Preferential Separation of Cytoplasmic-Polyhedrosis Virus (CPV) RNAs From Infected Midgut Cells

W. C. Richards and Y. Hayashi

Insect Pathology Research Institute, Department of the Environment, Sault Ste. Marie, Ontario, Canada

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Viral progeny, double-stranded RNA (dsRNA), and virus-specific single-stranded RNA (ssRNA) synthesized in midgut cells of Malacosoma disstria larvae infected with cytoplasmic-polyhedrosis virus (CPV), have been separated using Mg\(^{2+}\) for RNA aggregation, and lithium chloride for differential solubilization between the ds- and ssRNA.

Under high concentrations of Mg\(^{2+}\) (5 × 10\(^{-3}\) and 10\(^{-2}\) M MgSO\(_4\)), dsRNA was not affected, remaining positional at a sedimentation rate of 15S, but virus-specific ssRNA and host ribosomal RNA as single-stranded (rRNA), were separated from dsRNA by aggregation. After RNase treatment there was no appreciable contamination of virus specific ssRNA and rRNA.

It was found that ds and ssRNA could be separated in 2 M LiCl on the basis of their different solubilities. The ssRNA fraction obtained by such a procedure contained only 6% dsRNA, and the dsRNA fraction was free of ssRNA.

Further separation of dsRNA by both the Mg\(^{2+}\) and LiCl treatments was accomplished through prolonged centrifugation which yielded 12 and 15 S components; these are the same characteristics found for viral genome RNA appearing in the sedimentation profile.

INTRODUCTION

We have reported previously that both viral progeny double-stranded RNA (dsRNA) and virus-specific single-stranded RNA (ssRNA) can be synthesized in midgut cells infected with cytoplasmic-polyhedrosis virus (CPV). The dsRNA is viral progeny RNA, and it has been suggested that the ssRNA is virus messenger RNA since it is formed in the presence of sufficient actinomycin D to inhibit host-cell messenger RNA (mRNA) synthesis (Hayashi, 1970a; Hayashi and Donaghue, 1971). It appeared likely that a separation of ds- from ssRNA in infected cells could be achieved if one of them could be selectively removed or precipitated from the other by appropriate procedures.

It has been shown that many kinds of ssRNA including ribosomal RNA (rRNA) (Sporn and Dingman, 1963; Click and Hackett, 1966; Vaughan et al., 1967; Taylor et al., 1967) and viral RNA (Boedtker, 1960) may be aggregated in a solvent of high ionic strength, especially when Mg\(^{2+}\) is present, whereas no such aggregation is observed in buffers of low ionic strength. From these results, it would appear that the dsRNA synthesized in the infected cells might selectively separate from the ssRNA, if the dsRNA is not affected by high ionic strength.

Another approach is that suggested by Baltimore (1966), who selectively separated the dsRNA, which is the replicating form of poliovirus, by taking advantage of its specific solubility in high concentrations of lithium chloride.

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This paper describes methods for separating ds- and ssRNA of the cytoplasmic-polyhedrosis virus of *Malacosoma disstria*.

**Materials and Methods**

**Experimental animal and virus.** Insects used in these experiments were fourth-instar *Malacosoma disstria* larvae. The virus was *M. disstria* CPV, which is serologically (Krywienczyk et al., 1969) and biochemically (Hayashi and Durzan, 1971; Hayashi and Krywienczyk, 1972) different from that of *Bombyx mori*. The method of infecting the *M. disstria* larvae with CPV has been described (Hayashi and Bird, 1968).

**Radioisotope labeling and purification of virus.** These procedures were performed as described previously (Hayashi and Bird, 1968, 1970; Richards and Hayashi, 1971).

**Extraction of RNA from virions.** The method for extracting viral RNA has been reported (Hayashi, 1970a).

**Extraction of RNA from infected midgut.** Six days after the initial infection, each infected larva that had reached the fifth instar was injecting with 1 μg of actinomycin D (kindly donated by Merck, Sharp and Dohme Corporation), and 30 min later, each larva was reinjected with 5 μCi ³H-uridine (³H-6-uridine (³H-6-uridine, specific activity 10.4 Ci/mmol; New England Nuclear Corporation) (Hayashi and Kawarabata, 1970; Hayashi and Retnakaran, 1970). The insects were kept for 3 hr at 25°C, and the midguts were then removed. The midguts (100 each) were placed in a chilled glass-Teflon homogenizer containing 5 ml of TK buffer (0.03 M Tris·HCl, pH 7.5, 0.025 M KCl); bentonite (25 mg) was also added to inhibit RNase activity. After 15 strokes, the homogenized midguts were treated with an equal volume of water-saturated phenol to extract the RNA (Hayashi, 1970a). The RNA was precipitated in 3 volumes of ethanol at −15°C and collected by centrifugation, washed twice in 70% ethanol, and dissolved in TK buffer. This RNA was the starting material for all the experimental analyses.

**Separation of dsRNA and ssRNA.** A portion of the RNA dissolved in TK buffer (see above) was reprecipitated by the addition of 3 volumes of ethanol at −15°C. The precipitate was dissolved in SSC (0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4) mixed with an equal volume of 4 M LiCl (Baltimore, 1966) and kept overnight at 4°C. In such a system most of the ssRNA was precipitated by centrifugation while the dsRNA remained in solution in the supernatant.

The ssRNA fraction was dissolved in SSC, and bentonite was added to a final concentration of 20 μg/ml. To this suspension an equal volume of 4 M LiCl was added and the system held for 10 hr at 4°C. After centrifugation, the ssRNA which precipitated was dissolved in TK buffer and the bentonite treatment was repeated. The ssRNA suspension was then added to 3 volumes of ethanol and the resulting precipitate was dissolved in TK buffer and analyzed by sucrose gradient centrifugation.

The dsRNA fraction was treated with 3 volumes of ethanol which precipitated the dsRNA as a fibrous material. To further reduce any ssRNA contamination (about 5–7%) treatment with SSC and LiCl (see above) was performed. The resulting supernatant was added to 3 volumes of ethanol and the dsRNA which precipitated was dissolved in TK buffer and analyzed by sucrose gradient centrifugation.

**Sucrose gradient sedimentation analysis and RNase treatment.** Generally the RNA analysis was carried out using linear sucrose gradients (5–25%) in TK buffer by the use of a SW40 Ti rotor in a Spincro model L2 65-B at 5°C (Hayashi, 1970a). The conditions of the gradients for each experiment are specified in the figure captions. After sucrose gradient centrifugation, fractions were drop-collected (16 drops/tube) from the gradient column. Each fraction
was then adjusted to a final volume of 1 ml at a concentration of $5 \times 10^{-3}$ M MgSO$_4$ in TK buffer; it was then divided into two equal parts. One part was used for the determination of absorbance at 260 m$\mu$ and radioactivity and the other for RNase-resistant radioactivity by adding 1 $\mu$g RNase/fraction and incubating 30 min at 25°C. The acid-precipitable materials were recovered with yeast RNA (Hayashi, 1970a). Under the stated conditions, and at a concentration of $5 \times 10^{-3}$ M MgSO$_4$, it is calculated that more than 95% of the RNase resistant, double-stranded RNA was recovered (Hayashi and Kawarabata, unpublished). The radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

**RESULTS AND DISCUSSION**

**Effect of Mg$^{2+}$ on Viral RNA and Virus-Specific RNA Synthesized in Infected Cells**

High concentrations of Mg$^{2+}$ caused an increase in the sedimentation rate and/or aggregation of a number of species of ssRNA (Sporn and Dingman, 1963; Click and Hackett, 1966; Vaughan et al., 1967; Taylor et al., 1967). It was of interest to establish whether the same effect occurred with dsRNA. Figure 1 shows the rate of sedimentation of $^3$H-uridine labeled CPV-RNA in $5 \times 10^{-3}$ M MgSO$_4$. The results indicate clearly the presence of fragmented 15 and 12 S components, but no significant influence was observed in the sedimentation rate. This was expected because CPV-RNA has a double-helical configuration (Hayashi, 1970b). Figure 2 shows the effect of Mg$^{2+}$ on total RNA extracted from infected midgut cells. In the absence of Mg$^{2+}$ a typical sedimentation profile was obtained for ribosomal-RNA (rRNA) by absorbance measurements at 260 m$\mu$ and for newly synthesized virus specific RNA by radioisotope labeling (Hayashi, 1970a; Hayashi and Donaghue, 1971). After RNase treatment of each fraction, the 22 S component (tube 10) and part of the 15 S compo-

![Fig. 1. Effect of Mg$^{2+}$ on the sedimentation of viral genome RNA extracted from purified virions. No Mg$^{2+}$: viral genome RNA was dissolved in 1 ml TK buffer. Mg$^{2+}$ ($5 \times 10^{-3}$ M): viral genome RNA was dissolved in 1 ml TK buffer containing $5 \times 10^{-3}$ M MgSO$_4$. Sucrose gradients were made with the same buffer in which the viral genome RNA was dissolved. Sedimentation was for 19 hr at 23,000 rpm. ●●●●, Radioactivity.](image-url)
SEPARATION OF CYTOPLASMIC-POLYHEDROSIS VIRUS RNA

FIG. 2. Effect of Mg$^{2+}$ on the sedimentation of RNA extracted from infected midgut cells. No Mg$^{2+}$: RNA was dissolved in 1 ml TK buffer. Mg$^{2+}$ ($5 \times 10^{-3}$ M): RNA was dissolved in 1 ml TK buffer containing $5 \times 10^{-3}$ M MgSO$_4$. Sucrose gradients were made with the same buffer in which the RNA was dissolved. Sedimentation was the same as in Fig. 1. ●—●, Absorbance at 260 mp; ○—○, radioactivity (cpm); △...△, RNase-resistant radioactivity (cpm).

The method was used to separate the RNA into ssRNA and dsRNA. The sedimentation profile was markedly changed by the addition of $5 \times 10^{-3}$ M MgSO$_4$ to the RNA solution. The ssRNA, including rRNA (26 and 17 S) and virus specific RNA (22 and 15 S) tended to aggregate. However, as described above, dsRNA was not affected at concentrations of $5 \times 10^{-3}$ M MgSO$_4$. The aggregation effect of the ssRNA is more clearly shown in Fig. 3 by replotting the sedimentation profile from Fig. 2. In Fig. 3A representing rRNA, the 26 S increased to 33 S and the 17 S to 21 S, respectively. Most of the rRNA sedimented to the bottom of the centrifuge tube, and this loss is represented by the shaded areas in the graph. As can be seen in Fig. 3B virus specific ss RNA also aggregated. An 11 S component can be obtained from the polyribosomal fraction of infected midguts as additional RNA.

The effect on RNA of Mg$^{2+}$ at different concentrations was further examined and the results are presented in Fig. 4. The aggregation effect appeared dependent on the Mg$^{2+}$ concentration. The rate of aggregation, as indicated by the % of RNA recovered in the sucrose gradient profile at different concentrations of MgSO$_4$, is presented in Table 1. It is evident that in all instances Mg$^{2+}$ caused aggregation of the ssRNAs. In $10^{-2}$ M MgSO$_4$ about 70% of the host RNA and 80% of the virus-specific ssRNA did not appear in the gradient profile whereas dsRNA did not decrease in an appreciable amount by this treatment. Figure 4 clearly shows that the dsRNA was not affected by Mg$^{2+}$ and appeared as an independent peak with no cross-contamination of ssRNA. This method may be useful for...
Fig. 3. Effect of Mg$^{2+}$ on host rRNA and virus-specific ssRNA derived from the sucrose gradient profiles shown in Fig. 2. The shaded areas in effect represent the difference caused by aggregation. ●—●, Absorbance at 260 μ; ○--○, radioactivity (cpm).

Fig. 4. Effect of Mg$^{2+}$ at different concentrations on the sedimentation of RNA extracted from infected midgut cells. No Mg$^{2+}$: RNA was dissolved in 1 ml TK buffer. Mg$^{2+}$ (10^{-3} M): RNA was dissolved in 1 ml TK buffer containing 10^{-3} M MgSO_4. Mg$^{2+}$ (10^{-4} M): RNA was dissolved in 1 ml TK buffer containing 10^{-4} M MgSO_4. Sucrose gradients were made with the same buffer in which the RNA was dissolved. Sedimentation was the same as in Fig. 1. ●—●, Absorbance at 260 μ; ○--○, radioactivity (cpm); △...△, RNase-resistant radioactivity (cpm).
TABLE 1
EFFECT OF Mg2+ CONCENTRATIONS ON RECOVERY OF HOST AND VIRUS-SPECIFIC RNA IN SUCROSE GRADIENT CENTRIFUGATION

<table>
<thead>
<tr>
<th>MgSO4 Concentration (Moles)</th>
<th>RNA recovery (%)</th>
<th>Host RNA</th>
<th>Virus specific-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ssRNA</td>
<td>dsRNA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>87.0</td>
<td>83.7</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-3}$</td>
<td>52.5</td>
<td>26.9</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-2}$</td>
<td>31.0</td>
<td>16.2</td>
<td></td>
</tr>
</tbody>
</table>

* Deemed to be within range of experimental error.

following the synthesis of viral progeny RNA in infected cells.

Separation of ssRNA and dsRNA

The dsRNA can be obtained from the infected cells by the aggregation of the ssRNA with high concentrations of Mg2+.

The ssRNA which is presumed to be viral mRNA could not be separated by this procedure in a condition suitable for centrifugation studies. The separation of the ssRNA was accomplished with LiCl treatment and sucrose gradient centrifugation. Figure 5 shows the sedimentation profiles of the fractions obtained for ssRNA and dsRNA. Newly synthesized ssRNA measured by radioactivity consisted of two major components with sedimentation rates of 22 S and 15 S compared with host rRNA having sedimentation rates of 26 S and 17 S (Fig. 5A), while most of the dsRNA seems to have remained in the supernatant (see Materials and Methods) as shown in Fig. 5B. It is evident that after RNase treatment the ssRNA fraction contained only 6% of the enzyme-resistant RNA and that no enzyme-sensitive RNA could be detected from the dsRNA fraction. However, there was a small molecule of RNA corresponding

![Figure 5](image.png)

Fig. 5. Effect of LiCl on RNA extracted from infected midgut cells. Procedure as given in Materials and Methods. (A) Precipitate fraction, ssRNA dissolved in 1 ml TK buffer. (B) Supernatant fraction, dsRNA dissolved in 1 ml TK buffer. Sucrose gradients were made with the same buffer in which the RNA was dissolved. Sedimentation was for 16 hr at 30,000 rpm. •—•, Absorbance at 260 mμ; ○—○, radioactivity (cpm); △—△, RNase-resistant radioactivity (cpm).
to 4 S in the dsRNA fraction. A similar phenomenon has been observed in cells infected with poliovirus (Baltimore, 1966) and reovirus (Watanabe and Graham, 1967). From the results presented here it is evident that the separation of ssRNA and dsRNA with LiCl has been successful and that this procedure can be utilized in the characterization of ssRNA synthesized in infected cells.

**Further Separation of dsRNA**

As indicated in Fig. 1, viral genome RNA is comprised of two components in sucrose gradient analysis. Treatment with either LiCl or MgSO₄ did not result in the separation of the dsRNA from the infected midgut into these two components, but as might be expected such treatment followed by prolonged centrifugation was successful in giving a separation as shown in Fig. 6. As can be seen there were two distinguishable components with sedimentation rates of 15 and 12 S similar to those obtained from the virions (Fig. 1). This RNA can, therefore, be reasonably concluded to be viral progeny RNA that has been extracted from virions present in the infected cells.

It should be noted (Fig. 6) that by both treatments there was still some 4 S RNA remaining. Similar results have been reported by Sporn and Dingman (1963) with Mg²⁺. After LiCl and MgSO₄ treatment and prolonged sucrose gradient centrifugation, measurable dsRNA can be recovered with these procedures.

From these experiments we conclude that treatment with MgSO₄ is a useful quantita-
tive method to determine the synthesis of viral progeny dsRNA in infected cells; treatment with LiCl makes it possible to separate virus-specific ssRNA.

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