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Comparison of the Ribonucleic Acid Subunits of Reovirus, Cytoplasmic Polyhedrosis Virus, and Wound Tumor Virus[1]

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Double-stranded ribonucleic acid (RNA) from intact cytoplasmic polyhedrosis virus (CPV) and wound tumor virus (WTV) was analyzed by polyacrylamide gel electrophoresis. Using RNA from type 3 reovirus as a standard, it was calculated that CPV-RNA consisted of 9 subunits corresponding to a molecular weight of 12.7×10^6 and WTV-RNA consisted of 12 subunits corresponding to a molecular weight of 15.5×10^6.

The genomes of reovirus, cytoplasmic polyhedrosis virus (CPV) and wound tumor virus (WTV) have been shown to consist of helical, double-stranded ribonucleic acid (RNA; references 7, 12, 13, 16, 20). For reovirus RNA, contour lengths up to 7 μm have been occasionally observed (11) and a composite contour length up to 8.3 μm has been recently determined (25); however, RNA prepared by a variety of methods appears to consist of segments falling into three length distributions of 1.13, 0.66, and 0.38 μm (25). The molecular weights of these three groups of RNA fragments have been estimated as 2.4×10^6, 1.4×10^6, and 0.8×10^6, respectively (24, 26). When analyzed by polyacrylamide gel electrophoresis, reovirus RNA appears to consist of 10 individual subunits (24, 28). The lack of base sequence homology between the RNA subunits of different size suggests that breakage occurs at specific sites rather than at random (2, 26).

The molecular weight of WTV-RNA has been estimated at 15×10^6 (7). When WTV was osmotically shocked, filaments not greater than 1 μm in length were observed, although the RNA strands appeared to be complexed with protein (5). A more intensive analysis of WTV-RNA obtained by phenol extraction (14) has failed to give a nonrandom size distribution pattern of the sort observed for reovirus RNA.

The RNA obtained by phenol treatment of CPV has been reported to have two components (20), as observed by sedimentation analysis and by contour lengths of RNA in electron micrographs. One component has been shown to sediment at 15.5*S* and have a contour length of 1.3 μm, corresponding to a molecular weight of 3×10^6. The other component sedimented at 12.5*S* and had a contour length of 0.4 μm, corresponding to a molecular weight of 1×10^6.

In the present paper the RNA subunits of CPV and WTV were resolved by polyacrylamide electrophoresis and compared to the RNA components obtained from reovirus.

MATERIALS AND METHODS

Preparation of ^{32}P-labeled CPV RNA. Fifth-instar silkworm (*Bombyx morii*) larvae were infected with CPV by allowing them to feed on fresh mulberry leaves which had been painted with a suspension of polyhedra. Three days after infection the larvae were starved for 12 hr and then fed leaf discs on which about 0.35 mc of ^{32}P phosphoric acid (Mallinckrodt Chemical Works, St. Louis, Mo.) was dried. The larvae were starved for a further 12 hr after which time they were fed in the usual manner. ^{32}P-labeled virus could be obtained by allowing polyhedrosis to occur and isolating the virus by alkali treatment of polyhedra (17). ^{32}P-labeled RNA could be obtained by treatment of the virus with 1% sodium dodecyl sulfate (SDS) at 45 C for 10 min.

Alternatively, ^{32}P-labeled RNA could be obtained directly by phenol extraction of infected gut tissue. Viral double-stranded RNA was separated from single-stranded RNA species by hydroxyapatite chromatography (4, 17). The RNA was recovered by precipitation with acetone in the presence of potassium acetate (15).

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Preparation of ^{32}P-labeled WTV RNA from infected leaf hopper cells. Monolayer cultures of *Agallia constricta* (AC) cells infected with WTV were maintained in insect growth medium until confluent, trypsinized, and transferred to growth medium lacking KH_2PO_4 (10). One millicurie of carrier-free ^{32}P phosphoric acid (10 mc/ml) was added to each of two Falcon plastic culture flasks (25 cm^2) containing the new cell monolayers. After 5 days of incubation at 27 C the cells were still in the form of a viable monolayer. After removal of the ^{32}P medium, the AC cells were collected by twice washing the monolayer with Rinaldini's solution followed by trypsinization and two further washings. The cell pellet (0.1 ml) was suspended in 1 ml 0.3 M glycine; 0.03 M $MgCl_2$; 0.03 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.6) and the ^{32}P WTV was isolated by Genosolv-D treatment of cells followed by sucrose density gradient centrifugation. The viral peak fractions were collected and dialyzed against 3 liters of 0.1 M glycine; 0.01 M $MgCl_2$; 0.01 M Tris buffer (*p*H 7.6). ^{32}P-RNA was obtained by treating WTV with 1.0% SDS at 37 C for 10 min.

Polyacrylamide gel electrophoresis. The electrophoresis of RNA on 2.25% acrylamide, 1% agarose columns was carried out as described by Watanabe (27) with the following modifications. Running buffer was 0.05 M Tris buffer (*p*H 8.4), 10^{-4} M ethylenediaminetetraacetic acid (EDTA), and electrophoresis was carried out in columns (8 cm) at 5 ma per column (110 volts) for 8 hr at room temperature with bromphenol blue used as a tracking dye. Gels containing radioactive RNA were cut into 1-mm slices, dried on planchets at 45 C, and counted in a Beckman gas-flow counter. Alternatively, gels were stained with 1% methylene blue in 0.1 M acetate buffer (*p*H 4.0) and decolorized in water.

Sucrose gradient analysis of RNA. ^{32}P-RNA prepared either from intact CPV or WTV, or by phenol extraction of CPV-infected gut tissue was sedimented on a 5 to 20% sucrose gradient containing 0.1 M NaCl, 0.01 M Tris buffer (*p*H 7.6), 10^{-3} M EDTA, and 1% SDS in an SW41 rotor at 193,000 $\times$ *g* and at 20 C in a Spinco model L-2 centrifuge. The gradient was fractionated by collecting drops through the bottom of the tube. Portions of these fractions were dried on glass-fiber filters and counted in a Beckman liquid scintillation counter.

Phosphorus analysis. Phosphorus was determined (9) from samples of whole virus heated at 190 to 200 C for 30 min with 35% perchloric acid in 5 N H_2SO_4. The percentage of RNA was estimated from an assumed phosphorus content for double-stranded RNA of 8.6% and a protein determination (18) of a sample of virus.

Boundary sedimentation analysis. Virus sedimentation coefficients were determined with a Spinco model E analytical ultracentrifuge. For CPV, Schlieren optics and a 12-mm path-length Kel-F cell were used. All CPV studies were performed at 22 to 25 C and 9,500 $\times$ *g* by using a buffer of 0.01 M Tris (*p*H 7.4). For WTV, ultraviolet absorption optics and a 30-mm, path-length Kel-F cell were used. All WTV studies were performed at 4 to 6 C and 11,800 $\times$ *g* by using a buffer of 0.1 M glycine, 0.01 M $MgCl_2$, and 0.01 M Tris (*p*H 7.5).

RESULTS

Analysis of RNA subunits by polyacrylamide electrophoresis. When ^{32}P-labeled, double-stranded RNA from CPV obtained by 1% SDS treatment of intact virus was analyzed by electrophoresis in polyacrylamide gels, eight distinct peaks could be separated (Fig. 1). Peak I could be resolved into two components in stained gels which generally resulted in better resolution than gels sliced and counted for radioactivity. When ^{32}P-labeled WTV-RNA, obtained from intact virus was similarly treated, seven peaks were reproducibly separated (Fig. 2). As was the case with CPV, better resolution of the components could be obtained by dye staining of the gels. In this manner, peaks II and VI could be each visually resolved into two bands when unlabeled WTV-RNA isolated from plant tumors was subjected to the same degree of electrophoretic separation. Reovirus has been shown to contain about 15 to 20% of its nucleic acid content as a single-stranded, A-rich component (3, 22). To determine if this was the case with CPV and WTV, each virus was treated with 1% SDS and electrophoresed for 2 hr in 2.25% acrylamide gels, enough time for the bromphenol blue dye marker to migrate halfway down the gel. No comparable peak of radioactivity could be seen in the region where A-rich RNA was found for reovirus (i.e., R_F of 1.37 relative to

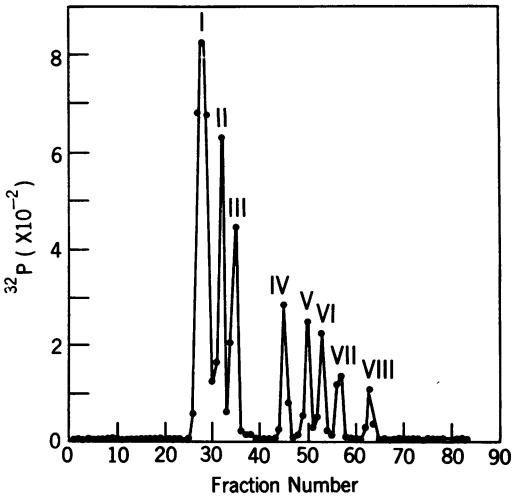

FIG. 1. *Electrophoretic separation of ^{32}P-labeled CPV-RNA subunits. Migration is from left to right in 2.25% polyacrylamide, 1% agarose gels of virus treated with 1% SDS.*

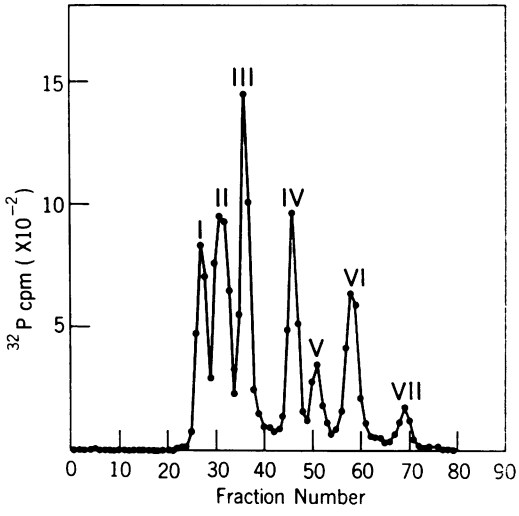

FIG. 2. *Electrophoretic separation of ^{32}P-labeled WTV-RNA subunits. The same conditions were used as in Fig. 1.*

bromphenol blue). Furthermore, 90% of the radioactivity present in a sample of ^{32}P-labeled virus dried directly on a planchet could be accounted for in the double-stranded RNA region when the gel was sliced and counted. About the same recovery of radioactivity was obtained after electrophoresis for 8 hr.

Assuming that the distance of migration of RNA in polyacrylamide gels is a direct function of its molecular weight (6), the molecular weight of the RNA subunits was estimated by using the migration of the three major RNA species of reovirus and their average molecular weights, i.e. 2.4×10^6, 1.4×10^6, and 0.8×10^6 as standards (24, 26). To eliminate any possible artifacts that may arise in comparing positions in different gels, unlabeled reovirus was treated with 1% SDS at 45 C for 10 min and run in the same gel with labeled CPV-RNA or WTV-RNA. The gels were stained so as to note the positions of reovirus RNA and then were sliced and counted. The molecular weights of the subunits were estimated from a plot of the distance migrated (Fig. 3).

To determine if the observed peaks represented more than one component, a comparison of the distribution of observed radioactivity was made with the theoretical radioactivity based on the molecular weight and the number of components in each peak (Table 1). Based on this analysis of the distribution of radioactivity, CPV-RNA would consist of nine subunits with a total molecular weight of 12.7×10^6. WTV-RNA would consist of 12 subunits with a total molecular weight of 15.5×10^6. The pattern of CPV-RNA obtained by phenol extraction of infected larval gut tissue and purified on hydroxyapatite did not vary from that obtained from intact virus in that about 73% of the total radioactivity of both preparations was associated with peaks I through III. When unlabeled WTV-RNA obtained from plant tumors was electrophoresed with trace amounts of high specific activity ^{32}P-WTV-RNA isolated from insect cell cultures, the positions of the carrier unlabeled RNA components observable by dye staining coincided with the ^{32}P-labeled RNA.

Sucrose density sedimentation of RNA. To investigate whether the smaller RNA subunits might have arisen from breakdown of the larger RNA subunits, labeled RNA from SDS-treated virus was subjected to sucrose gradient sedimentation. Figure 4 shows the results of such analysis of CPV-RNA. The RNA could be separated into two main classes and probably represents the 15.5*S* and 12.5*S* RNA components previously observed (20). Fractions 20 to 32 (L) and 35 to 42 (S) were pooled, dialyzed against 0.01 M Tris buffer (*p*H 7.4), flash evaporated, and run on acrylamide gels. Fraction L gave only the large molecular weight subunits (peaks I through III), and fraction S gave only the small molecular weight subunits (peaks IV through VIII).

Analysis of WTV-RNA on a sucrose gradient gave the profile shown in Fig. 5. The division

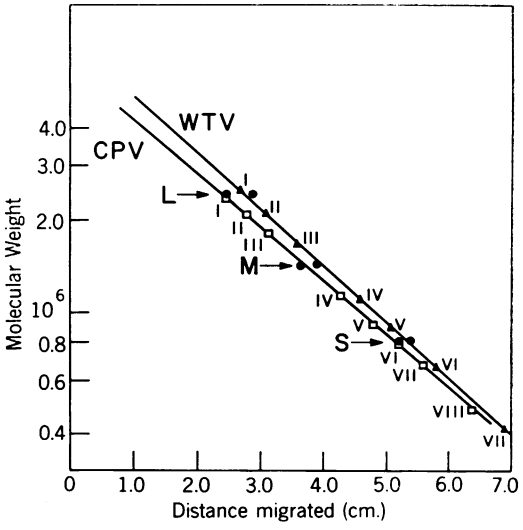

FIG. 3. *Semilog plot of the molecular weight versus the distance migrated in polyacrylamide gels. Symbols: □, CPV-RNA; △, WTV-RNA; ●, reovirus type 3 RNA. L, M, and S, represent positions of double-stranded RNA from reovirus 2.4, 1.4, and 0.8 × 10^6, respectively (26).*

TABLE 1. *Analysis of radioactivity in CPV-RNA and WTV-RNA subunits in Fig. 1 and 2*

CPV-RNA						WTV-RNA					
Peaks	Mol wt[a]	No. of pieces	Equivalent Mol wt	Counts/min[b]		Peaks	Mol wt[a]	No. of pieces	Equivalent Mol wt	Counts/min[c]	
				Observed	Estimated					Observed	Estimated
I	2.4	2	4.8	2,300	2,026	I	2.5	1	2.5	2,248	2,405
II	2.05	1	2.05	888	856	II	2.1	2	4.2	3,559	4,040
III	1.8	1	1.8	713	760	III	1.7	2	3.4	3,530	3,271
IV	1.15	1	1.15	391	485	IV	1.1	2	2.2	2,318	2,116
V	0.94	1	0.94	320	397	V	0.9	1	0.9	819	866
VI	0.8	1	0.8	315	338	VI	0.65	3	1.9	2,017	1,828
VII	0.64	1	0.64	258	270	VII	0.4	1	0.4	416	385
VIII	0.48	1	0.48	172	203						
Total		9	12.7	5,357		Total		12	15.5	14,907	

[a] Estimated from Fig. 3.
[b] Total counts per minute observed divided by total molecular weight. CPV counts per minute per 10^6 were 422.
[c] WTV-RNA counts per minute per 10^6 were 962.

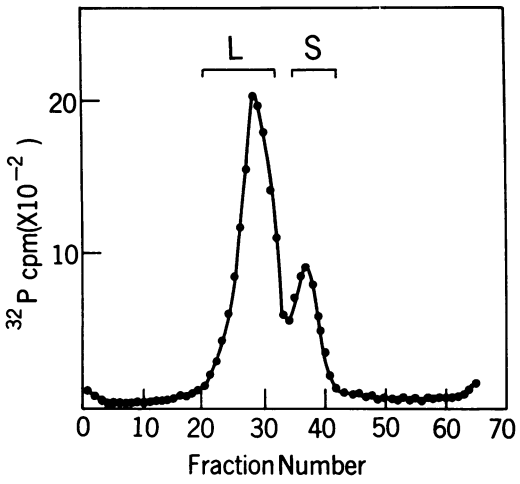

FIG. 4. *Profile of ^{32}P-labeled CPV-RNA on 5 to 20% sucrose gradient. Sedimentation was performed in the SW 41 rotor at 193,000 × g for 6 hr. Fractions L and S were subsequently analyzed by polyacrylamide electrophoresis. The bottom of the gradient is to the left.*

between the larger (2.4×10^6 to 1.7×10^6) and the smaller (1.1×10^6 to 0.4×10^6) RNA components observed with acrylamide gel separation is also apparent with the sucrose gradient profile; also reasonably good correlation exists between individual components seen with the two separation systems. The larger RNA components separated with acrylamide gels represented 62.6% of the radioactivity as compared to fractions 5–19 of the sucrose gradient which contained 60.4% of the radioactivity. When sucrose gradient fractions 5–19 (L) were pooled, dialyzed, flash evaporated, and then analyzed on an acrylamide gel, only the large RNA components (peaks I through III) were observable, even though the sample had been stored for one week at 4 C. This suggests that the larger subunits did not, as a result of breakdown, give rise to the smaller molecular weight subunits.

The sucrose gradient profile for the WTV ^{32}P-RNA reproducibly showed a higher degree of separation of components than for CPV ^{32}P-RNA. The much higher specific activity of the WTV ^{32}P-RNA obtainable with tissue culture as compared to the CPV ^{32}P-RNA isolated from whole insects accounts for the difference in resolution. In Fig. 5 there are small peaks at fractions 50 and 60, representing 3.7% of the total radioactivity; a similar sucrose gradient for a different preparation of WTV ^{32}P-RNA gave one small peak in the same general region of fraction 50, representing 1.5% of the total label. These small peaks are probably either degradation products of the larger components or impurities.

Boundary sedimentation analysis. CPV isolated from polyhedra was used for sedimentation studies; the average $S_{20,w}$ value of three determinations was 440 ± 20*S*. WTV isolated from plant tumors was used for all sedimentation studies; the average $S_{20,w}$ value for three determinations was 514 ± 10*S*.

Phosphorous determination. On the basis of phosphorous and protein determinations, the RNA content of CPV was 23 ± 1%. Phos-

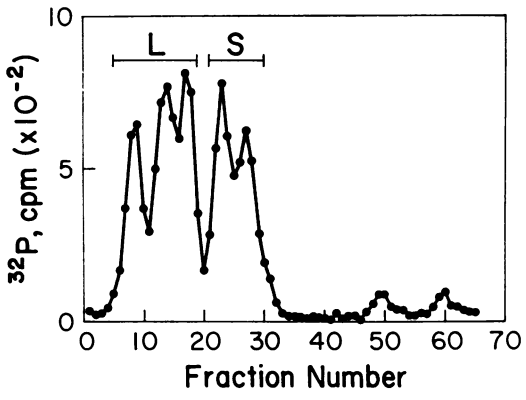

FIG. 5. *Sucrose density sedimentation of ^{32}P-labeled WTV-RNA. The same conditions were used as in Fig. 4 except that the gradient was centrifuged for 8 hr.*

phorous determinations for WTV were unsuccessful due to degradation during dialysis.

DISCUSSION

The RNA molecular weight of 15 × 10^6 previously estimated for WTV (7) agrees favorably with the value determined in this study. However, the molecular weight for CPV-RNA, calculated to be 4.7 × 10^6 by Miura (20), differs from the 12.7 × 10^6 determined in this investigation. To see if the values reported in this present study are reasonable determinations, the molecular weight of the viruses was calculated from the sedimentation coefficient of the virus, using an assumed $\bar{v}$ = 0.70 and the empirical equation of Marvin and Hofmann-Berling (19).

$$M = 1{,}150\left[\frac{S_{20,w}}{1-\bar{v}\rho}^{\bar{v}1/3}\right]^{3/2}$$

Using $S_{20,w}$ = 514 ± 10 for WTV, a molecular weight of 68 ± 2 × 10^6 was calculated. The RNA of 15.5 × 10^6 molecular weight would represent 23 ± 1% of the virus which is in favorable agreement with the reported values of 20% and a molecular weight for the RNA of 15 × 10^6 (7). A value of 22% RNA was also independently determined by using the ratio of sedimentation coefficients for WTV and its protein shell devoid of RNA, both isolated from plant tumors (21). Recently, the RNA and protein composition of WTV isolated from plant tumors has been extensively studied with 22.7% RNA being reported (M. E. Ahmed, Ph.D. Thesis, University of Illinois, 1969).

Similarly, from the sedimentation value of 440 ± 20*S* for CPV a molecular weight of (54 ± 4) × 10^6 is obtained. The RNA of 12.7 × 10^6 molecular weight would represent 23 ± 2% of the virus. From phosphorus analysis, a value of 23 ± 1% RNA was obtained, which agrees with the calculated estimation.

In reovirus each of the RNA subunits appears to be transcribed uninterrupted from end to end (2, 27), and the implication is that they are operons. By analogy, the RNA subunits of CPV and WTV may also be operons. This double-stranded RNA subunit structure may require a unique mechanism of viral replication. Indeed, as has been shown with reovirus (8, 23), CPV has a polymerase activity associated with the virion (17), as does WTV (D. R. Black, *unpublished results*).

One of the many questions that remain unanswered is how the RNA subunits of CPV or WTV can be linked together in the virion. The A-rich RNA found in reovirus could provide a mechanism for linking the subunits together (1). However, no evidence for an A-rich RNA has been found for CPV or WTV. Acid hydrolysis of a WTV pellet gave a purine to pyrimidine ratio of 1 (7), unlike the ratio of 1.5 for reovirus (22). Furthermore, no A-rich RNA could be demonstrated for CPV or WTV by disruption of labeled virus and analysis on either polyacrylamide gels or sucrose density gradients.

Finally, it is possible that the double-stranded RNA subunit structure of these viruses may be the result of a similar fragmentation process during isolation or disruption of these virions. It is interesting to note that for these viruses of diverse origins, probably genetically unrelated, the largest RNA component from each is of quite similar molecular weight (about 2.4 × 10^6), and, with CPV and WTV in particular, the RNA subunits are divided into two quite similar molecular weight groups (Fig. 3). Further investigation is necessary to determine whether the physical RNA subunits studied in this report have the biological or genetic significance, or both, that is presently inferred for reovirus.

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