Proteins Synthesized by Cucumber Cotyledons Infected with Two Strains of Cucumber Mosaic Virus

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SUMMARY

Protein synthesis in cucumber cotyledons infected with two strains of cucumber mosaic virus (CMV) differing in symptom type was investigated by the technique of radioactive double-labelling of polypeptides followed by analysis on SDS-polyacrylamide gels. Radioactive labelling in the presence of actinomycin D revealed several virus-stimulated proteins associated with infection. In addition to coat protein, three polypeptides with apparent mol. wt. larger than that of coat protein, together with two smaller species were detected. No qualitative difference was observed in the patterns of protein stimulation which might account for the difference in symptoms induced by the two strains.

INTRODUCTION

Radioactive double-labelling of the proteins of healthy and infected cells, followed by their analysis on SDS-containing polyacrylamide gels, has been employed successfully in the study of virus specified and/or induced proteins, both structural and non-structural, in virus-infected bacterial and animal cells (e.g. Garwes, Sillero & Ochoa, 1969; Mayol & Sinsheimer, 1970; Shapiro, Kos & Russell, 1973; Butterworth, 1973). However, the technique has not found general application in plant virology due partly to the difficulties encountered in restricting host cell protein synthesis.

Despite such difficulties, several virus-coded or induced polypeptides have been detected in tobacco leaf tissue infected with tobacco mosaic virus (TMV; Zaitlin & Hariharasubramanian, 1970, 1972; Singer & Condit, 1974), or with tobacco necrosis virus (Jones & Reichmann, 1973). In other cases, only coat protein was observed (Singer, 1971). The synthesis of a polypeptide distinct from that of coat protein has been reported in TMV-infected tobacco mesophyll protoplasts (Sakai & Takebe, 1972, 1974; Paterson & Knight, 1975). Recently, wheat embryo cell-free systems have been employed to characterize the in vitro products of the isolated RNA(s) of several plant viruses (Efron & Marcus, 1973; Shih & Kaesberg, 1973; Davies & Kaesberg, 1974; Roberts, Paterson & Sperling, 1974; Schwinghamer & Symons, 1975). Knowland (1974) has reported the synthesis of a major polypeptide of 140000 mol. wt. following injection of oocytes of the frog Xenopus laevis with TMV RNA, although it was not established whether or not this polypeptide contained a coat protein sequence.

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Cucumber mosaic virus (CMV; R/I : I "3 / 19 + 1-1/19 + 0-8/19 : S/S : S/Ap) has been shown to possess a divided genome (Kaper & West, 1972; Peden & Symons, 1973; Lot et al. 1974; Wood & Coutts, 1975), RNA from purified virus preparations being resolved into a maximum of six species, the three largest of which are needed for full infectivity. The coat protein cistron has been located on the smallest of these three both directly by translation of this species in a wheat embryo cell-free system (Schwinghamer & Symons, 1975) and indirectly by the production of pseudo-recombinants (Habili & Francki, 1974; Marchoux et al. 1974). Assuming there is little duplication of genetic information between these three species, there is sufficient information to code for approx. 33000 daltons of protein. The single coat protein, which in the strains studied previously has an estimated mol. wt. ranging between 24000 and 32000, thus accounts for less than 10% of the available coding potential.

This communication reports the first in vivo demonstration, by the double-labelling technique, of the induction of polypeptides in tissue infected with CMV. Cucumber cotyledons were infected with two strains of CMV differing in the severity of induced symptoms (W, mild; N, severe) and de novo polypeptide synthesis studied in excised discs.

**METHODS**

**Viruses and plants.** The source of virus strains, conditions of plant cultivation and the methods of virus propagation, purification and assay have been described previously (Ziemiecki & Wood, 1975a).

**Polyacrylamide gel electrophoresis.** Electrophoresis in the presence of SDS was carried out according to the procedure of Maizel (1966) using glass tubes (8.5 cm × 0.5 cm i.d.). Gels were electrophoresed at 10 mA/tube for 1 h before sample application, with 0.1 M-phosphate, pH 7.2, containing 0.1% SDS and 0.1% 2-mercaptoethanol as the electrophoresis buffer. Samples were disrupted by boiling for 5 min at 100 °C in 0.1 M-phosphate buffer, pH 7.2, containing 1% SDS and 1% 2-mercaptoethanol, brought to about 0.5 M with solid sucrose and layered on to the gels. Electrophoresis was performed at 8 mA/tube until the leading edge of the bromophenol blue marker was at the end of the gel. When desired, protein bands were visualized by staining overnight in a 0.25% solution of Coomassie brilliant blue (Sigma Chemical Co.) in water:ethanol:acetic acid, 5:5:1. Unbound dye was removed by washing in several changes of methanol:7% acetic acid, 1:1. Gels were stored in 7% acetic acid.

Alternatively, when radiolabelled materials were used, unstained gels were sliced into segments of approx. 1 mm thickness using a screw threaded plunger similar in design to that of Iandolo (1970). Gel slices were individually incubated for 18 h at room temperature in 0.5 ml Soluene 100 (Packard Instruments Ltd). Scintillation fluid (4 g 2,5-diphenoxazole (PPO), 0.12 g 1,4-bis-(5-phenyloxazol-2-yl) benzene (POPOP) per litre toluene) was added and the radioactivity (d/min) due to 3H and 14C determined for each slice using a Nuclear Chicago Isocap 300 liquid scintillation counter with computer facilities.

Mol. wt. of unknown proteins were calculated from a standard curve of mobility against log10 mol. wt. of the following proteins: bovine serum albumin (68000), human γ-globulin heavy chain (51600), light chain (23500), ovalbumin (43000), pepsin (35000), carbonic anhydrase (29000), trypsin (23500).

**Radioactive labelling of polypeptides.** Cotyledons of cucumber plants (14 to 16 days old) were inoculated with purified virus (dilution end point approx. 10^-4) and incubated in a small controlled environment cabinet (Fison's Scientific Apparatus Ltd, Model 140 G2)
at 20 ± 1 °C, with a 14 h photoperiod provided by warm white fluorescent tubes (15000 lux). After the desired time of infection, 7 mm discs of cotyledon tissue were taken (total weight approx. 1 g) and infiltrated in 5 ml of sterile distilled water containing 200 μCi 4,5-3H-L-leucine (The Radiochemical Centre, Amersham, U.K.). Infiltration was accomplished by evacuating three times for 30 s, using a vacuum pump (Millipore Corporation, Bedford, Massachusetts D1730, U.S.A.). The tissue discs were incubated for 15 h on a rotator (T.C. Drum Apparatus Laboratories and Electrical Engineering Co., Nottingham) in the environmental cabinet at 20 ± 1 °C. Healthy control cotyledon discs from buffer-inoculated plants were treated in an identical manner except that infiltration was in 5 ml of sterile distilled water containing 20 μCi 1-14C-L-leucine.

The procedure employed in experiments in which radioactive labelling was done in the presence of actinomycin D (‘Lyovac Cosmegen'; Merck, Sharp & Dohme Ltd) was similar, except that cotyledon tissue was treated with actinomycin D 24 h prior to commencement of labelling (3.5 days after inoculation). This was accomplished by injection of actinomycin D (10 μg/ml in sterile distilled water) into the mesophyll spaces of the cotyledons. In addition, the isotope infiltration solution contained 10 μg/ml actinomycin D. This concentration was adopted since it had previously been shown to be the lowest causing significant inhibition (about 75 %) of RNA synthesis in cucumber cotyledons (Barbara & Wood, 1974), and it caused a minimum of tissue damage and had little effect on the course of virus accumulation. Because actinomycin D appears to be a much less effective inhibitor of protein synthesis in plant cells than in animal and bacterial cells (Sänger & Knight, 1963; Sakai & Takebe, 1970), possibly attributable in part to the nature of plant tissues, a 24 h pre-treatment was used in an attempt to maximize inhibitory action.

Upon completion of the incubation period the discs were washed three times in 10 ml sterile distilled water, blotted dry, and gently homogenized in ice-cold 0.025 M-tris/HCl buffer, pH 7.5, containing 0.5 M-sucrose, 0.01 M-MgCl₂ and 0.005 M-2-mercaptoethanol. The slurry was squeezed through gauze and the solution subjected to differential centrifugation to yield four crude subcellular fractions; nuclear and chloroplast (25000 g pellet), mitochondrial (20000 g pellet), ribosomal (105000 g pellet) and soluble protein (105000 g supernatant fluid). The three particulate fractions were disrupted immediately by resuspending in 1 ml of 0.1 M-phosphate buffer, pH 7.2, containing 1% SDS and 1% 2-mercaptoethanol and boiling for 5 min at 100 °C. The soluble protein fraction was disrupted by boiling for 5 min with an equal vol. of 0.2 M-phosphate buffer, pH 7.2, containing 2% SDS and 2% 2-mercaptoethanol. Samples of disrupted healthy and infected fractions were mixed and either co-electrophoresed immediately or stored at −20 °C. Fractions stored at −20 °C were boiled for 5 min prior to use. Approx. 50 000 to 100 000 ct/min of each isotope were applied to gels to give a base ratio of about 1 to 3.

**Radiolabelled virus coat protein.** 14C-labelled coat protein was prepared by allowing excised tobacco leaves infected with CMV to take up 14C-leucine (about 20 μCi in 1 ml sterile distilled water). Following a 24 h incubation at 20 ± 1 °C in an environmental cabinet, CMV was purified by the standard method (Ziemiecki & Wood, 1975a).

### RESULTS

**Radioactive labelling in the absence of actinomycin D**

Because short-term labelling of TMV-infected leaf tissue in the absence of actinomycin D was successful for demonstrating TMV coat protein (Singer, 1971), a similar approach
Fig. 1. $^3$H/$^{14}$C ratio plots for all subcellular fractions of cucumber cotyledon tissue infected with the $W(a$ to $c)$ or $N(d)$ strain of CMV. The duration of labelling was 15 h, beginning 40 h ($a$, ‘early’ pulse), 88 h ($b$, ‘late’ pulse) or 4.5 days ($c$, $d$) after inoculation. Labelling was in the absence ($a$, $b$) or presence ($c$, $d$) of actinomycin D. Electrophoretic migration was from left to right, in 10% gels, with upper arrows indicating the position of reproducible peaks and the lower arrow coat protein.
was used to detect proteins specified or induced by infection of cucumber cotyledon tissue with the W strain of CMV.

To investigate the possibility of virus-specified or enhanced protein synthesis prior to the appearance of assayable virus, the first or ‘early’ pulse began 40 h after inoculation and was complete before extractable infectivity was detectable in significant amounts (Ziemiecki & Wood, 1975a). A second or ‘late’ pulse began 88 h after inoculation, when there was active synthesis of virus (Ziemiecki & Wood, 1975a). Several periods of labelling were tried; however, only the results of the 15 h pulse are presented because shorter labelling periods revealed nothing. The longest pulse period had little effect on the accumulation of virus as judged by infectivity assay.

The results of ‘early’ and ‘late’ pulses of 15 h duration are indicated in Fig. 1(a) and (b) respectively, the position of coat protein, determined by electrophoresis of 14C-labelled coat protein under identical experimental conditions, being indicated by the lower arrow.

No reproducible peak(s) or depression(s) in the ratio plot occurred in any of the subcellular fractions from either the ‘early’ (Fig. 1a) or ‘late’ pulse (Fig. 1b); however, the ribosomal fraction of the ‘late’ pulse consistently exhibited peaks in the regions corresponding to gel slices 10, 15 and 20 (Fig. 1b). These peaks varied in prominence, sometimes being indistinguishable from the ‘noise’ level. This was particularly true for the peak in the region of slice 10. Proteins in these positions in the gel corresponded to polypeptides of approximate mol. wt. 78,000 (slice 10), 63,000 (slice 15) and 50,000 (slice 20).

Radioactive labelling in the presence of actinomycin D

In an attempt to reduce the level of labelled amino-acid incorporation into host-directed proteins, radioactive labelling experiments were made in the presence of 10 μg/ml actinomycin D. Labelling experiments in the absence of actinomycin D suggested that a long labelling time during a period of active virus accumulation should yield the most profitable results. Consequently a 15 h labelling time, commencing 4.5 days after inoculation, was chosen.

The ratio plots for all the subcellular fractions of cotyledons infected with the W strain of CMV are shown in Fig. 1(c), where reproducible peaks are indicated by arrows. A peak corresponding in position to coat protein was found only in the particulate subcellular fractions (chloroplast and nuclear, mitochondrial, ribosomal). This peak often had a low mol. wt. shoulder, best seen in the chloroplast and nuclear fraction (about 26,000). The ribosomal fraction, in addition to coat protein, exhibited three peaks corresponding closely in position and mol. wt. to those detected in the ribosomal fraction of plants labelled in the absence of actinomycin D (Fig. 1b). The apparent mol. wt. of the three were approx. 78,000, 62,000 and 48,000.

The soluble protein fraction did not exhibit an increase in the region of the coat protein, although a peak corresponding to a protein of slightly lower mol. wt. (about 24,000), was readily detectable. This peak occasionally had a high mol. wt. shoulder (about 26,000). An increase in ratio in the region corresponding to a protein of approx. 48,000 was often observed, and may have been due to contamination from the ribosomal pellet. Although there were other peaks and depressions in the ratio plots they were discounted because of their irreproducibility.

Similar plots for the subcellular fractions of cotyledon tissue infected with the N strain of CMV are shown in Fig. 1(d). Infection with the N strain resulted in a very similar pattern of stimulation in all fractions (cf. Fig. 1c), a peak corresponding to coat protein again appearing only in particulate fractions. In addition to coat protein, three polypeptides with
A. ZIEMIECKI AND K. R. WOOD

Table I. Distribution of infectivity in the subcellular fractions of infected tissues

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>% of total infectivity</th>
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<tbody>
<tr>
<td></td>
<td>CMV-W</td>
</tr>
<tr>
<td>Chloroplast + nuclear</td>
<td>73</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>17</td>
</tr>
<tr>
<td>Ribosomal</td>
<td>10</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>0</td>
</tr>
</tbody>
</table>

the same apparent mol. wt. as the corresponding polypeptides detected in fractions of tissue infected with the W strain, were evident in the ribosomal fraction. The overall patterns of protein synthesis resulting from infection with either strain differed slightly in a quantitative manner, infection with the N strain evoking slightly greater stimulation of the coat protein.

The distribution of infectivity in subcellular fractions

Infected cotyledon tissue was treated with actinomycin D and, 5 days after inoculation, was subjected to extraction and fractionation procedures identical to those used in the labelling experiments, and the resultant subcellular fractions were assayed for CMV infectivity on primary leaves of cowpea.

The results of such infectivity studies are shown in Table I. The figures refer to the relative infectivity present in any fraction expressed as a percentage of total infectivity in all fractions. Infectivity was confined to the particulate fractions: chloroplast and nuclear, mitochondrial and ribosomal, a major part being in the chloroplast and nuclear fraction.

DISCUSSION

Radioactive double-labelling has demonstrated the appearance of polypeptides, either virus-coded or induced, in cucumber cotyledon tissue infected with two strains of CMV, W and N. The patterns of de novo protein synthesis in tissue infected by either strain were strikingly similar, both qualitatively and quantitatively, despite differences in symptom expression elicited by the two strains.

Although Singer (1971), labelling in the absence of actinomycin D, detected TMV coat protein together with two polypeptides of lower mol. wt., in the present study such a procedure failed to reveal major differences between healthy and infected material. Variable but low amounts of the three polypeptides present in the ribosomal fraction of the ‘late’ pulse (Fig. 1b) appeared quite consistently. Incorporation of label into host-directed proteins probably partially masked the appearance of any new proteins.

Double-labelling in the presence of actinomycin D revealed several polypeptides associated with virus infection, three of higher mol. wt. than coat protein (about 78,000, 62,000, 48,000), coat protein and two smaller mol. wt. polypeptides, possibly coat-related. It is possible that some of these polypeptides are related to those recently observed following in vitro translation of CMV RNAs with cell-free wheat germ extracts (Davies & Kaesberg, 1974; Schwinghamer & Symons, 1975).

A polypeptide with a mol. wt. corresponding to that of coat protein was observed in all three particulate fractions of tissue infected with both virus strains (Fig. 1c, d), in contrast to observations on fractions from TMV-infected tobacco (Singer, 1971) where coat protein was present in all fractions, and only in these fractions was infectivity observed (Table 1).
This would suggest that this polypeptide came from intact virus, and that either very little or no excess coat protein was synthesized or that any excess was degraded. It is also possible that any excess produced, under the conditions of extraction and fractionation, aggregated and appeared in the particulate fractions.

The major peak in the soluble protein fraction corresponded to a polypeptide of mol. wt. rather less than that of coat protein, while a polypeptide of intermediate mol. wt. appeared in some fractions, particularly the chloroplast and nuclear fractions. It is possible that these two represent incomplete, defective or degradation products of coat protein. Antiserum to CMV-W coat protein has previously revealed the presence of two low mol. wt. coat protein related antigens in similar soluble protein fractions (Ziemiecki & Wood, 1975b). Immunodiffusion analysis of soluble protein fractions prepared daily after inoculation suggested a gradual accumulation of the lower mol. wt. soluble antigen; in addition, a polypeptide with a mol. wt. slightly less than that of coat protein has occasionally been observed following electrophoresis of purified virus in SDS-gels (Ziemiecki, 1974). The presence of coat-related polypeptides of lower mol. wt. than coat protein has been observed in TMV-infected tobacco (Singer, 1971).

The nature and function of the three larger mol. wt. polypeptides detected in the ribosomal fractions remains unknown. Assuming little duplication of genetic information in the three species of RNA required for infectivity, the genome of CMV contains sufficient information to code for all three. One or more of the peaks could represent aggregates containing coat protein, though the disruption procedure employed would have been expected to overcome this. Another possibility is that one or all are intermediates in post-translational cleavage of a coat protein precursor, although there is again no evidence for this.

CMV-induced polymerase activity has been demonstrated in a particulate fraction of CMV-infected cucumber cotyledon tissue, and it has been suggested that the enzyme has a subunit structure (Clark, Peden & Symons, 1974). It is not inconceivable that one or more of the polypeptides detected in the ribosomal fraction represent subunit(s) of the viral polymerase. Hariharasubramanian et al. (1973) have demonstrated a protein of mol. wt. 34,500 in BMV-infected barley which they suggest represents a polypeptide subunit of a viral RNA polymerase of about 150,000.

A further possibility is that one or more of these polypeptides is functional in altering the specificity of ribosome binding in favour of the viral RNA, by interaction with either ribosome subunits or viral RNA or both, a situation perhaps similar to that postulated for the equestron in poliovirus infected cells (Cooper, Steiner-Pryor & Wright, 1973).

Finally, it is also quite feasible that the non-coat polypeptides observed in infected material represent a stimulation of host protein synthesis in response to virus infection. Further studies aimed at clarifying the situation are currently in progress.

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REFERENCES


Proteins in CMV-infected cucumber


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