This is to certify that the

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BLUEBERRY RED RINGSPOT VIRUS

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Major professor

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PHYSICAL AND CHEMICAL PROPERTIES

OF

BLUEBERRY RED RINGSPOT VIRUS

by

Jerri M. Gillett

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ABSTRACT

PHYSICAL AND CHEMICAL PROPERTIES OF BLUEBERRY RED RINGSPOT VIRUS

By

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Spherical, 45 nm virions purified from blueberry red ringspot diseased blueberry leaves formed two peaks in sucrose and CsCl gradients with sedimentation coefficients and buoyant densities of 212s, 275s and 1.30, 1.40 gm/cm^3, respectively. Antiserum made to formaldehyde fixed BBRRV virions used in ELISA failed to detect BBRRV until leaf material was partially purified. Tissue extracted in buffer containing nicotine produced as high, or higher, ELISA absorbance than partially purified samples. The BBRRV coat protein produced a major 44,000 molecular weight band in polyacrylamide gels. Purified BBRRV nucleic acid produced a thermal melting curve typical of double stranded nucleic acid and was degraded by DNase but not by RNase. Purified BBRRV was tested by ouchterlony gel diffusion against antiserum to CaMV, DaMV, FMV, and CERV; no relationship was found. A weak relationship between CaMV and BBRRV was found by ELISA. Attempts to associate purified virus with infectivity have been unsuccessful.

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INTRODUCTION

The symptoms of blueberry red ringspot disease (BBRR disease) were first reported by Hutchinson in 1950. In 1954 its virus-like nature was determined by Hutchinson and Varney when they showed that the disease could be graft transmitted. By propagating softwood cuttings taken from red ringspot infected budsticks that had been inserted into healthy blueberry plants (Stretch and Scott, 1977) blueberry plants were produced free of red ringspot disease. Since then, only limited work has been done with this disease.

Blueberry red ringspot disease is widespread in New Jersey blueberry plantings and has even been found in some New Jersey propagation stocks. The few incidences of BBRR disease in Michigan can generally be traced to plants received from New Jersey. Because this disease is not yet widespread in Michigan, there was an urgent need to study the disease now, before it had an opportunity to become widespread in Michigan blueberry plantings and propagation stocks.

The objectives of this thesis were: 1. to identify and isolate the causal virus of BBRR disease; 2. to make an antiserum to the virus; 3. to develop an assay to detect the virus in blueberry bushes; and 4. to determine sufficient physical-chemical properties of the virus to place BRRSV into a taxonomic category.

LITERATURE REVIEW

Blueberry Red Ringspot Disease

Blueberry red ringspot disease causes red spots and rings 2 to 6 mm in diameter on leaves of highbush blueberry, Vaccinium corymbosum L. and V. australe Small.. The symptoms appear on older leaves in mid- to late-summer and progress to younger leaves later in the growing season. The spots may coalesce, especially on older leaves, and are seen only on the top side of the leaf. Stems that are one year old or older may also exhibit red spots, rings, and blotches. Fruit from infected bushes may have circular light spots. A powdery mildew (Microsphaera alni DC ex Wint. var vaccinii (Schw.) Salm.) may cause similar leaf symptoms, but the red spots it causes can be seen on both sides of the leaf and the lower leaf surface often has a water-soaked appearance.

Blueberry red ringspot disease is of most economic importance in New Jersey where it is widespread in blueberry plantings. In 1981, Kim et al. reported that the disease was widespread in recent plantings in Arkansas. The disease has also been reported in Michigan, Connecticut,

Massachusetts, New York, North Carolina, and recently in Oregon (Converse and Ramsdell, 1982).

Vaccinium spp. are the only known hosts for BBRRV; there are no known herbaceous hosts (Kim et al, 1981). The disease caused by this virus has been observed in many highbush blueberry cultivars including: Blueray, Bluetta, Burlington, Cabot, Coville, Darrow, Earliblue, and Rubel. In New Jersey, symptoms of the disease have been observed on wild blueberry plants belonging to the V. australe Small and V. corymbosum L. group (Varney and Stretch, 1966). The virus is probably indigenous to New Jersey and has spread from the wild to commercial plantings. From there, it has probably spread to the other reported sites through propagation stock.

The symptoms of blueberry red ringspot disease are similar to those of ringspot disease in cranberry (V. macrocarpon Ait.). Electron microscope studies of affected cranberry show inclusion bodies similar to those found in blueberry tissue infected with red ringspot (K. S. Kim and A. W. Stretch, unpublished data). These two diseases may be caused by the same virus.

The present control strategies for BBRR disease are the use of virus-free planting stocks and rogueing of infected bushes. Although these strategies sound straight forward, they do present some problems. Once a propagation mother plant has become infected, it is very time consuming to

isolate disease-free propagation material from it. Stretch and Scott (1977) found that heat treatment of dormant rooted cuttings or cutting wood failed to yield disease-free plants. They did, however, produce some disease-free plants by propagating softwood cuttings from red ringspot infected bud sticks inserted into healthy blueberry plants. They advised that propagated plants should be observed for two to three years to ensure they are free of BBRR disease.

Another problem in the control strategy is identifying the red ringspot diseased bushes at an early, symptomless stage of infection so that they can be removed from the field before the virus is transmitted to other bushes. The literature is unclear regarding detection of this disease before symptoms appear. The virus can be detected by whip or bud grafting from suspected plants to very susceptible cultivars e.g. Cabot, Burlington, Darrow, or Blueray (Stretch and Varney, 1970). However, no mention is made about the reliability of this method for detecting symptomless infection. This method is also time consuming because even though the grafted indicator plants may express symptoms within three months, longer observation is recommended.

Recently, Hepp and Converse (1986) reported detecting BBRRV in syptomatic leaf tissue collected in August, September and October. They used a two-animal indirect ELISA. Samples of root, stem bark and flower buds from

dormant plants were also tested. Virus was found in stem bark and some flower bud samples. No virus was detected from root samples. The authors stated that background levels were higher for root samples and postulated this was due to the antiserum being cross-absorbed against healthy leaf sap and not against healthy root sap.

Enzyme Linked Immunosorbent Assay

If an appropriate antiserum is available, most blueberry viruses are assayed with enzyme linked immunosorbent assay (ELISA). The first report of the use of this assay to detect viruses in plant material is that of Clark and Adams, 1977. They found that double antibody sandwich ELISA (DAS-ELISA) proved to be an economical, quick, and sensitive assay that required only basic laboratory skills.

The DAS-ELISA that Clark and Adams used is still very much used in the plant sciences, however, many variations have been developed in an attempt to improve the assay. A common variation is indirect ELISA. It involves the use of pepsin derived antigen binding fragments, $F(ab')_2$. When whole immunoglobulin G (IgG) is digested with pepsin, the IgG is cleaved just below the two antigen binding arms, and the tail (F_c) portion of the IgG molecule fragments and can be dialyzed away. Barbara and Clark (1982) compared DAS-

ELISA to indirect ELISA and found that the indirect ELISA could be two to twenty-five times more sensitive than DAS-ELISA. This slight to extreme difference was largely due to lower and more consistent background absorbance values for indirect ELISA.

Taxonomy of Plant Viruses

Over the past 17 years, the plant viruses have been organized into 31 well established virus groups. The first 16 virus groups established were based on an Adensonian approach to taxonomy (Harrison et al., 1971). Forty-nine characteristics of 99 viruses were considered without weighting. Even though the data were incomplete, 16 distinct clusters, or groups, of viruses were evident. These original 16 groups are still valid today.

The subsequent 15 virus groups were established slightly differently. It became apparent that some characteristics were more taxonomically useful than others, and that some characteristics were more appropriate for distinguishing between viruses within a group than for distinguishing viruses between groups. Some characteristics have been recognized as being inappropriate for taxonomic consideration. For example, crude sap characteristics such as thermal inactivation, dilution end point, and longevity *in vitro* may be helpful to know in order to manipulate the

virus. However, these tests are hard to reproduce accurately and therefore are not good taxonomic criteria. Similarly, symptomatology is so variable depending on host and environment that it, too, is not a good taxonomic criterion.

Those properties that are useful for establishing taxonomic groups have become well identified (Hamilton et al.,1981; Francki, 1983) although they vary somewhat depending on the viral group under study and what is known about the individual viruses (i.e. the information that can be reasonably obtained). Particle morphology, that is, the size, shape, and presence or absence of an envelope around the viral particle, is generally an easy characteristic to obtain if an electron microscopy facility is available. These characteristics are often a strong indicator of one viral group such as the Caulimovirus group (large, 50 nm spheres without envelopes) or the Geminivirus group (geminate shaped particles). If the particles are small isometric particles 20 to 30 nm in diameter, the virus cannot be narrowed down to one viral group, however, this characteristic does disqualify about half of the viral groups for consideration.

A single characteristic should never be used to place a virus in a group. Some of the other characteristics that have proven useful for group establishment are: sedimentation properties of the particle(s), molecular

weight of the coat protein, number of polypeptides in the coat protein, type and strandedness of nucleic acid, number of nucleic acid species, and molecular weight of the nucleic acid.

The antigenic property of the virus particle is also a useful characteristic. It is best used to identify new viruses within a group but may also help to place a virus into a group. Depending on the shape of the viral particles, Ouchterlony gel double diffusion (for spherical viruses) or some form of liquid precipitation (for rod viruses) is applied to the virus in question using a number of available antisera. The closeness of the relationship between a pair of viruses is sometimes quantified as the serological differentiation index (SDI) i.e. the number of two fold dilution steps separating the homologous and heterologous titers. Recently, ELISA (Koenig, 1978; Bar-Joseph and Salomon, 1980; Rao et al., 1982) and immunoelectron microscopy (Milne, 1980) have been employed to establish serological relationships because of their greater sensitivity.

The Caulimovirus Group

In preliminary ultrastructure studies of BBRR diseas by K. S. Kim (unpublished data), 50 nm spheres were seen in ultrathin sections of BBRR diseased tissue. This suggested that BBRR disease might be caused by a virus belonging to the caulimovirus group. The type member of the caulimovirus group is cauliflower mosaic virus (CaMV).

The first pairing of CaMV with another virus was by Brunt in 1966. He demonstrated that CaMV and dahlia mosaic virus (DaMV) were serologically related, had similar morphology, produced similar inclusion bodies, and had similar aphid virus-vector relationships. In 1969, Hollings and Stone demonstrated that carnation etched ring virus (CERV) was also serologically related to these viruses. Because of these findings, the first Plant Virus Subcommittee of the International Committee for the Taxonomy of Viruses (ICTV) initiated a proposal for the establishment of a plant virus group characterized by isometric, DNA containing, aphid vectored viruses. Cauliflower mosaic virus was established as the type member and the group was named the Caulimovirus group (Harrison et al., 1971). The group was formally approved by the ICTV in 1976 (Fenner, 1976). The Caulimovirus group now has nine definitive and five tentative members (Hull,1984):

Definitive Members:

Cauliflower mosaic virus (CaMV)
Carnation etched ring virus (CERV)
Dahlia mosaic virus (DaMV)
Figwort mosaic virus (FMV)
Horseradish latent virus (HRLV)
Mirabilis mosaic virus (MMV)
Soybean chlorotic mottle (SoyCMV)
Strawberry vein banding virus (SVBV)
Thistle mottle virus (ThMV)

Tentative Members:

Blueberry red ringspot virus (BBRRV)
Cassava vein mosaic (CVMV)
Cestrum virus (CV)
Petunia vein clearing virus (PVCV)
Plantago virus 4 (PlV4)

Several characteristics set apart the Caulimovirus group from the other groups of plant viruses. Caulimoviruses are the only plant viruses with double stranded DNA (dsDNA). They are isometric and are 42 to 50 mm in diameter. This is larger than most of the other isometric plant viruses which measure between 20 and 30 nm. Only the Reoviridae and Tomato Spotted Wilt are larger than the Caulimoviruses. These two groups are easy to distinguish from Caulimoviruses because the Reoviridae have a dsRNA genome and Tomato Spotted Wilt has a ssRNA genome in a particle that is surrounded by a lipid-like envelope.

Another characteristic that sets apart the Caulimovirus group is the inclusion body type associated with the virions. When thin sections are viewed with a transmission electron microscope, the inclusion bodies appear roughly

spherical and consist of a finely granular electron dense matrix that may contain small electron-lucent regions (Fujisawa et al., 1967; Petzold, 1968; Kim et al., 1981). The virions are found embedded in the matrix but can also be found in the electron-lucent area. Generally, few virions are found in the cytoplasm outside of inclusion bodies although this varies greatly with different viral strains (Shalla et al., 1980) Individual virions of CERV (Lawson and Hearon, 1974) and BBRRV (Kim et al., 1981) have also been found in the nucleus, however, no inclusion bodies have been observed there.

Inclusion bodies can be helpful for diagnosis. If epidermal strips are peeled from the host and stained with 0.5 to 1.0% phloxine, the inclusion bodies can be visualized with a light microscope. This works best with succulent herbaceous hosts. Inclusion bodies can also be found in palisade and spongy parenchyma cells but have been seen, to a limited extent, in young tracheary and phloem cells (Fujisawa, 1967).

Where known, caulimoviruses are spread by infected vegetatively propagated plants and aphid vectors. While CaMV can be readily sap transmitted mechanically, transmission this way has not been possible for SVBV and is difficult for DaMV and CERV. Six of the nine definitive Caulimovirses are transmitted by aphids. The vector of the other members has not been determined. Aphids show little

specificity in transmission; up to 27 aphid species have been reported to transmit CaMV (Kennedy et al., 1962), and at least several aphid species have been reported to transmit DaMV (Brierly and Smith, 1950) and SVBV (Frazier, 1960; Frazier and Converse, 1980). The virus-vector relationship is an unconventional, non-persistent one. While the virus is acquired by the aphid in the normal non-persistent manner, retention is unconventional in that it can be quite long. Retention time is highly variable, from several minutes to three days and depends on the specific aphid species and viral strain (Day and Venables, 1961; Van Hoof, 1954; and Hamlyn, 1955). Despite the long retention times, other evidence supports the theory that Caulimoviruses are stylet-borne. Transmission of the virus is lost when the insect molts (Day and Venables, 1961). When CaMV was experimentally injected into the aphid hemolymph, the aphids were unable to transmit the virus which also supports a stylet-borne nature of transmission (Day and Venables, 1961).

One other interesting feature of the Caulimovirus vector relationship is the presence of a required accessory factor for transmission. When aphids are allowed to acquire purified virus by feeding through a membrane, they fail to transmit the virus, (Pirone and Megahed, 1966). However, if the aphids are allowed to probe on infected plants before

feeding through a membrane, they can then transmit the purified virus (Lung and Pirone, 1973; 1974).

The CaMV genomic DNA is approximately 8 kilobase pairs long and several strains have been sequenced (Franck et al., 1980; Gardner et al., 1981; and Balazs et al., 1982). The sequences show six to eight open reading frames on one strand while the other strand is non-coding. Genes coding for cell-to-cell spread, aphid transmission, coat protein precursor, reverse transcriptase, and inclusion body protein have been identified (Laquel et al., 1986; Covey and Hull, 1981; and Rakib et al., 1979). The genomic DNA is found in both linear and circular forms, however, only the circular form is infective (Hull and Shepherd, 1977). There are three to four discontinuities, one on the -stand and two to three on the strand. These discontinuities are in fixed positions for a given viral strain (Richins and Shepherd, 1983; Hull and Howell, 1978; Volovitch et al., 1978; Hull and Donson, 1982; Donson and Hull, 1983). At each discontinuity the 3'-end overlaps the 5'-end to a variable extent (Franck et al., 1980).

The protein coded for by the coat protein precursor gene has a molecular weight of 57,000. This is processed to a 42,000 MW protein which is believed to be the final coat protein (Frank et al., 1980; Al Ani, et al., 1979). The coat protein of MMV is reported as 32,000 MW (Brunt and Kitajima, 1973). The protein coat molecular weights of the

other Caulimoviruses are unknown.

The particle sedimentation coefficient of Caulimoviruses ranges from 200s for SVBV (Frazier and Converse, 1980) to 254s for MMV (Donson and Hull, 1983) and DaMV (Hull, 1984). The sedimentation coefficient for CaMV is 208s (Hull, 1984). Of the five members tested, they all produce one band in CsCl gradients of 1.35 or 1.38 g/cm^3 (CMI/ABB Description no. 295).

METHODS AND MATERIALS

Yield Studies

Berries from ten BBRRV-infected and ten non-infected 'Blueray' blueberry bushes of approximately equal size were harvested July 28 and August 11, 1986 at the Charles Hilton farm near Nunica, Mi. Harvesting was done two times with hand held vibrating harvesters and catching frames. Total weight of fruit harvested per bush, number of berried per cup (cup count), and gms per berry were recorded. The data were analyzed with the MSTAT statistical program using the analysis of variance (ANOVA) test.

Purification of Viral Particles

Mature, BBRRV symptom-bearing leaves were collected from 'Blueray' blueberry bushes near Nunica, MI and held at -20 C until processed. Several different schemes of purification were investigated. The first one was an improved method for purification of cauliflower mosaic virus (CaMV) as reported by Hull et al. 1976. Five hundred grams of blueberry leaves were ground with a Waring blender in a ratio of 6 ml/g leaves, 0.5 M potassium phosphate buffer pH 7.2 containing 0.75% sodium sulfite. The resulting juice

was expressed through cheesecloth. Urea and triton-X 100 were added to 1 M and 2.5 % (v/v), respectively. After stirring overnight at 4 C, the extract was given a low speed centrifugation (5,000 rpm for 10 min) in as IEC No. 872 rotor (IEC Co., Needham Heights, MA 02194). The resulting supernatent was given a high speed centrifugation in a Beckman No. 30 rotor (Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92634-3100) at 28,000 rpm (100,000 x g) for 90 min. The pellet was resuspended overnight in a small volume of resuspension buffer (0.01 M sodium phosphate buffer pH 7.2) and the preparation was given a second series of differential centrifugation. Linear-log 0-30% sucrose gradients were used to further purify the virus. (See Appendix for gradient recipe.) Gradients were centrifuged in a Beckman SW 41 rotor at 38,000 rpm (250,000 x g) for 60 min and then fractionated with a density gradient fractionator, ISCO Model 185 (Instrument Specialities Co., 4700 Superior St., Lincoln, NE 68505) and monitored with an ISCO Model UA-5 absorbance monitor at 254 nm. Initially, fractions were negatively stained with ammonium molybdate and examined in a transmission electron microscope (TEM) for the presence of virions. Those fractions containing virions were diluted 1:3 (1 part sucrose to 3 parts resuspension buffer) and centrifuged 4 to 5 hours at 38,000 rpm (100,000 x g) in a Beckman No. 40 rotor. The resulting pellets were resuspended in a small volume of resuspension buffer.

For antiserum production and buoyant density determinations, purification also included centrifugation of the virus preparation in CsCl gradients. The concentrated virus preparation from linear-log sucrose gradients was placed on CsCl step gradients consisting of 1 ml each of the following CsCl densities: 1.55, 1.45, 1.40, and 1.35 gm/cc. The gradients were centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm (150,000 x g) for 15 to 20 hr and fractionated as above.

Modifications of the above purification scheme were tested in order to increase yield and purity of virions. The first modification tried was based on a purification scheme of Lawson and Civerolo, 1980, for carnation etched ring virus (CERV) from Saponaria vaccaria leaves. Mature blueberry leaves symptomatic of BBRRV were extracted as above except the extraction buffer used was 0.1 M sodium phosphate buffer pH 7.2 containing 0.01 M 2-mercaptoethanol and 0.005 M thioglycollic acid. The resulting extract was made to 6% urea (w/v) and 2.5% (v/v) triton-X 100, as before. In addition, n-butanol was added to 8% (v/v). The extract was stirred overnight at 4 C and then given a low speed centrifugation as above. The supernatent was made to 8% (w/v) polyethelene glycol (MW 8,000) and to 1% (w/v) NaCl and then stored at 4 C for 4 to 18 hr. The polyethelene glycol precipitated virus was collected by low speed centrifugation. The resulting pellet was dissolved in

resuspension buffer (0.01 M sodium phosphate pH 7.2) using at least one tenth the starting volume. This was given a high speed centrifugation and the resulting pellet resuspended in a minimal volume of resuspension buffer. Sucrose gradients were employed as above.

Eventually the first low speed centrifugation following the overnight stirring with butanol, urea, and triton-X was replaced by expressing the crude homogenate through cheesecloth to decrease the time involved for purification.

Antiserum Production

In a first attempt at antiserum production (Kim et al. 1981), a series of six injections of purified virus emulsified in Freund's Complete (first injection) or Incomplete (subsequent injections) Adjuvant (Difco Laboratories, Detroit, MI 48202) was given intramuscularly to a female New Zealand white rabbit at 7-10 day intervals. Antigen amounts were 0.3 to 0.4 mg per injection. Test bleedings were begun after the fourth injection. A second attempt at antiserum production (Gillett and Ramsdell, 1984) was tried using purified virus that had been fixed in formaldehyde prior to emulsification and injection. To fix the purified virus, it was dialyzed against a 0.5 % (v/v) solution of formaldehyde for 24 hours. Emulsification in Freund's adjuvent and antigen concentration was as above.

Four intramuscular injections were administered. A test bleed was taken after the fourth infection. Four major (50ml) bleeds were made at 7-10 day intervals. The blood was placed at 37 C for 1 hour and then at 4 C for 12 hours. The serum was collected, freeze dried, and stored at -20 C until needed.

Antiserum titer was determined using gel double diffusion tests in agarose gel consisting of 0.8% (w/v) agarose containing 0.1% (w/v) NaN_3 and 0.85% (w/v) NaCl. Purified BBRRV (100 ug/ml) was used as test antigen and purified extract from healthy blueberry leaves (at an equivalent starting weight to final resuspension volume) was used as a healthy control antigen.

Enzyme-linked Immunosorbent Assay

Immunoglobulin Purification

Antiserum to BBRRV was produced in a rabbit as described above. The immunoglobulin (primarily IgG) was purified from crude serum according to Clark and Adams 1976. One ml of crude serum was added to 9 ml distilled water. Ten ml of saturated ammonium sulfate was then added slowly to the crude serum while stirring. After incubation for 30 to 60 min at room temperature, the precipitate was collected by a low speed centrifugation at 5,000 rpm for 5 min in a Beckman No. 30 rotor. The precipitate was resuspended in 2

ml 1/2 strength phosphate buffered saline (PBS, 0.015 M phosphate containing 0.8% (w/v) NaCl) and dialyzed three times against 500 ml 1/2 strength PBS. The IgG was then passed through a 6 ml bed of DEAE-23 cellulose (Sigma Chemical Co., St. Louis, MO 63178) which had been pre-equilibrated in 1/2 strength PBS. Two ml fractions were collected from the column and monitored for absorbance activity at 280 nm. The fraction(s) containing IgG were saved, adjusted to a concentration of 1 mg/ml (A_{280} of 1.4 = 1 mg/ml), and used to coat ElISA plates, to conjugate with alkaline phosphatase, and to make $F(ab')_2$ fragments.

Conjugation of IgG and Enzyme

To conjugate the IgG with alkaline phosphatase (Sigma No. P-5521), 2 mg of ammonium sulfate precipitated enzyme was added to 1 ml purified IgG and then dialyzed 3 times against 500 ml full strength (0.015 M) PBS. After dialysis, gluteraldehyde was added to a final concentration of 0.05 % (v/v). This was mixed well and incubated at room temperature 3 to 4 hr. To remove excess glutaraldehyde, the solution was dialyzed again as above. After dialysis, 5 mg/ml (w/v) of bovine serum albumin and 0.01 % (w/v) sodium azide were added. The finished conjugate was stored at 4 C.

Production of $F(ab')_2$ Fragments

When $F(ab')_2$ fragments were needed for indirect ELISA tests, they were produced according to Barbara and Clark, 1982. One ml (1 mg) of purified IgG was dialyzed three times against 500 ml of 0.07 M sodium acetate, pH 4.0, containing 0.05 M NaCl. After dialysis, 15 ul of a 3 mg/ml pepsin solution was added to the IgG and incubated overnight at 37 C. This was dialyzed against three 500 ml changes of PBS containing 0.01 % (w/v) sodium azide. The finished $F(ab')_2$ fragments were collected from the dialysis tubing and stored at 4 C.

Protein-A Alkaline Phosphatase

Protein-A conjugated with alkaline phosphatase for use in indirect ELISA was purchased from Zymed Laboratories Inc, South San Francisco, CA 94080. It was stored at 4 C as a 350 units/ml solution and diluted 1:1000 (v/v) in extraction buffer immediately before use.

Double Antibody Sandwich ELISA (DAS-ELISA)

A protocol of Clark and Adams, 1976, was followed. All ELISA solutions were added to the plate at 200 ul/well and the plate was sealed in plastic for all incubations. Purified IgG was diluted to a concentration of 1 ug/ml in sodium carbonate buffer, pH 9.6, (coating buffer), added to an Immulon^R 1, 96 well flat bottom polystyrene ELISA plate

(Dynatech Laboratories, Inc, Chantilly, VA 22021) and incubated at 37 C for 2 to 4 hr. It was then rinsed three times in PBS containing 0.05% tween-20 (PBS-tween). Blueberry leaf extract (see below) or other antigenic sample was added to the plate and incubated overnight at 4 C. The plate was again rinsed with PBS-tween as above except several extra rinses were used as needed to completely remove all traces of plant material from the wells. Enzyme conjugated IgG was diluted 1:800 in PBS-tween containing 2% (w/v) polyvinylpyrrolidone (PVP) and 0.2% (w/v) egg albumin (extraction buffer), added to the ELISA plate, and incubated 2 to 4 hr at 37 C. It was then rinsed in PBS-tween as above. The substrate p-nitrophenyl phosphate was diluted to 1 mg/ml in 0.97% (v/v) diethanolamine substrate buffer, pH 9.8, and added to the plate. The reaction was allowed to incubate at room temperature until the negative controls (background) started to increase in absorbance. This ranged from 30 to 180 min. If background levels were still low after several hr, the plate was stored overnight at 4 C, and another absorbance reading was taken. All plates were read in a Dynatech Microelisa^{R} Mini-Reader MR 590 for absorbance at 405 nm.

Indirect ELISA

In some cases, indirect ELISA was used instead of DAS-ELISA. The basic protocol of Barbara and Clark, 1982 was

followed. Immulon 1 ELISA plates were coated as above except $F(ab')_2$ fragments were used instead of whole IgG. The optimal $F(ab')_2$ concentration was periodically tested and was usually 2.5 ug/ml. Antigen samples were incubated as for DAS-ELISA. Instead of adding enzyme-conjugated IgG as in DAS-ELISA, unconjugated, whole IgG was diluted in extraction buffer usually at a 1 to 2 ug/ml concentration, added to the plate and incubated 2 to 3 hr at 37 C. The plate was rinsed in PBS-tween and a 1:1000 (v/v) dilution of Protein-A alkaline phosphatase in extraction buffer was added. The plate was incubated 2 to 3 hr at 37 C and then rinsed. Substrate addition and incubation was as for DAS-ELISA.

Interpretation of ELISA Data

For both DAS-ELISA and indirect ELISA, samples were considered positive if their absorbance values were equal to or above twice the mean of the appropriate negative control absorbance values.

Sample Preparation for ELISA

Crude Extraction of Samples

Blueberry leaves were ground 1:10 (w/v) in extraction buffer (PBS-tween containing 2% (w/v) polyvinylpyrrolidone and 0.2% (w/v) egg albumin) with a Tissuemizer^R SDT-182E2

homogenizer (Tekmar Co., Cicinnati, OH 45222), strained through cheesecloth, and then added to ELISA plates at 200 ul/well. All samples and buffer were kept on ice.

Partial Purification of Samples

Initially, the plant extract was carried through the first 2/3 of the purification protocol. One gram of frozen or unfrozen blueberry leaves was homogenized in 10 ml of 0.1 M sodium phosphate buffer, pH 7.2. Six per cent (w/v) urea, 2.5% (v/v) Triton-X 100, and 8% (v/v) n-butanol were added and the mixture shaken overnight at 4 C. The extract was then expressed through cheesecloth and PEG and NaCl were added to a final concentration of 8% (w/v) and 1% (w/v), respectively. After incubation overnight at 4 C, the extract was given a low speed centrifugation of 10,000 rpm for 30 minutes in an IEC No. 870 rotor and the resulting pellet resuspended in 1 ml of extraction buffer. This was then tested in an ELISA plate at 200 ul/well.

The method of Lawson and Hearon (1980) for purification of carnation etched ring virus (CERV) inclusion bodies was used in an attempt to purify inclusion bodies from BBRRV-infected blueberry leaves. One gram of tissue was ground in 10 ml of 0.1 M TrisHCl, pH 7.2, containing 0.025 M KCl and 0.001 M $MgCl_2$. Triton-X 100 was added to 5% (v/v) and the mixture shaken on ice in an orbital shaker at 140 rpm for 3 hr. The extract was strained through cheesecloth, given a

low speed centrifugation, and the resulting pellet was resuspended in 1 ml of extraction buffer. This protocol differs from the partial purification of BBRRV virions in the following ways: the initial homogenizing was changed from 0.1 M sodium phosphate to 0.1 M tris containing $MgCl_2$ and KCl, the addition of urea and n-butanol was omitted, the shaking time was shortened, and the PEG step was eliminated.

Crude Extraction of Leaf Samples in High Molarity Buffer

Non-infected, and three kinds of BBRRV infected blueberry leaves: symptomatic, symptomless from symptomatic branches and symptomless from symptomless branches, were ground 1:10 (w/v) in several different buffers according to Martin, 1988. He found that when the molarity of extraction buffer was increased and nicotine added, it increased the ELISA absorbance values of blueberry Oregon scorch virus infected blueberry samples (assayed with antiserum made to blueberry Oregon scorch virus). The buffers used to grind BBRRV were: standard recipe 0.015 M PBS extraction buffer with and without 0.5% (v/v) nicotine, 0.15 M PBS extraction buffer with and without 0.5% nicotine, and 0.1 M borate buffer, pH 8.0, with and without 0.5% (v/v) nicotine. Samples ground in these buffers were strained through cheesecloth and added to indirect ELISA plates. An aliquot of each tissue sample was also treated according to the

inclusion body purification and added to the plate for comparison.

Detection of BBRRV in Various Blueberry Tissues

Root, bark, blossom and buds were taken from BBRRV-infected and non-infected 'Blueray' blueberry bushes at the Hilton farm in Nunica, MI. These various samples were taken through the inclusion body protocol and tested in DAS-ELISA (1985) and indirect ELISA (1986).

Sedimentation Coefficient Determination

The sedimentation coefficient (s-value) of BBRRV was determined using linear-log sucrose density gradients of 0-30% sucrose. (See Appendix A for recipe.) Purified cauliflower mosaic virus, tobacco ringspot virus, and southern bean mosaic virus were used for markers. Approximately 0.25 mg of each marker and purified BBRRV were applied to individual gradients and centrifuged for 60 min at 38,000 rpm in a Beckman SW 41 rotor. Each gradient was fractionated as described above. The log of the distance migrated for each virus was plotted on the x-axis of semi-log paper. The sedimentation coefficient of the marker viruses was plotted on the y-axis. A straight line was drawn through these points and the s-value of BBRRV was determined from that line.

Buoyant Density of the Virus Particle

Blueberry leaves symptomatic of BBRRV were purified through sucrose density gradients. The fractions containing virus were diluted 1:3 (v/v) with 0.01 M sodium phosphate buffer, pH 7.2, (resuspension buffer) and pelleted from the sucrose in a Beckman No. 40 rotor at 38,000 rpm for 4 hr. The resulting pellet was resuspended in a small volume of the resuspension buffer and further purified in a step gradient of CsCl. The step gradient consisted of 1 ml each of CsCl solutions p = 1.55, 1.45, 1.40, and 1.35 gm/cc dissolved in resuspension buffer. The gradients were run in a Beckman SW 50.1 rotor at 35,000 rpm for 15 to 20 hr and fractionated as above into 0.25 ml fractions using Fluorinert^{R} as a chase solution. Refractive indices of the resulting CsCl fractions were determined in an Abbe 3L refractometer. Density of CsCl was determined from a formula from Bruner and Vinograd:

$$p^{25\ C} = 10.8601 \times (\text{refractive index}) - 13.4974\ .$$

Fractions were examined in the electron microscope to determine which ones contained virions.

Electrophoretic Mobility of Whole Particles

The electrophoretic mobility of whole virus particles was determined by the method of Tremaine and Wright, 1967. Electrophoresis was carried out in a slab gel consisting of 0.7% agarose in a 0.02 M Tris, 0.02 M sodium dibasic phosphate buffer that was adjusted to the desired pH with citric acid. Gels were cast by pipetting 5 ml of molten agarose solution onto a plexiglass plate (2.5 cm x 13.5 cm x 0.5 cm) and allowed to solidify and cool to room temperature. Two 1.5 mm diameter wells were cut in the gel, one above the other, along the width axis of the gel. The wells were cut approximately 5 mm apart and were centered with respect to the length of the gel.

Equal aliquots of BBRRV infected and non-infected blueberry leaves were purified as above and resuspended in equal volumes of 0.01 M sodium phosphate, pH 7.2. The samples were added at 15 ul per well. The concentration of the purified virus was 1 mg/ml.

Electrophoresis was carried out in a Gelman Deluxe Electrophoretic Chamber using a Bio-Rad Model 400 power pack at 180 volts, 4 to 8 milliamps for 4 hr. Electrophoresis tray buffer consisted of 0.02 M tris, 0.02 M sodium dibasic phosphate, adjusted to the desired pH with citric acid. Electrophoretic runs were carried out at pH 2, 3, 4, 5, 6, 7, and 8. Following electrophoresis, gels were stained 2 hr

overnight in 5% (v/v) acetic acid, 5% (v/v) glycerol, and 0.0125% (w/v) coomasie blue (Chrambach et al., 1967). Gels were destained in a solution of 5% (v/v) acetic acid, 10% (v/v) glycerol until background was reduced to a minimum.

Protein Coat Molecular Weight

The protein coat molecular weight was determined by the sodium dodecyl sulfate-polyacrylamide electrophoresis method of Laemmli 1970. Equal aliquots of BBRRV infected and non-infected blueberry leaves were purified through sucrose gradients. The resulting purified virus and plant protein were diluted 1:3 (v/v) with 4X sample buffer (see appendix for electrophoresis recipes) containing SDS. This was boiled 3 min and then cooled on ice. Marker proteins were treated similarly and were either from Bio Rad or Sigma (No. SDS-70L). Those from Bio Rad contained phosphorylase (MW 92,500), bovine serum albumin (MW 66,200), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), soybean trypsin inhibitor (MW 21,500), and lysozyme (MW 14,400) The markers from Sigma contained bovine serum albumin (MW 66,000), egg albumin (MW 36,000), glyceraldehyde-3-phosphate dehydrogenase (MW 36,000), carbonic anhydrase (MW 29,000), trypsinogen (MW 24,000), trypsin inhibitor (MW 20,100), and alpha-lactalbumin (MW 14,200).

All samples were applied to an SDS-polyacrylamide

vertical slab gel consisting of a 10 to 11 cm 12% acrylamide separating gel underneath a 5 to 6 cm 4% acrylamide stacking gel. Electrophoresis was carried out in a Bio Rad vertical slab gel electrophoresis chamber using a Fotodyne^{R} power pack at approximately 130 volts for 6 hr. The bromphenol blue tracking dye was run to the bottom or just off of the gel.

Gels were stained with either coomasie brilliant blue or silver. For the coomasie stain, gels were placed overnight in a solution of 10% (v/v) acetic acid, 50% (v/v) methanol, 40% (v/v) distilled water and 0.2% (w/v) coomasie brilliant blue. Gels were destained with several changes of 7% (v/v) glacial acetic acid, 10% (v/v) methanol, 83% (v/v) distilled water until background staining was reduced to a minimum (Chrambach, 1967).

For the silver stain (Morrissey, 1981), gels were placed overnight in 50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) distilled water. Gels were then rinsed several times with distilled water and placed for 30 min each in two changes of 500 ml distilled water containing 2.5 mg dithiothreitol (DTT). The DTT solution was decanted and a 0.1% (w/v) silver nitrate solution was added. This was decanted after 1 hr, the gel was quickly rinsed in distilled water, then rinsed in 3% (w/v) sodium carbonate and placed in 500 ml of 3% sodium carbonate containing 75 ul of 37% formaldehyde. The silver stain was allowed to develop for

15 to 45 min. After the desired stain intensity was reached, the reaction was stopped with 12 gm citric acid.

After either staining method, the stacking gel was removed and the distance from the top of the separating gel to the middle of each protein band was measured. The data were plotted on semi-log paper with molecular weight of the protein standards on the y-axis and the distance they migrated on the x-axis. A line was drawn and the molecular weight of BBRRV protein bands was determined from this line.

Nucleic Acid Studies

Isolation of BBRRV Nucleic Acid

Three hundred gm each of non-BBRRV infected and BBRRV infected leaves were taken through the virion purification described above in the "Purification of Viral Particles" section. Purification was carried out through the sucrose gradient step. Fifty gm each of non-CaMV infected and CaMV infected tendergreen mustard leaves were purified according to Hull et al, 1976. Each purified preparation was made (to final volume) 1 mM EDTA, 1% (w/v) SDS, and 0.5 mg/ml protease-K. They were then incubated 15 min at 65 C and cooled on ice. An equal volume of 10 mM tris-saturated phenol was added, the mixture vortexed gently for 1 minute, and then given a low speed centrifugation. The aqueous phase was collected and the nucleic acid extracted from it

with an equal volume of phenol/chloroform (1/1 (v/v)), and then with an equal volume of chloroform. The nucleic acids were precipitated from the final aqueous phase by adding sodium acetate to 0.3 M, at least 2 volumes of cold ethanol, and then storing at -20 C for 18 hr. The precipitate was collected by a low speed centrifugation and the pellet was dried in a stream of N_2. The pellet was resuspended in 10 mM tris, pH 7.3 containing 1 mM EDTA. If the nucleic acid was to be used for melting curve studies, the precipitate pellet was resuspended in 0.015 M sodium citrate containing 0.15 M NaCl.

Nucleases

RNase-free DNase and DNase-free RNase were kindly provided by Karen Haufler. They were prepared according to Maniatis et al, 1982. The DNase was made RNase free by heating at 100 C for 15 min. The RNase was made DNase free by eluting it from an agarose 5'-(4-aminophenyl-phosphoryl) uridine 2'(3') phosphate column with 0.02 M sodium acetate, pH 5.2.

Electrophoresis of Nucleic Acid

The purified nucleic acid samples were analyzed by electrophoresis in 0.7% (w/v) agarose, 2.2% (w/v) acrylamide tube gels according to the protocol of Civerolo and Lawson, 1978, and Hayward and Smith, 1972. Approximately 10 ug of

nucleic acid was added per 6 mm diameter x 11 cm long cylindrical gel that had been pre-electrophoresed for 30 min at 100 volts. Electrophoresis was carried out at 2.5 to 3.5 volt/cm for 10 to 13 hr in 0.05 M tris, 0.02 M sodium acetate, 0.002 M Na_2EDTA, 0.018 M NaCl buffer (TEAN) adjusted to pH 8.2 with glacial acetic acid. Gels were extruded and then stained in a solution of 1 ug ethidium bromide per ml sterile distilled water for 15 to 30 min and then examined in UV light. The gels were then placed in a 5 ug/ml DNase-free RNase solution, incubated for 45 min at room temperature and examined in UV light. The gels were then rinsed in sterile distilled water and placed in a 5 ug/ml RNase-free DNase solution containing 30 mM $MgCl_2$, incubated 45 min at room temperature and examined a final time in UV light.

Melting Curve Determination

Purified nucleic acid from CaMV and BBRRV were resuspended to 1.0 absorbance unit per ml in 1X SSC (0.15 M NaCl, 0.015 M sodium citrate). A thermal melting curve for each nucleic acid sample was then generated in a Gilford Dual Beam spectrophotometer with a cuvette holder equipped with a temperature programmer. Absorbance was monitored at 260 nm as the samples were heated slowly from 40 C to 99 C.

Determination of Serological Relationships

Agar gel double diffusion, as described for titering of BBRRV antiserum, was used to determine serological relationships. Purified (non-formaldehyde fixed) BBRRV, 100 ug/ml, was tested against antiserum to CaMV, carnation etched ring virus (CERV), dahlia mosaic virus (DaMV), and figwort mosaic virus (FMV). In the same way, 100 ug/ml purified CaMV was tested against the antiserum to BBRRV. All antisera were tested at the following dilutions 1:2, 1:4, 1:8, 1:16, 1:36, 1:64, and 1:128. Purified plant extract from non-infected tendergreen mustard leaves and non-infected 'Blueray' blueberry leaves were used as negative controls for CaMV and BBRRV respectively. No antigen was available for CERV, DaMV, or FMV. Twenty-four hr after adding the antiserum and antigen, the plates were examined in indirect light. To maximize sensitivity, the plates were then stained for several hr in a fresh solution of 9.86 mg L-Dopa (L 3,4-dihydroxyphenylalanine, Sigma Chemical Co. no. D-9628) per ml of 0.1 M phosphate buffer, pH 7.2, and examined again in indirect light.

Because ElISAs are generally more sensitive than agar gel double diffusion, a two-way indirect ELISA test using pepsin derived $F(ab')_2$ fragments was used to test for serological relationship between BBRRV and CaMV. The assays were carried out as described above except for the following

points. The assay was set up in test plate fashion. The $F(ab')_2$ fragments and whole IgG were used at 10, 5, and 2 ug/ml. Two ELISA plates were used, one plate with BBRRV $F(ab')_2$ fragments and BBRRV whole-IgG and a second plate with CaMV $F(ab')_2$ fragments and CaMV whole-IgG. The same four purified samples that were used in the agar gel double diffusion tests were diluted to 100 ug/ml in extraction buffer and added to each possible antiserum combination in both plates. Absorbance readings at 405 nm were taken at 15, 30, 60 min and after an overnight incubation at 4 C.

Association of Infectivity with Purified Virus

Two attempts were made to associate infectivity with viral particles. The first attempt was made in 1981. Purified BBRRV was adjusted in 0.01 M phosphate buffer, pH 7.2, to a concentration of 0.3 mg/ml based on the extinction coefficient E = 7 for CaMV (Hull, 1984) and rub-inoculated to ten 'Burlington' blueberry seedlings that had been dusted with 320 grit carborundum. Six seedlings received root and leaf inoculation and four seedlings received only leaf inoculation. Six additional seedlings were inoculated, but with buffer only: three with leaf inoculation and three with root and leaf inoculation.

The second attempt to associate infectivity with viral particles was done in January, 1987. Purified BBRRV was

taken through sucrose density gradients. The sucrose fraction containing virus was collected , put on ice, and immediately inoculated to 15 one-year-old 'Blueray' blueberry plants. Inoculation this time was by "slash" inoculation (Gonsalves, 1986). A new single edge razor blade was dipped into the sucrose/virus solution and then many (10 to 30) cuts several mm deep were made at a downward angle into the vascular tissue along the stem of each plant. The cuts were immediately wrapped in Parafilm^{R}. Estimated sucrose concentration of the fraction was 20% and virus concentration was 150 to 200 ug/ml. Ten additional control plants were similarly inoculated with a 20% sucrose solution.

All plants were observed for BBRRV symptoms. The rub-inoculated plants were tested by ELISA in 1986. Leaves were removed from the plants and taken through the inclusion body purification. These samples were then tested for BBRRV using indirect ELISA. A second ELISA was done in 1987. Leaves were removed from both the rub-inoculated and the slash inoculated plants and ground in 0.015 M extraction buffer containing 0.5% nicotine. These samples were also tested by indirect ELISA.

RESULTS

Yield Studies

The yield data are shown in Table 1. When ANOVA was applied to the whole bush harvest weight from the first harvest, no significant difference was found between BRRV infected and non-infected bushes (F-value = 2.19, p = 0.155). However, when ANOVA was applied to whole bush harvest weight from the second harvest or to combined harvests, both were significant (F-value = 25.05, p = 0.001 and F-value = 13.32, p = 0.001 respectively). A field observation at the time of harvest indicated that the diseased bushes were riper by approximately one week than the non-diseased bushes. This could account for the difference of significance between the two harvest dates. Total reduction in bush yield was 25%.

Berry size and weight were also reduced. There were more berries per cup harvested from diseased bushes than non-diseased bushes, indicating smaller berry size in fruit from diseased bushes. Cup counts for the first harvest were an average of 76.2 berries/cup from diseased bushes, 70.1 berries/cup from non-diseased bushes (F-value = 11.53, p = 0.003) and for the second harvest were an average of 109.6 berries/cup from diseased bushes and 91.8 berries/cup from non-diseased bushes (F-value = 28.35, p = 0.001). Berry size was reduced by 4.2% overall.

Table 1. Harvest data from ten BBRRV-infected and ten non-BBRRV-infected blueberry bushes in field plot near Nuncia, MI, 1986.

		Pounds of berries harvested per bush			Number of berries/cup		Grams of berries/cup	
Row-Bush	D/H[a]	July-28	Aug-11	Total	July-28	Aug-11	July-28	Aug-11
11-31	D	7.1	2.6	9.7	79	122	130	125
12-31	D	8.8	2.4	11.2	73	101	128	128
13-29	D	9.8	4.4	14.2	73	101	128	128
15-23	D	10.6	2.9	13.5	80	115	124	132
11-22	D	12.7	8.0	20.7	70	99	140	127
10-24	D	10.9	3.9	14.8	77	119	135	130
11-11	D	13.2	2.2	15.4	78	110	130	122
17-03	D	10.2	3.1	13.3	76	104	134	129
21-23	D	8.8	2.6	11.4	81	100	134	133
19-32	D	7.8	3.0	10.8	75	108	135	131
15-10	H	12.0	8.6	20.6	72	90	138	127
16-10	H	10.5	7.8	18.3	76	104	135	129
16-09	H	10.0	7.1	17.1	68	87	133	128
16-11	H	12.1	8.4	20.5	79	99	140	125
16-08	H	13.2	9.4	22.6	67	88	134	135
16-07	H	9.5	5.9	15.4	66	84	132	139
16-06	H	12.0	6.6	18.6	71	86	132	133
16-05	H	10.5	6.1	16.6	69	95	145	133
16-04	H	11.4	5.7	17.1	65	91	136	140
16-03	H	9.6	5.0	14.6	68	94	138	140

[a] D = BBRRV-infected bush, H = non-infected bush

Purification of Viral Particles

Using the starting purification scheme similar to that of Hull et al, 1979, a typical virus yield was 0.6 ug per starting gram of blueberry leaves. By adding n-butanol to the initial stirring step, as in the Lawson and Civerolo (1978) protocol, plus using 2-mercaptoethanol and thioglycolic acid instead of sodium sulfite as reducing agents, purification yield increased to 3-4 ug per gram of starting weight. No yield reduction was seen with the addition of a PEG concentration step or the substitution of expressing through cheesecloth for the initial low speed centrifugation step. These two changes greatly facilitated the purification process and reduced wear on the centrifuges. The final purification scheme is shown in Figure 1.

Two fairly sharp absorbance peaks consistently resulted from sucrose gradient centrifugation of purified virus preparations, (Figures 2 and 3). Electron microscopic examination of samples from these peaks revealed large spherical virions measuring 42-46 nm in diameter (Figure 4). There were also some virions present above and below the two peaks in the sucrose gradients but they were not as plentiful as in the peaks. In an attempt to further establish the location of virions in the gradient, two gradients, one loaded with a purified preparation from symptomatic leaves and one loaded with a purified

Extraction Buffer: 0.1 M Phosphate Buffer, pH 7.2
0.01 M 2-mercaptoethanol
0.005 M thioglycollic acid

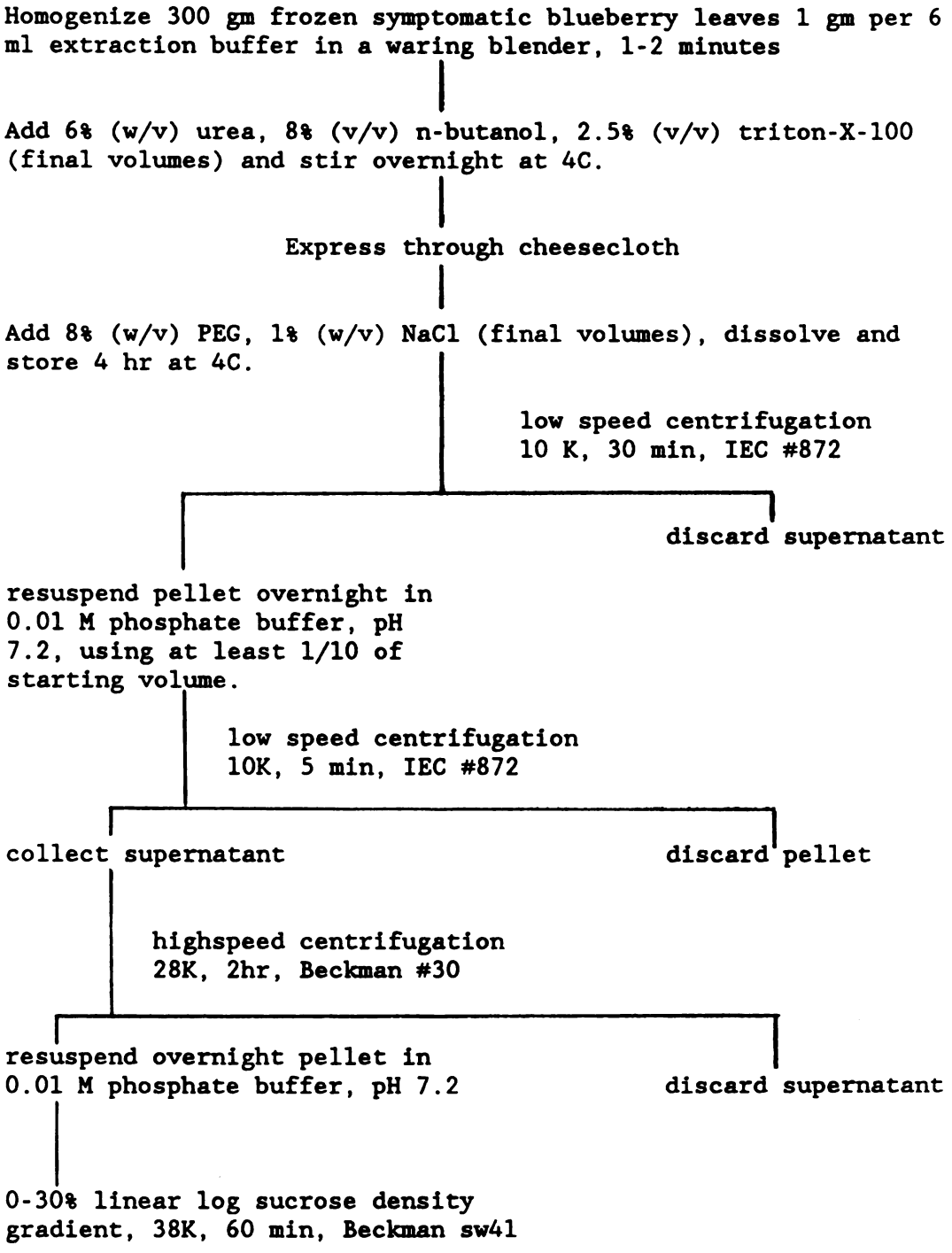
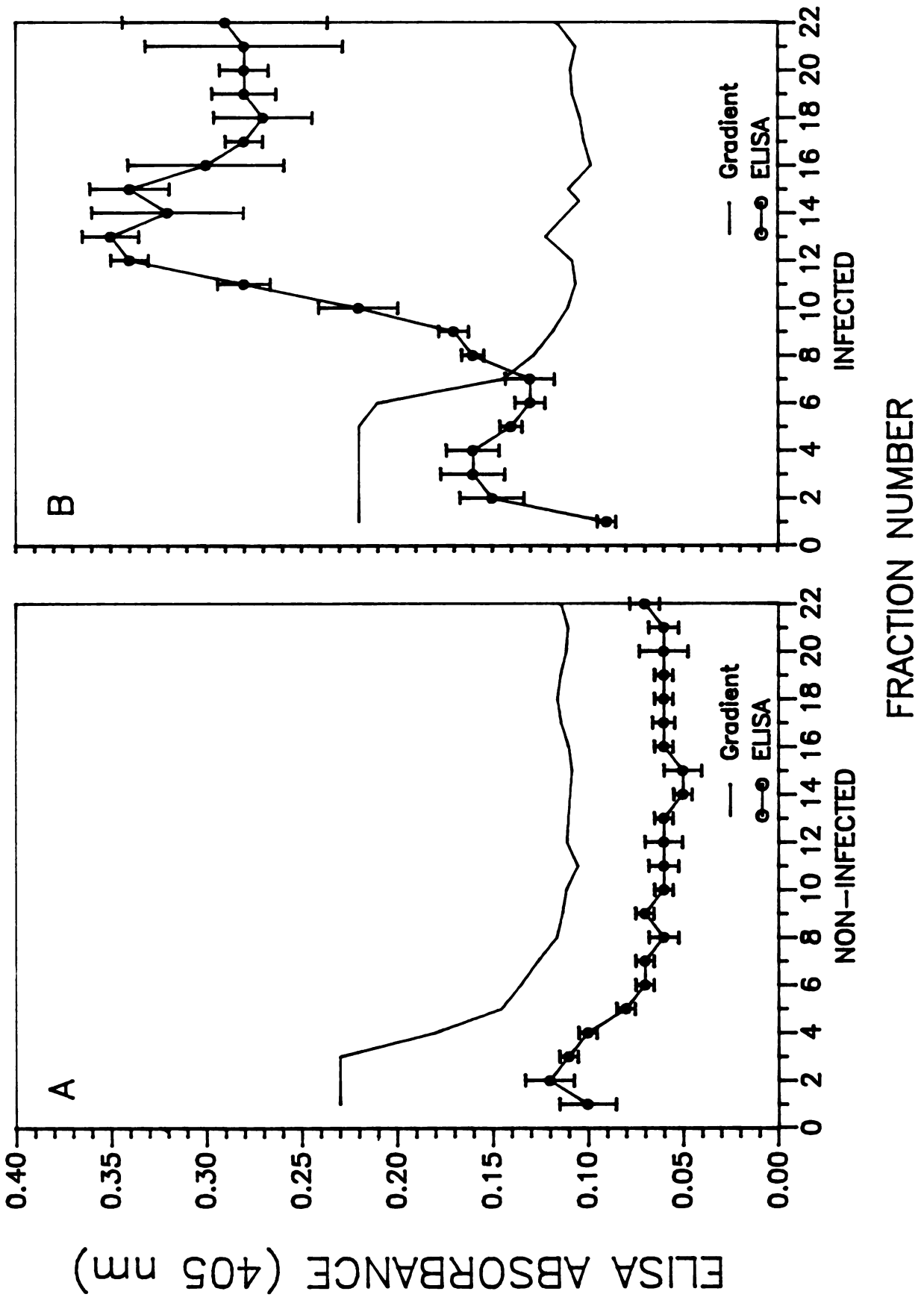


Figure 1. Purification of blueberry red ringspot virus.

Figure 2. Superimposed UV absorbance (254 nm) gradient profile and ELISA absorbance (405 nm) profile for purified BBRRV; A. From non-infected leaves. B. from BBRRV-infected leaves.


ELISA ABSORBANCE (405 nm)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A
B
Gradient
ELISA
0 2 4 6 8 10 12 14 16 18 20 22
NON-INFECTED
INFECTED
FRACTION NUMBER

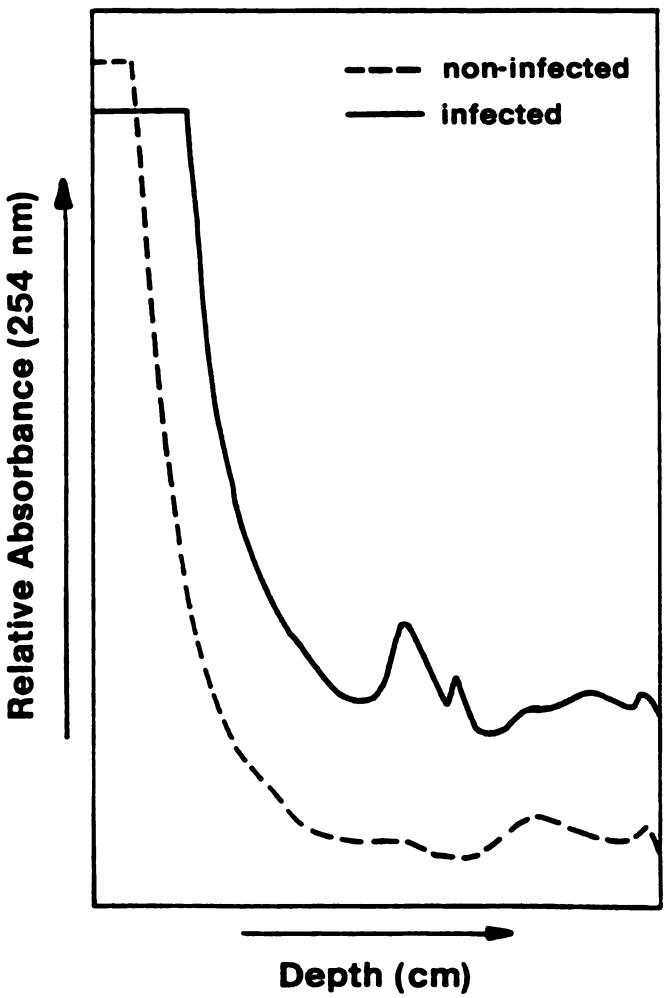


Figure 3. Superimposed UV absorbance (254 nm) linear-log sucrose gradient profiles from non-infected and BBRRV infected blueberry leaves.

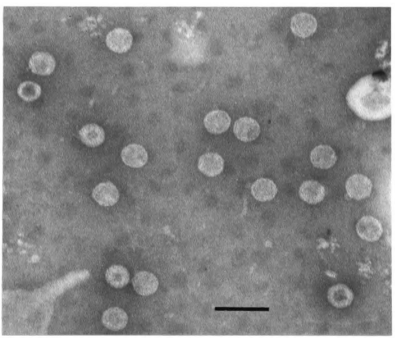


Figure 4. Transmission electron micrograph of ammonium molybdate stained purified BRRV virions. Bar = 100 nm.

preparation from non-infected leaves, were collected in 22 equal fractions. Samples from each of these fractions were tested by ELISA. The ultraviolet absorbance profile from these gradients was superimposed on a graph of the ELISA absorbance values for the corresponding fractions, (Figure 2). The ELISA absorbance values of the fractions from the purified non-infected leaves gradient was assumed to be due to non-specific reactions. If twice the mean of background is used as a threshold for positive values for the corresponding fractions from the infected leaf gradient, then fractions 8 through 22 are considered positive. This indicates that there is a large amount of virus in the final 60% of the gradient, but because the error bars are so large, it doesn't prove or disprove the number of peaks that are due to viral particles.

Figure 3 shows a superimposition of two gradient absorbance profiles. One gradient was loaded with a purification from BRRV symptomatic leaves and the other with a purification from non-infected leaves. Each gradient was loaded with a preparation from the same starting weight of blueberry leaves and scanned at the same absorbance level. Two diffuse peaks were evident in the profile from the non-infected leaves. The first peak, in the middle of the gradient, corresponded to the general area of the viral peaks, however, it was much smaller than the viral peaks. The first diffuse peak could have been a contaminating protein that co-migrated in the gradient with the BRRV

virions and thus is not seen as a separate entity because of its low concentration relative to virion concentration. Another explanation is that the concentration of this co-migrating protein increased with viral infection and resulted in the first "viral" peak.

A second diffuse peak could often be seen at the end of both the infected and non-infected preparation gradients. However, this was probably due to contaminating plant derived proteins because this peak was seen in equal concentrations in both gradients.

Antiserum Production

No antiserum was produced when purified virus emulsified in Freund's adjuvent was injected into a rabbit. However the virus was moderately immunogenic when fixed in 0.5% formaldehyde (v/v) prior to emulsification and injection (Gillett and Ramsdell, 1984). After four intramuscular injections, an antiserum was obtained with a titre of 1:128 against 100 ug/ml purified virus in agar gel double diffusion tests. Crude sap from diseased plants did not react in agar diffusion tests.

Sample Preparation

Crude Extraction of Sample

Simple homogenizing of blueberry leaves in extraction

buffer failed to distinguish between non-infected and infected symptomatic leaves by ELISA. It was presumed that the virions were mostly trapped in inclusion bodies and thus not available for binding to IgG in the ELISA plate. Thus, attempts were made to find an easy partial purification protocol that could be routinely used to treat samples before their testing by ELISA.

Partial Purification of Samples

The first protocol tested was the beginning 2/3 of the actual purification procedure. This procedure as described above, the extraction, addition of n-butanol, urea, triton-X, stirring, straining, PEG, and low speed centrifugation, usually resulted in a 100% detection of symptomatic leaf tissue with typical absorbance values of 0.50 vs. 0.06 for infected and non-infected leaves, respectively. Although the results were encouraging, the "mini-purification" was too cumbersome to carry out on numerous samples in one day and also did not yield positive absorbance values high enough above background to be as sensitive an assay as was desired. The assay was still unable to detect virus in symptomless tissue.

It was possible that the "mini-purification" was releasing the virions from the inclusion bodies and concentrating them. Thus, a protocol that purified inclusion bodies might also work for BBRRV dectection. This is why the slightly modified protocol of Lawson and Hearon,

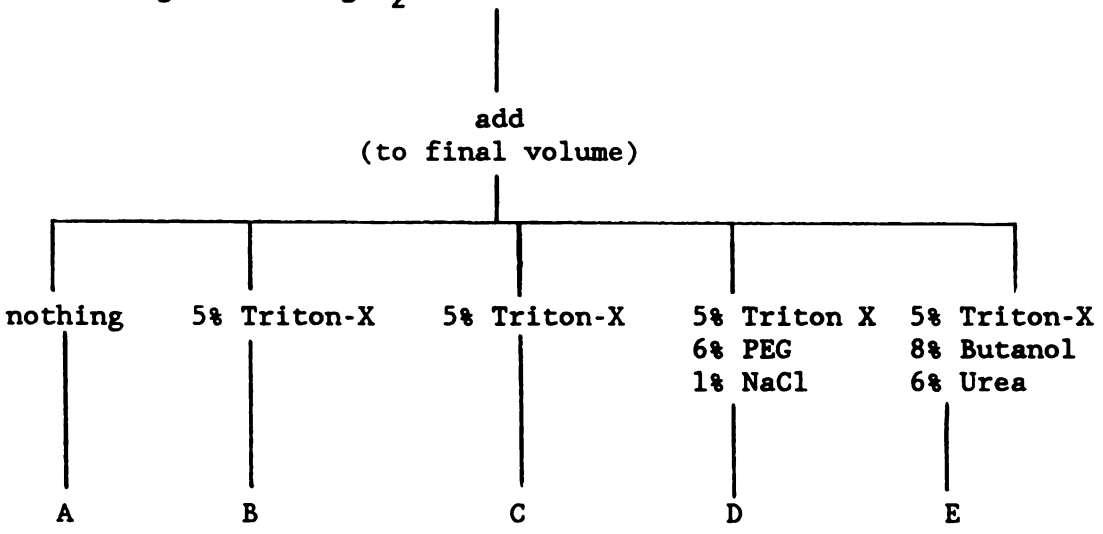
1980, for purifying CERV inclusion bodies was investigated. This simplified procedure could also generally detect 100% of the symptomatic leaf samples with average ELISA absorbance values of 0.25 vs. 0.02 for infected and non-infected leaves, respectively. This purification was also much easier to carry out than the partial purification of virions. However, the absorbance values were still not high enough to be a satisfactory assay for routine use. A comparison of these two protocols is shown in Table 2.

An attempt was made to maximize detection by modifying the inclusion body purification with elements of the viral particle purification. Modifications were tried according to the flow chart shown in Figure 5. Three variations were tested: resuspending the final pellet in half the normal amount of extraction buffer (sample C), adding 6% PEG and 1% NaCl (final volumes) along with the triton-x (sample D), and adding 8% butanol and 6% urea (final volumes) along with the triton-X (sample E). The resulting absorbance values are given in Table 3. A ratio derived by dividing the mean absorbance of the diseased sample by the mean absorbance of the healthy sample makes it easy to compare the benefits of each modification. The best modification (highest resulting ratio) was to simply resuspend the final pellet in less volume. The additions of PEG/NaCl or butanol/urea did not yield better absorbance readings in the inclusion body protocol.

Table 2. Comparison of partial purification of BBRRV to purification of inclusion bodies.

Treatment Step	Partial purification of virions	Purification of inclusion bodies
Homogenize blueberry leaves	2 gm in 10 ml 0.1 M phosphate, pH 7.2	2 gm in 10 ml 0.1 M Tris, pH 7.2 with 0.001 M MgCl2, 0.025 M KCl
Add (final volume)	2.5% triton-X 6% urea 8% n-butanol	5% triton-X
shake at 4C	overnight	3 hr
express through cheesecloth	yes	yes
add 8% PEG, 1% NaCl (final volumes)	yes	no
shake overnight, 4C	yes	no
low speed centrifugation	yes	yes
resuspend in 1 ml extraction buffer	yes	yes

With scissors, mince 10 gm blueberry leaf from each of three BBRRV-infected and three non-infected bushes. Divide into 2 gm aliquots. Homogzenize 1:10 (w/v) in 0.1 M Tris, pH 7.2, containing 0.001 M $MgCl_2$ and 0.025 M KCl.



Shake all samples 3 hr, 4C then express through cheesecloth and centrifuge at low speed. Resuspend each pellet in 2 ml extraction buffer except for sample C which is suspended in 1 ml extraction buffer. Test in indirect ELISA.

Figure 5. Modifications of inclusion body purification tested in an attempt to increase ELISA detection of BBRRV.

Table 3. ELISA absorbance values of samples from Figure 5.

Sample	Healthy tissue (duplicate wells) 1	2	3	Diseased tissue (duplicate wells) 1	2	3	Ratio of diseased to healthy (average)
A[a]	.11[f]	.10	.11	.14	.12	.11	1.2 : 1
	.10	.10	.10	.12	.12	.10	
B[b]	.08	.13	.10	.70	.78	.59	7.3 : 1
	.07	.10	.08	.66	.69	.52	
C[c]	.05	.05	.05	.49	.53	.48	9.0 : 1
	.05	.07	.05	.43	.48	.28	
D[d]	.05	.07	.06	.44	.60	.44	6.4 : 1
	.06	.11	.07	.46	.36	.38	
E[e]	.18	.17	.16	.42	.40	.37	2.3 : 1
	.17	.16	.14	.33	.35	.38	

[a]A = no additives

[b]B = Triton-X-100 5%, resuspended final pellet at 1 gm/1 ml

[c]C = Triton-X-100 5%, resuspended final pellet at 1 gm/0.5 ml

[d]D = Triton-X-100 5%, PEG 6%, NaCl 1%, resuspended final pellet at 1 gm/1ml

[e]E = Triton-X-100 5%, 8% Butanol, 6% Urea, resuspended final pellet at 1 gm/1ml

[f] Absorbance values read at A_{405}nm after 3 hour incubation of substrate.

DAS-ELISA v.s. Indirect ELISA

Since an indirect method of ELISA which used $F(ab')_2$ fragments had been reported to be more sensitive than the traditional DAS-ELISA (Barbara and Clark, 1982), the difference in the ability of these two assay methods to detect BRRV was tested. Indirect ELISA was more sensitive than DAS-ELISA, Table 4. Indirect ELISA absorbance values averageing 0.73 absorbance units for symptomatic blueberry leaf samples v.s. 0.03 absorbance units for non-infected leaves. With the same samples, average absorbance values from DAS-ELISA were 0.30 and 0.04 for symptomatic and non-infected leaves, respectively.

Extraction of Leaf Tissue in High Molarity Buffer

While developing an ELISA for blueberry Oregon scorch virus, Martin and Bristow, 1987, found that blueberry leaves ground in standard ELISA extraction buffer (0.015 M PBS) dropped the pH of the solution from 7.4 to 3.7. To overcome this pH drop which might interfere with antigen-antibody binding, stronger molarity buffers with and without nicotine were tested. Both the higher molarity buffer and the addition of nicotine produced higher ELISA absorbance values for scorch infected tissue.

Because of this phenomenon, samples of non-infected and BRRV infected blueberry leaves were ground in the standard 0.015 M extraction buffer and the pH tested. The pH of the extraction buffer was 7.4, the pH of the non-infected tissue

Table 4. Double antibody sandwich (DAS) ELISA versus indirect $F(ab')_2$ ELISA for detecting BBRRV in blueberry leaves.

Blueberry leaf sample[a]	$F(ab')_2$ ELISA[b] (duplicate wells)	DAS-ELISA[b] (duplicate wells)
non-infected-1	0.03/0.03[c]	0.05/0.04
non-infected-2	0.02/0.03	0.03/0.02
BBRRV infected-1	0.72/0.79	0.37/0.33
BBRRV infected-2	0.61/0.81	0.23/0.25

[a] Samples taken through inclusion body purification

[b] Optimal antiserum reagents were used as follows: for $F(ab')_2$ ELISA: $F(ab')_2$ = 2 ug/ml, IgG = 2 ug/ml; for DAS-ELISA: coating IgG = 1 ug/ml, conjugated IgG = 1:800 dilution.

[c] Absorbance values read at A_{405}nm after 2 hr incubation of substrate.

was 5.9, and the pH of the infected tissue was 3.8. This could explain why the previous trials of grinding BRRV infected leaves in standard extraction buffer failed to yield any positive absorbance values. Because of this, BRRV infected tissue was again ELISA-tested after a simple grinding in buffer, this time using the buffers used by Martin.

The resulting absorbance values are shown in Table 5. Of the three buffers tested, simply adding 0.5% (v/v) nicotine to the standard 0.015 M ELISA extraction buffer yielded the highest ratio of diseased to healthy absorbance values. This also worked as well or better than inclusion body purification. An aliquot of BRRV symptomatic tissue was divided in two equal parts, half was ground in extraction buffer containing 0.5% nicotine and half was taken through the inclusion body purification. When they were tested by ELISA, the ratio of absorbance values for infected/non-infected tissue was 9:1 for inclusion body purification and 10.6:1 for grinding in extraction buffer with nicotine. It is interesting to note that all methods failed to detect virus in symptomless leaf samples taken from symptomless branches on the bush.

Detection of BRRV in Various Blueberry Tissues

In April 1985, five samples each of roots, buds, and blossoms from infected and non-infected bushes were ground in standard ELISA extraction buffer (no nicotine) and tested

Table 5. The effect of high molarity buffers with or without nicotine alkaloid on detection of BBRRV in crude blueberry leaf extracts by $F(ab')_2$ ELISA at A_{405}.

	0.015M		0.15M		0.1M	
Leaf samples	EB[a] without (ratio)[d]	EB with (ratio)	EB without (ratio)	EB with (ratio)	Borate[c] without (ratio)	Borate with (ratio)
non-infected	.045	.07	.08	.065	.10	.055
symptomatic	.045 (1:1)	.55 (7.8:1)	.26 (3.2:1)	.40 (6.2:1)	.21 (2.1:1)	.22 (4:1)
non-symptomatic	.045 (1:1)	.08 (1.1:1)	.085 (1:1)	.08 (1.2:1)	.04 (.4:1)	.065 (1.2:1)

[a] EB = extraction buffer: 0.015 or 0.15 M phosphate, 0.8% (w/v) NaCl, 2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) egg albumin.

[b] with or without 0.5% (v/v) nicotine alkaloid added to the buffer

[c] Borate = 0.1 M borate buffer, pH 7, with 2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) egg albumin.

[d] ratio of absorbance reading of sample to absorbance reading of non-infected control; ratio of 2:1 or greater is positive sample.

with DAS-ELISA. No positives were found. Tissue from these samples was then treated according to the inclusion body protocol and tested in DAS-ELISA. Again, no positives were found for the blossom and bud samples. However, if the ELISA substrate was allowed to incubate overnight at 4 C, some of the root samples showed very weak positive absorbance values. Twice the mean absorbance of the non-infected root samples was 0.49 (if the mean plus three standard deviations were to be used, x+3s, that value was 0.39). The absorbance values, in duplicate, for the five infected plant root samples were 0.43/0.47, 0.49/0.48, 0.37/0.42, 0.27/0.26, 0.49/0.51. By using as a criterion for the positive-negative threshold twice the mean of healthy, one sample was barely positive. By the more liberal x+3s standard, three samples were positive.

In February of the following year, four root, four vegetative bud, three fruit bud, and three bark samples were tested. Samples were again taken through the inclusion body purification but this time tested in indirect ELISA. Zero of four root samples and zero of three fruit bud samples were positive. Of the three bark samples, only one was positive (absorbance 0.08/0.02 diseased and healthy, respectively), although all three bark samples had symptomatic red spots on them. Of the four vegetative bud samples, one was positive, (absorbance 0.06/0.02 diseased and healthy respectively). The vegetative buds were very small and had to be carved off of the branch, therefore,

they had bark attached to them. This could account for the vegetative bud sample being positive.

Of the two root samplings, it is surprising that the second set had no positive values. This may indicate that there was virus in the roots but the distribution of it may have been uneven. More sampling is needed to draw any strong conclusion concerning the location of BRRV in the plant.

Sedimentation Coefficient Determination

The absorbance profiles for the three marker viruses and BRRV are shown in Figure 6. Blueberry red ringspot virus consistently had two virus peaks in linear-log (0-30%) sucrose gradients. The sedimentation coefficient of each peak was 212 $\pm$ 5% and 275 (by extrapolation), respectively (Figure 7.) The reported sedimentation coefficients for members of the caulimovirus group range from 208 for CaMV to 254 for dahlia mosaic virus.

Buoyant Density of the Virus Particle

Further purification of the virus preparation through CsCl step gradients revealed two sharp peaks of densities, 1.30 and 1.40 gm/cc, Figure 8. Both of these peaks contained a high concentration of virions. Electron microscopy revealed no discernable morphological difference

Figure 6. Sedimentation coefficient determination. UV absorbance profile of sucrose gradient for three viruses of known s value and for purified BBRRV (TRSV-M = tobacco ringspot virus, middle component; TRSV-B = TRSV, bottom component; SBMV = southern bean mosaic virus; CaMV = cauliflower mosaic virus; BBRRV1 and 2 = first and second peak from BBRRV gradient).

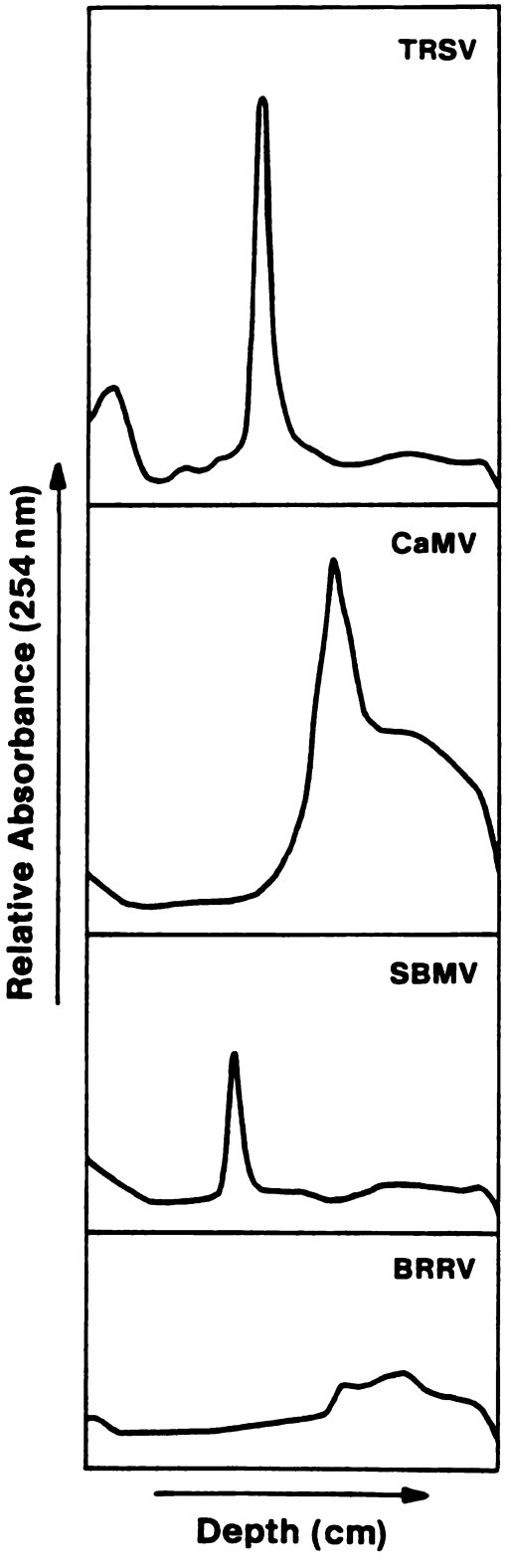
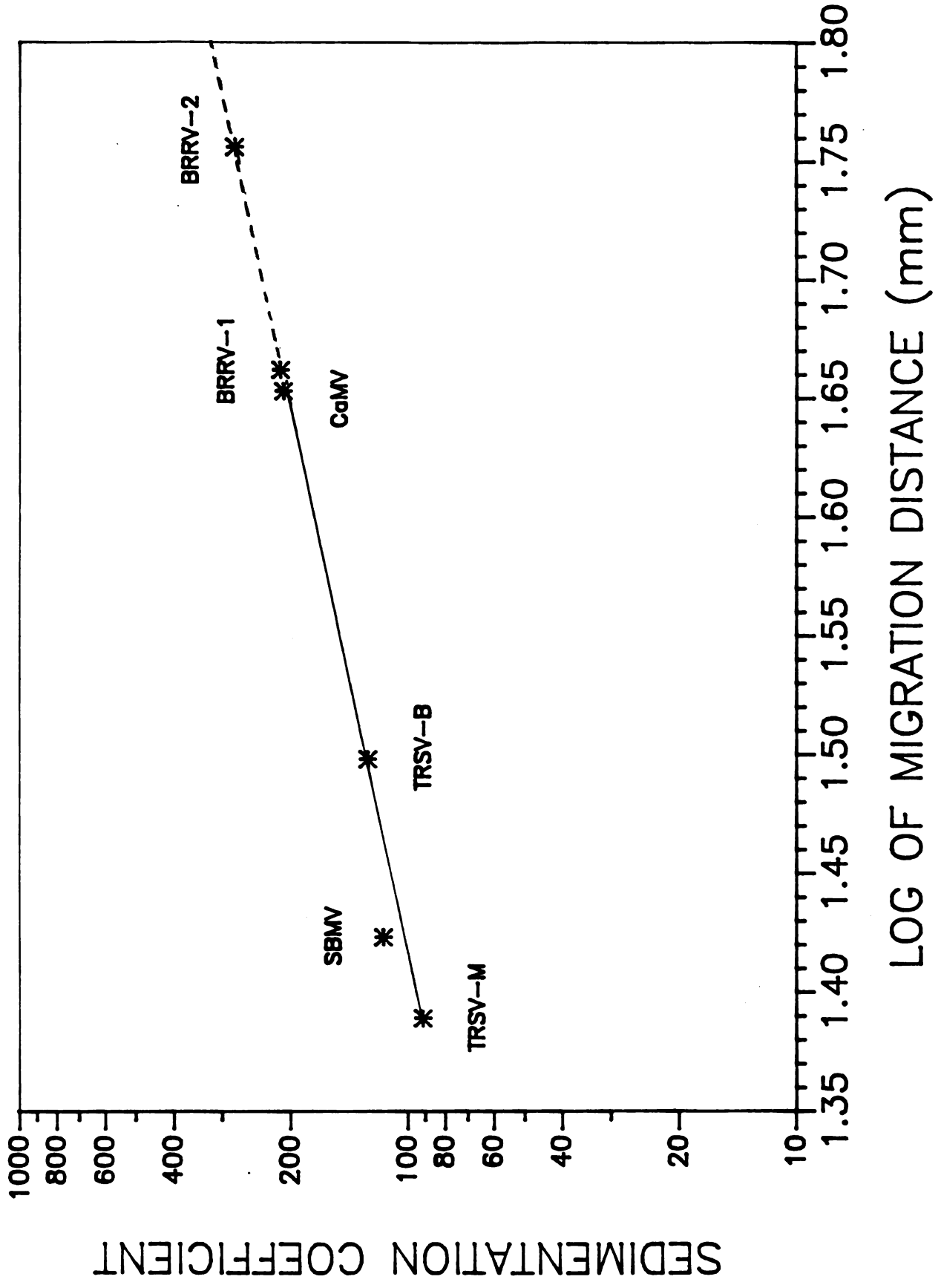

TRSV
CaMV
SBMV
BRRV
Relative Absorbance (254 nm)
Depth (cm)

Figure 7. Sedimentation coefficient determination: distance migrated vs. sedimentation coefficient.


SEDIMENTATION COEFFICIENT
1000
800
600
400
200
100
80
60
40
20
10
1.35
1.40
1.45
1.50
1.55
1.60
1.65
1.70
1.75
1.80
LOG OF MIGRATION DISTANCE (mm)
BRRV-2
BRRV-1
CaMV
TRSV-B
SBMV
TRSV-M

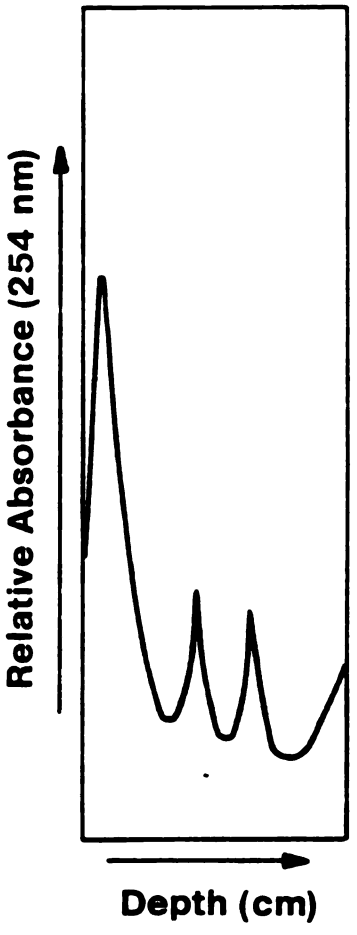


Figure 8. Buoyant density determination: Purified BBRRV ultracentrifuged in isopycnic gradients.

between the two species of particles, however, both peaks contained broken particles. Fractions either side or between the peaks did not contain virions. A sibling tube containing CaMV resulted in a single peak with a density of 1.35 g/cc (Figure 9), which is the reported buoyant density of CaMV, Hull 1984. A preparation from non-BRRV infected blueberry plants purified similarly to BRRV and then run in CsCl gradients did not reveal any virions. Only one peak with a density of 1.20 g/cc was observed.

Electrophoretic Mobility of Whole Particle

At pH 6, 7, and 8, both the purified virus and purified non-infected plant extract had material that migrated toward the anode and absorbed the CBB stain. However, the purified virus sample had an extra spot of intensly stained material, presumed to be the virus particles. This spot was still present at pH 5.0, and had migrated less distance toward the anode than at the higher pH's. At pH 5.0, no material was seen migrating from the well containing purified non-infected plant material. At pH 4.0, neither well produced any stained material outside the wells. This was determined to be the isoelectric point of purified BRRV particles. At pH 3.0, a faintly stained line from the well containing purified virus could be seen migrating in the direction of the cathode, while no stained material was seen migrating from the well containing purified non-infected plant

material. Finally, at pH 2.0, both samples contained stained material migrating to the cathode.

An electrophoretic mobility measurement was made from the trial at pH 5.0 according to the following formula:

$$u = \frac{\text{distance of migration (cm)/electrophoresis time(sec)}}{\text{voltage applied (volt)/ gel length (cm)}}$$

The electrophoretic mobility, u, for BRRV was calculated to be 21.5×10^{-6} $cm^2 sec^{-1} volt^{-1}$.

Protein Coat Molecular Weight

Multiple electrophoretic runs were carried out using two different marker kits and two different batches of purified virus and plant proteins. All trials yielded similar results. Two representative gels are shown in Figures 9 and 10. Figure 9 was stained with coomasie brilliant blue (CBB) and Figure 10 was stained with silver. The importance of a purified non-infected plant control is clearly demonstrated. Several (in CBB) to many (in silver) bands can be seen in the non-infected plant control lanes. However, one major band at approximately 44,000 MW (Figure 11) and a faint band at 101,000 MW (by extrapolation) were consistently found in the lanes loaded with purified virus and not found in the non-infected control lanes. The major coat protein MW of CaMV and mirabilis mosaic virus is

Figure 9. Polyacrylamide vertical slab gel stained with coomasie brilliant blue: lane 1 = marker proteins; lane 2 = SDS-treated purified BBRRV coat protein from blueberry; lane 3 = SDS-treated purified non-infected blueberry.

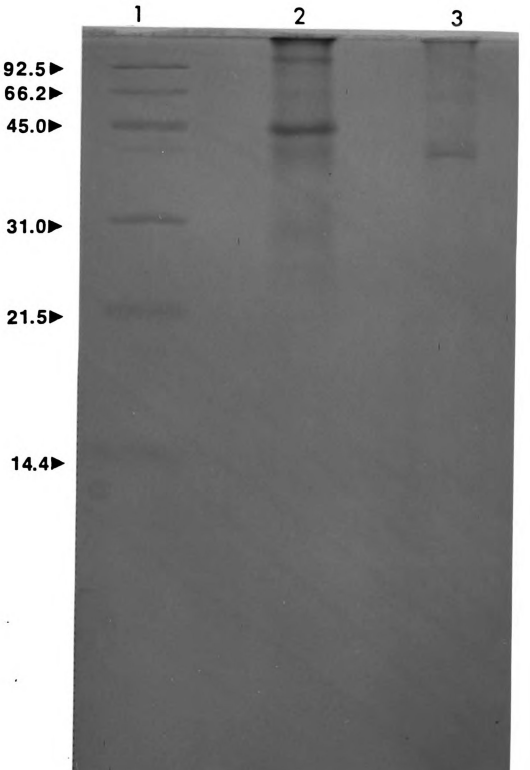


Figure 9

Figure 10. Polyacrylamide vertical slab gel stained with silver: lanes 1, 2 = marker proteins; lanes 3,5,7,9 = blank; lanes 4,8 = 2 and 4 ul, respectively, SDS-treated purified BBRRV coat protein from blueberry lanes; 6, 10 = 2 and 4 ul, respectively, SDS-treated purified non-infected blueberry.

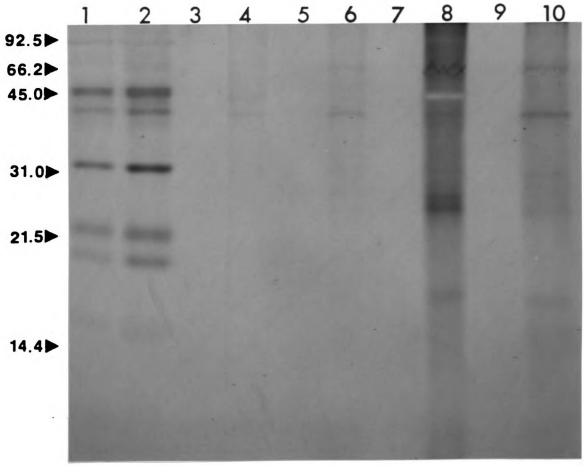
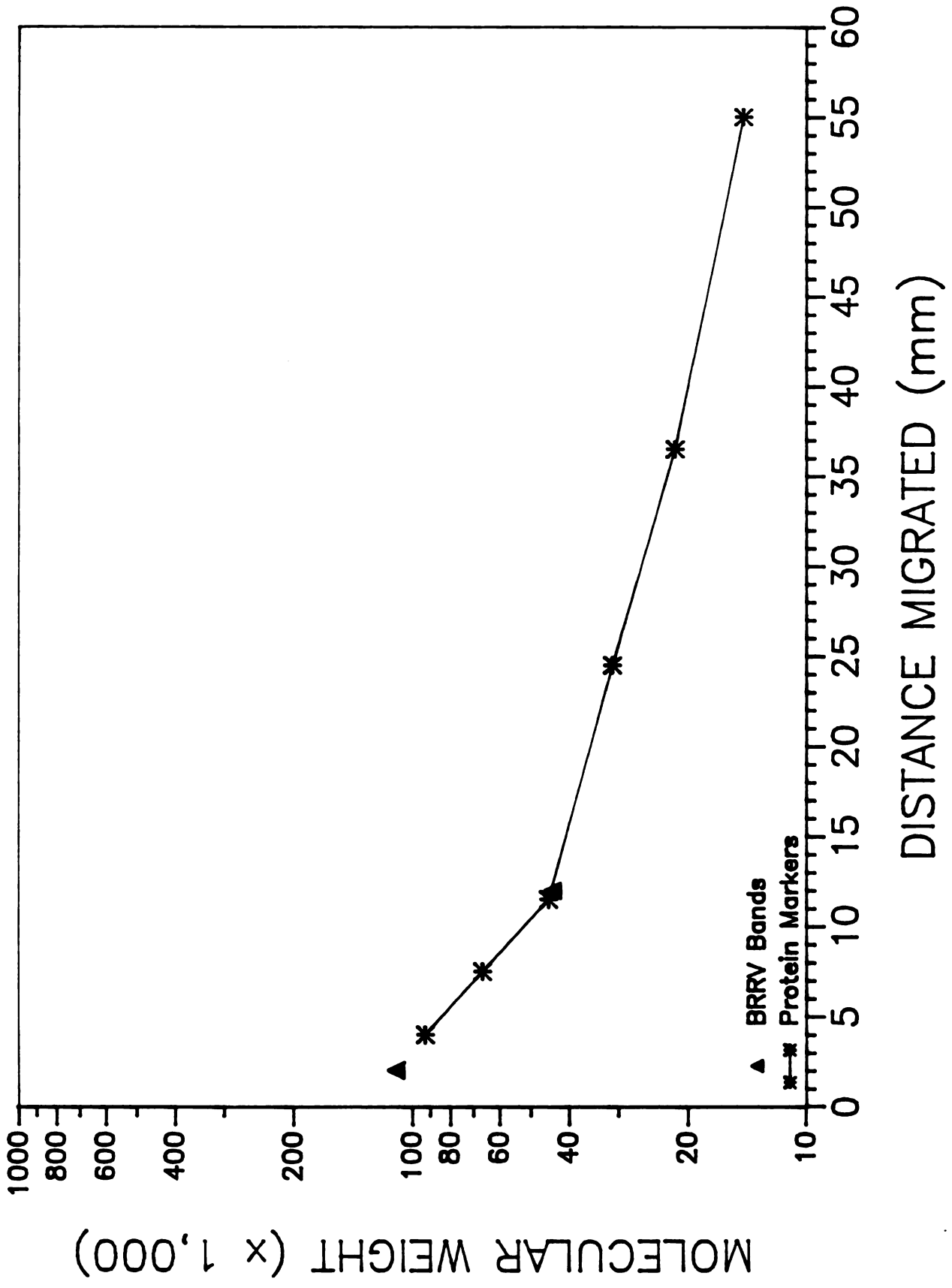


Figure 10

Figure 11. Coat protein determination: distance migrated vs. molecular weight of protein markers and SDS-treated BRRV; measurements taken from gel pictured in Figure 9.


MOLECULAR WEIGHT (x 1,000)
1000
800
600
400
200
100
80
60
40
20
10
0
5
10
15
20
25
30
35
40
45
50
55
60
DISTANCE MIGRATED (mm)
BRRV Bands
Protein Markers

reported as 42,000 and 32,000 MW, respectively. For BRRV, the 44,000 MW band is probably due to the coat protein and the 101,000 MW band could be due to inclusion body protein or some other protein associated with the virus.

Nucleic Acid Studies

Electrophoresis of Nucleic Acid

There were no bands visible in the tubes loaded with purified material from non-infected blueberry and mustard plants. The BRRV and CaMV nucleic acid each produced 2 to 3 bands per gel. In the case of CaMV, the bands were probably due to circular and linear forms of the nucleic acid (Civerolo and Lawson, 1978). Not enough is known about BRRV nucleic acid to draw conslusions about the nature of it's bands. The nucleic acid from the two viral samples seemed to co-migrate, however, they only moved into the top third of the gel so resolution was minimized.

The important data from this experiment indicate that the bands were not affected by DNase-free RNase but were totally degraded by RNase-free DNase. This indicates that the nucleic acid in BRRV is DNA.

Melting curve Studies

The resulting melting curves for CaMV and BRRV nucleic acid are shown in Figure 12. The sharp rise in each indicates they are double stranded. The Tm for CaMV was 92 (reported value is 87, Hull 1984), the Tm for BRRV was 86.

Serological Relationships

Purified BRRV from infected blueberry leaves (100 ug/ml) and purified plant sap from non-infected leaves gave no reactions in agar gel double diffusion tests with antiserum to CaMV, CERV, DaMV, and FMV. However, in its homologous test, BRRV reacted against BRRV antiserum dilutions of 1:16, 1:32 and 1:64 (Table 6, Figure 13). There were no reactions of CaMV or negative controls to BRRV antiserum at any dilution. Purified CaMV reacted to the 1:2 and 1:4 dilutions of DaMV antiserum (Figure 14) and also to its homologous antiserum at dilutions 1:2 through 1:128. At CaMV antiserum dilutions 1:2 and 1:4, a healthy control reaction was seen for all four purified samples, Figure 15-A. The healthy reaction disappeared at antiserum dilutions of 1:8 and higher, Figure 15-B. Table 6 is a summary of the dilution end points for the various antiserum and antigen combinations. As determined by agar gel double diffusion tests, there is no obvious relationship between BRRV and CaMV, CEV, DaMV, or FMV.

Figure 12. **Thermal melting curves for purified BBRRV and CaMV nucleic acid.**

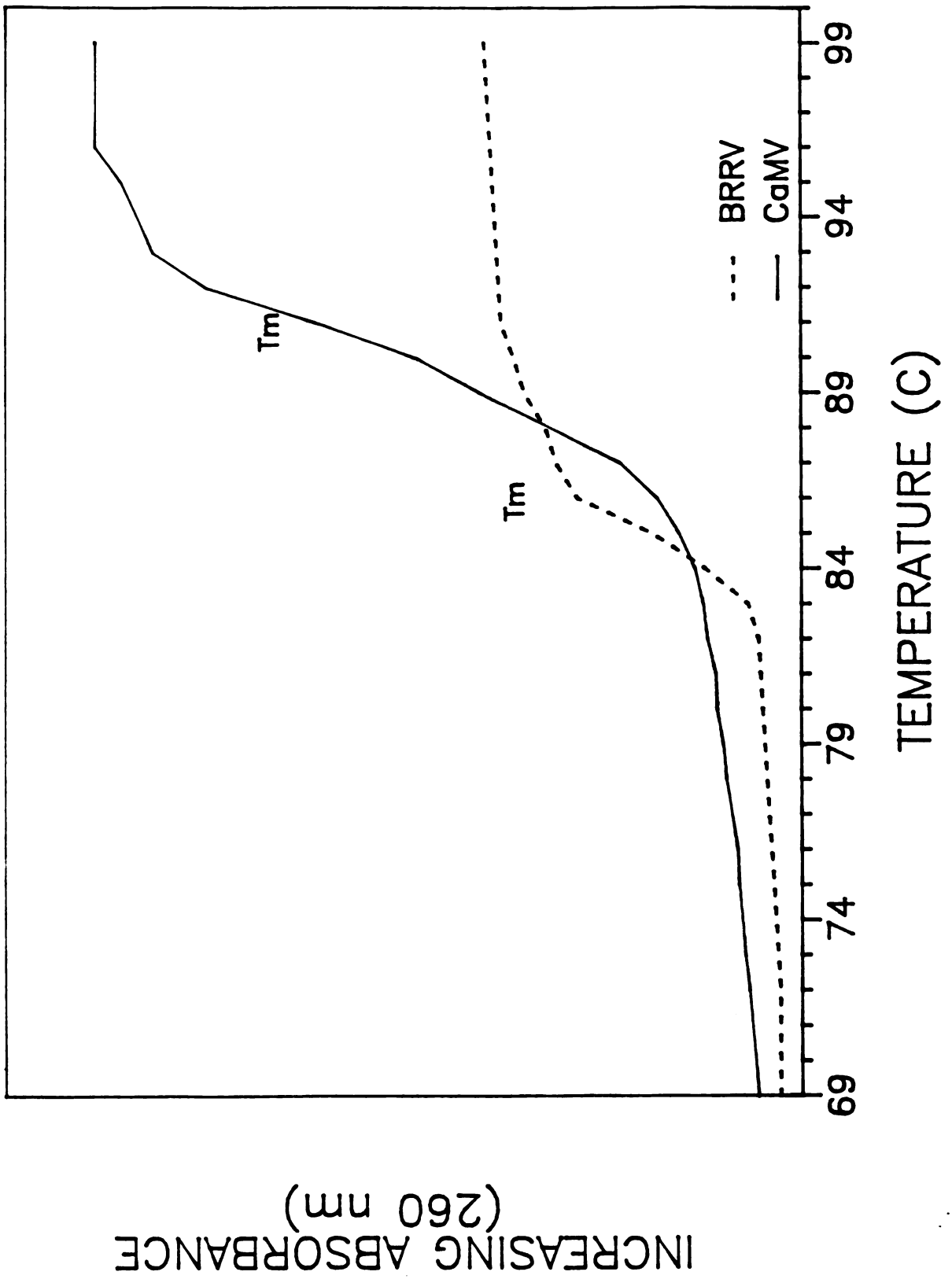

INCREASING ABSORBANCE
(260 nm)
TEMPERATURE (C)
69
74
79
84
89
94
99
BRRV
CaMV
Tm
Tm

Table 6. Titre of purified CaMV, BBRRV, mustard leaves and blueberry leaves to antiserum against CaMV, BBRRV, and DaMV.

	Antiserum to:[a]																			
	CaMV							BBRRV							DaMV					
Purified antigen[b]	2[c]	4	8	16	32	64	128	2	4	8	16	32	64	128	2	4	8	16	32	64	128
CaMV	+[d]	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Mustard	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BBRRV	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Blueberry	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Antisera to CERV and FMV were also tested but failed to precipitate with any of the antigens.

[b] Purified tendergreen mustard leaves and purified non-infected blueberry leaves were used as negative controls. The concentration of the negative controls was adjusted to the same starting gm/ml as the purified virus. Purified virus was 100 ug/ml.

[c] Antisera were diluted two-fold from 1:2 through 1:128.

[d] + = precipiten line formed; - = no line formed.

Figure 13. Agarose gel double diffusion test using BBRRV antiserum at a 1:2 dilution; 1 = purified non-infected blueberry, 2 = purified BBRRV at 100 ug/ml, 3 = purified CaMV at 100 ug/ml, 4 = purified non-infected tendergreen mustard.

Figure 14. Agrose gel double diffusion test using DaMV antiserum at a 1:2 dilution; well designations as in Figure 13.

Figure 15. Agarose gel double diffusion test using CaMV antiserum, A = at a 1:2 dilution and B = at a 1:4 dilution, well designation as in Figure 13.

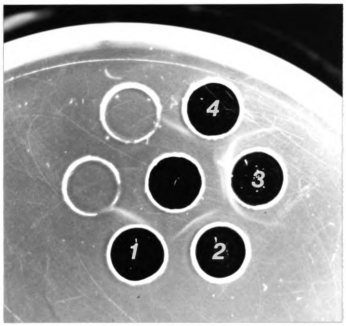


Figure 15-A

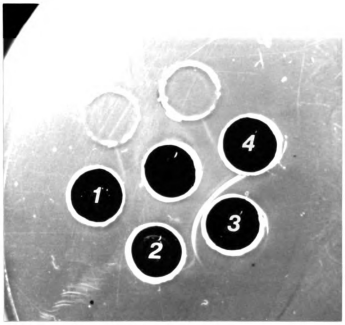


Figure 15-B

However, depending on how one defines serological relationships, BRRV and CaMV may be slightly related. In the $F(ab')_2$ ELISA using BRRV antiserum, a positive reaction occured for both purified BRRV and CaMV, Table 7. Although there was a strong background absorbance for purified non-infected blueberry, the absorbance reading of the purified BRRV sample was 2.2 times higher than the negative control at the highest $F(ab')_2$ and IgG dilutions (10 ug/ml). The heterologous purified CaMV reaction was actually stronger relative to its negative control of purified non-infected mustard plants than was the homologous BRRV reaction. At the $F(ab')_2$ dilution of 10 ug/ml and IgG dilutions of 10, 5, and 2 ug/ml, the CaMV absorbance was 11 to 12 times the absorbance of the corresponding negative control. At the next dilution of $F(ab')_2$, 5 ug/ml, the absorbance quickly dropped to two or less times the negative control absorbance readings.

In the $F(ab')_2$ ELISA test using CaMV antiserum, the homologous CaMV absorbance reaction was strong for all the antiserum dilutions and ranged from 10 to 21 times the absorbance of the negative control, (Table 8). However, in this case, the heterologous BRRV absorbance reaction never went above 1.5 to 1.8 times the corresponding negative control. This was at the most dilute CaMV antiserum dilutions and perhaps the absorbance would have been higher with even more dilute antiserum, but this was not tested.

Table 7. Absorbance values (A_{405}nm) of homologous and heterolgous reactions of CaMV and BBRRV to BBRRV antiserum.

		BBRRV Antiserum								
		Concentration of F(ab')2								
		10			5			2		
Purified antigen[a]	Absorbance read at (min)	Concentration of whole IgG (ug/ml)								
		10	5	2	10	5	2	10	5	2
CaMV	15	04	03	02	02	02	02	02	02	02
	30	07	06	04	03	02	03	03	03	02
	60	12	09	06	03	03	03	04	03	02
	on	57	33	24	07	04	05	06	04	02
mustard	15	05	02	02	02	02	02	02	02	05
	30	05	02	03	03	02	02	02	03	05
	60	05	03	03	03	02	03	03	03	05
	on	04	03	02	03	02	05	03	04	02
BBRRV	15	44	35	25	10	10	08	07	08	05
	30	114	108	69	24	23	20	18	19	08
	60	165	161	115	46	43	37	32	33	13
	on	175	176	176	158	155	145	128	131	55
blueberry	15	20	22	15	07	06	07	08	08	05
	30	57	55	37	16	15	12	17	17	11
	60	102	99	64	29	26	22	29	31	18
	on	174	176	172	119	112	96	119	127	77

[a] Purified tendergreen mustard leaves and purified non-infected blueberry leaves were used as negative controls. The concentration of the negative controls was adjusted to the same starting gm/ml as the purified virus. Purified virus was 100 ug/ml.

[b] ON = absorbance reading taken after overnight incubation of ELISA plate at 4C.

Table 8. Absorbance values (A_{405}nm) of homologous and heterolgous reactions of CaMV and BBRRV to CaMV antiserum.

		CaMV Antiserum								
		Concentration of F(ab')2								
		10			5			2		
Purified antigen[a]	Absorbance read at (min)	Concentration of whole IgG (ug/ml)								
		10	5	2	10	5	2	10	5	2
CaMV	15	74	67	59	74	71	62	70	83	71
	30	163	159	152	165	162	147	158	165	149
	60	176	175	174	177	177	175	176	177	174
	on	175	175	175	176	176	176	175	176	175
mustard	15	07	05	03	05	05	04	04	05	05
	30	15	11	07	11	09	07	10	10	07
	60	27	19	11	19	15	11	17	17	12
	on	121	92	51	86	69	47	76	74	52
BBRRV	15	02	02	02	02	02	02	03	02	03
	30	03	03	02	03	03	03	04	03	03
	60	03	03	03	03	03	03	05	04	04
	on	05	05	04	07	06	07	14	13	14
blueberry	15	02	02	02	02	03	02	02	02	02
	30	03	03	03	03	04	03	03	03	03
	60	04	04	04	04	04	03	04	03	04
	on	11	09	08	08	12	06	09	07	09

[a] Purified tendergreen mustard leaves and purified non-infected blueberry leaves were used as negative controls. The concentration of the negative controls was adjusted to the same starting gm/ml as the purified virus. Purified virus was 100 ug/ml.

[b] ON = absorbance reading taken after overnight incubation of ELISA plate at 4C.

In summary, CaMV reacted very strongly in the homologous $F(ab')_2$ ELISA test and strongly at the lowest dilutions of antiserum in the heterologous ELISA test. The background absorbance from purified non-infected mustard plants was very low in both tests. The reactions of BRRV were weaker than those of CaMV in both tests and BRRV reacted positively only in its homologous test. The background absorbance from purified non-infected blueberry plants was generally high.

The exact relationship of BRRV to CaMV is unclear according to these data. According to the standard agar gel double diffusion test, there is no serological relationship between the two viruses. However, if one uses the more sensitive $F(ab')_2$ ELISA test with antiserum reagents at high concentrations, a weak relationship between the two viruses may exist.

Association of Infectivity With Purified Virus

In 1982, one of the rub-inoculated 'Burlington' seedlings that had been inoculated on its leaves and roots produced red spots on a stem typical of those caused by BRRV. However, this plant never produced any leaf symptoms that year or in subsequent years. No possible symptoms were seen on any other rub or slash inoculated plant and none had positive ELISA absorbance values in either ELISA test.

DISCUSSION

Prior to this research, the only reference to yield loss due to blueberry red ringspot virus was in the introduction of a 1963 paper by Moore and Stretch. They stated that no detrimental plant growth had been observed in BBRRV infected plants. However, they did report one grower's observation that BBRRV-infected 'Burlington' bushes were "less productive and produced smaller berries than non-infected bushes." The research presented here confirms that grower's observation (Table 1). Blueberry red ringspot virus does not seem to stunt bush growth or cause any leaf or stem deformities. That is what makes this virus so insidious; it does not appear to damage the bush and yet it can cause dramatic crop loss (25%).

When the field data were taken in 1986, an observation was made that fruit from the BBRRV-infected bushes ripened approximately one week earlier than fruit from non-infected bushes. This could cause additional yield loss in a field containing both BBRRV-infected and non-infected bushes. Harvesting would have to be timed carefully to maximize berry recovery from the early ripening infected bushes but also to maximize recovery from the slower ripening non-

infected bushes. Another effect of this virus is the reduction it causes in berry size. It is interesting to note that the symptoms of BBRR disease first appear in the middle of the season, long after fruit set, and that the symptoms increase in intensity as the season progresses. The disease symptoms peak by the time the fruit is mature. It seems likely that the virus causes a drain on the plant's resources and this causes a reduction in fruit size.

This relatively late onset of BBRR disease symptoms is an interesting feature of the disease. Generally, plant viruses appear early in the season and can be found first in the actively growing meristematic areas of a plant. However, BBRRV symptoms occur first on the oldest leaves and gradually move to leaves upward on the stem. Then, at the peak of symptom development in September, all of the leaves undergo natural senescence. It is not known if the newly made virus moves out of the leaves and into the stems and roots before the leaves fall off the bush or if a majority of the newly made virus is lost in the leaf litter. It could be beneficial to have those answers to help determine when and where is the best time to sample the bush for early detection of the virus. At this point, no BBRRV has been detected in symptomless tissue and this is a major flaw in ELISA tests. To eradicate the virus from nursery stock and propagated plants, one needs to be able to detect the virus at the early stages of infection, i.e. in the first year or

two of infection. No studies have been done to determine how long it takes after natural infection for symptoms to develop.

It seems likely that ELISA failed to detect BBRRV in the initial experiments when crude extracts of blueberry leaves were made in low molarity extraction buffer without nicotine because the pH of the solution dropped to such low levels. The low pH could have interfered with the antigen-antibody binding in the ELISA plate. Both extremely high and low pH have been found to dissociate antigen-antibody complexes. Bar-Joseph and Salomon (1980) found that exposing both homologous and heterologous reactions of tocacco mosaic virus in ELISA plates to low pH (2.2) for 30 minutes did not dissociate the complex. However, exposure of the plate to high pH (12.1) did cause almost total dissociation. Prior to this, Bar-Joseph et al. (1979) reported that ELISA plates could be reused for detecting citrus tristeza virus by treating the plates with a 0.2 M glycine-HCl solution of pH 2.2 because this caused the antigen-antibody complexes to dissociate.

There could have been other effects of the low pH. The isoelectric point of BBRRV is at pH 4. It is possible that the virions in the crude extract were simply falling out of solution because of the low pH. The low pH could also be the result of H ions being released by polyphenyl oxidase activity. The intermediate products of this reaction can

readily bind with proteins and thus inactivate the virions and also precipitate them. This is probably why nicotine was so helpful in virus detection by ELISA. When nicotine was added to the extraction medium, it bound to the intermediates and inactivated them before they could inactivate the virus. Nicotine also kept the pH of the extract from dropping.

The data from the sample preparation of BBRRV infected blueberry leaves indicate that neither partial purification of the virion nor inclusion body purification is worthwhile to increase ELISA detection of BBRRV, and that simple extraction of the tissue in buffer containing nicotine is the most efficient and sensitive method to detect BBRRV in blueberry leaf tissue at this time. No studies have been done using buffer containing nicotine to extract BBRRV from any tissue other than leaf.

One last avenue of sample treatment could be investigated. Lawson and Hearon (1977) treated purified CERV inclusion bodies with several proteases. The inclusion bodies were then fixed, embedded and studied in ultra thin section in the electron microscope. They found that although Protease VIII would digest both the matrix protein of the inclusion body and the virion coat protein, the matrix protein was digested first. Protease VIII worked best at an alkaline pH. It would be interesting to extract BBRRV-infected blueberry leaves, either crudely or with a

partial purification, and keep the extract at a high pH. Protease VIII would then be added and the extract allowed to incubate for an appropriate amount of time to digest the matrix protein but not the virion protein. The pH of the solution would then be adjusted back to a neutral pH and used in ELISA.

For detection of BBRRV, indirect ELISA is more sensitive than DAS-ELISA. Other researchers have also found that an indirect form of ELISA is more sensitive than DAS-ELISA (Barbara and Clark, 1982). It is theorized that the process of conjugating the enzyme to the antibody hinders later binding of the antibody to the antigen. This could be due to the harsh effects of the glutaraldehyde on the proteins or due to the enzyme interfering with the antigen binding site. Several researchers have studied these effects. Koenig (1978) found that replacing the coating antibody with a heterologous antibody did not decrease ELISA absorbance value as severely as did replacing the conjugated antibody with a heterologous one. Ghabrial and Shepherd (1980) studied the relative benefit of ELISA and radioimmunosorbent assay (RISA) to detect CaMV and several other plant viruses. The major difference between RISA and ELISA is that RISA employs a radioactively labeled immunoglobulin (in this case ^{125}I) instead of one labeled with an enzyme. The RISA wells are then analyzed in a gamma counter instead of a spectrophotometer. A chloramine-T method is used to link

the radioactive iodine to the globulin. The chloramine-T replaces hydrogen on tyrosine residues with the radioactive iodine. This process is much gentler on the immunoglobulin than is glutaraldehyde fixation. Ghabrial and Shepherd took ^{125}I-labeled-IgG and then conjugated alkaline phosphatase to it. Both the ^{125}I-IgG and the ^{125}I-IgG conjugated with enzyme were tested in RISA. Counts per minute were reduced in the wells containing the ^{125}I-IgG with enzyme conjugated to it. A reciprocal test was done with ELISA. The addition of ^{125}I to the enzyme-conjugated-IgG did not reduce absorbance values when the IgG was used in ELISA.

A satisfactory yield of BBRRV can be obtained from the purification scheme shown in Figure 1. The addition of butanol, 2-mercaptoethanol, and thioglycolic acid to the initial extraction step increased total yield of virus by five-fold. The two peaks found in linear-log and CsCl gradients are puzzling. No other caulimovirus has two such peaks. Whether there are two different kinds of BBRRV particles is still unknown. When ELISA was used to test the fractions collected from sucrose gradients, no difference in absorbance was found between the fractions from the two viral peaks (Figure 2). This was due, in part, to the error bars being very large. When sucrose gradient profiles from BBRRV-infected and non-infected purified preparations are superimposed (Figure 3), it is clear that the two viral peaks are due to the BBRRV infection. There is a small,

diffuse peak in the gradient from the non-infected tissue that corresponds to the same area as the viral peak. Although unlikely, the material in this peak may increase in concentration with virus infection and may cause a second peak in the viral preparation gradients or at least contribute to it. The two viral peaks could also be from broken particles, although not very many of these are seen in the electron microscope. The gradient profile could also be affected by differing amounts of inclusion body matrix being attached to the virions. There is a continuum of material that reacts to the BBRRV antiserum throughout the bottom 60% of the sucrose gradient.

Aside from this two-peak characteristic, BBRRV has several characteristics very similar to caulimoviruses. The known sedimentation coefficients of the caulimoviruses are: CaMV-208, CERV-206, DaMV-254, MMV-254, SVBV-200, ThMV-210, CVMV-246, PlV4-208. The first sedimentation peak of BBRRV in sucrose gradients sediments at 212 $\pm$ 5% which is within the caulimovirus range of values. The second sedimentation peak of BBRRV in sucrose, 275, is somewhat heavier than the caulimoviruses but is still very close to their range. No caulimovirus has been reported as producing two buoyant density peaks in cesium gradients. The reported densities for the caulimoviruses CaMV, CERV, FMV, and MMV are all 1.35 gm/cc. The density for ThMV is 1.38. The two buoyant density peaks from purified BBRRV are 1.30 and 1.40, which

fall just on either side of the values for the other caulimoviruses. Two caulimoviruses have values reported for their coat protein molecular weight: CaMV-42,000 and MMV-32,000 daltons. The molecular weight of the major band from purified BBRRV is 44,000 daltons. It is assumed that this is the coat protein band. The nature of the faint band with a molecular weight of approximately 101,000 daltons is unknown. The inclusion body protein for CaMV has a molecular weight of approximately 66,000 daltons.

The fact that BBRRV contains double-stranded DNA is another strong indicator that this virus belongs to the caulimovirus group. No other group of plant viruses have DNA that is double stranded. Further studies should be carried out on BBRRV DNA to determine its molecular weight and to determine if it is circular and has single stranded breaks. Sequence homology studies, although interesting, would probably not help determine the relationship between BBRRV and the other caulimoviruses. Nucleic acid hybridization tests have shown that there is little or no homology between the DNA of CaMV, CERV, DaMV. FMV, MMV, and ThMV (Richins and Shepherd, 1983 and Hull, 1984).

There must be at least limited nucleic acid sequence homology in the coat protein coding region between several of the caulimoviruses because when whole virions are tested serologically, a relationship exists between CaMV, CERV, DaMV, SVBV, and HRLV (Hull, 1984). The ouchterlony gel

double diffusion tests reported in this thesis did not find any serological relationship between BBRRV and antiserum to CaMV, CERV, DaMV, and FMV. It is unfortunate that positive antigen controls were not available for the three latter viruses. Because CaMV did react to the DaMV antiserum, it can be assumed that this antiserum was at an appropriate dilution to detect any potential relationship to BBRRV. However, CaMV should have reacted to CERV antiserum but it did not. This does not disprove a lack of relationship between BBRRV and CERV but it does cast doubt on that particular serological test. A CERV-positive antigen would have been very useful but was unattainable. When antiserum reagents in ELISA were manipulated, a slight serological relationship between CaMV and BBRRV could be seen.

Attempts to associate infectivity with purified virus particles have been unsuccessful so far. This means that Koch's postulates have not been fulfilled. It is hoped that the slash-inoculated plants will show some symptoms or be ELISA positive in 1988. The fact that 45 nm spheres can be seen in inclusion bodies when red lesions from BBRR diseased blueberry leaves are fixed, embedded, and studied in ultra-thin section in the electron microscope, is strong evidence that these virions are the causal agent of the disease.

Further studies on the ecology of BBRRV need to be done. No vector of BBRRV has been identified. Because BBRRV is a caulimovirus, a likely vector is the blueberry

aphid, Illinoia pepperii (MacGillivray). Moore and Stretch, 1963, reported a progressive increase in BBRR disease incidence in a New Jersey field of blueberries over a six year period and observed that the virus tended to spread down the row. This is evidence that the vector could be an aphid. However, BBRR disease spreads in New Jersey but does not seem to spread in Michigan. Michigan has abundant numbers of Illinoia pepperi and so one would expect this disease to spread in Michigan also. One kind of insect present in abundant numbers in New Jersey blueberry fields that is seldom seen in Michigan is a mealybug. Samples of these insects taken from New Jersey have been sent to the United States Department of Agriculture in Beltsville, Maryland for identification and are in the genus, Dysmicoccus. If Dysmicoccus is the vector of BBRRV, it, too, could cause a down-the-row pattern of spread. Certainly an important proven method of spread of BBRRV is through vegetative propagation of plants (Moore and Stretch, 1963). Regardless of what the insect vector may be, care should be taken to not propagate from infected plants.

Blueberry red ringspot virus is an interesting virus for several reasons. It is of economic significance in New Jersey where it is present in abundant numbers of blueberry plants and may be causing significant amounts of crop loss. It is also of economic significance in blueberry growing areas where it is not present in abundant numbers of plants,

e.g. Michigan, because it is in some planting stocks and cannot be easily detected and therefore has a high potential for spread. It is important to stop state-to-state spread of this disease. The identity of a vector of BBRRV should be interesting. Aside from the obvious practical aspects of this knowledge, BBRRV could be a caulimovirus that is spread by something other than an aphid. Finally, any plant virus that contains double stranded DNA is of interest because plant viruses of this type are relatively few in number. Also, double stranded DNA genomes lend themselves to manipulation in studies involving molecular biology more readily than RNA genomes do.

APPENDICES

APPENDIX A

Linear-Log Sucrose Gradient Recipe[a]

% sucrose	vol./tube[b]	start vol.	finish vol.	make to
30	1.3	45.0	37.2	42.9
26	3.3	42.9	23.1	28.6
21	2.4	28.6	14.2	18.6
16	1.7	18.6	8.4	13.5
10	1.4	13.5	5.1	
0	1.0			

[a] Brakke, M.F. and N. vanPelt. 1970. Linear-log sucrose gradients for estimating sedimentation coefficients of plant viruses and nucleic acids. Anal. Biochem. 38: 57-65.

[b] This recipe makes six SW 41 tube gradients. All volumes are in ml.

APPENDIX B

Electrophoresis Solutions

Stock Solutions

A. Acrylamide

29.2 g acrylamide

0.8 g N'N'-BIS methylene acrylamide

Bring volume up to 100 ml with distilles water. Store at 4 C in dark bottle.

B. 1.5 M Tris-HCl, pH 8.8

18.15 g Tris base

50 ml distilled water

Adjust to pH 8.8 with 1 N HCl

Bring volume up to 100 ml with distilled water.

C. 0.5 M Tris-HCl, pH 6.8

3.0 g Tris base

50 ml distilled water

Adjust pH to 6.8 with 1 N HCl

Bring volume up to 50 ml with distilled water.

Seperating Gel (12% acrylamide)

13.5 ml distilled water

10.0 ml 1.5 M Tris-HCl, pH 8.8

0.4 ml of a 10% SDS solution (sodium dodecyl sulfate)

16.0 ml stock solution A

0.1 ml of a 10% ammonium persulfate solution (fresh)

0.02 ml TEMED (N,N,N',N'-Tetramethylethylenediamine)

Stacking Gel (4.0 % acrylamide)

6.1 ml distilled water

2.5 ml 0.5 M Tris-HCl, pH 6.8

0.1 ml of a 10 % (w/v) SDS solution

1.3 ml stock solution A

0.05 ml of a 10% ammonium persulfate solution (fresh)

0.005 ml TEMED

Sample Buffer (4X: use one part buffer to 3 parts sample)

10.0 ml glycerol

2.0 ml 2-mercaptoethanol

2.0 g SDS

12.5 ml 0.5 M Tris-HCl, pH 6.8

2.0 mg bromphenol blue

Electrode Buffer, pH 8.3

3.0 g Tris base

14.4 g glycine

1.0 g SDS

Adjust pH to 8.3 with 1 N HCl (if needed)

Bring up to 1 liter with distilled water.

Solutions according to:

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

LITERATURE CITED

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Al Ani, R,. P. Pfeiffer, and G. Lebeurier. 1979. The structure of cauliflower mosaic virus - II. Identity and location of the viral polypeptides. Virology. 93:188-197.

Balazs, E., H. Guilley, G. Jonard, and K. Richards. 1982. Nucleotide sequence of DNA from an altered-virulence isolate D/H of the cauliflower mosaic virus. Gene 19:239-249.

Bar-Joseph, M., M. Moscovitz, and Y. Sharafi. 1979. Reuse of coated enzyme-linked immunosorbent assay plates. Phytopath. 69:424-426.

Bar-Joseph, M., and R. Salomon. 1980. Heterologous reactivity of tobacco mosaic virus strains in ELISA. J. Gen. Virol. 47:509-512.

Barbara, D.J., and M.F. Clark. 1982. A simple indirect ELISA using $F(ab')_2$ fragments of immunoglobulin. J. Gen. Virol. 58:315-322.

Bitterlin, M.W., D. Gonsalves and R. Seorza. 1987. Improved mechanical transmission of tomato ringspot virus to Prunus seedlings. Phytopathology 77: 560-563.

Brakke, M.K. and N. vanPelt. 1970. Linear-log sucrose gradients for estimating sedimentation coefficients of plant viruses and nucleic acids. Anal. Biochem. 38: 57-65.

Brierly, P. and F.F. Smith. 1950. Some vectors, hosts, and properties of dahlia mosaic virus. Plant Dis. Rep. 34:363-370.

Bruner, R., and J. Vinograd. 1965. The evaluation of standard sedimentation coefficients of sodium RNA and sodium DNA from sedimentation velocity data in concentrated NaCl and CsCl solutions. Biochim. Biophys. Acta. 180:18-29.

Brunt, A.A. 1966. Partial purification, morphology, and serology of dahlia mosaic virus. Virology 28:778-779.

Brunt, A.A. and K. Kitajima. 1973. Intracellular location and some properties of mirabilis mosaic virus, a new member of the CaMV group of viruses. Phytopath. Z. 76:265-275.

Chrambach, A., R.A. Reisfeld, M. Wyckoff, and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Analytical Biochemistry 20: 150-154.

Civerolo, E.L. and R.H. Lawson. 1978. Topological forms of cauliflower mosaic virus nucleic acid. Phytopath. 68:101-109.

Clark, M.F. and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:1-9.

Converse, R.H., and D.C. Ramsdell. 1982. Occurence of tomato and tobacco ringspot viruses and of dagger and other nematodes associated with cultivated highbrush blueberries in Oregon. Plant Disease 66:710-712.

Covey, N.C. and R. Hull. 1981. Transcription of cauliflower mosaic virus DNA. Detection of transcripts, properties, and location of the gene encoding the virus inclusion body protein. Virology 111:463-474.
Day, M.F. and P. G. Venables. 1961. Aust. J. Biol. Sci. 14:187-197.

Donson, J. and R. Hull. 1983. Physical mapping and molecular cloning of cauliflower mosaic virus DNA. J. Gen. Virol. 64:2281-2288.

Fenner, F. 1976. Classification and nomenclature of viruses; second report of the international committee on taxonomy of viruses. Intervirology 7: 1-115.

Franck, A., H. Guilley, G. Jonard, K. Richards and L. Hirth. 1980. Nucleotide sequence of cauliflower mosaic virus DNA. Cell 21:285-294.

Francki, R.I.B. 1983. Current problems in plant virus taxonomy. In: A Critical Appraisal of Viral Taxonomy. Ed. R.E.F. Matthews. CRC Press, Boca Raton, Florida.

Frazier, N.W. 1960. Differential transmission of four strains of strawberry vein banding virus by four aphid vectors. Plant Dis. Rep. 44:436-437.

Frazier, N.W. and R. H. Converse. 1980. Strawberry Vein Banding Virus. Commonw. Mycol. Inst. Desc. Plant Viruses. No. 219. 4 pp.

Fujisawa, I., M. Rubio-Huertos, C. Matsui, and A. Yamozuchi. Intracellular appearance of cauliflower mosaic virus particles. 1967. Phytopath. 37:1130-1132.

Gardner, R.C., A.J. Howarth, P. Hahn, M. Brown-Luedi, R.J. Shepherd and J. Messing. 1981. The complete nucleotide sequence of an infectious clone of CaMV by M13mp17 shotgun cloning. Nucleic Acids Res. 9:2871-2888.

Ghabrial, S.A., and R.J. Shepherd. 1980. A sensitive radioimmunosorbent assay for detection of plant viruses. J. Gen. Virol. 48:311-317.

Gillett J. M. and D.C. Ramsdell. 1984. Detecting the inclusion forming blueberry red ringspot virus with ELISA. Phytopathlolgy 74:862 (abst).

Hamilton, R.I., J.R. Edwardson, R.I.B. Francki, H.T. Hsu, R. Hull, R. Koening, and R.G. Milne. 1981. Guidelines for the identification and characterization of plant viruses. J. Gen. Virol., 54:223-241.

Hamlyn, B.M.G. 1955. Aphid transmission of cauliflower mosaic on turnips. Plant Pathol. 4:13-16.

Harrison, B.D., J.T. Finch, A.J. Gibbs, M. Hollings, R.J. Shepherd, V. Valento, and C. Wetter. 1971. Sixteen groups of plant viruses. Virology 45:356-241.

Hayward, H. and S. Smith. 1972. The chromosome of bacteriophage T5 1. analysis of the single stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biology 63:383-395.

Hepp, R.F. and R.H. Converse. 1987. Blueberry red ringspot virus detection in crude sap of highbush blueberry. Plant Disease 71:536-539.

Hollings, M. and O. M. Stone. 1969. Rep. Glasshouse Crops. Res. Inst. 102.

Hull, R. 1984. Caulimovirus Group. Commonw. Mycol. Inst. Desc. Plant Viruses. No. 295. 4 pp.

Hull, R. and S.H. Howell 1978. Structure of cauliflower mosaic virus genomw II. Variation of DNA structure and sequence between isolates. Virology 86:482-493.

Hull, R. and R.J. Shepherd. 1977. The structure of cauliflower moasiac virus genome. Virology 79:216-230.

Hull, R. and J. Donson 1982. Physical mapping of the DNA's of carnation etched ring virus and figwort mosaic virus. J. Gen. Virol 60:125-134.

Hull, R., R.J. Shepherd, and J.D. Harvey. 1976. Cauliflower mosaic virus: an improved purification procedure and some properties of the virus particles. J. Gen. Virol. 31:93-100.

Hutchinson, M.T. 1950. Can you recognize the symptoms of stunt disease? Proceedings of the Nineteenth Annual Blueberry Open House 19:9-11.

Hutchinson, M.T., and E. H. Varney. 1954. Ringspot: a virus disease of cultivated blueberry. Plant Disease Reporter 38: 260-263.

Kennedy, J.S., M.E. Day and V.F. Eastop. 1962. A Conspectus of Aphids as Vectors of Plant Viruses. Commonw. Entomol. Inst. London.

Kim, K.S., D.C. Ramsdell, J.M. Gillett, and J.P. Fulton. 1981. Virions and ultrastructural changes associated with blueberry red ringspot disease. Phytopath. 71:673-678.

Koenig, R., 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. J. Gen. Virol. 40:309-318.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Laquel, V.Z. and L. Hirth. 1986. The 80K polypeptide associated with the replication complexes of cauliflower mosaic virus is recognized by antibodies to gene V translation product. J. Gen. Virol. 67:197-201.

Lawson, R.H., and E.L. Civerolo. 1978. Carnation etched ring virus: purification, stability of inclusions, and properties of the nucleic acid. Phytopath. 68:181-188.

Lawson, R.H. and S.S. Hearon. 1974. Ultrastructure of carnation etched ring virus-infected Sapponaria vaccaria and Dianthus caryophyllus. J. Ultrastruc. Res. 48:201-215.

Lawson, R.H. and S.S. Hearon. 1977. Ultrastructure of extracted carnation etched ring virus inclusion bodies treated with proteolytic enzymes and DNase. Phytopath. 67: 1217-1226.

Lawson, R.H. and S.S. Hearon. 1980. Carnation etched ring virus inclusions: serology and ultrastructure of alkaline treated inclusions. Phytopath. 70:327-332.

Lung, M.C.Y. and T.P. Pirone 1973. Studies on the reason for differential transmissibility of cauliflower mosaic virus isolates by aphids. Phytopath. 63:910-914.

Lung, M.C.Y. and T.P. Pirone. 1974. Aquisition factor required for aphid transmission of purified cauliflower mosaic virus. Virology 60:260-264.

Maniatis, T, E.F. Fritsch, J. Sambrood. Molecular Cloning: a laboratory manual. 1982. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 545pp.

Martin, R.R. and P.R. Bristow. 1987. A Carlavirus associated with blueberry scorch disease. Phytopath. 78: in press.

Milne, R.G. 1980. Some observations and experiments on immunosorbent electron microscopy of plant viruses. Act. Hort. 110:129-135.

Morrissey, J.H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Analalytical Biochemistry 117: 307-310.

Moore, J.N. and A.W. Stretch. 1963. Incidence of red ringspot virus in experimental and commercial blueberry plantations in New Jersey. Plant Dis. Reptr. 47:294-297.

Petzold, H. 1968. Elektronenmikroskopishe untersuchugen an von dahlienmosaikvirus inficierten pflanien. Phytopatholo. Z. 63:201-218.

Pirone, T.P. and Megahed, E.S. 1966. Aphid tansmissibility of some purified viruses and viral RNA's. Virology 30:631-637.

Ramsdell, D.C., K.S. Kim, and J.P. Fulton. 1987. IN: Virus Disease of Small Fruits, USDA Agriculture Handbook No. 631.

Rao, A.L.N., Hatta, T., and Francki, R.I.B. 1982. Comparative studies on tomato aspermy and cucumber mosaic viruses. VII. Serological relationships reinvestigated. Virology 116:318-326.

Richins, R. and R.J. Shepherd 1983. Physical maps of the genomes of dahlia mosaic virus and mirabilis mosaic virus-two members of the caulimovirus group. Virology 124:208-214.

Shalla, T.A., R.J. Shepherd, and L.J. Petersen. 1980. Comparative cytology of nine isolates of cauliflower mosaic virus. Virology 102: 381-388.

Shepherd, R.J., and R.J. Wakeman. 1971. Observations on the size and morphology of cauliflower mosaic virus DNA. Phytopath. 61:188-193.

Stretch, A.W. and D.H. Scott. 1977. Production of highbush blueberries free of red ringspot disease. Plant Disease 61: 605-607.

Stretch and Varney, 1970. Red ringspot of blueberry. In: Virus Diseases of Small Fruits and Grapevines. N.W. Fraizer, Ed. University of CA, Berkly, CA. 290pp.

Tremaine, J.H. and N.S. Wright. 1967. Cross-reactive antibodies in antisera to two strains of southern bean mosaic virus. Virology 31: 481-488.

Van Hoof, H.A. 1954. Difference in the transmission of cauliflower mosaic virus by Myzus persicae Sulzer and Brevicor brassicae L. Tijdschi. Planterziekten 60:267-272.

Varney, E.H. and A.W. Stretch. 1966. Diseases and their control. IN: Blueberry Culture, Eck, P., and N.F. Childees (eds.), Quinn and Boden Co., Inc., Rahway, N.S. 378pp.

Volovitch, M., G. Drugeon, and P. Yot. 1978. Studies on the single-stranded discontinuities of the cauliflower mosaic virus genome. Nucl. Acids Res. 5: 2913-2925.