Evidence for Three Functional RNA Species in several Strains of Cucumber Mosaic Virus

By H. LOT*, G. MARCHOUX AND J. MARROU
Station de Pathologie végétale, Centre de Recherches d’Avignon,
I.N.R.A. 84 Montfavet, France

J. M. KAPER AND C. K. WEST
Plant Protection Institute, Agricultural Research Service,
U.S. Department of Agriculture, Beltsville, Maryland 20705, U.S.A.

LOU S V AN VLOTEN-DOTING
Department of Biochemistry, State University of Leiden,
Wassenaarseweg 64, Leiden, The Netherlands

AND R. HULL
John Innes Institute, Colney Lane, Norwich, NOR 7oF, U.K.

(Accepted 23 August 1973)

SUMMARY

The RNA content of four different CMV strains was investigated. All RNA preparations always contained the four major RNA species described by Kaper & West (1972), while most preparations also contained several minor RNA species. The four largest RNAs were separated by sucrose density-gradient centrifuging and by electrophoresis on polyacrylamide gels. Combination experiments suggest, for all strains, that the genome consists of the three largest RNAs (1 + 2 + 3), while RNA 4 is not required for infectivity.

The distribution of RNAs in the capsids could not be established unequivocally, and the situation possibly differs in different strains.

INTRODUCTION

Cucumber mosaic virus (CMV) preparations contain a single sedimenting nucleoprotein component with a mol. wt. of 5 to 5.5 x 10^6 containing about 1 x 10^6 daltons of RNA (Kaper, Diener & Scott, 1965; Dupont et al. 1968; van Regenmortel, Hendry & Baltz, 1972). However, the RNA from CMV has several sedimenting components with estimated mol. wt. of 1.00, 0.77 and 0.33 x 10^6 (Kaper et al. 1965). Infectivity was associated with the fastest-sedimenting component and the two smaller components were thought to be degradation products. Electrophoresis of CMV-RNA in polyacrylamide gels resolves six species, the fastest-migrating two being in small amount (Kaper & West, 1972).

CMV therefore appears to be similar to the members of the bromovirus group, broad-bean mottle, brome mosaic and cowpea chlorotic mottle viruses, each of which has a single

* Present address: Station de Pathologie, CRAAG, Domaine Duclos, Petit-Boug, Guadeloupe, French West Indies.
sedimenting nucleoprotein component yet several RNA components (Kodama & Bancroft, 1964; Bockstahler & Kaesberg, 1965; Bancroft et al. 1968). Electrophoresis of RNAs of bromoviruses on polyacrylamide gels resolves four species for each virus; the heaviest two or three species are needed for infection (Bancroft, 1971; Lane & Kaesberg, 1971; Bancroft & Flack, 1972; Hull, 1972). Preparations of each of these viruses consist of a mixed population of nucleoprotein particles containing either of the two heavier RNA species alone; brome mosaic and cowpea chlorotic mottle virus preparations also have particles which contain one piece of each of the two lighter RNA species, whereas broad-bean mottle virus has particles which contain several, probably four, pieces of the lightest RNA. This gives density heterogeneity (Lane & Kaesberg, 1971; Bancroft & Flack, 1972; Hull, 1972). Lot & Kaper (1973) showed that CMV is also heterogeneous in CsCl density-gradient equilibrium experiments although to a much lesser degree than brome mosaic virus. For this reason, and because the nucleocapsid had to be stabilized by formaldehyde, which destroys the infectivity, the differences in buoyant density could not be used for separation purposes.

CMV has some properties in common with alfalfa mosaic virus (Hull, 1969; Bol, van Vloten-Doting & Jaspars, 1971). Alfalfa mosaic virus has four nucleic acid species which correspond to four nucleoprotein components (Bol et al. 1971). The total genome of alfalfa mosaic virus is contained in the three largest RNA species but these are not infectious unless either coat protein or the smallest RNA species is present (van Vloten-Doting, Dinjan-Versteegh & Jaspars, 1970; Bol et al. 1971).

In this paper we present data obtained in four different laboratories on the RNAs found in several strains of CMV and show that the three largest RNA species are needed for infection. Neither the coat protein nor the smaller RNA species play a role in the infectivity of this virus.

**METHODS**

*Strains and purification.* Strain CMV-S, isolated from squash and originally obtained from Dr M. H. V. van Regenmortel was grown in squash (*Cucurbita pepo. L. var. Caserta Bush*) for 7 to 8 days and purified by the method of van Regenmortel (1964) ('CMV-S1') or cultured in tobacco (*Nicotiana tabacum L. var. Samsun NN*) and purified like alfalfa mosaic virus as described by van Vloten-Doting & Jaspars (1972) ('CMV-S2'). In the latter case, after the second high-speed centrifuging, the virus was further purified by centrifuging in an MSE BXIV zonal rotor using isokinetic gradients of 14 to 30% sucrose in 10⁻² M-NaH₂PO₄, 10⁻³ M-EDTA, adjusted to pH 7.0. The virus was then concentrated by ultracentrifuging. The pellets were redissolved in 10⁻² M-NaH₂PO₄, 10⁻³ M-EDTA and 10⁻³ M-NaNO₃, pH 7.0. Strain CMV-D, isolated from tomato was propagated in tobacco (*Nicotiana tabacum L. var. Xanthi-nc*) for 6 days and purified by the method described by Lot et al. (1972). CMV-Q, isolated from pepper and received from Dr R. I. B. Francki, was cultured in squash and purified as CMV-S1. CMV-TAV-898, (one of the two TAV isolates described by Hollings, Stone & Brunt, 1968) was received from Dr M. Hollings and was grown in *Nicotiana glutinosa × Nicotiana clevelandii* (Christie, 1969) for 10 days. It was purified essentially as described by van Vloten-Doting & Jaspars (1972) but using high-speed centrifuging instead of polyethylene glycol (PEG) concentration.

*Preparation of virus RNA.* RNA from CMV-S₁ and CMV-Q preparations was extracted from a 3.5 mg/ml solution of purified virus using phenol, 0.1 M-NaH₂PO₄ buffer, pH 7.0, 0.15% SDS and 0.02 mg/ml bentonite followed by a tenfold dilution of the aqueous phase and two cycles of ethanol precipitation and resuspension in distilled H₂O. CMV-D RNA
was also purified by the SDS-phenol method of Lot (1972). CMV-TAV RNA was isolated by direct dissociation of the virus with SDS using the method described for broad bean mottle virus (Hull, 1972). RNA was extracted from CMV-S2 (10 mg/ml) either as described earlier for alfalfa mosaic virus (van Vloten-Doting & Jaspars, 1967) but, prior to phenol extraction of a virus solution, SDS was added to a concentration of 0.6% instead of pyrophosphate or by direct dissociation of the virus using SDS.

**Preparation of protein.** CMV-S2 coat protein was prepared like alfalfa mosaic virus-protein by dissociating the virus with MgCl₂ (Kruseman et al., 1971).

**U.v. absorption.** For CMV and CMV-RNA the extinction coefficients (E₉₅₀₇) used were 5 and 25 respectively and for CMV protein an extinction coefficient (E₉₆₀₇) of 0.7 was used.

**Rate zonal and isopycnic centrifuging in sucrose density gradient.** For studying the distribution of RNA species in nucleoproteins, CMV preparations were centrifuged on a linear sucrose gradient in a MSE BXIV zonal rotor as described above.

Isopycnic gradients were formed by layering 1.3 ml each of 60, 65 and 70% sucrose dissolved in buffered D₂O in Spinco SW 39 tubes and stood for 24 h at room temperature. CMV-TAV (1.5 mg) was loaded on to each gradient which was centrifuged for 72 h at 35000 rev/min and 25 °C in a Spinco SW 39 rotor. Banding density was determined refractometrically (Hull & Lane, 1973). For velocity gradient centrifuging of RNA preparations, linear gradients of 6 to 28% or 10 to 30% were centrifuged in a Spinco SW 25 rotor or MSE BXIV zonal rotor, respectively, using conditions described in the legends to the figures.

**Infectivity assays.** Local lesion assays were made on primary leaves of *Vigna sinensis* var. Black Eye. In most experiments, plants 7 to 12 days old were shaded 12 to 24 h before inoculation. Leaves were lightly dusted with carborundum prior to inoculation with either a cotton-tipped applicator or a glass spatula.

In some experiments, opposite half-leaves were inoculated with the sample to be assayed and unfractonated CMV RNA of known infectivity level at an arbitrary concentration (the standard). The lesion number of the sample was expressed as a percentage of the standard and recorded as an ‘infectivity index’. In most experiments, assays were done as complete or incomplete blocks (Kleczkowski, 1950). When complete leaves were used, leaves from ten plants were inoculated with a sample and the opposite leaves were inoculated with a standard inoculum.

**Gel electrophoresis of RNA.** Electrophoresis was done in 2.4%, 2.6% or 3% gels using the buffer system of Loening (1967) or of Peacock & Dingman (1968). Some assays, especially with strain D, were done on 2.5% polyacrylamide, 0.5% agarose gel according to the technique of Peacock & Dingman (1968). Except when specified, gels and buffer systems contained 0.1% SDS. In some experiments gels and samples contained 1 M- or 6 M-urea. Approximately 50 μg RNA, unheated, or heated 10 min at 50 °C, containing 1% SDS was applied on each gel and electrophoresis was carried out for 2 to 4 h at 3 to 5 mA per tube at 4 °C or at room temperature. In specified experiments, samples of RNA were prepared by mixing virus nucleoprotein (1 to 2 mg/ml) with a solution containing phosphate buffer and 1 to 1.5% SDS. Under these conditions, CMV dissociates completely into RNA and protein (Boatman & Kaper, 1973). Gels were either immediately scanned or soaked overnight in 7.5% acetic acid and scanned at 260 nm on a Gilford 2400 spectrophotometer equipped with a linear transport.

For extraction purposes (CMV-S₁ and CMV-D), electrophoresis was done in most experiments in 2.5% polyacrylamide, 0.5% agarose gel without SDS, or in 2.6% to 3% polyacrylamide gels without SDS, for 3 to 4 h at 4 °C and 4 mA per tube. Gels were stained
with 0.3% toluidine blue. After destaining in acetate buffer (10^{-2}\text{ M-sodium acetate, } 10^{-3}\text{ M-magnesium acetate pH 5.5}), partially stained bands were excised and pooled according to species. The RNA was extracted from the gels by phenol in 10^{-3}\text{ M-tris, } 10^{-3}\text{ M-Na}\text{EDTA, } 10^{-1}\text{ M-NaCl, pH 7.4.}\) After washing with ether, RNA was precipitated twice with ethanol. The electrophoresis and extraction of species of CMV-TAV RNA was as described for BBMV (Hull, 1972).

For electrophoresis of CMV-S_v RNA’s, an apparatus with the specifications described by Popescu, Lazarus & Goldblum (1971) was used. Electrophoresis of RNA liberated by disrupting the virus was performed in 2.4% polyacrylamide gels and Loening buffer diluted 1 in 10 with distilled water. Samples of 0.1 ml of 2 mg/ml virus were loaded on the gel in 1% SDS, 15% sucrose followed by an overlay of 2 ml 0.01% SDS, 5% sucrose.

**RESULTS**

**Nomenclature of CMV RNAs**

In previous publications by Diener, Scott & Kaper (1964), Kaper et al. (1965), Kaper & Geelen (1971), Kaper & West (1972) and Kaper & Waterworth (1973), the CMV RNAs were called A, B, C and C_2. In this publication we will use numbers for the RNAs as has been previously done for brome mosaic, broad-bean mottle and cowpea chlorotic mottle viruses. To avoid any confusion we list below the old name and the new number for each CMV RNA species together with the mol. wt. ($\times 10^{-6}$) (according to Kaper & West, 1972; Kaper & Waterworth, 1973). RNA C_2 = RNA-L, mol. wt. 1.07; RNA C_1 = RNA-2, mol. wt. 0.95; RNA B = RNA-3, mol. wt. 0.69; RNA A = RNA-4, mol. wt. 0.33; RNA O = RNA-5, mol. wt. 0.11; RNA OO = RNA-6, mol. wt. 0.01.

**Infectivity of fractions and mixtures of fractions from sucrose density-gradient centrifuging of CMV RNA**

Three peaks were found when either CMV-S_1 RNA or CMV-D RNA was centrifuged down a sucrose density gradient. For each strain, material from the fastest sedimenting band had the highest specific infectivity, that from the middle band had intermediate specific infectivity and that from the slowest band was not infectious (Diener et al. 1964; Lot, 1972).

In a more systematic approach using CMV-S_1, it was found that mixtures of some RNA fractions were more infectious than the fractions alone (Fig. 1, Table 1). In Table 1 it can be seen that the infectivity of fraction 19 (Fig. 1) is lower than that of the unfractonated RNA and is increased when a mixture of the smaller CMV RNAs is added but is not increased by the addition of an equivalent amount of TYMV RNA.

A better separation of CMV RNA components was achieved by using a zonal rotor (Fig. 2). Analysis by polyacrylamide gel electrophoresis of fractions from zonal rotor centrifuging showed that RNAs 3 and 4 were well separated but that RNAs 1 and 2 still formed one peak (Fig. 3A to D). The infectivity of the individual fractions was very low but the addition of RNA-3 to the mixture of RNA-1 + 2 increased the infectivity considerably. RNA-3 could not be substituted by RNA-4 and the addition of RNA-4 to the mixture of the other three RNAs had no effect (Table 2). No infectivity was associated with RNA-4 in any of the experiments in which it was tested.

Thus RNA-3 and either or both RNA-1 and RNA-2 are required for infectivity.
Three functional RNA species in CMV

Fig. 1. Infectivity of fractions and combinations of fractions after sucrose density gradient centrifuging. Extinction profile of 1.0 mg of CMV-S1 RNA after centrifuging on a linear gradient of 6 to 28% sucrose in 0.02 M-KH₂PO₄, pH 7.0. Fractions of 1.0 ml were collected. Specific infectivity, expressed as % of infectivity of unfractioned RNA at the same concentration (2 μg/ml), is indicated by the shaded area.

Fig. 2. Extinction profile of a sucrose density gradient loaded with about 3 mg CMV-S RNA. Before centrifuging the RNA was precipitated with ethanol. The pellet was redissolved in 1 ml dimethyl sulphoxide. After 30 min at 25 °C, 9 vol. of cold buffer were added, and the solution was applied to a BXIV MSE zonal rotor. Centrifuging was for 17 h at 41000 rev/min and 4 °C. Gradient was from 10% to 30% sucrose in 10⁻² M-NaH₂PO₄, 10⁻³ M-EDTA, 10⁻¹ M-NaCl, pH 7.0.

Table 1. Infectivity of fractions* and combinations† of fractions from Fig. 1

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Lesions</th>
<th>Infectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>(19) Unfractionated RNA</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>(4+7+12+16) Unfractionated RNA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(19)+(4+7+12+16) Unfractionated RNA</td>
<td>49</td>
<td>83</td>
</tr>
<tr>
<td>(19)+(4+7+12+16)</td>
<td>31</td>
<td>310</td>
</tr>
<tr>
<td>(19)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>(19)+(TYMV-RNA)†</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>Unfractionated RNA</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>(19)+(4+7+12+16)</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>(19)+(TYMV-RNA)</td>
<td>9</td>
<td>211</td>
</tr>
</tbody>
</table>

* Fractions 4, 7, 12, 16 and 19 were selected to represent each centrifuge peak from Fig. 1.
† RNAs were combined in such a way that the natural ratio was reflected in complete as well as incomplete mixtures. Concentration of RNA 1+2 (fraction 19) 56 μg/ml.
‡ Concentration of TYMV RNA = 1.44 μg/ml.
Fig. 3. Densitograms of polyacrylamide gels run with fractions of a gradient comparable to the one shown in Fig. 2. In this case 15 mg CMV-S RNA was applied to the gradient. From the fractions 0.5 ml was loaded on to the gels. After 0.5 h of electrophoresis the sucrose layer was removed, and electrophoresis was continued for 4 h. Gel A shows the CMV RNA before centrifuging. Gels B, C and D show the RNA composition of fractions comparable to 36, 30 and 21 of Fig. 2 respectively.

Table 2. Infectivity of combinations of CMV-S2 RNA species separated by zonal centrifuging

<table>
<thead>
<tr>
<th>RNA species*</th>
<th>Lesion numbers†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>-‡</td>
</tr>
<tr>
<td>1+2+3</td>
<td>85</td>
</tr>
<tr>
<td>1+2+3+4‡</td>
<td>90</td>
</tr>
</tbody>
</table>

* Undiluted fractions indicated by (o) in Fig. 2 were used. Final concentration of each RNA species was held constant.
† Average of seven half-leaves of cowpea.
‡ Infectivity of RNA-4 not tested in this experiment. Previous experiments had shown it to be non-infective on its own.

Analytical gel electrophoresis of RNAs from different CMV strains

Electrophoresis of CMV strains D, Q and TAV always revealed the six RNAs described for CMV-S by Kaper & West (1972) (Fig. 4A to E). Only in the case of CMV-S2 were RNA 5 and 6 missing (L. van Vloten-Doting, unpublished results, and Fig. 4B); this may be due to differences in the growing and isolation of the virus. The mol. wt. of the RNAs from CMV-D and Q are not significantly different from those of CMV-S (Kaper & Waterworth, 1973; Marchoux, Douine & Lot, 1973; J. M. Kaper, unpublished results). The RNAs of CMV-TAV, however, are significantly larger: $1 = 1.23 \times 10^6$; $2 = 1.17 \times 10^6$; $3 = 0.97 \times 10^6$; $4 = 0.50 \times 10^6$ and $5 = 0.17 \times 10^6$. CMV-TAV RNAs, modified with formaldehyde using the technique of Hull & Lane (1973) appeared even larger, being $1 = 36$, $2 = 27$, $3 = 4$ and $0.49 \times 10^6$ for 1 to 4 respectively, using formaldehyde modified Escherichia coli ribosomal RNAs as standards. Supporting evidence for these mol. wt. estimates came from co-electrophoresis of CMV-TAV RNA with those of brome mosaic and alfalfa mosaic viruses and using the published mol. wt. estimates of the RNAs of these two viruses (Bol, 1969; Hull, Rees & Short, 1969; Lane & Kaesberg, 1971). However the differences between mol. wt. estimates of CMV-TAV RNA species and those of other CMV strains may reflect the fact that these estimates were made in different laboratories with different techniques.

The relative proportions of RNA species from CMV-S1, D and Q, were estimated by measurements of peak areas from gel traces. RNA-1+2 are 28 to 33% of total RNA; RNA-3 40 to 45% and RNA-4 21 to 25%. For CMV-S2, the ratio of RNA 3 to 4 from
Fig. 4. Densitograms of polyacrylamide gels showing the RNA contents of different strains. (A) CMV-S₁ RNA on 2.4% polyacrylamide gel. Electrophoresis for 2 h at 5 mA/tube. (B) CMV-S₂ RNA on 3.5% polyacrylamide gel. Electrophoresis for 4 h at 5 mA/tube. (C) CMV-D RNA on 2.5% polyacrylamide 0.5% agarose gel. Electrophoresis for 4 h at 2 mA/tube. (D) CMV-Q RNA on 2.4% polyacrylamide gel. Electrophoresis for 2 h at 5 mA/tube. (E) CMV-TAV RNA on 2.6% polyacrylamide gel. Electrophoresis for 4 h at 3 mA/tube. (F) CMV-S₂ RNA eluted from 2.5% polyacrylamide gel. Electrophoresis for 7 h at 12 mA/tube. The RNAs in (A), (C) and (D) were prepared using phenol and those in (B), (E) and (F) using SDS disruption. To show the separation of RNAs 1 and 2 it was necessary to electrophorese RNAs 5 and 6 off the gel in (E) and RNA 6 off the gel in (C) and (D). The shaded areas in (F) were used in the experiment described in Table 3.
Table 3. Infectivity of combinations of CMV RNA components separated by gel electrophoresis

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Extracted from gel</th>
<th>Eluted from gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S₄⁺</td>
<td>D⁺</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 + 2</td>
<td>27</td>
<td>135</td>
</tr>
<tr>
<td>1 + 3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2 + 3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1 + 2 + 3</td>
<td>107</td>
<td>367</td>
</tr>
</tbody>
</table>

* Average of 7 half-leaves CMV-S₁ and CMV-S₂, 10 half-leaves CMV-TAV and 10 leaves CMV-D.
† RNAs were combined in a 1:1 vol. ratio as their relative proportions are the same as in the unfractionated RNA.
⁺ 2.5% polyacrylamide, 0.5% agarose gel (Fig. 4C).
§ 2.6% polyacrylamide (Fig. 4E).
|| 2.5% polyacrylamide (Fig. 4F).

Table 4. Infectivity of CMV-D RNA species after re-electrophoresis of RNA fractions eluted from gel*

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Lesion numbers†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>1 + 2</td>
<td>35</td>
</tr>
<tr>
<td>1 + 3</td>
<td>14</td>
</tr>
<tr>
<td>2 + 3</td>
<td>9</td>
</tr>
<tr>
<td>1 + 2 + 3</td>
<td>341</td>
</tr>
</tbody>
</table>

* RNA (1 + 2) bands were excised from gels after a first electrophoresis. RNA was extracted, concentrated and re-run on polyacrylamide-agarose gel. RNA 1 and 2 were then extracted separately. RNA 3 was also re-run after extraction from the first gel electrophoresis.
† Average on ten leaves of cowpea.

SDS-dissociated virus was 2:1:1:0. As the ratio of their mol. wt. is 2:12:1:0 it seems likely that these RNA species are in equimolar proportions. However, the ratios of RNA-3 to 4 from phenol prepared RNA from this strain was variable and significantly smaller. The ratio of RNA-3 to 4 for SDS-dissociated CMV-TAV was larger, being approx. 3:1.

RNA-1 and 2 were never separated well enough to enable reliable estimates of their relative proportions to be made. It is evident from the densitograms (Fig. 4) that the ratio of RNA-1 to 2 varied considerably between strains. However, there was also variation in this ratio between preparations of some of the strains, possibly due to differences in culture and isolation methods.

Infectivity of combinations of RNAs separated by gel electrophoresis

In Table 3 are shown the results of combination experiments performed with RNAs extracted or eluted from polyacrylamide or agarose-polyacrylamide gels. In all cases the mixture of RNA-1 + 2 + 3 had the highest infectivity. The mixtures of RNA-1 + 3 and RNA 1 + 2, however, always showed an intermediate infectivity suggesting possible con-
Three functional RNA species in CMV

Fig. 5. Effect of CMV coat protein on infectivity caused by CMV-S RNA 1+2 or CMV-S RNA 1+2+3. RNA (12 μg/ml) was combined shortly before inoculation, in a 1:1 vol. ratio with protein solutions of increasing concentration. ×—×, protein added to a mixture of RNA 1 and 2; ○—○, protein added to a mixture of RNA 1, 2 and 3. The ratio of RNA molecules to protein molecules was calculated using a mol. wt. of 24,000 for the protein subunit (Hill & Shepherd, 1972; van Regenmortel et al. 1972) and an average mol. wt. of 1 × 10^6 for the three large RNAs (Kaper & West, 1972). Lesion number is the average of seven half-leaves.

tamination with RNA-2 and RNA-3, respectively. For strain D the bands containing RNA-1+2 and RNA 3 were extracted, concentrated and re-run on further gels; RNAs 1, 2 and 3 were then extracted separately. The infectivity assay (Table 4) showed that the addition of RNA-3 to RNAs 1+2 gave a tenfold increase in infectivity compared with a 2- to 12-fold increase after just one cycle of separation (Table 3).

In none of the strains was RNA-4 necessary for infection.

To investigate the effect of coat protein on the infectivity of CMV-S RNA, increasing amounts of protein were added to mixtures of RNAs 1+2 and RNAs 1+2+3 (Fig. 5). It can be seen that CMV coat protein had no significant effect on the infectivity of CMV RNA.

Attempts to correlate the RNA components with nucleoprotein components

CMV sediments as a single peak on sucrose density gradients. However, analysis of the RNA content of fractions showed that the slower-sedimenting fractions were enhanced in RNA-2 (Fig. 6C, G) while the faster-sedimenting fractions contained relatively more RNA-1 (Fig. 6E and I); particles containing RNA-3 and 4 were found in all fractions but were more abundant in the fastest sedimenting fractions. Fig. 6A and F supports the conclusion drawn from Table 3 that RNA-1 is indispensable for infectivity.

Because CMV degrades in high salt concentrations (Kaper et al. 1965) it is not possible to use isopycnic CsCl gradients for separation of nucleoproteins.

CMV-TAV formed a single band at ρ = 1.34 g/ml on sucrose/D_2O isopycnic gradients (Fig. 7). Analysis of the RNA content of fractions from such a gradient (Table 5) showed that the denser fractions were richer in RNA-1 and the lighter in RNA-3.
Fig. 6. Demonstration that particles containing RNA 1 sediment faster than those containing RNAs 2, 3 and 4. (A) extinction profile of a sucrose density gradient loaded with 637 mg CMV. Centrifuging for 2.5 h at 45,000 rev/min and 4 °C in a MSE BXIV zonal rotor. ○—○, extinction; ×—×, specific infectivity at 4 μg/ml. B, C, D and E show the RNA content of unpurified CMV (B), fraction 29 (C), fraction 30 (D) and fraction 32 (E), as revealed by polyacrylamide gel electrophoresis in the presence of SDS. The fractions 29 and 30 were combined with comparable fractions of two other gradients and concentrated. The resulting material (27 mg) was applied to a second gradient shown in (F). Centrifuging for 3 h 25 min, 45,000 rev/min and 4 °C in an MSE BXIV zonal rotor. ○—○, extinction profile; ×—×, specific infectivity (at 4 μg/ml). (G), (H) and (I) show the RNA content of fractions 36 (G), 41 (H) and 43 (I) as revealed by polyacrylamide gel electrophoresis in the presence of SDS.
Three functional RNA species in CMV

Table 5. Distribution of RNA species in CMV-TAV fractions from isopycnic gradient

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>43.7</td>
<td>23.2</td>
<td>28.5</td>
<td>4.6</td>
</tr>
<tr>
<td>B</td>
<td>38.8</td>
<td>24.6</td>
<td>31.0</td>
<td>5.6</td>
</tr>
<tr>
<td>C</td>
<td>33.4</td>
<td>27.5</td>
<td>33.7</td>
<td>5.4</td>
</tr>
<tr>
<td>D</td>
<td>33.8</td>
<td>27.0</td>
<td>34.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Control</td>
<td>38.1</td>
<td>25.1</td>
<td>32.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* Fractions from Fig. 7.
† The amount of each RNA was estimated by measuring each peak area from polyacrylamide gel electrophoresis. The virus in the fractions was concentrated by precipitation with 10% PEG and then was dissociated by SDS.

DISCUSSION

From the results presented it is evident that all the CMV strains examined possess the four major RNA components described by Kaper & West (1972). The highest infectivity is always associated with a mixture of the three largest RNAs. The fact that RNA-1 + 2 is always infectious too, could suggest that RNA-3 is not really required for infectivity. Bancroft (1971) described a similar situation for cowpea chlorotic mottle virus but Bancroft & Flack (1972) concluded that the infectivity of cowpea chlorotic mottle virus RNA-1 + 2 was due to contamination with RNA-3. However, Hull (1972) was unable to show with BBMV that RNA 3 was necessary for infection. In the case of CMV, the results suggest that RNA 1 + 2 + 3 constitute the genome. In contrast to the situation, in alfalfa mosaic virus this genome does not need coat protein for activation. So although CMV has a number of physical and biological properties in common with alfalfa mosaic virus (Hull, 1969; Bol et al. 1970) the requirements for infectivity are similar to brome mosaic and cowpea chlorotic mottle viruses (Lane & Kaesberg, 1971; Bancroft & Flack, 1972).

Another indication of the multicomponent nature of viruses is the effect of dilution on infectivity (see van Vloten-Doting, Kruseman & Jaspars, 1968). In several experiments, however, the dilution curve for CMV was of the ‘single-hit’ type (unpublished results). A similar situation has been reported for brome mosaic virus (Lane & Kaesberg, 1971), broad-bean mottle virus (Kodama & Bancroft, 1964) and for cowpea mosaic virus.
A van Kammen, personal communication) all three being multicomponent viruses, the first two with a tripartite and latter with a bipartite genome. A possible explanation could be that, in these cases, one of the essential components is present in a limiting amount.

The situation concerning the distribution of RNA in the nucleocapsids is not clear. The simplest possibility is that, like brome mosaic and cowpea chlorotic mottle viruses (Lane & Kaesberg, 1971; Bancroft & Flack, 1972) there are three types of nucleoprotein particle, the densest containing RNA-1, an intermediate one containing RNA-3 + 4 and the lightest containing RNA-2. The analysis of velocity gradient bands (Fig. 6) and of isopycnic banding of CMV-TAV in sucrose/D_2O gradients (Fig. 7) indicates that this might be the case with CMV. The equimolar proportions of RNAs-3 and 4 in strain S_2 of CMV would seem to add further support to this suggestion. However, RNA-3 and 4 of CMV-TAV are not found in equimolar proportions. Furthermore for CMV-S, Lot & Kaper (1973) suggest that capsids contain, in descending order of buoyant density: RNA-1, RNA-2 + 5, RNA-3 + 4. For CMV-S_2 this could not be the case as RNA-5 is missing and also the capsids containing RNA-2 sediment slower than those containing RNA 3 + 4. These differences could be explained by suggesting that there are two kinds of RNA-2; the functional molecule and a degradation product of RNA-1 (together with RNA-5). If this is so, then in CMV-S_2 the true species 2 dominates and in CMV-S_1 it is the degradation product which is most common. This hypothesis of in vivo degradation could also explain the fluctuations of infectivity during the course of infection (Marrou, Marchoux & Migliori, 1971; Barbara & Wood, 1972). However, the possibility that RNA-5 is a host RNA, encapsidated by the virus capsid under certain circumstances, cannot be overlooked since pseudovirions have been reported for tobacco mosaic virus (Siegel, 1971).

L.V.V.-D. would like to thank Dr E. M. J. Jaspars for enthusiastic discussions and Lyda Neeleman for assistance with the experimental work; R.H. would like to thank Dawn Aldous for technical assistance, and G.M. and J.M. give thanks to C. Esvan and Marcelle Clement.

REFERENCES


Three functional RNA species in CMV


(Received 25 May 1973)