Factors Influencing the Infection of Cowpea Mesophyll Protoplasts by Alfalfa Mosaic Virus

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SUMMARY

Judged by the yield of infective virus, good infection of cowpea mesophyll protoplasts with alfalfa mosaic virus is obtained when protoplasts are resuspended at 0°C in 0.5 M-mannitol, 0.01 M-potassium phosphate, pH 5.6, containing 1 µg/ml poly-L-ornithine and about 2/zg/ml virus. At 25°C, virus infectivity is first detectable 12 h after inoculation and increases exponentially in the next 12 h. Forty hours after inoculation about 1 to 5 × 10⁶ virus particles are produced per living protoplast. As with intact leaves, a mixture of bottom component, middle component and top component b nucleoprotein is required for the infection of protoplasts.

Virus multiplication is not sensitive to chloramphenicol (200 µg/ml) but is completely inhibited by cycloheximide (10 µg/ml) applied at any point in the growth cycle. Actinomycin D (10 µg/ml) interferes with an early step in virus replication.

INTRODUCTION

Alfalfa mosaic virus (AMV) is a virus with a tripartite RNA genome. The genome fragments, called bottom (B)–, middle (M)–, and top component b (Tb)–RNA, are separately encapsidated in bacilliform particles of different lengths. In addition, virus preparations contain a fourth class of particle, top component a (Ta), containing 2 RNA molecules of identical size. To initiate infection, a mixture of the 3 genome fragments has to be supplemented with a small amount of coat protein, or its messenger, Ta–RNA (Bol, van Vloten-Doting & Jaspars, 1971). Coat protein is present in polyribosomes extracted from infected leaves (Bol, Bakhuizen & Rutgers, 1976) and in preparations of the soluble virus replicase (C. M. Clerx-van Haaster and J. F. Bol, unpublished results). Thus it is conceivable that the coat protein has a regulatory function in the translation and/or replication of virus RNA.

To permit a more detailed investigation of the role of the coat protein in the infection process, we initiated a study of AMV replication in cowpea mesophyll protoplasts. Preference for this system was given because of its practical advantages and the fact that a preliminary experiment, done in cooperation with Dr T. Hibi, had shown that cowpea protoplasts could be successfully infected with AMV (Hibi, Rezelman & Van Kammen, 1975). Here we report the requirements for satisfactory infection of cowpea protoplasts and the sensitivity to antibiotics of virus replication. The conditions for efficient inoculation are somewhat different from those reported by Motoyoshi, Hull & Flack (1975) for the infection of tobacco mesophyll protoplasts by AMV.
METHODS

Preparation of protoplasts. *Vigna unguiculata* (L.) Walp cv. Blackeye Early Ramshorn seeds were germinated for 2 days in moistened vermiculite and the seedlings were cultured for 8 days in a growth chamber (Weiss Klimaprüfschrank) as described by Hibi *et al.* (1975). The lower epidermis of primary leaves was peeled off with forceps and 5 half leaves were floated, peeled side down, in Petri dishes (15 cm diam.) each containing 40 ml of a solution of 0.025% Macerozyme R-10 (Kinki Yakult Manuf. Co.) and 0.5% Cellulase Onozuka R-10 (Kinki Yakult Manuf. Co.) in 0.5 M-D-mannitol, pH 5.5. After a 3.5 h incubation at 25°C, the dishes were gently swirled and the released protoplasts passed through a 150 mesh stainless steel filter. The protoplasts were collected by centrifugation of the filtrate at 600 g for 2 min and washed three times by resuspending them in 0.5 M-mannitol and sedimenting at 600 g for 2 min. Haemocytometer counts showed an average yield of 4 × 10^6 protoplasts/g leaf, and the percentage of living protoplasts as determined by fluorescein diacetate staining (Widholm, 1972) was over 90%.

Inoculation of protoplasts. Unless otherwise stated the following standard procedure was used: 5 × 10^6 protoplasts were sedimented from the 0.5 M-mannitol solution and resuspended in 1 ml 0.5 M-mannitol containing 1 μg/ml poly-L-ornithine (New England Nuclear Co., mol. wt. approx. 150000), 2 μg/ml virus and 0.01 M-potassium phosphate, pH 5.6, which had been pre-incubated at 0°C for 10 min. The suspension was kept at 0°C for 15 min; thereafter the protoplasts were washed by three cycles of centrifugation at 600 g for 2 min and resuspending in sterile 0.5 M-mannitol to remove the non-adsorbed virus. In preliminary experiments the washing solution contained 10 mM-CaCl₂, but this was omitted because it induced clumping of the protoplasts. After the washing procedure, virus was no longer detectable by local lesion assay.

Incubation of inoculated protoplasts. In the initial experiments the protoplasts were incubated essentially as described by Aoki & Takebe (1969) in a medium containing 0.5 M-mannitol, 1.0 mM-KNO₃, 0.1 mM-MgSO₄, 0.1 mM-CaCl₂, 1.0 μM-KI, 0.01 μM-CuSO₄, and 0.2 mM-KH₂PO₄, pH 5.4. As mentioned in the text, a simplified medium was used in later experiments, containing 0.5 M-mannitol and 10 mM-CaCl₂. In all experiments the medium contained either 5 μg/ml tetracycline or 200 μg/ml chloramphenicol to suppress bacterial growth. Portions of 10 ml containing 5 × 10^6 protoplasts were incubated in 100 ml Erlenmeyer flasks at 25°C under continuous illumination (about 2500 lux). Unless mentioned otherwise, the incubation was for 40 h.

Infectivity assay. Protoplasts from a 10 ml portion were collected by centrifugation at 600 g for 2 min. The pelleted material could be stored at −80°C without any detectable change in infectivity. The pelleted material was resuspended in 1 ml of 0.1 M-potassium phosphate buffer, pH 7.0, and homogenized in a Potter mini-homogenizer. The homogenate was tested undiluted, and in 10-fold and 100-fold dilutions in a local lesion assay on 7 half leaves of *Phaseolus vulgaris* L. cv. Berna (Kleczkowski, 1950). Appropriate numbers of lesions induced per half leaf by the 10-fold or 100-fold dilutions, were multiplied by the dilution factor.

Purification of virus nucleoproteins. AMV (strain 425) was isolated as described by VanVolten-Doting & Jaspars (1972) and purified from plant contaminants by sucrose gradient centrifugation (Verhagen & Bol, 1972). Virus preparations retained their infectivity towards protoplasts for more than one year when stored at −80°C and 1 mg/ml in 0.01 M-phosphate buffer, pH 7.0.

To separate the virus nucleoproteins, about 25 mg of virus material was centrifuged for
Infection of protoplasts by AMV

Fig. 1. Virus production in cowpea protoplasts inoculated at 25°C with AMV (5 µg/ml) in 0.01 M-potassium phosphate buffers (○—○) or 0.01 M-potassium citrate buffers (●—●) of different pH. Virus production was measured 40 h after inoculation by infectivity assay.

6 h at 32000 rev/min and 4°C in a 10 to 30% sucrose gradient in a Beckman 15 Ti zonal rotor (Bol & Lak-Kaashoek, 1974).

Fractions enriched in either B-, M- or Tb-nucleoprotein were subjected to a second centrifugation cycle. Prior to the inoculation of protoplasts, fractions of the gradients containing purified B-, M- or Tb-nucleoprotein were dialysed extensively against 0.5 M-mannitol.

Preparation of fluorescent antibodies. An antiserum raised against AMV (strain 425) with a titre of 1/64 as determined by the gel double-diffusion method (Ouchterlony, 1962), was used to conjugate antibodies with fluorescein isothiocyanate according to Motoyoshi et al. (1973). However, the preparation thus obtained did not give specific staining of protoplasts isolated from systemically infected tobacco or cowpea leaves or protoplasts infected in vitro. Attempts to prepare antisera with a higher titre were unsuccessful.

RESULTS

Influence of pH and buffer salts

Kubo, Harrison & Robinson (1974) reported that infection of tobacco protoplasts with tobacco rattle virus was more efficient when the inoculation was done in phosphate buffer instead of citrate buffer. Fig. 1 shows the production of AMV in cowpea protoplasts after inoculation in potassium phosphate or citrate buffers (0.01 M) at a range of pH values. In both buffers most infection was obtained around pH 5.6, but in phosphate buffer the efficiency was much greater. Increasing the molarity of the phosphate buffer from 0.01 to 0.025 M did not influence the results but above this concentration there was a rapid decline in the efficiency of infection (data not shown). In all subsequent experiments the inoculation was done on 0.01 M-phosphate buffer, pH 5.6.

Influence of inoculation temperature

To study the effect of temperature, the pre-incubation of virus and poly-L-ornithine as well as the inoculation of protoplasts with this mixture was done at 0°C and 25°C. Table 1 shows that virus production in protoplasts was greatly enhanced when the inoculation was
Table 1. The effect of temperature on infection of cowpea protoplasts by AMV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-incubation temperature (°C)</th>
<th>Inoculation temperature (°C)</th>
<th>Average lesion number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>25</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0</td>
<td>2300</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>8310</td>
</tr>
</tbody>
</table>

* Protoplasts were homogenized 40 h after inoculation in 1 ml buffer and the homogenate was assayed for infectivity undiluted and at 10-fold and 100-fold dilutions. The average number of lesions in seven half leaves induced by the appropriate dilution was multiplied by the dilution factor.

Fig. 2. (a) Virus production in cowpea protoplasts inoculated for 15 min with AMV/poly-L-ornithine mixtures which had been pre-incubated for different times. (A pre-incubation of '0 min' means that protoplasts were resuspended in 0.5 M-mannitol containing the virus and that poly-L-ornithine was added immediately afterwards.) (b) Virus production in cowpea protoplasts inoculated for different times with virus/poly-L-ornithine mixtures which had been pre-incubated for 10 min. Virus production was measured 40 h after inoculation by infectivity assay. (The difference in ordinate units between (a) and (b) is caused by a difference in sensitivity of the batches of ‘Berna’ beans used.)

done at 0 °C (treatment 2) instead of 25 °C (treatment 1). The enhancement was even more pronounced when the virus/poly-L-ornithine mixture was also pre-cooled (treatment 3). All subsequent inoculations were therefore done at 0 °C.

Influence of inoculation time

Incubation of virus and poly-L-ornithine prior to the inoculation of protoplasts was done by analogy with the procedure used by Hibi et al. (1975) to infect cowpea protoplasts with cowpea mosaic virus (CPMV). To see if this pre-incubation is a prerequisite for AMV infection, the time of the pre-incubation was varied (Fig. 2a). A pre-incubation of ‘0 min’ means that sedimented protoplasts were resuspended in 10 ml of a cold solution of 0.5 M-mannitol and 0.01 M-phosphate, pH 5.6, containing the virus, and that poly-L-ornithine was added immediately thereafter. Fig. 2a shows that the virus production obtained in this way was comparable to that induced by virus/poly-L-ornithine mixtures which had been pre-incubated for various times. Thus pre-incubation of AMV and poly-L-ornithine is not required for successful infection of protoplasts. For convenience, however, virus/poly-L-ornithine mixtures in mannitol/phosphate solution were kept for about 10 min in an ice-bath, before inoculation was started by resuspending protoplasts in the solution.
Infection of protoplasts by AMV

Fig. 3. (a) Virus production in cowpea protoplasts inoculated with solutions containing 1 μg/ml poly-L-ornithine and different concentrations of AMV. (b) Virus production in cowpea protoplasts inoculated with solutions containing different concentrations of poly-L-ornithine and 1 μg/ml AMV (●—●), 2 μg/ml AMV (▲—▲), or 5 μg/ml AMV (■—■). Virus production was measured 40 h after inoculation by infectivity assay.

Fig. 2(b) shows that adsorption of virus to protoplasts occurs within 5 min, because the same amount of virus was produced when the protoplasts were exposed to virus for either 5 or 25 min.

Influence of poly-L-ornithine and virus concentration

Because there is evidence that poly-L-ornithine in the inoculum interacts with negatively charged virus particles (cf. Takebe, 1975; Kubo et al. 1976) the optimal concentrations of virus and poly-L-ornithine may be interdependent. In the experiment shown in Fig. 3(a) the virus concentration in the inoculum was varied at a fixed poly-L-ornithine concentration of 1 μg/ml. Apparently, the virus concentration is rather critical, most infection being obtained in this experiment at 1 μg/ml. In several experiments with different batches of virus maximal infection was obtained at concentrations ranging from 1 to 3 μg/ml, whereas at 5 μg/ml virus the efficiency was always much lower. To establish the optimal combination of virus and poly-L-ornithine, the polycation concentration in the inoculum was varied at virus concentrations of 1, 2 and 5 μg/ml. The upper limit of poly-L-ornithine concentration was set by the fact that protoplasts were severely damaged by exposure to concentrations above 1 μg/ml. Fig. 3(b) shows that poly-L-ornithine is an absolute requirement for infection; in its absence no virus was produced at any virus concentration. At virus concentrations of 2 and 5 μg/ml maximum infection was obtained at 1 μg/ml poly-L-ornithine; at 1 μg/ml virus 0.75 μg/ml of the polycation was most efficient. In most of the subsequent experiments virus and poly-L-ornithine concentrations of 2 and 1 μg/ml, respectively, were used.

Growth curve

To follow the time course of virus production, protoplasts were infected with AMV and at 6 h intervals samples were taken and assayed for infectivity. Fig. 4 shows that virus infectivity is first detectable 12 h after infection, and increases exponentially in the next 12 h. As the increase in infectivity levels off after this period, it can be concluded that most of the virus is produced within 24 h after inoculation of the protoplasts.
Fig. 4. Virus production in protoplasts incubated for different times after inoculation with AMV. Virus production was measured by infectivity assay.

Table 2. Infection of cowpea protoplasts after inoculation with purified AMV nucleoproteins

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Inoculum</th>
<th>Protoplast extract†</th>
<th>Inoculum</th>
<th>Protoplast extract†</th>
<th>Protoplast extract supplemented with missing nucleoproteins‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Tb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>B + M</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>189</td>
<td>153</td>
</tr>
<tr>
<td>B + Tb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>121</td>
</tr>
<tr>
<td>M + Tb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>B + M + Tb</td>
<td>64</td>
<td>5457</td>
<td>78</td>
<td>2767</td>
<td></td>
</tr>
<tr>
<td>AMV</td>
<td>—§</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Purified nucleoproteins were present in the inoculum each at 1 μg/ml; the concentration of the unfractionated AMV preparation was 3 μg/ml.
† Assayed as described in the footnote of Table 1.
‡ Protoplasts were homogenized 40 h after inoculation and the virus nucleoproteins absent in the inoculum were added to the homogenates at a concentration of 1 μg/ml.
§ Not tested.

Inoculation with purified nucleoproteins

Systemic infection in tobacco leaves or the induction of local lesions in bean leaves is obtained only when B-, M- and Tb-nucleoproteins are present in the inoculum (Van Vloten-Doting, Dingjan-Versteegh & Jaspars, 1970). To confirm that these requirements also hold for the infection of protoplasts, inocula were prepared which contained either purified B-, M- and Tb-nucleoprotein or mixtures of these components. The preparations were inoculated to bean leaves as well as to protoplasts; the inoculated protoplasts were assayed for infectivity after an incubation of 40 h. Table 2, experiment 1, shows that the biological...
activity of the inocula towards bean leaves and protoplasts is the same: virus production in protoplasts as measured by local lesion assay is obtained only when B, M and Tb are present in the inoculum. It has been shown by Dingjan-Versteegh, Van Vloten-Doting & Jaspars (1972) that genetic information in M is responsible for the formation of local lesions on bean leaves. One might imagine the possibility that B and Tb together contain the genetic information for the virus replicase and coat protein, and that information in M-RNA is not relevant for the replication in protoplasts. Hence it is conceivable that inoculation of protoplasts with a mixture of B and Tb induces the synthesis of these components and that this synthesis escapes detection by local lesion assay because of the absence of M. To rule out such a possibility the homogenates prepared from the protoplasts 40 h after their inoculation, were assayed for infectivity either directly or after the addition of the virus nucleoproteins which had been omitted in the respective inocula. The results (Table 2, experiment 2) indicate that incomplete inocula did not give rise to the synthesis of the corresponding virus nucleoproteins. Moreover, this experiment shows that similar amounts of virus are produced when protoplasts are inoculated with an artificial mixture of B, M and Tb or with a complete virus preparation. This confirms that Ta-nucleoprotein is not required for the initiation of infection in protoplasts.

**Influence of antibiotics**

A more detailed study of the virus growth cycle can be made by following the incorporation of radioactively labelled precursors into virus RNA and protein. For this, the possibility of suppressing the respective host processes without interfering with the virus replication is a prerequisite. The effects of actinomycin D, cycloheximide and chloramphenicol on AMV replication were tested by incubating freshly inoculated protoplasts in media containing the respective drugs. Table 3 shows that under these conditions actinomycin D greatly interferes with virus production, that cycloheximide completely inhibits virus replication, and that chloramphenicol slightly stimulates virus production. This stimulation may be due to more efficient suppression of bacterial growth, or virus protein synthesis may be favoured by the inhibition of host protein synthesis in mitochondria and chloroplasts. The effect of cycloheximide suggests that virus protein synthesis is making use of cytoplasmic ribosomes.

The inhibition by actinomycin D was unexpected. It suggests that DNA-dependent RNA synthesis is required for the initiation of infection. However, Kassanis, White & Woods (1975) reported that the inhibition of virus multiplication by a number of antibiotics may be due to their ability to chelate metal ions from protoplast membranes, and that inhibition is largely prevented by adding a divalent metal to the medium. Table 4 shows that comparable results are obtained when AMV-infected protoplasts are incubated in a medium

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**Table 3. Effect of antibiotics on AMV multiplication in cowpea protoplasts**

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Average lesion number†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1997</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>137</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>3380</td>
</tr>
</tbody>
</table>

* Immediately after inoculation the protoplasts were transferred into media containing no inhibitor, 10 µg/ml actinomycin D, 10 µg/ml cycloheximide, or 200 µg/ml chloramphenicol, respectively.
† Determined as described in the footnote of Table 1.
Table 4. AMV production in cowpea protoplasts incubated in different media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Average lesion number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete†</td>
<td>2857</td>
</tr>
<tr>
<td>0.5 M-mannitol + 10 mM-CaCl₂</td>
<td>2300</td>
</tr>
<tr>
<td>0.5 M-mannitol + 10 mM-MnCl₂</td>
<td>21</td>
</tr>
</tbody>
</table>

* Determined as described in the footnote of Table 1.
† Medium according to Aoki & Takebe (1969) as described in Methods. All media contained 200 μg/ml chloramphenicol to suppress bacterial growth.

Fig. 5. Effects of (a) actinomycin D and (b) cycloheximide on AMV multiplication in cowpea protoplasts. The drugs were added at 6 h intervals to samples of inoculated protoplasts; 40 h after inoculation all samples were assayed for infectivity. Open and closed symbols represent two independent experiments.

according to Aoki & Takebe (1969) or in 0.5 M-mannitol, pH 5.5, containing 10 mM-CaCl₂. Substituting MnCl₂ for CaCl₂, however, reduced the viability of the protoplasts as determined by fluorescein diacetate staining and interfered with virus multiplication. In subsequent experiments protoplasts were incubated in mannitol-CaCl₂ medium, supplemented with 200 μg/ml chloramphenicol to suppress bacterial growth. Addition of actinomycin D or cycloheximide to freshly inoculated protoplasts in this medium yielded results comparable to those presented in Table 3, indicating that the effect is not due to the possible Ca-chelating ability of the drugs. To determine the sensitive step in the growth cycle, actinomycin D or cycloheximide were added at six-hourly intervals to samples of inoculated protoplasts. All samples were assayed for infectivity 40 h after inoculation. Fig. 5(a) shows that actinomycin D influences virus multiplication only when added immediately after inoculation; addition after 6 h or later has no effect. The curve obtained with cycloheximide (Fig. 5b) closely resembles the virus growth curve (Fig. 4). This indicates that addition of cycloheximide at any time in the growth cycle results in an immediate cessation of virus production.
DISCUSSION

The investigation of the inoculation conditions showed that the induction of AMV multiplication in cowpea protoplasts is most efficient when protoplasts are resuspended at 0°C in 0.5 M mannitol, 0.01 M potassium phosphate, pH 5.6, containing 1 μg/ml poly-L-ornithine and about 2 μg/ml AMV. Two factors appeared to be of major importance: temperature and virus concentration. From the fact that inoculation of tobacco protoplasts with cowpea chlorotic mottle virus is equally efficient at 0°C and 25°C, Motoyoshi, Watts & Bancroft (1974) concluded that an energy-dependent process such as pinocytosis is not likely to be involved in virus uptake. The increase in efficiency observed when cowpea protoplasts were inoculated with AMV at 0°C may be due to a better protection of the virus against enzymatic degradation, as it is known that AMV is highly susceptible to the action of ribonucleases and proteolytic enzymes (Bol & Veldstra, 1969; Bol, Kraal & Brederode, 1974). Moreover, no infectivity was detected after the inoculated protoplasts were submitted to the washing procedure. This means that either the amount of adsorbed virus is too low to be detectable, or that adsorption results in a conformational change rendering the virus RNA susceptible to degradation during subsequent protoplast homogenization. In this case, inoculation at 0°C might favour the survival of the RNA at the entry into the eclipse phase.

The virus concentration in the inoculum appeared to be rather critical. This may be related to the absolute requirement for poly-L-ornithine in the inoculum. At the pH of the inoculum, 5.6, AMV particles will be negatively charged, as the isoelectric point of the virus is below pH 5.0 (see Hull, 1969). There is evidence that poly-L-ornithine interacts with virus particles thus neutralizing their negative charge and facilitating the adsorption of the virus-polycation aggregate on to the protoplast surface which may also be negatively charged (see Takebe, 1975). The low efficiency of AMV concentrations in the inoculum above 3 μg/ml might be due to a requirement for poly-L-ornithine concentrations above 1 μg/ml, which are, however, deleterious to protoplasts. Aggregate formation between AMV and poly-L-ornithine is also suggested by the increase in infection when the inoculum is made in phosphate buffer instead of citrate buffer. Kubo et al. (1976) reported that such an increase in infection is correlated with the finding that, in phosphate buffer, virus-polycation aggregates are smaller and thus more numerous than in citrate buffer. That pre-inoculation incubation of AMV and poly-L-ornithine is not essential for infection may be explained by the observation that adsorption of the possible virus/poly-L-ornithine aggregates to the protoplasts occurs within 5 min. Thus, there may be enough time during the inoculation period of 15 min to allow complex formation between virus and polycation.

Motoyoshi et al. (1975) reported that the best conditions for infection of tobacco mesophyll protoplasts with AMV were pH 5.2, 2.5 μg/ml virus and 1 μg/ml poly-L-ornithine. They also noticed a reduced infection at higher virus concentration. The fact that in our hands pH 5.6 was found to be optimal for the infection of cowpea protoplasts may reflect the difference between the plasmalemma of cowpea and tobacco mesophyll protoplasts as suggested by Hibi et al. (1975).

Because our attempts to prepare antisera against AMV with titres high enough to allow the preparation of fluorescent antibodies were unsuccessful (see Methods), it was not possible to determine the percentage of the protoplasts that became infected under the conditions described above. However, by comparing the infectivity of protoplast homogenates with that of standard virus concentrations inoculated in each experiment, and taking the average mol. wt. of the virus particles to be 6 x 10^6 (Heijtink, 1974), it was estimated that 40 h after
inoculation 1 to $5 \times 10^6$ virus particles are produced per living cell. One should realize, however, that this figure refers to a mixture of four components, three of which are necessary for infection. Moreover, the estimates are based on the assumption that the specific infectivity of purified virus and newly synthesized virus are the same. The fact that our estimates are in the same order of magnitude as those made in other virus-protoplast systems (see Takebe, 1975) suggest that a reasonable percentage of the protoplasts become infected. Moreover, the amount of virus produced seems sufficient to permit a study of the multiplication process.

Prior to an investigation of the crucial role of the coat protein in virus replication, one has to confirm that the same molecular species which are necessary for infection of intact leaves are required for protoplast infection. The results reported in this paper show that B-, M- and Tb-nucleoprotein are essential for infection of protoplasts, as they are for intact leaves. Recent attempts to inoculate cowpea protoplasts with AMV-RNA according to the method described by Beier & Bruening (1976) gave small yields of virus. Our next aim will be to demonstrate that the addition of coat protein to a mixture of B–RNA, M–RNA and Tb–RNA is required for the infection of protoplasts.

An interesting phenomenon is the observation that AMV multiplication is inhibited when actinomycin D is present during the first 6 h after inoculation. Addition at later times has no effect, demonstrating that actinomycin D does not interfere with virus RNA or protein synthesis. This suggests that an early step in AMV-replication is dependent on the synthesis of a host specific product. Actinomycin D also interferes similarly with the multiplication of potato virus X in tobacco protoplasts (Otsuki et al. 1974), CPMV in cowpea protoplasts (P. J. M. Rottier, personal communication) and tobacco mosaic virus in tobacco leaf disks (Dawson & Schlegel, 1976).

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