An Improved Method for the Preparation of the Four Ribonucleic Acids of Cowpea Chlorotic Mottle Virus

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Gel Preparation, Individual CCMV RNAs

The four components of the genome of cowpea chlorotic mottle virus have been prepared in a highly active and highly purified state by a method based on their resolution by polyacrylamide gel electrophoresis. Activity and purity have been confirmed by gel electrophoresis under denaturing and non-denaturing conditions, infectivity tests on whole plants, translation in an mRNA dependent rabbit reticulocyte lysate and fingerprinting of T_1 ribonuclease digests after labelling with ^{32}P at the 5′ ends of the oligonucleotide products. The quality of the RNAs obtained was superior to those previously obtained by zonal centrifugation but only comparatively small batches of whole RNA can be handled with ease.

Introduction

The purification of intact ribonucleic acids (RNAs) from biological materials still presents many difficulties. These are particularly severe when, as is most often the case, more than one molecular species of RNA is present, adding problems of resolution to those of degradation.

The degradation of RNA by nucleases and extremes of pH is universally recognised and can be controlled. It is less widely known that often in biochemical experimentation, polyvalent metal ions which can degrade RNAs by splitting internucleotide bonds, also bring about aggregation and/or conformational changes, properties which they share with some monovalant metal ions and polyamines.

In a thorough study of cowpea chlorotic mottle virus (CCMV) Verduin [1] has shown that all four components of the RNA genome degrade into small heterogeneous pieces within the isolated virion. He gave circumstantial evidence for a free radical mediated mechanism of this process and showed that the viral coat protein and polyvalent cations are involved. Protein-free CCMV RNA is not degraded under otherwise similar conditions and low concentrations of chelating agents prevent degradation within the stored, intact virion.

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The hydrolysis of internucleotide bonds in RNA under comparatively mild conditions by a number of metal ions has been recorded [2–5]. Recently [6] it was shown that at pH 6.5 and 65° tobacco mosaic virus RNA is rapidly inactivated by low concentrations of polyvalent metal ions and that the inactivation is correlated with the breaking of internucleotide bonds.

There are two other phenomena involving cations which can have a bearing on the resolution of RNA mixtures. Low concentrations of divalent cations and polyamines (ca. 1 mM) and higher concentrations of monovalent cations (ca. 100 mM) were shown to promote conformational changes in all four of the RNAs of CCMV [7] rendering them physically heterogeneous and more difficult to resolve. The cations can also promote aggregation, particularly dimerisation of RNAs [8], thus adding further to the heterogeneity.

We have devised a procedure for the isolation and resolution of the four RNAs of CCMV which, while avoiding contamination by nucleases, reduced interference by cations to a minimum. And, since we were unable to avoid cross-contamination of these RNAs using our earlier method [9], which involved resolution on a sucrose density gradient in a zonal rotor to obtain relatively large quantities of the RNA components, they were resolved by polyacrylamide gel electrophoresis.

The integrity of the products was tested by gel electrophoresis, infectivity assays, translation in a rabbit reticulocyte lysate and examination of fingerprints of complete T_1 ribonuclease digests.

Materials and Methods

RNA from CCMV

Type CCMV was prepared from cowpea (*Vigna unguiculata* (L.) Walp. var. Blackeye) inoculated seven days after sowing and harvested after 10–15 days further growth. Virus was extracted from the leaves by the method of Bancroft *et al.* [10] and within 24 h RNA prepared from it by a modification of a phenol method devised by Verduin [1], who first dissociated the virus with SDS in buffer at pH 5 in the presence of EDTA and bentonite. To maximise the chelation of metals by EDTA we carried out this step at pH 8.5. The modified procedure was as follows. Virus (ca. 20 mg/ml) suspended in 0.1 M acetate buffer pH 4.7 containing 0.1 M EDTA was mixed with two volumes of a mixture containing 0.2 M NaCl, 2% w/v SDS and 0.2% w/v bentonite in 0.2 M tris-HCl pH 8.5. The final volume was 25 ml and the final concentration of virus was 0.5–1% w/v. The suspension was stirred for 30 min at room temperature and one volume (25 ml) of a mixture of water-saturated phenol, m-cresol and 8-hydroxyquinoline (v : v : w = 7.5 : 1.0 : 0.075) and one volume (25 ml) of chloroform added. Stirring was continued for 15 min and the suspension centrifuged at 12 000 × *g* for 10 min at 5°. The aqueous phase was pipetted off and submitted to two further extractions with the phenol-chloroform mixture. It was then extracted four times with ether to remove phenol, bubbled with O_2-free N_2 to remove ether and the RNA precipitated with 3 volumes of ethanol. When stored in 5 mg batches under 75% v/v ethanol at −20° this RNA remained undegraded and highly infective after many months.

Resolution of RNA components on polyacrylamide gels

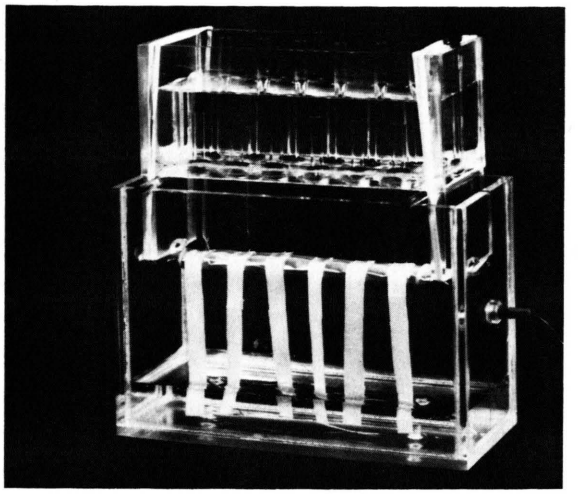
The polyacrylamide gel electrophoresis method of Loening [11] was used to separate the four RNA components and to estimate the recovery of each of them.

a) The molecular proportions of RNA components in the virus

Fresh virus was stood in 12.5% SDS for 60 min at room temperature. Samples (50 µl) containing 250 µg virus (ca. 50 µg RNA) were loaded onto 2.6% w/v disc gels (7 cm × 0.2 cm²) and run for 2.5–3 h at 4 mA per tube (7 v/cm) using tris-phosphate buffer ph 7.6. Under these conditions the whole of the RNA was released from the virus and resolved on the gel. The freed coat protein moved in a band ahead of component 4. The RNA bands were located by photography in ultraviolet light as described by Lipkin *et al.* [12] for the detection of ultraviolet absorbing compounds on paper. The reflex document paper used, Ilford Ilfoprint SR 4, was protected by enclosing the gels in thin-walled polythene bags. An exposure time of 10 sec at a distance of 75 cm from the light source was sufficient. To excise the nucleic acid bands the gels were frozen by immersing the bags briefly in a freezing mixture (solid CO_2-acetone). The RNA bands were located with the aid of the corresponding photographic prints and cut out with a sterile razor blade. Corresponding bands from three gels were pooled, blended with a little water and sonicated until the gel was dispersed to give a water-clear liquid of low viscosity (2–5 min). This solution was made to volume in 1.0 N-HCl stood at room temperature for 24 h. The A_{260} value was read against a similarly treated blank gel, in a spectrophotometer. This permitted the rapid estimation of the relative amounts of nucleotide derived from each RNA component and, assuming accepted molecular weight values for the RNA components, values for molecular proportions were calculated.

b) The isolation of components from whole RNA

Starting with whole RNA, components were resolved on 2.6% polyacrylamide gels loading samples of up to 75 µg RNA in 25 µl 15% sucrose onto each gel and running and excising bands as in a). Each gel slice was moulded into the base of an agarose disc gel (7 cm × 0.2 cm²) made with 1% LGT agarose (Marine Colloids Inc., Miles Laboratories Ltd.) in 0.1 M tris, 25 mM sodium acetate, 1 mM EDTA pH 7.3. The RNA was transferred onto a strip (10 cm × 1.0 cm) of Whatman GF 81 glass fibre paper in the apparatus shown in Fig. 1 which is an adaptation of a standard disc gel electrophoresis apparatus used in this laboratory. The gels were run at 150 V in the tris-acetate buffer for 75, 60, 45 and 20 min to transfer components 1, 2, 3 and 4 respectively. The wet papers were then rolled in batches of 3 and centrifuged at 2 000 rpm for 5 min in spin



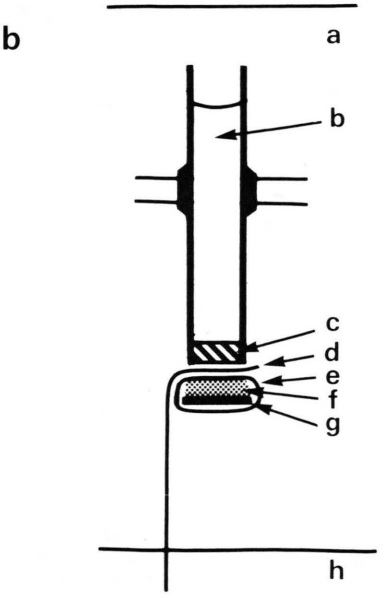


Fig. 1. a) Standard disc gel apparatus set up for RNA extraction. b) Schematic diagram of electrophoretic apparatus. a, Cathode buffer chamber; b, 1% LGT Agarose; c, 2.6% polyacrylamide gel slice; d, Whatman GF 81 paper strip; e, Polythene cover; f, Foam cushion; g, Glass support; h, Anode buffer chamber.

thimbles in an MSE Minor bench centrifuge to collect the RNA solution. Each batch was washed twice with freshly distilled water. Insoluble matter from the glass fibre paper pelleted during the centrifugation. The RNA was precipitated by adding 3 volumes of ethanol and 40% potassium acetate to a final concentration of 2% w/v.

Polyacrylamide gel electrophoresis

2.6% polyacrylamide gels run and prepared according to Loening [11] were used for the resolution of components as described above and also to examine the quality of RNA preparations both before and after the resolution of the four components. For the latter purpose the RNAs were run both with and without the following pretreatments:

1) after heating for 3 min at 80°,
2) after standing in 95% formamide for 30 min and reprecipitating with ethanol,
3) after standing for 30 min in 95% dimethyl sulphoxide and reprecipitating with ethanol,
4) after denaturation with glyoxal according to McMaster and Carmichael [13].

Some samples were also run under denaturing conditions on 4% (w/v) polyacrylamide in formamide according to Pinder, Staynov and Gratzer [14].

Infectivity on whole plants

RNAs were assayed for infectivity on carborundum-dusted half leaves of *Chenopodium hybridum.* All were applied at a concentration of 160 µg RNA/ml.

Translation in vitro

Total and fractionated CCMV RNAs were translated in an mRNA dependent rabbit reticulocyte lysate prepared essentially as described by Pelham and Jackson [15]. The translation mixture contained 80% v/v reticulocyte lysate 20 µM hemin, 50 µg/ml creatine kinase, 10 mM creatine phosphate, 150 units/ml micrococcal nuclease (Boehringer), 1 mM $CaCl_2$, 2 mM EGTA, 100 mM KCl, 0.5 mM $MgCl_2$, 50 µg/ml. Calf liver tRNA (Boehringer), 50–300 µM of each amino acid (except leucine) and 100 µCi/ml L-[4,5-^{3}H]leucine (154 Ci/mmol) (The Radiochemical Centre, Amersham). Incubations were performed in a final volume of 20 µl containing 25–30 µg/ml CCMV RNA. 2 µl samples were assayed for incorporation of tritium and translation products were analysed on SDS-polyacrylamide slab gels [16] and fluorographed as described by Laskey and Mills [17].

Fingerprinting of T_1 ribonuclease digests

Fingerprints of all four RNA components were made by digesting to completion with T_1 ribonuclease, labelling [18] the products at the 5′ end with ^{32}P and running on a two-dimensional polyacrylamide gel [19].

Digestion

The incubation mixture (10 µl) contained 1–2 µl RNA solution (10–15 µg RNA for components 1, 2 and 3 and 5 µg RNA for component 4), 4 µl T_1 ribonuclease (Boehringer, 40 units), 2 µl calf intestine alkaline phosphatase (Boehringer, 0.1 unit) to remove terminal phosphate groups, 1 µl 0.1 M tris-HCl buffer pH 8.0 and water to 10 µl. This mixture was incubated for 1 h at 37°, 2 µl 40 mM nitrilotriacetic adjusted to pH 7.5 were then added and the mixture stood at room temperature for 20 min to inactivate the phosphatase and then heated to 100° for 2 min. This material can be stored at – 20° before further treatment.

Labelling

To the T_1 ribonuclease digest were added 1 µl Tris-HCl pH 8.1 containing 0.1 M Mg^{2+} and 0.2 M mercaptoethanol, obtained by adding 14 µl mercaptoethanol to each ml buffer at the time of use and 5 µl polynucleotide phosphokinase (Boehringer) containing about 25 units. This mixture was added to 25–30 µCi [γ-^{32}P]ATP (Amersham) dried *in vacuo* from a solution in 50% v/v aqueous ethanol, and incubated at 37° for 30 min.

The remaining [γ-^{32}P]ATP and the newly formed ADP were degraded by incubating with 3 µl 10 mM glucose, 1 µl 10 mM cold ATP, 1 µl hexokinase (Boehringer 0.007 unit), 1 µl myokinase (Boehringer 0.018 unit) for 20 min at 37°. Finally the mixture was heated at 100° for 3 min.

Two dimensional polyacrylamide gel electrophoresis

The procedure of Lee and Wimmer [19] was followed. The first dimension (8% gel) was run at 4° on a 20 × 20 cm slab and the second dimension (22% gel) was run at 14° in a preparative apparatus (40 × 20 cm slab) Autoradiography was done at room temperature for 7–20 h using Kodak Kodirex X-ray film.

Results

All the data in this paper were obtained with RNA prepared from a single preparation of type CCMV (John Innes) known to be highly infective and free from large quantities of degradation products. Table I shows that the virus contained nearly equimolar proportions of the four components and gives the recoveries of the four components which decrease sharply with the increase of molecular weight.

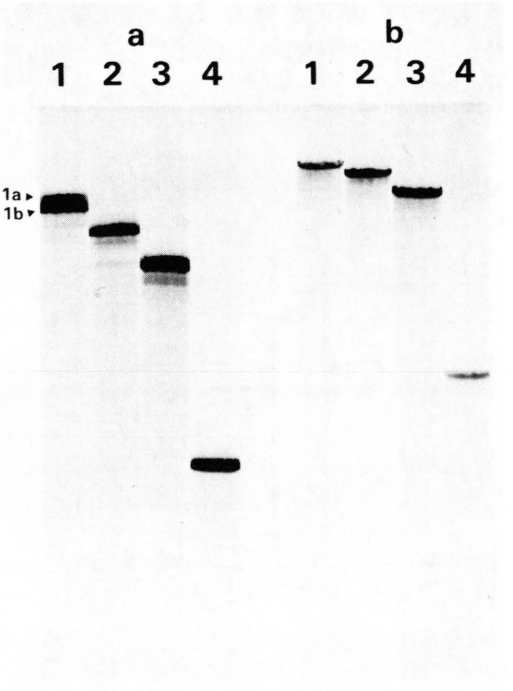


Fig. 2. CCMV RNA components 1, 2, 3 and 4, resolved as described in Materials and Methods, checked for purity and degradation on a) 2.6% polyacrylamide gels according to Loening [11], b) denatured with glyoxal according to McMaster and Carmichael [13] and electrophoresed as in a).

Table I. Recoveries of the resolved components of CCMV RNA.

Component	% of total RNA of the virus	Molecular weight × 10^{-6} after Bancroft [20]	Molecular ratio	Recovery % of resolved component
1	35	1.15	1.0	17
2	30	1.00	1.0	30
3	26	0.85	1.0	57
4	9	0.32	0.94	95

Each resolved component was examined for contaminants by electrophoresis on 2.6% polyacrylamide gels before and after denaturation with DMSO and glyoxal [13]. As may be seen in Fig. 2 the gels showed no evidence of cross-contamination of components or the presence of aggregates.

All components contained small proportions of products which appeared to be derived by the removal of a comparatively short sequence of the parent polynucleotide.

When the viral RNA was prepared as described here components 2, 3 and 4 contained only one electrophoretically distinguishable conformer. However in all preparations component 1 which, when treated with cations [7], had three electrophoretically detectable conformers, had two components (1a and 1b, Fig. 2) just separable on 2.6% polyacrylamide gels. These remain after pretreatment by heating at 80°, standing in formamide and standing in dimethyl sulphoxide. But after pretreatment with methyl glyoxal or on running on 4% polyacrylamide gels made in 98% formamide there is only one electrophoretically detectable component.

Infectivity tests were a more critical measure of the purity of the RNA components. The results in Table II show retention of infectivity in all components when inoculated in an equimolar mixture of the three largest (components 1, 2 and 3) and virtually complete inactivity of each of them when applied alone. Component 4 was not included since it is not required for infection. However the combinations RNA 1 + RNA 2, RNA 1 + RNA 3 and RNA 2 + RNA 3 all showed a low level of activity suggesting cross-contamination of components. This could be at a very low level since it has been shown that in all three of the above combinations the addition of a relatively small proportion of the appropriate third component causes a large increase in infectivity [21].

Table II. Specific infectivities of RNAs 1, 2 and 3, either singly or in mixtures of equal amounts.

Inoculum concentration optical density 4.0 i.e. 0.16 mg/ml	
RNA component	Total lesions produced on 12 half leaves
1	1
2	3
3	–
1 + 2	18
1 + 3	12
2 + 3	8
1 + 2 + 3	95
Unfractionated RNA which includes RNA 4	66

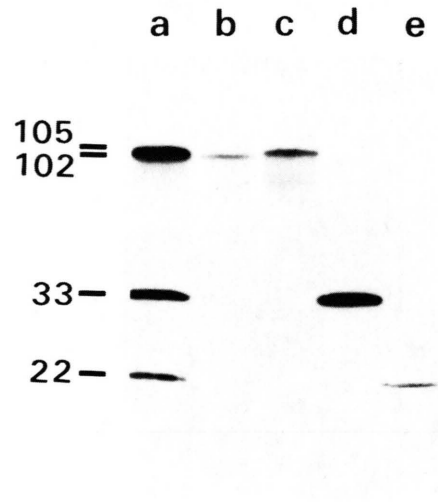


Fig. 3. Fluorogram showing translation products of fractionated CCMV RNAs. Channel (A) total CCMV RNA, unfractionated; (B) CCMV RNA 1; (C) CCMV RNA 2; (D) CCMV RNA 3; and (E) CCMV RNA 4. The RNAs were fractionated and translated as described in Materials and Methods. Translation products were electrophoresed on a 15% polyacrylamide SDS slab gel and fluorographed. The estimated mol. wt. $\times 10^{-3}$ of major translation products is indicated.

The dominant translatable component in the separated RNAs corresponded to the appropriate component in the unfractionated RNA as is shown in Fig. 3. There was little evidence of cross-contamination of components, although components 1 and 2 in particular gave a proportion of protein products with a smaller molecular weight than would be expected.

It is likely that the additional protein products arose from the translation of degradation products of the intact RNAs and/or partial readthrough of them. A deeper analysis of the protein products would not only clarify this matter but would check the homogeneity of the predominant RNA components of the preparations which we take to be the intact components, 1, 2, 3 and 4 of the CCMV genome. The results obtained so far indicate size homogeneity of the predominant protein products corresponding to the observed size homogeneity of the RNAs concerned but leave the question of composition (and sequence) unanswered.

Scrutiny of fingerprints of T_1 ribonuclease digests of the four RNA components (Fig. 4) showed that

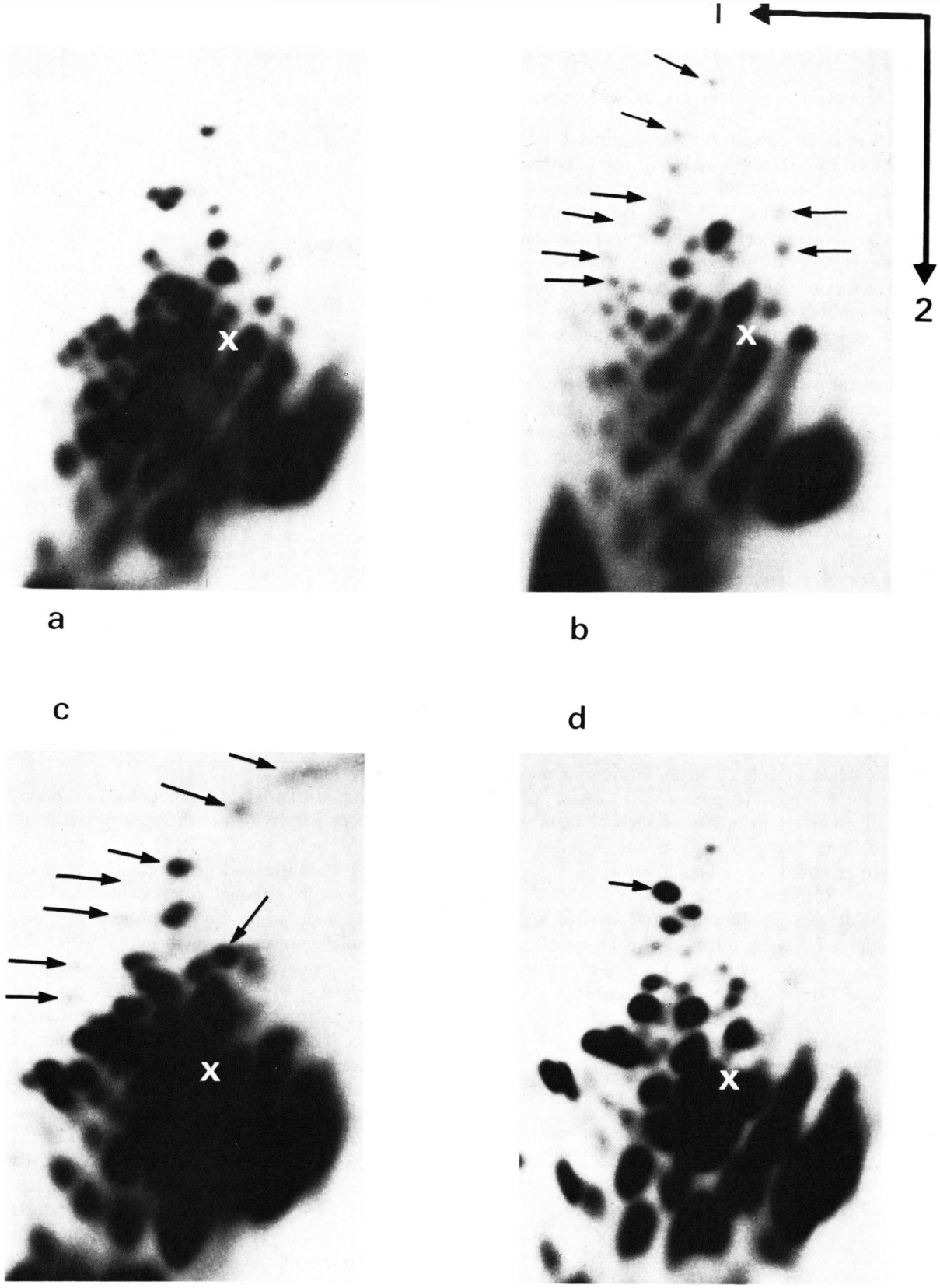


Fig. 4. Fingerprints of complete RNA T_1 digests of CCMV RNA components isolated as described in the text. a) RNA 1; b) RNA 2; c) RNA 3; d) RNA 4. Spots indicated by the arrows (→) are oligonucleotides which occur only in the specified RNA species. X = Bromphenol blue marker.

there was virtually no cross-contamination. However, while it is not relevant in the present exercise, which is primarily concerned with the resolution of the RNAs according to size, it is of longer term importance to explain the presence of a number of relatively weak spots on the maps. Apart from possible contamination by nucleases, they are unlikely to be due to inadequacies of the method or to degradation products of the viral RNA but could be due to inhomogeneity of each RNA component arising from contamination by a small proportion of viral mutants in the type strain, or to the possibility that pure lines of virus have a more complex make-up than hitherto suspected.

Discussion

Our early attempts to purify the components of CCMV RNA by polyacrylamide gel electrophoresis were abandoned in favour of density gradient centrifugation because of difficulties encountered in recovering the RNA from the gels. Using a sucrose gradient on a zonal rotor [9] we obtained high yields of the constituent RNAs but cross-contamination was considerable and could not be eliminated. This led to the reappraisal of gel electrophoresis as a method of resolution described in this paper.

The resolution of the viral RNAs on 2.6% polyacrylamide gels was improved by using the procedures described here. But recovery of RNA from the gels by leaching with water or buffers was poor and the product was contaminated with acrylamide polymer which is not easily separable from RNA [22].

There are numerous devices which seek to extract RNA from gels by electrophoresis into an aqueous phase bounded by a semipermeable membrane, usually dialysis membrane, positioned so as to prevent the RNA from reaching the anode. In our experience [23] this incurs large losses (up to 60% of the RNA) which arise from passage of the RNA through the membrane, although this does not occur when the membrane is used simply for dialysis.

As described above, we dispensed with membranes and trapped the RNA in strips of several materials of which glass fibre paper was the most suitable since it incurred the smallest loss of RNA. The larger the RNA the smaller the recovery, possibly as a result of irreversible adsorption of part of the RNA onto the carrier strip.

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