Physical and Chemical Properties of the Particles and Ribonucleic Acid of Blueberry Leaf Mottle Virus

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ABSTRACT

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The properties of blueberry leaf mottle virus (BBLMV), a serologically closely related isolate from grapevine in New York state (NY isolate), and the serologically distantly related grapevine Bulgarian latent virus (GBLV) from Europe were compared. Of these three isolates, only BBLMV infected highbush blueberry (cultivar Rubel) seedlings that were inoculated with purified virus. In linear-log sucrose density gradients, the middle (M) and bottom (B) components of both BBLMV and GBLV had estimated sedimentation coefficients of 120 S and 128 S, respectively. The M and B components of the NY isolate were not resolved and produced a shouldered peak that sedimented between 120 and 128 S. The proportion of M to B component was greater for BBLMV preparations while the converse was true for GBLV. When the M and B components of BBLMV were separated by sucrose density gradient centrifugation and inoculated separately to *Chenopodium quinoa*, they were one seventh as infectious as when the M and B inoculum components were used in combination. Combined M and B components of BBLMV had E_{max} = 260 nm, E_{min} = 240 nm, and $E_{260/280}$ = 1.69; top component (T) had $E_{260/280}$ = 0.94. Centrifugation of M and B components of BBLMV to near equilibrium in CsCl revealed a larger M-component peak and a smaller B-component peak at buoyant densities of 1.471 and 1.497 g/cm^3, respectively. GBLV was resolved into a sharp M-component peak which was twice as large as the sharp B-component peak, with buoyant densities of 1.475 and 1.492 g/cm^3, respectively. The CsCl gradient profile of the NY isolate was similar to that of BBLMV, but the peaks were broader and had buoyant densities of 1.470 and 1.488 g/cm^3, respectively. The molecular weight of the coat protein subunit for all three virus isolates was 54,000 in 5% SDS polyacrylamide gels. The nucleic acid of BBLMV was degraded by RNase, but not by DNase. The RNA was single stranded and exhibited a T_M = 60 C and 15.4% hyperchromicity when melted over a temperature range of 30–99 C. The RNA-1 and RNA-2 of all three virus isolates was resolved in 2.4% polyacrylamide gels, and had molecular weights of 2.35 and 2.15 × 10^6, respectively. Blueberry leaf mottle virus is distinct from its serologically distant relative, GBLV, and we propose that BBLMV be included as an independent member of the nepovirus group. The NY isolate is so closely related to BBLMV that it should be considered a closely related strain of BBLMV.

Additional key words: Vaccinium, nepovirus.

Recently blueberry leaf mottle was reported as a new virus disease of highbush blueberry, *Vaccinium corymbosum* L. (12). The causal agent is designated as a new virus and the name of blueberry leaf mottle virus (BBLMV) is proposed for this new member of the nepovirus group (12). Purified virus preparations separated into three components in sucrose density gradients: top (T), middle (M), and bottom (B), with sedimentation coefficients ($S_{20,w}$) of 53, 120, and 128 S, respectively. Negatively stained M and B virus components measured 28 nm in diameter. In agar gel diffusion tests, a distant serological relationship was detected between BBLMV and grapevine Bulgarian latent virus (GBLV) (12); with BBLMV antiserum, the homologous titer was 1/1,024 and the heterologous was 1/128; with GBLV antiserum, the homologous titer was 1/2,048 and the heterologous was 1/128. A close relationship was detected between BBLMV and a virus isolated from a grapevine in New York state (15). With BBLMV antiserum, the heterologous titer was 1/512; with the NY isolate antiserum the homologous titer was 1/1,024 and the heterologous titer was 1/512 when tested against BBLMV. In our preliminary report, the herbaceous host range for BBLMV, as determined by using sap from infected *Nicotiana clevelandii* as inoculum, was found to be broader than reported for GBLV or for the NY isolate (10,15). Some host responses to BBLMV infection differed from those published for the other two viruses. This report contains a detailed description of the physical and chemical properties of the particles of BBLMV compared with those for GBLV from Europe (GBLV) and the grapevine isolate from New York state (NY isolate).

MATERIALS AND METHODS

Virus sources. Blueberry leaf mottle virus originally was isolated from a diseased highbush blueberry (cultivar Rubel) bush from southwestern Michigan, and a local lesion isolate was maintained in *Chenopodium quinoa.* Isolates of GBLV and the NY isolate were kindly supplied by G. P. Martelli, Bari, Italy, and D. Gonsalves, Ithaca, NY, respectively, and were maintained as local lesion isolates in *C. quinoa.*

Purification of virus particles. *C. quinoa* was the source of materials for purification of all three isolates. *Nicotiana clevelandii* was tested as a propagative host and although yields were comparable to those from *C. quinoa,* the latter was chosen because of its rapid growth.

The method of purification used for both BBLMV and GBLV was as follows. All steps were performed at 0–4 C. *C. quinoa* leaves and stems were harvested 7–10 days after inoculation, homogenized in 2 ml/g cold 0.05 M boric acid-borax buffer containing 0.1% (w/v) each of sodium thioglycollate (TGA) and sodium diethyldithiocarbamate (DIECA), pH 7, and the sap was expressed and frozen. After being thawed at 4 C, the sap was centrifuged at 10,000 rpm for 15 min. Chloroform and *n*-butanol, (each at 10% [v/v]) were added to the supernatant and stirred for 1 hr. After low-speed centrifugation, the aqueous supernatant was collected and polyethylene glycol (mol wt 6,000 [PEG]) and NaCl were added to 8 and 1% (w/v), respectively. After the PEG dissolved, the mixture was centrifuged at low speed and the pellet was resuspended for several hours in 10% of the initial volume with 0.05 M tris-HCl buffer, pH 7.4. After an additional low-speed centrifugation, the supernatant was ultracentrifuged in a Beckman No. 40 rotor at 38,000 rpm for 90 min. The pellet was resuspended overnight in tris-HCl buffer and then centrifuged through 5–30%

linear or linear-log (2) sucrose density gradients made in tris-HCl buffer. Sucrose density gradients (SDG) were centrifuged in a Beckman SW 41 rotor at 38,000 RPM for 90 min. Gradients were scanned at 254 nm and fractionated with ISCO equipment (Instrumentation Specialities Inc., Lincoln, NE 68504).

An alternate method of purification was developed to obtain good yields of the NY isolate: the extraction buffer was 0.5 M boric acid-borax, pH 7, with 0.1% (w/v) each of the TGA and DIECA. The expressed sap was frozen overnight and after being thawed at 4 C, it was centrifuged at low speed. Ammonium sulfate was added to the supernatant at 20% (w/v) and stirred for 6 hr. After low-speed centrifugation, the supernatant was ultracentrifuged in a Beckman No. 30 rotor at 27,000 rpm for 2.5 hr. The presence of 20% ammonium sulfate apparently interfered with virus precipitation by PEG, since no virus was recovered when these treatments were used in combination. The pellet was resuspended overnight in 0.05 M tris-HCl buffer, pH 7.4. After low-speed centrifugation, the supernatant was centrifuged through sucrose density gradients as previously described.

Inoculation of blueberry seedlings with the three virus isolates. To determine if GBLV and the NY isolate could infect blueberries, purified preparations of these two isolates were adjusted to a concentration of 1.0 A_{260}/ml in 0.05 M tris-HCl buffer and each of the virus isolates was rub-inoculated to the leaves and roots of 35 Rubel seedlings. Seedlings were dusted with 22-μm (600-mesh) carborundum prior to inoculation. Control plants were inoculated with buffer or with BBLMV.

Separation of BBLMV components and infectivity enhancement. Purified BBLMV was centrifuged through three successive 5–30% sucrose density gradients to separate M and B components for infectivity enhancement tests. The T component could be collected from the first SDG run only. The separated components were adjusted to 7 μg/ml with 0.05 M tris-HCl buffer, pH 7.4, assuming $E^{0.1\%}_{260\ nm} = 1.0$ for the T component and $E^{0.1\%}_{260\ nm} = 10$ for M and B components. Each component alone and in combination with the other components was used to rub-inoculate carborundum-dusted *C. quinoa* plants.

Determination of buoyant density. Particles of the three virus isolates were purified through SDG and dialyzed overnight against three changes of 0.05 M tris-HCl buffer, pH 7.4. Each preparation was adjusted to 0.3 A_{260}/ml; mixed with 4.5 ml of CsCl in 0.05 M tris buffer, pH 7.4, at an initial density of 1.564 g/cm^3; and centrifuged in a Beckman SW 39L rotor at 30,000 rpm for 42 hr at 4 C. The gradients were scanned at 254 nm and 0.25-ml fractions were collected by using ISCO equipment. The refractive index of each fraction was determined with a Bausch and Lomb Abbé 60 refractometer at 25 C and the values obtained were converted to density (4).

SDS-polyacrylamide gel electrophoresis of coat protein subunits. The molecular weight of the coat protein subunit was determined by SDS-polyacrylamide gel electrophoresis. Virus protein was prepared by diluting a 1 mg/ml suspension of purified virus 1:1 (v/v) with dissociation buffer (1% 2-mercaptoethanol, 4 M urea, and 1% SDS in 0.1 M sodium phosphate buffer, pH 7.2). Each sample was heated in a boiling water bath for 90 sec. Protein standards were bovine serum albumin, carbonic anhydrase, myoglobin, and ovalbumin. The standards and virus subunit proteins were applied separately to 3, 5, and 7.5% gels in 5-mm i.d. glass tubes and electrophoresed. Gels and tray buffer were made according to the method of Dunker and Ruekert (5).

Extraction of nucleic acid. Virus particles were purified through SDG, concentrated from the sucrose by ultracentrifugation, and resuspended in 0.05 M tris-HCl buffer, pH 7.4. A mixture of 1 ml of virus preparation (2 mg/ml) and 1 ml of dissociation buffer (0.05 M tris-HCl, 1% SDS, 0.025 M EDTA, 0.15 M NaCl, pH 9) was incubated at 50 C for 30 min. The treated sample was divided into six 0.33-ml aliquots and layered onto linear-log sucrose density gradients made with RNase-free sucrose (Schwarz-Mann Co., Inc., Orangeburg, NY 10962) in 1 × SSC buffer, pH 7, containing 6 μg/ml purified bentonite (6). After centrifugation in an SW 41 rotor at 38,000 rpm for 5 hr at 4–6 C, the gradients were scanned at 254 nm and fractionated with ISCO equipment. The single nucleic acid peak was collected. It was not possible to resolve two RNA peaks in sucrose density gradients. Sodium acetate was added to 0.15 M and the nucleic acid was precipitated with two volumes of 95% ethanol. The mixture was allowed to stand at −20 C overnight. The nucleic acid was collected by low-speed centrifugation at 10,000 rpm for 10 min at −20 C, resuspended in 1 × SSC buffer, and used immediately or stored at −20 C.

Effects of nucleases on BBLMV nucleic acid. Two 0.15-ml volumes of BBLMV nucleic acid (about 30 μg) were mixed with 6 μg/ml of either RNase or DNase (both isolated from bovine pancrease; Sigma, St. Louis, MO 63178) in 1 × SSC buffer, pH 7, and incubated at room temperature for 30 min. A control sample was incubated with a corresponding amount of 1 × SSC buffer. The samples were layered onto linear-log sucrose density gradients made in 1 × SSC buffer, pH 7, with 6 μg/ml purified bentonite. After centrifugation at 38,000 rpm in an SW 41 rotor for 5 hr at 4–6 C, the gradients were scanned at 254 nm and fractionated.

Thermal denaturation of BBLMV nucleic acid. The BBLMV nucleic acid was scanned from 220 to 340 nm in a Gilford Model

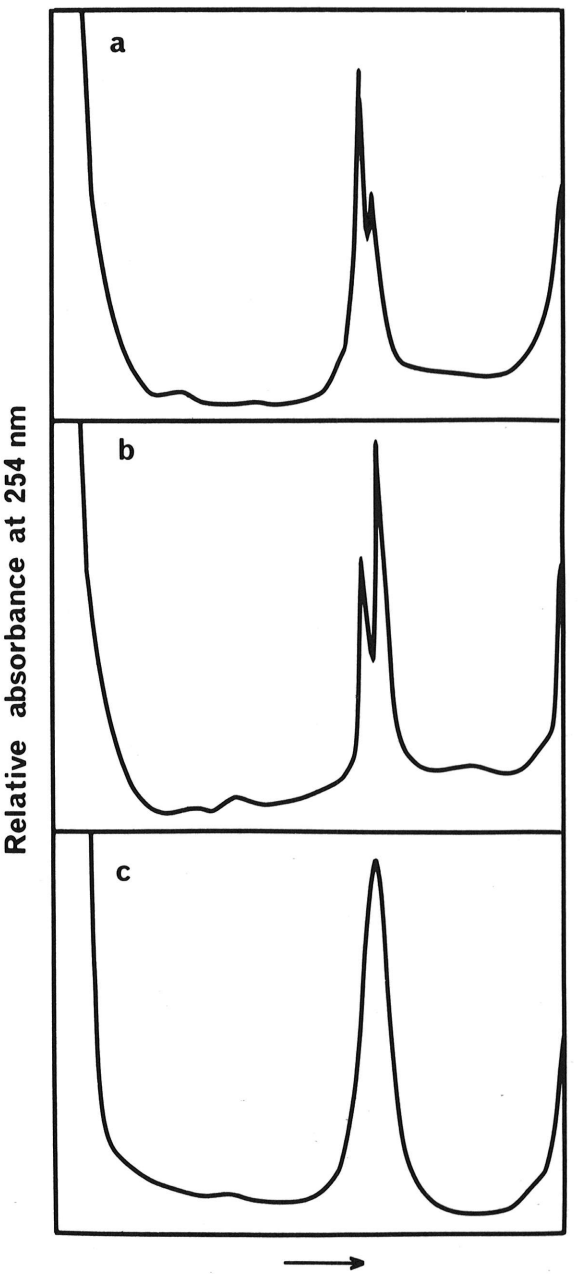

Fig. 1. Linear-log sucrose gradient absorbance profiles of: **a,** blueberry leaf mottle virus; **b,** grapevine Bulgarian latent virus (European isolate); and **c,** grapevine Bulgarian latent virus (New York state isolate).

250 UV-Vis wavelength scanner (Gilford Laboratory Instruments, Oberlin, OH 44074) to determine its purity. Viral RNA in 1 × SSC buffer was placed in a thermal cuvette and double-stranded DNA of *Micrococcus lysodeikticus* in 1 × SSC buffer was placed in the adjacent thermal cuvette. The thermoprogrammer (Model 2527) on the Gilford Model 250 spectrophotometer was set at gradient of 1 C/min from 30 to 99 C and the absorbance at 260 nm was plotted at 1-min intervals.

Polyacrylamide gel electrophoresis of nucleic acid. The molecular weight of the nucleic acid of each isolate was estimated by reference to the RNA(s) of cherry leaf roll (mol wt 2.4 and 2.1 × 10^6 daltons), brome mosaic (mol wt 1.1, 1.0, 0.7, and 0.3 × 10^6 daltons), southern bean mosaic (mol wt 1.4 × 10^6 daltons), and tobacco mosaic viruses (mol wt 2.05 × 10^6 daltons). Purified virus was degraded by adding an equal volume of dissociation buffer (0.05 M tris-HCl, 0.025 M EDTA, and 0.5 M Na Cl, pH 9). The mixtures, in samples of 50 to 80 μl, were layered onto 2.4% polyacrylamide gels (acrylamide:bis ratio, 20:1) cast in 5 mm i.d. glass tubes and run according to the method of Loening (9) without SDS in the gel or tray buffer. Electrophoresis was at 4 mA/gel and 70 V for 2.5 hr at room temperature. The gels were extruded and stained in 0.03% toluidine blue in distilled water, then destained in water. Migration of the nucleic acids was measured with a ruler or by scanning the gels at 565 nm in a Gilford Model 250 spectrophotometer equipped with a gel transport system.

Sedimentation coefficient of BBLMV nucleic acid under nondenaturing and denaturing conditions. *Nondenaturing conditions.* Suspensions of particles of blueberry leaf mottle, brome mosaic, cowpea mosaic, southern bean mosaic, and tobacco mosaic viruses at a concentration of 1 mg/ml were each added to an equal volume of dissociation buffer (0.05 M tris-HCl, 1% SDS, 0.025 M EDTA, 0.15 M NaCl, pH 9) and incubated for 30 min at 37 C. Each virus preparation was layered onto a linear-log sucrose density gradient containing RNase-free sucrose and 6 μg/ml purified bentonite. Gradients were centrifuged in an SW 41 rotor at 38,000 rpm for 5 hr at 4 C, then scanned at 254 nm and fractionated.

Denaturing conditions. One volume of each virus preparation was added to an equal volume of dissociation buffer (0.1 M ammonium carbonate, 1% SDS, 0.001 M EDTA, and 200 μg/ml purified bentonite) (13) and incubated for 16 hr at 4 C. Nucleic acid was formaldehyde treated according to the method of Boedtker (1). Each sample was layered onto a linear-log sucrose density gradient made with 1 × SSC buffer and 6 μg/ml purified bentonite. Gradients were centrifuged in an SW 41 rotor at 38,000 rpm for 5 hr at 4–6 C, scanned at 254 nm and fractionated. The molecular weight of BBLMV nucleic acid was calculated from the formula, $S = 0.083\ M^{0.38}$ (3).

TABLE 1. Infectivity of separated components of blueberry leaf mottle virus[a]

Inoculum	Local lesions on four *Chenopodium quinoa* plants[b]	
	Sum	Mean
T	0	0
M	16	1.0
B	20	1.3
M + B	119	7.4
T + M + B	122	7.6

[a] Nucleoprotein components were separated by three successive sucrose density gradients. Concentrations of all components were adjusted to 7 μg/ml in 0.05 Tris-HCl, pH 7.4.

[b] Four *Chenopodium quinoa* plants (four leaves per plant) were inoculated with each component or combination of components.

RESULTS

Purification. All three isolates were stable to freezing in *C. quinoa* sap, but slow thawing at 4 C was essential to obtain good yields. Freezing and slow thawing resulted in partial clarification of the extract while further clarification was obtained by treatment with 10% each (v/v) of chloroform and *n*-butanol. Precipitation of BBLMV and GBLV with 8% PEG and 1% NaCl gave yields comparable to those obtained by ultracentrifugation. The purification method of the NY isolate gave yields comparable to those of BBLMV and GBLV on the basis of absorbance scans (Fig. 1). Yields were about 0.5 mg virus per 100 gm of infected leaf material. All three methods resulted in a small amount of T component, which was collected and examined in the electron microscope. Negatively stained T-component particles were penetrated by 2% phosphotungstic acid; M and B component particles were not penetrated by the stain. The M and B components of BBLMV and GBLV were just resolved in SDG, with consistently more M than B component in purified BBLMV preparations and more B than M component in GBLV preparations. The M and B peaks of the NY-isolate preparations were not resolved. Ultraviolet absorbance scans of the combined M and B components of BBLMV showed $E_{max} = 260$ nm, $E_{min} = 240$ nm, and $E_{260/280} = 1.69$. Purified T component showed $E_{260/280} = 0.94$.

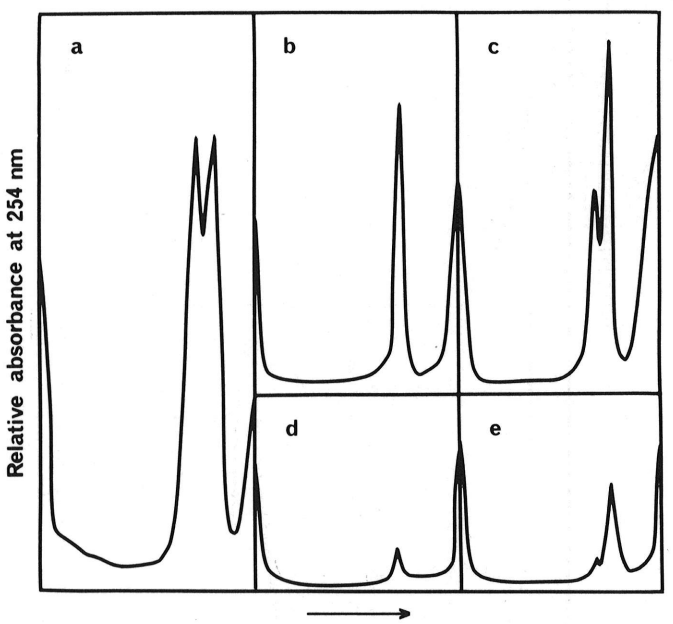

Fig. 2. Separation of blueberry leaf mottle virus by separate collection and passage of the middle (M) and bottom (B) components through three consecutive 5–30% linear-log sucrose gradient centrifugations. **a,** First gradient; **b,** middle (M) component separated after centrifugation of M in the second gradient; **c,** B contaminated with M after centrifugation of B in the second gradient; **d,** M component after centrifugation in the third gradient; and **e,** B with a trace of contaminating M after centrifugation in a third gradient.

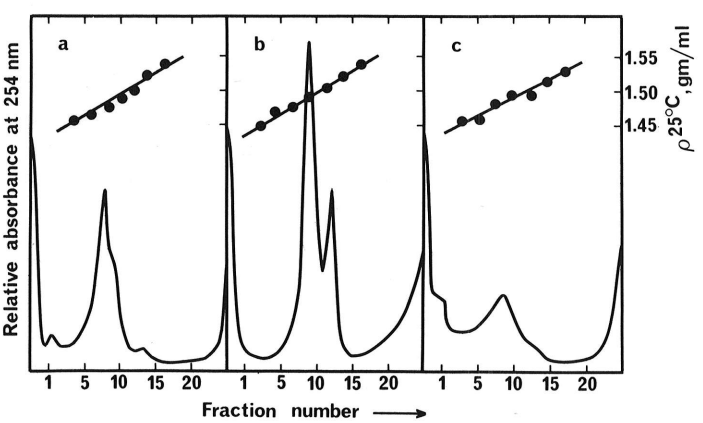

Fig. 3 Absorbance profiles and densities of CsCl gradients containing particles of: **a,** blueberry leaf mottle virus; **b,** grapevine Bulgarian latent virus (European isolate); and **c,** grapevine Bulgarian latent virus (New York state isolate).

Purified virus stored well in 0.05 M tris-HCl buffer, pH 7.4. Aliquots stored for 6 wk or longer at 4 C showed no evidence of aggregation or loss when assayed by SDG centrifugation.

Infection of blueberry seedlings by inoculation with the purified virus isolates. Indexing of rub-inoculated blueberry seedlings on *C. quinoa*, 4 mo after inoculation, revealed that 5/35, 0/35, and 0/35 seedlings were infected by BBLMV, GBLV, and NY isolate, respectively.

Separation of BBLMV nucleoprotein components and tests for infectivity enhancement. Three successive SDG separations of M and B components of BBLMV gave good separation with a concomitant loss of virus (Fig. 2). The T component was not infective (Table 1). The infectivity of separate inoculum preparations of M and B components was low. When M and B inoculum preparations were combined, a sevenfold enhancement in infectivity resulted. The addition of T to M and B components did not enhance infectivity. The infectivity of individual inoculum preparations of M and B components presumably was caused by contamination of one component with the other. Absolute separation of these two components could not be achieved due to their close proximity in SDG.

Buoyant densities in CsCl. GBLV particles consistently gave the sharpest profile in CsCl gradients when ultracentrifuged in an SW 39L rotor (Fig. 3). The buoyant densities of M and B of GBLV were 1.475 and 1.492 g/cm^3, respectively. Those reported by Martelli et al (10) were 1.479 and 1.489, respectively. Whereas in SDG, the B component of GBLV was predominant over M; in CsCl, M component predominated over B. Perhaps B component degraded to a greater degree than did M in CsCl. The buoyant densities of M and B components of BBLMV were 1.471 and 1.497, respectively. The M component of BBLMV exhibited a shoulder on the denser side of the peak and was considerably larger than the B component. The buoyant densities of M and B components of the NY-isolate were similar to those of BBLMV, except the peaks were broader. Martelli et al (10) reported that the M and B components of GBLV contained 38 and 40% RNA, respectively. Our data indicate that the M and B components of BBLMV contain 37 and 41%, respectively, and those of the NY isolate, 37 and 40%, respectively, based on calculations from a formula (14).

Molecular weight of coat protein subunits. In 5% SDS polyacrylamide gel, the coat protein subunit of each isolate migrated as a single band and the molecular weight for all three isolates was about 54,000. This value agrees with the value published by Martelli et al (10).

Effect of nucleases on BBLMV nucleic acid. Incubation with DNase had no effect on the gradient profile of extracted BBLMV nucleic acid, but RNase removed the nucleic acid peak (Fig. 4). The nucleic acid peak of BBLMV is RNA. Martelli et al (10) indicated that GBLV contained RNA, based on the loss of infectivity of virus preparations as a result of incubation with RNase.

Hyperchromicity of BBLMV-RNA. The absorbance of the purified RNA of BBLMV was scanned from 220 to 340 nm to determine its purity. The $A_{260/280}$ ratio was >2 which indicates pure RNA. The melting profile of BBLMV fits that of a single-stranded RNA, exhibiting a gradual increase in absorbance at 260 nm over the temperature gradient (Fig. 5). The T_M of BBLMV-RNA in 1 × SSC was 60 C and the percent hyperchromicity (%H) was 15.4. The DNA of *M. lysodeikticus* exhibited a cooperative melting curve, typical of double-stranded DNA, exhibiting a T_M of 88.5 and a %H = 58.7.

Separation of RNA-1 and RNA-2 on polyacrylamide gels. Martelli et al (10) speculated that GBLV-RNA was "possibly occurring as two species with very similar mol. wt." We have successfully separated RNA-1 and RNA-2 in 2.4% polyacrylamide gels for all three isolates (Fig. 6). The molecular weight of RNA-1 and RNA-2 for the three isolates are 2.35 and 2.15 × 10^6, respectively. The two RNAs consistently fall within the confines of RNA-1 and RNA-2 of cherry leaf roll virus that have molecular weights of 2.4 and 2.1 × 10^6, respectively (8). Although we were not able to obtain sufficient amounts of separated M or B component of BBLMV from which to extract the RNA, it is reasonable to assume that RNA-1 corresponds to B component and RNA-2 corresponds to M component.

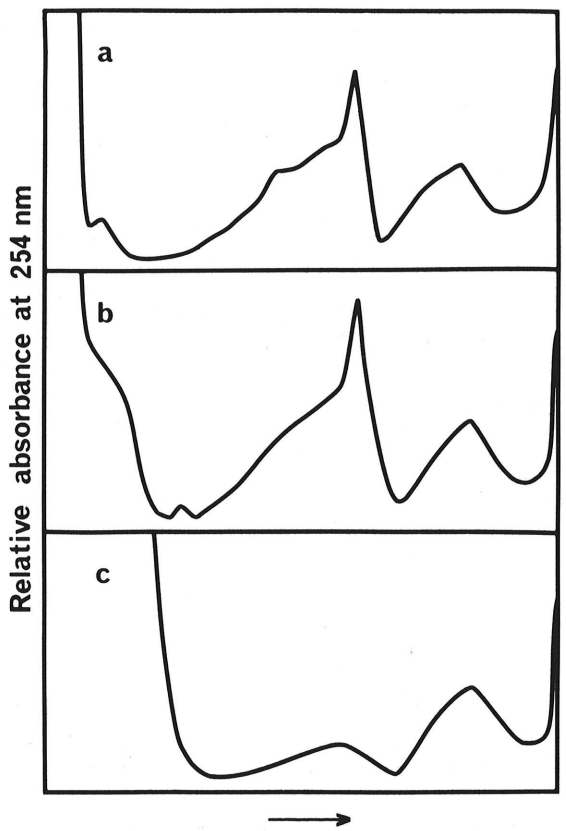

Fig. 4 Absorbance pattern of blueberry leaf mottle virus nucleic acid. Patterns from top to bottom are those of: **a,** untreated nucleic acid; **b,** after treatment with DNase; and **c,** after treatment with RNase.

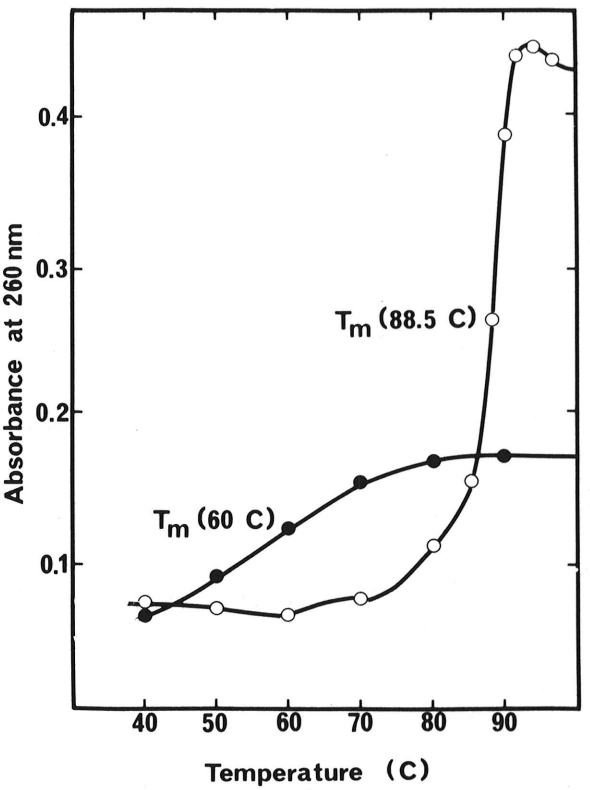

Fig. 5. Thermal denaturation curve for blueberry leaf mottle virus RNA (•—•) and *Micrococcus lysodeikticus* DNA in 1 × SSC buffer, pH 7 (o—o).

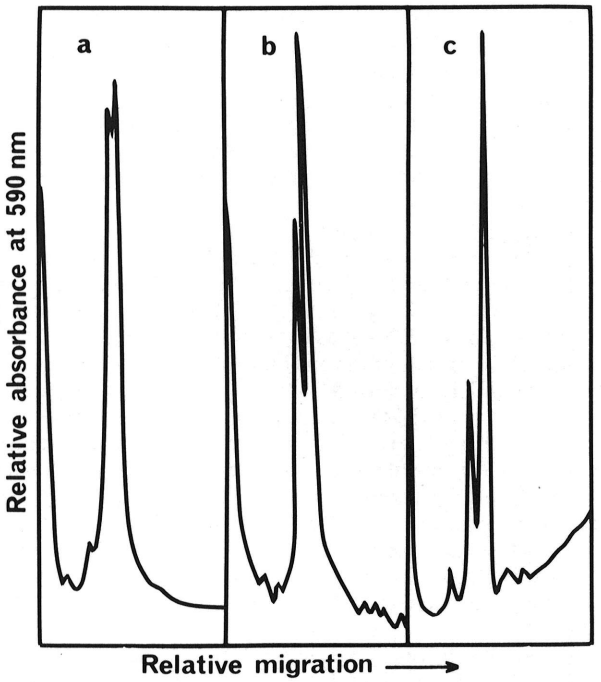

Fig. 6. Electrophoretograms of 2.4% polyacrylamide gels containing the RNA of **a,** blueberry leaf mottle virus; **b,** grapevine Bulgarian latent virus (European isolate); and **c,** grapevine Bulgarian latent virus (New York state isolate).

Sedimentation coefficient and molecular weight of BBLMV-RNA in sucrose density gradients. Neither formaldehyde-treated nor untreated BBLMV-RNA separated into two distinct peaks in linear-log sucrose density gradients. Rather, a slight shoulder formed on the main peak. The $S_{20,w}$ of the RNA was 32.5 and 21.8 S respectively, under nondenaturing and denaturing conditions, estimated by comparison with the sedimentation ratios of viral RNA markers. The molecular weight of the RNA, calculated from the $S_{20,w}$ value of formaldehyde-treated BBLMV-RNA, was 2.33×10^6 daltons.

DISCUSSION

The physical and chemical properties of BBLMV particles are consistent with those of the nepovirus group. Nematode vector transmission experiments are being conducted at this time with *Xiphinema americanum.* Confirmation that the test nematodes are *X. americanum,* sensu stricto, is pending. Low populations of *X. americanum* generally are associated with highbush blueberry plantings in Michigan. At present, BBLMV should be considered a tentative member of the nepovirus group.

The sedimentation coefficient of the M component is determined by the molecular weight of the RNA-2 strand. With BBLMV, the molecular weight of the RNA-2 is close to that of RNA-1, which accounts for the fact that the M and B components sediment at nearly the same rate. Complete separation of the M and B components is virtually impossible, but the infectivity enhancement when M and B are combined clearly suggests that both are needed for infectivity.

In serological tests, we were unable to detect any relationship between BBLMV and tomato ringspot virus, peach rosette mosaic virus, or cherry leaf roll virus, three other nepoviruses that have M components with sedimentation coefficients approaching 120 S. Serologically, the virus was distantly related to GBLV; the degree of relationship was similar to that which exists between grapevine fanleaf and arabis mosaic viruses (12).

For this reason, we considered it appropriate to use the name "blueberry leaf mottle virus" rather than to designate the virus as the blueberry strain of GBLV. In this study, comparisons of the physical and chemical properties of the two viruses revealed many similarities, but also several distinguishing features. Lacking absolute criteria for the separation of the viral "species" from a viral "strain," we feel that the degree of serological relatedness is probably the most significant criterion. As noted by Harrison and Murant (7) several of the definitive members of the nepovirus group can be classified into serologically related subgroups. According to Quaquarelli et al (11), BBLMV could be added to a subgroup along with tomato ringspot virus and GBLV as tentative members of the nepovirus group. In that subgroup, the M and B components contain RNAs with similar molecular weights (2.1 and $2.2–2.3 \times 10^6$ daltons, respectively).

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