Sequential Synthesis of Double-stranded and Single-stranded RNA by Cell-free Extracts of Barley Leaves Infected with Brome Mosaic Virus

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SUMMARY

A particulate cell-free fraction of barley leaves infected with brome mosaic virus had RNA polymerase activity in the presence of actinomycin D and EDTA. The RNA produced during a short pulse (3½ min.) of $^3$H-labelled UTP was mostly resistant to RNase in high salt concentration, whereas longer labelling periods gave an increasingly RNase-sensitive product. ‘Pulse-chase’ experiments showed that the first product labelled sedimented at about 14 s, was presumably a double-stranded RNA and was the precursor of single-stranded RNA, most of which sedimented more slowly. Production of the single-stranded RNA depended upon the presence of all four ribonucleotides. Little complete radioactive brome mosaic virus RNA could be found, possibly because of the effect of nucleases in the reaction mixture. When heated, the 14 s RNase-resistant RNA gave polydisperse single-stranded RNA, some of which sedimented at a similar rate to the large component of brome mosaic virus RNA. Most of the RNase-resistant RNA did not behave like replicative intermediates that have been described.

INTRODUCTION

When incubated with the ingredients necessary for RNA synthesis, a particulate fraction prepared from barley leaves infected with brome mosaic virus (BrMV) incorporates $^3$HUTP into RNA in the presence of actinomycin D (Semal & Hamilton, 1968). The product obtained was mostly double-stranded (Semal, 1970a). Further analysis of the system has been hampered by the low efficiency with which it incorporates labelled nucleotides.

It was shown recently (Semal, 1970b) that modifications of the method for grinding the leaves, and addition of EDTA, notably increased the efficiency of nucleotide incorporation into RNA by the above system. The deproteinized product obtained with the modified technique was partially resistant to RNase in ×2 standard saline citrate (0.15M-NaCl, 0.015M-sodium citrate, pH 7.0: SSC), but was completely digested by this enzyme in ×0.05: SSC.

We report here experiments on the rate and amount of RNA synthesis by cell-free extracts of BrMV-infected barley leaves, together with the properties of the labelled RNA product obtained when $^3$HUTP is present.

METHODS

Plants and viruses. Barley seedlings var. Herta were grown in a greenhouse and were inoculated at the one-leaf stage with the juice of brome mosaic virus-infected leaves mixed
with carborundum. The inoculated plants were placed in a cabinet at 24° with continuous light. Under these conditions, symptoms appeared at the base of the second leaf 4 days after inoculation of the first leaf.

**Preparation of the crude RNA polymerase fraction.** The basal part of the second leaf of barley plants was excised at 3 days after inoculation (4 days in winter); this stage of infection corresponds to the maximum activity of the virus-induced RNA polymerase, and to the early stages of detectable virus growth (Semal & Kummert, 1970). Fresh leaf tissue was cut into small pieces; 2 g. samples were ground in a cold pestle and mortar with 40 ml. extracting medium containing 0.05 M-tris-HCl, 0.01 M-KCl, 0.008 M-MgCl₂, 0.004 M-mercaptoethanol and 0.001 M-EDTA (pH 7.4). The extract was filtered through three layers of fine cloth and centrifuged for 15 min. at 10000 g. Pellets were discarded and supernatants were centrifuged for 15 min. at 10,000 g. The pellets were resuspended in incubation medium (2 ml./leaf sample) similar to the extraction medium (except that pH was 8.6), and this we call the crude RNA polymerase preparation.

**Cell-free synthesis of RNA.** The reaction mixture for RNA synthesis in vitro consisted of the crude RNA polymerase preparation in incubation medium together with 0.1 volume of the necessary ingredients in water to make the following final concentrations: 20 μg./ml. of actinomycin D; 1.25 mg./ml. of the tricyclohexamine salt of phosphoenolpyruvic acid; 20 μg./ml. of pyruvate kinase; 500 μg./ml. of each of the three unlabelled ribonucleotides; and 50 μc/ml. of [³H]UTP (1 to 2 c/mm). The mixture was incubated at 30°. Samples (0.4 ml.) of the mixture were removed at intervals and processed as indicated.

RNA was extracted from the incubation mixture in the following way. Samples were mixed with 2 ml. of water-saturated phenol, 4.5 ml. of 0.5 % sodium dodecyl sulphate in × 1.1 SSC, and 2.5 mg. of unlabelled UTP. The tubes were shaken vigorously for 5 min. at 20 to 25°, cooled, centrifuged for 10 min. at 10,000 g, and two 2-ml. samples of each supernatant were collected. These were each mixed with 0.25 ml. of 2 M-sodium acetate pH 5.2, and 2 volumes of ethanol. The mixture was kept overnight at 4° (in some experiments 2 hr at −20°), and the precipitates were collected by centrifugation for 15 min. at 10,000 g, washed with 95% ethanol, resuspended in 2 ml. of × 2 SSC and precipitated in the presence of carrier protein by addition of trichloracetic acid (containing 1 % of sodium pyrophosphate) to a final concentration of 5 %. The insoluble material was collected on Millipore filters, type BDWP. The filters were washed successively with 5 % trichloracetic acid and 95 % ethanol, dried, and placed in vials containing 10 ml. of scintillation fluid consisting of 5 g. PPO (2.5-diphenyloxazol) and 0.3 g. of POPOP (p-bis-(5-phenyloxazolyl)-benzene) in 1 l. of toluene. The radioactivity was measured with a Nuclear Chicago scintillation counter. In all experiments reported in Fig. 1 and Fig. 2, samples were deproteinized immediately after addition of the labelled precursor to the enzyme fraction, and consistently gave a radioactivity of 100 to 200 counts/min./2 ml. sample of RNA solution; the actual t = 0 values were subtracted from the other results in each individual experiment.

RNase resistance was assayed by incubating RNA samples (2 ml.) in × 2: SSC for 30 min. at 37° with 5 μg./ml. of pancreatic RNase. The reaction was stopped by adjustment to 5 % trichloracetic acid. Carrier protein was added, and the precipitate was collected on Millipore filter and tested as above for radioactivity. In some experiments, the RNase resistance of the incorporated [³H]UTP was also tested before deproteinization, using the technique described earlier (Semal, 1970a).

**Fractionation in sucrose gradients.** Several RNA pellets of ethanol precipitation were resuspended in standard acetate medium, containing 0.1 M-sodium acetate, 0.001 M-MgCl₂ and 0.001 M-CaCl₂, pH 5.5 (Bockstahler & Kaesberg, 1965); the suspensions were pooled and
clarified by low-speed centrifugation. The clarified solution (0.3 ml.) was layered on top of a 5 to 20 % (w/v) sucrose gradient in the standard acetate solution containing 1 µg./ml. of polyvinyl sulphate, and was centrifuged for 16 hr in the SW 25 rotor of a Spinco centrifuge. The tubes were then pierced at the bottom and 5-drop fractions were collected. Fractions were adjusted to × 2: SSC; one sample of each fraction was precipitated with trichloracetic acid, while another was first incubated with RNase. The radioactivity of the precipitates, was then measured as described above.

Chemicals. Actinomycin was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. Common chemicals and RNase-free sucrose were purchased from Merck Co. Unlabelled nucleotides were obtained from Schwarz BioResearch Inc.; tricyclohexamine salt of phosphoenol pyruvic acid, pyruvate kinase and pancreatic RNase A were from Sigma Chemicals Co. PPO and POPOP were purchased from Packard Instrument Co. Uridine-5'-triphosphate ([3H]UTP, 1 to 2 c/mM) was obtained from The Radiochemical Centre, Amersham.

RESULTS

Specificity of [3H]UTP incorporation in the presence of EDTA and actinomycin D

Crude RNA polymerase preparations were made from healthy barley leaves, or from leaves of brome mosaic virus-infected barley seedlings at 4 days after inoculation (winter conditions), and incubated for increasing periods of time with [3H]UTP. The labelled RNA product was isolated and tested for RNase resistance in × 2: SSC. The results shown in Fig. 1 indicate that under these conditions, RNA synthesis was obtained with extracts of infected leaves only. During the first minutes of incubation, label was incorporated into an RNA product which was largely resistant to RNase in × 2: SSC; with longer periods

![Graph](image-url)
of labelling, an increasing proportion of the label in the product was sensitive to RNase. Incorporation of label ceased completely after 45 min.

Other experiments gave similar results, with some variation in the period (3½ to 10 min.) after which RNase sensitivity was first detected. This type of response was regularly, obtained, provided the leaves used in the experiments were harvested before showing noticeable symptoms, i.e. at 3 days after inoculation (4 days in winter). In two experiments with plants at 4 days after inoculation and showing clear symptoms, the labelled product remained completely resistant to RNase, even after 30 min. of incubation with [3H]UTP (see below). Preincubation of the crude enzyme preparation for 15 min. at 30° with all standard ingredients except ribonucleotides, followed by incubation with [3H]UTP together with unlabelled ATP, CTP and GTP, did not modify the pattern of RNase resistance shown in Fig. 1.

Pulse-chase experiments

The standard RNA-synthesizing system (3 days of infection) was given [3H]UTP for 3½ min.; then a 100-fold excess of non-radioactive UTP was added and the mixture was incubated further; controls did not receive unlabelled UTP, and were corrected for volume.

![Graph](image)

**Fig. 2.** Pulse-chase analysis of the RNA product of incubation of the crude RNA polymerase preparation with [3H]UTP. Results expressed as acid-insoluble counts x 10^-3/min./sample (2 ml.) of RNA solution. Four independent experiments: O—O, total radioactivity; •—•, RNase-resistant radioactivity; 5 µg./ml. RNase for 30 min. at 37° in ×2: SSC; □—□, total radioactivity in the presence of non-radioactive UTP added at 3½ min.; ■—■, RNase-resistant radioactivity in the presence of non-radioactive UTP added at 3½ min.

Samples were removed at 7, 10 and 15 min. after the start of the experiment; RNA was extracted and tested for resistance to RNase in ×2: SSC. The results of four independent experiments are shown in Fig. 2. Addition of an excess of non-radioactive UTP suppressed any further incorporation of radioactivity into the RNA product. There was some loss of acid-insoluble radioactivity after unlabelled UTP was added, but most noticeable was the rapid increase in RNase sensitivity of the product after 3½ min. chase. Part of the radio-
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activity incorporated during the pulse resisted RNase treatment, even after a prolonged chase (up to 90 min. in other experiments). This shift from RNase resistance to RNase-sensitivity was regularly obtained with leaves at an early stage of infection. However, in pulse-chase experiments with leaves bearing clear symptoms, when no RNase-sensitive product was obtained upon incubation with [³H]UTP for 10 to 15 min. (see preceding section), the entire pulse-labelled material remained resistant to RNase even after a long incubation under 'chase' conditions.

Effect of ribonucleotides during the chase, on the RNase resistance of the pulse-labelled product

In order to investigate the nature of the shift from RNase resistance to RNase sensitivity observed during the chase, and the possible rôle of RNA synthesis in this phenomenon, the standard RNA-synthesizing mixture was given a 3·5 min. pulse of radioactive UTP and was then rapidly cooled. After centrifugation for 15 min. at 10,000 g, the supernatants were

Table 1. [³H]UTP ‘pulse-chase’ experiments

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Duration of post-incubation (min.)</th>
<th>Post-incubation*</th>
<th>Without nucleotides</th>
<th>With the four ribonucleotides</th>
<th>With UTP alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No RNase†</td>
<td>RNase†</td>
<td>No RNase†</td>
</tr>
<tr>
<td>1</td>
<td>0·0</td>
<td></td>
<td>4,022†</td>
<td>3,584</td>
<td>4,064</td>
</tr>
<tr>
<td></td>
<td>6·0</td>
<td></td>
<td>4,692</td>
<td>4,234</td>
<td>4,247</td>
</tr>
<tr>
<td></td>
<td>10·0</td>
<td></td>
<td>5,142</td>
<td>3,577</td>
<td>4,041</td>
</tr>
<tr>
<td></td>
<td>15·0</td>
<td></td>
<td>4,100</td>
<td>3,949</td>
<td>3,245</td>
</tr>
<tr>
<td>2</td>
<td>0·0</td>
<td></td>
<td>13,200</td>
<td>7,944</td>
<td>12,968</td>
</tr>
<tr>
<td></td>
<td>3·5</td>
<td></td>
<td>15,024</td>
<td>8,137</td>
<td>13,405</td>
</tr>
<tr>
<td></td>
<td>6·5</td>
<td></td>
<td>14,856</td>
<td>8,477</td>
<td>14,856</td>
</tr>
</tbody>
</table>

* After a 3·5 min. pulse of [³H]UTP, the 10,000 g pellet of the reaction mixture was resuspended and post-incubated as indicated.
† RNA incubated with 5 µg./ml. RNase for 30 min. at 37° in × 2: SSC.
‡ Acid-insoluble counts/min./sample (2 ml.) of RNA solution.

discarded and the pellets were resuspended in fresh incubation medium with actinomycin D and ATP-generating system. Samples of the mixture were then incubated at 30°: (1) without nucleotides; (2) with 500 µg./ml. of each of the four ribonucleotides; (3) with UTP alone (500 µg./ml.). The results presented in Table 1 indicate that the shift from RNase resistance to RNase sensitivity was obtained only in the presence of the four ribonucleotides; controls without nucleotides or with UTP alone remained RNase-resistant.

Sedimentation properties of the labelled RNA products

The crude RNA polymerase preparation (3 days of infection) was incubated for 3½ or 7 min., with [³H]UTP. RNA was extracted and centrifuged in a 5 to 20% sucrose gradient; fractions were tested for total and RNase-resistant acid-insoluble radio-activity. BrMV-RNA (L, large component; M, medium component; S, small component) was used as external extinction marker. Results of Fig. 3 show that most of the label incorporated after a 3·5 min. pulse (Fig. 3a) was associated with RNase-resistant RNA, most of which sedimented at about 14 S with some more quickly. A similar sedimentation pattern was obtained with labelling periods of 7 min. (Fig. 3b), but larger quantities of RNase-
sensitive RNA were present, mainly in the lower part of the gradient. The RNase-resistant material was heterogeneous, with minor peaks in the region between 14 and 27 s.

A similar experiment was done with a 10 min. pulse of radioactive UTP. The results are shown in Fig. 3c and indicate that the RNase-resistant fraction sedimented mainly at 14 s, with some heterogeneous faster sedimenting material. The RNase-sensitive product was spread throughout the gradient and included some slowly sedimenting material.

Fig. 3. Sedimentation profile in a 5 to 20% sucrose gradient (right to left) of the RNA isolated from the crude RNA polymerase preparation after incubation with [3H]UTP. Centrifugation for 16 hr at 25,000 rev./min. O—O, Total radioactivity; ——, RNase-resistant radioactivity (5 μg/ml RNase for 30 min. at 37° in x2: SSC). (a) 3-5 min. pulse, (b) 7-0 min. pulse, (c) 10-0 min. pulse, (d) 3½ min. pulse, followed by chase of the particulate fraction (10,000g pellet) for 3½ min. in the presence of the four ribonucleotides. S, M, L: small, medium and large component of BrMV-RNA respectively, used as external extinction markers.
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The RNA obtained after a pulse-chase experiment was also analysed in sucrose density gradient. The RNA-synthesizing fraction was given a 3½ min. pulse of \(^{3}H\)UTP; the tubes were rapidly cooled and were centrifuged for 15 min. at 10,000 g. The pellets were resuspended in standard incubation medium containing 500 \(\mu g./ml\). of each of the four ribonucleotides, and were incubated for another 3½ min. Most of the RNase-sensitive product, when centrifuged in sucrose gradient, stayed in the upper part of the gradient (Fig. 3 d), suggesting that the displaced product either was of rather small size, or was degraded during the chase. The residual RNase-resistant material was heterogeneous and most sedimented around 14 s. It should be noted that Fig. 3 d is not directly comparable to the other results of Fig. 3, as only the pulse-labelled product sedimenting at 10,000 g was considered, and the UTP concentration during the chase of Fig. 3 d was about 30 times higher than UTP concentration in the standard incorporation mixture. This might increase RNA synthesis during the chase, the low concentration of UTP being a limiting factor during incubation with \(^{3}H\)UTP (see also Girard, 1969).

Fractionation of the labelled RNA products with lithium chloride

The crude RNA polymerase preparation was incubated as usual with \(^{3}H\)UTP; samples were removed at intervals and the RNA was extracted and isolated by ethanol precipitation. The pellets were resuspended, adjusted to 2 M-LiCl and kept overnight at 4 °. The material was then centrifuged for 10 min. at 10,000 g, and supernatants and pellets were tested separately for acid-insoluble radioactivity. Results are shown in Table 2. Some of the radioactively labelled RNA was soluble and some insoluble in LiCl. The insoluble RNA became increasingly sensitive to RNase in \(\times 2:\) SSC with increasing incubation times.

Table 2. Lithium chloride fractionation of the RNA product of \(^{3}H\)UTP incorporation

<table>
<thead>
<tr>
<th>Duration of the radioactive 'pulse' (min.)</th>
<th>Product soluble in 2 M-LiCl</th>
<th>No RNase</th>
<th>No RNase</th>
<th>RNase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RNase</td>
</tr>
<tr>
<td></td>
<td>No RNase</td>
<td>1375</td>
<td>967</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'5</td>
<td>2749</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7'0</td>
<td>3431</td>
<td>3852</td>
<td>1333</td>
</tr>
<tr>
<td></td>
<td>10'0</td>
<td>4048</td>
<td>5765</td>
<td>1560</td>
</tr>
<tr>
<td></td>
<td>15'0</td>
<td>6373</td>
<td>7106</td>
<td>1593</td>
</tr>
</tbody>
</table>

* RNA incubated with 5 \(\mu g./ml\). RNase for 30 min. at 37 ° in \(\times 2:\) SSC.
† Acid-insoluble counts/min./sample (2 ml.) of RNA solution.

LiCl supernatants of a 7 min. pulse experiment were reprecipitated with ethanol, using BMV-RNA as carrier. Pellets thus obtained were resuspended in standard acetate medium, pooled, and layered on a 5 to 20 % sucrose gradient. As shown in Fig. 4, LiCl-soluble material sedimented as a RNase-resistant peak at about 14 s, with little RNase-sensitive material distributed throughout the gradient, thus resembling the sedimentation pattern of the unfractionated 7 min. pulse-labelled RNA (Fig. 2 b) freed from the major part of its RNase-sensitive components.

Effect of RNase on the 'native' product of \(^{3}H\)UTP incorporation

As the resistance of the product of the RNA polymerase reaction to RNase could be an artifact caused by the deproteinization of a 'replicative complex' (Borst & Weissmann, 1965), we tested the RNase resistance of the product of \(^{3}H\)UTP incorporation before
deproteinization by phenol, as described earlier (Semal, 1970a). Results of Table 3 indicate that the fraction of the product which sedimented at 10,000g (representing most of total RNase-resistant product) was largely resistant to RNase in ×2: SSC before phenol treatment.

![Sedimentation profile](image)

Fig. 4. Sedimentation profile in a 5 to 20% sucrose gradient (right to left) of the LiCl-soluble RNA isolated from the crude RNA polymerase preparation after incubation for 70 min. with [3H]UTP. Centrifugation for 16 hr at 25,000 rev./min. ○, Total radioactivity; ●, RNase-resistant radioactivity (5 μg./ml. RNase for 30 min. at 37°C in ×2: SSC).

Table 3. RNase resistance of the product of [3H]UTP incorporation before deproteinization

<table>
<thead>
<tr>
<th>Duration of radioactive pulse</th>
<th>Particle-bound product* resuspended</th>
<th>In × 2: SSC</th>
<th>In × 0.05: SSC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No RNase</td>
<td>RNase†</td>
<td>No RNase</td>
</tr>
<tr>
<td>7 min.</td>
<td>2991‡</td>
<td>2485</td>
<td>2033</td>
</tr>
<tr>
<td></td>
<td>3265</td>
<td>2785</td>
<td>2336</td>
</tr>
<tr>
<td>10 min.</td>
<td>3763</td>
<td>3145</td>
<td>2495</td>
</tr>
<tr>
<td></td>
<td>3814</td>
<td>3185</td>
<td>2603</td>
</tr>
</tbody>
</table>

* Product associated with the 10,000g pellet of the reaction mixture.
† 40 μg./ml. RNase for 30 min. at 37°C.
‡ Acid-insoluble counts/min./sample (2 ml.) of RNA solution.

**Thermal denaturation of the labelled RNA product**

The standard RNA-synthesizing system was incubated for 5 min. with [3H]UTP. RNA was extracted and heated at increasing temperatures for 10 min. in ×2: SSC; after rapid cooling in ice, samples were digested with RNase. As shown in Table 4, heating the labelled product up to 100°C in ×2: SSC had no major effect on its resistance to RNase, while heating at 105°C induced almost complete sensitivity to this enzyme. When heated in
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x 0.1: SSC, however, complete RNase sensitivity was obtained at 100°; a sample of the material thus treated was layered on a 5 to 20% sucrose gradient, and was compared for sedimentation properties to the untreated original product. The results (Fig. 5) show that the RNA strands dissociate into single-stranded material, some of which sediments together with, or possibly faster than the large particle of BrMV-RNA.

Table 4. Effect of temperature on RNase resistance of the product of a 5 min. pulse of [3H]UTP

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>No RNase</th>
<th>RNase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>6538†</td>
<td>4775</td>
</tr>
<tr>
<td>40</td>
<td>6924</td>
<td>4706</td>
</tr>
<tr>
<td>50</td>
<td>6802</td>
<td>5557</td>
</tr>
<tr>
<td>60</td>
<td>7282</td>
<td>5402</td>
</tr>
<tr>
<td>70</td>
<td>6723</td>
<td>4669</td>
</tr>
<tr>
<td>80</td>
<td>6284</td>
<td>4855</td>
</tr>
<tr>
<td>90</td>
<td>6460</td>
<td>4949</td>
</tr>
<tr>
<td>100</td>
<td>5828</td>
<td>4406</td>
</tr>
<tr>
<td>105</td>
<td>6195</td>
<td>252</td>
</tr>
</tbody>
</table>

* RNA incubated with 5 μg./ml. RNase for 10 min. at 37° in x 0.2: SSC.
† Acid-insoluble counts/min./sample (2 ml.) of RNA solution.

Fig. 5. Sedimentation profile in a 5 to 20% sucrose gradient (right to left) of the heat-denatured RNA product of a 5 min. pulse of [3H]UTP. Centrifugation for 16 hr at 23,000 rev./min. Untreated RNA; O—O, RNA heated for 10 min. at 100° in x 0.1: SSC; ——, heated RNA incubated with RNase (5 μg./ml. RNase for 30 min. at 37° in x 2: SSC). S, M, L: small, medium and large component of BrMV-RNA used as external extinction marker.
DISCUSSION

The EDTA-induced stimulation of [\textsuperscript{3}H]UTP incorporation by a particulate crude RNA polymerase fraction in the presence of actinomycin D, was obtained specifically with BMV-infected barley leaves (Fig. 1); the nature of this EDTA effect was not investigated further.

Total incorporation of the labelled UTP precursor was almost linear with time during the first 7 to 10 min. of incubation. The radioactive product of a short pulse of radioactivity was highly resistant to RNase in \times 2: SSC, but the proportion of RNase-resistant RNA decreased with increasing times of labelling. This shift towards RNase sensitivity was noticeable after somewhat variable times of incubation with [\textsuperscript{3}H]UTP (3.5 to 10 min. in different experiments), possibly because of variation in the speed of the reaction.

The rate of increase of RNase sensitivity suggested that RNase-resistant RNA was the precursor of RNase-sensitive RNA. Pulse-chase experiments confirmed this, as part of the radioactivity of the RNase-resistant product of a 3.5 min. pulse could be chased into an RNase-sensitive material upon addition of non-radioactive precursor. These results were consistently obtained using preparations made from leaf material in early stages of infection, before appearance of clear symptoms. This stage of infection gave the highest activity of the BMV-induced RNA polymerase, as measured without EDTA (Semal & Kummert, 1970).

Pre-incubation of the crude polymerase fraction without nucleotides did not change the sequential synthesis of double- and single-stranded RNA, suggesting that this pattern depended upon continuous RNA synthesis. Furthermore, loss of RNase-sensitive radioactivity from the pulse-labelled RNase-resistant RNA did not take place in the absence of the four ribonucleotides (Table 1), as expected if RNA synthesis is a prerequisite for the release of labelled single-stranded polynucleotides from a double-stranded precursor molecule. The data of our pulse-chase experiments are in general agreement with those obtained with crude RNA polymerase fractions obtained from cells infected with picornaviruses (see Plagemann & Swim, 1966; Girard, 1969). The results with picornaviruses were interpreted as displacement of single-stranded RNA from replicative intermediate (RI) structures by the growing RNA molecules. The residual undisplaced RNA was considered as replicative form (RF) (Girard, 1969).

With picornaviruses, virus RNA, as characterized by sedimentation velocity, was the main single-stranded product of the crude RNA polymerase reaction. In the BrMV-barley system described here, the single-stranded radioactive product was heterogeneous in size after a chase, and most of it did not sediment at the same rate as the three classes of BrMV-RNA. This may be due to the action of nucleases in the mixture, which seem active in our system during the chase as suggested by the loss of acid-precipitable material (Fig. 2) and by the small size of the displaced single-stranded product (Fig. 3d).

The major product of pulse-labelling was isolated as RNase-resistant RNA sedimenting around 14 s; this is in agreement with the s values obtained for the double-stranded RNA synthesized \textit{in vivo} in BMV-infected barley leaves (Hiruki, 1969), and for the product synthesized by cell-free extracts of infected leaves incubated without EDTA (Semal & Hamilton, 1968).

Heating the RNA polymerase product gave a polydisperse RNase-sensitive material, part of which sedimented in the region of the large component of BrMV-RNA, and may be complete virus RNA molecules.

Some RNase-resistant RNA sedimented faster than the 14 s double-stranded product (Fig. 3a, b) and was associated with single-stranded RNA; the fractions involved may con-
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tain structures comparable to RI, as suggested also by the results of the 3½ min. pulse in Table 2, showing that part of the RNase-resistant product was insoluble in 2M-LiCl.

There are indications, however, that part of the RNase-resistant structures which release single-stranded RNA in our system do not possess the typical properties of RI after deproteinization. During the experiment reported in Table 2, when the product of the 3·5 min. pulse was chased for 3½ min. with an excess of unlabelled UTP, 59% of the label was found to be RNase-sensitive after the chase, although only 33% of the 3·5 min. pulse product was insoluble in 2M-LiCl. Thus it appears that part of the material isolated as LiCl-soluble RNA at 3½ min. may have released single-stranded RNA during the chase. Also the deproteinized RNA product of a 5 min. pulse showed only one melting temperature in contrast with two in other systems for typical RI molecules (Plagemann & Swim, 1968) or for RNA polymerase reaction products (Ehrenfeld, Maizel & Summers, 1970). The fact that structures able to synthesize and release single-stranded polynucleotides are deproteinized as RNA with properties of RF in our system, may possibly reflect the degradation of RI molecules by nucleases present in the crude polymerase preparation.

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