Tobacco Rattle Virus in Tobacco Mesophyll Protoplasts: Infection and Virus Multiplication

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SUMMARY

Poly-L-ornithine greatly increased infection of protoplasts by tobacco rattle virus (TRV), and had the largest effect when incubated with virus particles for at least 10 min before inoculation. Using a final concentration of 1 µg/ml TRV particles and 1 to 1.5 µg/ml poly-L-ornithine in 0.025 M-phosphate buffer, pH 6.0, to inoculate mesophyll protoplasts of tobacco cv. Xanthi by the ‘indirect’ method, up to 98% of the intact protoplasts became infected. When the protoplasts were stored overnight at 5 °C before inoculation, 95% became infected. In protoplasts kept at 22 °C after inoculation, about half the yield of infective particles was produced during the first day and almost all the remainder during the second. The final yield was about $2 \times 10^8$ long virus particles and $6 \times 10^5$ short particles per infected protoplast. Fluorescent antibody staining showed that TRV coat protein antigen accumulated throughout the cytoplasm. In electron micrographs, the long TRV particles were associated with mitochondria whereas the short particles were generally dispersed in the cytoplasm. Infective RNA was produced after inoculation with long particles but TRV coat protein antigen, and long and short TRV particles, were made only in protoplasts inoculated with both kinds of particle; infection was not detected in protoplasts inoculated with short particles alone.

INTRODUCTION

The finding of Takebe & Otsuki (1969) that tobacco mesophyll protoplasts can be reliably prepared and infected with tobacco mosaic virus (TMV) has given the plant virologist a technique for synchronous infection of the majority of plant cells in a preparation. This technique has since been applied to several other plant viruses, including tobacco rattle virus (TRV), infection with which can be considerably enhanced by substituting phosphate buffer for citrate buffer in the inoculum (Kubo, Harrison & Robinson, 1974). In this paper we describe (1) the effects of other factors on TRV infection, (2) the course of accumulation of TRV in infected protoplasts, and (3) the use of the protoplast system to verify the properties of the particles that contain the two parts of the genome of TRV (Harrison & Nixon, 1959; Lister, 1966; Frost, Harrison & Woods, 1967).

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METHODS

Preparation of protoplasts. Tobacco (Nicotiana tabacum) plants of cv. Xanthi (not Xanthi-nc) and of White Burley cv. Judy’s Pride were used as sources of protoplasts. The plants were grown in 15 cm diam. pots containing a peat-sand mixture and were kept in a glasshouse in which the temperature averaged 25 °C by day and 18 °C by night. The glasshouse was shaded in summer but at other times natural light was supplemented by illumination from a low-pressure sodium lamp.

Leaves approaching their full size were preferred as sources of protoplasts. They were usually the 8th to 10th leaves above the cotyledons of 45- to 50-day-old plants. Most batches of protoplasts were prepared from four or more leaves taken from different plants. The leaves were detached, allowed to wilt, their lower epidermis removed, and leaf pieces were exposed first to pectinase and then to cellulase preparations, essentially as described by Takebe, Otsuki & Aoki (1968) and Otsuki et al. (1974). Pectinase (0.5 % solution of Macerozyme R-10; Kinki Yakult Manufacturing Co.) treatment was in three stages of 5, 30 and 30 min at 25 °C, and cells released during the third stage were treated with cellulase (2 % solution of Cellulase Onozuka R-10; Kinki Yakult Manufacturing Co.) for 30 to 45 min at 35 °C. Before use, the protoplasts were washed three times by sedimenting for 100 s in a centrifuge set to run at 80 g and resuspending in fresh 0.7 M-mannitol solution. Such protoplasts were nearly all derived from palisade cells but a few came from spongy mesophyll cells.

Virus purification. The CAM isolate (Harrison & Woods, 1966) of TRV; R/I:2.4/5+0.7/5:E/E:S/Ne was used throughout. It was purified from systemically infected leaves of Nicotiana clevelandii as described by Cooper & Mayo (1972). Long and short particles in such preparations were separated by two cycles of sedimentation in sucrose density gradients containing phosphate buffer (0.017 M, pH 7.3) and 0.002 M-EDTA.

Inoculation and incubation of protoplasts. Unless otherwise stated, inoculum virus was thoroughly mixed with poly-L-ornithine (mol. wt. 120000; Sigma), and incubated for 10 to 20 min at 25 °C as described by Otsuki, Takebe, Honda & Matsui et al. (1972), in the presence of either citrate buffer, pH 5-2, or phosphate buffer, pH 6-0. Freshly sedimented protoplasts were either resuspended in this inoculum, the ‘direct’ method of inoculation, or resuspended in 10 ml of 0.7 M-mannitol and then immediately mixed with an equal vol. of double-strength inoculum, the ‘indirect’ method. The final concentrations in the inoculation mixture were usually: TRV 1 μg/ml, poly-L-ornithine 1 μg/ml, protoplasts 1 to 3 × 10^6/ml, and either 0.025 M-phosphate or 0.01 M-citrate. After 10 min at 25 °C, the protoplasts were freed from excess inoculum by two washes in 0.7 M-mannitol containing 0.1 mM-CaCl_2. Finally, the protoplasts were resuspended in the incubation medium of Otsuki, Shimomura & Takebe (1972), containing Mycostatin (25 units/ml) and carbenicillin (250 μg/ml), and transferred to 50 ml conical flasks (10 ml/flask). These flasks were kept in controlled environment cabinets at 22 °C and illuminated continuously at an intensity of 3000 lux.

Staining with fluorescent antibody. The globulin fraction of antiserum to TRV particles was conjugated with fluorescein isothiocyanate (FITC) as described by Otsuki & Takebe (1969) to give a molecular ratio of FITC:globulin of about 1.6. Before use, it was absorbed with the acetone-insoluble fraction of tobacco leaf tissue. For staining, protoplasts were spread on a glass slide (previously coated with Meyer’s albumin) by a rotary motion and then dried in a current of warm air. They were fixed in 90 % ethanol, stained for 1 h at 37 °C with the conjugated antiserum and washed with phosphate-buffered saline. They were then mounted in
40% glycerol and examined using a Reichert Zetopan microscope equipped for fluorescence microscopy.

The FITC-conjugated CAM antiserum did not stain uninoculated protoplasts, and staining of protoplasts inoculated 2 days previously was much decreased by pre-treating them with unconjugated anti-CAM γ-globulin. The inoculated protoplasts did not stain with FITC-conjugated TMV antiserum. The staining was therefore specific for TRV antigen.

Unless otherwise stated, the protoplasts were sampled 2 days after inoculation for staining with fluorescent antibody.

Serological assay of virus. Virus particle antigen in protoplast extracts was assayed using the latex flocculation test as described by Abu Salih, Murant & Daft (1968). Using latex treated with anti-CAM γ-globulin, and incubating the antiserum-antigen mixtures for 30 min on black glass plates, the lowest detectable concentration of virus particles was 0.5 μg/ml. The endpoint was the same using either purified virus preparations or extracts of uninoculated protoplasts to which known amounts of purified virus were added before freezing, storage, thawing and clarifying by low-speed sedimentation.

Infectivity assays. To assay infective TRV particles, samples of protoplasts were sedimented at 800 g, resuspended in 1 ml 0.017 M-phosphate buffer, pH 7.3 and disrupted in a ground-glass tissue grinder. The extracts were then stored at −20 ºC. Just before assay, they were thawed, clarified by low-speed sedimentation, diluted serially with phosphate buffer and inoculated on to carborundum-dusted leaves of Chenopodium amaranticolor, with the treatments distributed among leaves and plants using a Latin square layout.

Infective TRV-RNA was obtained from samples of protoplasts, resuspended in 1 ml 0.15 M-NaCl, 0.015 M-Na citrate, pH 7.2 (SSC), containing 0.5% SDS, by emulsifying with 1 ml water-saturated phenol containing 10% (v/v) m-cresol and 0.1% 8-hydroxyquinoline. The phases were separated by centrifuging, the phenol phase re-extracted with 1 ml SSC+SDS and the two aqueous layers combined. RNA was then precipitated by adding 5 ml ethanol and standing overnight at −20 ºC. The precipitate was washed twice with 70% ethanol, then dissolved in 2 ml 0.017 M-phosphate buffer, pH 8.0, and a series of dilutions prepared in the same buffer. Infectivity was assayed using Chenopodium amaranticolor plants as above, except that the inocula were kept in an ice bath and applied to the leaves with muslin pads, and plastic gloves were worn.

Electron microscopy. Ratios of the numbers of long and short particles in preparations were determined by photographing phosphotungstate-treated samples at ×20000 and counting particles in the electron micrographs using a binocular dissecting microscope fitted with a micrometer eyepiece.

Protoplasts were fixed, embedded, sectioned and stained essentially as described by Jones, Kinninmonth & Roberts (1973) for tissue fragments.

RESULTS
Factors affecting infection

Poly-L-ornithine

Very little infection occurred when poly-L-ornithine was omitted from the inoculum (Table 1). The result was the same using the direct or indirect methods of inoculation, and with citrate or phosphate buffer in the inoculum. The optimum concentration of poly-L-ornithine for infection in these experiments was 1 to 2 μg/ml; 3 μg/ml often damaged the protoplasts and 2 μg/ml did so in some experiments. In most experiments in which other factors were varied, therefore, poly-L-ornithine was used at 1 μg/ml. It is worth noting that
Table 1. *Effect of poly-L-ornithine on infection of protoplasts by TRV*

<table>
<thead>
<tr>
<th>Expt.*</th>
<th>Inoculation method</th>
<th>Buffer</th>
<th>Poly-L-ornithine (µg/ml)</th>
<th>% of intact fluorescing</th>
<th>Protoplasts†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (Judy’s Pride)</td>
<td>Direct</td>
<td>Citrate, pH 5.2</td>
<td>0</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>87</td>
<td>13</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>2</td>
<td>86</td>
<td>13</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>3</td>
<td>74</td>
<td>4</td>
</tr>
<tr>
<td>2. (Xanthi)</td>
<td>Direct</td>
<td>Citrate, pH 5.2</td>
<td>0.5</td>
<td>89</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>84</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>76</td>
<td>23</td>
</tr>
<tr>
<td>3. (Xanthi)</td>
<td>Indirect</td>
<td>Phosphate, pH 6.0</td>
<td>0</td>
<td>92</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>91</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.75</td>
<td>91</td>
<td>90</td>
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<td></td>
<td></td>
<td>1</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>91</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

* Tobacco cultivar in parentheses.
† Final virus concentration in inoculum was 4 µg/ml (Expt. 1 and 2) or 1 µg/ml (Expt. 3).
‡ Sampled 26 h (Expt. 1) or 48 h (Expt. 2 and 3) after inoculation.

A few protoplasts became infected in some experiments (Expt. 3, Table 1) when poly-L-ornithine was omitted from the inoculum.

The time for which poly-L-ornithine and TRV were incubated together before inoculation was also important. In Fig. 1 are shown the results of an experiment in which this time was varied from 20 s to 30 min, inoculation was by the indirect method and the inoculum contained 1 µg/ml TRV, 1 µg/ml poly-L-ornithine and phosphate buffer. The percentage of antibody-stained protoplasts increased greatly with increase of incubation period from 20 s to 10 min, and slightly with a further increase from 10 to 30 min. In other experiments an incubation period of 10 to 20 min was used.

**Inoculation procedure**

Motoyoshi, Watts & Bancroft (1974) reported that the direct method of inoculation gives a greater percentage of infection of tobacco mesophyll protoplasts by cowpea chlorotic mottle virus than the indirect method. In our experiments with TRV, however, there was little difference. In three experiments with inoculum containing citrate buffer, the average percentages of infection were 35 for the indirect method and 30 for the direct method. Also, in four tests with inocula containing phosphate and 0.2 to 4 µg/ml TRV particles, the average percentages were 87 and 85 for the indirect and direct methods, respectively.

In a further experiment, protoplasts were prepared, and inoculated either immediately or after 20 h storage in 0.7 M-mannitol at 5 °C. Inoculation was by the indirect method using inoculum containing 1 µg/ml TRV, poly-L-ornithine and phosphate buffer. Inoculation of the fresh protoplasts gave 98% infection, and that of the stored protoplasts gave 95%. This is an important result because it shows that protoplasts retain their susceptibility to infection by TRV for considerable periods, and is a useful point to note when designing experiments.
**TRV in tobacco mesophyll protoplasts**

Fig. 1. Effect of time of pre-inoculation incubation of tobacco rattle virus and poly-L-ornithine on infection of protoplasts. Inoculum contained virus and poly-L-ornithine each at final concentrations of 1 μg/ml, 0.025 M-phosphate buffer, pH 6 and 0.7 M-mannitol.

Table 2. *Comparative susceptibility to TRV infection of protoplasts of two tobacco cultivars*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Inoculation conditions</th>
<th>Cultivar</th>
<th>Virus (μg/ml)</th>
<th>Poly-L-ornithine (μg/ml)</th>
<th>% of intact protoplasts fluorescing*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Direct; citrate, pH 5.2</td>
<td>Judy’s Pride</td>
<td>4</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthi</td>
<td>4</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>Indirect; phosphate, pH 6.0</td>
<td>Judy’s Pride</td>
<td>0.04</td>
<td>1</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthi</td>
<td>0.2</td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>1</td>
<td>69</td>
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<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td>1</td>
<td>94</td>
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<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>96</td>
</tr>
</tbody>
</table>

* Sampled 48 h after inoculation.
Fig. 2. Multiplication of tobacco rattle virus in protoplasts. ●—●, infectivity of buffer extracts in arbitrary units; ○—○, percentage of intact protoplasts staining with fluorescent antibody. The inoculum mixture contained virus at a final concentration of 2 μg/ml, poly-L-ornithine at 1 μg/ml, and 0.01 M-citrate buffer.

**Tobacco cultivar**

In early experiments, White Burley tobacco plants derived from seed originally obtained from Rothamsted Experimental Station (cv. Judy's Pride) and the John Innes Institute, respectively, were compared as sources of protoplasts. The plants from the two seed lots looked different but their protoplasts did not differ in susceptibility to infection. In further tests the Rothamsted strain of White Burley tobacco was compared with the cultivar Xanthi (Table 2). The Xanthi protoplasts were slightly but consistently the more susceptible to infection. However, more than half the protoplasts of each sort became infected when the inoculum contained as little as 0.04 μg/ml TRV in the presence of phosphate. Xanthi protoplasts were also somewhat less delicate and so more resistant to damage during manipulation and incubation.

**TRV multiplication in protoplasts**

The increase in infectivity of protoplast extracts with increasing time after inoculation is shown in Fig. 2. Nearly half of the infective particles were produced in the first day after inoculation and the remainder during the second day. There seemed to be some asynchrony of infection because the percentage of antibody-stained protoplasts increased over a somewhat extended period. About half the final number of fluorescing protoplasts was stained one day after inoculation and there was perhaps a slight increase even between 2 and 3 days after inoculation. At all times the fluorescence was cytoplasmic. During the first day it was weaker than later, and after two days took the form of numerous small fluorescent spots dispersed through the cytoplasm, with the chloroplasts represented by non-fluorescent blobs (Fig. 3). Electron micrographs of sections of protoplasts sampled 2 to 3 days after inoculation confirmed the occurrence of many TRV particles in the cytoplasm (Fig. 4). However, the long and short particles were quite differently distributed within the cytoplasm, as they
are in systemically infected leaf tissue of *Nicotiana clevelandii* (Harrison & Roberts, 1968). The long particles were associated end-on with mitochondria, whereas the short particles were generally distributed through the cytoplasm. Virtually all the mitochondria in a protoplast had many long TRV particles associated with them in this manner, and are probably the fluorescent spots seen after staining with conjugated antibody.

In the experiment providing the data for Fig. 2 there was a small initial decrease in infectivity. However, this is an artefact, because in other experiments in which the protoplasts were carefully washed after inoculation, very little or no infectivity was detected 1 h after inoculation and infectivity subsequently increased by 2000-fold or more. Other experiments also showed that the first detectable increase in infectivity at 22 °C occurred between 7 and 9 h after inoculation, and that a very few protoplasts became stainable by antibody during the same period.

The virus yield of infected protoplasts was estimated in two ways: (1) by comparing the infectivity of protoplast extracts with that of known concentrations of purified TRV, and (2) serologically, using the latex flocculation test. The estimates derived from infectivity assays were two to fivefold greater than those made serologically. This discrepancy may derive from the tacit assumption that the specific infectivity of the virus in protoplast extracts is the same as that of purified TRV, when in fact the purified virus is likely to have a lower specific infectivity. For this reason the serological estimate is considered more accurate, and using this and the particle ratio determined by electron microscopy, the calculated yield was nearly $10^6$ TRV particles per infected protoplast at 2 days after inoculation (Table 3).

*Interaction of long and short particles in TRV replication*

Earlier work using inoculated leaves has shown that long TRV particles produce lesions containing infective RNA, that short particles do not induce any detectable infection, and
Fig. 4. Electron micrograph of a section of a protoplast 3 days after inoculation with tobacco rattle virus. Several short virus particles are indicated by arrows; the long particles are associated with mitochondria.
Table 3. Yield of long (L) and short (S) TRV particles per infected protoplast

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Protoplasts fluorescing %</th>
<th>Virus per 10⁶ infected protoplasts (μg) *</th>
<th>Ratio of particle numbers (S/L)</th>
<th>Particles per infected protoplast (x 10⁻⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>15.7</td>
<td>1.6</td>
<td>2.3 (S) + 1.4 (L)</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>31.4</td>
<td>2.8</td>
<td>6.5 (S) + 2.3 (L)</td>
</tr>
</tbody>
</table>

* Estimated serologically using the latex flocculation test, and assuming 82% infection of both samples.

Table 4. Virus nucleoprotein and RNA yields of protoplasts inoculated with long and short TRV particles, separately and together

<table>
<thead>
<tr>
<th>Particles in inoculum</th>
<th>Assay dilution</th>
<th>Long* (2 μg/ml)</th>
<th>Short* (2 μg/ml) + short (2 μg/ml)</th>
<th>Long (2 μg/ml) + short (20 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenopoecium amaranticolor leaves</td>
<td>1/1</td>
<td>174†</td>
<td>0</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>1/5</td>
<td>33</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Tobacco protoplasts†</td>
<td>(24 h)</td>
<td>0</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>(48 h)</td>
<td>0</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Virus nucleoprotein yield</td>
<td>(24 h)</td>
<td>1/2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/10</td>
<td>1</td>
<td>0</td>
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<tr>
<td></td>
<td>(48 h)</td>
<td>1/2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Virus RNA yield</td>
<td>(24 h)</td>
<td>1/1</td>
<td>168</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/5</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(48 h)</td>
<td>1/1</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/5</td>
<td>29</td>
<td>0</td>
</tr>
</tbody>
</table>

* Electron microscope examination showed that the inoculum of long particles contained some short particles (9.5% by number). The preparation of short particles contained < 0.05% of long particles.
† Figures are total lesions in 8 half leaves (nucleoprotein assay) or whole leaves (RNA assay) of Chenopoecium amaranticolor. Figures for virus nucleoprotein yields and RNA yields, or for yields at different times after inoculation, are not strictly comparable.
‡ Judy’s Pride tobacco protoplasts were inoculated by the direct method with pH 5.2 citrate in the inoculum.

that both long and short particles are needed to produce infections in which both kinds of particles are made (Harrison & Nixon, 1959; Lister, 1966; Frost et al. 1967). Such experiments deal with the behaviour of TRV in cells into which the virus spreads from the cells that are infected at inoculation; the initially infected cells in leaves are too few for infectivity assays to measure the events in these cells. This limitation does not apply to the tobacco protoplast system, and experiments were therefore made to examine the production of infective TRV particles, infective RNA and TRV particle antigen in cells infected at inoculation.
The results of such a test (Table 4) indicate that infective TRV particles (buffer extracts) are produced only in the protoplasts inoculated with long plus short particles, but that infective RNA (phenol extracts) accumulates also in protoplasts inoculated with long particles only. The few infective particles produced in the protoplasts inoculated with the preparation of long particles are presumed to result from the observed contamination of the long-particle preparation with some short particles. No infective particles or RNA were produced in protoplasts inoculated with short particles alone. The assays made either 1 day or 2 days after inoculation lead to the same general conclusion that TRV behaves in the same way in inoculated protoplasts and in secondarily infected leaf cells.

The results in Table 4 also indicate that virus antigen is produced only when infective TRV particles are made, and that only a small proportion of protoplasts was infected by long plus short particles in this experiment. Further tests showed that a good deal of infectivity was lost during fractionation of the long and short particles used in the inocula. However, when the percentage infection of protoplasts was increased by increasing the particle concentrations in the inocula, TRV particles and antigen were produced in considerable amounts in protoplasts inoculated with the preparation of long particles alone, presumably because the short-particle contamination in the long-particle preparation had become increasingly important. Further cycles of density gradient sedimentation failed to diminish this short-particle contamination to less than 2% by weight.

DISCUSSION

We have already shown that infection of protoplasts by TRV is increased greatly by adding phosphate buffer to the inoculum (Kubo et al. 1974) and in this paper we describe the effects of some other factors. The requirement for poly-L-ornithine fits the rule established for other viruses with isoelectric points below the inoculum pH, and its lesser importance for viruses with higher isoelectric points (Motoyoshi & Hull, 1974). The conditions required to give a large percentage of protoplasts infected with TRV resemble those for potato virus X (Otsuki et al. 1974) more closely than those for other viruses. Both TRV and potato virus X give at least as good infection at pH 6.0 as at pH 5.2, both show a slight response to lengthening the pre-inoculation incubation of virus+poly-L-ornithine beyond 10 min and, using citrate in the inoculum, the optimum concentration of each virus is at least 5 μg/ml. Perhaps some at least of these similarities relate to the unusually small electrophoretic mobilities of particles of these two viruses near pH 6.0 (Bawden & Kleczkowski, 1959; Harrison & Nixon, 1959).

On the basis of our results, we recommend the following conditions for obtaining a large percentage of infection of protoplasts with TRV: Xanthi tobacco leaves as a source of protoplasts; virus pre-incubated with poly-L-ornithine for 20 min; and inoculated by the indirect method using inoculum containing both TRV and poly-L-ornithine at final concentrations of 1 μg/ml, and 0.025 M-phosphate buffer, pH 6.0. This combination usually results in 90% infection or more, and it would be interesting to know whether similar percentages would be obtained with potato virus X in similar conditions.

In our experiments on the time course of accumulation of TRV, the infectivity of extracts and the percentage of fluorescing protoplasts seemed to increase more gradually than with TMV or cowpea chlorotic mottle virus (Takebe & Otsuki, 1969; Motoyoshi et al. 1973). In part this probably reflects the lower incubation temperature (22 °C) used in our experiments than that (25 or 28 °C) used in work with the other viruses. However, it may also reflect a special property of TRV, namely that the characteristic long particles can initiate
infections in which no virus particle antigen is produced, and possibly some of these infections are converted to antigen producers by involvement of the RNA from the short particles at a later stage of infection. The time at which the short particles become involved in virus replication might differ from protoplast to protoplast.

The pattern of distribution of virus antigen in TRV-infected protoplasts differs to a greater or lesser extent from that indicated by the fluorescence shown by antibody-stained protoplasts infected with TMV, or with cowpea chlorotic mottle, cucumber mosaic or pea enation mosaic viruses (Takebe & Otsuki, 1969; Motoyoshi et al. 1973; Otsuki & Takebe, 1973; Motoyoshi & Hull, 1974). Also, at the ultrastructural level, TRV infections differ from all the others, TRV long particles being associated with mitochondria whereas the short particles are generally dispersed in the cytoplasm. This is the same distribution as is found in TRV-infected cells in intact leaves (Harrison & Roberts, 1968) but protoplasts should provide much the superior system for further investigation of this phenomenon.

Our estimates of virus yield per infected protoplast based on antigen assay are probably correct to within a factor of two, and are considered more accurate than those based on infectivity. This is largely because the specific infectivity of any virus sample used as a standard in the assays may differ from that of virus in the protoplast extracts. The same is probably also true for most other plant viruses.

The particle-mixing experiments led to the same general conclusions about the roles of long and short particles in TRV infections as came from experiments using intact leaves. Thus protoplasts should be a very useful system for studying these roles in greater depth. However, the experiments also emphasize the greater need to have contamination-free samples of the two kinds of particle in work using protoplasts. In part this may reflect the much greater proportion of protoplasts that can be infected than of leaf cells, but another factor is probably introduced by the use of poly-L-ornithine in inocula for protoplasts. This material may aggregate TRV particles in inocula and so increase the chance that short particles will enter the cells along with the long particles, even when the short particles constitute only a small proportion of virus in the inoculum.

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