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Characterization of the Double-stranded RNA Isolated from Cowpea Mosaic Virus-infected *Vigna* Leaves

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SUMMARY

A standard procedure for the isolation of virus specific double-stranded RNA from cowpea mosaic virus-infected *Vigna* leaves is described. The double stranded RNA is characterized by its buoyant density in caesium sulphate and its melting temperature (T_m). The buoyant density and the T_m are compared with those of other double-stranded RNA's on basis of their guanine+cytosine content. The frequency distribution of the lengths of the molecules, determined by electron microscopy, indicate the occurrence of double-stranded RNA molecules specific for each of the two RNA's of cowpea mosaic virus. During hybridization with labelled cowpea mosaic virus RNA, the virus RNA is specifically incorporated into the double-stranded structure.

INTRODUCTION

Purified infectious preparations of cowpea mosaic virus (CPMV) consist of three components with sedimentation coefficients of 58S, 95S and 115S. These are referred to as top (T), middle (M) and bottom component (B) and contain 0, 24 and 33 % RNA, respectively. The different components can be separated and purified by zonal density gradient and equilibrium sedimentation in CsCl. The separate components are non-infectious, whereas mixtures of the components are infectious (Van Kammen, 1968). RNA's isolated from purified middle and bottom component are homogeneous in the ultracentrifuge and have sedimentation coefficients of 26S and 34S, respectively, corresponding with mol. wts. of $1 \cdot 45 \times 10^6$ and $2 \cdot 55 \times 10^6$. The buoyant density of CPMV-RNA is 1·628 g/cm^3 in Cs_2SO_4 (Van Kammen & Van Griensven, 1970).

To study the formation of the RNA of the M and B components, a RNase-resistant RNA fraction, which was presumably double-stranded RNA, was isolated from CPMV-infected cowpea leaves (Van Griensven & Van Kammen, 1969). This RNA was found associated with the nucleoli-chloroplast fraction of a homogenate of infected leaves. It was resistant to RNase in 0·15 M-NaCl, but not in 0·015 M-NaCl. It sedimented at 15 and 18 to 19S, respectively, suggesting the occurrence of two sizes of double-stranded RNA molecules, one corresponding with M and the other with B component RNA. Recently,

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hybridization experiments with the double-stranded RNA have been reported (Van Kammen, 1971). In this paper, further physical properties of this RNA are presented and compared with those of the replicative forms of other RNA viruses.

METHODS

Virus. The NIGERIAN strain isolate of CPMV was propagated in *Vigna unguiculata* (L) Walp var. 'Blackeye Early Ramshorne'; the YELLOW strain isolate from Nigeria was used and was purified as described (Van Kammen, 1968). Turnip yellow mosaic virus (TYMV), obtained from Dr E. M. J. Jaspars (Department of Biochemistry, University of Leyden) was grown in chinese cabbage plants, *Brassica chinensis* L. cv. Granaat and purified by the method of Steere (1956). Preparations of virus-RNA (CPMV–RNA and TYMV–RNA) were isolated by the phenol-SDS method. Three per cent (v/v) diethylpyrocarbonate was added to the extraction buffer as an inhibitor of RNases (Solymosy *et al.* 1968).

Isolation of double-stranded RNA from CPMV-infected Vigna leaves. The isolation procedure described by Van Griensven & Van Kammen (1969) was modified in a few respects: instead of bentonite, 3 % (v/v) diethylpyrocarbonate was added to the buffer used for the extraction of the RNA to act as an inhibitor of RNase; and RNase was not used to eliminate single-stranded RNA. After extraction and purification of the nucleic acids from the chloroplast-nucleoli fraction of CPMV-infected *Vigna* leaves, the nucleic acids were treated with DNase to destroy DNA. Then the solution was made 4 M in sodium chloride and frozen at −20 °C. After slowly thawing at 4 °C, the precipitated single-stranded RNA was eliminated by low-speed centrifuging. The resulting supernatant fluid was subjected to gel filtration on a Sephadex G 200 column (35 × 2·5 cm) which was equilibrated with 1 × SSC (0·15 M-NaCl, 0·015 M-Na citrate, pH 7·2). The column was eluted with 1 × SSC. The peak that eluted just after the void volume represented the high mol. wt. material including the double-stranded RNA. Its contents were collected, concentrated by vacuum evaporation and subjected to equilibrium sedimentation in Cs_2SO_4, for further purification.

Equilibrium density gradient sedimentation. Double-stranded RNA was mixed with Cs_2SO_4 solution in 1 × SSC to obtain 4·5 ml of solution with an average density of 1·60 g/cm^3. The mixture was centrifuged in a Spinco SW 39·1 rotor for 72 h at 35 000 rev/min at 15 °C. After the run, the bottom of the tube was pierced and 8 drop (0·2 ml) fractions were collected. The density of the fractions (ρ^{20}) was determined pycnometrically using calibrated micropipettes. Extinction and in the case of [^{32}P]-labelled RNA, radioactivity of the fractions were measured as described (Van Griensven & Van Kammen, 1969).

Determination of the melting temperature. The thermal transition curve of double-stranded RNA dissolved in 1 × SSC was measured by determining the change in E at 260 nm in a thermostated cuvette in a Zeiss PMQ II spectrophotometer with increasing temperature. The temperature in the cuvette was measured by means of a Cu–Co thermoresistor in a glass capillary tube through the stopper of the cuvette. The reference was melting ice. The temperature increase was about 1 °C/min.

The thermal transition point was also determined by measuring the temperature dependency of the RNase resistance of double-stranded RNA. [^{32}P]-labelled double-stranded RNA was dialysed, exhaustively, against a solution of 1 × SSC, 0·005 M-Na_3EDTA, pH 7·2, and finally against 1 × SSC. Samples containing equal amounts of RNA in 1·0 ml 1 × SSC were heated at different temperatures in sealed tubes for 20 min. The tubes were cooled in acetone-dry ice, opened and each sample was incubated with 100 μg RNase A and 1500 units RNase T_1 for 30 min at 37 °C. Trichloroacetic acid (TCA) was added to a final

concentration of 5 % and RNase resistance was determined as TCA-precipitable radioactivity.

Electron microscopy. The RNA was prepared for electron microscopy according to the Kleinschmidt spreading technique as modified by Borst *et al.* (1969). Approximately 0·1 ml of a 0·5 M-ammonium acetate solution containing 2 to 4 μg RNA/ml and 0·02 % cytochrome C was allowed to flow down a freshly split piece of mica (1 × 2 cm) on a hypophase of 0·5 M-ammonium acetate in a Petri-dish covered with parafilm (ϕ 20 cm). The surface film was picked up on carbon-coated formvar films on 200-mesh copper grids. The grids were dried, and shadowed on a turntable rotating at a speed of 3000 rev/min at an angle of 7° with 7 mg Pt–Ir alloy. The alloy was evaporated from a 1 mm tungsten wire at a pressure of 5×10^{-4} Torr at a mean distance of 5 cm from the grids. The grids were examined in a Siemens Elmiskop I and photographs were made at a magnification of 10 000, using an accelerating voltage of 60 kV. Measurements of molecules were made with a map measurer on prints at a final magnification of 60 000.

Hybridization. The procedure for the hybridization of the double-stranded RNA with [^{32}P]-labelled CPMV RNA has been described (Van Kammen, 1971). The amount of hybridization was corrected for the minor RNase resistance of the single-stranded virus RNA.

Labelling. The procedure for labelling of *Vigna* leaves with $NaH_2[^{32}PO_4]$ for the preparation of labelled virus RNA or double-stranded RNA, has been described (Van Griensven & Van Kammen, 1969).

Chemicals. $NaH[^{32}PO_4]$, carrier free was obtained from Philips Duphar (The Netherlands). RNase A, 5 × crystallized, protease free (from bovine pancreas) and RNase T_1 grade III, ammonium sulphate suspension (from *Aspergillus oryzae*) were both obtained from Sigma. Cs_2SO_4 p.a. and CsCl p.a. were obtained from Merck.

RESULTS AND DISCUSSION

After treatment with DNase and fractionation with 4 M-sodium chloride, the residual RNA from the chloroplast-nucleoli fraction of CPMV-infected leaves was subjected to gel filtration on a Sephadex G 200 column. A small peak eluted immediately after the void volume. After centrifuging to equilibrium in a Cs_2SO_4 density gradient, the RNA was found mainly in a band at a density of 1·595 g/cm^3 (Fig. 1, lower). Sometimes a smaller band was also found at an average density of 1·630 g/cm^3 (Fig. 1, upper).

We have reported that single-stranded CPMV–RNA had a buoyant density of 1·628 g/cm^3 in Cs_2SO_4 solutions (Van Griensven & Van Kammen, 1970). Therefore, the material banding at a density of 1·630 g/cm^3 was presumably contaminating single-stranded RNA. A buoyant density of 1·595 g/cm^3 may be expected for double-stranded CPMV–RNA. In Fig. 2 data from the literature on the buoyant densities (ρ^{20}) of double-stranded virus RNA's were plotted versus their G–C content. Sueoka, Marmur & Doty (1959) published that the density of DNA was dependent on its G–C content.

There was a linear relationship between the buoyant density and the G–C-content of double-stranded RNA. A change of 1 % in G–C content corresponded with a change of 0·0015 g/cm^3 in buoyant density of the RNA. The G–C content of CPMV–RNA was 40 % (Van Griensven & Van Kammen, 1970). This should correspond with a buoyant density of 1·59 according to the relationship plotted in Fig. 2. The experimental density of 1·595 g/cm^3 agreed well with this expected value. Equilibrium sedimentation in Cs_2SO_4 was used as a final purification step in the preparation of double-stranded CPMV–RNA because it was very useful for detecting contaminating single-stranded RNA.

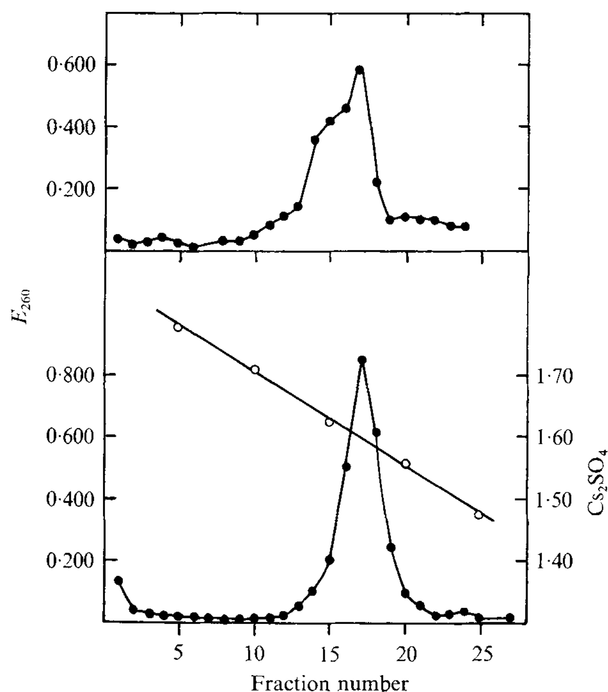

Fig. 1. Banding of the double-stranded RNA fraction from CPMV-infected *Vigna* leaves in Cs_2SO_4 after sedimentation for 72 h at 35 000 rev/min at 15 °C in a Spinco SW 39·1 rotor (●—●). The lower pattern is of a rather pure preparation; the upper pattern of a preparation contaminated with single-stranded RNA. Density of CsCl (○—○).

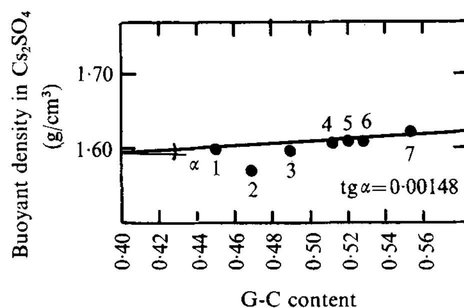

Fig. 2. The buoyant density (ρ^{20}) of double-stranded RNA (d.s. RNA) in Cs_2SO_4 versus its G–C content

	G–C content	ρ^{20} g/cm³	Reference
1 = d.s. TMV–RNA	0·45	1·601	Burdon *et al.* (1964)
2 = d.s. EMC–RNA	0·47	1·57	Montagnier & Sanders (1963)
3 = d.s. poliovirus–RNA	0·49	1·60	Bishop & Koch (1967)
4 = d.s. R17–RNA	0·51	1·606	Erikson & Franklin (1966)
5 = d.s. MS2–RNA	0·52	1·609	Weissmann *et al.* (1964)
6 = d.s. fr–RNA	0·53	1·609	Kaerner & Hoffmann-Berling (1964)
7 = d.s. TYMV–RNA	0·55	1·617	Bové (1967); Bockstahler (1968)

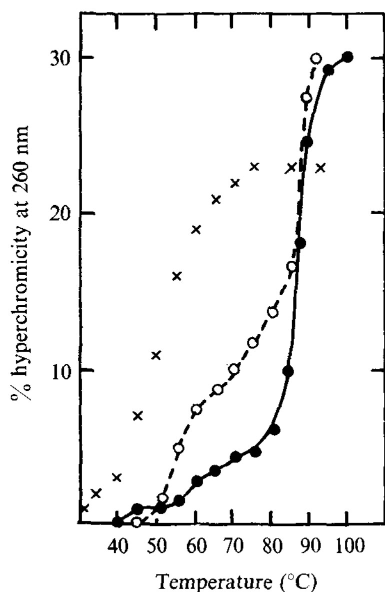

Fig. 3

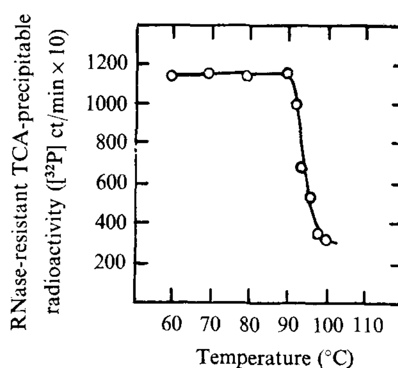

Fig. 4

Fig. 3. The temperature-dependent hyperchromicity of RNA fractions from a Cs_2SO_4 gradient (see Fig. 1), isolated from CPMV-infected *Vigna* leaves. × ×, indicates the hyperchromicity of single-stranded CPMV in 1 × SSC. ●—●, RNA $\rho_{av.}$ = 1·60 (double-stranded); ○ -- ○, RNA, $\rho_{av.}$ = 1·63 (single-stranded contamination).

Fig. 4. The thermal helix-coil transition curve of CPMV-specific double-stranded RNA determined by RNase resistance. Equal samples of [32P]-labelled double-stranded RNA in 1 × SSC were heated for 20 min at the indicated temperature, chilled quickly and incubated with RNase. RNase resistance was measured as TCA-precipitable radioactivity.

Table 1. *The sensitivity to ribonuclease of fractions of RNA isolated from a Cs_2SO_4 gradient as measured by the increase in E_{260}*

Fraction	E_{260} Before RNase incubation	E_{260} After RNase incubation*	Increase in E_{260} (%)
Double-stranded RNA (buoyant density 1·595 g/cm³)	0·197	0·198	0
Contaminating single-stranded RNA (buoyant density 1·63 g/cm³)	0·206	0·226	9·7
CPMV–RNA	0·323	0·424	31·3

* Incubation for 5 min with 100 μg RNase A + 150 units RNase T_1/3 ml.

The nature of the RNA fractions isolated from the Cs_2SO_4 gradient was confirmed by further tests. The RNA with a buoyant density of 1·595 g/cm³ was resistant against RNase; it did not show an increase in E_{260} if incubated with a mixture of RNase A and RNase T_1 in 1 × SSC (Table 1), whereas incubation with these RNases increased the E_{260} of single-stranded RNA more than 30 %. The material banding at a density of 1·63 g/cm³ in Cs_2SO_4 showed only slight hyperchromicity on incubation with RNases (Table 1) because it was presumably contaminated with RNase resistant material from the 1·595 g/cm³ band. These data confirmed our earlier findings on the RNase resistance of the double-stranded RNA

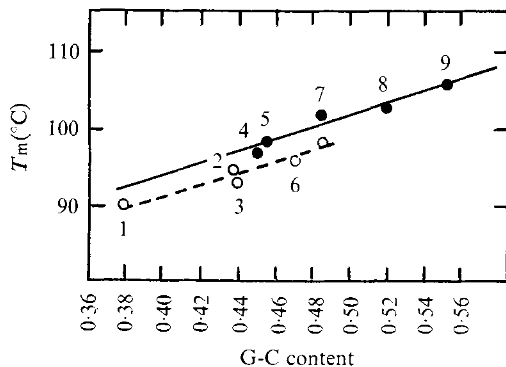

Fig. 5. The melting temperature (T_m) of double stranded-RNA's plotted versus their G–C content. The T_m's were determined in 0·15 M-sodium chloride by measuring the hyperchromicity at 260 nm (○) or the RNase resistance (●) in dependency on the temperature.

	G–C content	T_m (by hyper-chromicity)	T_m (by RNase resistance)	Reference
1 = Wound tumor virus RNA	0·38	90	—	Gomatos & Tamm (1963)
2 = Rice dwarf virus RNA	0·438	95	—	Miura, Kimura & Suzuki (1966)
3 = Reovirus RNA	0·44	93	—	Gomatos & Tamm (1963)
4 = d.s. TMV-RNA	0·45	—	97	Burdon *et al.* (1964)
5 = d.s. AMV-RNA	0·455	—	98	Pinck, Hirth & Bernardi (1968)
6 = d.s. EMC-RNA	0·47	96	—	Montagnier & Sanders (1963)
7 = d.s. Newcastle disease virus-RNA	0·485	98	102	Kingsbury (1966)
8 = d.s. MS2-RNA	0·52	—	103	Weissmann *et al.* (1964)
9 = d.s. TYMV-RNA	0·553	—	106	Bové (1967); Bockstahler (1967)

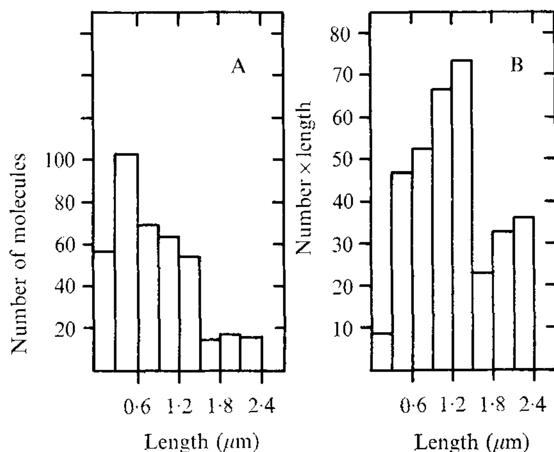

Fig. 6. A histogram of the length distribution of 397 CPMV specific double-stranded RNA molecules. The RNA was prepared for electron microscopy according to the Kleinschmidt procedure (see Methods). Measurements of molecules at a final magnification of ×60 000. (A) Length versus number of molecules. (B) Length versus number of molecules × length.

Table 2. *Hybridization of double-stranded CPMV–RNA with [^{32}P]-labelled CPMV–RNA and TYMV–RNA*

Composition of the hybridization mixture: Double-stranded CPMV–RNA (μg)	Composition of the hybridization mixture: Virus RNA added	Radioactivity of the added RNA [^{32}P] (ct/min)	Radioactivity in the double-stranded RNA after hybridization (ct/min)
2	2·5 μg CPMV–RNA	3·250	360
2	5·0 μg CPMV–RNA	6·500	580
2	10·0 μg CPMV–RNA	13·000	610
2	50·0 μg TYMV–RNA	365·000	0

from CPMV-infected leaves (Van Griensven & Van Kammen, 1969). The RNA found at a density of 1·595 g/cm^3 had a much higher thermal hypochromicity than that of single-stranded RNA. The helix-coil transition in 1 × SSC occurred in a well-defined rather small temperature range (Fig. 3). The T_m in 1 × SSC was 87 °C. The contaminating RNA with an average density of 1·63 g/cm^3 showed a less abrupt transition (Fig. 3). Fig. 4 presents the results of a determination of the melting temperature measured by RNase resistance in 1 × SSC. Samples of [^{32}P]-labelled RNase resistant RNA were heated at the different temperatures indicated and, after rapid cooling, incubated with RNases. The RNase resistance was measured as TCA-precipitable radioactivity to give a melting temperature (T_m) of 94 °C.

Fig. 5 shows a plot of a number of known T_m's versus G–C content. The straight line through the points was calculated by the least squares method. A T_m of 94 °C corresponded with a G–C content of about 0·40, which was in good agreement with the G–C content of CPMV–RNA (see above). The broken line in Fig. 5 represented the relationship between the G–C content and the T_m as determined by the thermal hypochromicity. Melting-points determined by temperature-dependent RNase resistance were consistently higher than those determined by thermal hypochromicity, possibly because of a partial reversion of the thermal transition caused by the cooling before RNase incubation. The experimental value of T_m of 87 °C determined by thermal hypochromicity fits reasonably well on the line given in Fig. 5. The discrepancy between the experimental and the predicted value of 90 °C might be partly explained by the experimental conditions, and partly by the considerable heterogeneity in size of the double-stranded RNA molecules (see Fig. 6).

Fig. 6A shows a frequency distribution of the lengths of 397 molecules of RNase-resistant RNA with a density of 1·595 g/cm^3 in Cs_2SO_4. The molecules varied in length from 0·1 μm to 2·4 μm. The large number of small molecules clearly shows that the material was considerably degraded. In Fig. 6B the relative frequency distribution of the length of the molecules (the amount of RNA (number of molecules × length) having a certain length) was plotted. Such a relative frequency distribution was analogous to an extinction distribution pattern in a sucrose gradient because it reflected the amount of RNA rather than the number of molecules. The distribution curve shows two maxima, one in the size range of 1·2 to 1·4 μm and another in the range 2·0 to 2·4 μm. Middle and bottom component RNA had mol. wts. of $1·45 \times 10^6$ and $2·55 \times 10^6$, which corresponded with chain lengths of about 4500 and 7900 nucleotides, respectively. The distance between nucleotides in double-stranded RNA has been determined by various methods to be 3·17 Å for double-stranded R17-RNA (Granboulan & Franklin, 1966); 2·7 Å for double-stranded reovirus RNA (Arnott *et al.* 1968) and 2·75 Å for double-stranded TMV–RNA (Nicolaieff *et al.* 1970).

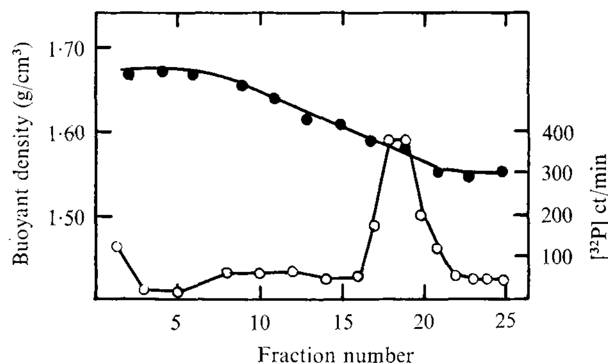

Fig. 7. Banding of the production of hybridization between double-stranded RNA from CPMV-infected *Vigna* leaves and [^{32}P]-labelled CPMV–RNA, after equilibrium density gradient sedimentation in Cs_2SO_4. After hybridization the reaction mixture was incubated with RNase and subjected to Cs_2SO_4 density gradient sedimentation in a Spinco SW 25 I rotor at 20 000 rev/min for 96 h at 15 °C. ○—○, radioactivity; ●—●, density, (ρ^{20}) of Cs_2SO_4 in g/cm³.

Assuming that the molecules in the size range 1·2 to 1·4 μm corresponded with double-stranded middle component RNA and those in the range 2·0 to 2·4 μm were double-stranded bottom component RNA, we calculated 2·9 and 2·8 Å to be the distance between nucleotides, which was in good agreement with the published values. This presents independent evidence for the earlier proposition (Van Griensven & Van Kammen, 1969) that middle component and bottom component RNA each induce the formation of its own replicative form in the host cell. Whether the double-stranded RNA existed as such in the infected cell or arose because the isolation procedure induced hydrogen bonding was an unanswered question. The virus specificity of the double-stranded RNA was demonstrated by hybridization experiments with [^{32}P]- or [^{3}H]-labelled CPMV–RNA (Van Kammen, 1971).

In Table 2 the hybridization of 2 μg of double-stranded RNA with [^{32}P]-labelled CPMV–RNA and TYMV–RNA was compared. It can be seen that there was no hybridization with TYMV–RNA, whereas a considerable exchange with labelled CPMV takes place after annealing. When the reaction product after annealing was incubated with RNase and then subjected to equilibrium centrifugation in Cs_2SO_4, the radioactive hybrid RNA, banded at a density of about 1·59 (Fig. 7), indicating that labelled CPMV–RNA had become incorporated into the double-stranded RNA during the hybridization procedure.

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