The Infection of Tobacco Protoplasts with Cowpea Chlorotic Mottle Virus and its RNA

By F. MOTOYOSHI, J. B. BANCROFT, J. W. WATTS AND J. BURGESS

John Innes Institute, Colney Lane, Norwich, NOR 70F, U.K.

(Accepted 12 March 1973)

SUMMARY

Tobacco protoplasts were infected with cowpea chlorotic mottle virus and its RNA in the presence of poly-L-ornithine to yield $10^8$ to $10^9$ particles per infected protoplast representing a probable increase in virus concentration of at least 1000-fold by 24 to 72 h after inoculation. The optimum inoculum input for virus was about 0.5 µg/ml and for RNA about 1.2 µg/ml; about 60 and 7%, respectively, of the protoplasts became infected, as judged by fluorescent antibody staining. Data relating to growth curves, inoculum and poly-L-ornithine concentrations, and pH effects are presented along with electron micrographs of sectioned protoplasts.

INTRODUCTION

Tobacco protoplasts have been infected with tobacco mosaic virus (TMV) (Takebe & Otsuki, 1969; Coutts, Cocking & Kassanis, 1972) and TMV-RNA (Aoki & Takebe, 1969). Preliminary reports indicate that cucumber mosaic virus (Otsuki & Takebe, 1971) and potato virus X (Otsuki & Takebe, 1972) may also be used successfully and F. Motoyoshi & R. Hull (unpublished observations) have recently had positive results with pea mottle mosaic virus. Cowpea chlorotic mottle virus (CCMV) multiplies in the inoculated leaves of tobacco but does not become systemic. We describe the infection of tobacco protoplasts with CCMV and CCMV-RNA and consider some conditions required for maximum infection.

METHODS

The preparation of good plant cell protoplasts requires particular attention to detail. Thus, although procedures have been published by Takebe et al. (1968), Takebe & Otsuki (1969), Aoki & Takebe (1969) as well as by Coutts et al. (1972), we describe our procedures, which differ in detail from those of previous workers.

Tobacco leaves. Fully expanded leaves (about 30 cm long) of about 40 to 60-day-old Nicotiana tabacum var. White Burley were used for protoplast preparation. Plants were grown in a 1:1 mixture of John Innes No. 2 Compost and peat compost in 6-inch-pots in a glasshouse at 22 °C. They were given a supplementary high-nitrogen feed (Fisons No. 5P) weekly. Natural light was used in the summer, but in winter supplementary light was provided by GEC Solarcolor sodium lamps for 15 h per day (10000 to 20000 lux at bench level).

Detached leaves were washed with distilled water and were dried in a sterile-air cabinet until a partial loss of turgor resulted, facilitating epidermal peeling. The lower epidermis
was peeled off with sharp forceps and the stripped leaves were cut into pieces about 4 cm² which were immersed in 0.7 M-d-mannitol to avoid desiccation before the start of maceration.

Maceration. The maceration medium consisted of 1% (w/v) Macerozyme (Macerozyme ME43, Kinki Yakult Co., Nishinomiya, Japan), 0.7 M-mannitol, and 0.5% potassium dextran sulphate (sulphur content 17.8%, mol. wt. ~ 2800, Meito Sangyo Co., Nagoya, Japan), the pH being adjusted to 5.8 with N HCl. The stripped pieces (about 6 g) from 1 leaf were immersed in 20 ml of the maceration medium in a 250 ml Erlenmeyer flask, infiltrated for 2 min in a vacuum desiccator by means of an electric vacuum pump, and then shaken for 10 min in a reciprocal shaker (3 cm stroke) in a water bath at 25 °C at a frequency of 120 excursions/min. After shaking, the maceration medium containing cell debris was discarded and was replaced by 50 ml of fresh medium. After shaking for a further 20 to 30 min, the medium, which contained mostly spongy mesophyll cells, was discarded and replaced by 80 ml fresh medium. The preparation was then shaken for an additional 1.5 to 2 h and contained mostly palisade cells.

Cellulase treatment. The palisade cells were separated from the undigested upper epidermis by filtration through a nylon mesh, collected by centrifuging (130 g, 1 min) in 40 ml round-bottomed centrifuge tubes and washed once in 0.7 M-mannitol followed by centrifuging. The pelleted cells were suspended in 100 ml of 2% (w/v) Cellulase Onozuka (CLA 679, Kinki Yakult Co.) and 0.7 M-mannitol at pH 5.2 in a 100 ml flask. The suspension was shaken for 1.5 to 2 h at 35 °C at a frequency of 80 excursions/min.

Washing and collection of protoplasts. The protoplast suspension in the cellulase solution was filtered through gauze (cheesecloth) and centrifuged (50 g, 2 min) in 40 ml round-bottomed centrifuge tubes. The protoplast pellet was suspended in 0.7 M-mannitol and washed 1 to 3 times. The protoplasts were then suspended in 0.7 M-mannitol to a concentration of 3 to 10 x 10⁵/ml (determined in a haemocytometer) and the suspension was divided into 10 ml portions in 15 ml centrifuge tubes.

Infection of protoplasts. A CCMV solution at 20 to 40 mg/ml, purified according to Bancroft et al. (1972) in 0.1 M-acetate buffer, pH 4.7, was diluted to 5 μg/ml (unless a concentration series was being run) in 0.02 M-potassium citrate buffer, pH 5.2, containing 0.7 M-mannitol (CBM). If the effect of pH was being studied, the pH was adjusted accordingly. Poly-L-ornithine (mol. wt. ~ 120000, Pilot Chemical Co., Boston, Mass.) was made to 10 μg/ml (unless a concentration series was being run) in CBM. 2 ml of the poly-L-ornithine solution was diluted to 8 ml with CBM in a 100 ml centrifuge tube, and then 2 ml of the 5 μg/ml CCMV solution was added to the poly-L-ornithine solution. This 10 ml CCMV-poly-L-ornithine mixture was shaken at 80 excursions per min at 25 °C for 10 min. A protoplast pellet which had been prepared in one 15 ml centrifuge tube was suspended in 10 ml of 0.7 M-mannitol, added to 10 ml of the incubated CCMV-poly-L-ornithine mixture and shaken at 25 °C for 10 min. Final concentrations were 0.5 μg/ml CCMV, 1 μg/ml poly-L-ornithine, 0.01 M-CBM and 1.5 to 5.0 x 10⁵ protoplasts/ml.

If brome mosaic virus (BMV) or tobacco mosaic virus (TMV) were used as inocula, the above procedure was followed at final virus concentrations of 0.5 μg/ml and 1 μg/ml, respectively.

CCMV-RNA, whose infectivity was completely abolished with snake venom phosphodiesterase, was prepared from CCMV by phenol extraction as described (Bancroft et al. 1968) to a concentration of 1 to 3 mg/ml in water and was diluted to 12 μg/ml with CBM, unless a dosage response experiment was to be done. Two ml poly-L-ornithine (10 μg/ml) was diluted to 8 ml with CBM in a 100 ml centrifuge tube and then 2 ml of the RNA solution was added to the poly-L-ornithine solution. The RNA-poly-L-ornithine mixture was shaken
at 25 °C for 2 min on the same shaker as used for the virus. Incubation of the RNA-poly-L-ornithine mixture with the protoplasts was carried out as for the virus. The final concentration of RNA was 1.2 µg/ml.

**Culture of inoculated protoplasts.** The inoculated protoplasts were centrifuged (50 g, 2 min) and the pellet was washed 3 times by centrifuging in autoclaved 0.7 M-mannitol containing 10^{-4} M-CaCl_2. The washed protoplasts were transferred aseptically into 10 ml of solution (Takebe et al. 1968) from which 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid (Aoki & Takebe, 1969) were omitted and in which the concentration of mannitol was 0.7 M. They were cultured in 50 ml Erlenmeyer flasks stoppered with aluminum foil at 25 °C under continuous fluorescent light (3000 lux) for the desired period of up to 72 h. The numbers of protoplasts for each 10 ml treatment or time interval were counted haemocytometrically. Two ml portions of the 10 ml samples were removed for fluorescent antibody staining. The protoplasts in the remaining 8 ml (in most cases) were concentrated by centrifuging (50 g, 2 min) and washed once by centrifuging in 0.7 M-mannitol. The pellet was suspended to 1 ml in 0.1 M-acetate buffer pH 5.0 (water was used for TMV) and homogenized in a ground glass homogenizing tube by means of a MSE homogenizer for 2 min at about 1 °C. All protoplasts were broken as judged by light microscopy.

**Fluorescent antibody production.** This procedure was based on that of Otsuki & Takebe (1969). The immunoglobin G fraction was isolated by (NH_4)_2SO_4 precipitation from 27 ml of anti-CCMV rabbit serum with a ring-test titre of 1/1000 to 1/2000. 27 ml of phosphate buffered saline (PBS, 0.01 M-phosphate buffer, pH 7.0-8.5 % NaCl) was added to the serum. 54 ml of saturated (NH_4)_2SO_4 was added dropwise and the mixture stirred for 30 min. The precipitate was collected by centrifuging at 8000 g, for 10 min, dissolved in 50 ml PBS, reprecipitated by 33 % saturated (NH_4)_2SO_4, collected, dissolved in 25 ml PBS, reprecipitated as before, dissolved in about 2 ml PBS and dialysed for 24 h against PBS. The final vol. was 2.1 ml containing about 3.8 % protein as measured spectrophotometrically. The ring precipitin titre was 1/2560.

The globulin (1 ml) was diluted to 3.8 ml (1 % protein) with 0.02 M-Na_2CO_3-Na HCO_3 buffer, pH 9.8 and dialysed against 0.01 % (w/v) fluorescein isothiocyanate (FITC) in the same buffer for 24 h. The FITC-conjugated protein was separated from uncoupled dye by passage through a 1.7 × 14 cm Sephadex G-25 column. The conjugated protein was recovered in 1.8 ml solution with a ring precipitin titre of 1/200. The FITC/protein molar ratio was 1.9:1 as calculated from optical densities at 280 nm and 495 nm.

In order to minimize nonspecific staining, the conjugated globulin was treated with acetone-extracted powder of pea leaves. 12 g of leaves yielded about 1 g of dry powder collected on a Büchner funnel. 100 mg of powder was washed 3 times with 40 ml portions of PBS and collected (1200 g, 7 min). A 1.7 ml portion of the conjugated globulin was diluted 4-fold with PBS and added to 67 mg of the pea powder. The mixture was shaken at 37 °C for 20 min on a shaker, and then centrifuged (1200 g, 7 min). The supernatant fraction was transferred to 33 mg of pea powder and the procedure was repeated. The supernatant fraction, which was able to stain CCMV-antigen in tobacco protoplasts up to a dilution of 1/32, was stored at −20 °C.

The FITC-BMV conjugated-globulin was prepared as above. The FITC-TMV conjugated-globulin was kindly supplied by Dr I. Takebe, Institute for Plant Virus Research, Chiba, Japan.

**Protoplast staining.** A 2 ml portion from the 10 ml sample of cultured protoplasts was diluted to 10 ml with 0.7 M-mannitol and collected by centrifuging (50 g, 2 min). The pellet was resuspended in 0.25 to 0.5 ml of 0.7 M-mannitol and one drop of the thick suspension
was placed on the surface of a glass slide coated with Mayer's albumin. The suspension was
dried in warm air and the protoplasts were then fixed in 95% ethanol at room temperature
for 10 min. The specimen was washed for 30 min in 0.01 M-PBS, pH 7, with stirring, blotted,
and the protoplasts were covered by a drop of the conjugated globulin solution. The slide
was incubated overnight at 4 °C in a moist chamber, then washed for 1 h in PBS with
stirring. The stained protoplasts were then mounted with PBS-glycerol (9 parts 0.01 M-PBS
+ 1 part glycerol).

A Vicker's fluorescent microscope (M41 Photoplan) equipped with a HBO 200 mercury
vapour lamp, with exciter filter BG12 and a barrier filter OG4 + GG9 was used to observe
the stained specimens. Usually, 500 protoplasts were scored for staining at 200 × with
transmitted light fluorescence. Photographs were taken at 500 × with incident light
fluorescence, using Kodak Tri-pan X film, with 4 min exposures.

The specificity of the CCMV fluorescent antibodies was confirmed by the fact that they
stained neither non-infected nor TMV-infected protoplasts, nor did they stain specimens
first treated overnight at 4 °C with unconjugated anti-CCMV globulin. Further, fluorescent
antibodies previously cross-absorbed by CCMV did not stain infected specimens. The
TMV fluorescent antibodies did not stain CCMV-infected protoplasts.

Virus assay. Infectivity assays were made on Chenopodium hybridum L. Each sample,
usually at a dilution of from a half to one-sixth, was assayed on a total of at least eight half-
leaves on two plants directly against freshly purified virus inocula on opposite half-leaves
of a concentration (from 1 to 4 μg/ml) adjusted so that there was not a great disparity in
lesion numbers. This method gave figures which were consistently in the same order of
magnitude as seen in the Results section.

The number of particles per protoplast, quoted in the Results section, were based on a
mol. wt. of 4.6 × 10^6 for CCMV (Bancroft et al. 1968).

Electron microscopy. Samples for electron microscopy were fixed in glutaraldehyde, post-
fixed in osmic acid, dehydrated in an ethanol series and embedded in Araldite, as described
by Burgess et al. (1973b). Specimens were taken after 10 min, 30 min and 1, 24 and 48 h
incubation periods.

RESULTS

Growth curves

The results from CCMV growth curve experiments in tobacco protoplasts infected with
virus at 0.5 μg/ml or RNA at 1-2 μg/ml both in the presence of 1 μg/ml poly-L-ornithine
are given in Table I and Fig. 1. Virus was detected by infectivity tests 9 to 18 h after infec-
tion, depending on the experiment, if with a virus inoculum and by 18 to 24 h with a RNA
inoculum. The difference in time of virus detection resulted from the relatively low number
of protoplasts infected with the RNA inoculum. Regardless of the type of inoculum, yields
approximating to 10^6 particles per protoplast were obtained 24 h after infection; by 48 to
72 h, the yields reached 10^7 particles per protoplast. Fig. 2 shows the appearance of a
protoplast stained with fluorescent antibody 24 h after infection with RNA. Fluorescence
associated with virus antigen is detected in the cytoplasmic area in contrast to the chloro-
plasts which remain dark. These data show that the virus multiplied, reaching levels esti-
mated by the likeliest method (footnote, Table 1) of at least 1000 fold over the initial
protoplast-associated inoculum level. Protoplast disintegration also occurred (Table 1,
Experiment 4), particularly by 72 h after infection as shown by virus left in the supernatant
after protoplast sedimentation.
Table 1. Growth curves of cowpea chlorotic mottle virus in tobacco protoplasts infected with virus or RNA

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Time after infection (h)</th>
<th>Inoculum*</th>
<th>Virus yield from protoplasts (µg)</th>
<th>Virus yield from supernatant (µg)</th>
<th>% infected protoplasts†</th>
<th>No. of input particles (× 10^-6)</th>
<th>Virus particles per infected protoplast§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Virus</td>
<td>0</td>
<td>—</td>
<td>12.6</td>
<td>—</td>
<td>0 (3.9 × 10^6)</td>
</tr>
</tbody>
</table>
| 6        | 12                       | Virus      | 0.3                             | —                               | 12.2                   | 6.4                             | 0.1
| 24       | 18                       | Virus      | 14.5                            | —                               | 19.0                   | 4.4                             | 5.7
| 2        | 1                        | Virus      | 0                               | —                               | 5.9                    | —                               | 0 (1.1 × 10^6) |
| 9        | 18                       | Virus      | 0.7                             | —                               | 3.8                    | —                               | 0.1
| 24       | 12                       | Virus      | 7.4                             | —                               | 4.1                    | —                               | 0.55
| 3        | 1                        | Virus      | 0                               | —                               | 0.4                    | 3.0                             | 1.1
| 9        | 18                       | Virus      | 0.52                            | —                               | 15.0                   | 2.9                             | 0.2
| 18       | 12                       | Virus      | 4.3                             | —                               | 25.0                   | 2.8                             | 1.0
| 24       | 1                        | RNA        | 0                               | —                               | 2.9                    | —                               | 0 (7.2 × 10^9) |
| 9        | 18                       | RNA        | 0.04                            | —                               | 3.4                    | —                               | 0.4
| 24       | 1                        | RNA        | 1.4                             | —                               | 7                      | 3.3                             | 1.0
| 4        | 1                        | Virus      | 0                               | 0                               | 2.4                    | —                               | 0 (1.8 × 10^9) |
| 9        | 18                       | Virus      | 0.6                             | 28                               | 2.1                    | —                               | 5.8
| 24       | 48                       | Virus      | 63.9                            | 53                               | 4.2                    | —                               | 10.8
| 72       | 1                        | Virus      | 87.5                            | 28.9                             | 4.9                    | 1.9                             | 15.2
| 5        | 1                        | Virus      | 0                               | 0                               | 2.8                    | —                               | 0 (2.1 × 10^9) |
| 9        | 18                       | Virus      | 5.8                             | 43                               | 2.9                    | 1.0                             | 0.7
| 24       | 48                       | Virus      | 27                              | 48                               | 1.9                    | 1.0                             | 4.5
| 72       | 1                        | Virus      | 38                              | 61                               | 1.3                    | 0.8                             | 7.8
| 1        | 1                        | RNA        | 0                               | 0                               | 3.5                    | —                               | 0 (9.6 × 10^6) |
| 9        | 18                       | RNA        | 0                               | 0                               | 3.7                    | —                               | 0
| 24       | 48                       | RNA        | 1.6                             | 2.8                              | 2.3                    | 147                             | 3.6
| 72       | 7.6                      | RNA        | 13.0                            | 1.3                              | 32                     | 7.5                             |

* Virus at 0.5 µg/ml, RNA at 1.2 µg/ml. Both with 1 µg/ml poly-L-ornithine: pH 5.2.
† As scored from fluorescent antibody staining.
‡ 4 ml used for experiment 1; 8 ml for the remainder.
§ The number of particles initially added (6.5 × 10^6 virus/ml and 6 × 10^11 RNA/ml) divided by the initial number of protoplasts/ml that eventually became infected. For example, the virus inoculum in Expt 5 at 24 h
\[
\frac{6.5 \times 10^6}{0.48 \times 1.4 \times 10^5} = 9.7 \times 10^4
\]
particles per protoplast. The 2.8 × 10^6 protoplasts/ml is divided by 2 to give 1.4 × 10^3 because an equal vol. of virus is added to the protoplasts before centrifuging. Implicit in this calculation is the unlikely assumption that all added inoculum becomes attached to protoplasts.

|| We cannot detect less than about 0.01 µg/ml (13 × 10^6 particles) CCMV on 8 half-leaves of Chenopodium hybridum. Assuming that every protoplast that eventually stained was actually infected at 0 h, or some other time at which infectivity could not be detected, then the upper limit of the number of virus particles per protoplast can be estimated. For example in Expt 1, 19% of 4 ml × 4.4 × 10^4 protoplasts contained less than 13 × 10^6 virus particles per ml because we can detect more than that. Therefore, 1 protoplast would contain no more than 3.9 × 10^3 particles. The values for the other experiments are given in brackets.
Virus dosage

Table 2 shows the response of protoplasts to various different concentrations of the CCMV inoculum in the presence of 1 μg poly-l-ornithine/ml. The maximum virus yield and percentage stained protoplasts occurred at about 0.5 μg virus/ml. The variation in the number of virus particles per protoplast in this table and subsequent ones results from accumulated errors in estimating virus yield, percentage infected protoplasts and total number of protoplasts.

The effect of poly-l-ornithine on infection and on the virus

Poly-l-ornithine is necessary for the efficient infection of tobacco protoplasts with CCMV (Table 3). Greatest infection efficiency occurred at levels from 1 to 3 μg/ml as measured by infectivity tests and from 2 to 3 μg/ml as measured by staining.

Poly-l-ornithine precipitates CCMV at poly-l-ornithine:virus ratios (w/w) of approximately 2. This was similar to the ratio used during inoculation (Table 4). The pattern of the precipitation response is not unlike that found in a serological reaction. Presumably, the attraction is electrostatic between the amino groups of the homopolymer and the carboxyl groups of the virus both of which would be strongly and oppositely charged at pH 5.2. The precipitates have a high degree of order as seen in the electron microscope (Fig. 3).
CCMV infected protoplasts

Fig. 2. Micrograph of fluorescent antibody stained tobacco protoplast infected with CCMV-RNA and cultured for 24 h.

Table 2. The response of tobacco protoplasts to different amounts of cowpea chlorotic mottle virus

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Virus dosage (µg/ml)</th>
<th>Virus yield from protoplasts (µg)*</th>
<th>% infected protoplasts†</th>
<th>No. of protoplasts/ml (× 10⁶)‡</th>
<th>Virus particles yielded per infected protoplast (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>7.7</td>
<td>17</td>
<td>1.2</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>8.1</td>
<td>42</td>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>9.5</td>
<td>42</td>
<td>1.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>11.0</td>
<td>63</td>
<td>1.1</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
<td>0.2</td>
<td>0</td>
<td>1.6</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>1.4</td>
<td>2</td>
<td>2.2</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>26</td>
<td>2.3</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.9</td>
<td>16</td>
<td>2.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* After 24 h infection.
† As scored by fluorescent antibody staining.
‡ 8 ml used.

The effect of pH

The virus infects the protoplasts equally well from pH 4.7 to 5.7 (Table 5). Since the specific infectivity of the virus is not diminished at pH 4.2, nor appreciably at pH 6.2 and at pH 6.7 is still 40% of that found at pH 4.2 to 5.7, the effect is probably on uptake of the virus since the protoplasts withstand these pH levels.
Table 3. The effect of poly-L-ornithine on the infection of tobacco protoplasts with cowpea chlorotic mottle virus

<table>
<thead>
<tr>
<th>Poly-L-ornithine (µg/ml)</th>
<th>Virus yield from protoplasts (µg)*</th>
<th>% infected protoplasts†</th>
<th>No. of protoplasts/ml (× 10⁶)‡</th>
<th>Virus particles yielded per infected protoplast (× 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.38</td>
<td>0</td>
<td>2.2</td>
<td>—</td>
</tr>
<tr>
<td>0.25</td>
<td>1.7</td>
<td>14</td>
<td>2.0</td>
<td>10.4</td>
</tr>
<tr>
<td>0.5</td>
<td>21.3</td>
<td>29</td>
<td>1.9</td>
<td>6.3</td>
</tr>
<tr>
<td>1</td>
<td>36.9</td>
<td>46</td>
<td>1.5</td>
<td>8.7</td>
</tr>
<tr>
<td>2</td>
<td>31.9</td>
<td>80</td>
<td>1.7</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>38.0</td>
<td>78</td>
<td>2.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* After 24 h infection. Virus inoculum at 0.5 µg/ml.
† As scored by fluorescent antibody staining.
‡ 8 ml used.

Table 4. The effect of poly-L-ornithine on CCMV

<table>
<thead>
<tr>
<th>Ratio poly-L-ornithine:CCMV</th>
<th>Turbidity at 480 mμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>0.101</td>
</tr>
<tr>
<td>0.2:1</td>
<td>4.790</td>
</tr>
<tr>
<td>2:1</td>
<td>9.010</td>
</tr>
<tr>
<td>20:1</td>
<td>0.398</td>
</tr>
</tbody>
</table>

* The virus was at 2 mg/ml in 0.01 M-citrate buffer, pH 5.2, and was incubated at 25 °C for 10 min.

RNA dosage and the effects of poly-L-ornithine

The RNA dosage experiments are shown in Table 6. Clearly, the best infection efficiencies occur at levels from 0.12 to 1.2 µg/ml. The best poly-L-ornithine concentrations with 1.2 µg RNA/ml are between 1 and 2 µg/ml (Table 7). The levels of infectivity with RNA are generally but not always low (compare Expt 2, Table 6 with Table 7 in which the same RNA inoculum was used) for reasons that are not yet clear.

The effect of poly-L-ornithine on the solubility of the RNA is shown in Table 8. The level at which the polypeptide is used with RNA inoculum is, unlike the case with the virus, insufficient to cause much precipitation.

Brome mosaic virus and tobacco mosaic virus

Brome mosaic virus does not multiply in tobacco plants nor does it multiply in the same protoplast preparation which supports the multiplication of both tobacco mosaic virus and CCMV (Table 9).

Electron microscopy

Specimens fixed within the first hour of incubation following exposure to the virus showed surface damage and peripheral vesiculation thought to be due to the presence of poly-L-ornithine in the virus inoculum (Burgess, Motoyoshi & Fleming, 1973a). At this stage, virus particles were only seen at the surface of the plasmalemma or at points of damage to the plasmalemma membrane (Fig. 4).

After 24 h incubation, large numbers of virus particles, which appeared as deeply stained rings, 25 nm in diameter (Hills & Plaskitt, 1968), and which were not seen in control
**CCMV infected protoplasts**

Fig. 3. Crystals of a 2:1 (w/w) poly-L-ornithine-CCMV mixture stained with 2% uranyl acetate, pH 4.1 (picture by G. J. Hills).

Fig. 4. Section through the surface of a tobacco mesophyll protoplast fixed 30 min after infection with CCMV. Virus particles are present within membrane-bound spaces and on the protoplast surface at a point of damage to the plasmalemma.
Table 5. The effect of pH on the infection of tobacco protoplasts with cowpea chlorotic mottle virus*

<table>
<thead>
<tr>
<th>pH</th>
<th>Virus particles (μg)†</th>
<th>% infected protoplasts‡</th>
<th>No. of protoplasts/ml (× 10^-9)§</th>
<th>Virus particles yielded per infected protoplast (× 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>11.7</td>
<td>39</td>
<td>2.7</td>
<td>1.8</td>
</tr>
<tr>
<td>4.7</td>
<td>28.4</td>
<td>68</td>
<td>3.3</td>
<td>2.0</td>
</tr>
<tr>
<td>5.2</td>
<td>29.0</td>
<td>67</td>
<td>3.7</td>
<td>1.9</td>
</tr>
<tr>
<td>5.7</td>
<td>33.4</td>
<td>52</td>
<td>4.2</td>
<td>2.5</td>
</tr>
<tr>
<td>6.2</td>
<td>15.4</td>
<td>14</td>
<td>4.5</td>
<td>4.0</td>
</tr>
<tr>
<td>6.7</td>
<td>0.7</td>
<td>0</td>
<td>3.1</td>
<td>?</td>
</tr>
</tbody>
</table>

* The specific infectivity of CCMV at pH 6.2 and 6.7 is 90% and 40%, respectively, of that at pH 4.2 to 5.7 (Bancroft et al. 1968).
† After 24 h infection. Virus inoculum at 0.5 μg/ml.
‡ As scored by fluorescent antibody staining.
§ 8 ml used.

Table 6. The response of tobacco protoplasts to different amounts of cowpea chlorotic mottle virus RNA

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>RNA dosage (μg/ml)</th>
<th>Virus yield from protoplasts (μg)*</th>
<th>% infected protoplasts‡</th>
<th>No. of protoplasts/ml (× 10^-9)§</th>
<th>Virus particles yielded per infected protoplast (× 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12</td>
<td>--</td>
<td>2.0</td>
<td>2.1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>--</td>
<td>7.0</td>
<td>2.9</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>--</td>
<td>0.8</td>
<td>4.8</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>--</td>
<td>0</td>
<td>3.1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>90.0</td>
<td>--</td>
<td>0</td>
<td>4.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.5 (virus)</td>
<td>--</td>
<td>22</td>
<td>3.1</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>2.6</td>
<td>2.0</td>
<td>1.4</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>2.0</td>
<td>7.0</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0.65</td>
<td>0.4</td>
<td>1.7</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>0.60</td>
<td>0</td>
<td>1.7</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>90.0</td>
<td>0.65</td>
<td>0</td>
<td>1.7</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.5 (virus)</td>
<td>1.3</td>
<td>65</td>
<td>1.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* After 24 h infection.
† As scored by fluorescent antibody staining.
‡ 8 ml used.

Table 7. The effect of poly-L-ornithine on the infection of tobacco protoplasts with cowpea chlorotic mottle virus RNA

<table>
<thead>
<tr>
<th>Poly-L-ornithine (μg/ml)</th>
<th>Inoculum</th>
<th>Virus yield from protoplasts (μg)†</th>
<th>% infected protoplasts‡</th>
<th>No. of protoplasts/ml (× 10^-9)§</th>
<th>Virus particles yielded per infected protoplast (× 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>RNA *</td>
<td>0.1</td>
<td>5</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>0.5</td>
<td>RNA *</td>
<td>2.0</td>
<td>9</td>
<td>1.1</td>
<td>6.6</td>
</tr>
<tr>
<td>1</td>
<td>RNA *</td>
<td>3.8</td>
<td>31</td>
<td>1.1</td>
<td>3.6</td>
</tr>
<tr>
<td>2</td>
<td>RNA *</td>
<td>3.6</td>
<td>29</td>
<td>0.78</td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td>RNA *</td>
<td>1.7</td>
<td>19</td>
<td>0.46</td>
<td>6.3</td>
</tr>
<tr>
<td>4</td>
<td>Virus †</td>
<td>6.7</td>
<td>73</td>
<td>0.93</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* 1.2 μg/ml.
† 0.5 μg/ml.
‡ After 24 h infection.
§ As scored by fluorescent antibody staining.
‖ 4 ml used.
Table 8. The effect of poly-L-ornithine on CCMV-RNA

<table>
<thead>
<tr>
<th>Ratio poly-L-ornithine:RNA</th>
<th>% RNA left in supernatant fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 *</td>
<td>100</td>
</tr>
<tr>
<td>0:1:1</td>
<td>100</td>
</tr>
<tr>
<td>1:1</td>
<td>79</td>
</tr>
<tr>
<td>2:1</td>
<td>56</td>
</tr>
<tr>
<td>5:1</td>
<td>15</td>
</tr>
<tr>
<td>8:1</td>
<td>8</td>
</tr>
</tbody>
</table>

* The RNA was used at 54 µg/ml in 0.01 M-citrate pH 5.2 and left standing at 1 °C for 2 h before centrifuging for 15 min at 5000 rev/min.

Table 9. Comparative behaviour of tobacco mosaic, brome mosaic and cowpea chlorotic mottle viruses in tobacco leaf protoplasts

<table>
<thead>
<tr>
<th>Time after infection (h)</th>
<th>Inoculum</th>
<th>% lesions</th>
<th>Virus yield (µg)</th>
<th>% infected protoplasts;</th>
<th>No. of protoplasts/ml (x 10^-6)§</th>
<th>Virus particles yielded per infected protoplast (x 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCMV*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>CCMV*</td>
<td>100</td>
<td>30</td>
<td>45</td>
<td>1.6</td>
<td>6.6</td>
</tr>
<tr>
<td>1</td>
<td>BMV *</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>BMV *</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>TMV †</td>
<td>0.7</td>
<td>4</td>
<td>0</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>TMV †</td>
<td>100</td>
<td>516</td>
<td>40</td>
<td>2.6</td>
<td>9.3</td>
</tr>
</tbody>
</table>

* 0.5 µg/ml + 1 µg/ml poly-L-ornithine.
† 1.0 µg/ml + 1 µg/ml poly-L-ornithine.
‡ As scored by fluorescent antibody staining.
§ 8 ml used.

specimens, were visible in the cytoplasm of infected protoplasts and within small vacuolar areas where they sometimes appeared to associate into chains (Fig. 5). The membrane periphery of these small vacuoles was frequently fragmented. The virus was not seen in crystalline form, and it was not associated with the nucleus, mitochondria or plastids. A specific feature of infected protoplasts was the appearance within the cytoplasm of regions containing a specialized type of endoplasmic reticulum (Fig. 6). The lumen of the reticulum membrane contained amorphous electron dense material and was unusually distended. In its most distended parts it also contained small vesicles (Fig. 7). The endoplasmic reticulum in infected protoplasts also formed enclosed rings of membrane (Fig. 8). It seemed likely that these rings might give rise to the small virus-containing vacuoles, since at least a proportion of these were delineated by a distended double membrane (Fig. 5).

The only consistent changes in the appearance of infected protoplasts after 48 h incubation were an increase in the number of virus particles present in the cytoplasm (Fig. 9) and the occurrence of particles within the large central vacuole. No signs of degeneration of the cytoplasm were detected at this time.

Amongst the uni-nucleate protoplasts in any preparation there always appeared a few which possessed several nuclei, presumably due to spontaneous fusion during preparation (Withers & Cocking, 1972). These became infected and showed all the characteristics of infection described above (Fig. 10).
Fig. 5. Part of the cytoplasm of an infected tobacco protoplast fixed after 24 h incubation. Virus particles are distributed randomly throughout the cytoplasm and also appear within small vacuoles.

Fig. 6. A local region of an unusual type of endoplasmic reticulum within an infected tobacco protoplast after 24 h incubation. The lumen of the membrane is distended and contains electron dense material.
Fig. 7. Similar to Fig. 6. Vesicles appear within the lumen of the membrane at its most extended parts.

Fig. 8. A group of enclosed rings of endoplasmic reticulum membrane in an infected tobacco protoplast after 24 h incubation. Virus particles are clearly visible within the cytoplasm.
Fig. 9. Section through the peripheral cytoplasm of an infected tobacco protoplast after 48 h incubation. Almost every small particle in the cytoplasm at this stage is a virus particle.

Fig. 10. A multinucleate infected protoplast fixed 48 h after exposure to CCMV. Several regions of specialized endoplasmic reticulum are visible at this magnification (arrows).
DISCUSSION

There are four types of evidence which indicate that CCMV multiplies in tobacco protoplasts. The infectivity data suggest that about \(10^9\) times more virus particles are recovered from protoplasts than are initially absorbed by them. These data have the disadvantage that the initial absorption level of the virus is merely an estimate as will be discussed. The fluorescent staining antibody results show that there can be an increase from 0 up to 80% in the number of infected protoplasts in the best case. Levels of about 60% staining are common. Electron microscopic observations show cell sections almost devoid of virus particles the first hour after inoculation in comparison to cell sections showing large numbers of particles 24 h after infection. Further, in preliminary labelling experiments, we have found that virus purified in optical amounts (206 \(\mu\)g) from infected protoplasts incorporated \(^{35S}\) up to about \(7.5 \times 10^6\) ct/min/mg. The virus yield in this experiment was \(5.6 \times 10^6\) particles per infected protoplast after 72 h infection which is in reasonable agreement with the yields estimated by infectivity measurements. The purified virus showed the usual three density components in CsCl (Bancroft & Flack, 1972).

The efficiency of infection by virus in the protoplast system is much higher than in tobacco plants since we were unable to infect intact tobacco plants with \(0.5 \mu\)g/ml of virus in the presence or absence of poly-L-ornithine. The infection efficiency is also higher in protoplasts than in Chenopodium hybridum since it takes more than about \(1 \times 10^6\) particles to infect the local lesion host whereas high infection levels occurred in protoplasts at an input dosage of from 3 to \(5 \times 10^5\) particles per protoplast. The latter levels are probably at least 100 times higher than the numbers of particles which are actually initially absorbed by the protoplasts. The true initial absorption level is difficult to estimate directly because, unlike the situation with TMV (Table 9, Takebe & Otsuki, 1969), no infectivity can be detected at zero time with CCMV even if the samples are undiluted. With TMV, we estimate that about \(7 \times 10^4\) particles are absorbed by each protoplast that becomes infected. This is higher than the figure of Takebe & Otsuki (1969) and Takebe, Otsuki & Aoki (1971) who found that approximately 100 particles were absorbed per protoplast. These estimates agree with the \(10\) to \(10^2\) particles per protoplast obtained by indirect methods involving electron microscopy (Hibi & Yora, 1972). The best way to get the upper limit of absorption would be by the use of highly radioactive virus. In any event, even if the least favourable and most unlikely assumption is made that all input CCMV is protoplast associated, there is still about a 15-fold increase in virus concentration by 72 h after infection and the actual increase is probably \(10\) to \(100\) times more than that as judged by the least detectable infectivity levels which themselves are probably upper limits. The levels of virus reached are in the order of those found for TMV. Brome mosaic virus does not multiply detectably in intact tobacco under our conditions nor in protoplasts isolated from tobacco suggesting, but not proving, that the inability of the virus to multiply is not because it does not enter the cells.

The principal difference between the infection results with CCMV and TMV is that only about \(1.2 \mu\)g/ml of CCMV-RNA is required for optimal infection whereas \(0.5\) to \(1.5\) mg/ml of TMV-RNA is apparently necessary to reach about the same percentage of stained protoplasts (Aoki & Takebe, 1969). The difference in dosage level may be related to the fact that the specific infectivity of TMV-RNA is only about 0.1 to 5% that of encapsidated RNA on intact plants, depending on assay conditions (Sarkar, 1963), while that of CCMV-RNA is about 50% of the RNA in the virus (Bancroft et al. 1968). The relatively low plating efficiency of the physically tripartite (Bancroft & Flack, 1972) CCMV on plants as compared to that of TMV which is about \(10^2\) to \(10^3\) times more efficient under normal assay
conditions does not account for this difference since both viruses have about the same apparent infection efficiency on tobacco protoplasts. Apparently, TMV-RNA is intrinsically less efficient than CCMV-RNA for reasons that are not clear, but which may be related to the relatively large size of the TMV-RNA, even though three unique pieces of CCMV-RNA are required for infection. Once infection has occurred with CCMV-RNA, complete virus particles are made just as they are with TMV-RNA (Aoki & Takebe, 1969).

Poly-L-ornithine (at about 1 μg/ml) is required for infection by both CCMV and its RNA. Since under normal concentrations the virus is precipitated by the polycation whereas the RNA is not, the effect of poly-L-ornithine is probably on the protoplast rather than on the conditions or type of inoculum used. That is, 1 μg/ml of poly-L-ornithine should be about optimal in causing just the right amount of protoplast injury for any type of inoculum.

CCMV does not become systemic in tobacco but is confined to the inoculated leaf on which it incites very indistinct primary chlorotic lesions which gradually enlarge and coalesce. The virus yield is about 4.6 × 10⁶ particles per cell 15 days after inoculation which is about the level in the protoplasts. It is not known why the virus is limited to the inoculated leaf but since no necrotic reaction is visible, the limitation is presumably different from that controlled by the N gene in tobacco for TMV the effects of which are not apparent in tobacco protoplasts (Otsuki, Shimomura & Takebe, 1972a).

Ultrastructural aspects of virus infection of protoplasts have until now been studied using TMV. Early work with tomato fruit protoplasts failed to show any detailed structural characteristics of infection although it was claimed that the virus entered the host by a pinocytotic process (Cocking, 1966, Cocking & Pojnar, 1969), a phenomenon that has yet to be convincingly demonstrated in higher plants (Burgess et al. 1973a). More recently, Hibi & Yora (1972) and Otsuki et al. (1972b) have examined TMV infection of tobacco mesophyll protoplasts by electron microscopy. These authors do not note any changes, such as described for CCMV, within the host in response to infection apart from the appearance of newly synthesized virus. The results with CCMV in tobacco protoplasts generally agree with those reported by Hills & Plaskitt (1968) in cells from intact cowpea leaves infected for about 3 weeks except that in the latter case, virus was found in the nucleus. This may result from the long infection period since no nuclear-associated virus is found in lesion tissue from Chenopodium hybridum infected for 4 days (G. J. Hills & K. A. Plaskitt, unpublished data).

The infection of tobacco protoplasts is a reliable process when care is taken in protoplast preparation. We have run 27 consecutive experiments with CCMV in all of which at least the control inoculum has infected the protoplasts. We have also scaled the procedure up to prepare usable amounts of the pure virus. The CCMV-protoplast system is at least as efficient as that for TMV and in addition to providing useful comparative data with a spherical virus, is the best available so far for studies on the biochemistry of viruses with divided genomes.

We wish to thank Dr I. Takebe and Mr Y. Otsuki for their kind help and advice and Denise Allen, Elizabeth Fleming and Janet King for technical assistance.

REFERENCES
CCMV infected protoplasts


(Received 14 February 1973)