Physical and Chemical Properties of Blueberry Shoestring Virus

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I wish to thank R. Stace-Smith, J. Tremaine, and W. Ronald for valuable suggestions and criticisms and for Model E analytical ultracentrifuge runs, while I was a visiting scientist at the Agriculture Canada Research Station, Vancouver, British Columbia. I also thank B. Schroeder for electron microscopy, L. Johns for graphic art work, S. MacDiarmid for photography, and F. Cupples for typing the manuscript. I am grateful to L. Russell (SEA/FR, Beltsville, MD for aphid taxonomy assistance and to M. K. C. Sun for technical assistance.

This research was supported in part by a grant from the Michigan Blueberry Growers Association, Grand Junction, 49056.

Michigan State University Agricultural Experiment Station Journal Series Article 8892.

Accepted for publication 9 April 1979.

ABSTRACT

RAMSDELL, D. C. 1979. Physical and chemical properties of blueberry shoestring virus. Phytopathology 69:1087-1091.

Blueberry shoestring virus (BBSSV) is an isometric virus 27 nm in diameter. The sedimentation coefficient is 120S. Buoyant density of the virions in CsCl is 1.392 g/cm^3. In Cs_2SO_4 virions form two bands, at buoyant densities of 1.273 and 1.355 g/cm^3. Particles are comprised of 20% RNA and 80% protein. The single RNA component is single stranded, based on thermal denaturation studies. Molecular weights of the RNA and protein subunit are 1.45×10^6 and 30,000, respectively, as determined by polyacrylamide gel electrophoresis. Electrophoretic mobility of the virus at pH 5 in 0.02 M buffer is 0.42×10^{-5} cm^{2}sec^{-1}volt^{-1} and the isoelectric point is between pH 4.5 and 4.7. Reversible temperature-dependent self-aggregation of the virus occurs at 20 C but not 4 C. Although apparently serologically unrelated to other viruses, BBSSV belongs with viruses in the southern bean mosaic group, based on the above properties and stabilization by divalent metallic cations. The cryptogram is R/1:1.45/20:S/S:S/Ve, Ap.

Additional key words: Highbush blueberry, Vaccinium virus disease, purification.

Blueberry shoestring virus (BBSSV) was reported as the causal agent of shoestring disease of highbush blueberry, *Vaccinium corymbosum* L. (16,17). In these two publications, preliminary methods of purification, electron microscopy, serology, infectivity, and some properties of the virus were reported.

This paper reports an improved purification technique, physical and chemical properties of BBSSV, its nucleic acid and protein moeities, and classification of the virus.

MATERIALS AND METHODS

Virus purification. Because BBSSV is not sap-transmissible to herbaceous hosts (16,17), purification was done directly from naturally infected blueberry. Blossom tissue for virus purification was collected from shoestring-diseased Jersey highbush blueberry bushes near Holland, MI, transported on ice (0C) in polystyrene cold chests to East Lansing, and frozen at −20 C until processed. All virus purification procedures were done at 0 to 4 C. After several purification procedures were tried, the following method was adopted: One-hundred grams of blossoms were homogenized in 300 ml of buffer in a Waring Blendor for 3–5 min in cold 0.1 M potassium phosphate buffer containing 0.01 M 2-mercaptoethanol and 0.005 M thioglycolic acid, pH 7.0. Triton X-100 was added to the homogenate (8% v/v) and stirred for 2 hr. Chloroform and *n*-butanol (5% each v/v) were added to sap squeezed through cheesecloth, and the mixture was stirred for 15 min. The emulsion was broken by centrifugation at 7,500 rpm for 15 min in a Sorval GSA rotor. The upper aqueous phase was retained and the solution was made 8% (w/v) with polyethylene glycol (MW 6000) and 0.1 M in NaCl while stirring. After 4–6 hr, the preparation was centrifuged for 30 min at 10,000 rpm in a Sorval SS-34 rotor. The pellet was resuspended with a glass rod in 10% of the starting volume that included 0.05 M phosphate buffer containing 0.001 M dithiothreitol, pH 7.0 (P-DTT). After 30 min, the preparation was given a low-speed centrifugation for 30 min. The supernatant was then ultracentrifuged in a Beckman 40 rotor for 2 hr at 36,000 rpm. The pellet was resuspended in 0.3 ml of P-DTT buffer per tube and allowed to stand overnight. Then 0.3 ml of the preparation was loaded onto centrifuge tubes containing a 5–30% linear sucrose gradient made in P-DTT buffer and centrifuged in a Beckman SW 41 rotor at 38,000 rpm for 90 min. The single virus band was collected with an ISCO density gradient fractionator and UV-analyzer (Instrumentation Specialties Co., Lincoln, NE 68504). The sucrose fractions containing the virus were diluted threefold with 0.05 M phosphate buffer (PB), pH 7, and ultracentrifuged for 3 hr in a No. 40 rotor at 36,000 rpm. The pellet was resuspended in 0.5 ml of PB per tube, using a glass rod, and then allowed to stand overnight. Dialysis, used to remove sucrose, caused a large loss of virus, probably because virus adhered to the walls of the dialysis tubing.

Determination of ultraviolet extinction coefficient. Virus that had been purified by two sucrose density gradients was dialyzed against glass-distilled water, pelleted, and resuspended in distilled water. The A_{260} was obtained and 0.25 ml of the solution was pipetted onto preweighed glass cover slips and then dried in a desiccator over silica gel under vacuum before reweighing.

Electron microscopy. Purified preparations of BBSSV from frozen blossoms were mounted on Formvar-coated grids, stained in 2% ammonium molybdate, pH 7.0, and examined in a Philips 300 TEM. Tobacco mosaic virus (TMV) was used as an internal size reference (11).

Determination of the sedimentation coefficient of the virus. Sedimentation coefficients of BBSSV and other viruses were determined with the SW 41 rotor, in linear-log gradients (4) prepared in PB. Brome mosaic (15), southern bean mosaic (25), and tomato bushy stunt (21) viruses were used as markers. Marker viruses and BBSSV, at concentrations of 0.1 mg in 0.4 ml of buffer, were layered on gradients, run singly in sister tubes, and combined in the same tube. Centrifugation was for 90 min at 38,000 rpm. After the gradients were scanned, the log of the depth sedimented was plotted vs. the log of the sedimentation coefficients of the known marker viruses to obtain an accurate estimate of the sedimentation coefficient of BBSSV.

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Determination of buoyant density of virions in CsCl and Cs_2SO_4. Buoyant density of BBSSV was determined by equilibrium banding in both the SW 50.1 rotor and in the Model E analytical ultracentrifuge (8). For determinations made in the SW 50.1 rotor, 1.8 ml of saturated CsCl in distilled water was mixed with 3.2 ml of 0.05 M phosphate buffer, pH 7, containing BBSSV at about 50 μg/ml. The virus was centrifuged at 45,000 rpm for 24 hr at 4 C. Gradient columns were scanned at 254 nm, and 0.3-ml fractions were collected and read in an Abbe 3L refractometer at 25 C. Refractive index was converted to density (g/cm^3) (5).

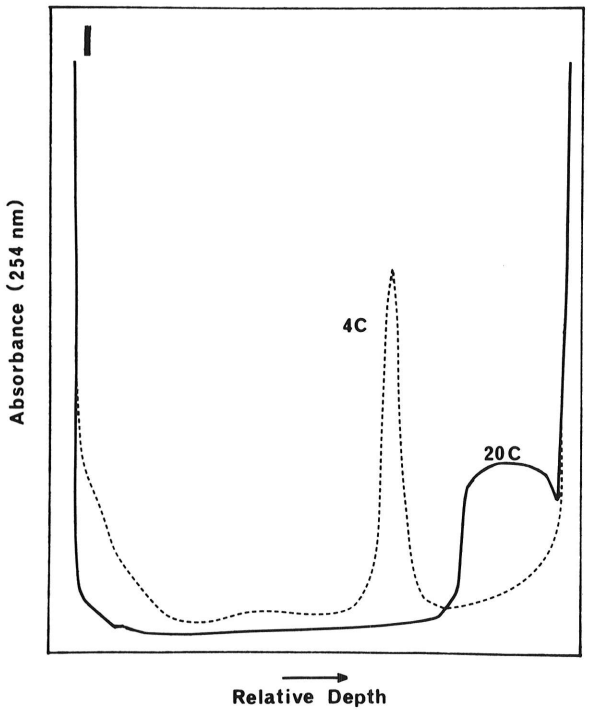

Fig. 1. Absorbance profile at 254 nm of blueberry shoestring virus centrifuged in linear-log sucrose gradients in the SW 41 rotor at 38,000 rpm for 90 min at 4 C (-----) and 20 C (———), showing temperature-dependent aggregation of virions.

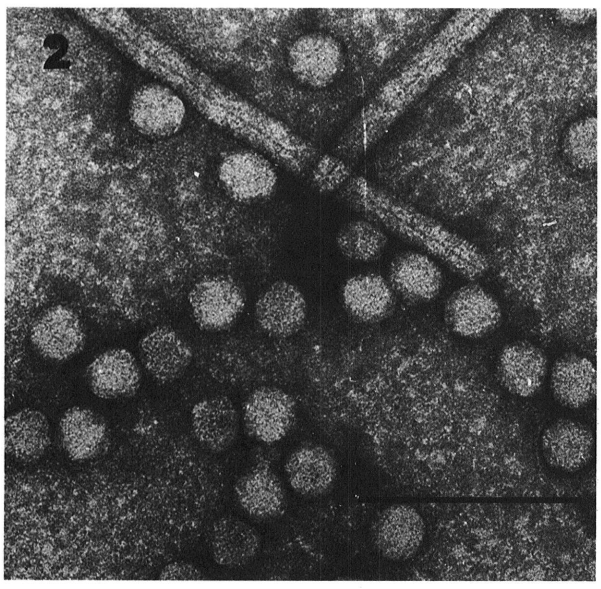

Fig. 2. Electron micrograph of blueberry shoestring virus purified from shoestring-diseased blueberry blossoms. Tobacco mosaic virus was used as an internal standard. Scale bar represents 100 nm.

For determination of the buoyant density in CsCl in the Model E analytical ultracentrifuge, a starting density of 1.379 g/cm^3 CsCl in 0.05 M phosphate buffer, pH 7, was used for BBSSV. Southern bean mosaic virus also was run in sister tubes in a starting density of 1.387 g/cm^3. Both viruses were run at concentrations of about 50 μg each in Kel F 4° single-sector cells in an An D rotor. After 20 hr at 44, 770 rpm at 10 C, viruses were photographed at a Schlieren angle of 65°. Centrifugation was continued overnight at 25 C and photographs were taken.

Buoyant density in a solution of Cs_2SO_4 was determined in 0.05 M Tris-acetate buffer, pH 7, with a starting density of 1.33 g/cm^3. About 200 μg of virus in 0.1 ml of buffer was layered onto 4.9 ml of Cs_2SO_4 solution in SW 39L tubes and centrifuged at 30,000 rpm for 27 hr at 4 C. Gradient columns were scanned at 254 nm, and 0.25-ml fractions were collected and read in an Abbe 3L refractometer at 25 C. Refractive indices were converted to density by the formula of Ludlum and Warner (19).

Electrophoretic mobility and isoelectric point of BBSSV. Electrophoretic mobility and the isoelectric point of BBSSV were obtained with horizontal 0.7% agarose gels made in 0.02 M dibasic sodium phosphate and 0.02 Tris buffer titrated to various pH values (4–6.25) with citric acid. The tank buffer was the same. Agarose solution (5 ml) was layered onto a plexiglass slab 2 × 13.5 cm and 0.5 cm thick. Holes 1.5 mm in diameter were punched in the solidified gel, and 15 μg of BBSSV in 0.05 M phosphate buffer, pH 7, was put in the well. Southern bean mosaic virus and sowbane mosaic virus were run in companion wells. A horizontal slab electrophoresis apparatus was used (Gelman Instrument Co. Ann

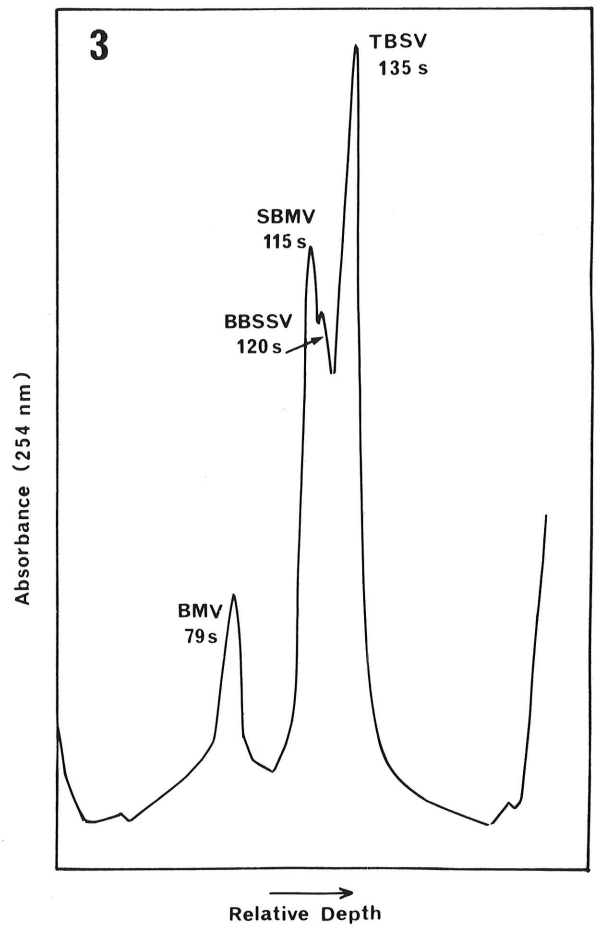

Fig. 3. Absorbance profile at 254 nm of brome mosaic, southern bean mosaic, and tomato bushy stunt virus as markers, and blueberry shoestring virus (BBSSV) as a result of a comparative run in a linear-log sucrose density gradient to determine the sedimentation coefficient of BBSSV. Gradients were centrifuged in the SW 41 rotor at 38,000 rpm for 90 min at 4 C.

Arbor, MI 48106). Gels and buffers were electrophoresed at room temperature at various pH values for 4 hr at 200 V and 3.5–7 mA. Gels were stained overnight in 50% acetic acid and 5% glycerol in water containing 0.1% Coomassie brilliant blue. Destaining was done by soaking gels overnight in 5% acetic acid and 10% glycerol in water.

Extraction of viral nucleic acid. The nucleic acid (NA) extraction method used was modified from the published method (1). All glassware was heated to 225 C in an oven for 2 hr before use. One volume (4 ml) of BBSSV (2 mg) was added to one volume of 0.01 M EDTA, 0.01 M sodium acetate, pH 5.1, two volumes 80% saturated, redistilled phenol in water, and 0.3 volume of 5% sodium dodecyl sulfate (SDS). The mixture was heated to 65 C, shaken for 5 min in a water bath, and chilled on ice. Two volumes of chloroform was added and the mixture was shaken for 5 min. Glass centrifuge tubes were silanized before use. After low-speed centrifugation at 10,000 rpm for 20 min, the aqueous phase was retained and the organic phase was reextracted with one volume of EDTA-sodium acetate plus 0.3 volume of 5% SDS. After low-speed centrifugation, the aqueous phases were pooled and reextracted twice again with one volume of 80% saturated phenol in water plus one volume of chloroform. Then 2.5 volumes of 95% ethanol (redistilled) plus 1/30 volume of 3 M sodium acetate were added to the aqueous phase and allowed to stand overnight at −20 C. The pellet was (i) collected by centrifugation at 10,000 rpm for 10 min at −20 C, (ii) resuspended in 15 ml of 0.1 M sodium acetate in 66% ethanol, pH 6, (iii) recentrifuged at 10,000 rpm for 10 min at −20 C, (iv) resuspended in 1 ml of glass distilled water, and (v) stored at −70 C until used in further tests.

Orcinol and diphenylamine tests with extracted nucleic acid. Orcinol (24) and diphenylamine (6,24) tests were performed with extracted viral NA.

Thermal denaturation with extracted nucleic acid. Fifty micrograms of BBSSV NA in 0.05 M phosphate buffer, pH 7, containing 0.1 M NaCl, was placed in two thermal cuvettes of a Gilford Model 2527 thermoprogrammer (Gilford Laboratory Instruments Co. Oberlin, OH 44074). *Drosophila melanogaster* DNA was placed in a third cuvette as a standard. A programmed temperature gradient of 0.5 C/min was generated, and absorbance readings at 260 nm were plotted from 50 to 99 C.

Effect of RNase on viral nucleic acid. Blueberry shoestring virus (2 mg/ml in 0.05 M Tris acetate pH 7 buffer) was added to an equal volume of dissociation buffer (9) (0.2 M Tris, 40 mM EDTA, and 2% SDS, pH 7) and allowed to incubate at room temperature for 30 min. After 30 min, 3 μg/ml of pancreatic ribonuclease (Nutritional Biochemicals Div. ICN, Cleveland, OH 44128) was added to an aliquot of the dissociated virus. These aliquots and those not containing RNase were incubated for an additional 30 min at room temperature. RNase treated and untreated samples (0.2 ml) were loaded each onto single SW 41 tubes containing linear-log sucrose gradients made in SSC buffer (0.15 M NaCl and 0.015 M sodium citrate, pH 7), made for viral RNA to be centrifuged at 6 C (4). The gradients were centrifuged at 4 C for 5 hr at 38,000 rpm and scanned with ISCO equipment.

Determination of nucleic acid by phosphorus analysis. Inorganic phosphorus content was determined according to the method of Chen et al (7) from samples of BBSSV that were purified in Tris buffer. Virus samples were heated in an equal quantity of 70% perchloric acid at 190–200 C for 15 min.

Polyacrylamide gel electrophoresis of nucleic acid. The molecular weight of BBSSV NA was estimated relative to viral NA standards of tobacco mosaic, brome mosaic, and southern bean mosaic virus RNAs. These were prepared for use in 2.6% polyacrylamide gels (18) in 5 mm i.d. glass tubes by dissociating 1 mg/ml virus concentrations in an equal volume of 0.2 M Tris, 40 mM EDTA and 2% SDS, pH 7 (15). The viruses were incubated in dissociation buffer for 30 min at room temperture. Electrophoresis was done for 4 hr at 2 mA/gel at room temperature. Gels were stained in 0.02% toluidine blue-0 in 40% ethylene glycol (monomethyl ether) overnight and destained in distilled water (2).

Polyacrylamide gel electrophoresis of protein subunits. The molecular weight of BBSSV coat protein subunit was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Virus protein preparations and protein marker standards were prepared by diluting each sample 1:1 (v/v) with dissociation buffer, consisting of 1% 2-mercaptoethanol, 4 M urea, 1% SDS in 0.1 M sodium phosphate, pH 7.2, and by heating each sample for 90 sec in a boiling water bath. Protein marker standards were carbonic anhydrase, bovine serum albumin, myoglobin, ovalbumin, and SBMV protein subunit. The markers and BBSSV subunit protein were applied separately to 3, 5, and 7.5% gels in 5-mm i.d. glass tubes. Gels and tray buffer were made according to the method of Dunker and Rueckert (10). Gels were electrophoresed for 5 hr at 5

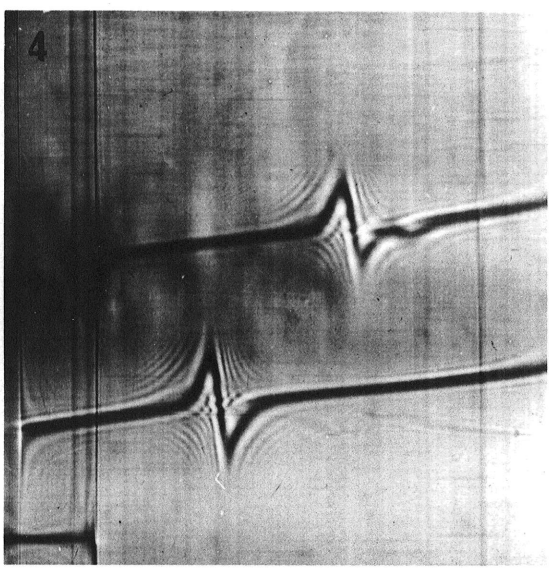

Fig. 4. Schlieren patterns of blueberry shoestring virus (BBSSV) (top) and southern bean mosaic virus (bottom) after centrifugation to near equilibrium in CsCl at 44,770 rpm at 25 C. Note some particle heterogeneity present in BBSSV.

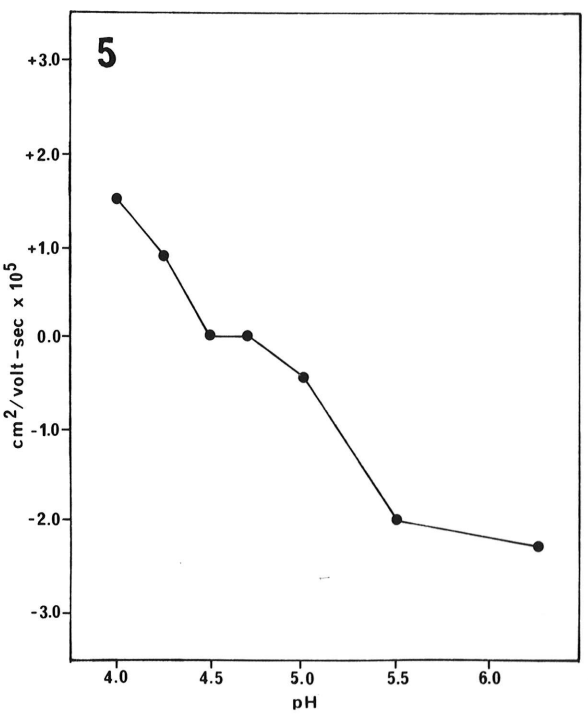

Fig. 5. Electrophoretic mobility of blueberry shoestring virus as a function of pH in 0.02 M dibasic sodium phosphate and 0.02 M Tris, titrated to various pH values with citric acid.

mA/gel at room temperature. Gels were stained overnight in 0.2% Coomasie brilliant blue (w/v) in 10% acetic acid and 50% methanol in water. Destaining was done with a series of changes of 7% acetic acid and 10% methanol in water.

RESULTS

Purification. All purification steps were done at 4 C, because self-aggregation of the virions occurs at higher temperatures (Fig. 1). This is reversible after several hours at 4 C, however. A critical step early in the purification scheme is addition of Triton X-100. The minimal amount needed for maximal virus yield is 8% (v/v). Various amounts of chloroform and butanol were tried as a clarification procedure. Excellent clarification and the highest yield were achieved by adding 5% of each (v/v) to homogenized sap. Use of larger amounts of these solvents resulted in serious loss of virus. Polyethylene glycol (MW 6000) at 8% (w/v) was necessary for maximum virus precipitation. The yield of virus from this method is about 5 mg/kg of blossoms, which is three to five times the yield of the two previously reported methods (16,17).

Properties of virions. The molar extinction coefficient for BBSSV (uncorrected for light scattering) is $E_{260}^{0.1\%} = 5.2$.

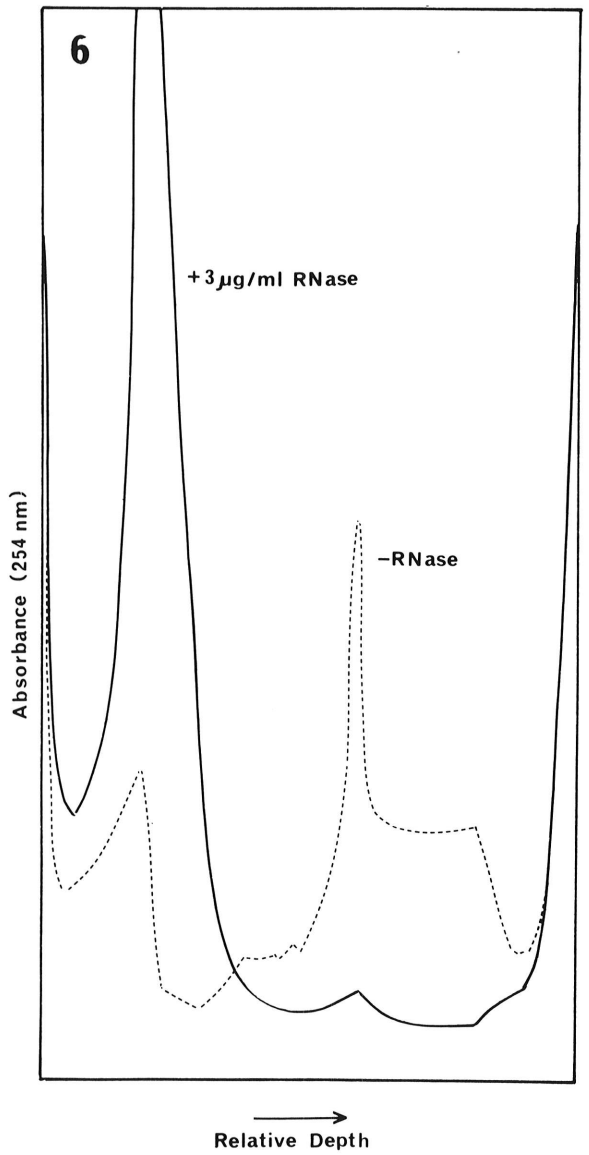

Fig. 6. Effect of pancreatic ribonuclease on blueberry shoestring virus (BBSSV) RNA. Photometric scanning pattern of BBSSV-RNA (-----) and BBSSV-RNA after exposure to 3 μg/ml of RNase (———). Linear-log sucrose gradients were run in the SW 41 rotor at 38,000 rpm for 5 hr at 4 C.

Previously, we (16,17) reported the diameter of BBSSV as 24 nm ± 1.2. We used a TMV standard and based our comparative measurement on a TMV width of 15 nm. In the present work (Fig. 2) we used a TMV width of 18 nm (11), and the diameter of BBSSV, fixed in 2% uranyl acetate, is 27 nm.

Previously (17), we gave an estimated sedimentation coefficient of 78.6 ± 3.1. In the present study, the sedimentation coefficient of BBSSV was estimated at 120S (Fig. 3). In linear-log gradients, the virus was slightly faster than southern bean mosaic virus (SBMV), which has an $s_{20})_w = 115$ (25). Our earlier estimation was based on two comparison runs using 10–40% linear sucrose gradients in the SW 25.1 rotor (3). In the previous work, SBMV and TMV were used as marker viruses, and sedimenting virus bands were measured visually with a ruler.

The buoyant density value obtained from UV-scans of CsCl gradients centrifuged to near equilibrium at 4 C in the SW 50.1 rotor was 1.395 g/cm^3. The values calculated from the model E analytical ultracentrifuge equilibrium sedimentation runs at 10 and 25 C were 1.384 and 1.397 g/cm^3, respectively. The Schlieren pattern of BBSSV shows a slight amount of heterogeneity at 25 C (Fig. 4) and at 10 C (not shown). The average vaule is 1.392 g/cm^3. The buoyant density of SBMV from the same runs at 10 and 25 C was calculated as 1.370 and 1.362 g/cm^3, which agrees well with the values of Magdoff-Fairchild (20).

The virus formed two bands in Cs_2SO_4 gradients at 4 C. Buoyant density of the top band was 1.273 and that of the bottom band was 1.355 g/cm^3.

The electrophoretic mobility of BBSSV at pH 5 was 0.42×10^{-5} cm^{2}volt^{-1} sec^{-1}. The isoelectric point was between pH 4.5 and 4.7 (Fig. 5). Sowbane mosaic and SBMV at pH 5 had electrophoretic mobilities of 0.23×10^{-5} and -0.42×10^{-5} cm^{2}volt^{-1}sec^{-1}, respectively.

Properties of viral NA. The extracted NA yield was 25–40% of theoretical yield. The $A_{260/280}$ value of extracted NA was 2.0, indicating a high degree of purity. The small starting amount of virus and NA adhering to the sides of the glass centrifuge tubes probably contributed to losses.

BBSSV NA reacted positively with orcinol and negatively with diphenylamine reagent, indicating that BBSSV NA is RNA. Exposure of BBSSV NA to 3 μg/ml of pancreatic RNase for 30 min at room temperature resulted in degradation of the NA peak in linear-log sucrose gradients (Fig. 6). This confirmed that BBSSV NA is indeed RNA.

The melting curve (Fig. 7) of BBSSV fits that of a single-stranded

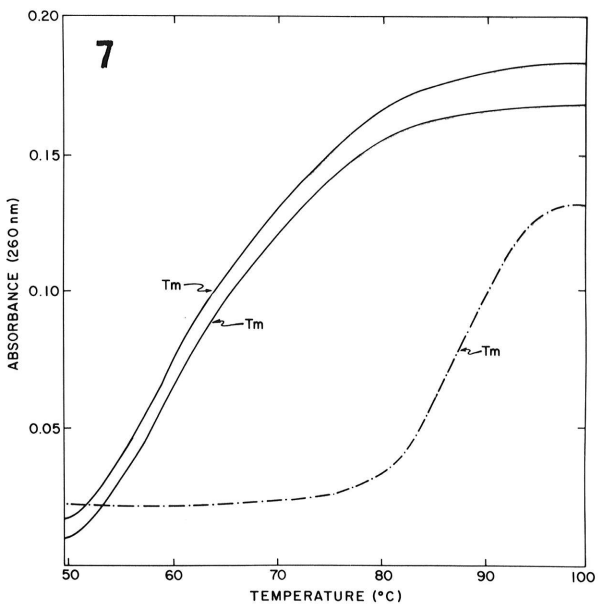

Fig. 7. Thermal denaturation curve for blueberry shoestring virus RNA in 0.1 M NaCl (———) and *Drosophila melanogaster* DNA (-·-·-). A temperature increase of 0.5 C/min was used.

RNA and shows a gradual increase in absorbance at 260 nm with increase in temperature. The DNA melting curve under the same conditions is sigmoidal and characteristic of double-stranded NA. The mean percent hyper-chromicity (H%) (Δ absorbance 99.5 C/50 C) for BBSSV is 13.6%, and the thermal melting point (Tm) is 63.6 C. *Drosophila melanogaster* DNA H% is 38.5%, and the Tm, 87.3 C.

The RNA content determined in two experiments using phosphorus analyses was 21.1 and 19.2% for an average of 20% RNA. This contrasts with the value of about 30% RNA that is obtained when calculations are made from Sehgal's formula (22) correlating % RNA with buoyant density in CsCl.

Polyacrylamide gel electrophoresis of RNA and protein subunit. A single RNA component was observed from dissociated BBSSV run in 2.6% polyacrylamide gels. The molecular weight of BBSSV RNA was estimated at 1.45×10^6 by comparing its mobility with RNAs from brome mosaic, tobacco mosaic, and southern bean mosaic viruses.

A single protein subunit component was observed from dissociated BBSSV run in SDS-polyacrylamide gels. The molecular weight of BBSSV protein was determined in 3, 5, and 7.5% gels by plotting the relative mobility of BBSSV protein and known protein standards against the logarithm of the molecular weight of the standards (23). Hedrick and Smith plots (13) were constructed to test for anomalous electrophoretic movement of the protein in gels of different concentrations. Because BBSSV protein and marker proteins passed through a common intercept (relative mobility = 1.0) at zero gel concentration (after arbitrarily setting the relative mobility of myoglobin as 1.0), it was concluded that BBSSV and marker proteins behaved normally.

DISCUSSION

Because the plant material used as a purification source was from diseased bushes, more than one strain of BBSSV may have been purified. There has not been any evidence of contaminating other viruses in purified preparations, however.

The phenomenon of reversible self-aggregation of purified BBSSV at temperatures above 4 C is not new. An isolate of carnation ringspot virus undergoes reversible temperature-dependent aggregation (27).

BBSSV is not serologically related to other described viruses with similar physical and chemical properties. It has characteristics in common with Hull's (14) SBMV group of small, spherical viruses with one RNA component; like the southern bean mosaic, cocksfoot mottle, rice yellow mottle, sowbane mosaic, and turnip rosette viruses in this grouping, BBSSV forms one band in CsCl and two or more in Cs_2SO_4 gradients. Electron microscopy of ultrathin sections of shoestring-diseased blueberry (12) show that BBSSV forms crystalline arrays in the host's cytoplasm in a manner similar to the five viruses in Hull's grouping. BBSSV is resistant to 1% SDS alone but is dissociated in the presence of 1% SDS plus 10 mM EDTA (D. Ramsdell, *unpublished*), indicating particle stabilization by divalent, metallic cations. This places BBSSV in a grouping with tomato bushy stunt, southern bean mosaic, cocksfoot mottle, and sowbane mosaic virus, according to a dendrogram proposed by Tremaine (26).

Preliminary transmission experiments indicate that the vector is the blueberry aphid *Masonaphis pepperi* MacGillivray (Ramsdell, *unpublished*).

The cryptogram for BBSSV is R/1:1.45/20:S/S/Ve, Ap.

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