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The Two-step Purification of Ribosomal RNA and Plant Viral RNA by Polyacrylamide Slab Gel Electrophoresis

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Abstract

The requirement for purified plant viral RNAs for sequence characterization by hybridization analysis using complementary DNA led to the development of a routine two-step procedure for their purification. The method was worked out using *Escherichia coli* ribosomal RNAs and then applied to four plant viral RNAs, all of which contain four major RNA components. The first of the two steps involves the electrophoresis of native RNA on 2·8% polyacrylamide slab gels, the location of the RNA bands by brief staining and the recovery of the RNA from each gel band by electrophoretic elution. The eluted RNA, concentrated by ethanol precipitation, was then run on a second gel after a denaturation step to release nicked and aggregated RNA fragments. Each RNA band was again located by staining and recovered by electrophoretic elution and ethanol precipitation. The 16-S and 23-S ribosomal RNAs and the plant viral RNAs were obtained in overall yields of 40 and 8–16% respectively. RNAs so purified have been successfully used for the preparation of complementary DNA by two different methods.

Introduction

Of the 22 groups or families of plant viruses classified so far, nine contain viruses with two or more single-strand RNA species encapsidated in two or more viral particles (Fenner 1976). In the case of these nine groups or families, the viral genome is functionally divided since the coinfection of either two or three RNA species is required for infectivity (Jaspars 1974; Van Vloten-Doting 1976). These viruses are called the bipartite and tripartite viruses respectively.

Cucumber mosaic virus (CMV), the main virus considered below, is an example of a tripartite virus and is the type member of the cucumoviros group (Fenner 1976). When CMV RNA is fractionated by electrophoresis on 2·4% polyacrylamide gels, four major and at least two minor RNA species are found (Kaper and West 1972; Peden and Symons 1973; Lot *et al.* 1974; Lot and Kaper 1976) and these are designated RNAs 1–6 in order of decreasing molecular weight. The four major RNAs, RNAs 1–4, have molecular weights of $1{\cdot}35\times10^6$, $1{\cdot}16\times10^6$, $0{\cdot}85\times10^6$ and $0{\cdot}35\times10^6$ respectively, whilst the two minor RNAs, RNAs 5 and 6, have molecular weights of about $0{\cdot}12\times10^6$ and $0{\cdot}05\times10^6$ respectively (Peden and Symons 1973). All the essential genes for virus multiplication are located on RNAs 1–3 and the coat protein gene is present on both RNAs 3 and 4 (Peden and Symons 1973; Habili and Francki 1974; Lot *et al.* 1974; Schwinghamer and Symons 1975, 1977).

It is obviously of considerable importance to investigate the primary structure of the RNAs of these bi- and tripartite viruses. Such information is necessary for the proper classification of these viruses and for understanding the molecular basis of their replication. Our current approach is to prepare complementary DNA (cDNA) to purified viral RNAs and to use this in hybridization analysis with the viral RNAs. In this way we are characterizing the extent of sequence homology between the RNAs within several tripartite viruses as well as between the corresponding RNAs in three strains of cucumber mosaic virus (Gould and Symons 1977; Gould *et al.* 1977; Gonda and Symons, unpublished data).

The requirement for purified viral RNAs for the preparation of cDNA to be used in this sensitive and powerful hybridization analysis led to the development of the routine and reliable method described here for the rigorous purification of plant viral RNAs. The overall procedure involves two polyacrylamide slab gel electrophoresis steps with the electrophoretic elution of RNA after each step. It has evolved from methods previously described by us using both polyacrylamide slab gels and sucrose gradient centrifugation (Schwinghamer and Symons 1975, 1977). The method was worked out using *Escherichia coli* ribosomal RNA and then applied to CMV RNA. It has also been used successfully in the purification of the RNAs of other tripartite viruses.

Rationale and Outline of the Two-step Purification Procedure

In the first electrophoretic step, RNA in its native state (2–5 mg) was fractionated on one or two 2·8% polyacrylamide slab gels (18 by 16 cm, 4–5 mm thick) by electrophoresis overnight at 40 mA per gel (faster running at higher currents caused vertical streaking of the RNA bands). The RNA bands were located by brief staining with toluidine blue followed by destaining with water and were then excised. Since CMV RNA contains significant amounts of nicked and aggregated RNA (Schwinghamer and Symons 1975), it was important not to denature the RNA prior to the first step since these nicked and aggregated RNA fragments would be released on denaturation and would contaminate RNA species of lower molecular weight.

In the past we have recovered RNA from acrylamide gels by phenol–sodium dodecyl sulphate (SDS) extraction of gel pieces followed by ethanol precipitation of the RNA from the aqueous extract (Peden and Symons 1973; Schwinghamer and Symons 1977). The appreciable quantities of polyacrylamide also obtained were then removed by centrifugation of the RNA on a sucrose density gradient (Schwinghamer and Symons 1977). In the present method, a simple electrophoretic elution device was used in which RNA was completely eluted in 2–4 h from homogenized gel pieces by electrophoresis into a chamber sealed with dialysis tubing and holding about 3 ml of buffer. The toluidine blue also migrated into the chamber and was removed by extraction of the aqueous phase with phenol–chloroform before ethanol precipitation of the RNA. There was only very minor contamination of the RNA by polyacrylamide whilst the phenol–chloroform extraction served to remove any contaminating ribonucleases.

For the second step, the RNA species isolated from the first step were heated at 80°C for 4 min in 1 mM EDTA, pH 7, in order to release nicked and aggregated RNA fragments prior to the second electrophoretic separation on a smaller 2·8% polyacrylamide slab gel. Each RNA band was again located by brief staining followed

by destaining in water. The usual pattern obtained was a major band with a blur of lower-molecular-weight RNA running faster than it. The RNA was recovered by electrophoretic elution as before and was checked for purity and intactness by electrophoresis in analytical polyacrylamide tube gels.

Materials and Methods

The Q- and P-strains of CMV (Q-CMV and P-CMV) were purified as described by Peden and Symons (1973) and the M-strain (M-CMV) as described by Mossop *et al.* (1976). Tomato aspermy virus (TAV) and alfalfa mosaic virus (AMV) were purified by the methods of Habili and Francki (1974) and Van Vloten-Doting and Jaspars (1972) respectively. Viral RNA was isolated from purified virus by phenol–SDS extraction (Peden and Symons 1973) and stored at −15°C in 0·1 mM EDTA, pH 7. Ribosomal RNA (both unlabelled and labelled with ^{32}P) was purified by phenol–SDS extraction of ribosomes from *E. coli* MRE600, kindly provided by D. J. Eckermann. [^{125}I]Ribosomal RNA was prepared by the method of Prensky (1976). Acrylamide and methylene bisacrylamide were recrystallized before use (Loening 1967). Formamide was purified by stirring with dry mixed-bed ion-exchange resin for several hours followed by partial freezing at 0°C; the purified formamide was stored at −15°C.

Sterile techniques were routinely used to reduce possible contamination by ribonucleases. Solutions and glassware were sterilized by autoclaving and forceps and spatulas by heating in a bunsen flame. Perspex apparatus was soaked in 0·1 M NaOH and well washed with sterile water before use. Disposable plastic syringes (5 ml) and 18-gauge needles were autoclaved (the barrels and plungers of these syringes must be kept separate). Glass plates used in the slab gel apparatus were well scrubbed after use and stored in alcoholic NaOH (alcohol saturated with NaOH); before use, they were well washed with water, 1 M HCl, water, ethanol, and acetone.

First Slab Gel Electrophoresis of Native RNA

In general, the simple vertical slab gel apparatus and techniques of De Wachter and Fiers (1971) have been used. Electrophoresis buffer was 40 mM tris–acetate, 20 mM sodium acetate, 2 mM EDTA, pH 7·5 (Loening 1967). Large slab gels with 20 by 20 cm glass plates and 4·5 mm Perspex spacers were suitable for 1·5–3 mg of RNA. The spacers were sealed with a thin layer of silicone grease on their outside edges and the plates held firmly together with three lots of masking tape plus spring clips. After sealing the bottom of the plates with parafilm, a bottom layer of about 15 ml of 7·5% acrylamide–0·375% bisacrylamide was poured in and allowed to set. This was prepared by adding 40 µl of TEMED* and 0·4 ml of freshly prepared 10% ammonium persulphate to 20 ml of degassed 7·5% acrylamide–0·375% bisacrylamide in electrophoresis buffer. For the main 2·8% gel, 120 µl of TEMED and 1·2 ml of 10% ammonium persulphate were added to 140 ml of a degassed, freshly prepared solution of 2·8% acrylamide–0·14% bisacrylamide in electrophoresis buffer and the solution run into the glass plates to about 2·5–3·0 cm from the top. The liquid was overlaid to a depth of about 0·5 cm with t-butanol and the gel allowed to set for 1–2 h. The t-butanol was then removed and replaced with electrophoresis buffer.

The slab gel was connected to the upper buffer vessel with a paper wick between two pieces of plastic sheeting to prevent evaporation. Three layers of Whatman 3 MM paper were used and were well washed with 0·1 M NaOH and electrophoresis buffer before use. About 1500 ml of electrophoresis buffer were used in the two electrode vessels for each gel. Pre-electrophoresis was for about 1 h at 30–40 mA per gel. RNA samples (1·5–3 mg) in 0·5–1·5 ml 10–20% sucrose, 1 mM EDTA, pH 7, containing a trace of bromophenol blue, were loaded on top of the gel with an autozero pipette connected to a 1-ml plastic syringe. After the wick had been carefully replaced, electrophoresis was carried out overnight at room temperaure at 40 mA per gel.

At the end of the run, the top glass plate was carefully levered off and the gel transferred onto a piece of commercial fibreglass mesh (25 by 50 cm) which was resting in a plastic dish with the ends of the mesh running over the edges of the dish. The fibreglass mesh allowed easy handling of the slab gels. The mesh and dish were washed with 0·5 M NaOH and then water before use. The gel was stained with 0·01% toluidine blue in water for 1–2 min and then destained in the dark with

* TEMED = *N, N, N′, N′*-tetramethyl-ethylenediamine.

several changes of water over 2–3 h. The gel was transferred to a sheet of alkali-washed Perspex and the stained bands carefully cut out with a scalpel blade. Each gel strip was cut into pieces and transferred to two 1·3 by 10 cm test tubes, snap frozen in ethanol–dry ice and stored at −15°C. Each gel strip from these large gels was divided into two because two separate elution tubes were used for the elution of RNA in each band (see below).

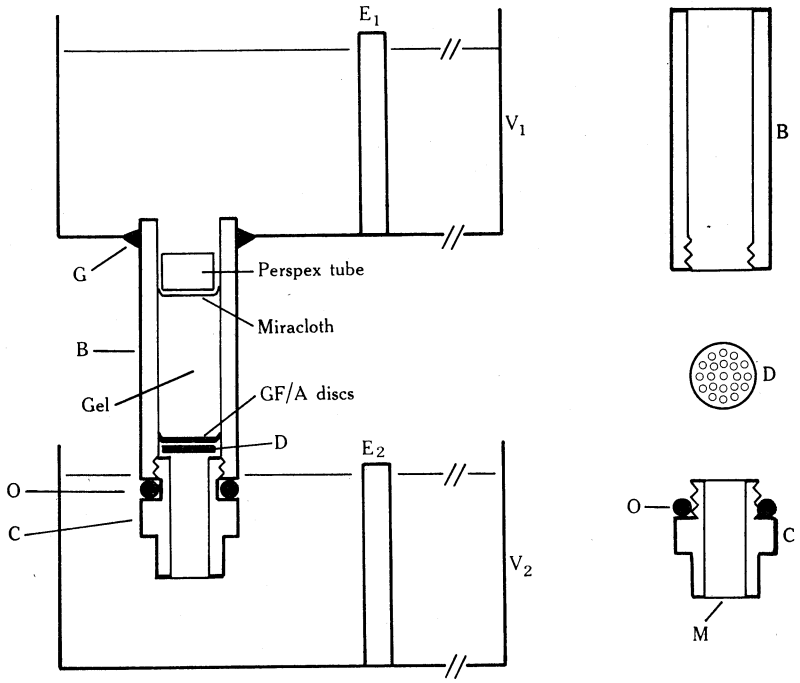


Fig. 1. Apparatus for the electrophoretic elution of RNA. V_1 and V_2 are the upper and lower electrode vessels, respectively, and were made from commercial round Perspex dishes 17 cm in diameter and 8·5 cm deep. Platinum electrodes E were attached to Perspex poles in the centre of each vessel. Four radial holes were drilled to take grommets G with a 2·5-cm diameter opening. The Perspex barrel B was 7·0 cm long with an outside diameter of 2·55 cm and an internal diameter of 1·7 cm and contained a female thread as shown (1·0 cm long, 1·5 mm pitch). The Perspex disc D was 1·65 cm in diameter, 0·2 cm thick and contained about 20 holes of 0·15 cm diameter. The elution chamber C was 3·0 cm long with a 1·0-cm male thread (1·5 mm pitch), an internal bore of 1·1 cm and an O-ring O. Dialysis membrane M was sealed to the bottom of chamber C with cyanoacrylate ester glue. Barrel B and chamber C were screwed together as shown to give the elution tube. For further details see Materials and Methods. At the end of each run, the dialysis membrane was removed by soaking chamber C in 0·1 M NaOH.

Electrophoretic Elution of RNA

The simple apparatus of Fig. 1 was used and proved to be very convenient. Dialysis tubing M was glued to the bottom of chamber C with cyanoacrylate ester glue and allowed to set for at least 2 h. Excess dialysis membrane was trimmed off and the chamber well washed with electrophoresis buffer (see below). The chamber was screwed into barrel B to give the elution tube which was inserted into a rubber grommet G set in the upper reservoir vessel V_1. The latter was a commercial circular Perspex dish 17 cm in diameter and 8·5 cm deep and contained four holes with a platinum electrode E_1 fixed on a Perspex pole in the centre. The inside of the dish and elution tubes were rinsed several times with 0·1 M NaOH and then with water just prior to use.

Sterile electrophoresis buffer containing 0·2% SDS was then added to each elution tube to about 0·5 cm above the top of chamber C and a single perforated disc D was dropped into place. Three well-washed 1·9-cm diameter circles of Whatman GF/A glass fibre (punched out of 2·5-cm diameter GF/A discs and a little larger than the diameter of barrel B) were pushed into place on top of disc D with a spatula. The gel sample (one-half of each RNA band) was then added to the barrel by forcing it through an 18-gauge needle attached to a 5-ml plastic disposable syringe, care being taken not to disturb the glass fibre discs. A few millilitres of electrophoresis buffer plus SDS were added with a Pasteur pipette and the gel stirred with the pipette to ensure even suspension. In order to hold the gel suspension in place, a 2·5-cm diameter circle of well-washed Miracloth (Calbiochem) was placed across the top of barrel B and then forced down in the barrel and held in place on top of the gel suspension with a piece of hollow Perspex tubing (outside diameter 1·6 cm). The upper reservoir was filled with 800 ml of electrophoresis buffer plus 0·2% SDS and the elution chamber immersed in a lower electrode reservoir of the same size (Fig. 1).

Electrophoretic elution was carried out in subdued light at 40–45 mA per elution tube for 3–4 h. All the toluidine blue stain migrated into chamber C and was completely eluted in 20–30 min. At the end of the run the upper reservoir buffer was drawn off and the colourless gel suspension removed. The chamber C was carefully unscrewed, the disc D removed (the glass fibre filters remained in place) and the chamber contents removed with a Pasteur pipette; some of the contents were drawn into the pipette and blown out several times to ensure rinsing of the dialysis membrane. Finally each chamber was rinsed with 0·5 ml of 2·0 M sodium acetate. Tests with both [^{32}P]- and [^{125}I]ribosomal RNA showed that this rinsing procedure removed essentially all the eluted RNA from the elution chamber.

The toluidine blue was removed by vortex mixing the RNA solution from each chamber with 0·5 ml chloroform plus 0·5 ml water-saturated phenol followed by centrifugation to separate the phases; all the blue colour was now in the bottom phase. The RNA was then precipitated from the upper aqueous phase by the addition of 2·0–2·5 volumes (about 8 ml) of cold ethanol and storage overnight at −15°C. Centrifugation at 10000 rev/min for 15 min at −5°C gave a typical RNA gel pellet (where more than about 200 μg of RNA was present) with essentially no visible contamination by polyacrylamide. After draining the RNA pellet and removing the residual liquid *in vacuo*, the pellet was dissolved in 0·4 ml of 1 mM EDTA, pH 7.

Second Slab Gel Electrophoresis Step of Denatured RNA

For the second step two sizes of slab gels have been used. For running a single RNA sample, 2·8% gels were poured into 15 by 16 cm glass plates with 3-mm Perspex spacers with the usual bottom sealing layer of 7·5% gel (see above). However, it was usually more convenient to prepare a 2·8% gel in 20 by 20 cm glass plates with 3-mm spacers and to provide two compartments by pushing a tightly fitting piece of plastic tubing (1·5 cm long) down in the centre between the glass plates and 1–2 mm into the gel just prior to the pre-electrophoresis. In this way, two samples could be run on each gel with the additional advantage that the mobilities of the two RNAs could be compared.

For this second run, each RNA sample in 1 mM EDTA, pH 7, was heated at 80°C for 4 min in order to release aggregated and nicked RNA, prior to adding 40% sucrose in 1 mM EDTA, pH 7, to a concentration of 10–20% together with a trace of bromophenol blue and applying the sample to the gel. Electrophoresis was carried out overnight at 20–30 mA per gel at room temperature. RNA bands were recovered by staining and the RNA electrophoretically eluted and recovered by ethanol precipitation as already described. To ensure removal of residual phenol and SDS, the precipitated RNA was dissolved in 1·0 ml of 0·2 M NaCl, 50 mM sodium acetate, 1 mM EDTA, pH 5·0, and reprecipitated by the addition of 2·0 ml ethanol and storage overnight at −15°C. The final RNA precipitate was dried *in vacuo*, dissolved in 100–200 μl 0·1 mM EDTA, pH 7, and stored at −15°C.

The purity and intactness of the purified RNAs were checked by electrophoresis on 0·6 by 9 cm 2·4% polyacrylamide tube gels. Approximately 3 μg of RNA in 0·1 mM EDTA, pH 7, was dried *in vacuo*, dissolved in 30 μl of 99% formamide, 1 mM EDTA, and heated at 60°C for 1·0 min to ensure complete denaturation. The sample was then applied to the top of the tube gel and subjected to electrophoresis at 5 mA per gel for 2·5 h. After staining in 0·05% toluidine blue for 10 min and destaining in repeated changes of water, the gel was scanned at 600 nm using a Gilford spectrophotometer with linear transport attachment.

Results and Discussion

Purification of 23-S and 16-S E. coli *Ribosomal RNA*

The two-step slab gel procedure (see Materials and Methods) was worked out using RNA extracted from *E. coli* ribosomes. The fractionation obtained on the first 2·8% slab polyacrylamide gel is shown in Fig. 2*a*. The well-separated 23-S and 16-S RNA bands were cut out and the RNA electrophoretically eluted as described in Materials and Methods. There was an 88% recovery of 23-S plus 16-S RNA at this stage (Table 1); 5-S RNA, which makes up 2·4% by weight of ribosomal RNA, was not recovered. After the second electrophoretic step (gel pattern not shown), the final yield of 23-S plus 16-S RNA was 40% relative to the original 2·14 mg (Table 1); the greater loss of RNA in the second step can be attributed mostly to the removal of nicked and aggregated fragments by heat denaturation of the 23-S and 16-S RNAs prior to the second slab gel (Schwinghamer and Symons 1975). The expected 2 : 1 ratio by weight (molar 1 : 1 ratio) for the 23-S and 16-S RNAs was found after both steps (Table 1) which indicates no differential loss of one of the RNAs.

Table 1. Recovery of 23-S and 16-S ribosomal RNA after the two electrophoretic elution steps

The two-step procedure for the polyacrylamide slab gels and electrophoretic elution were carried out as described in Materials and Methods

RNA	Initial RNA (μg)	RNA after first elution		RNA after second elution		
		μg	% of initial RNA	μg	% of initial RNA	% Intactness of RNA[A]
16S	—	640	30	285	13	89
23S	—	1250	58	570	27	91
Total	2140	1890	88	855	40	—

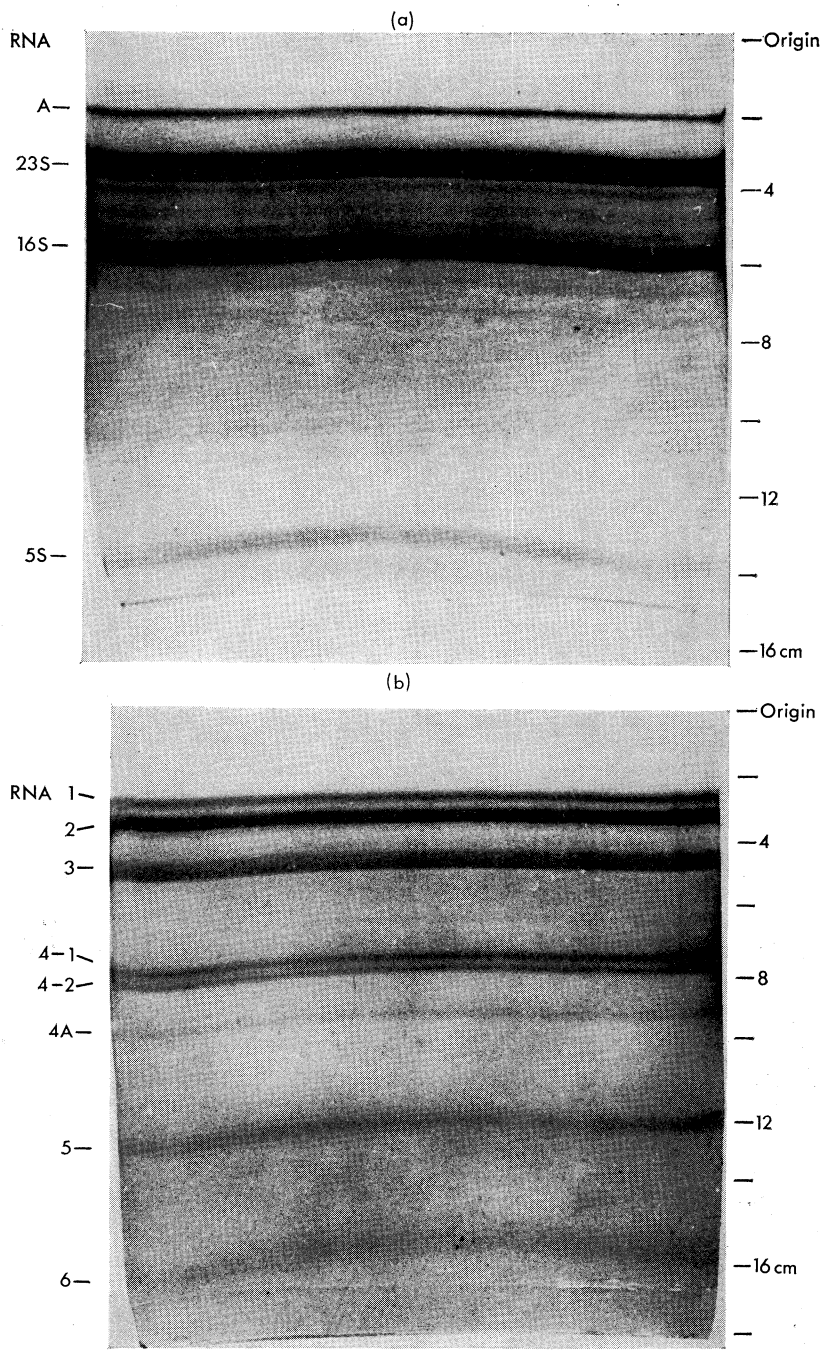
[A] Data obtained from scans of stained gels in Fig. 3. Values calculated from the area under the main peak relative to total area (main peak plus shoulder on leading side of peak).

The purified RNAs appeared free of contaminating polyacrylamide as shown by the absence of visible contaminants in the opaque RNA gel after ethanol precipitation. In addition, spectral analysis of the final RNAs showed typical RNA spectra with $A_{260} : A_{230}$ ratios of 2·3–2·7 which compared very well with the usual ratio of 2·7 found for the starting material. Finally, the purity and intactness of each RNA was checked by electrophoresis on 2·4% aqueous tube gels after denaturation of the RNA by heating in 99% formamide, 1 mM EDTA, pH 7, at 60°C for 1 min. Scans of the stained gels (Fig. 3) showed a single sharp peak for the 23-S and 16-S RNAs

Fig. 2. Polyacrylamide slab gel electrophoresis of (*a*) native *E. coli* ribosomal RNA and (*b*) native CMV RNA. For (*a*) ribosomal RNA (2·3 mg in 0·8 ml of 0·1 mM EDTA, pH 7) was mixed with 0·4 ml of 40% (w/v) sucrose in 1 mM EDTA, pH 7, and layered on top of a large 2·8% polyacrylamide slab gel prepared as described in Materials and Methods. For (*b*) RNA (2·2 mg in 0·6 ml of 0·1 mM EDTA, pH 7) was mixed with 0·3 ml of 40% (w/v) sucrose in 1 mM EDTA, pH 7, and layered on top of a large 2·8% polyacrylamide slab gel prepared as described in Materials and Methods. For both (*a*) and (*b*) electrophoresis was at 40 mA for 14·5 h at room temperature. The gels were stained in 0·01% toluidine blue in water for 10 min and destained in several changes of water. These gels were prepared especially for photography and were stained for 10 min rather than the usual 1–2 min. In (*a*) the minor band A has not been characterized.

with a small amount of material running just ahead of each peak; these presumed breakdown products represented about 10% of the total RNA as determined from the gel scans (Fig. 3; Table 1).

Hence, the two-step procedure provided 23-S and 16-S RNA which was essentially pure and approximately 90% covalently intact in an overall yield of 40%.



Purification of CMV RNAs

The fractionation of Q-CMV RNA on the first polyacrylamide slab gel gave the pattern shown in Fig. 2*b*, which is typical of many that have been obtained. RNAs 1 and 2 (molecular weights $1 \cdot 35 \times 10^6$ and $1 \cdot 16 \times 10^6$ respectively) were clearly separated and could be excised separately without difficulty. RNA 3 was a single band whereas RNA 4 was always resolved into two bands designated 4-1 and 4-2 as shown (Fig. 2*b*). Usually RNA 4-2 was in excess of RNA 4-1 although the reverse was true for the

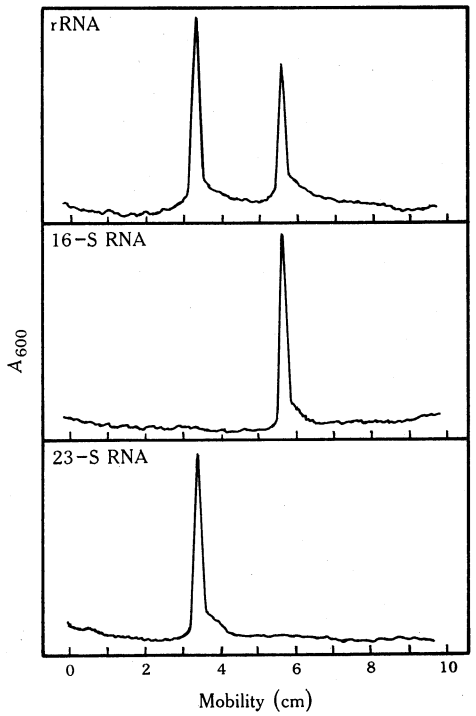


Fig. 3. Check on purity and intactness of 16-S and 23-S ribosomal RNAs after two electrophoretic purification steps. RNA samples (*c.* 4 μg) in 99% formamide, 1 mM EDTA, pH 7, were heated at 60°C for 1·0 min before electrophoresis on analytical 2·4% polyacrylamide tube gels as described in Materials and Methods. After staining for 10 min in 0·05% toluidine blue in water and destaining in water, the gels were scanned at 600 nm.

CMV RNA preparation used for the data of Table 2. Neither we (Peden and Symons 1973; Schwinghamer and Symons 1975, 1977) nor others (Lot *et al.* 1974; Lot and Kaper 1976) have reported the splitting of RNA 4 into two bands although the presence of two components is evident from published gel patterns (Lot *et al.* 1974; Lot and Kaper 1976; Schwinghamer and Symons 1977). The minor bands of RNAs 4A, 5 and 6 are always present in our preparations of Q-CMV.

The results for the two-step purification of CMV RNA are given in Table 2. Starting with 5·32 mg of RNA which was run on two large slab gels (see Materials and Methods), total recovery of RNA after the first step was about 50%. Part at least of this low recovery could be attributed to appreciable quantities of RNA fragments which formed a light blue background to the main RNA bands seen on the stained slab gels. An even greater loss occurred after the second slab gel step which involved the denaturation by heat of the RNA before electrophoresis. We have previously shown that CMV RNA contains appreciable amounts of nicked and aggregated RNA molecules which can be dissociated by heat (Schwinghamer and Symons 1975). The usual pattern obtained with the second gel was a major band of RNA with a blur of lower-molecular-weight RNA running faster than it.

The final recovery of the eight RNA bands totals 16% relative to starting RNA (Table 2) and is to be compared with 40% for the two ribosomal RNA species (Table 1). Spectral analysis of the purified RNAs showed typical RNA spectra but the $A_{260} : A_{230}$ ratios varied from about 2·0 to 2·7 (starting RNA, 2·7), the lower ratios being obtained with small amounts of RNA where traces of contaminating polyacrylamide had a greater influence on the spectra. Analysis of the purified RNAs after denaturation in formamide, on 2·4% aqueous tube gels gave the scanning patterns shown in Fig. 4. The scans for RNAs 3, 4-1 and 4-2 showed single sharp peaks with a small amount of material running as a shoulder at the front of each peak; these presumed breakdown products represented 9–16% of the total RNA in each peak (Table 2).

Table 2. Recovery of CMV RNAs after the two electrophoretic elution steps

The two-step procedure for the polyacrylamide slab gels and electrophoretic elution were carried out as described in Materials and Methods. Two large slab gels were used in the first step. The molecular weights are taken from Peden and Symons (1973)

CMV RNA	$10^{-6}\times$ Molecular weight	Initial RNA	RNA after first elution		RNA after second elution		
			μg	% of initial RNA	μg	% of initial RNA	% Intactness of RNA[A]
1	1·35	—	230	4·3	45	0·8	63
2	1·16	—	930	17·5	195	3·7	69
3	0·85	—	715	13·4	275	5·2	84
4-1	0·38	—	390	7·3	180	3·4	88
4-2	0·35	—	150	2·8	50	0·9	91
4A	0·20	—	145	2·7	35	0·7	—
5	0·12	—	65	1·2	25	0·5	—
6	0·05	—	60	1·1	25	0·5	—
Total	—	5320	2685	50	830	16	—

[A] Data obtained from scans of stained gels in Fig. 4. Values calculated from the area under the main peak relative to total area (main peak plus shoulder on leading side of peak).

In contrast to the substantially covalently intact ribosomal RNAs (Fig. 3; Table 1) and Q-CMV RNAs 3, 4-1 and 4-2 (Fig. 4; Table 2), Q-CMV RNAs 1 and 2 were more degraded at the end of the two-step procedure as shown by the gel scans in Fig. 4 which indicated 63 and 69% intactness respectively (Table 2). It would seem that these two Q-CMV RNAs are more sensitive than the others to break down. Wherever substantially intact Q-CMV RNAs 1 and 2 are required, an additional purification step involving sedimentation on a sucrose gradient would be required (Schwinghamer and Symons 1975, 1977).

The gel scans of Fig. 4 showed no evidence of significant cross-contamination of any one RNA species by any of the others. However, in the case of RNA 1 minor contamination by RNA 2 would be obscured by the breakdown fragments of RNA 1. Cross-contamination of RNA species is considered further below.

RNAs 4-1, 4-2, 4A and 6 have yet to be fully characterized. Hybridization studies using cDNA prepared against RNAs 4-1 and 4-2 have shown no detectable sequence differences between the RNAs (Cates and Symons, unpublished data). It is therefore possible that RNAs 4-1 and 4-2 may represent two conformational forms of the same

RNA. Preliminary experiments indicate that RNA 4A is a mixture of breakdown fragments of RNAs 1–4 as is RNA 6 which also contains some host RNA (Cates and Symons, unpublished data).

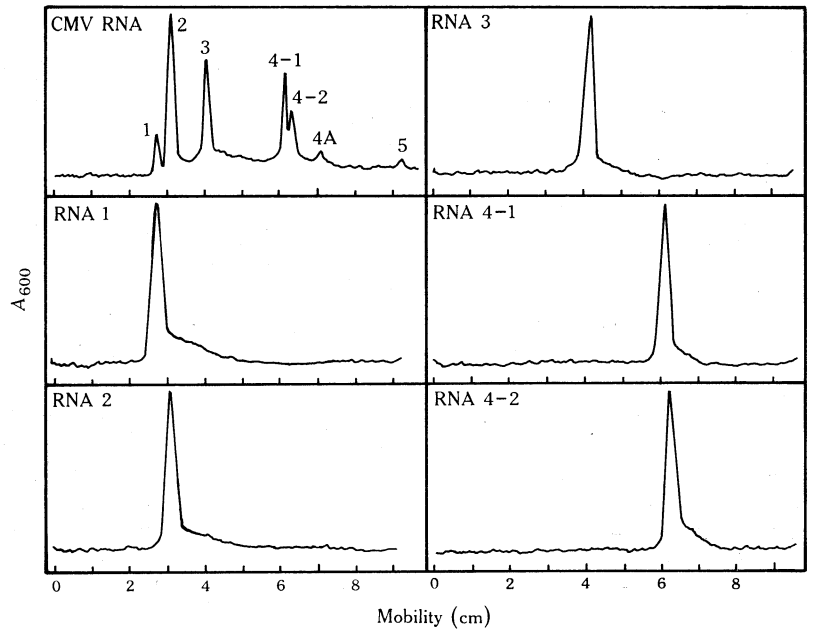


Fig. 4. Check on purity and intactness of CMV RNAs 1, 2, 3, 4-1 and 4-2 after two electrophoretic purification steps. RNA samples (3–5 μg) in 99% formamide, 1 mM EDTA, pH 7, were heated at 60°C for 1·0 min before electrophoresis on analytical 2·4% polyacrylamide tube gels as described in Materials and Methods. After staining for 10 min in 0·05% toluidine blue in water and destaining in water, the gels were scanned at 600 nm.

Purification of Other Viral RNAs

The two-step purification procedure has been successfully applied to two other strains of CMV, P-CMV and M-CMV (Mossop *et al.* 1976), and the results are summarized in Table 3. The recoveries of the four RNA species were 10·3 and 8·2%, respectively, relative to the total starting RNA and were therefore less than the recovery obtained with Q-CMV RNAs (Table 2). All RNAs showed a high degree of intactness which varied from 78 to 87%.

The versatility of the purification method described here was shown in the purification of the four RNA species of alfalfa mosaic virus (AMV). AMV RNAs are separately encapsidated in virions of different molecular weights which can be separated by electrophoresis on polyacrylamide gels (Bol *et al.* 1971; Bol and Lak-Kaashoek 1974). Hence, 30 mg of purified AMV was fractionated by electrophoresis on a mixture of tube and slab 3·0% polyacrylamide gels and the four major viral bands located by brief staining with toluidine blue. Each band was then subjected to the electrophoretic elution step in which the presence of 0·2% SDS would be expected to disrupt the intact virions and release the RNA; however, any intact virions which remained and were eluted would have been deproteinized during the phenol–chloro-

form extraction step. The second slab gel and elution step proceeded as usual to give the four AMV RNAs in an overall yield of 8·0% with an intactness which ranged from 78 to 90% (Table 3).

Table 3. Recovery and intactness of P-CMV, M-CMV and AMV RNAs after the two purification steps

Details are as described in Table 2. n.d., Not determined

Viral RNA strain	Initial RNA (mg)	RNA species	RNA after second elution		
			μg	% of initial RNA	% Intactness of RNA[A]
P-CMV	4·8	1	145	3·0	85
		2	168	3·5	84
		3	92	1·9	87
		4	92	1·9	85
Total			497	10·3	—
M-CMV	5·2	1	55	1·1	83
		2	194	3·7	82
		3	164	3·2	78
		4	10	0·2	n.d.
Total			423	8·2	—
AMV[B]	5·3	1	214	4·0	78
		2	62	1·2	80
		3	112	2·1	90
		4	40	0·7	87
Total			428	8·0	—

[A] See Table 2 for details.

[B] A total of 30 mg of AMV [equivalent to 5·3 mg of RNA (Hull *et al.* 1969)] was subjected to electrophoresis on a mixture of 3·0% polyacrylamide tube gels (Bol *et al.* 1971; Bol and Lak-Kaashoek 1974) and large slab gels (10 mg AMV per gel run at 60 mA per gel at 4°C for 24 h). Virus bands were located by staining with 0·01% toluidine blue for 2 min and destaining in water.

Further Comments on the Purification Procedure

The main aim of this work has been to develop a simple, routine procedure for the rigorous purification of the RNAs of multipartite viruses for sequence characterization. The CMV, AMV and tomato aspermy virus (TAV) RNAs so purified have been successfully used for the preparation of cDNA by Gould and Symons (1977) using the method of Taylor *et al.* (1976). This method makes use of a DNase I digest of DNA to provide primers on the viral RNA for the synthesis of cDNA by the avian myeloblastosis reverse transcriptase. In addition, cDNA has been prepared by the oligo(dT)-primed synthesis with reverse transcriptase using purified Q-CMV RNAs 5 and 6 to which a poly(A) tract has been added at the 3′-end (Gould *et al.* 1977; Cates and Symons, unpublished data).

The low yields of viral RNA obtained after the two-step procedure (8–16%, Tables 2 and 3) are disappointing when compared to the 40% yield obtained with *E. coli* ribosomal RNA (Table 1). However, it is diffcult to see how these yields can be appreciably improved because of the extensive nicking and fragmentation of the RNA as isolated from purified virions. Purification of CMV from infected plants about 7 days after inoculation instead of the usual 12–14 days should decrease RNA breakdown although total virus yield would be less. This approach has been successful

in the case of AMV where RNA was extensively degraded when isolated from virus purified 12 days after infection but showed a many-fold increase in intactness when virus was purified 4·5 days after infection (Symons, unpublished data).

In order to inhibit any contaminating ribonucleases, 0·2% SDS was included in the electrophoretic elution buffer (Ceri and Maeba 1973). However, the overall yields of the ribosomal and Q-CMV RNAs after the two-step procedure were essentially the same whether or not SDS was present (results not shown). Nevertheless, it was considered desirable to retain the SDS as a routine component of the buffer.

The time taken for the electrophoretic elution of RNA was determined using [^{32}P]- and [^{125}I]ribosomal RNA. Under the conditions described in Materials and Methods, RNA elution from the gel was complete after about 2 h. A routine time of 4 h was adopted to allow for variation in the volume of gel being eluted and for possible variation between RNAs in the rate of elution. Electrophoretic elution was faster in the absence of 0·2% SDS and was complete in about 2 h at a lower current of 30 mA per gel elution tube as compared with about 2 h at 45 mA per tube in the presence of 0·2% SDS.

Schuerch *et al.* (1975) used ethidium bromide to localize RNA bands after polyacrylamide slab gel electrophoresis because they found that several other dyes, including toluidine blue, degraded both double-stranded and single-stranded RNA. However, in the present work, staining with 0·04% ethidium bromide for 5 min gave the same overall yield of ribosomal RNAs after two electrophoretic steps as the usual staining with 0·01% toluidine blue for 1–2 min. In addition, the yields of eluted CMV RNA species were found to be the same after staining with either ethidium bromide or toluidine blue. Because toluidine blue gave better staining of minor components in CMV RNA, it has been used routinely in this work. It is important to note that Schuerch *et al.* (1975) stained their gels for 2 h with 0·01% dye whereas 1–2 min was used in the present work with the same dye concentration. In addition, destaining of gels was carried out in the dark in covered dishes whilst the electrophoretic elution step was done in subdued light.

Finally, it is most important to note that the method described gave each RNA purified on the basis of size. Thus contaminating RNA of the same size class as that of the desired RNA would not have been removed. This is of considerable importance in the case of Q-CMV RNA which contains significant amounts of fragmented molecules (Schwinghamer and Symons 1975; this work). For example, Q-CMV RNA 3, purified as described here, was shown by hybridization studies using cDNA to contain about 18% contamination by fragments derived from RNAs 1 and 2 (Gould and Symons 1977). In the same work it was shown that Q-CMV RNA 1 was contaminated by 8% RNA 2. RNA 4 was contaminated by 7% RNA 3 sequences and less than 1% by fragments of RNAs 1 and 2. However, RNA 2, which showed no contamination by full length RNA 1 molecules, was contaminated to 20% by RNA 1 sequences (Gould and Symons 1977).

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References

Bol, J. F., Van Vloten-Doting, L., and Jaspars, E. M. J. (1971). A functional equivalence of a top component RNA and coat protein in the initiation of infection by alfalfa mosaic virus. *Virology* **46**, 73–85.

Bol, J. F., and Lak-Kaashoek, M. (1974). Composition of alfalfa mosaic virus nucleoproteins. *Virology* **60**, 476–84.

Ceri, H., and Maeba, P. Y. (1973). Association of a ribonuclease with the 50-S ribosomal subunit of *Escherichia coli* MRE 600. *Biochim. Biophys. Acta* **312**, 337–48.

De Wachter, R., and Fiers, W. (1971). Fractionation of RNA by electrophoresis on polyacrylamide slab gels. *Methods Enzymol.* **21**, 167–78.

Fenner, F. (1976). Classification and nomenclature of viruses. *Intervirology* **7**, 20–115.

Gould, A. R., Palukaitis, P., Symons, R. H., and Mossop, D. W. (1977). Characterization of a satellite RNA associated with cucumber mosaic virus. *Virology* (In press.)

Gould, A. R., and Symons, R. H. (1977). Determination of the sequence homology between the four RNA species of cucumber mosaic virus by hybridization analysis with complementary DNA. *Nucleic Acids Res.* **4**, 3787–802.

Habili, N., and Francki, R. I. B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. 1. Physical and chemical properties. *Virology* **57**, 392–401.

Hull, R., Hills, G. J., and Markham, R. (1969). Studies on alfalfa mosaic virus. II. The structure of the virus components. *Virology* **37**, 416–28.

Jaspars, E. M. J. (1974). Plant viruses with a multi-partite genome. *Adv. Virus Res.* **19**, 37–149.

Kaper, J. M., and West, C. K. (1972). Polyacrylamide gel separation and molecular weight determination of the components of cucumber mosaic virus. *Prep. Biochem.* **2**, 251–63.

Loening, U. E. (1967). The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. *Biochem. J.* **102**, 251–7.

Lot, H., and Kaper, J. M. (1976). Physical and chemical differentiation of three strains of cucumber mosaic virus and peanut stunt virus. *Virology* **74**, 209–22.

Lot, H., Marchoux, G., Marrou, J., Kaper, J. M., West, C. K., Van Vloten-Doting, L., and Hull, R. (1974). Evidence for three functional RNA species in several strains of cucumber mosaic virus. *J. Gen. Virol.* **22**, 81–93.

Mossop, D. W., Francki, R. I. B., and Grivell, C. J. (1976). Comparative studies on tomato aspermy and cucumber mosaic viruses. V. Purification and properties of a cucumber mosaic virus inducing severe chlorosis. *Virology* **74**, 544–6.

Peden, K. W. C., and Symons, R. H. (1973). Cucumber mosaic virus contains a functionally divided genome. *Virology* **53**, 487–92.

Prensky, W. (1976). The radioiodination of RNA and DNA to high specific activities. *Methods Cell Biol.* **13**, 121–52.

Schuerch, A. J., Mitchell, W. R., and Joklik, W. K. (1975). Isolation of intact individual species of single- and double-stranded RNA after fractionation by polyacrylamide gel electrophoresis. *Anal. Biochem.* **65**, 331–45.

Schwinghamer, M. W., and Symons, R. H. (1975). Fractionation of cucumber mosaic virus RNA and its translation in a wheat embryo system. *Virology* **63**, 252–62.

Schwinghamer, M. W., and Symons, R. H. (1977). Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell-free systems and in toad oocytes. *Virology* **79**, 88–108.

Taylor, J. M., Illmensee, R., and Summers, J. (1976). Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. *Biochim. Biophys. Acta* **442**, 324–30.

Van Vloten-Doting, L. (1976). Similarities and differences between viruses with a tri-partite genome. *Ann. Microbiol. (Paris)* **127A**, 119–29.

Van Vloten-Doting, L., and Jaspars, E. M. J. (1972). The uncoating of alfalfa mosaic virus by its own RNA. *Virology* **48**, 699–708.

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