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Double-stranded Ribonucleic Acid from Cytoplasmic Polyhedrosis Virus of the Silkworm

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Ribonucleic acid (RNA) was extracted by phenol treatment from cytoplasmic polyhedrosis virus isolated from the midgut of infected silkworms. This RNA appears as threads when precipitated in alcohol. Two components having different sedimentation constants were observed. The molecular weight of the RNA preparation obtained by sedimentation coefficient (weight-averaged) and intrinsic viscosity was about 2×10^6 to 3×10^6. It was one-half to one-third the size of the calculated molecular weight for an entire RNA molecule in a virion. Electron micrographs of this RNA preparation showed two peaks in the distribution of contour length, at 0.4 and 1.3 μm, which would correspond to molecular weights of 10^6 and 3×10^6, respectively. The extracted RNA seemed to split into segments at a preferential breaking point. This RNA was soluble in concentrated salt solution, differing from single stranded high-molecular-weight RNA. The base composition of this RNA was complementary in the ratios of adenosine to uridine and guanosine to cytosine. It contained 43% guanosine plus cytosine. Based on its filamentous appearance by electron microscopy, typical pattern of optical rotatory dispersion and circular dichroism, sharp transition of the optical properties on heating, great hyperchromicity on degradation, nonreactivity with formaldehyde, and resistance to ribonucleases, it is concluded that this RNA is double-stranded and has regular base pairings of guanosine-cytosine and adenosine-uridine.

Cytoplasmic polyhedrosis disease in insects results in inclusion bodies, polyhedra, which contain many virus particles. When the nucleic acid extracted from the cytoplasmic polyhedra formed in the midgut epithelium of the infected silkworm was precipitated in ethyl alcohol, fibrous precipitates were obtained in addition to flocculent precipitates (14), both of which were proven to be ribonucleic acid (RNA) by color reactions for sugar components and by digestion with nucleases. The fibrous precipitate fraction, however, was different from the ordinary cellular RNA species in its elution profile on a methylated albumin column and in its complementary base composition (15).

Recently, virus particles were liberated from cytoplasmic polyhedra with the use of a carbonate buffer (*p*H 10.8) and were purified by ultracentrifugation (16). The nucleic acid extracted by phenol treatment from this virus preparation produced only a fibrous precipitate in alcohol, and no flocculent precipitate. The properties of this fibrous nucleic acid have now been studied, and it has been concluded that this nucleic acid is a double-stranded RNA like the RNA from reovirus (11, 22), from wound tumor virus (2, 43), and from rice dwarf virus (29, 36).

MATERIALS AND METHODS

Purification of virus. On the 1st day of 5th instar, silkworms, *Bombyx mori* (L.), were injected in the posterior region with virus suspension, which was obtained by dissolving cytoplasmic polyhedra in 0.05 M Na_2CO_3–0.05 M NaCl (S. Kawase and S. Miyajima, J. Invert. Pathol., *in press*). A few days later, midguts of the infected silkworm were removed and immediately put in dry ice. About 100 g of the frozen material was thawed, added to 350 ml of cold water, and homogenized in a Waring Blendor (2 min at high speed). The homogenate was filtered through gauze, and the sap filtrate centrifuged at $8,000 \times g$ for 10 min to obtain a residue of polyhedra. Precipitated polyhedra were then washed several times with cold water (until the supernatant fluid became clear). Finally, the wet pellet was suspended in 40 ml of a carbonate buffer (*p*H 10.8, 20 C; 9:1 mixture of 0.2 M Na_2CO_3 and 0.2 M $NaHCO_3$). After 1 hr at room temperature, the solution was diluted with three volumes of distilled water and centrifuged at $8,000 \times g$ for 30 min. When a white polyhedra precipitate still remained, the precipitate

was treated again with 20 ml of carbonate buffer, diluted with distilled water, and centrifuged. The combined supernatant extract, in which cytoplasmic polyhedrosis viurs (CPV) was suspended, was centrifuged at 65,000 × *g* for 1 hr.

The virus pellet was suspended in 40 ml of distilled water and centrifuged at 2,000 × *g* for 10 min to remove insoluble materials. A representative picture of the virus is shown in Fig. 1. The virus preparation was almost homogeneous, as shown by many electron micrographs and sedimentation patterns, although there were some empty virus particles. The infectivity of this preparation was proved by injecting silkworm larvae (Kawase and Miyajima, *in press*).

Extraction of nucleic acid. The phenol treatment used for extraction of nucleic acid was essentially the same as that described by Gierer and Schramm (12) for purifying RNA from tobacco mosaic virus (TMV). The CPV suspension was added to an equal volume of 90% phenol, and the mixture was shaken vigorously for 10 min in the cold. Centrifugation at 1,000 × *g* for 10 min resulted in three separate layers. The top layer was removed with a pipette, and the phenol treatment was repeated for 2 min. The top water layer, with one drop of 1 M NaCl added, was poured into three volumes of cold ethyl alcohol. The jellylike precipitate was spooled up with a glass rod (Fig. 2). It was then immersed in 70% ethyl alcohol and stored in a freezer until use. For physical measurements, the precipitate was dissolved in a suitable solvent and dialyzed in the cold against that solvent. For analysis, the precipitate was dipped in ethyl alcohol, ether-ethyl alcohol (1:1), and ether, and it was then air-dried.

Analysis of nucleotide composition and analysis of phosphorus. Three methods were used for nucleotide composition analysis. (i) By the acid hydrolysis method, RNA was hydrolyzed with 1 N HCl at 100 C for 1 hr. The composition of the resulting pyrimidine nucleotides and purines was determined by one-dimensional paper chromatography (24) as in a previous paper (28). (ii) By the alkaline hydrolysis method, RNA was digested with 0.3 N KOH at 37 C for 18 hr. The composition of the resulting mixture of nucleotides was determined by two-dimensional paper chromatography (4) as in previous papers (29, 31). (iii) Instead of alkaline hydrolysis, ribonuclease (RNase) T2 digestion was achieved (20); 1 mg of RNA was dissolved in 0.1 ml of 0.05 M acetate buffer (*p*H 4.5) and incubated with 5 units of RNase T2 at 37 C overnight. In all cases, Whatman no. 1 analytical filter paper was used for the separation of nucleotides or bases.

Analysis of phosphorus was performed by the method of Chen, Toribara, and Warner (7).

Spectrophotometry. Ultraviolet (UV) spectra were obtained with a Zeiss spectrophotometer (PMQ-11) and Nihon Bunko spectrophotometer (JASCO model ORD/UV-5).

Optical rotatory dispersion and circular dichroism were measured with a Nihon Bunko spectrophotometer. On raising the temperature continuously by circulating water from a water bath into the jacket surrounding a cuvette, the UV absorption at 258 nm, optical rotatory dispersion at 282 nm, and circular dichroism at 260 nm were recorded. The solvent for RNA was 0.01 × SSC (0.15 M NaCl plus 0.015 M sodium citrate). From the experiences of Marmur and Doty (25, 26) with deoxyribonucleic acid (DNA), and from the present use of CPV RNA, it was concluded that one can observe thermal transition at a low temperature if the salt concentration of the nucleic acid solution is low, as it is in 0.01 × SSC. The RNA was at a concentration of approximately 40 μg/ml and was contained in a 4-ml quartz cuvette, which had a 1-cm light path and contained a small tipped thermister.

Reaction with formaldehyde. The conditions used for the reaction with formaldehyde were as described by Fraenkel-Conrat (9). To 3 ml of RNA solution (approximately 30 μg of RNA/ml in 0.1 M NaCl) was added 0.15 ml of 37% formaldehyde, to bring the final formaldehyde concentration to 1.8%. The control (time zero) was prepared with 0.15 ml of water instead of formaldehyde. The mixtures were kept in stoppered test tubes at 37 C, and the UV absorption was measured at intervals.

Digestion with RNase. A solution of pancreatic RNase IA (5 μliters, chromatographically purified; Worthington Biochemical Corp., Freehold, N.J.) was added to a solution containing approximately 50 μg of RNA in 2 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 7.6). (In the control, 5 μliters of water was added.) RNase T1 (1 μg, chromatographically purified; Sankyo, Ltd.) was added to a solution containing about 1 mg of RNA dissolved in 0.1 ml of 0.05 M Tris buffer (*p*H 7.5) plus 2×10^{-3} M ethylenediaminetetraacetic acid (EDTA). After incubation of the mixture at 25 C, the increase in optical density at 260 nm was measured.

Determination of sedimentation constant and intrinsic viscosity. Ultracentrifugal analysis of nucleic acid in SSC was performed by using a Spinco model E analytical ultracentrifuge with schlieren optics and UV optics at 20 C. The sedimentation constant at infinite dilution was obtained by extrapolation of several values, measured at different concentrations of RNA.

Viscosity was measured with a Couette-type viscometer at an extremely low velocity gradient (0.07 per sec) under conditions corresponding to those for sedimentation, because the viscosity of CPV RNA solution was markedly influenced by the shear rate. Intrinsic viscosity was found by extrapolating the reduced viscosity values to zero concentration.

Electron microscopy of nucleic acid. Electron microscopy of nucleic acid was performed by the protein monolayer method (19). The suspension containing nucleic acid (5 μg/ml), 2 M ammonium acetate, and 0.01% cytochrome *c* was spread on a surface of double-distilled water. The protein monolayer was transferred to the carbon supporting film by touching the grid to the surface of the water. The drop of water was dried by dipping the surface of the grid into ethyl alcohol. The contrast was enhanced by rotatory shadowing with an alloy of 80% platinum and 20% palladium. Photographs were taken in electron microscope (Hitachi-HS-7).

RESULTS AND DISCUSSION

Characteristics and column chromatography of nucleic acid. The nucleic acid extracted from CPV by phenol treatment was precipitated as a jelly when its aqueous solution was poured into more than two volumes of ethyl alcohol. It could be spooled onto a glass rod (Fig. 2). When it was dehydrated by successive dipping in ethyl alcohol

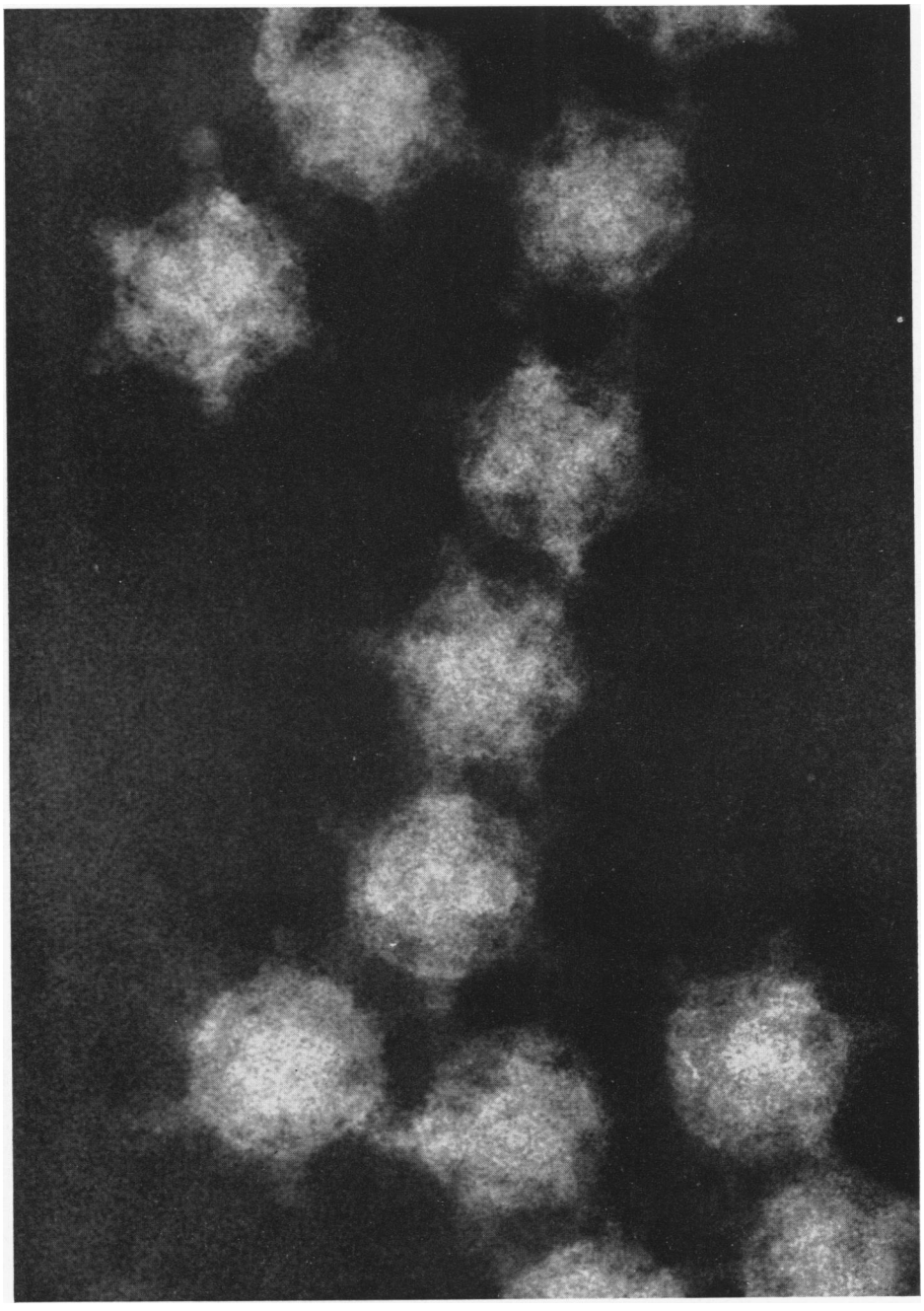

FIG. 1. *Purified cytoplasmic polyhedrosis virus particles. Negative staining with phosphotungstate.* × *600,000.*

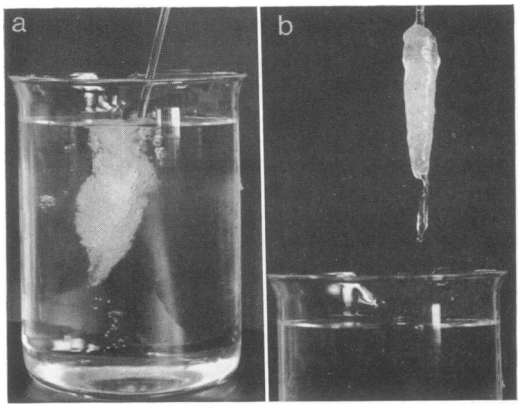

FIG. 2. *Jellylike precipitate of the extracted CPV RNA in 70% ethyl alcohol (a), spooled up by a glass rod (b).*

and ether, it appeared fibrous. In this respect, it is similar to DNA, whereas other RNA preparations of ribosomal RNA, transfer RNA, or TMV RNA, deposit as flocculent precipitates in alcohol.

Flocculent precipitates did not appear in the present preparation from CPV. The RNA preparation extracted from crude polyhedra contained both fibrous and flocculent precipitates, which were separated on methylated albumin-kieselguhr column chromatography or on gel filtration (Sephadex G-25). The CPV RNA extracted from the purified virus appeared as one component on methylated albumin column chromatography (17) or on Sephadex G-25 gel filtration.

Evidence of RNA. The fibrous nucleic acid prepared from CPV was confirmed as RNA by the following tests. The orcinol reaction (27) gave a typical green color for ribose, and the optical density at 665 nm was almost the same as that of the same amount of ribosomal RNA prepared from rat liver. The diphenylamine reaction for deoxyribose (45) did not give any color for CPV nucleic acid. The fibrous material was digested thoroughly by alkali (0.3 N KOH at 37 C) or by RNase T2 into mononucleotides; it was digested by pancreatic RNase IA or by RNase T1, although it was resistant to small amounts of RNase in comparison with other natural RNA preparations.

Ultracentrifugation and viscosity. The schlieren pattern obtained in the ultracentrifuge showed a sharp boundary, similar to that obtained with DNA (33) but different from that obtained with other RNA types. The schlieren patterns or UV-absorption photographs obtained with a dilute solution of CPV RNA gave two boundaries (Fig. 3). Their sedimentation coefficients, $S_{20,w}$, at

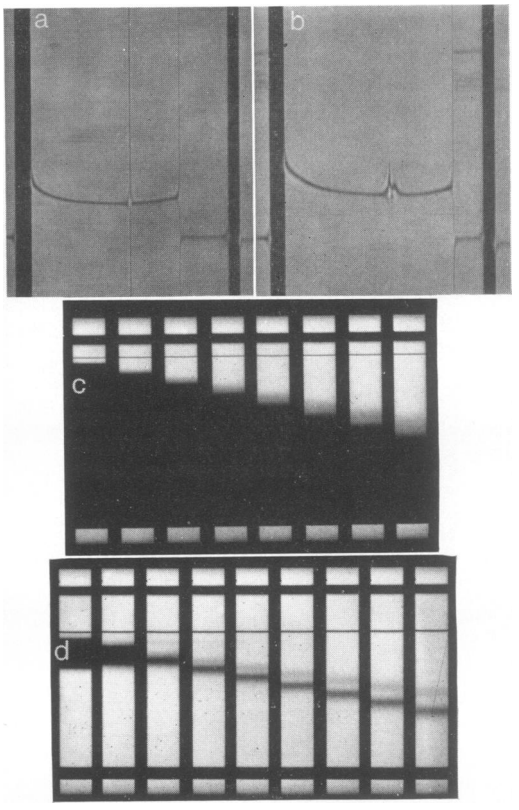

FIG. 3. (a, b). *Schlieren pattern of CPV RNA. RNA was dissolved in SSC at 1.1 mg/ml (a) or 0.30 mg/ml (b). The picture was taken 32 min after reaching a speed of 50,740 rev/min at 20 C. (c) Sedimentation patterns, obtained with ultraviolet optics, of CPV RNA (45 μg/ml) in SSC. Pictures were taken every 8 min at 44,770 rev/min at 20 C. The dark regions correspond to optically dense zones. (d) Sedimentaion patterns, obtained with synthetic boundary cell and ultraviolet optics, of CPV RNA (73 μg/ml). CsCl (3.3 M) solution (ρ = 1.44) was used as a solvent. Pictures were taken every 8 min at 42,040 rev/min at 20 C. The dark bands correspond to optically dense zones.*

infinite dilution were 15.8*S* and 12.5*S*, respectively. The amount of the former was about nine times that of the latter, judged from the optical density.

These results agree satisfactorily with those obtained by band centrifugation (44), which gave two distinct bands in a concentrated CsCl solution at 20 C (Fig. 3d). Sedimentation constants ($S_{20,w}$) obtained in this experiment were 15.4*S* for the major component and 12.1*S* for the minor component, respectively. The proportion of the major component was about 90%.

A preliminary experiment on the viscosity of this RNA solution showed some features which

are observed for a double-stranded DNA; the viscosity was much higher than that of ribosomal RNA in a similar concentration, and it was characterized by dependence on the rate of shear, as in ordinary DNA. The intrinsic viscosity of this RNA in SSC was determined as 8.70 dl/g by using a Couette-type viscosimeter in low-velocity gradient (0.07 per sec) and extrapolating from several measurements of relative viscosity in different concentrations of RNA.

From the viscosity and from the average sedimentation coefficient (15.5*S*), the average molecular weight of CPV RNA was calculated according to the Sheraga-Mandelkern equation (37) as 2.3×10^6, assuming that the coefficients in the equation are the same as those for double-stranded DNA ($\beta = 2.5 \times 10^6$; partial specific volume = 0.55 cm^3/g). When the molecular weight of this RNA is calculated by using Franklin's empirical equation for double-stranded RNA (10), the molecular weight of CPV RNA is obtained as 3.1×10^6 from the average sedimentation coefficient (15.5*S*).

The molecular weight of a CPV virion was calculated as 2.87×10^7 from the measurement of the sedimentation constant (371*S*) and partial specific volume (0.703 cm^3/g) assumed as a sphere. The phosphorus content of this virion was 1.4%. As the phosphorus content in RNA was 8.59%, the RNA content in a virion would be 16.2%. Thus, the molecular weight of RNA in a virion is calculated to be 4.7×10^6, if the RNA is a single molecule in a virion. The present RNA preparation was one-half to one-third the size of the calculated molecular weight. If the RNA had been split, there would be only one break for one intact molecule. However, it remains to be investigated whether this RNA preparation is intact in size or is split during the extraction.

In spite of such a high molecular weight, this RNA was soluble in concentrated salt solution (2 M NaCl). This characteristic is quite different from large single-stranded RNA (30, 32).

Electron microscopy. One of the representative electron micrographs of CPV RNA is shown in Fig. 4. The general appearance was similar to DNA and to double-stranded RNA from reovirus and wound tumor virus (18). The distribution of the contour length of the filaments was bimodal. It has a peak of 0.4 μm and another at 1.3 μm (Fig. 5). Using the length of 10 nucleotides as 0.305 pm, which was obtained from X-ray analysis for the double-stranded RNA from rice dwarf virus (36), we determined that the molecular weight of the RNA whose contour length is 0.4 μm would be about 10^6 and that the molecular weight of the RNA whose contour length is 1.3 μm would be about 3×10^6.

These two groups might correspond to the two bands found by ultracentrifugation. However, it is not known whether these nucleic acids derived from the virus are intact or partially degraded. These sizes of nucleic acids are smaller than the calculated size if RNA in the virus is a single molecule. The observation on the double-stranded RNA of reovirus and wound tumor virus (18) exhibited multimodal distribution in their contour length, and it has been suggested recently that the reovirus RNA consists of fragments (8).

Even if CPV RNA was degraded, the discrete distribution in size of the RNA suggests that there are preferential breaking points in its molecule. The ratio of a larger component to a smaller component in the CPV RNA preparation seems to be different in the sedimentation experiments from that seen in electron microscopy. Although the reason for this has not been determined, a larger component might be dissociated into smaller segments or smaller segments might be associated together in some way.

Base composition. CPV RNA was hydrolyzed with alkali (0.3 N KOH) at 37 C for 18 hr or with RNase T2 at 37 C for 18 hr at *p*H 4.5. The resulting nucleotides were separated by two-dimensional paper chromatography.

There were four nucleotides: guanylic acid, adenylic acid, cytidylic acid, and uridylic acid. They were identified spectroscopically after elution. The RNA was also hydrolyzed with 1 N HCl at 100 C for 1 hr. The resulting pyrimidine nucleotides and purine bases were separated by one-dimensional paper chromatography. The hydrolysis under these conditions was almost complete for this nucleic acid, and the results confirmed that the CPV nucleic acid is RNA.

The molar ratios of these base components are listed in Table 1. A remarkable feature of the composition of this RNA is seen in the complementarity of two pairs: a pair for guanylic acid and cytidylic acid and another pair for adenylic acid and uridylic acid. This suggests regular base pairings of guanine (G) and cytosine (C) and of adenine (A) and uracil (U) in CPV RNA.

The G + C content of CPV RNA was 43% of the total nucleotides. This is comparable to 44% G + C RNA of rice dwarf virus (29), but is somewhat different from that for the other two double-stranded species of RNA: 40% or 48% for reovirus (13, 39) and 39% for wound tumor virus (2).

UV absorption. The UV-absorption curve of CPV RNA in 0.2 M NaCl at 20 C is typical for RNA (Fig. 9). However, the wavelength of minimum absorption, 232 nm, is slightly longer than that of single-stranded nucleic acid. The minimum absorption shifted to a shorter wavelength when the RNA was denatured or hydrolyzed (Table

2), although the maximum was constant. The ratio of absorbance at maximum to that at minimum increased with degradation of the native structure of CPV RNA. This phenomenon was also observed for double-stranded RNA of rice dwarf virus.

The UV absorbance at 260 nm per mole of phosphorus, ϵ(P), determined by the method of Chargaff and Zamenhof, (6), was 6,600 for CPV RNA in 0.2 M NaCl at 20 C. This is considerably lower than the values for a single-stranded RNA or for a single-stranded DNA under comparable

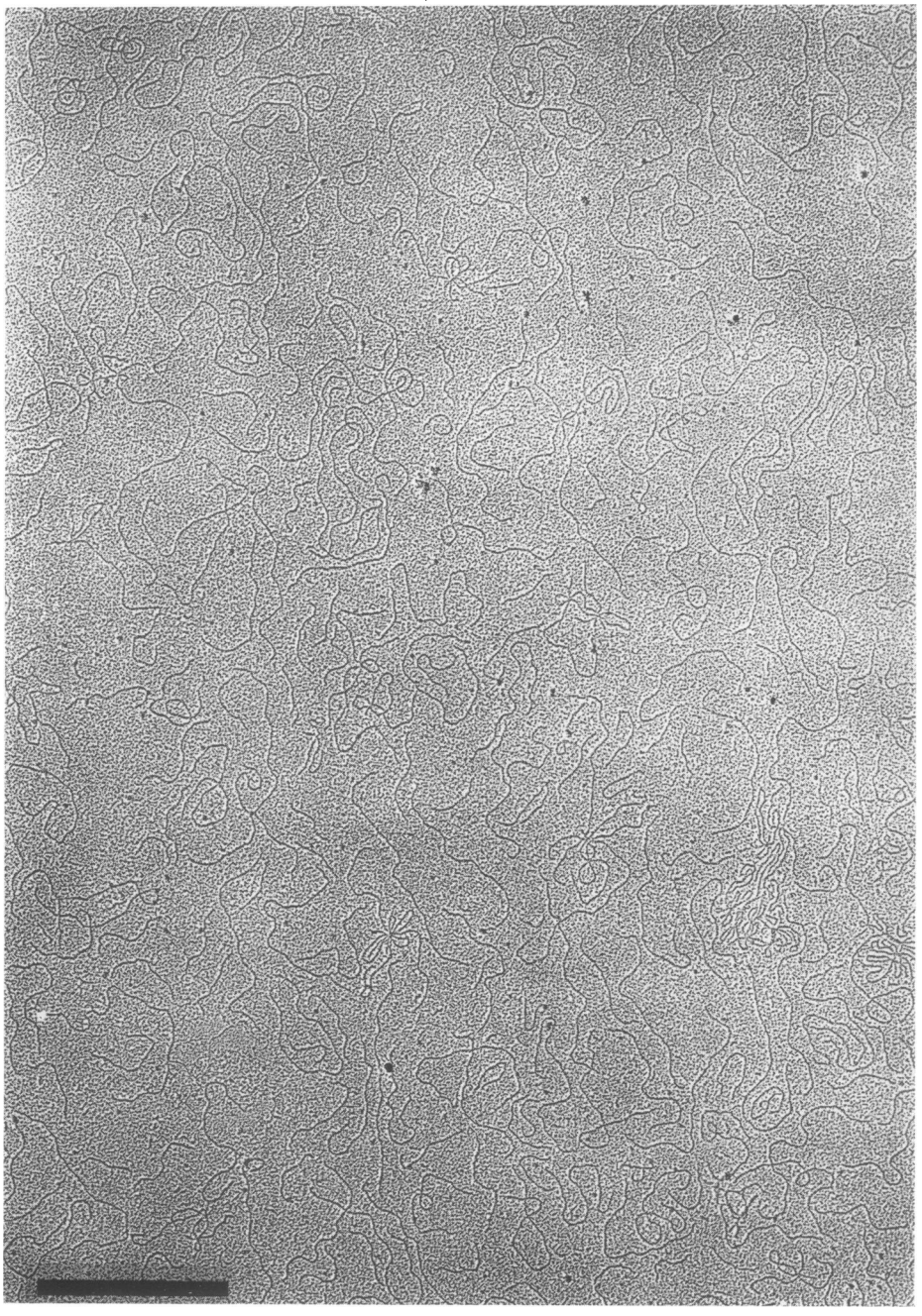

FIG. 4. *Electron micrograph of CPV RNA.* × *25,500. The black bar indicates 1 μm.*

conditions. This value for CPV RNA is nearer to the ϵ(P) of natural DNA; it was similar to that of double-stranded rice dwarf virus RNA. Heat-denatured CPV RNA gave a higher ϵ(P) value under the same conditions. Hydrolysis with alkali or with RNase T2 split the RNA into mononucleotides, and ϵ(P) of the hydrolysate increased further. The hyperchromicity of CPV RNA observed in alkaline digestion was considerably greater than that of ordinary single-stranded RNA. This suggests that CPV RNA has a more ordered structure than single-stranded RNA.

Optical rotatory dispersion and circular dichroism. For CPV RNA, the multiple Cotton effects displayed peaks at 282 and 228 nm and troughs at 250 and 220 nm (Fig. 6). The magnitude of the Cotton effect was the largest observed in nucleic acids. The circular dichroism curve of CPV RNA (Fig. 7) showed an intense positive band at 260 nm; close to the crossover point of the Cotton effect the intensity of the band was more prominent than that of a single-stranded RNA. These features are quite similar to those of the double-stranded rice dwarf virus RNA (34), but are different from normal single-stranded RNA or double-stranded DNA (3, 35).

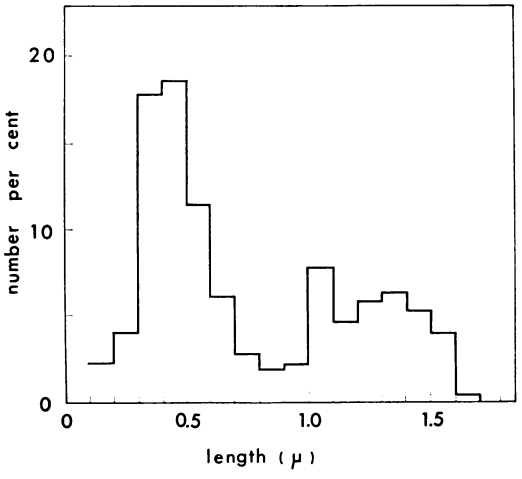

FIG. 5. *Length distribution of CPV RNA in electron micrographs, expressed as percentages of 450 fibers.*

TABLE 1. *Nucleotide composition of CPV RNA*[a]

Nucleotide	Hydrolysis		
	Acid (1 N HCl, 100 C, 1 hr)	Alkali (0.3 N KOH, 37 C, 18 hr)	RNase T2 (37 C, 18 hr)
Guanylic acid	21.8	21.3	20.3
Adenylic acid	27.8	29.1	27.8
Cytidylic acid	21.2	21.2	21.1
Uridylic acid	29.2	28.4	29.8
G/C	1.03	1.01	0.96
A/U	0.95	1.03	0.94
Pu/Py[b]	0.98	1.02	0.93
(A + C)/(G + U)	0.96	1.01	0.97
G + C (%)	43.0	42.5	41.4

[a] Expressed as moles per 100 moles of guanylic, adenylic, cytidylic, and uridylic acid, adjusted to 100% recovery. The actual recovery of bases, compared to total phosphorus, was 98% for acid hydrolysis, 92% for alkaline hydrolysis, and 90% for RNase T_2 hydrolysis. Three to five analyses were averaged.

[b] Pu, purine; Py, pyrimidine.

TABLE 2. *Ultraviolet absorption of CPV RNA and denatured preparations in 0.2 M NaCl at 20 C*

Prepn	Absorption maximum	Absorption minimum	Emission ratio	ϵ(P) at 258 nm
	nm	*nm*		
Native CPV RNA	258	232	2.23	6,600
Heat-denatured	258	230	2.56	9,380
Alkali-hydrolyzed	258	227	3.00	11,200
RNase T_2-hydrolyzed	259	228	2.98	11,400

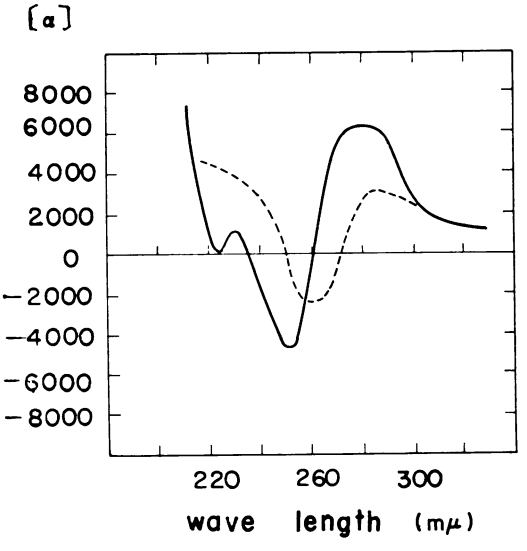

FIG. 6. *Ultraviolet rotatory dispersion of CPV RNA (solid line) and of heat-denatured CPV RNA (dashed line) in 0.01 × SSC solution at room temperature. The RNA concentration was 40 μg/ml. Heat denaturation was performed in 0.01 × SSC at 100 C for 10 min, followed by quenching in ice cold.*

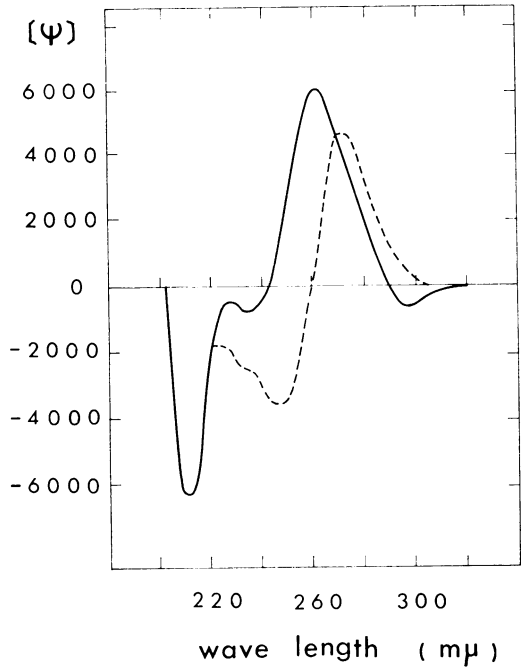

FIG. 7. *Circular dichroic absorption curves of CPV RNA (solid line) and of heat-denatured CPV RNA (dashed line) (40 μg/ml) in 0.0.1 × SSC at room temperature. Heat denaturation was as in Fig. 6.*

After CPV RNA was denatured by heating at 100 C for 10 min in 0.01 × SSC (and quickly cooled in ice), the magnitude of the Cotton effect and the intensity of the circular dichroism band decreased to the value obtained in a normal single-stranded RNA (Fig. 6, 7). These results suggest that the original CPV RNA possesses a specially ordered structure which is lost with heat denaturation.

Thermal denaturation. The UV absorption of CPV RNA at 258 nm showed a steep rise around 80 C when heated in 0.01 × SSC (Fig. 8). Similar sharp changes were also observed in optical rotatory dispersion at 282 nm and in circular dichroism at 260 nm. The temperature corresponding to the midpoint of the absorbance rise, the melting temperature (T_m), was 80 C for CPV RNA. This is quite similar to the T_m of rice dwarf virus RNA (29, 34). The appearance of the absorption rise is similar to that of natural double-stranded DNA. Under the same conditions, transfer RNA or ribosomal RNA showed a temperature-dependent increase in UV absorption over a wide range. Similarly, the UV absorption of single-stranded DNA (40) or thermally denatured DNA (38) exhibited a marked function of temperature over a wide range. After CPV RNA was heated at 100 C for 10 min in 0.01 × SSC and quickly quenched in ice, the mode of increase in UV absorption became similar to that of single-stranded DNA and to other types of RNA. These results suggest that CPV RNA is a double-stranded molecule and that heating and rapid cooling produced a single-stranded state.

It is known that the T_m of DNA is linearly related to its base composition (25, 26). The G + C content of CPV RNA is 43% and its T_m is 80 C in 0.01 × SSC. However, the T_m of calf thymus DNA, which has a G + C content (5) similar to that of CPV RNA (42%), was 61 C in 0.01 × SSC. It is apparently lower than the T_m of CPV RNA. Double-stranded RNA might be more resistant to thermal agitation than the double-stranded DNA having the same base ratio.

Since CPV RNA melts at a higher temperature in salt solutions more concentrated than 0.01 × SSC, it was difficult to cover the whole melting curve and to determine an accurate T_m value.

Nonreactability with formaldehyde. Formaldehyde is believed to react with free amino groups in the bases of a nucleic acid molecule and to produce an increase in UV absorption and a shift of the wavelength of the absorption maximum (9, 42). Changes induced in UV absorption of the RNA by 1.8% formaldehyde at 37 C in 0.1 M NaCl are shown in Fig. 9. Whereas single-stranded nucleic acids exhibited a marked increase (23 to 26%) and a shift to a longer wavelength in their UV absorption (4 to 6 nm), CPV RNA showed almost no increment and no shift in its UV-absorption maximum; CPV RNA is thus like other double-stranded RNA or DNA (11, 29, 40). When CPV RNA was denatured by heating at 100 C for 10 min in 0.01 × SSC and then quickly cooled in ice, it reacted with formaldehyde in the same manner as does transfer RNA or ribosomal RNA. It is possible that amino groups of the bases in the original CPV RNA are occupied, owing to base pairing as is the case for double-stranded DNA. After heat denaturation of the RNA, these amino groups would be exposed, allowing reaction with formaldehyde.

Resistance to RNase. In order to observe degradation of small amounts of RNA by RNase treatment the hyperchromic effect at 260 nm was measured (Table 3). When a dilute pancreatic RNase solution (61 ng/ml) was used, the increase in UV absorption was slight for CPV RNA, as compared with transfer RNA. However, increased RNase treatment (long incubation wih 10 times the amount of RNase) induced almost complete hydrolysis of the CPV RNA. The increase in absorption at 260 nm rose to 48% for CPV RNA, a larger increase than that for transfer RNA. If CPV RNA has a regular structure, and if there is a large structural gap between the original ordered

structure and the digest, it would be plausible to expect a larger hyperchromic effect than those observed for the other types of RNA. Heat-denatured CPV RNA (heated at 100 C for 10 min in 0.01 × SSC and quickly cooled in ice) showed a hyperchromicity for RNase treatment similar to that of transfer RNA. These results seem to show that natural CPV RNA is more resistant to dilute RNase than is either transfer RNA or heat-denatured CPV RNA.

The above observations indicate that the RNA of CPV is double-stranded. Various physical and chemical characteristics of CPV RNA are similar to those of a double-stranded DNA or to those of other double-stranded RNA preparations (13, 29), but are different from those of transfer RNA or of ribosomal RNA, which are single-stranded molecules in which the intramolecular base pairings are considered incomplete (11). From the fact of nonreactivity with formaldehyde, almost all the amino groups of the bases in CPV RNA would seem to participate in hydrogen bonding between the two strands. Regular base pairings would occur in the pairs of A-U and G-C, since both the base ratios of A/U and G/C for CPV RNA were unity.

APPENDIX

Additional evidence indicating CPV RNA to be a double-stranded helical molecule has been obtained by X-ray analysis and absorption in the far infrared. This evidence is summarized below by Y. Murakami, K. Shuto, Y. Mitsui, Y. Iitaka, and M. Tsuboi, of the Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan. Their cooperation is gratefully acknowledged.

X-ray diffraction photographs of fibers of sodium RNA of silkworm poylhedrosis virus were obtained with a microcamera and Ni-filtered CuKα radiation. The specimen-to-film distance was 29 mm, and flat films were used. The humidity of the atmosphere around the sample was controlled (at 75%) by the method previously described (36). The fibers so far obtained were much less crystalline than fibers of rice dwarf virus RNA. In the fiber diagram (Fig. 10), only diffuse reflections can be seen; no sharp spots are observed. However, all of these diffuse reflections are situated where strong spots are observed in the fiber diagrams of rice dwarf virus RNA (36) and other double helical RNA molecules (1, 21, 22, 41, 43). For example, strong reflections of RNA molecules with ξ values of 0.07 to 0.09 A^{-1} and 0.17 to 0.18 A^{-1} on the equator, those of 0.05 to 0.06 A^{-1}, 0.12 A^{-1}, and 0.17 A^{-1} on the second-layer line (ζ = 0.07 A^{-1}), those of 0.07 to 0.13 A^{-1} on the seventh-layer line (ζ = 0.23

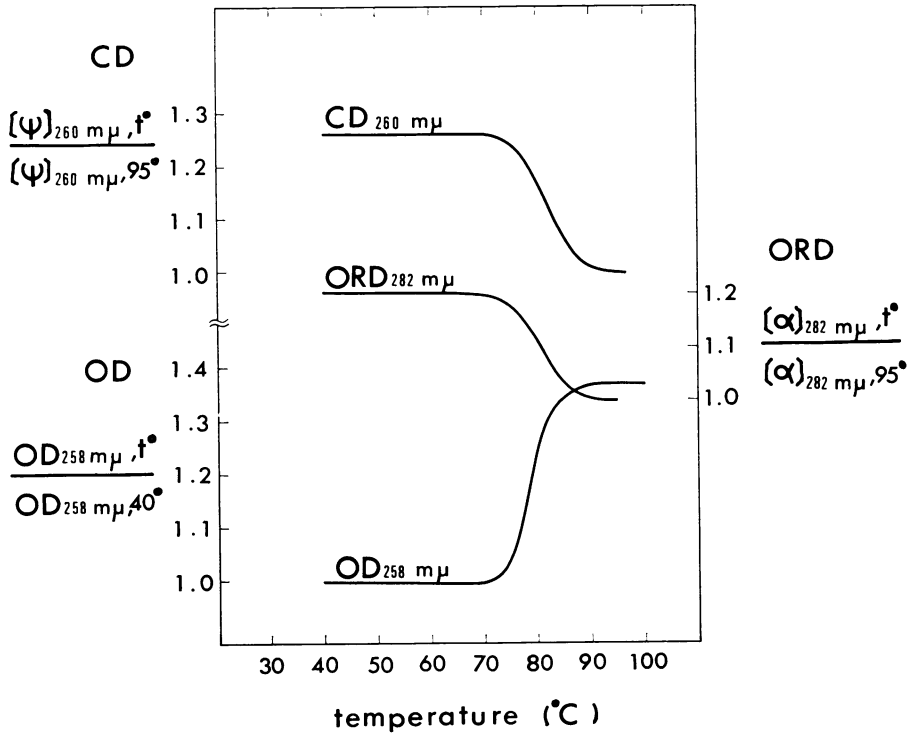

FIG. 8. *Variation in ultraviolet absorption at 260 nm, the peak of the Cotton effect (282 nm), and circular dichroic band at d 260 nm of CPV RNA as a function of temperature in 0.01 × SSC. The concentration of RNA was 40 μg/ml.*

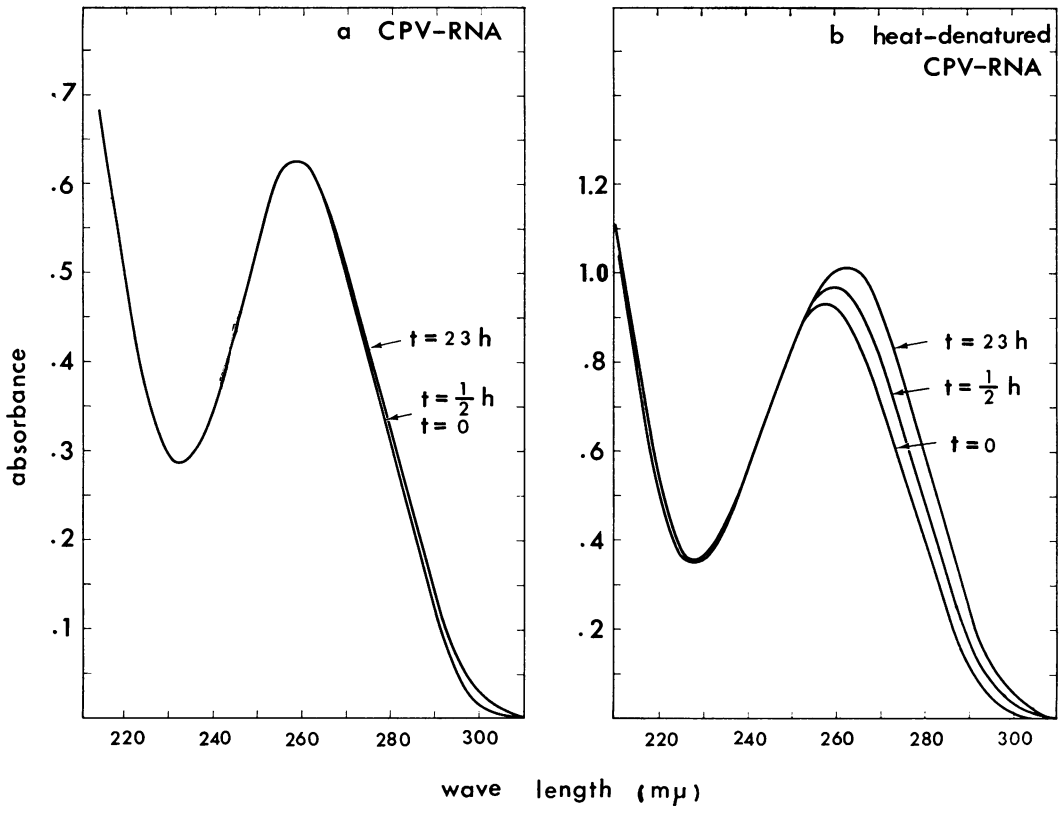

FIG. 9. *Variation in ultraviolet absorption of nucleic acid after reaction with formaldehyde. Nucleic acid (34 μg/ml) in 0.1 M NaCl was incubated with 1.8% formaldehyde at 37 C. After 30 min or 23 hr, the UV absorption was measured. Time zero samples were examined without added formaldehyde.*

TABLE 3. *Percentage increase in ultraviolet absorbance at 260 nm after ribonuclease treatment*[a]

Sample	Amt of RNase							
	50 ng		125 ng			1,250 ng		
	30 min	60 min	30 min	60 min	24 hr	30 min	60 min	24 hr
CPV RNA	0	1	5	8	19	28	37	48
Denatured CPV RNA			21	21	21			
Transfer RNA	21	23	24	25	25	24	25	24

[a] RNA (50 μg) was dissolved in 2 ml of 0.05 M Tris-chloride buffer (*p*H 7.6) containing 2 mM EDTA. The indicated amounts of pancreatic RNase I were added. After incubation, UV absorption at 260 nm was measured.

A^{-1}), and a reflection of 0.08 A^{-1} on the ninth-layer line (ζ = 0.30 A^{-1}) are identified in Fig. 10. (It has not been possible to determine whether the pattern belongs to α-, β-, or some other type found in other RNA preparations.)

An infrared absorption spectrum of an oriented film of the CPV RNA was obtained at 75% relative

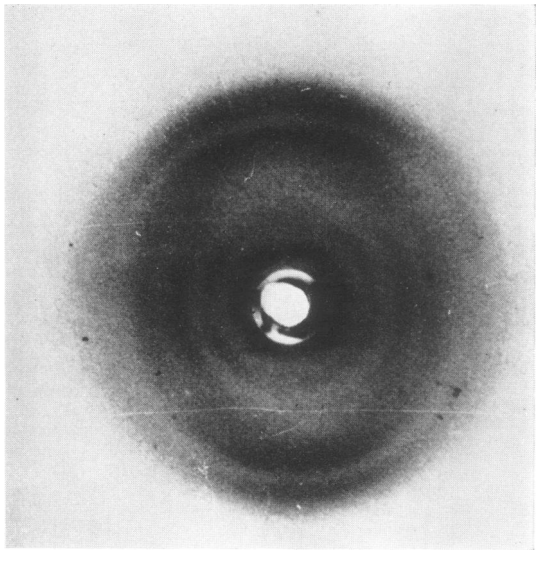

FIG. *10. X-ray diffraction photograph of sodium RNA from silkworm polyhedrosis virus at 75% relative humidity.*

humidity with polarized radiation. The general features of the spectrum in the spectral region examined (1,800 to 700 cm^{-1}) were quite similar to those of rice dwarf virus RNA (36). The strong bands at 1,710 cm^{-1} (in-plane vibrations of bases) and 1,240 cm^{-1} (PO_2^- antisymmetric stretching vibration) showed perpendicular dichroism, and that at 1,085 cm^{-1} (PO_2^- symmetric stretching vibration) paralleled dichroism. The dichroism ratios were 0.75, 0.67, and 1.40, respectively. These facts indicate that the orientation of the PO_2^- group in the molecular structure of this RNA is similar to that of rice dwarf virus RNA (36), and that it is different from the PO_2^- orientation in the DNA structures.

Unfortunately, our X-ray and infrared data are not yet complete enough for discussion of the structure in detail, but they are enough to give substantial support to the idea that this RNA has a double-helical structure similar to that of rice dwarf virus RNA.

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