A Method for the Purification of Large Quantities of Biologically Active Ribonucleic Acid Components from Cowpea Chlorotic Mottle Virus, a Multicomponent Plant Virus

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Zonal Resolution, Multicomponent Virus Ribonucleic Acids

Cowpea chlorotic mottle virus RNA has been prepared in comparatively high yield (at least 50%) by a modified phenol extraction method. The preparation, which has high biological activity, has been resolved into four components by zonal centrifugation on a 15—40% (w/v) sucrose density gradient. The components obtained have been tested for biological activity against whole plants and plant protoplasts. Each of the two largest components RNA 1 and RNA 2 was by itself infective (50—90% of the specific infectivity of the whole genome) and produced virus-specific proteins (coat protein and P2) and RNAs ("RNA 3" and "RNA 4"). Contamination by small proportions (<10%) of neighbouring RNAs is presumed to be involved in this infectivity. The two smallest components were obtained in an almost pure form.

Introduction

Much of the work on viral RNAs and messenger RNAs is done on the μg scale, often using tracer techniques. Structural and functional studies of biologically active RNA could be facilitated and extended if large (mg) quantities could be obtained. Many plant viruses can readily be prepared on a large scale and this has been exploited by a method described here for the isolation of the RNA components from the divided genome of cowpea chlorotic mottle virus (CCMV). The components have been obtained in quantity, in a biologically active and relatively pure form.

CCMV contains four RNA components distributed between three virions composed of identical shells of sub-units[1], with slightly differing buoyant densities in CsCl. The approximate molecular weights of these components range between approx. 0.3×10^6 and approx. 1.1×10^6. Small amounts of them were separated by electrophoresis on polyacrylamide gels and the fractions were used for biological studies[1,2]. Lane and Kaesberg[3], working with the related brome mosaic virus (BMV), made similar observations.

CCMV is unusual in giving low yields of RNA[4], when extracted with phenol. The modifications of the phenol method described here greatly improve the yield and release about 70% of the RNA from the coat protein leading to the isolation of at least 50% of it. Cox, Hirst Godwin and Huvos[5] devised a method for the preparative resolution of RNA fragments with a molecular mass range of $0.2 - 0.5 \times 10^6$ by zonal centrifugation. This method has now been applied to the separation of the components of the genome of CCMV. From 10 mg of the whole genome mg quantities of the individual components were separated, thus yielding large enough quantities for thorough checks of their chemical purity and integrity and their infective properties in two biological systems.

Materials and Methods

RNA from CCMV

Type CCMV was prepared from cowpea (*Vigna unguiculata* (L). Walp. var. Blackeye), inoculated ten days after germination and harvested after 10 to 15 days further growth. Virus was prepared from these leaves by the method of Bancroft *et al.*[6]. In the early stages of this work yields of about 20% of the total RNA were obtained. Later, after the introduction of modifications, it was possible to

recover, routinely, about 50% of the RNA. In a few cases considerably higher recoveries were recorded. By making allowances for mechanical losses of the aqueous layer during the preparation, it was estimated that $65-70\%$ of the viral RNA was transferred to the aqueous phase when the modified procedure was used.

All glass vessels used for the preparation were baked overnight at 200 °C and only sterile, plugged pipettes were used. Gloves were not worn but care was taken not to touch any parts of the glassware which were likely to come into contact with the preparation. The virus was treated in batches of $200-250$ mg in a 200 ml glass-stoppered tube. About 5 ml of a suspension of bentonite (50 mg/ml) in 0.1 M sodium acetate (pH 6.0) and 6 ml of a 20% (w/v) aqueous solution of S.D.S. were added. The toal volume was brought to 36 ml with distilled water. All aqueous solutions were made with sterile distilled water.

After standing at room temperature for 30 min 36 ml of a mixture of redistilled phenol, *m*-cresol, water and 8-hydroxyquinoline, in the proportions 500 g : 70 ml : 55 ml : 0.5 g, were added [8]. The mixture was shaken by hand at 4 °C for 15 min. A few drops of octan-2-ol were added to break the froth and the suspension was spun on a Serval Superspeed centrifuge, in glass tubes, in a SS 34 head at 10000 rpm for 30 min at 4 °C. The aqueous layer was pipetted off and shaken by hand with half a volume of the phenol mixture for 5 min at 4 °C. The mixture was centrifuged as before but for only 20 min. This treatment was repeated once more and the aqueous layer extracted with ether to remove phenol [9]. Four or five extractions with ether in the proportion of 5 vol ether per vol RNA solution were necessary. Ether in the aqueous layer was removed by bubbling nitrogen through the solution and the RNA was recovered by precipitation with $2-3$ vol ethanol. After standing at -20 °C for a few hours, it was recovered by centrifuging. The solid pellet was stored at -20 °C and remained stable for at least a year.

Separation of RNA components by zonal centrifugation

A 10 mg sample of CCMV RNA dissolved in 5 ml buffered 7% (w/v) sucrose containing 0.1 M-KCl, 10 mM-MgCl$_2$ and 40 mM-Tris chloride pH 7.2 was applied to a $15-40\%$ (w/v) sucrose gradient containing the same salts and buffer, overlaid with 250 ml of buffer, in a M.S.E. B XIV zonal rotor and spun for 6 h at 45 K — rev/min at 4 °C using an M.S.E. Superspeed 65 centrifuge. The rotor was unloaded by displacement with 50% (w/v) sucrose.

The first 200 ml were discarded then 95×5 ml samples collected by hand and the E_{260} measured against water in a Pye Unicam SP 500 spectrophotometer. The RNA was precipitated by adding $2-3$ vol of ethanol to each fraction and 40% (w/v) potassium acetate solution to give a final concentration of 2% (w/v).

Polyacrylamide gel electrophoresis

To detect the presence of the viral RNA components and the degree of contamination by degradation products the precipitated material was dissolved in a small volume of water and samples containing 10 μg RNA in 50 μl were applied to 2.6% (w/v) polyacrylamide disc gels and run for 2.5 h at 4 mA per tube, using Loening's [10] tris-phosphate buffer pH 7.6. UV absorbing bands were located in a Joyce-Loebl Ultraviolet Scanner and the gels were then stained with toluidine blue and photographed. To detect hidden breaks, samples were run on 4% (w/v) polyacrylamide gels in formamide according to the method of Pinder, Staynov and Gratzer [11].

Biological activity on whole plants

The RNA preparations were assayed for infectivity on celite-dusted half-leaves of *Chenopodium hybridum* at three concentrations (E_{260}, 0.25, 0.50 or 1.0), with whole RNA, the complete genome, as a control on opposite half leaves. The whole RNA had a specific infectivity which was 90% that of the virus on a weight basis.

Biological activity in mesophyll protoplasts

Mesophyll protoplasts were prepared from leaves in *Nicotiana tabacum* (var. White Burley) [12]. They were washed with 0.7 M mannitol and inoculated with RNA, using the technique of Watts, Cooper and King [13]. RNA and poly-L-ornithine (mol. wt. 120000) were mixed in equal amounts to give a final concentration of 100 μg each/ml in 0.1 M potassium citrate buffer (pH 5.2) at 0 °C and diluted with 0.01 M potassium citrate in 0.7 M mannitol to give a final concentration of 1 μg RNA/ml for inoculation. 20 ml of this solution was at once used to resuspend 2×10^6 freshly pelleted protoplasts. These were left for 10 min, washed three times with 0.7 M mannitol and cultured at 25 °C in continuous white fluorescent light (600 lx), using the method of Motoyoshi, Watts and Bancroft [12].

Labelling of CCMV-RNA infected and healthy protoplasts

Protein labelling was achieved in RNA-inoculated protoplasts using an incubation medium containing

[³H]leucine according to the procedure of Sakai and Takebe[14]. Healthy protoplasts were treated in the same way but [³H]leucine was replaced by [¹⁴C]leucine. RNA labelling and extraction was also done by the method of Sakai and Takebe[14]. The labelled proteins and RNAs were resolved by polyacrylamide gel electrophoresis.

Results

The gradient profile (Fig. 1) and P.A.G.E. patterns of the fractions show that CCMV RNA component 4 was completely resolved and yielded 0.8 mg of RNA only lightly contaminated with degradation products of a lower molecular weight and containing no higher molecular weight species. Component 3 was completely separated from component 4 but overlapped to a small extent with components 2 and 1. 80 – 90% of component 3 was found in fractions 66 – 72 and was recovered in a essentially pure form. Components 1 and 2 were not well resolved but fraction 76 contained 0.4 mg of component 2 in a fairly pure state. 0.4 mg of component 1 was obtained by pooling fractions 82 to 84 inclusive.

Stained gels were used to select the fractions indicated in Table I for biological testing. Those containing the minimum of degradation products

Table I. The separation of the components of CCMV RNA by zonal centrifugation.

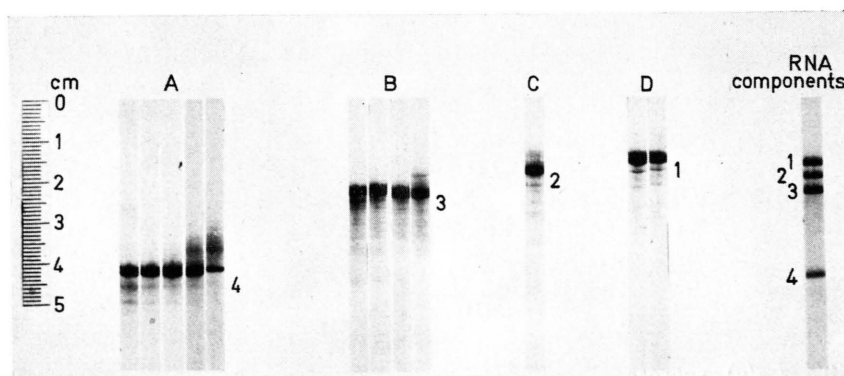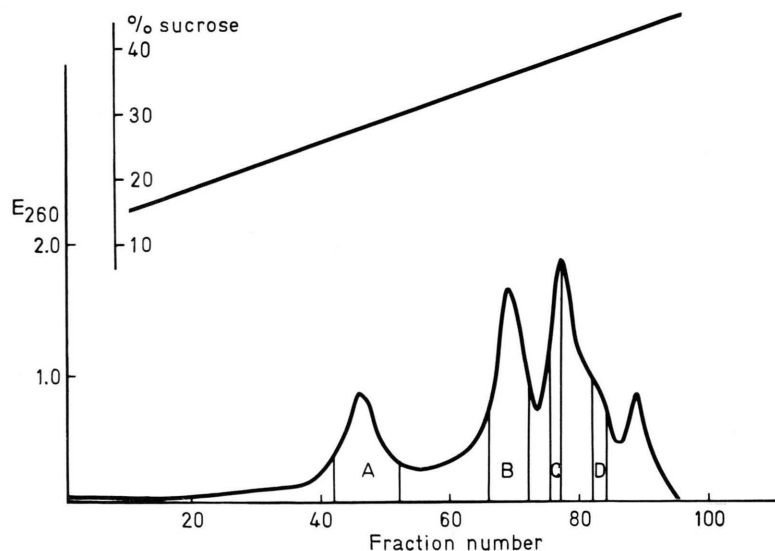| Component	Gradient region (Fig. 1)	Fractions taken	% of components			
			1	2	3	4
1	D	82 – 84	88	9	4	0
2	C	76	5 – 10	80 – 90	5 – 10	0
3	B	66 – 72	0	0	100	0
4	A	44 – 52	0	0	0	100

Fig. 1. As described in the text, 10 mg of the complete genome of CCMV in 7 ml were run on a 15 – 40% (w/v) sucrose gradient. Each 5 ml fraction from the run was precipitated with ethanol and run on PAGE to select the purest fractions. A, RNA 4, fractions 44 through 52; B, RNA 3, fractions 66 through 72; C, RNA 2, fraction 76; D, RNA 1, fraction 82, 83 and 84.

were checked for a absence of hidden breaks by running on formamide gels.

The results of the assays for specific infectivity obtained by inoculating half-leaves of *C. hybridum* with RNA components 1 and 2 are shown in Table II. At the three concentrations tested the specific infectivities were high compared with whole RNA. This is of interest because the quantities of contaminating RNA species are minimal.

Table II. Specific infectivities of RNA 1 and RNA 2 compared with whole RNA. Total number of local lesions produced on half-leaves * of *Chenopodium hybridum* at three concentrations.

Optical density of RNA	0.25	0.50	1.00
Expt. 1			
RNA 1 **	44 (45)	34 (103)	55 (86)
complete genome	97	33	64
Expt. 2			
RNA 2 **	98 (49)	129 (49)	158 (47)
complete genome	202	262	338

* 6 half-leaves, Expt. 1; 10 half-leaves, Expt. 2.
** Figures in parenthesis are infectivities of RNA 1 or RNA 2 compared with whole RNA expressed as a %.

Table III. The optical densities of dilution end points for last traces of infectivity of CCMV RNAs.

RNA component	Optical density
1	$\frac{1}{32}$
2	$\frac{1}{32}$
3	1
whole RNA	$\frac{1}{256}$

The dilution end-points (Table III) of the RNA components at which the last traces of infectivity were detected show that RNA 3 is relatively pure in terms of infectivity, whereas RNA 1 and RNA 2 were contaminated to a greater degree.

Table IV gives the results obtained by infecting protoplasts from the leaves of *Nicotiana tabacum* with each of the four RNA components. There was no detectable synthesis of any virus-specific proteins using RNAs 3 and 4, but RNAs 1 and 2 both induced two proteins P1 (coat protein) and P2 (possibly RNA polymerase) [15], using four different preparations of protoplasts. RNA 2 was the most active in every way, but, qualitatively, RNA 1 gave all the same effects. In a labelling experiment using component 1 as the inoculum for a very active preparation of protoplasts, three products, "coat protein" (P1), protein P2 and "RNA 3" were clearly seen. There was also some indication of a third protein, which is larger than P1 and P2. A small amount of RNA "4" occurred and there may have been small amounts of RNAs "1" and "2".

Discussion

There is a need to obtain RNA components from viruses with divided genomes in quantity and in a pure undegraded state, since this would provide the opportunity to deepen the study of viral messenger RNAs, and, in particular, the roles of the components of the divided genome. Zonal centrifugation is a relatively cheap, safe and quick method for this purpose. The experiments described above show that good resolution of CCMV RNA into its components can be achieved in a single run leading to a high yield of almost pure components 3 and 4 and allowing the selection of fractions containing component 1 and component 2 each in a highly enriched

Table IV. Virus and virus-specific proteins and nucleic acids produced by tobacco mesophyll protoplasts inoculated with CCMV RNAs.

Experiment	Material inoculated	% Infectivity	Proteins synthesised			RNAs synthesised			
			P 1	P 2 Coat Pr.	P 3	1	2	3	4
1	RNA 1	3.4	+	+ +	−				
	RNA 2	14.7	+ + +	+ + +	−				
	RNA 3	0.8	−	−	−				
	RNA 4	0.5	−	−	−				
2	RNA 1		+ + +	+ + + +	+	(+)	(+ +)	(+ + + + + +)	(+)

form. The results obtained with these fractions are consistent with the known requirements for infectivity: The presence of RNAs 1, 2 and 3 [1].

The immediate need is for increased purification of the resolved components, particularly components 1 and 2. However, although the dilution end points for infectivity (Table III) indicate that RNA 3 is purer than RNA 1 and RNA 2 in infectivity terms, the purity of even this component is not yet adequate for a critical evaluation of its biological role. Work is in progress to improve the purity of all components.

The complete genome of type CCMV contains the four RNA components in about equimolar amounts (Dickerson and Trim, unpublished results). The results presented here suggest however that equimolar amounts even of the three RNA species essential for infection $(1+2+3)$ are not required for optimal infectivity, since very high infectivity compared with the complete genome, was obtained with either component 1 or 2 when these constituted, on a molar basis, at least 80% of the mixture and the remaining two required components each represented not more than 10% of the residue.

When more highly purified components can be prepared in qantity, the relative molar amounts of RNA species 1, 2 and 3 required to initiate infection will become open to investigation.

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