10 µg/ml) (immediately after infection), for 24 h. The protoplasts were incubated for an additional day and assayed by the fluorescent antibody technique. We found that in 2TU-treated half leaves 60 to 70% fewer cells were infected than in the untreated ones (21% of the protoplasts were infected in the presence of 2TU and 58% in the absence of 2TU).

Our results therefore show that in synchronously infected protoplasts, 2TU treatment had no effect on TMV multiplication, even when applied long before inoculation. This contrasts with the inhibition of multiplication reported in 2TU-treated whole leaves (Francki, 1962; Dawson & Schlegel, 1976; Meyer et al., 1977). However, in both systems the virus produced is of reduced specific infectivity. Taken together, these results suggest that virus multiplication in primarily infected cells (in both leaves and protoplasts) is not inhibited by 2TU, but that virus multiplication in 2TU-treated leaves is inhibited because the spread of infection is inhibited. This conclusion is supported by the lower percentage of infected protoplasts found in 2TU-treated leaves.

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REFERENCES


Short communications


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