Induced Ribonucleic Acids in Cells Infected with Vesicular Stomatitis Virus

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SUMMARY

RNA molecules labelled in BHK 21 cells following infection with vesicular stomatitis virus in the presence of Actinomycin D and $^{32}$PO$_4$ were examined. A complex pattern was obtained when the RNA was centrifuged in sucrose gradients, at least five radioactive peaks being found. These had sedimentation coefficients in sucrose gradients in $0.1 \text{ m-}$sodium acetate, pH 5.0, of approx. 38 S, 28 S, 20 S, 12 S and less than 4 S. Two, 12, 16 and 8% respectively of the radioactive RNA was resistant to ribonuclease 1 $\mu$g./ml. Treatment of the unfractionated induced RNA with ribonuclease 1 $\mu$g./ml yielded a product which had a heterogeneous sedimentation profile (7 to 11 S) in sucrose gradients. Recentrifuging individual fractions of this peak in separate gradients confirmed that the ribonuclease-resistant RNA was heterogeneous. The ribonuclease-resistant RNA also gave a heterogeneous profile in caesium sulphate gradients, with a density range of 1.61 to 1.65 g./ml. The induced RNA which sedimented most slowly in the sucrose gradients could be separated from the labelled cellular 4 S RNA by filtration through Sephadex G-75. The base composition of this RNA was significantly different from those of the faster sedimenting induced RNA molecules. Its association with vesicular stomatitis virus infection was confirmed by the failure to detect a similar slowly sedimenting RNA in uninfected cells or in cells infected with foot-and-mouth disease virus.

INTRODUCTION

Our previous studies showed that the RNA of the infective component of vesicular stomatitis virus generally sedimented in sucrose gradients as a fairly homogeneous peak at approx. 38 S (Brown et al. 1967). We also drew attention to the fact that several preparations of the virus gave RNA which had a heterogeneous profile in sucrose gradients. Since vesicular stomatitis virus exhibits the phenomenon of autointerference (Cooper & Bellett, 1959) it seemed possible, by analogy with influenza virus, that the virus RNA in these virus preparations might be present within the virus particle as a linear complex of RNA subunits or segments rather than in the form of a single molecule. If this supposition is correct, it would be logical to expect that double-stranded RNA molecules corresponding to the different segments would be present in cells infected with the virus. To test this hypothesis we investigated the nature of the virus-specified RNA molecules found in BHK 21 cells infected with the INDIANA strain of the virus, and found evidence for the presence of several double-stranded RNA molecules. In addition, we found a small single-stranded RNA molecule (< 25S), specific to virus infection and with a very high uridylic acid content, in the infected cells.
METHODS

Virus. The INDIANA strain of virus was used throughout. This had been plaque-purified and grew well in BHK 21 cells. Titres of $10^{6.5}$ ID50/ml. were reached in 5 hr (Crick, Cartwright & Brown, 1969). The virus was titrated by intracerebral inoculation of 7-day-old mice with serial tenfold dilutions of the virus.

Preparation of virus-induced RNA. Monolayers of BHK cells were incubated for 30 min. at 37° with 20 ml. of virus containing $10^{11}$ ID50 in Roux flasks rocked gently throughout the incubation period. Monolayers were then washed thoroughly with three 20 ml. amounts of a phosphate-deficient medium (Earle’s saline with 0.01 M-tris, pH 7.6, replacing the phosphate; the phosphate concentration was less than $4 \times 10^{-6}$ M) and incubated with gentle rocking in 20 ml. of the same medium containing Actinomycin D 1 µg./ml. and $^{32}$PO₄. The medium was then removed and titrated to ensure that the virus had grown satisfactorily. The monolayer was washed twice with Eagle’s medium to remove excess radioactivity and then extracted either directly with phenol saturated with 0.05M-acetate, pH 5.0, or with 0.5% sodium dodecyl sulphate followed by acetate-saturated phenol. In each method the aqueous layer was extracted a second time with acetate-saturated phenol before precipitating the RNA by adding 2 vol. cold ethanol and storing overnight at $-20°$.

Purification and fractionation of virus-induced RNA. The precipitated RNA was collected by centrifuging at 1000 g for 5 min. and dissolved in 0.1 M-acetate, pH 5.0, containing 0.1% sodium dodecyl sulphate. The solution was filtered through either Sephadex G-25 or G-200 in equilibrium with the same buffer solution and the radioactive peak emerging in the void volume was centrifuged in 5 to 25% sucrose gradients prepared in 0.1 M-acetate, pH 5.0. In the extraction using sodium dodecyl sulphate, the DNA was first hydrolysed with deoxyribonuclease before filtering through Sephadex. Centrifugation was continued for 15 hr at 20,000 rev./min. and the gradients then fractionated by puncturing a hole in the bottom of each tube and collecting 1 ml. fractions with an automatic pumping device. Portions of each fraction were used for optical extinction and radioactivity measurements. Appropriate fractions were combined, mixed with 0.5 mg. BHK cell RNA and precipitated with 2 vol. of ethanol at $-20°$.

Analysis of RNA by filtration through Sephadex G-75. Certain of the RNA fractions precipitated in ethanol were dissolved in 0.1 M-acetate, pH 5.0, containing 0.1% sodium dodecyl sulphate and filtered through a Sephadex G-75 column (35 x 1.5 cm.) in equilibrium with the same buffer solution. Two ml. fractions were collected and analysed for optical extinction at 260 nm. and radioactivity. Appropriate fractions were combined, mixed with 0.5 mg. BHK cell RNA and precipitated with 2 vol. of ethanol at $-20°$.

Determination of base composition of RNA. The RNA precipitates were drained well and then hydrolysed in 0.15 ml. 0.3N-KOH at 37° for 18 hr. After cooling to 0°, the solutions were adjusted to pH 3.5 with HClO₄ and the precipitate of KClO₄ removed. The nucleotide solutions were applied to Whatman 3 MM paper strips (75 x 7.5 cm.) that had been moistened in 0.25 M-citric acid trisodium citrate buffer, pH 3.5, and electrophoresis was continued for 18 hr at an initial current of 2 mA. per 7.5 cm. strip. The papers were dried and the ultraviolet-absorbing regions placed in vials containing 5 ml. of scintillation fluid (5 g. PPO and 0.3 g. dimethyl POPOP in 1 l. of toluene)
and counted in a Packard Tri-Carb liquid scintillation counter no. 3310. In some experiments the electrophoreograms were scanned along their entire length to ensure that the radioactivity was associated with the nucleotide regions only.

**Estimation of proportion of ribonuclease-resistant RNA in sucrose gradient fractions.** Each precipitated fraction was freed from sodium dodecyl sulphate by washing three times with 70% (v/v) ethanol in 0.1M-acetate, pH 5.0. The precipitates were then dissolved in 2 ml. 0.1M-acetate and 2 μg. pancreatic ribonuclease added. After 15 min. incubation at room temperature the solutions were each filtered through a Sephadex G-200 column (45 x 1 cm. in equilibrium with 0.1M-acetate, pH 5.0, containing 0.1% sodium dodecyl sulphate) and 2 ml. fractions collected. The distribution of radioactivity in the fractions gave an estimate of the proportion of ribonuclease-resistant RNA because only this fraction passed through the column in the void volume. The hydrolysed fragments were eluted as a separate peak at 50 to 60 ml. Good recovery of radioactivity was obtained.

**Estimation of density of RNA fractions.** The RNA was dissolved in Cs₂SO₄ solution, d = 1.6 g./ml., containing 1% formaldehyde (Szybalski, 1966) and centrifuged at 35,000 rev./min. for 60 hr in the SW 39 Spinco rotor. One-drop fractions were analysed for radioactivity and optical extinction at 260 nm. and every fifth drop was used for density determination.

**RESULTS**

**Fractionation of induced RNA by sucrose gradient centrifugation**

Monolayers which had been infected for 5 hr in the presence of Actinomycin D and ³²PO₄ were extracted with phenol saturated with 0.05M-acetate, pH 5.0, and the RNA precipitated with 2 vol. cold ethanol and stored at −20°. The precipitate was dissolved in 0.1M-acetate, pH 5.0, containing 0.1% sodium dodecyl sulphate and separate portions filtered through Sephadex G-200 or G-25 before centrifuging in 5 to 25% sucrose gradients. All the distributions of radioactivity showed that several species of RNA were present in the infected cells (Fig. 1). Both the G-200 and G-25 filtrates gave similar patterns except in the upper part of the gradients.

The peak at 38 S was presumed to correspond to the RNA of the infective virus component. The observation that this peak contained less radioactivity than the other peaks in all the experiments is probably explained by the small amount of virus in the cells. Our experiments on the replication of vesicular stomatitis virus in BHK 21 cells have shown that the amount of infective virus in the cells is less than 10% of that in the medium at all stages of the multiplication cycle (Crick et al. 1969). This would be expected if the virus matured only as it passed through the cell membrane. In addition, peaks with sedimentation constants of about 28 S, 20 S and 12 S were observed in both gradients (Fig. 1). Although there was considerable overlapping due to the complexity of the mixture, the peaks were shown to correspond to a different entity by re-centrifuging them through separate gradients. Their association with virus replication was shown by the base composition data (Table 1). The composition of each was similar to that of virus RNA but different from cell RNA.

The Sephadex G-25 filtrate also gave a peak of radioactivity in fractions 21 to 25 of the gradient. The distribution of radioactivity suggested that molecules sedimenting more slowly than the cellular 4 S RNA were also present. This peak was examined in more detail by filtration through Sephadex G-75 and G-200 (see below).
Fig. 1. Centrifugation in a 5 to 25% sucrose gradient of \( ^{32} \)P-labelled RNA extracted from BHK cells infected with vesicular stomatitis virus. (a) RNA filtered through Sephadex G-25 before centrifugation; (b) RNA filtered through Sephadex G-200 before centrifugation. ●—●, optical extinction at 260 nm.; ×—×, \( ^{32} \)P.

Table 1. Base composition of the RNA molecules synthesized in BHK cells infected with vesicular stomatitis virus and the virus and cell RNAs

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>C (%)</th>
<th>A (%)</th>
<th>G (%)</th>
<th>U (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38 S</td>
<td>21.1</td>
<td>30.3</td>
<td>20.8</td>
<td>27.7</td>
</tr>
<tr>
<td>28 S</td>
<td>21.5</td>
<td>30.9</td>
<td>21.5</td>
<td>26.0</td>
</tr>
<tr>
<td>20 S</td>
<td>21.4</td>
<td>30.3</td>
<td>20.6</td>
<td>27.5</td>
</tr>
<tr>
<td>12 S</td>
<td>20.4</td>
<td>30.8</td>
<td>21.8</td>
<td>27.1</td>
</tr>
<tr>
<td>&lt; 2 S</td>
<td>17.4</td>
<td>18.7</td>
<td>24.1</td>
<td>30.8</td>
</tr>
<tr>
<td>Purified virus*</td>
<td>21.1</td>
<td>29.3</td>
<td>20.9</td>
<td>28.7</td>
</tr>
<tr>
<td>28 S ribosomal†</td>
<td>29.6</td>
<td>18.4</td>
<td>34.6</td>
<td>17.7</td>
</tr>
<tr>
<td>16 S ribosomal†</td>
<td>25.9</td>
<td>22.1</td>
<td>30.5</td>
<td>21.5</td>
</tr>
<tr>
<td>4 S (labelled in presence of Actinomycin)</td>
<td>76.0</td>
<td>11.7</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Actinomycin-resistant‡</td>
<td>21.5</td>
<td>25.8</td>
<td>30.1</td>
<td>22.5</td>
</tr>
</tbody>
</table>

Ribonuclease-resistance of the induced RNA

The method most often used for estimating the proportion of ribonuclease-resistant RNA in peaks isolated from sucrose gradients consists of the precipitation of the enzyme-treated fractions with 10% trichloracetic acid followed by collection and estimation of the radioactivity of the acid-insoluble precipitate. Even using ribonuclease 1 μg./ml. with single-stranded RNAs isolated from purified viruses we were unable to convert the RNA completely into an acid-soluble form, although sucrose-gradient centrifugation of the same enzyme-treated samples showed that all the RNA had been converted into slowly sedimenting molecules. As an alternative method we filtered the enzyme-treated RNA fractions through Sephadex G-200 to separate unhydrolysed RNA from the products of the enzyme treatment. We found that the proportion of radioactivity eluting from the G-200 columns in the void volume was less than 2% for the 38 S and 28 S RNA, 12% for the 20 S RNA and 16% for the 12 S RNA. The slowly sedimenting peak at the top of the gradient of the G-25 filtrate had about 8% ribo-
nuclease-resistant radioactivity. RNA resistant to the enzyme was therefore present in significant amounts in a number of the RNA peaks.

**Heterogeneity of the ribonuclease-resistant RNA in sucrose and caesium sulphate gradients**

The RNA isolated from infected cells 5 hr after infection was hydrolysed with ribonuclease 1 μg./ml. for 15 min. at 20° and then sedimented in a 5 to 25% sucrose gradient in 0·1 M-acetate, pH 5·0, for 15 hr at 20,000 rev./min. The distribution of radioactivity showed that most of the ^32P was at the top of the gradient but there was a distinct shoulder on the leading edge of the peak. To allow a more precise examination of the ribonuclease-resistant RNA, the products from the enzyme-treated RNA were first filtered through Sephadex G-200 and the radioactivity eluting in the void volume was mixed with BHK cell RNA (to act as internal marker) before centrifuging it in a 5 to 25% gradient (Fig. 2a). A heterogeneous profile was obtained at 7 to 11 S. The heterogeneity of the peak was confirmed by centrifuging different fractions of the peak in separate gradients (Fig. 2b).
The ribonuclease-resistant RNA was also sedimented to equilibrium in a caesium sulphate gradient. This showed one peak at the position of single-stranded RNA (1.65 g./ml.) but there was also a trail of radioactivity on the less dense side of the peak (Fig. 3). The profile obtained with the single-stranded RNA isolated from purified virus particles is included for comparison. We do not know the reason for the heterogeneous profile obtained with the ribonuclease-resistant RNA.

**Fig. 4.** Sephadex G-75 filtration of the slowly sedimenting RNA induced in BHK cells by infection with vesicular stomatitis virus. • • •, Optical extinction at 260 nm.; × × × × × ×, \( ^{32} P \); peak A = ribosomal RNA and traces of the larger virus-induced RNAs; peak B = 4S RNA; peak C = slowly sedimenting virus-induced RNA.

**Nature of the slowly sedimenting RNA**

We at first assumed the difference between the radioactive profiles obtained by sucrose-gradient centrifugation of the Sephadex G-200 and G-25 filtrates to be due to the presence in the G-25 filtrate of labelled cellular 4S RNA (Fig. 1). This would have been present because of terminal labelling which occurs even in the presence of Actinomycin D. However, comparison of the radioactivity and optical extinction profiles suggested that molecules smaller than 4S RNA were present in the slowly sedimenting fractions. These fractions were precipitated overnight at \(-20^\circ\) with 2 vol. cold ethanol after adding carrier BHK to cell RNA. The precipitate was dissolved in 0.1 M-acetate, pH 5.0, containing 0.1% sodium dodecyl sulphate and passed through a Sephadex G-75 column. The distribution of optical extinction at 260 nm. and radioactivity in the eluate showed the presence of three peaks of RNA (Fig. 4). The first peak, A, contained ribosomal RNA together with a small amount of virus-induced
RNA. Peak B contained 4 S RNA and the third peak, C, was the slowly sedimenting RNA. The radioactivity of peak A was due to traces of virus-induced RNA incompletely separated by sucrose-gradient centrifugation. Similar results were obtained when Sephadex G-200 was used instead of G-75. Uninfected cells or cells which had been infected with foot-and-mouth disease virus and then incubated under the same conditions in phosphate-low medium containing Actinomycin D and ^3PO_4 for 5 hr did not give a similar peak eluting after the 4 S RNA when filtered through G-75 columns.

Fractions containing the 4 S RNA and the smaller RNA molecules eluted from the G-75 column were mixed with BHK cell RNA and precipitated with 2 vol. ethanol at -20°. The precipitates were hydrolysed with 0.3 N-KOH and the distribution of ^3P in the nucleotides was determined by paper electrophoresis. The high proportion of radioactivity in the cytidylic acid fraction of the 4 S RNA confirmed that this radioactivity was due to terminal labelling. The RNA eluted after the 4 S RNA had a base composition which was significantly different from those of the faster sedimenting RNA molecules induced by virus infection (Table I). The composition of different preparations of the slowly sedimenting RNA varied to a greater extent than we normally encounter with different samples of other RNA molecules. In all our experiments, however, the slowly sedimenting RNA had a significantly lower adenylic acid and higher uridylic acid content than the other virus-induced RNA molecules.

**DISCUSSION**

The evidence we present in this paper shows that cells infected with vesicular stomatitis virus contain a complex of RNA molecules. This was not unexpected because our earlier studies (Brown *et al.* 1967) had suggested that the RNA of the virus particle might consist of more than one molecule. We had found that the virus RNA often sedimented as a heterogeneous peak in sucrose gradients. This peak contained molecules with sedimentation constants ranging from 25 to 50 S. The ribonuclease-resistant RNA found in infected cells also gave a heterogeneous profile in sucrose gradients and the heterogeneity of the peak was confirmed by recentrifuging individual fractions of the peak. The sedimentation constant of the individual molecules ranged from 7 to 11 S. If the replication of vesicular stomatitis virus RNA proceeds via a double-stranded replicating form, a 7 S double-stranded RNA molecule would be expected to correspond to two single strands of approx. 11 S. Similarly, an 11 S double-stranded molecule would be expected to consist of two single strands of approx. 18 S. These results suggest that the basic unit of the virus RNA is not the 38 S molecule most often found in the virus but a complex of four 18 S molecules or eight 11 S molecules. This situation is analogous to that with influenza virus RNA and it is interesting in this connexion that both viruses exhibit autointerference. The autointerfering particle in vesicular stomatitis virus contains RNA with a sedimentation constant of 18 to 25 S (Huang & Wagner, 1966; Brown *et al.* 1967).

We have also made the unusual observation that infected cells contain a small RNA molecule with a high content of uridylic acid and a low content of adenylic acid. This molecule was not found in uninfected BHK cells nor was it present in BHK cells infected with foot-and-mouth disease virus. Its significance in the replication of vesicular stomatitis virus is not clear at present.
REFERENCES


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