Isolation and Characterization of Oat Blue Dwarf Virus Ribonucleic Acid

D. R. Pring, R. J. Zeyen, and E. E. Banttari

Postdoctoral Fellow, Department of Plant Pathology, University of Nebraska, Lincoln 68503; and Research Associate and Associate Professor, respectively, Department of Plant Pathology, University of Minnesota, St. Paul 55101. Present address of Senior author: USDA, Plant Virus Laboratory, University of Florida, Gainesville 32601.

Journal Series Paper No. 3367, Nebraska Agricultural Experiment Station, Lincoln, and Scientific Journal Series Paper No. 7957, Minnesota Agricultural Experiment Station, St. Paul. Supported in part by the Plant Science Research Division, ARS, USDA, Cooperative Agreement 12-14-100-10443 (34).

Mention of a trademark name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA, nor does it imply its approval to the exclusion of other products that may also be suitable.

Accepted for publication 3 October 1972.

ABSTRACT

Purified preparations of oat blue dwarf virus (OBDV) yielded a single virus zone with a sedimentation coefficient (S) of 119 S, using brome mosaic virus and southern bean mosaic virus as sedimentation markers. Material collected from the zone was infectious when injected into *Macrosteles fascifrons*, (Stal), the vector of OBDV. Viral RNA was released from the particles in each of five bentonite-containing buffers at pH 9.0, and yielded single-stranded RNA with a sedimentation coefficient of 31.9 S. The viral RNA was alkali-labile, susceptible to ribonuclease (0.1 μg/ml) degradation, and was resistant to deoxyribonuclease (50 μg/ml). Formaldehyde-treated OBDV-RNA sedimented at 21.1 S, and the molecular weight of the RNA was estimated to be 2.13×10^6 daltons.

Phytopathology 63:393-396

The oat blue dwarf virus (OBDV) is a spherical virus (28-30 nm) (2) which is obligately transmitted by the aster leafhopper (*Macrosteles fascifrons* Stål) (1, 3, 15). In plant hosts, the virus is phloem-limited, of low titer (4, 22), and has a wide host range (22). Procedures useful in the purification of the virus have been published (2, 21). We report here further characterization of the virus particle and the nonphenol preparation and characterization of its associated nucleic acid.

MATERIALS AND METHODS.—*Virus purification and preparation.*—Oat blue dwarf virus was propagated in oat plants (*Avena sativa* L. 'Rodney') and purified by use of a cellulose column chromatography system (21), combined with sucrose density-gradient centrifugation, as previously described by Banttari & Zeyen (2). Purified preparations of the virus were filtered through a Millipore filter (Millipore Corp., Bedford, Mass.), and portions of the preparations were injected into virus-free leafhoppers and assayed for infectivity (3). The remainder of the filtered OBDV preparations were then shipped by air to Lincoln, Nebr., and were received 24-36 hr later.

Infectivity assays.—Portions of the purified-filtered preparations were injected into 100 virus-free leafhoppers, and these were placed, two/plant, on 50 oat seedlings. After 8 days, the surviving insects were placed on 35 seedlings and allowed to feed for 8 more days.

Determination of sedimentation coefficient (S) of OBDV.—The OBDV preparations were analyzed on linear-log sucrose density gradients designed for the Beckman (Beckman Instruments, Inc., Palo Alto, Calif.) SW 41 rotor (6). Brome mosaic virus (BMV) and southern bean mosaic virus (SBMV) were used as S value markers. Values of 79 and 115 S, respectively, were assumed for the marker viruses (6). The gradients were centrifuged at 14 C for 45 min at 259,000 *g* (39,000 rpm) in a Beckman L265B ultracentrifuge before fractionation with an ISCO (Instrumentation Specialties Co., Lincoln, Nebr.) UA-2 or UA-4 ultraviolet analyzer coupled to a Model D fractionator and an external recorder. Sedimentation coefficients were determined by the plotting of log depth against log S value for the marker virus species.

Preparation of OBDV nucleic acid.—Approximately 0.25 OD_{260} units of OBDV were placed in a total of 0.5 ml of each of five buffer systems containing 100-500 μg of EDTA-treated bentonite (10) per ml. The buffer systems included those useful in the nonphenol preparation of BMV-RNA (0.02 M Tris [tris (hydroxymethyl) amino methane], pH 9.0, with 0.1 M NaCl) (7), tobacco mosaic virus (TMV) RNA (0.02 M Tris-HCl, pH 9.0, with 0.001 M Na_2-EDTA) (7), wheat streak mosaic virus (WSMV) and maize dwarf mosaic virus (MDMV) RNA (0.1 M ammonium carbonate, pH 9.0, with 0.001 M Na_2-EDTA and 1% sodium dodecyl sulfate) (7, 19). In addition, OBDV preparations were also placed in 0.25 M sodium orthoborate, pH 9.0, with 0.001 M Na_2-EDTA and 1% N-lauroyl sarcosine, and in 0.02 M Tris-HCl, pH 9.0, containing 0.001 M Na_2-EDTA and 0.1 M NaCl. The solutions were allowed to incubate 12-24 hr at 4 C prior to layering on linear-log sucrose density gradients. Marker RNA species consisted of TMV-RNA and BMV-RNA and were prepared as previously described (7). Values of 13.8, 20.5, and 25.3 S were assumed for the sedimentation coefficients of BMV-RNA, whereas a value of 31.1 S was assumed for TMV-RNA (7). Gradients for nucleic acids were made in 0.15 M sodium chloride, 0.015 M sodium citrate (SSC), pH 7.0.

For enzyme assays with OBDV nucleic acids,

aliquots of the virus-dissociation solution (12-hr incubation) were dialyzed against 1 liter of SSC for 24 hr. The preparation was then centrifuged for 20 min at 8,700 *g* to sediment the bentonite. The supernatant fluid was used for enzyme treatments. Ribonuclease (EC 2.7.7.16, Sigma I-A, Sigma Chem. Co., St. Louis, Mo.) was added to 0.1 μg/ml, or deoxyribonuclease (EC 3.1.4.5, deoxyribonuclease I, DPFF, Worthington Biochemical Corp., Freehold, N. J.) to 50 μg/ml, 0.001 M $MgCl_2$. The solutions were incubated 30 min at 37 C and 10 min at 25 C, respectively, prior to density-gradient centrifugation.

Aliquots of the virus-deproteinization solution were exposed to formaldehyde to determine the extent of secondary structure and to expose hydrogen-bonded segments of the OBDV nucleic acid. The virus was allowed to incubate in the dissociation buffer for 12 hr at 4 C. The preparation was then dialyzed against 250 ml of 1.1 M formaldehyde, 0.001 M Na_2-EDTA, pH 7.0, for 8-15 hr at 40 C (7) prior to density-gradient centrifugation. Aliquots of the virus-dissociation solution were also exposed to 0.1 N NaOH, 30 min at 25 C, to determine alkaline resistance of OBDV nucleic acid.

RESULTS.—*Infectivity assays.*—Two infectivity assays were attempted; data from the initial assay were discarded due to excessive insect mortality. In the second assay, 33 of 85 plants exposed became infected with OBDV, indicating that the virus particles injected into the insects were infective.

Sedimentation of OBDV.—Oat blue dwarf virus particles sedimented as a single zone (Fig. 1, line c) in linear-log sucrose density gradients made in 0.05 M neutral phosphate. Southern bean mosaic virus (Fig. 1, line b) sedimented slightly slower than OBDV, whereas BMV sedimented substantially slower than

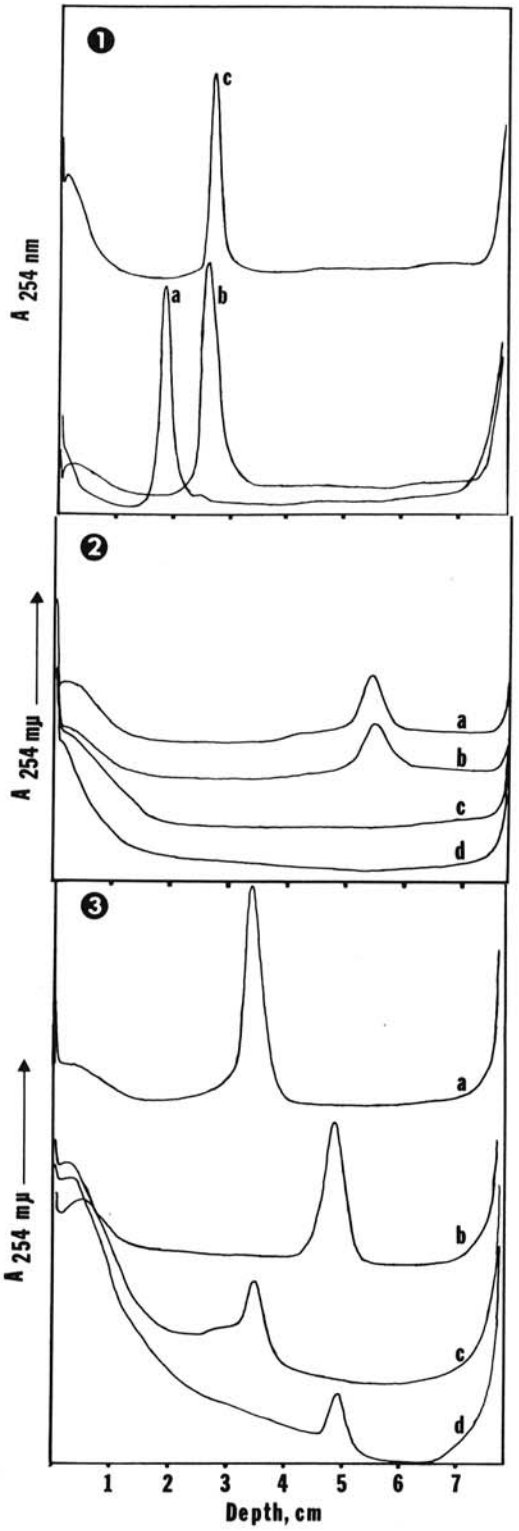

Fig. 1-3. 1) Photometric scanning patterns from linear-log sucrose density-gradient centrifugation of (a) brome mosaic virus; (b) southern bean mosaic virus; and (c) oat blue dwarf virus. The gradients were run in a Beckman SW-41 rotor for 45 min at 259,000 *g* (39,000 rpm) at 14 C, and the rotor was allowed to come to rest without braking before fractionation. **2)** Photometric scanning patterns from linear-log sucrose density-gradient centrifugation of (a) RNA prepared from oat blue dwarf virus (OBDV); (b) OBDV-RNA exposed to 50 μg deoxyribonuclease/ml; (c) OBDV-RNA exposed to 0.1 μg ribonuclease/ml; and (d) OBDV-RNA exposed to 0.1 N NaOH. The gradients were run in a Beckman SW-41 rotor for 8.0 hr at 259,000 *g* (39,000 rpm) at 14 C, and the rotor was allowed to come to rest without braking prior to fractionation. **3)** Photometric scanning patterns from linear-log sucrose density-gradient centrifugation of (a) tobacco mosaic virus (TMV) RNA following formaldehyde denaturation; (b) native TMV-RNA; (c) oat blue dwarf virus (OBDV) RNA following formaldehyde denaturation; and (d) native OBDV-RNA. The OBDV-RNA preparations were scanned with a range of 0-0.2 optical density, whereas 0-0.5 range was used for TMV-RNA. The gradients were run in a Beckman SW-41 rotor at 259,000 *g* (39,000 rpm) 7.0 hr at 14 C, and the rotor was allowed to come to rest without braking prior to fractionation.

either SBMV or OBDV (Fig. 1, line a). The sedimentation coefficient of OBDV was estimated to be 119 S. In linear-log gradients made in 0.01 M sodium acetate, pH 6.0, OBDV sedimented at about the same rate as did papaya mosaic virus, which exhibits a sedimentation coefficient of 118 S (11). Some contamination of the OBDV preparation (Fig. 1) was apparent as ultraviolet-absorbing material near the meniscus.

Properties of OBDV nucleic acid.—Purified OBDV, incubated in each of the five buffer systems, yielded a single species of nucleic acid (Fig. 2, line a). The size and shape of the zone, and the amount of apparent viral protein near the meniscus, was the same for each buffer system. Eleven trials with OBDV nucleic acid in SSC yielded a mean sedimentation coefficient of 31.9 S with a range of 30.9-32.4 S. The TMV-RNA buffer system (0.02 M Tris-HCl, pH 9.0, 0.001 M Na_2-EDTA) was routinely used in further experiments.

Exposure of the dialyzed preparation to 50 μg deoxyribonuclease/ml (30 min at 25 C), 0.001 M $MgCl_2$, had no effect on the OBDV nucleic acid (Fig. 2, line b), whereas exposure to 0.1 μg ribonuclease/ml (30 min at 37 C) (Fig. 2, line c) or to 0.1 N NaOH (30 min at 25 C) (Fig. 2, line d) resulted in degradation of the nucleic acid. The above experiments show the nucleic acid of OBDV to be RNA.

Formaldehyde denaturation of TMV-RNA and OBDV-RNA substantially reduced the sedimentation of both RNA species. Native (Fig. 3, line b) or formaldehyde-denatured (Fig. 3, line a) TMV-RNA yielded a single zone, whereas evidence of a smaller RNA or of degradation of a portion of OBDV-RNA was apparent after formaldehyde denaturation (Fig. 3, line c), but not before (Fig. 3, line d). The shoulder OBDV-RNA species sedimented at about 17 S after formaldehyde treatment. Some of the scanning patterns of native OBDV-RNA preparations also showed a slight baseline shift (Fig. 2, line a) in about the 24-S region. The irregular appearance of slower-sedimenting RNA zones after formaldehyde denaturation has also been observed with other viral RNA preparations (TMV-RNA, barley stripe mosaic virus RNA) when stored in dissociation buffer for several weeks. It may be suggested that some of the OBDV preparations were partially degraded during transit. Alternatively, the baseline shifts associated with these preparations may in fact represent the presence of more than one RNA species associated with the particles.

The mean sedimentation coefficient of formaldehyde-treated OBDV-RNA (five trials) was 21.1 S, with a range of 20.2 to 21.9 S. Substitution of the 21.1 S-value into the formula of Brakke & Van Pelt, $S = 0.083\ M^{0.38}$ (7) gives an estimated molecular weight of 2.13×10^6 daltons.

DISCUSSION.—These experiments show that OBDV displays a sedimentation coefficient of about 119 S in 0.05 M neutral phosphate or in 0.01 M sodium acetate, pH 6.0. Several other spherical viruses of similar size exhibit similar sedimentation properties: barley yellow dwarf virus (BYDV) (115-118 S) (20), southern bean mosaic virus (SBMV) (115 S) (16), and tobacco necrosis virus (TNV) (118 S) (13). Estimates of molecular weight for two of these viruses range from 6.6×10^6 daltons for SBMV (16) to 7×10^6 daltons for TNV (13). The RNA prepared from OBDV sedimented slightly faster than did TMV-RNA, before or after formaldehyde denaturation. The estimated molecular weight of OBDV-RNA was 2.13×10^6 daltons, or higher than estimates of SBMV-RNA (1.4×10^6 daltons) (8) or of TNV-RNA ($1.3\text{-}1.6 \times 10^6$ daltons) (13). Ultraviolet spectral analyses of the OBDV preparations suggested a 260:280 ratio of 1.50-1.70, but the preparations contained some extraneous protein. Thus, it was not possible to accurately estimate the percentage nucleic acid of the particle, but the high molecular weight of the RNA may suggest that it comprises a considerable portion of the mass of the sphere. The 260:280 ratio of purified OBDV was previously determined to be 1.63 (2).

Oat blue dwarf virus and BYDV share several common characteristics, even though they do not share a common insect vector. Both viruses are phloem-limited (4, 12, 23), have similar sedimentation coefficients, and are circulative, if not propagative, in their respective vectors (3, 17). The diameter of the particles is similar (2, 20), although Jensen (12) reported the BYDV sphere to be 24 nm in diam when viewed in situ. The molecular weight of BYDV nucleic acid also appears to be similar to that of OBDV-RNA (M. K. Brakke, *unpublished data*). Information concerning other insect vectored phloem-restricted viruses is unavailable.

The sedimentation properties of OBDV-RNA and TMV-RNA before and after formaldehyde denaturation indicate that the RNA species have a similar degree of secondary structure, in that both RNA species exhibited about a 34% reduction in sedimentation coefficient upon denaturation. The percentage reduction of $S_{20,w}$ values of other formaldehyde-treated viral RNA species appears to be somewhat associated with molecular weight estimate: WSMV-RNA (2.8×10^6 daltons) (7) and MDMV-RNA (2.7×10^6 daltons) (19) exhibited about a 41% reduction in $S_{10,w}$ value, whereas the $S_{20,w}$ value of the similarly treated small component of BMV-RNA (3×10^5 daltons) was reduced only 25% (7). That variation in conformation of plant viral RNA species exists, however, is demonstrated by the 29% reduction in sedimentation coefficient of formaldehyde-treated barley stripe mosaic virus RNA (18), whereas the $S_{20,w}$ value of the large component of similarly treated BMV-RNA was reduced about 38% (7). In this regard, it is relevant that the apparent difference in molecular weights for these RNA species is only about $1\text{-}2 \times 10^5$ daltons (14, 18).

The ease with which OBDV-RNA is released from the virions is striking. The only common components of the buffer systems described above is the pH (9.0), the presence of EDTA-treated bentonite, and the inclusion of either 0.001 M Na_2-EDTA or 0.10 M NaCl. No attempts were made with only a pH 9.0

buffer and bentonite. The bentonite preparation procedure includes two washings with 0.01 sodium acetate, pH 6.0, after the bentonite is exposed to 0.10 M Na_2-EDTA. The EDTA concentration remaining associated with the bentonite is unknown, but only 0.01 ml was added to the final 0.50 ml volume of virus. Thus, the concentration of EDTA contributed by the bentonite is probably low. The addition of either sodium dodecyl sulfate or N-lauroyl sarcosine to the virus preparation was without effect. The addition of one or more of the above components (detergents, chelating agents, or salts) has been found necessary for the complete liberation of BMV-RNA (5), WSMV-RNA (7), and MDMV-RNA (19). The deproteinization of OBDV, however, is easily accomplished at pH 9.0 in a variety of buffer systems, independent of ionic strength. The OBDV virus particle may be an interesting model for studies on dissociation and reassociation of virus components.

Unequivocal proof of the nature of the RNA described herein must by definition include the demonstration of infectivity. In the absence of such data, only indirect evidence supports the assumption that the RNA released by the single-sedimenting OBDV virus zone is indeed the entire RNA genome. The formaldehyde-induced heterogeneity of OBDV-RNA may in fact represent the presence of more than one species. Small differences in molecular weight of RNA species may not be detected in density-gradient centrifugation patterns of native or denatured nucleic acids. Formaldehyde-denatured large components of BMV-RNA (25.3 S, 20.5 S) were not resolved in linear-log density gradients (7), even though the molecular weight of the components differ by about 3×10^5 daltons. The complexity of apparent homogenous RNA species, as recently demonstrated by polyacrylamide gel electrophoresis (9, 14), would suggest that this procedure may be very useful in the re-examination of viral nucleic acids. The formaldehyde-induced heterogeneity of OBDV-RNA and WSMV-RNA (7), as well as the heterogeneity of native MDMV-RNA compared to other viral RNA species (19), may indicate a more complex nature than was originally hypothesized.

LITERATURE CITED

1. BANTTARI, E. E., & M. B. MOORE. 1962. Virus cause of blue dwarf of oats and its transmission to barley and flax. Phytopathology 52:897-902.
2. BANTTARI, E. E., & R. J. ZEYEN. 1969. Chromatographic purification of the oat blue dwarf virus. Phytopathology 59:183-186.
3. BANTTARI, E. E., & R. J. ZEYEN. 1970. Transmission of oat blue dwarf virus by the aster leafhopper following natural acquisition or inoculation. Phytopathology 60:399-402.
4. BANTTARI, E. E., & R. J. ZEYEN. 1971. Histology and ultrastructure of flax crinkle. Phytopathology 61:1249-1252.
5. BRAKKE, M. K. 1971. Degradation of brome mosaic and tobacco mosaic viruses in bentonite. Virology 46:575-585.
6. BRAKKE, M. K., & NANCY VAN PELT. 1970. Linear-log sucrose gradients for estimating sedimentation coefficients of plant viruses and nucleic acids. Anal. Biochem. 38:56-64.
7. BRAKKE, M. K., & NANCY VAN PELT. 1970. Properties of infectious ribonucleic acid from wheat streak mosaic virus. Virology 42:699-706.
8. DIENER, T. O. 1965. Isolation of infectious ribonucleic acid from southern bean mosaic virus. Virology 27:425-429.
9. FOWLKS, E., & R. J. YOUNG. 1970. Detection of heterogeneity in plant viral RNA by polyacrylamide gel electrophoresis. Virology 42:548-550.
10. FRAENKEL-CONRAT, H., B. SINGER, & A. TSUGITA. 1961. Purification of viral RNA by means of bentonite. Virology 14:54-58.
11. HIEBERT, S. 1970. Some properties of papaya mosaic virus and its isolated constituents. Phytopathology 60:1295 (Abstr.).
12. JENSEN, S. L. 1969. Occurrence of virus particles in the phloem tissue of BYDV-infected barley. Virology 38:83-91.
13. KASSANIS, B. 1970. Tobacco necrosis virus. Descriptions of plant viruses, No. 14. Commonwealth Mycol. Inst., Ass. Applied Biol.
14. LANE, L. C., & P. KAESBERG. 1971. Multiple genetic components in bromegrass mosaic virus. Nature 232:40-43.
15. LONG, D. L., & R. G. TIMIAN. 1971. Acquisition through artificial membranes and transmission of oat blue dwarf virus by Macrosteles fascifrons. Phytopathology 61:1230-1232.
16. MILLER, G. L., & W. C. PRICE. 1946. Physical and chemical studies on southern bean mosaic virus. I. Size, shape, hydration and elementary composition. Arch. Biochem. Biophys. 10:467-477.
17. PALIWAL, Y. C., & R. C. SINHA. 1970. On the mechanism of persistence and distribution of barley yellow dwarf virus in an aphid vector. Virology 42:668-680.
18. PRING, D. R. 1972. Barley stripe mosaic virus replicative form RNA: preparation and characterization. Virology 48:22-29.
19. PRING, D. R., & W. G. LANGENBERG. 1972. Preparation and properties of maize dwarf mosaic virus ribonucleic acid. Phytopathology 62:253-255.
20. ROCHOW, W. F., & M. K. BRAKKE. 1964. Purification of barley yellow dwarf virus. Virology 24:310-322.
21. VENEKAMP, J. H., & W. H. M. MOSCH. 1964. Chromatographic purification of plant viruses on cellulose columns with polyethylene glycol containing solutions as solvents. Netherlands J. Plant Pathol. 70:85-89.
22. WESTDAL, P. H. 1968. Host range studies of oat blue dwarf virus. Can. J. Bot. 46:1431-1435.
23. ZEYEN, R. J., & E. E. BANTTARI. 1969. Electron and light microscopy of oat tissue infected with the oat blue dwarf virus. Phytopathology 59:1059 (Abstr.).