

**Localization of Viral RNA Polymerase Activity and its
Products in Extracts of Barley Leaves Infected with Bromegrass
Mosaic Virus**

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ABSTRACT

The pulse-labeled, double-stranded RNA product of the virus-specific RNA polymerase activity in extracts of barley leaves infected with bromegrass mosaic virus was found to be bound to particulate material which sedimented at 10,000 *g*. However, after a chase, part of the pulse-chased product was released in the 10,000 *g* supernatant as single-stranded RNA.

Further fractionation of the standard RNA polymerase preparation by centrifugation in a discontinuous sucrose gradient showed that both the virus-specific, actinomycin-resistant, RNA polymerase activity and the pulse-labeled RNA products accumulated at the interphase between the 25% and the 45% sucrose. Whole organelles were not present in the active fraction,

which contained essentially chloroplast debris together with various membranous and vesicular structures. Similar structures were found in comparable preparations made from healthy leaves, the latter being devoid of actinomycin-resistant RNA polymerase activity.

Treatment with sodium deoxycholate dissociated most of the pulse-labeled RNA product from the 10,000 *g* pellet to which it was bound, while most of the green pigment remained sedimentable at 10,000 *g* after such treatment. It is suggested that the subcellular structures involved in the synthesis of virus-specific RNA are distinct from the bulk of pigment-bearing chloroplast debris.

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Virus-induced RNA polymerase activity associated with an RNA template has been characterized in cell-free extracts of barley leaves infected with bromegrass mosaic virus (BMV) (7, 6). Most of the ribonuclease-resistant fraction of the reaction product was homologous to genome BMV-RNA (10); a sequential synthesis of double-stranded and single-stranded RNA was characterized in the system under consideration (4, 9). In the above experiments, the crude RNA polymerase preparation consisted of the fraction of the leaf homogenate sedimenting between 5 min × 1,000 *g* and 10 min × 10,000 *g* (standard RNA polymerase preparation). The present paper deals with the localization of the RNA polymerase activity and of its products within the various particulate components of the standard RNA polymerase preparation.

MATERIALS AND METHODS.—*RNA synthesis.*—The standard RNA polymerase preparation was obtained from BMV-infected barley leaves, and RNA synthesis was measured by the incorporation of [³H]-UTP, as described by Kummert and Semal (4).

The extraction medium (pH 7.6) contained 0.05 M Tris-HCl, 0.01 M KCl, 0.004 M mercaptoethanol, 0.001 M EDTA, and 40 μg/ml of yeast RNA. The incubation medium was similar to the extraction medium, except that it contained 0.008 M MgCl₂ and the pH was 8.6. The reaction mixture for RNA synthesis consisted of the RNA polymerase preparation in the incubation medium together with the necessary ingredients to make the following concentrations: 20 μg/ml of actinomycin D (AMD) (a gift from Merck, Sharp & Dohme Research

Laboratories, Rahway, N. J.), 1.25 mg/ml of the tricyclohexylamine salt of phosphoenolpyruvic acid, 20 μg/ml of pyruvate kinase; 500 μg/ml of each of the three unlabeled ribonucleotides; and 50 μCi/ml of [³H]-UTP (1 to 2 C/mole from The Radiochemical Center, Amersham, Great Britain).

In one type of experiment, the standard RNA polymerase preparation from leaves of plants inoculated 3 days previously with BMV was fractionated further, and the RNA polymerase activity of the subfractions was determined. One ml of the standard RNA polymerase preparation was layered on top of a discontinuous sucrose gradient (made of 4 ml of 60% sucrose, 4 ml of 45% sucrose and 3 ml of 25% sucrose in extraction buffer). The tubes (9.5-cm-long tubes with a total volume of 14 ml) were centrifuged for 30 min at 35,000 rpm in the swing-out rotor of an MSE centrifuge, at a rotor temp of 6-10 C. Fractions (0.5 ml) were collected from the top of the gradient, using an ISCO fractionator. Each subfraction was mixed with 4 ml of extraction medium, and then centrifuged for 10 min at 10,000 *g*. Supernatants were discarded; the pellets were resuspended in incubation medium, and mixed with the necessary ingredients for RNA synthesis in the presence of [³H]-UTP and 20 μg/ml of actinomycin D (AMD) as described by Kummert and Semal (4). After 3 min incubation at 30 C, the fractions were deproteinized with phenol and detergent (4). RNA was precipitated from the water phase by mixing with two volumes of ethanol in the presence of 50 μg/ml of carrier yeast RNA; the pellets were dissolved in

2XSSC (SCC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The acid-insoluble material associated with each fraction was collected on Millipore filters and radioactivity was determined by liquid scintillation in a Nuclear Chicago liquid spectrometer.

In other experiments, the standard RNA polymerase preparation was immediately resuspended in incubation medium, mixed with [³H]-UTP, AMD, and the necessary ingredients for RNA synthesis, and incubated for 2 min at 30 C. The reaction was terminated by rapid cooling and by addition of EDTA (final concn 0.02 M) and unlabeled UTP (final concn 10 mg/ml).

The RNA polymerase preparation was then centrifuged at 4 C for 10 min at 10,000 g. The supernatant was discarded and the pellet was resuspended in 1 ml of extraction medium and layered on top of a discontinuous sucrose gradient (see above) or on top of a continuous sucrose gradient (made of 1 ml of 60% sucrose overlaid with 10 ml of a linear 25-60% sucrose gradient in extraction medium). The tubes were centrifuged for 30 min at 35,000 rpm in the MSE centrifuge. Fractions (0.5 ml) were collected and deproteinized with phenol and detergent; the RNA was precipitated with two volumes of ethanol in the presence of 50 µg/ml of carrier yeast RNA. Pellets were resuspended in 2XSSC, brought to 5% trichloroacetic acid, and the acid-insoluble material was collected on Millipore filters. Radioactivity was determined in the liquid scintillation spectrometer.

In some experiments, the RNA polymerase preparation was incubated for 2 min with [³H]-UTP, AMD, and the necessary ingredient for RNA synthesis. The reaction was stopped and the preparation was centrifuged for 10 min at 10,000 g. The supernatant was discarded and the pellet was resuspended in extraction buffer containing one of the following detergents: 0.5% Triton X-100 (Rohm & Haas, Philadelphia), 2.0% Nonidet P40 (Shell Nederland Chemie, Rotterdam, The Netherlands), 0.5% Na deoxycholate, and 0.5% Lubrol (Imperial Chemical Industries, Brussels, Belgium). After centrifugation for 10 min at 10,000 g, the RNA was extracted separately from the pellets and the supernatants.

Electron microscopy.—For observations by electron microscopy, unlabeled RNA polymerase preparations were centrifuged in the discontinuous sucrose gradient, and the material at the sucrose interphase fractions was diluted with extraction medium, and centrifuged for 10 min at 10,000 g. The pellets were fixed overnight with 5% glutaraldehyde in 0.05 M CaCl₂, postfixed with osmium fixative (1% OsO₄, 1% potassium dichromate, 0.05 M CaCl₂, pH 6.5) for 4 h at room temperature, washed with distilled water, stained overnight with 2% uranyl acetate in water, dehydrated in alcohol and embedded in Spurr medium (11). Ultrathin sections, cut with a Reichert Ultramicrotome OMU 2, were stained with lead by Karnovsky method II (3), and examined with a Siemens Elmiskop I.

RESULTS.—*Association of the products with*

particulate components of the standard RNA polymerase preparation.—As shown earlier (4), the deproteinized pulse-labeled RNA products synthesized by the standard RNA polymerase preparation from BMV-infected leaves were largely resistant to pancreatic RNase in 2XSSC, while after a chase upon addition of a large excess of unlabeled nucleotide precursor to the incubation mixture, part of the product became sensitive to RNase in 2XSSC. In order to investigate the binding of the products to particulate components in the standard RNA polymerase preparation, the RNA of a polymerase preparation from plants inoculated 3 days previously with BMV was pulse-labeled for 2 min, or pulse-labeled for 2 min followed by a 2 min chase. After incubation, the reaction was terminated by rapid cooling, and addition of EDTA and unlabeled UTP. The samples were then centrifuged for 10 min at 10,000 g. The supernatants were collected and the pellets were resuspended in extraction medium. Supernatant and pellets were separately deproteinized with phenol and detergent, and the acid-insoluble radioactivity was determined. In control tubes, supernatants and pellets were re-mixed before deproteinization. Results are presented in Table 1. The major part of the highly RNase-resistant pulse-labeled product sedimented at 10,000 g and most of the pulse-chased product was released as an essentially RNase-sensitive material in the 10,000 g supernatant.

Fractionation of the standard RNA polymerase preparation by centrifugation in a sucrose gradient and pulse-labeling of the fractions.—The standard RNA polymerase preparation from 3-day

TABLE 1. Sedimenting properties of the labeled products synthesized by the standard RNA polymerase preparation from bromegrass mosaic virus-infected barley leaves^a

	Pulse-labeled products		Pulse-chased products	
	no RNase :	RNase ^b	no RNase :	RNase ^b
10,000 g pellet	4,933	3,849	2,157	894
10,000 g supernatant	1,101	642	2,985	335
Control	5,250 ^c	4,630	4,648	1,134

^aThe RNA of the standard RNA polymerase preparation was pulse-labeled for 2 min, or pulse-labeled for 2 min followed by a 2-min chase. The reaction was stopped and the preparation was centrifuged for 10 min at 10,000 g. The RNA was extracted separately from the pellet and the supernatant; in control tubes, supernatant and pellet were re-mixed, and the RNA was extracted from the mixture. Results are expressed as acid-insoluble counts per min per tube in the RNA fraction.

^bThe deproteinized RNA was resuspended in 2XSSC (0.3 M NaCl + 0.03 M sodium citrate, pH 7.0); it was then incubated for 30 min at 37 C with RNase (5 µg/ml).

^cIn the same experiment, a comparative preparation from healthy barley leaves incorporated 312 counts per min per tube in the RNA fraction.

BMV-infected leaves was fractionated by centrifugation in a discontinuous sucrose gradient. Several bands were observed after centrifugation. A white flocculent band remained on top on the 25% sucrose phase (band I). A thin, sharply defined, dark-green layer of sticky material (band II) accumulated at the interphase of the 25-45% sucrose, with some diffuse whitish material floating above band II. The 45% sucrose phase appeared yellow-green throughout and a second dark-green layer accumulated at the interphase between the 45% and the 60% sucrose; the whole 60% sucrose phase remained translucent and very little material pelleted to the bottom of the tubes. A similar distribution

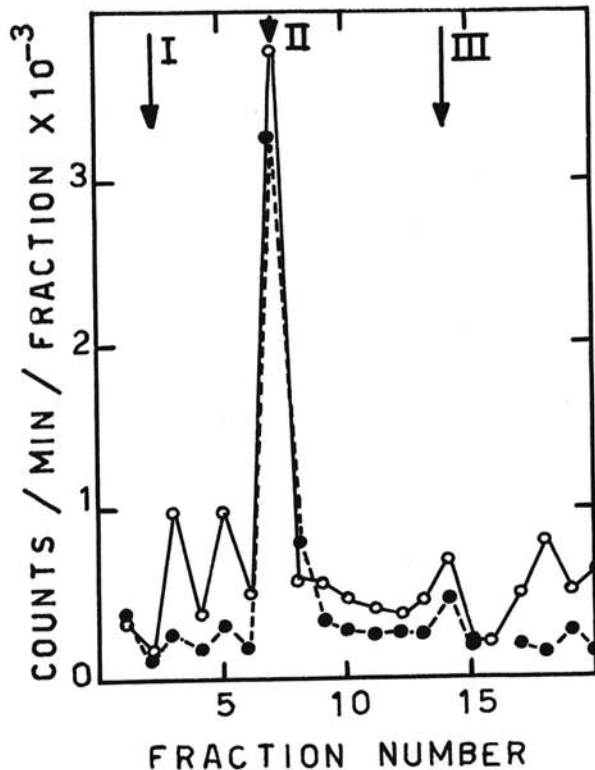


Fig. 1. Distribution of RNA polymerase activity in the standard RNA polymerase preparation from BMV-infected leaves. The polymerase preparation was centrifuged for 30 min at 35,000 rpm through a discontinuous sucrose gradient (made of 4 ml of 60% sucrose, 4 ml of 45% sucrose, 3 ml of 25% sucrose, and 1 ml of sample without sucrose, respectively). Fractions were collected from the top of the gradient, diluted, centrifuged for 10 min at 10,000 g, and the pellets were assayed for RNA polymerase activity by incubation for 3 min in the presence of [³H]-UTP and actinomycin D; only the upper 20 fractions of the gradient were analysed. Arrows indicate the bands formed at the interphases: white band I (0 - 25% sucrose), green band II (25 - 45% sucrose), and green band III (45 and 60% sucrose).

○—○ total acid-insoluble radioactivity (cpm) in the RNA;
 ●—● acid-insoluble radioactivity (cpm after incubation for 30 min at 37 C with RNase (5 µg/ml) in 2XSSC (0.3 M NaCl, 0.03M sodium citrate, pH 7.0).

pattern was observed in comparable preparations made from healthy leaves. The distribution of the green material between band II and band III was variable from experiment to experiment.

When the standard RNA polymerase preparation was centrifuged in a discontinuous sucrose gradient and the fractions were tested for RNA polymerase activity in the presence of AMD, a major peak of activity was observed, corresponding to band II (Fig. 1). Some RNA polymerase activity was also associated with slower-sedimenting material but the products thus obtained were entirely RNase-sensitive while the product of the RNA polymerase activity associated with band II was highly resistant to RNase in 2XSSC (Fig. 1).

Fractionation of the standard RNA polymerase preparations by centrifugation in sucrose gradient.—The standard RNA polymerase preparation from leaves infected for 3 days with BMV was incubated for 2 min with [³H]-UTP. After centrifugation for 10 min at 10,000 g, the pellet was resuspended in extraction medium and centrifuged in either a discontinuous or a continuous sucrose gradient. The results (Fig. 2-A) show that radioactivity in the discontinuous sucrose gradient was mainly associated with the fraction containing green band II. In the continuous sucrose gradient (Fig. 2-B), a green zone with diffuse edges was obtained and all radioactivity was associated with this zone.

In four independent experiments with discontinuous sucrose gradients, the major peak of radioactivity was associated with band II, and a minor peak (the radioactivity of which ranged from 10 to 60% of that of the major peak) was found at the interphase between the 45% and the 60% sucrose solution (band III). The distribution of the green material between band II and III was not correlated with the distribution of the radioactivity among these two bands.

Fig. 3 shows the results obtained with comparable polymerase preparations made from either healthy leaves or leaves from 3-day BMV-infected barley. After centrifugation of the pulse-labeled preparations in a discontinuous sucrose gradient, no radioactivity was obtained with the extracts from healthy leaves, while the pulse-labeled extract from infected leaves contained radioactivity associated with band II. Thus, radioactivity associated with the latter band was entirely specific for the BMV-infected material.

Dissociation of the pulse-labeled product from the 10,000 g pellet.—The standard RNA polymerase preparation from leaves infected for 3 days with BMV was incubated for 2 min with [³H]-UTP. The reaction was terminated by rapid cooling and addition of EDTA and unlabeled UTP. After centrifugation for 10 min at 10,000 g, the pellets were resuspended in 0.5 ml of extraction buffer and treated for 5 min at 20 C with various detergents. After cooling, the material was centrifuged for 10 min at 10,000 g and the acid-insoluble radioactivity of the pellet and of the supernatant was determined. Results appear in Table 2. With control samples or

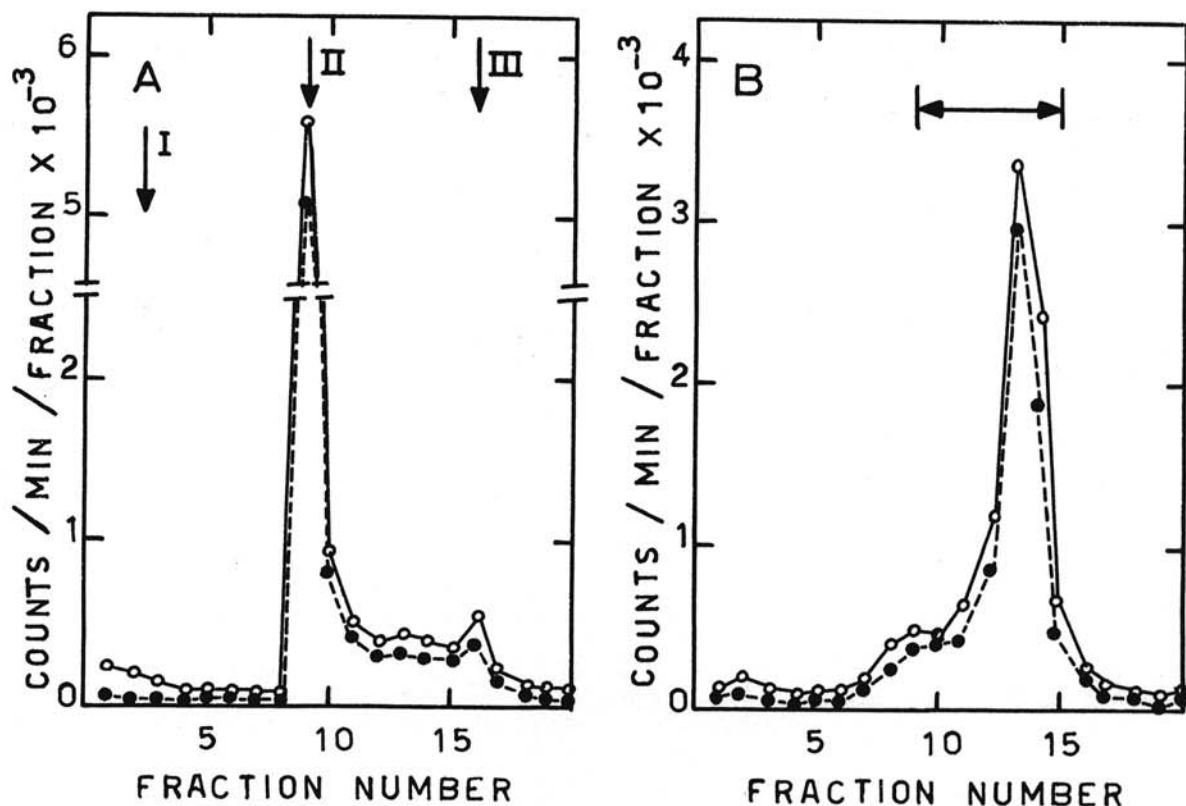


Fig. 2-A, B. Distribution of the standard RNA polymerase preparation from BMV-infected barley leaves. The standard RNA polymerase preparation was incubated for 2 min in the presence of [³H]-UTP and actinomycin D, and then centrifuged for 30 min at 35,000 rpm through a discontinuous or a continuous sucrose gradient. Fractions were collected from the top of the gradient and tested for radioactivity; only the upper 20 fractions of each gradient were analysed. Results are expressed as acid-insoluble radioactivity (cpm) incorporated in the RNA. A) Discontinuous sucrose gradient (made of 4 ml of 45% sucrose, 3 ml of 25% sucrose, and 1 ml of sample without sucrose, respectively). Arrows indicate the bands formed at the interphase: white band I (0 - 25% sucrose), green band II (25 - 45% sucrose), green band III (45 and 60% sucrose). B) Continuous sucrose gradient (made of 1 ml of 60% sucrose, 11 ml of continuous 25-60% sucrose, and 1 ml of sample without sucrose). Arrows delineate the zone containing the green pigments. ○—○ total acid-insoluble radioactivity (cpm) in the RNA; ●—● acid-insoluble radioactivity (cpm) after incubation of 30 min at 37 C with RNase (5 μg/ml) in 2XSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0).

with samples treated with 0.5% Lubrol, the pulse-labeled product remained firmly bound through the whole procedure to material sedimenting at 10,000 g. In samples treated with 0.5% Triton X-100 or with 2% Nonidet P40, the green pigment dissociated entirely from the 10,000 g pellet, together with about 50% of the original radioactivity. In samples treated with 0.5% sodium deoxycholate, the major part of the radioactive product was released in the 10,000 g supernatant, while most of the green pigments remained bound to the 10,000 g pellet.

Electron microscopy of the interphase materials.—Electron microscopy of the content of Band I revealed essentially mitochondrial material. Band II contained membrane material, disrupted chloroplast grana and lamellae, and various vesicular bodies (Fig. 4). No consistent difference could be seen between the content of band II from either healthy or BMV-infected leaves. The material of band III appeared similar to that of band II, except for the

larger size of the various membranous and vesicular components in band III.

DISCUSSION.—The analysis of the sub-cellular structures of the standard RNA polymerase preparation involved in the synthesis of virus-specific RNA by virtue of an RNA polymerase associated with an RNA template, has been performed in two different ways: (i) by tracing the virus-specific, AMD-resistant RNA polymerase activity, and (ii) by tracing its labeled double-stranded newly-made product.

As shown in Table 1, the pulse-labeled, double-stranded RNA product of the reaction is associated with material sedimenting at 10,000 g while the pulse-chased RNA is partly released into the 10,000 g supernatant as a product which is now essentially RNase-sensitive in 2XSSC. These results are consistent with others (5), which showed that part of the deproteinized RNA product synthesized by the pulse-chased standard RNA polymerase

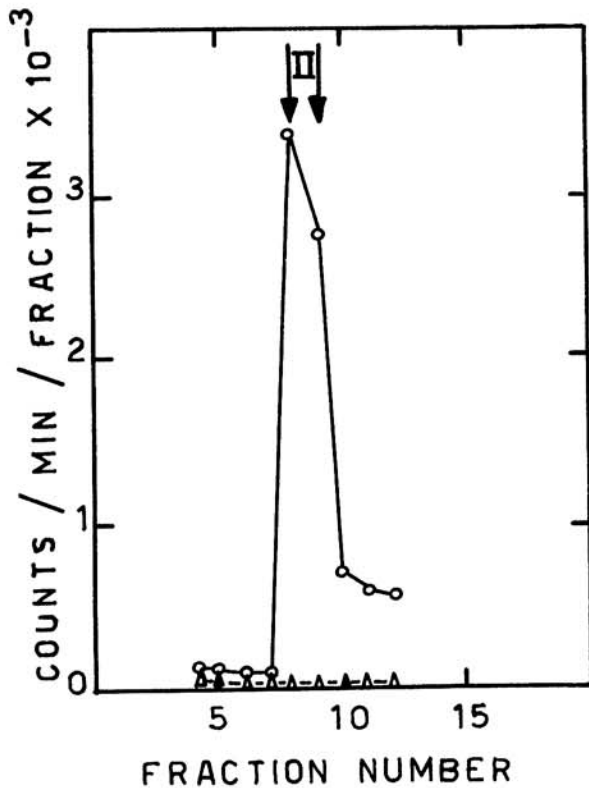


Fig. 3. Distribution of the standard RNA polymerase preparation from either healthy or BMV-infected barley leaves. The standard RNA polymerase preparation was incubated for 2 min in the presence of [3 H]-UTP and actinomycin D, and was then centrifuged for 30 min at 35,000 rpm through a discontinuous sucrose gradient (made of 4 ml of 60% sucrose, 4 ml of 45% sucrose, 3 ml of 25% sucrose, and 1 ml of sample without sucrose, respectively). Fractions were collected from the top of the gradient and tested for radioactivity; only nine fractions, comprising the 25 - 45% interphase (green band II), were analysed. Results are expressed as acid-insoluble radioactivity (cpm) incorporated in the RNA.

- total acid-insoluble radioactivity (cpm) in the RNA, for a preparation from BMV-infected barley leaves.
 ▲—▲ total acid-insoluble radioactivity (cpm) in the RNA, for a preparation from healthy barley leaves.

preparation migrated in the region of single-stranded BMV-RNA when submitted to electrophoresis in polyacrylamide gels.

Further fractionation of the standard RNA polymerase preparation in a discontinuous sucrose gradient showed that the RNA polymerase activity responsible for the *in vitro* synthesis of virus-specific double-stranded RNA was not linked to intact mitochondria.

Most of the BMV-specific RNA polymerase activity and pulse-labeled RNA products were found at the interphase between the 25% and the 45% sucrose components of the discontinuous sucrose gradient. The fact that both activity and products

TABLE 2. Effect of treatment with detergents on the sedimentation properties of the pulse-labeled, particle-bound products synthesized by the standard RNA polymerase preparation from bromegrass mosaic virus-infected barley leaves^a

Detergent	Pellet		Supernatant	
	no RNase	RNase ^b	no RNase	RNase ^b
none	3,134	2,837	182	111
0.5% Triton X-100	1,240	1,094	1,311	1,230
2.0% Nonidet P40	1,305	1,216	1,469	1,256
0.5% Na deoxycholate	600	419	2,923	2,492
0.5% Lubrol	2,988	2,797	469	467

^aThe RNA of the standard RNA polymerase preparation was pulse-labeled for 2 min. The reaction was stopped and the preparation was centrifuged for 10 min at 10,000 *g*. Supernatants were discarded and pellets were resuspended in extraction buffer containing detergents as indicated, and incubated for 5 min at 20 C. After centrifugation for 10 min at 10,000 *g*, the RNA was extracted separately from the pellet and the supernatant. Results are expressed as acid-insoluble counts per min per tube in the RNA fraction.

^bThe deproteinized RNA was resuspended in 2XSSC (0.3 M NaCl + 0.3 M sodium citrate, pH 7.0) and incubated for 30 min at 37 C with RNase (5 μ g/ml).

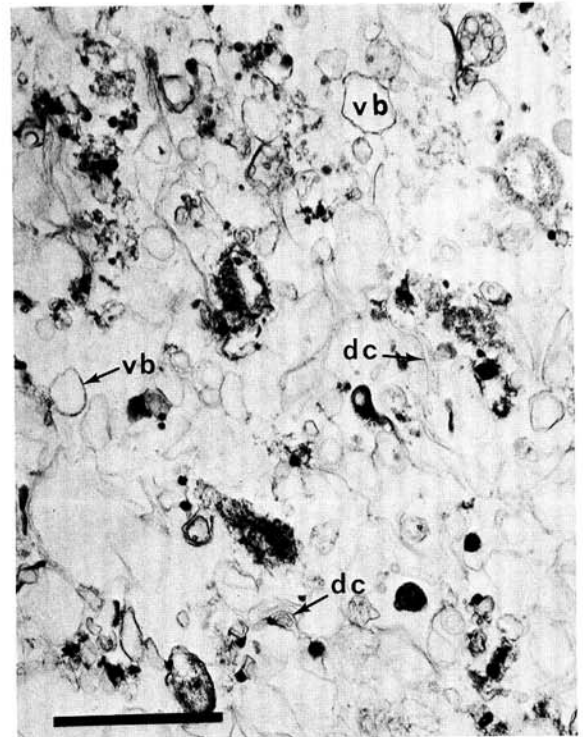


Fig. 4. Ultrathin section of the material of the 25 - 45% sucrose interphase (band II) obtained after centrifugation of the standard RNA polymerase preparation from BMV-infected leaves, for 30 min at 35,000 rpm through a discontinuous sucrose gradient. Pellets obtained by centrifugation of the interphase material were fixed, postfixed, and embedded. Ultrathin sections were stained with lead ($\times 24,000$). dc = disrupted chloroplasts. vb = vesicular bodies.

were found in the same fraction suggests that the pulse-labeled products remained bound to the template-enzyme complex.

The active RNA polymerase fraction contained chloroplast debris and various membranous and vesicular structures, but no intact organelles; no virus-specific sub-cellular structures were found in this fraction, when compared to the similar fraction prepared from healthy leaves.

With turnip yellow mosaic virus, specific vesicles with a double membrane, originating from chloroplasts, were identified in the leaf fraction which synthesized virus-specific double-stranded RNA (1); such structures were not observed in our preparations. In other papers dealing with the synthesis of virus-specific double-stranded RNA, the contents of the active fractions were related to the presence of mitochondria or light-weight chloroplasts (2, 6).

In the case of BMV, there are indications that the bulk of the chloroplast debris which bears the green pigments does not follow the fate of the pulse-labeled RNA product upon centrifugation through a discontinuous sucrose gradient. Indeed, the major part of the radioactivity was always found in band II, even when most of the pigments were found in band III. Also, upon treatment with sodium deoxycholate, the major part of the radioactivity was dissociated from the 10,000 g pellet, while the bulk of the green material remained sedimentable at 10,000 g.

It remains to be seen whether the virus-specific RNA polymerase in extracts of BMV-infected leaves is linked to a special class of chloroplast debris, or is associated with a non-chloroplastic structure. Hopefully, it will be possible to modify the fractionation procedure in order to solve this problem.

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