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Relationships between the RNA Components of Chronic Bee-Paralysis Virus and those of Chronic Bee-Paralysis Virus Associate

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SUMMARY

The RNA components of chronic bee-paralysis virus (CPV) and those of chronic bee-paralysis virus associate (CPVA) have been compared. CPV has five single-stranded RNA components, two larger RNAs designated 1 (mol. wt. 1.35×10^6 ; 4200 nucleotides) and 2 (mol. wt. 0.9×10^6 ; 2800 nucleotides), and three smaller RNAs designated 3a, 3b and 3c, each with mol. wt. 0.35×10^6 (1100 nucleotides). CPVA has three single-stranded RNA components designated A, B and C, each with mol. wt. 0.35×10^6 (1100 nucleotides). Gel electrophoretic and T_1 fingerprint analyses have shown that RNAs 3a, 3b and 3c are very similar to, and probably identical to, RNAs A, B and C respectively, i.e. these three RNAs appear to be encapsidated in both CPV and CPVA particles. A positive correlation was observed between the proportion of RNAs 3a, 3b and 3c in CPV particles and the amount of CPVA (and hence RNAs A, B and C) which had replicated. Fingerprint analysis has shown that RNAs A, B and C are distinct but related and also that about 50% of the sequence of each these three RNAs is homologous with RNA 2. It is therefore possible that, although CPV and CPVA are serologically unrelated, the RNAs of CPVA have evolved from CPV RNA 2.

INTRODUCTION

Chronic bee-paralysis virus (CPV), which causes a trembling condition soon followed by death in the adult honey bee, *Apis mellifera* (Bailey *et al.*, 1963), consists of ellipsoidal particles falling into four size classes with modal lengths of 30, 40, 55 and 65 nm, each approximately 20 nm wide. These components have sedimentation coefficients of 82S, 97S to 106S, 110S to 124S and 125S to 136S respectively and are found in the same relative proportions in preparations from naturally infected bees, from artificially infected bees and from bees injected with terminal dilutions of bottom component only. In caesium chloride gradients, all size classes have a buoyant density of 1.33 g/ml. The particles contain single-stranded RNA with a base composition of 20% G, 24% A, 28% C and 28% U. Serologically, the components are indistinguishable (Bailey, 1976; Bailey *et al.*, 1968).

Chronic bee-paralysis virus associate (CPVA) is frequently found in bees infected with CPV (Bailey, 1976). It consists of 17 nm isometric particles which are serologically unrelated to those of CPV. The virus particles have a buoyant density of 1.38 g/ml in caesium chloride, a sedimentation coefficient of 41S, and contain single-stranded RNA. CPVA cannot multiply when injected into bees unless CPV is included in the inoculum, which suggests that CPVA and CPV are related as satellite and helper viruses (Bailey *et al.*, 1980). However, CPVA sometimes multiplies in bees injected with CPV preparations which contain no detectable CPVA. In this paper, we report the identification of several RNA components from CPV and CPVA and the results of comparison of these RNAs by T_1 oligonucleotide fingerprinting.

METHODS

Propagation and purification of viruses. CPV and CPVA were propagated by injection of individual pupae (queens, drones and workers) with terminally infective dilutions of extracts (Bailey, 1976) from infected bees and maintenance of these pupae at 35°C for up to 1 week, as described by Bailey & Woods (1974, 1977). Viruses were

extracted and purified by sucrose density gradient centrifugation as described by Bailey *et al.* (1980). Relative proportions of CPV and CPVA in these preparations were determined from A_{254} profiles of the sucrose gradients measured with an ISCO UA 2 analyser.

Analysis of CPV and CPVA RNA components. RNA was analysed by polyacrylamide gel electrophoresis (PAGE) in slab gels ($16 \times 16 \times 0.15$ cm) containing 4% acrylamide, 0.04% *N,N*-methylenebisacrylamide, 8 M-urea, 0.1% SDS and TAE buffer (0.04 M-Tris-HCl, 0.02 M-sodium acetate, 0.016 M- Na_2EDTA , adjusted to pH 8.0 with acetic acid). Samples were prepared by disruption of virus particles in 1% SDS at 60 °C for 20 min, with or without subsequent glyoxalation (McMaster & Carmichael, 1977). Electrophoresis was carried out at 80 V for 17 to 20 h with a top electrode buffer of TAE + 0.1% SDS and a bottom electrode buffer of TAE + 0.1% SDS + 8 M-urea. Gels were then washed for 1 to 2 h in distilled water and stained in 0.01% aqueous toluidine blue. Tracks cut out from stained gels were scanned at 550 nm with a Gilford model 240 spectrophotometer equipped with a model 2410-S linear transport. RNA molecular weights were determined by co-electrophoresis of glyoxalated samples with the following glyoxalated RNA standards: cucumber mosaic virus RNAs (Francki *et al.*, 1979), wheat germ ribosomal RNA (Loening, 1968) and denatured *Penicillium stoloniferum* virus F RNA 3 (Buck & Ratti, 1977).

Purification of RNA components of CPV and CPVA. RNA samples were subjected to PAGE as described above. Instead of staining with toluidine blue, the RNA bands were visualized by placing the gel on a fluorescent TLC plate (Polygram; Camlab, Cambridge, U.K.) and illuminating from above with u.v. light of 254 nm. Bands appearing as shadows on the TLC plate were cut out. RNA, extracted from the gel slices by the method of Schuerch *et al.* (1975), was stored in $0.1 \times$ TNE buffer (0.005 M-Tris-HCl, 0.015 M-NaCl, 0.01 mM- Na_2EDTA , adjusted to pH 7.9 with HCl) at -20 °C.

RNA fingerprinting. RNA samples (1 μg) in $0.1 \times$ TNE were incubated at 37 °C for 30 min with T_1 ribonuclease (Sankyo, 3 units), bacterial alkaline phosphatase (Worthington, 0.2 μg), polynucleotide kinase (Boehringer, Mannheim, 1 unit) and [γ - ^{32}P]ATP (Amersham International, 50 μCi) (Szekely & Sanger, 1969). The labelled T_1 oligonucleotides thus generated were fractionated in two dimensions by PAGE according to Kennedy (1976). Fingerprints were autoradiographed for 1 to 3 days using Kodak Blue Brand X-ray film.

RESULTS

RNA components of CPV and CPVA

The RNA components of CPV and CPVA were analysed by electrophoresis in 4% polyacrylamide gels containing 8 M-urea. Five major RNA components were seen in CPV, and three components in CPVA (Fig. 1*a, b*). The CPV RNAs were designated 1, 2, 3a, 3b and 3c, and the CPVA RNAs A, B and C, in order of increasing electrophoretic mobility. In mixtures, CPVA RNAs A, B, and C co-migrated with CPV RNAs 3a, 3b and 3c respectively.

In 8 M-urea at room temperature, the RNA components may not be completely denatured (Reijnders *et al.*, 1973). In order to determine their mol. wt., therefore, the RNA components were glyoxalated (McMaster & Carmichael, 1977; Murant *et al.*, 1981) to remove all secondary structure, prior to co-electrophoresis with glyoxalated marker RNAs. After this treatment, CPV RNA was seen to consist of only three major components, with RNAs 3a, 3b and 3c co-migrating. Similarly, after glyoxalation, the three RNA species of CPVA co-migrated and the resulting single RNA band was shown to co-migrate with the single band produced by the glyoxalated RNAs 3a, 3b and 3c of CPV (Fig. 1*c*). The mol. wt. of CPV RNAs 1, 2 and 3 were estimated to be 1.35×10^6 (4200 nucleotides), 0.9×10^6 (2800 nucleotides) and 0.35×10^6 (1100 nucleotides) respectively. The mol. wt. of the CPVA RNAs was therefore 0.35×10^6 (1100 nucleotides).

These results suggest that the three smallest RNAs of CPV might be identical to the three RNAs of CPVA, in view of their indistinguishable size and their common property of migrating, when partially denatured, as three distinct bands. Two explanations of this latter property are possible: firstly, that three RNAs of indistinguishable size but different sequence are present, and that these can form secondary structures sufficiently different to cause a difference in electrophoretic mobility; or, secondly, that a single RNA species is present which is capable of forming three different secondary structures with different mobilities.

To determine which of these explanations is correct CPVA RNA was subjected to PAGE under partially denaturing conditions (8 M-urea, room temperature) and the three resulting RNA bands were extracted from the gel. Samples of the three separated RNAs in $0.1 \times$ TNE buffer were heated to 100 °C for 2 min to remove all secondary structure and then slowly cooled to room temperature over a period of 90 min to allow secondary structures to reform. When analysed by

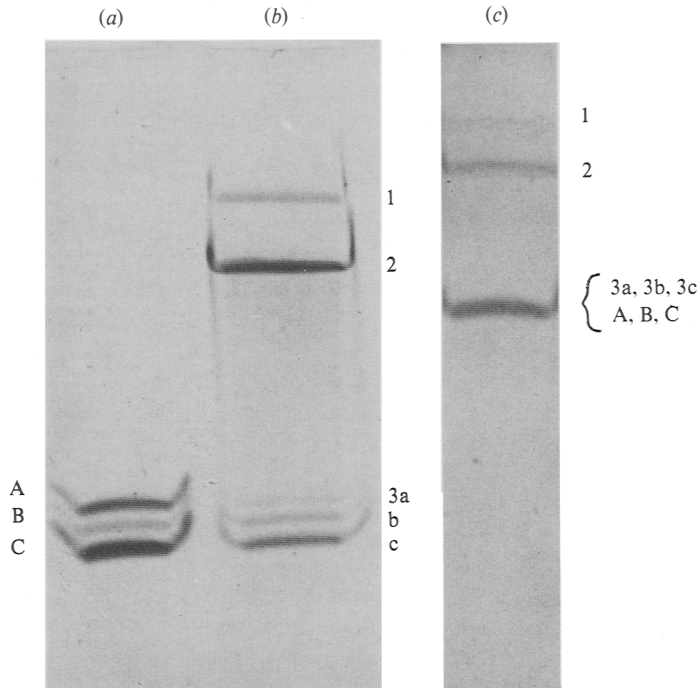


Fig. 1. Urea-PAGE of CPV and CPVA RNA components. Electrophoresis was from top to bottom and gels were stained with toluidine blue. (a) CPVA RNA; (b) CPV RNA; (c) mixture of glyoxalated CPVA RNA and glyoxalated CPV RNA.

PAGE, each of the heated samples gave a single band with a mobility identical to that of the corresponding untreated RNA. It was concluded that the RNA of CPVA consists of three components of different sequence and secondary structure, but of indistinguishable size. If the RNA had been a single species with three possible secondary structures each of the purified, heated and cooled RNA samples should have appeared as three bands in the gel due to interconversion of the three structures.

Fingerprinting of CPV and CPVA RNA components

The initial approach to separating CPV RNAs was based on resolution of the virus particles by sucrose density gradient centrifugation (SDGC) in the hope that RNAs 1, 2 and 3 (a, b and c) might be encapsidated separately in particles of different size. However, although good separation of the particle size classes was obtained by SDGC, very little separation of the RNA components was achieved. RNAs 1, 2 and 3 (a, b and c) were found in all the fractions in similar proportions. Therefore, PAGE was used to separate the RNA components of CPV and those of CPVA. Separated RNA components were then extracted from the gels as described in Methods. Each RNA was digested to completion with T_1 ribonuclease, the T_1 oligonucleotides were separated by two-dimensional PAGE and the gel was autoradiographed. The resultant fingerprints were reproducible, but there was some variation in relative intensities of spots, depending on the activity of polynucleotide kinase. Because of this variation at least two autoradiographic exposures of each gel were obtained in order to obtain maximum resolution in each area of the fingerprint and to facilitate comparisons between fingerprints of different RNAs.

The upper regions of fingerprints contain small T_1 oligonucleotides, common to all RNAs. The lower regions, shown in Fig. 2 and 3, contain the large T_1 oligonucleotides, specific to each RNA. The degree of homology between two RNAs can be estimated from the proportion of specific oligonucleotides of identical mobility which their fingerprints have in common. This involves two assumptions. Firstly, the small proportion (10 to 15%) of the total RNA sequence

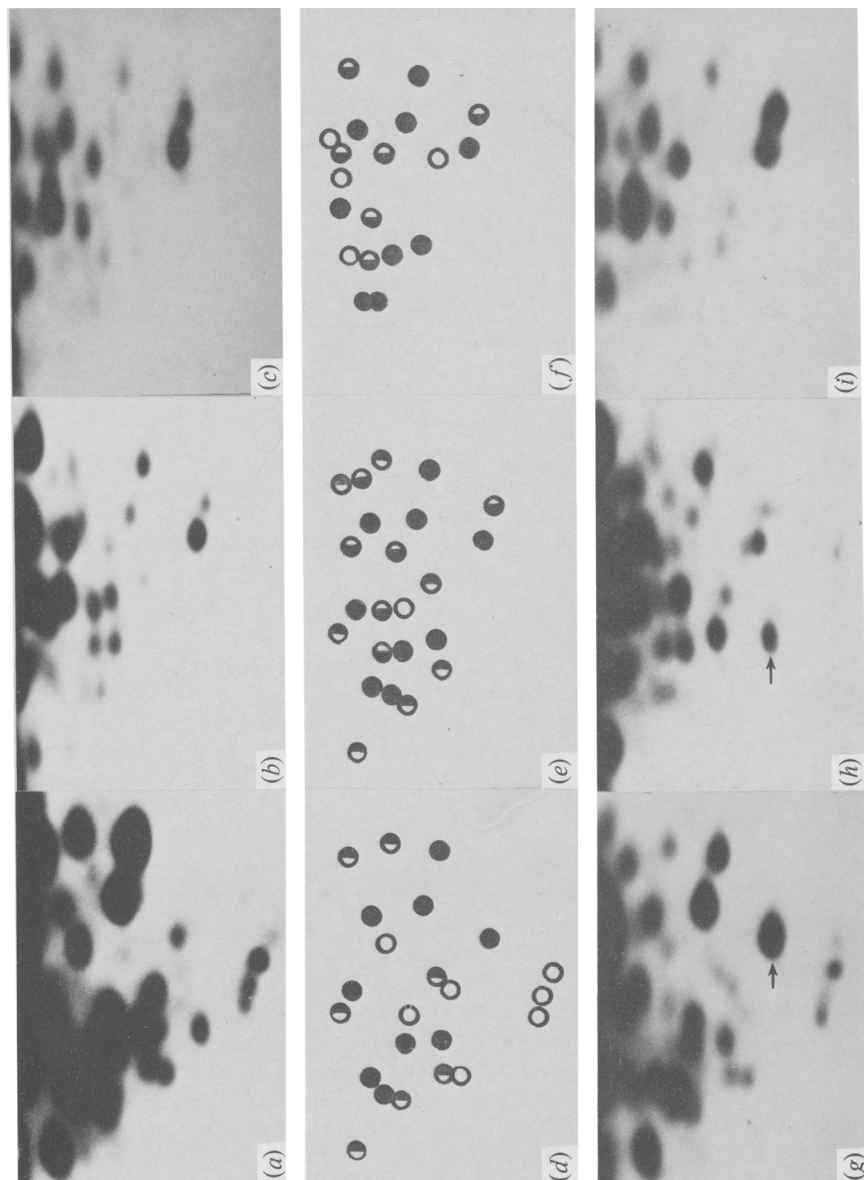


Fig. 2. T_1 oligonucleotide fingerprints of CPVA RNAs A, B and C and CPV RNAs 3a, 3b and 3c. Autoradiograms show: (a) RNA A; (b) RNA B; (c) RNA C; (g) RNA 3a; (h) RNA 3b; (i) RNA 3c. Diagrammatic representations are: (d) RNA A; (e) RNA B; (f) RNA C. Oligonucleotide spots are classified as: ○, unique to RNAs A, B or C; ●, common to RNAs A and B; ●, common to RNAs B and C; ●, common to RNAs A, B and C. In these and subsequent fingerprints, migration in the first dimension is from right to left and in the second dimension from bottom to top. Only the large, specific oligonucleotides are represented. The top of each autoradiogram indicates the approximate position of the bromophenol blue marker dye.

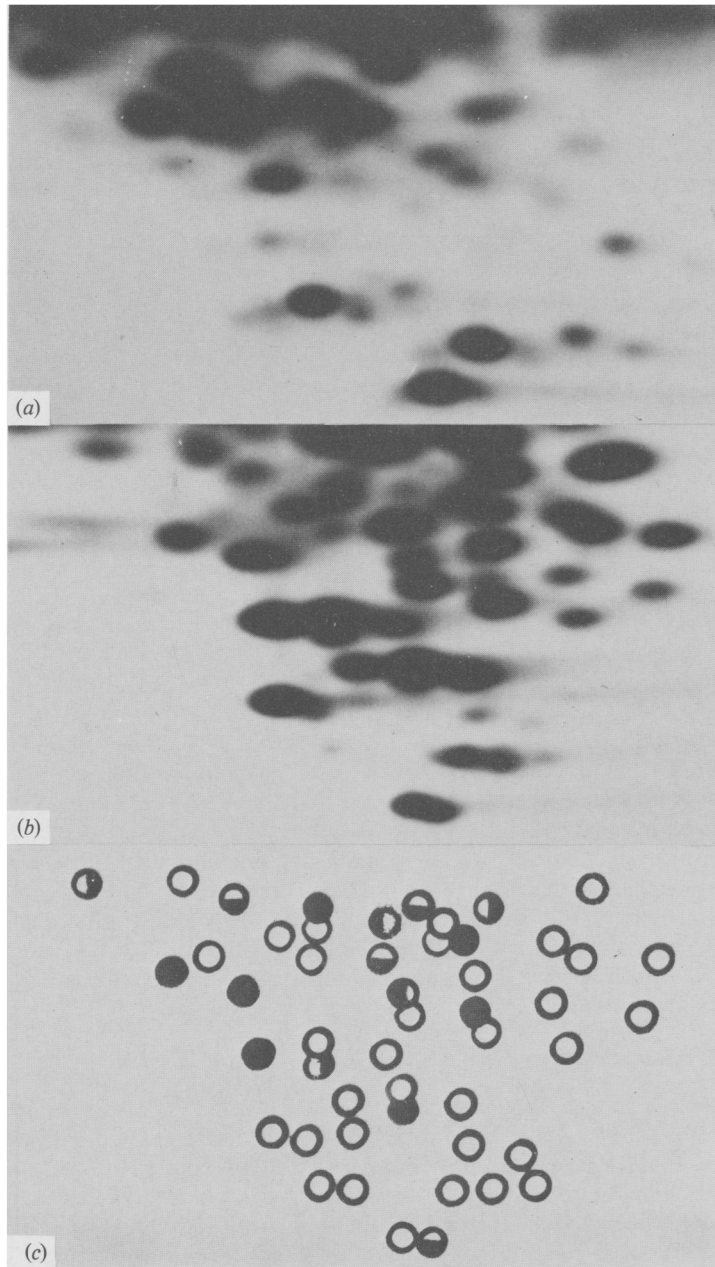


Fig. 3. T_1 oligonucleotide fingerprints of CPV RNAs 1 and 2. Autoradiograms show: (a) RNA 1; (b) RNA 2. (c) Diagrammatic representation of RNA 2 fingerprint showing oligonucleotide spots shared by: \odot , RNAs A, B or C only; \bullet , RNAs A and B; \bullet , RNAs B and C; \bullet , RNAs A, B and C. RNA 2 oligonucleotides not found in RNAs A, B and C are represented as open circles.

that appears in the 'specific' region of the fingerprint is taken to be representative of the whole sequence. Secondly, oligonucleotides with indistinguishable mobilities are taken to be identical and not just sequence isomers. A small number of overlapping T_1 oligonucleotide spots may be discounted as insignificant for this reason, unless their identity is confirmed by sequencing.

The fingerprints of CPVA RNAs A, B and C (Fig. 2a, b, c) were distinct, confirming that these three RNAs have unique sequences and are not merely conformational variants of the

same molecule. However, the three fingerprints also showed a number of similarities. Nine oligonucleotides were common to the fingerprints of all three RNAs, whereas others were found in fingerprints of two of the RNAs, but not that of the third RNA. Common and unique oligonucleotides are shown diagrammatically in Fig. 2(*d, e, f*). It is clear that all three RNAs are related; individual pairs of RNAs share 40 to 70% sequence homology.

The fingerprints of CPV RNAs 3a, 3b and 3c (Fig. 2*g, h, i*) were very similar to those of CPVA RNAs A, B and C (Fig. 2*a, b, c*). All the major T₁ oligonucleotides in the fingerprints of RNAs A, B and C could be identified in the fingerprints of RNAs 3a, 3b and 3c respectively. The fingerprints of the latter three RNAs showed a variable degree of contamination by RNA 2 oligonucleotides (Fig. 3*b*). When RNA was prepared from a CPV preparation which had been stored at 4 °C for 1 week, nearly all of the RNA 2 oligonucleotides could be identified in fingerprints of the RNA 3 subspecies; these probably arose from degradation products of RNA 2 with the same mobilities as the RNA 3 subspecies, since these RNAs were electrophoretically homogeneous. However, when RNA was prepared from freshly isolated and purified CPV preparations, contamination with RNA 2 degradation products was slight. Only one major oligonucleotide (arrowed in Fig. 2*g, h*) in the fingerprints of RNA 3a and 3b, and none in that of RNA 3c, could be ascribed to contamination by RNA 2 degradation products.

The similarities of the fingerprints of CPV RNAs 3a, 3b and 3c with those of CPVA RNAs A, B and C respectively, together with their identical molecular weights and identical mobilities when electrophoresed under partially denaturing conditions, suggest that these three pairs of RNAs are closely similar and probably identical, i.e. CPV RNAs are also encapsidated in CPV particles. The presence of these RNAs in CPV is not the result of contamination of the preparations by CPVA particles. No CPVA could be detected in CPV preparations, after separation by SDGC, either by electron microscopy or by PAGE analysis of virus polypeptides (Bailey & Woods, 1977; Bailey *et al.*, 1980).

Fingerprints of CPV RNAs 1 and 2 (Fig. 3*a, b*) were obviously different and co-fingerprints of the two RNAs showed that each RNA had at least 25 oligonucleotides not found in the other RNA. Comparison of the fingerprints of RNA 2 with those of RNAs A, B and C (and RNAs 3a, 3b and 3c), and with co-fingerprints of pairs of these RNAs, showed that about half of the oligonucleotides in each of the RNA A, B and C fingerprints were present in the RNA 2 fingerprint. These included seven of the nine oligonucleotides common to RNAs A, B and C, as well as some found in two or only one of the RNAs, and are shown diagrammatically in Fig. 3(*c*). It is important to note that contamination of the fingerprints of RNAs A, B and C by oligonucleotides derived from RNA 2 degradation products produced *in vitro* is not possible, since RNA 2 was absent from CPVA.

Relative abundance of RNA components in CPV

Viruses were extracted from single queen pupae and CPV and CPVA were purified by SDGC (Bailey *et al.*, 1980). Ten such pairs of CPV and CPVA RNA samples without glyoxalation were analysed by urea-PAGE. Scans of representative CPV RNA tracks cut from stained gels, and the absorbance profiles of the sucrose gradients from which they were taken, are shown in Fig. 4.

The amount of CPVA produced varied considerably from individual to individual, and the amount of CPV produced was inversely related to this (L. Bailey & B. V. Ball, unpublished results). It was found that the relative proportions of the RNA components in CPV were related to the amount of CPVA which had multiplied in that individual. When large amounts of CPVA were present (Fig. 4*a*), CPV contained principally RNAs 3a, 3b and 3c, although RNAs 1 and 2 were always detectable. Conversely, CPV from queens containing small amounts of CPVA consisted mainly of RNAs 1 and 2 (Fig. 4*b*). An extreme case occurred in drone and worker pupae, in which CPVA is usually barely detectable. Here, RNAs 3a, 3b, and 3c were very much reduced in amount though never entirely absent. In the case illustrated (Fig. 4*c*) only RNA 3b can be seen on the scan.

In Fig. 4(*a, b*) RNA 3c is seen to be more abundant than RNAs 3a and 3b. This was not always the case, however. Some samples have been shown to contain mainly RNA 3a or 3b. The relative proportions of the three RNAs in CPV were not always mirrored by the proportions of

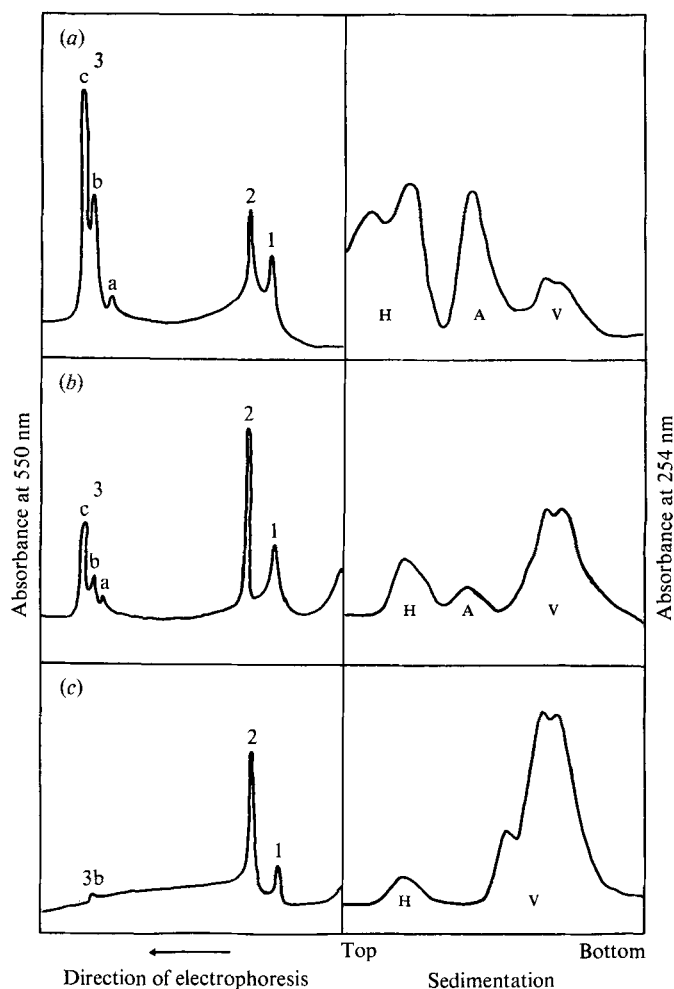


Fig. 4. PAGE of CPV RNA samples (left) and sucrose density gradient centrifugation profiles of virus samples from which they were derived (right). Gels were stained with toluidine blue and scanned at 550 nm. Density gradients were scanned at 254 nm with an ISCO u.v. monitor. V, CPV; A, CPVA; H, host material. Samples were from (a, b) two different queen pupae and (c) from five drone pupae.

these RNAs in CPVA (Fig. 1). In all CPV samples examined, RNA 2 was seen to be more predominant than RNA 1, but the relative proportion of these RNAs was variable.

DISCUSSION

The results presented here show that CPV and CPVA contain five and three RNA components respectively and that the smallest RNAs of CPV are very similar, and probably identical, to those of CPVA. It appears that these three RNAs can be packaged in either of two serologically unrelated and morphologically different particles. The positive correlation observed between the proportion of RNAs 3a, 3b and 3c in CPV and the amount of CPVA (and hence RNAs A, B and C) which had multiplied is consistent with these results. In some cases these RNAs can be the major constituents of CPV particles, showing that the packaging may be highly efficient. The packaging of CPVA RNAs in CPV particles explains the appearance of CPVA in bees injected with purified CPV (Bailey *et al.*, 1980).

Substantial homology between CPVA RNAs A, B and C has been established by T_1 fingerprinting. Several oligonucleotides are common to all three RNAs, while other oligonucleotides are found in only two of the three RNAs, and each has some unique oligonucleotides. About half

the sequence of each of these CPVA RNAs is present in CPV RNA 2, although the sequences so represented are partly different in each case (Fig. 3c). It is therefore possible that these RNAs have evolved from RNA 2, perhaps via a subgenomic messenger RNA derived from RNA 2. The possibility should also be considered that there are more than three forms of CPVA RNA but that only three resolved, and that there are three (or more) forms of RNA 2 each related to the CPVA RNAs. Some support for this suggestion comes from the presence of several minor spots in the RNA fingerprints. Although these could be partly due to differing efficiencies of labelling of different nucleotides (Szekely & Sanger, 1969) they could also indicate sequence heterogeneity. Further evidence of this possibility, or otherwise, could be obtained by analysis of cDNA clones prepared from each of the RNA species resolved by gel electrophoresis.

CPVA is serologically unrelated to CPV and multiplies only in the presence of CPV. Assuming that the capsid polypeptide of CPVA is encoded by one of its RNA components, CPVA could be regarded as a satellite virus, as defined by Mossop & Francki (1978) who distinguished between satellite viruses, satellite RNAs and defective-interfering RNAs. However, CPVA RNA is also encapsidated by CPV. The encapsidation of the RNA of one virus in the capsid of another (genomic masking) has been observed to take place efficiently between related viruses, but inefficiently, or not at all, between unrelated viruses *in vivo* (Peterson & Brakke, 1973; Dodds & Hamilton, 1976). The efficient encapsidation of CPVA RNA by CPV may be due to the presence of common sequences in CPVA RNAs and CPV RNA 2, which could include initiation sites for CPV capsid assembly. It is interesting to speculate that CPVA could represent an intermediate stage in the evolution of a satellite virus from its helper genome. However, support for this possibility and for the apparent similarity of CPV to a number of multicomponent plant viruses (Hilleman & Morris, 1981) must await further studies of the genome structure, strategy and gene expression in this virus system.

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