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Two carlaviruses, pea streak and red clover vein mosaic viruses, were identified as principal streak-inciting viruses of peas (Pisum sativum L.) in the Pacific Northwest. Research was directed toward the development of new and significant information to facilitate the development of control strategies.

The ecology of legume carlaviruses was studied by surveying legumes of the region as potential inoculum reservoirs and by investigations of aphid vectoring characteristics, including the transmission characteristics of purified PSV and RCVMV. In studies cooperative with this thesis, alfalfa was determined to be the principal inoculum reservoir of PSV in the Pacific Northwest. Two perennial clover species, Trifolium pratense and T. repens, were determined to be inoculum reservoirs for RCVMV in western Oregon, an area in which this virus is consistently epiphytotic on peas. The pea aphid, Acyrthosiphon pisum [Harris] a proven vector of RCVMV, was observed as transient populations

(T. repens) or colonizing populations (T. pratense) on these reservoir hosts in May and June, prior to aphid flights to, and RCVMV infections of, western Oregon pea plantings. Repeated successful aphid transmission of purified PSV and RCVMV provided the first definitive evidence indicating non-participation of a 'helper agent' in the aphid transmission of these viruses.

Biological and biochemical properties of Northwest legume carlaviruses were compared to type isolates of PSV and RCVMV. Northwestern isolates of these viruses differed from U.S. type cultures only in minor characteristics and were serologically identical to type cultures. Modifications of published procedures for carlavirus purification resulted in improved reliability and virus yield. Molecular weights of purified RCVMV and PSV coat proteins were estimated by SDS polyacrylamide-gel electrophoresis to be 31,000 and 29,000 daltons, respectively. Previously reported lability of PSV type culture protein was confirmed and determined to be also characteristic of Northwestern isolates of PSV. The RCVMV reference isolate (ATCC PV/110) was found to contain a sub-isolate which typically produced multi-banded profiles during isopycnic centrifugation. Moreover, a small, non-infectious RNA species was typically associated with particle fragments of upper (light) bands. In contrast, wild type isolates consistently exhibited single bands during isopycnic centrifugation, suggesting that the sub-isolate from the reference isolate was unstable during normal purification procedures.

THE IDENTITY, BIOLOGICAL AND MOLECULAR CHARACTERISTICS AND SEROLOGY OF CARLAVIRUSES INFECTIOUS TO PEAS GROWN IN THE PACIFIC NORTHWEST

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Redacted for privacy

Head of Department of Botany and Plant Pathology

Redacted for privacy

Dean of Graduate School

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THE IDENTITY, BIOLOGICAL AND MOLECULAR CHARACTERISTICS AND SEROLOGY OF CARLAVIRUSES INFECTIOUS TO PEAS GROWN IN THE PACIFIC NORTHWEST

INTRODUCTION

Pea crops of the Pacific Northwest are periodically subject to epiphytotics of at least three plant viruses capable of inducing rapid and systemic plant necrosis. One of these, alfalfa mosaic virus, is not treated in this thesis. The other two, pea streak and red clover vein mosaic viruses had previously been confused in identity and geographic distribution, and there was little understanding of their respective inoculum reservoirs or ecological parameters affecting epiphytotic periodicity. Definitive characterization of these two viruses, then, was the purpose of this thesis.

Despite very similar particle morphology and host ranges, it was known that pea streak and red clover vein mosaic viruses could be differentiated by their biological and serological properties, and recently, during this thesis research, the biochemical differentiation was independently published. This new information was immediately evaluated and concluded to be reliable. The thesis research was then directed toward tasks that would supply essential information supplementary to that already published. Specifically, work was begun on the identification and characterization of pea streak-inciting carlaviruses collected from the Northwest over a fifteen year period.

Once identification was established, we reasoned, specific information useful to the development of disease control strategies could be sought. Studies were, therefore, designed to compare the

biological and biochemical properties of Pacific Northwest isolates of PSV and RCVMV to their respective type isolates. In addition efforts were undertaken to determine the ecological parameters which affect the incidence of these viruses in the Northwest. This involved a survey of perennial legumes for the presence of either PSV or RCVMV and an investigation of seed transmission of these two viruses in plant hosts determined to be principal inoculum reservoirs.

Both PSV and RCVMV were known to be aphid-borne in the nonpersistent manner. Detailed characterization of their aphid transmission properties had not been reported and an aphid acquisition factor required for aphid transmission had recently been hyptothesized for carlaviruses. Using recently developed purification procedures for carlaviruses, studies were therefore undertaken to determine whether or not aphid transmissibility of purified PSV and RCVMV were possible. Specifically, we sought to definitively determine whether or not an acquisition factor or 'helper agent' was required for aphid transmission and these viruses.

In addition to vector relationships, substantial efforts were made to study an interesting morphological-biochemical phenomenon associated with purified RCVMV. Previous workers had found two isolates of RCVMV which exhibited multiple bands in sucrose density gradients and others reported that a second RNA species was associated with RCVMV. Although both of these characteristics were considered unusual for a carlavirus, no satisfactory explanations were offered. It was found that a sub-isolate of the RCVMV reference isolate exhibited both these

characteristics. Since two other isolates of RCVMV exhibiting neither of these characteristics were available for comparison, a study was conducted to elucidate the nature of the multiple banded sucrose density gradient profiles and the origin of the second RNA species as previously reported for RCVMV.

LITERATURE REVIEW

PEA STREAK VIRUS

There are numerous virus pathogens of peas which can induce stem streaking, among which are red clover vein mosaic virus and pea streak virus (4). Pea streak virus (PSV), a carlavirus (5), consists of slightly flexuous, rod-shaped particles with dimensions of 620x12 nm, and contain single-stranded RNA. It is cosmopolitan and affects numerous species in the Leguminoseae family. PSV is described by the cryptogram, R/1:2.55/5.4:E/E:S/Ap (67).

Pea streak virus has been reported in New Zealand, Europe, and North America (5) and it is considered to be of economic importance in those areas where peas are grown, causing considerable damage in peas (27, 76).

Pea streak virus was first described by W. J. Zaumeyer in 1938 (74). Earlier reports describe pea streak inducing viruses but it is difficult to ascertain whether or not viruses described were indeed PSV. Zaumeyer differentiated between pea streak virus-1 (PSV-1) and alfalfa mosaic by host range, and physical properties. The exact origin of PSV-1 was not known, but was assumed to have been an Arlington, Virginia alfalfa stand from which the virus was borne by pea aphids. It is interesting that although PSV has been isolated from alfalfa by Hampton (31) since 1965, the association of PSV with alfalfa, had not been determined elsewhere until 1978 (40). The host

range described for PSV-1 by Zaumeyer closely resembles the host range of PSV isolate ID-3-2 from Idaho presented in this thesis. The physical properties cited for PSV-1 also generally agree with reported values of PSV type strains.

The streaking disease of peas prevelant in Wisconsin was definitively described by Hagedorn and Walker in 1949. Wisconsin isolates designated PSV-W since that time have been considered type isolates for comparison with PSV-like viruses (27).

Hagedorn and Walker (27) found that PSV-W produced a latent infection in several clover species and induced severe necrosis in both peas (Pisum sativum L.) and broad bean (Vicia faba L.). The host range study of PSV-W by Hagedorn and Walker confirmed Zaumeyer's 1938 conclusions that PSV-1 was unable to infect bean plants (Vulgaris sp.). In addition these workers determined the physical properties of PSV-W. In comparison to PSV-1, PSV-W appeared to be more stable to heat and aging had a higher titer in pea sap than was determined for PSV-1. Hagedorn and Walker failed to transmit PSV-W by pea aphids, though field observations indicated a strong possibility of an insect vector (27). Later investigations by Skotland and Hagedorn (57) indicated a very limited capability of the pea aphid to transmit PSV-W.

Carlaviruses are generally aphid vectored in a non-persistent manner, unfortunately the first attempts to characterize the mode of aphid transmissions were hampered by repeated failure to readily transmit the virus by aphids (27). Conclusion, in practical terms, was that the pea aphid was not a principal vector. The pea aphid,

Acrythosiphon pisum [Harris] an efficient vector of pea enation mosaic virus, was first shown to transmit PSV-W at very low rates by Skotland & Hagedorn (1954). Tests included aphid transmission rates of PSV from pea to pea and from clover to pea. The rates of transmission were low and large numbers of aphids were required to achieve measurable levels of transmission (52). A year later, a hypothesis to explain the low rate of aphid transmission was proposed by Skotland and Hagedorn (58). Light microscopy of stylet tracks indicated that aphids were phloem feeders and rarely fed on intracellular material. It was suggested that virus transmission resulted from incidental cell damage occurring from insertion of the insect's stylets contaminated by prior feeding on infected plants (58). It was later concluded that the low transmission rate of PSV-W was an artifact of experimental factors, and that A. pisum was an efficient vector of PSV under controlled conditions (29). Hampton and Sylvester (1969) demonstrated that A. pisum efficiently transmitted PSV-W. They investigated the interactive effects between PSV and AMV on the aphid transmission of each virus since these viruses were frequently found in complex in naturally infected alfalfa. The results indicated that a slight reduction of the transmission rate of PSV occurred in the presence of AMV (29).

Only moderate strain variation has been reported between isolates of PSV. By 1960 several efforts had been made to consolidate the PSV literature. Workers began re-examining previously described PSV-like isolates and found that some such as Steinkleevirus (sweet clover virus)

were PSV strains (71). Kim and Hagedorn (34) collected streak-inducing viruses from U. S. pea fields and studied their various properties in contrast to PSV-W, but failed to definitely identify the isolates studied as true PSV isolates (34). An Idaho virus isolate (I-5) related but not identical to PSV-W was also described by Zaumeyer & Patino in the same year (76).

Studies reporting the properties of PSV in sap indicated that the virus is fairly stable. The thermal inactivation point was above 60 C for most isolates and the Wisconsin isolate was reported to be heat stable up to 70-80 C. The dilution end point is between 10^{-6} and 10^{-7}. Although unbuffered sap extracts remained infective for between 2-7 days for most isolates, the Wisconsin isolate was reported to persist in sap for up to 60 days (5).

The physiology of PSV infected plants in peas has been investigated only superficially. Hagedorn and Walker (27) studied the effects of temperature on disease and symptom development and found that lower temperatures arrested streak symptom development, whereas elevated temperatures stimulated rapid and severe necrosis leading to the death of the plant. Maduewesi and Hagedorn (1965) monitored the movement of PSV within the pea plant by assaying plant sections at various time intervals on Chenopodium amaranticolor. It was found that the virus could systemically invade plant tissue from a primary infection focus within 45 hrs. It was also shown that the virus initially moved upward toward the growing point and then downward toward the roots. A study of nutritional factors affecting virus movement was attempted.

Of the supplemental elements studied only phosphorus influenced virus movement (37).

Pea streak virus was partially purified in 1966 by Rosenkranz and Hagedorn. Infected pea tissue was homogenized in distilled water and acid clarified, followed by three cycles of differential centrifugation. The modal length was estimated to be 586±29 nm and the sedimentation coefficient was determined to be 136-137S. The computed nucleic acid content was 5.4% (54). Infectious nucleic acid prepared by phenol-SDS extraction was rendered non-fectious by the addition of pancreatic ribonuclease indicating that PSV-W nucleic acid was RNA. Intact virons showed little loss of infectivity from the addition of ribonuclease (39). Veerisetty and Brakke (1978) later confirmed these findings (67).

PSV is characteristically strongly antigenic. PSV strains tested to date have all been closely related serologically. Wetter and Quantz (1962) produced PSV antiserum with a titer of 1/16,384 in microprecipitin tests (73). PSV does not readily diffuse through agar and there are no reports of successful reactions in Ouchterlony plates. Weber and Hampton (1978) reported successful immunoprecipitin reactions for PSV in agar gels containing 1% SDS, after virus disruption in 1% SDS (69).

An improved purification scheme was developed recently by Veerisetty and Brakke (1978). The procedure involved homogenizing frozen infected pea tissue in an alkaline solution of sodium borate followed by precipitation of the virus by polyethylene glycol (MW 6000).

The virus was resuspended and subjected to both differential and sucrose density centrifugation. This procedure produced yields of between 0.3 and 1.0 mg of virus/ gm of pea tissue. Purified virus particles were 620 nm in modal length and had a sedimentation coefficient of 160-161S (67).

Biochemical evidence for differentiation between RCVMV and PSV was reported by Veerisetty and Brakke who demonstrated differences in both capsid subunits and nucleic acid size. This work also provided data for completing the cryptogram for PSV. PSV was found to contain a single stranded RNA sedimenting at 37.6S and weighing 2.55×10^3 daltons. Intact viral protein was estimated to have a molecular weight of 33.5×10^6 daltons by SDS polyacrylamide gel electrophoresis, but protein subunits were also degraded into fragments by unknown processes (67).

There are no reports of PSV being seed transmitted in any of its known hosts.

An investigation of PSV-infected pea tissue by both light and electron microscopy was done by Bos and Rubio-Huertos (1972). Light microscopy of epidermal strips revealed amorphous inclusion bodies which were often vaculated and located near the nucleus. PSV did not form crystalline inclusion bodies. Examination of thin sections by electron microscopy demonstrated large number of virus particles in the ground cytoplasm which often appeared in dense packets of limited size with distinct borders. Like other carlaviruses studied, PSV was membrane associated, the viroplasm often being limited by the nuclear

membrane or tonoplast. Transverse sections of PSV inclusions revealed whorls of virus that increased in density towards interior (7).

Comprehensive information on PSV was summarized by Bos (4) in the Descriptions of Plant Viruses, CMI/AAB.

RED CLOVER MOSAIC VIRUS

Red clover vein mosaic virus (RCVMV) consisting of 650 nm slightly fexuous rod-shaped particles is a member of the carlavirus group of plant viruses. It causes a streak-like disease in peas (Pisum sativum L.) in western Oregon and western Washington, where it occurs naturally in red clover in this area. Its host range is primarily limited to members of the Legumenoseae with a few exceptions (26, 56). RCVMV is described by the following cryptogram - R/1:2.59/(5):E/E:S/Ap (67). RCVMV has been reported in North America, Europe and South Africa (62).

The virus now accepted as RCVMV was described in 1937 by Osborne (42), a researcher at a medical center in New Jersey. Vein mosaic symptoms in red clover were described and transmission of the pathogen was accomplished by sap inoculation onto healthy clover plants. Osborn also transmitted the virus to peas and bell bean (Vicia faba) and described the symptoms induced in these hosts. The isolate described in this report had a thermal inactivation point of 60 C and a longevity in sap of three days. The pea aphid (Acrythosiphon pisum [Harris] was identified as a vector. Aphid transmission characteristics were described, characteristics which would later be termed non-persistant or stylet-borne. Efforts to identify a second aphid species as a potential vector failed. Osborn produced evidence that RCVMV was a unique pathogen separate from know viruses including a strain of pea streak virus.

Hagedorn and Walker in 1949 described what they believed to be a new virus of Pisum and named it Wisconsin pea stunt virus (27). This virus was later (1951) determined to be the same as Osborn's RCVMV (26). Pea stunt (not descriptive of symptoms induced by Northwest isolates of RCVMV), now conclusively identified as RCVMV, was discovered to be widespread in the red clover fields of Wisconsin, and isolates were classified according to symptomatology on clover (25). Ramamurthi (1958) demonstrated that Gomphrena globosa was capable of reacting to mechanical inoculation by producing local lesions. The logarithm of the number of local lesions was linearly related to the virus concentration in crude sap. While local lesion induction by RCVMV was subject to environmental factors, the potential use of G. globosa as a possible quantitative assay plant was discussed (52).

The following year in 1959, Sander studied the biological properties of RCVMV. It was determined that 0.1M cysteine at pH 4.0 was an optimum buffer for the production of local lesions on G. globosa. Seed transmission was reported in Vica faba but not in Pisum sativum. Peas infected with RCVMV were found to photosynthesize at a reduced rate and to respire at an increase rate. Clover cuttings were shown to exhibit a decreased hardiness when infected, and it was projected that a similar hardiness reduction would occur in the field. Sander studied the leaf hairs of infected red clover plants by light microscopy and found both amorphous and crystalline inclusion bodies. The amorphous type were more common (56).

The host range of RCVMV was reviewed by Varma and tested by

Hampton et al. (28). Hosts include plant species in the Amaranthaceae, Chenopodiaceae, Legumenosae, and Solanaceae. Various types of symptoms may be induced in these hosts by RCVMV, among which are vein mosaic, mosaic, streaking, and stunting. The virus has been found to produce latent infections in some hosts. Symptom expression is reportedly dependent on the environmental conditions (62).

Before the extent of RCVMV strain variation had been determined, some workers failed to distinguish between strains which induced streaking in peas and the morphologically similar pea streak virus (18, 19, 75). Kim and Hagedorn (1958) in an attempt to study streak inducing viruses of the United States of America, described but failed to identify an isolate from Minnesota which, by the descriptive evidence presented, was probably RCVMV (34). Wetter and co-workers (72, 73) distinguished between pea streak and red clover vein mosaic virus and identified previously described viruses as either PSV or RCVMV. Bos reviewed identification inaccuracies in the literature in 1970 (6) and Veerisetty and Brakke (1978) were able to add biochemical data in addition to serological and morphological evidence to support the proposed differentiation between PSV and RCVMV (67).

RCVMV is moderately stable in plant sap (62). Extracts of infected peas were usually inactivated when heated for ten minutes at 60 C, maintained infectivity for only 24 hr at 20C, and were rendered non-infectous when diluted beyond 10^{-3}.

As with many rod-shaped viruses, the first attempts to purify RCVMV often met with the great loss of virus due to precipitation

inducted by aggregation. Pea has been the most commonly used RCVMV propagation host.

Varma, et al. (63) in 1970 reported the first biochemical and physiochemical characteristics of RCVMV. By electron microscopy the periodicity of the nucleoprotein was determined to be 33 angstroms. After testing some twenty-three different solutions these workers found that .005M sodium borate pH 7.0 was a satisfactory resuspension medium. Their final purification procedure yielded larger quantities of purified RCVMV than any previous report. Photoreactivation of virions damaged by ultra-violet radiation was also reported.

RCVMV is an excellent immunogen. Several workers have reported detectable precipitin reactions with dilutions of antiserum up to 1/1024 (5, 63, 75). Because of the size and morphology of RCVMV, diffusion through agar plates is very limited unless the particles are disrupted. Sonically disrupted virus was found by Varma (63) to diffuse through agar and from visible precipitin reactions with antibodies in Ouchterlony plates. Weber and Hampton (69) have reported successful immunoprecipitin band formation in 1% SDS agar gels.

Little or no tolerance to RCVMV in pea, a crop in which this virus can do considerable damage, have been discerned to date (19). The severity of damage to pea plants and pods by RCVMV infection is reportedly related to the time of inoculation (17). Plants infected when young were reported to out-grow the symptoms, thus becoming symptomless carriers. Plants that became infected when about four weeks old are susceptible to pod damage by RCVMV, whereas plants

inoculated at or near maturity usually developed no pod symptoms (17).

Seed transmission of RCVMV has been demonstrated in two hosts, Vicia faba and Trifolium pratense (56). A probable isolate of RCVMV, reported as pea streak virus, was located on the seed coats of peas but was not detectable in pea seedlings from infected mother plants (18).

Some RCVMV strain differentiation was reported by Bos in 1971 (6). In this work, symptoms in pea and clover induced by several strains of RCVMV were compared. Electron microscopy of the isolates studied revealed slight differences in particle modal lengths of these strains. Particle lability during purification was reported for two of the isolates studied. Evidence supporting Osborn's and Wetter's earlier observations on the differentiation of RCVMV from PSV was also presented.

Histological and ultrastructural changes of pea tissue infected with RCVMV were investigated by Rubio-Huertos and Bos (55). The strains of RCVMV studied produced intracellular crystalline inclusion bodies. No amorphous inclusions as reported by Sanders were found (56). The inclusion bodies consisted of proteinacous spherical particles with a size periodicity of 11-13 nm. The cytoplasmic distribution of virus particles was typical of carlaviruses. The virus was usually found in dense packets closely associated with cellular membranes.

An improved procedure for the purification of carlaviruses was recently published and is quite effective for the purification of RCVMV (67). This improved purification scheme led to the completion of the cryptogram and provided other biochemical properties.

Pea Streak and Red Clover Vein Mosaic Viruses as Compared to Other Members of the Carlavirus Group

Carlaviruses comprise a taxonomic group of plant viruses presently containing 14 members of which carnation latent virus (70) is the type virus. The modal particle lengths of the carlaviruses range between 610 nm for hop latent virus (50) and 685 nm for chrysanthemum virus B (33). All the members consist of slight flexuous rod-shaped particles similar to those of PSV and RCVMV (see Figure 1-5).

Nine of the 14 viruses are cosmopolitan and the host ranges of carlaviruses are characteristically restricted. Many are latent or produce mild symptoms in their host, hence the names, alfalfa latent (66), carnation latent (70), cowpea mild mottle (10), hop latent (50) and narcissus latent (11). PSV and RCVMV are notable exceptions in this regard; however, even these two are latent in many hosts (Table 1-1).

Both PSV and RCVMV are typical of other carlaviruses with respect to their stability in unbuffered, undiluted sap. Generally carlaviruses are inactivated after a few days (2-4) and most members are rendered non-infectious after a 10^{-3} X dilution sap. As a group they are fairly stable to heat with the average thermal inactivation point between 65-75 C. The viruses are all stable enough to be mechanically transferred and all of those for which vectors have been determined, have been aphid-borne in the non-persistent manner.

The biochemical properties are largely unknown due to the lack of effective purification procedures until very recently (64). Alfalfa latent virus, PSV and RCVMV are the only carlaviruses for which complete cryptograms have been published (67).

Chapter One

BIOLOGICAL PROPERTIES AND ECOLOGY OF LEGUME CARLAVIRUSES OF THE PACIFIC NORTHWEST

INTRODUCTION

Because Pisum resistance to streak-inducing viruses is complexly inherited and only vaguely understood, control of these viruses in the Pacific Northwest can be expected to require advanced knowledge of the biological properties and ecology of both red clover vein mosaic (RCVMV) and pea streak (PSV) viruses, the two dominant pea-streaking pathogens of this area. Biological properties investigated herein were viral characteristics exhibited either within host plants or within host extracts. Ecological investigations were focused on interactions of the virus with the environment, specifically on disease cycles. Such information seems logically prerequisite to the development of strategies for minimizing crop losses or, ultimately, complete control.

Reasonably complete generalized information has been published on the biological properties of legume carlaviruses (56) (54) and this information has been useful to our attempt to explain why RCVMV and PSV are periodically devastating to Northwest pea crops. However, specific information was developed in this study for specific carlavirus isolates collected by this laboratory for over 15 years. Properties of these isolates were compared to those of type isolates, to detect and elucidate any characteristics unique to local isolates, particularly

those which could affect control strategies. We also gave particular attention to symptomatology, and to the host range of pea streak and red clover vein mosaic viruses.

The ecology of the pea-streaking carlaviruses had previously been only partially investigated. It is generally recognized that PSV and RCVMV can cause epiphytotics in Northwest pea fields, and some information had indicated that aphid spp could transmit the viruses in a non-persistent manner. While likely vectors have been known or suspected for some time, the source of the inoculum has been enigmatic until recently when the inoculum reservoir of PSV in Southern Idaho was determined. There are currently no effective control measures against PSV or RCVMV in the Northwestern U.S.A. Consequently, when environmental factors favor epiphytotics of the pea streak disease, the pea industry and pea breeding programs suffer economic loss; sometimes devastating loss. However, the recent elucidation of alfalfa as a primary inoculum source of PSV, and evidence in this thesis that red clover is a primary inoculum source of RCVMV, provide a foundation for effective measures against these viruses. This information, coupled with knowledge of active aphid vectors, provides new insights which demonstrate the practical significance of plant virus ecology.

It has been observed that virus source hosts are often infected with two or more viruses. For example, alfalfa plants are frequently found to be simultaneously infected with PSV and alfalfa mosaic virus (AMV) (29). The possibility that the presence of an

unrelated virus in the natural legume population infected with either RCVMV or PSV could influence aphid transmission from the inoculum reservoir to peas therefore was investigated in this thesis.

In summary then, this first chapter presents comparisons of type isolates and Northwest isolates of PSV and RCVMV, under seven chapter sections. These sections are entitled: 1. Symptomatology and host range, 2. Stability in sap, 3. Electron microscopy, 4. Field assays, 5. Aphid transmission of carlaviruses in multiple infections, 6. Strain comparisons, and 7. Seed transmission.

METHODS

Isolate origins. The two carlavirus isolates from the Northwest most often used experimentally in this thesis were RCVMV isolate WA-7641 and PSV isolate ID-3-2. Both isolates were collected by Dr. Hampton of Oregon State University. Isolate, ID-3-2 was derived from pea aphids, Acrythosiphon pisum [Harris] feeding on alfalfa in Idaho in 1974. This isolate was then transferred to six alfalfa plants as greenhouse inoculum reservoirs.

RCVMV (WA-7641) was derived in 1976 from an infected pea plant collected in the vicinity of Mt. Vernon, Washington. Isolate WA-7641 was subsequently increased in greenhouse-grown pea seedlings and infected tissue was desicated for reference purposes. The sample was originally contaminated with white clover mosaic virus (WCMV). Aphids were used to free isolate WA-7641 from WCMV, which has no known vectors. The intriguing occurrence and origin of WCMV in the

original plant is conjectured in this chapter (see Results section, Aphid transmission of carlaviruses in mixed infections). Type cultures of both PSV and RCVMV were obtained for comparison purposes. RCVMV (ATC/PV 110) was obtained from the American Type Culture Collection in Washington, D. C. The Wisconsin isolate of PSV was supplied by Dr. D. J., Hagedorn University of Wisconsin, Madison.

Symptomatology and host range. Pea cv. Cascade was inoculated with PSV isolates Wisconsin and ID-3-2 or RCVMV isolates ATC or WA-7641. Symptom development was monitored on a daily basis for two weeks.

A standard selection of host plants for testing carlaviruses was chosen (28). Test plants were grown until they were old enough to survive mechanical inoculation and still young enough to maximize the response to potential infections. Mechanical inoculations were accomplished by homogenizing either fresh or desicated tissue in a mortar with approximately equal volumes of 0.1M sodium phosphate, pH 7.0. Test plant were dusted with carborundum power (#400) and plant extract was abraded across the leaf surfaces using a cotton swab. Symptoms on inoculated leaves were generally recorded 5-7 days and systemic symptoms were recorded 14-21 days after inoculation. Suspected latent infections were tested by back inoculation onto peas or onto local lesion indicator plants.

Sap stability. The American Type Culture and isolate WA-7641 of RCVMV and the Wisconsin and ID-3-2 isolates of PSV were tested for sap stability. All treated extracts were assayed for infectivity on 10 to 12-day-old pea seedlings, cv. Cascade. Each treatment was assayed on six plants. Percentage of infected plants was recorded 14 to 21 days after inoculation.

For dilution end point determinations, infected pea plants were ground in a mortar, and the extract was pressed through cheesecloth and diluted serially with glass distilled water. Each dilution of sap extract was immediately inoculated onto pea plants for infectivity determinations (41).

Longevity of viruses in sap was determined in undiluted sap prepared as described above. Sap was placed on test tubes, sealed, and subsequently opened and assayed for infectivity over a period of four weeks (41).

Thermal inactivation points were determined by exposing sap from infected pea plants to 10 min periods at various temperatures. Each heat treated extract was then inoculated onto pea seedlings (41).

Electron microscopy. Infected plant tissue was ground in 3.5% glutaraldehyde. A single drop of the extract was transferred to a formvar and carbon coated copper grid. After two min the extract was removed with filter paper and negatively stained for two min with a drop of 2% phosphotungstate (pH 7.0). Tobacco mosaic virus was frequently added as an internal calibration standard, assuming a TMV

modal length of 300 nm.

Field assays. To assay legume plants from the field, specimens were collected and placed in plastic bags with a moist paper towel. Specimens were identified and their location recorded. Symptoms indicative of a viral infection were noted. Plants were stored at 4 C and mechanically inoculated onto pea seedlings cv. Cascade within four days, to establish reference isolates and also to increase virus titer and facilitate serological diagnosis. Carlavirus identifications were based on symptomatology in test hosts, particle morphology, and SDS immunodiffusion (51) results.

Aphid transmission of carlavirus occurring in mixed infections. In all experiments described, a local biotype of the pea aphid, Acrythosiphon pisum [Harris] was used. Aphids were reared on bell bean, Vica faba L. Aphid nymphs, 5-7 days old were starved two to four hours to enhance feeding proneness during experiments.

The first experiment was designed to test for an effect of the presence of alfalfa mosaic virus (AMV) on the transmission efficiency of PSV by aphids. AMV taken from a mixed infection of PSV in alfalfa (see Table 1-4) was freed of PSV by passage through cowpea and reinoculated onto alfalfa. Several months later, a latent systemic infection of AMV was verified. Alfalfa infected with PSV isolate ID-3-2 was available from previous work. Alfalfa plants used in these experiments were

from a genetic mixture named Duprei.

Fasted aphids were allowed a 5-10 min feeding on either healthy or AMV-infected alfalfa. Aphids were then transferred to a PSV infected alfalfa for a second access feeding of 5-10 min. The aphids were then transferred to a pea seedling cv. Cascade. Control experiments eliminating the pre-access feeding were performed. After an overnight inoculation period, aphids were removed and the plants fumigated with nicotine. Infections were determined by symptomatology and verified by electron microscopy when symptoms were equivocal.

During the course of this dissertation, it was determined that WCMV was associated with RCVMV in naturally infected peas. An experiment designed to test for possible transfer of WCMV in the presence of RCVMV by the aphid vector was conducted. For this purpose, a multiple infection of WCMV and RCVMV was established in pea cv. Cascade from the original material containing both the RCVMV isolate WA-7641 and WCMV. Pea aphids were then allowed 2-5 min access feedings on the infected plant. Feeding aphids were transferred to pea seedlings cv. Cascade and allowed an overnight inoculation period. Aphids were removed and after 14 days and the plants were assayed for the presence of both RCVMV and WCMV. Assays were conducted by serological techniques and by electron microscopy.

Strain comparisons. Isolates of pea-streak-inducing viruses collected from the Northwest by Drs. R. E. Ford and R. O. Hampton and by the author were inoculated into pea, and Gomphrena globosa L., to study

regional variations among the pea-necrosing carlaviruses. Symptomatology, particle morphology and serological identity were investigated. Comparisons were made among the Northwest isolates and to type cultures of RCVMV and PSV.

The following experiment was designed to survey virulence in the isolates of PSV from Idaho and to select an optimum propagative pea cultivar. Alfalfa plants infected with PSV isolates ID-3(1-4) were chosen as virus-source hosts. Eight pea cultivars were selected for use as host plants. Alfalfa inoculum and test plants were prepared for mechanical inoculations as described earlier. Sixteen days after inoculation, final records for infection severity were taken. A score of (0) indicated no visible symptoms, (1) indicated wilting of upper leaves, (2) indicated noticeable stem necrosis or discoloration, and a score of (3) indicated a severe necrosis or death. Each cultivar was rated for relative susceptibility to PSV.

To determine the extent of strain variation among Northwest isolates of RCVMV, four isolates of RCVMV from Oregon, RCVMV isolate WA-7641 and the American Type Culture were compared. Oregon isolates of RCVMV, identified by serology and particle morphology, were collected from red clover and transferred to pea cv. Cascade. A limited host range study was performed including pea cv. 447, and two local lesion indicators, Gomphrena globosa and Chenopodium amaranticolor.

Seed transmission. To assay seed transmissibility of RCVMV in red clover, red clover seeds were collected from several plants from a

Corvallis field where infection was detected at about 50% of the plants tested (se Table 1-3). Alfalfa cv. Ranger seeds from a field planted near Toucket, Washington in 1964 were collected to assay seed transmission of PSV. PSV had previously been detected in this field (Table 1-4).

Seeds were scarified by gentle rubbing between sheets of fine sandpaper and planted in moist vermiculite. Three weeks after planting seedlings were removed, bulked in groups of two to eight, homogenized and assayed for infectivity on plants of pea cv. Cascade. Presence of virus in seedling homogenate, as evidence of viral transmission through seeds, was based on symptoms induced on test plants and by electron microscopy if symptoms were equivocal.

RESULTS & DISCUSSION

Symptomatology and host range. Systemic infections of PSV and RCVMV in pea were unequivocally distinguishable in the greenhouse by symptomatology. For convenience, however, their development in inoculated peas will be generalized and discussed together.

Pea seedlings (one to three bifoliate stage) inoculated in the greenhouse with either PSV isolate ID-3-2 or RCVMV isolate WA-7641 had usually developed necrosis on inoculated leaves after five days. Control plants abraded with buffered plant extract showed mechanical damage but necrosis was limited to abraded leaves in the same period (Figure 1-1). Stunting was apparent in the virus inoculated plants and inoculated leaves were dying seven days after inoculation. Wilting of leaves above the PSV inoculated leaves could be observed by the ninth or tenth day. Wilting of individual leaves tended to occur randomly, early in the systemic infection process, with normal leaves typically occuring between those that were collapsed (Figure 1-2). RCVMV generally induced systemic symptoms consisting of stunting by days nine or ten. Between days 11-13, stem streaking was apparent in the PSV infected plants. Stem streaking appeared as a darkening or graying due to internal necrosis. The first systemic symptoms of RCVMV showed as epinasty of the upper leaves; next vein clearing occurred and the stipules curled downwards (Figure 1-3). RCVMV isolate WA-7641 was unable to induce any systemic necrosis. The symptomatology in pea induced by the Wisconsin isolate of PSV was identical to that induced by isolate ID-3-2. RCVMV isolate ATC induced symptoms in peas similar

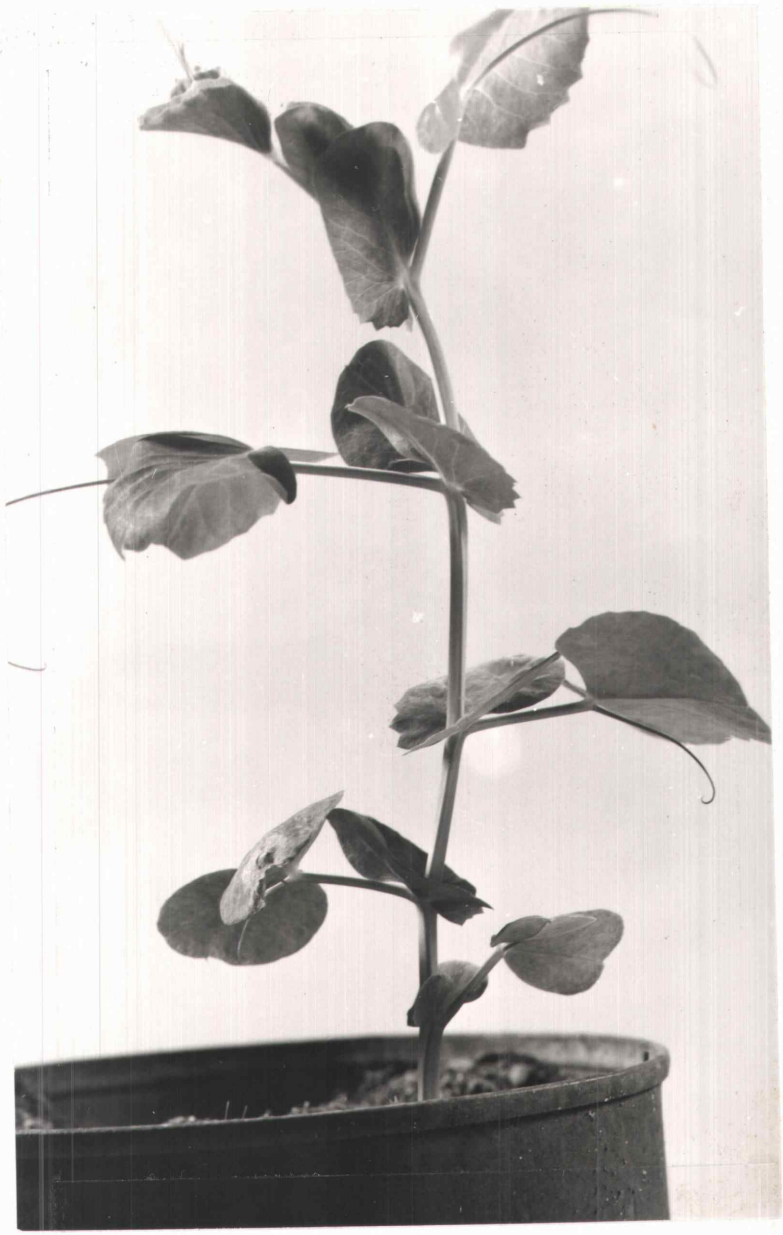


FIGURE 1-1. Pea, Pisum sativum L., healthy plant.

FIGURE 1-2. Pea, Pisum sativum L. cv. Cascade, infected with red clover vein mosaic virus.

Note: plant stunting, leaf epinasty, and leaf rolling.

FIGURE 1-3. Pea, Pisum sativum L. cv. Cascade, infected with pea streak virus, ID-3-2.

Note: collapse of leaf at third node (arrow).

to, though more severe, than isolate WA-7641. In addition, RCVMV isolate ATC occasionally induced stem necrosis similar to PSV infections, though no leaf collapse occurred.

Isolates of both RCVMV and PSV occasionally lost virulence, in which case isolates were re-initiated from desicated reference inoculum. The most severe and reliable symptoms induced by PSV or RCVMV typically occurred in either fall or spring. Declining virulence of virus isolates was most often observed in summer or winter. Moreover, mere transfers over several months sometimes resulted in decreased virulence. Shading or growth chamber environments were of little value in enhancing symptom severity.

The host ranges of RCVMV isolate WA-7641 and PSV isolate ID-3-2 compared to their respective type cultures were quite similar (Table 1-1). The four isolates studied were infectious to relatively few plant species, most of which were legumes. Local lesion induction in Chenopodium amaranticolor or Gomphrena globosa was an important point of differentiation among isolates. The ability to induce local lesion on Chenopodium or Gomphrena was a stable characteristic for the type isolates, whereas Northwest isolates of PSV and RCVMV were unable to induce local lesions on Chenopodium. RCVMV isolate WA-7641 did not induce local lesions on Gomphrena, and PSV isolate ID-3-2 sometimes induced local lesions on this host, though this was an unstable characteristic.

A new plant host was discovered by our host range study of RCVMV. RCVMV was reported to be non-infectious to cucumber, Cucumis sativa L.

HOST SPECIES	VIRUS ISOLATES			
	PSV(Wisc)	PSV(ID-3-2)	RCVMV(WA-7641)	RCVMV(ATC)
Antirrhinum majus L.	-/-	-/-*	-/-	o
Chenopodium amaranticolor	$LL_{c,n}$/-	-/-*	-/-	LL_c/-
Cucumis sativum	-/-	-/-*	1/s	1/-
Glycine max cv. Bragg	-/-	-/-*	-/-	o
cv. Davis	-/-	-/-*	-/-	o
Gomphrena globosa	LL_n/-	(LL_n)/-	-/-	LL_n/-
Lycopersicon esculentum	-/-	-/-*	-/-	o
Medicago sativa	-/s	-/s	-/-	o
Nicotiana glutinosa	-/-	-/-*	-/-	o
Nicotiana tabacum	-/-	-/-*	-/-	o
Petunia hybrida	-/-	-/-*	-/-	o
Phaseolus vulgaris				
cv. Bountiful	-/-	-/-*	-/-	o
cv. Black Turtle	-/-	-/-*	-/-	o
cv. Pinto 111	-/-	-/-*	-/-	o
Phlox drummondii	-/-	-/-*	-/-	o
Pisum sativum cv. Perfected Wales	LN/N,Str,K	LN/N,Str,K	LN/Ros,Ep,VC	LN/Ros,Ep,VC
cv. Dark Skin Perf.	LN/N,Str,K	LN/N,Str,K	LN/Ros,Ep,VC	LN/Ros,Ep,VC
Spinacea oleracea	-/-	-/-*	-/-	o
Tritolium pratense	-/s	-/s	-/s	o
Trifolium repens	-/-	-/-	-/s	o
Vicia faba	LN/N,Str,K	LN/N,Str,K	LL_n/s	LL_n/Str,N
Vigna unguiculata	-/-	-/-*	-/-	o

TABLE 1-1. Symptoms induced by PSV and RCVMV in standardized hosts useful for identification and differentiation of legume viruses[1].

Symbols are: Localized reactions/Systemic reactions; o = Not tested; - = No symptoms; ( ) = Variable symptoms not always expressed; * = No back test for latent infection; Ep = Epinasty; K = Killed; l = Latent, localized; LL_c = Local lesion, chlorotic; LL_n = Local lesion, necrotic; LN = Leaf necrosis; N = Necrosis; Ros = Rosetting; s = Latent, systemic; Str = Streaking stem necrosis; VC = Vein clearing.

1. For a complete description of host species used including National Seed Storage Laboratory seedlot accession numbers see Hampton et al. (25).

(6, 75), whereas we established that this host was susceptible to latent infection with both RCVMV isolates ATC and WA-7641 (Table 1-1). Such susceptibility could hardly be regarded as unimportant. For instance, it is interesting to note that early workers (27, 74) concluded that alfalfa, Medicago sativum L. was not susceptible to PSV. PSV, though latent, has in fact proven to be latently infectious to alfalfa (Table 1-1) and plays an important role in the ecology of the virus (30). The early failures to detect PSV in alfalfa were presumably due to the relatively long incubation periods required before virus titer increased to a level where detection by bioassay was possible.

Sap stability. Results of sap stability tests for RCVMV and PSV isolates are summarized in Table 1-2. All isolates were inactivated within 24 hr in crude sap. Dilution end points of all four were 10^{-5}, notwithstanding data to the contrary reported elsewhere (5, 62). Thermal inactivation determinations revealed that PSV is significantly more heat stable than RCVMV. Inactivation points of the two viruses were separated by approximately twenty degrees C, a difference reported by earlier investigators (5, 62).

Electron microscopy. Standard leaf dip preparations from both PSV and RCVMV-infected pea plants provided an abundance of carlavirus-like particles (Figure 1-4). RCVMV isolates ATC and WA-7641 were morphologically indistinguishable as were also Wisconsin and ID-3-2 isolates

TABLE 1-2. Pea streak and red clover vein mosaic virus stability in sap.

	VIRUS STABILITY IN SAP			
TEST	PEA STREAK		RED CLOVER VEIN MOSAIC	
	ID-3-2	WISCONSIN	WA-7641	ATC
Longevity in sap	24 hr	24 hr (60 day*)	24 hr	24 hr
Dilution end point	10^{-5}	10^{-5} (10^{-7}*)	10^{-5}	10^{-5} (10^{-3}*)
Thermal inactivation point	75°C	80°C	60°C	60°C (65°C)

*Value reported in literature.

of PSV. There was, however, a consistant 30 nm difference between modal lengths of RCVMV and PSV particles. PSV invariably consisted of unimodal slightly flexous 628 nm rods (Figure 1-5), whereas RCVMV particles showed greater variability and comprised a modal length of 660 nm (Figure 1-5). These measurements confirm earlier reports (6, 63).

Field assay results. The results of field assays from Oregon are summarized in Table 1-3. No PSV was detected in legumes collected in the Corvallis vicinity. RCVMV was initially isolated from peas in Western Oregon and; in the course of this study, was subsequently found to occur in volunteer red clover and white clover plants. RCVMV isolates taken from these hosts induced mild symptoms under greenhouse conditions despite inducing severe necrosis on peas grown in the field.

The discovery of two perennial hosts of RCVMV is significant. These two hosts are considered by us to be the most likely sources from which aphid vectors actively transfer virus to the Oregon pea fields. Pea aphids were observed feeding on red clover early in June, a few weeks before the pea fields became heavily infected. White clover mosaic virus was also detected in 3/6 of the white clover samples tested.

Alfalfa has been known within our research program to be the principal inoculum reservoir of PSV for some time (30). In the course of this thesis study, we confirmed this by determining that alfalfa stands in southeastern Washington and southern Idaho typically contain

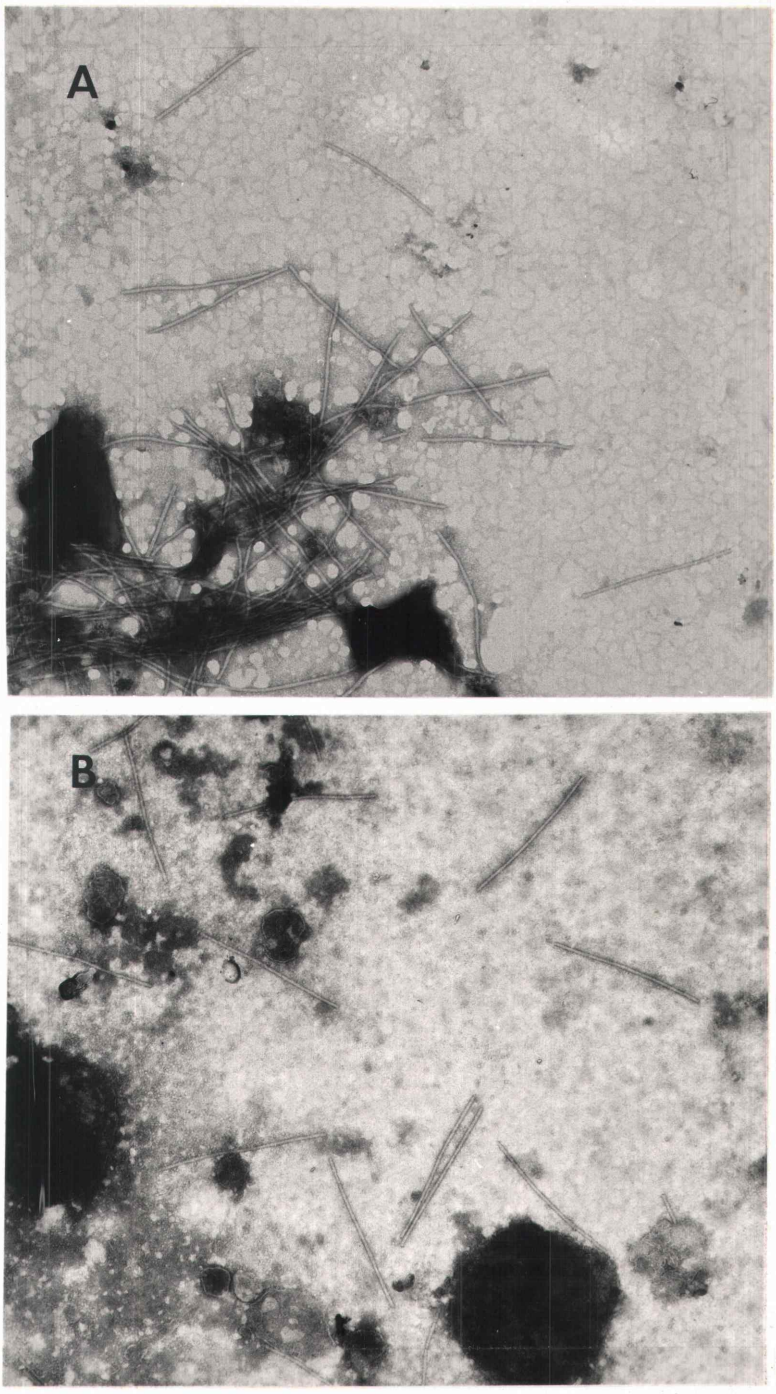


FIGURE 1-4. Leaf dip preparations of a. pea streak virus isolate ID-3-2 and b. red clover vein mosaic virus isolate WA-7641.

Tissue was ground in 3.5% glutaraldehyde and negatively stained with phosphotungstate pH 7.0. 30,500X.

FIGURE 1-5. Particle length frequencies a. red clover vein mosaic virus isolate WA-7641; b. pea streak virus isolate ID-3-2.

The modal length of RCVMV was 660 nm; however, significant numbers of particles were between 670 and 690 nm in length. Other isolates of RCVMV including the type culture had a skewed particle length distribution and the inclusion of additional particle measurements indicated that particle length variability was characteristic of RCVMV.

PSV had a unimodal length of 628 nm.

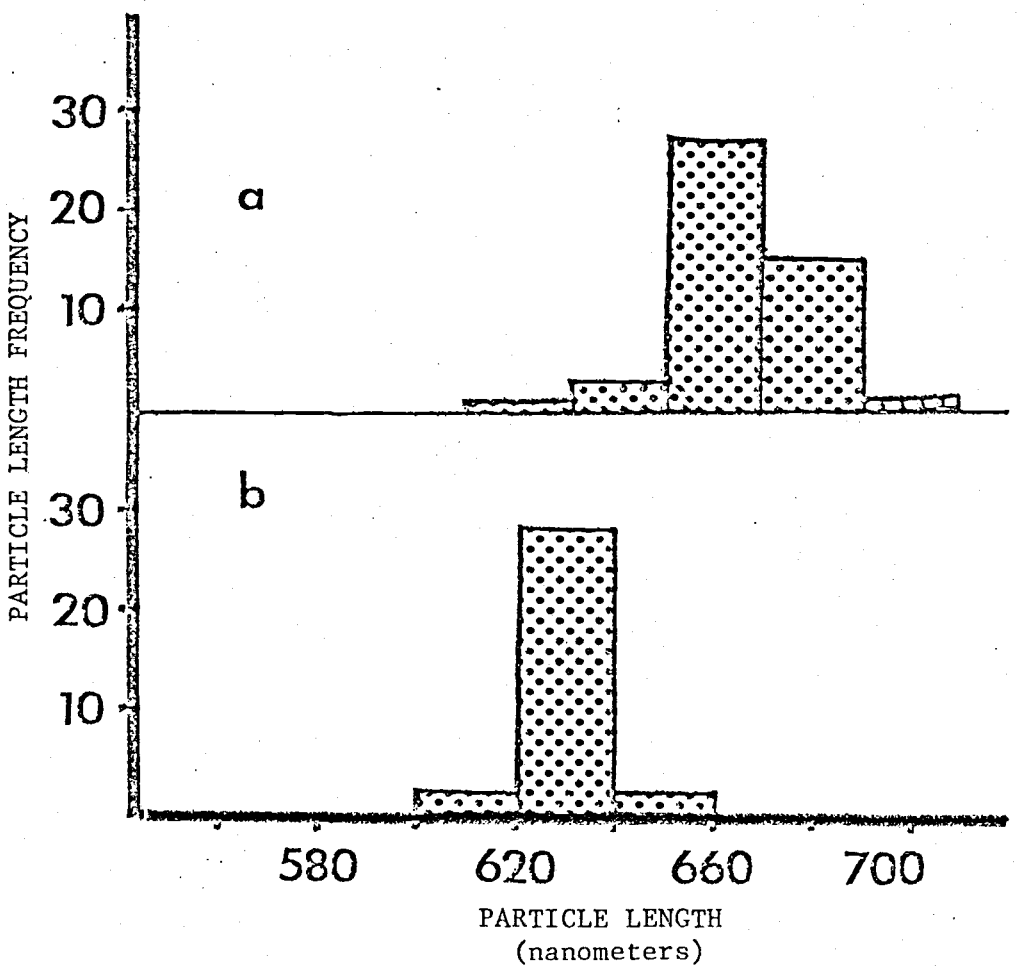

30
20
10
a
30
20
10
b
PARTICLE LENGTH FREQUENCY
580
620
660
700
PARTICLE LENGTH
(nanometers)

FIGURE 1-5

TABLE 1-3. Field assay results from Corvallis, Oregon.

TEST PLANTS	YEAR	SAMPLES TESTED	RCVMV	PSV
Pisum sativum L.	1977	8	2	0
	1978	9	8	0
Medicago sativa L.	1979	6	0	0
Trifolium dubium L.	1976	3	0	0
	1979	3	0	0
T. pratense L.	1978	9	4	0
	1979	6	3[1]	0
T. repense L.	1979	6	2[1]	0
T. subterraneum L.	1976	4	0	0
	1979	2	0	0

Plants were collected and sap extracts mechanically inoculated onto pea for assay work. RCVMV was identified by a combination of symptomatology, particle morphology and serology.

[1]White clover mosaic virus was positively identified in one of the two white clover plants infected with RCVMV.

significant levels of PSV latent infection (Table 1-4). A second virus causing latent infection in alfalfa was alfalfa mosaic virus (AMV), found to be associated with PSV in all of eight fields tested in the Gardena Bench area of southeastern Washington.

The primary purpose of the Washington alfalfa survey (Table 1-4) was to search for alfalfa latent virus (ALV). ALV is a recently discovered carlavirus infectious to legumes (66). No ALV was detected in 24 alfalfa samples collected from Washington. An assay for ALV from 6 alfalfa plants from Salem, Oregon were also negative (Table 1-3).

The presence of multiple infections of WCMV with RCVMV and of AMV with PSV in potential perennial inoculum reservoirs led to speculation that the aphid transmission of carlaviruses to peas are affected by the presence of the non-carlaviruses.

Aphid transmission of carlavirus in multiple infections. Hampton and Sylvester demonstrated that the presence of AMV lowered the transmission rate of PSV from peas (29). The effects of the presence of AMV on the transmission of PSV from alfalfa by the pea aphid were further investigated in this study. Seven trials testing for relative effects on transmission efficiency of aphids allowed a preaccess feeding on alfalfa infected with AMV are summarized in Table 1-5. There were no discernible differences in transmission efficiency due to the relatively low aphid transmission rates of PSV from alfalfa (below 4%). The low transmission rate was unexpected as preliminary work completed six months earlier indicated repeatable transmissions of PSV of 33% from the same alfalfa plants using 5 aphids per plant.

TABLE 1-4. Field assay for carlaviruses in alfalfa cultivars from the Gardena Bench area of Washington.

VIRUSES DETECTED IN ALFALFA CULTIVARS (GARDENA)						
Test Plants			Virus Incidence			
alfalfa cultivar	age of stand, yr	no. of samples	PSV	RCVMV	ALV	PSV+AMV
Ranger	5	6	3	0	0	3
Ranger	14	6	1	0	0	1
Vernal	1	4	0	0	0	0
Vernal	18	4	4	0	0	4
Agate	2	4	0	0	0	0
TOTAL		24	8			8

PSV titer fluctuation in alfalfa has been demonstrated under greenhouse conditions (30). The potential for AMV to play a role in the periodic fluctuations in the incidence of pea streak virus in pea fields grown near alfalfa needs to be investigated further.

The desicated reference isolate from which RCVMV (WA-7641) was obtained was contaminated with WCMV. Contamination of WA-7641 with WCMV by our handling was unlikely. Since WCMV has no reported vectors, an experiment designed to test the hypothesis that WCMV may be transmitted in the presence of RCVMV by genomic masking was conducted. This phenomenon is not without precedent (16). While both viruses were transmitted to pea by mechanical inoculation, aphids were unable to transmit WCMV to any of 15 pea plants with 5 aphids transferred to each test plant. RCVMV was transmitted from these doubly infected source plants at a rate of 20%.

Strain comparison. Before and during Dr. Hampton's tenure at Oregon State University, legume tissue containing streak inducing pea viruses from the Pacific Northwest were desicated and stored for reference purposes.

Thirteen carlavirus isolates from this reference collection were studied and their respective characteristics compared (Table 1-6). There appeared to be an overlap between PSV and severe strains of RCVMV with regard to their ability to induce stem necrosis in peas. In the field, RCVMV isolates from the Corvallis vicinity induced severe necrosis, but upon transfer to peas under greenhouse conditions

TABLE 1-5. An investigation of the effect of prior access to alfalfa mosaic virus infected alfalfa plants on the transmission of PSV by aphids.

ACCESS #1 host/virus	ACCESS #2 host/virus	No. of aphids [1]	No. of test plants [2]	No. of plants inf. with PSV
---	Alfalfa/ PSV ID-3-2	197	63	3
Alfalfa/ healthy	Alfalfa/ PSV ID-3-2	174	74	4
Alfalfa/ AMV	Alfalfa/ PSV ID-3-2	222	90	3

1. Acyrthosiphon pisum Harris.
2. Pisum sativum cv. Cascade.

only mild symptoms were usually induced in peas. Isolate B-5 (Table 1-6) typified this phenomenon. Whereas induction of stem streaking in peas was an overlapping characteristic, the alternating leaf collapse was as unique to PSV as epinasty, leaf curl and vein chlorosis were for RCVMV infections in pea. There were measurable particle length differences between PSV and RCVMV isolates as identified by SDS-immunodiffusion. In no case was an isolate consisting of 660 nm particles serologically identified as PSV nor was any isolate with 630 nm particles serologically reactive to RCVMV antiserum. The uniqueness of modal length may be attributable to characteristic sizes of RCVMV and PSV nucleic acid molecules (65). The differentiation of PSV and RCVMV by symptomatology, and serology confirms the results reported by Bos et al. in 1971 (6).

None of the Northwest isolates of carlaviruses induced chlorotic local lesions on Chenopodium as are characteristically induced by the Wisconsin isolate of PSV or the ATC isolate of RCVMV. Some isolates of PSV from the Northwest exhibited transient ability to induce local lesions on Gomphrena globosa. This characteristic was usually lost on serial passage through pea plants in the greenhouse. ID-3-2 #2, ID-3-2 #4 and ID-3-3 #1 were three PSV isolates which induced local lesions on G. globosa ID-3-2 #1 which was different from ID-3-2 #2 only in the alfalfa plant in which it was cultured gave no local lesions on G. globosa (Table 1-6).

The virulence of four isolates of PSV, ID-3-(1-4) on eight cultivars of pea is presented in Table 1-7. In summary, virulence was measured by the disease severity index for each pea cultivar.

TABLE 1-6. Comparison of Northwest carlavirus isolates to type cultures, PSV isolate Wisconsin and RCVMV isolate ATC.

Isolate	Symptomatology		Particle length	Serology
	Pisum sativum	*Gomphrena globosa*		
95 PW 1-23-63	LN/Str	-	628nm	PSV
198 PW 8-7-63	LN/Str	-	628nm	PSV
304 HB LL 8-7-63	LN/Str	-	628nm	PSV
Idaho #6 1973	LN/Str	-	660nm	RCVMV
Idaho #8 1973	LN/Str,Ep,Ros	-		RCVMV
ID-1-1	LN/Str	-	628nm	PSV
ID-3-1 #1	LN/Str	-	628nm	PSV
ID-3-1 #2	LN/Str	-	628nm	PSV
ID-3-2 #1	LN/Str	-	628nm	PSV
ID-3-2 #2	LN/Str	LLn	628nm	PSV
ID-3-3 #1	LN/Str	LLn	628nm	PSV
ID-3-3 #4	LN/Str	LLn	628nm	PSV
B-5 Corvallis	1/s	-	660nm	RCVMV
RCVMV WA-7641	LN/Ep,Ros	-	660nm	RCVMV
RCVMV ATC	LN/Str,Ep,Ros	LLn	660nm	RCVMV
PSV Wisconsin	LN/Str	LLn	628nm	PSV

Symbols are: /=localized reactions/systemic reactions; - = no visible symptoms; Ep=epinasty; l=latent; LN=leaf necrosis; Ros=rosetting; Str=streaking; s=symptomless infection.

Cultivar, 447 was the least susceptible with a total disease rating of six out of a possible 15. The highest five cultivars were scored between 9.5 and 10 of which cv. Cascade was selected as the most suitable cultivar for further experimentation.

On the basis of this experiment ID-3-2 in alfalfa plant #2 was the most severe inoculum with a disease rating of 20.5 out of a possible 24, and ID-3-3 in alfalfa plant #1 was the least virulent with a score of only 2.5. Inoculum ID-3-3 in alfalfa plants 1 and 3 showed significant variation in disease severity on pea. Differences in virulence between virus isolates were assumed to be partially dependent upon virus titer. Virus titer has been known to fluctuate in alfalfa (30, 31) and this may explain the variation between ID-3-3 #1 and #3.

Results from these studies facilitated comparisons among six isolates of RCVMV, including four Oregon isolates from red clover plants, RCVMV isolates WA-7641 and ATC. These comparisons showed that RCVMV from the Northwest, unlike RCVMV isolate ATC, failed to produce any local lesions on either G. globosa or Chenopodium amaranticolor. Despite this unique difference, other biological properties such as particle morphology, serological identity, and symptomatology on Pisum were essentially identical among the six.

Seed transmission. A preliminary investigation was made to determine if evidence for seed transmission of either RCVMV or PSV could be easily obtained. A total of 578 red clover seedlings were assayed on peas. No pea seedlings showing symptoms indicative of RCVMV infection were detected (Table 1-7).

TABLE 1-7. A survey of virulence of PSV isolates from Idaho upon eight different cultivars of pea (Pisum sativum L.)

PSV Isolates[1]	CULTIVARS							
	Perfected Wales	Cascade	447	Roger's 69-225 Freezer	Roger's Aurora #661	Gallitan Valley H302-2 (Luna)	Roger's Perfected Freezer 60	Roger's Canner #68,273
ID-3-2#2	2.5	3	2	2	3	3	3	2
ID-3-3#3	3	3	2	2	3	1	3	2
ID-3-3#1	0	0	0	1	0	0	0	1.5
ID-3-2#5	2.5	1.5	1	2	2	1.5	1.5	2
ID-3-4#4	1.5	2	1	2	2	2.5	2.5	2.5

1. Four isolates were studied ID-3-(1-4). Each isolate derived from natural aphid populations feeding on alfalfa stands in southern Idaho had been transferred to alfalfa plants.

(0)=no symptoms, (1)=wilting of upper leaves, (2)=stem necrosis, (3)=death.

TABLE 1-8. Assay of alfalfa and clover seedlings for seed transmission of carlaviruses.

TESTING FOR SEED TRANSMISSION				
Virus	Source	No. of Seedlings Tested	Host Pl.	Results
Pea streak virus	Alfalfa Gardina Bench, Washington	96	Pisum	0/96
		15	" "	0/15
		136	" "	0/136
		21	" "	0/21
		60	" "	0/60
Red clover vein mosaic virus	red clover Corvallis, OR	68	Pisum	0/68
		357	" "	0/357
		90	" "	0/90
		63	" "	0/63

A total of 328 alfalfa seedlings were mechanically inoculated onto peas to assay for possible seed transmission of PSV. After two weeks several inoculated peas exhibited stem necrosis. In all cases assay by electron microscopy revealed no filamentous rods indicative of an infection of PSV. Seed transmission of AMV in alfalfa has been reported and local lesion production on Chenopodium amaranticolor and on cowpeas (Vigna sinensis [L.]) confirmed the presence of AMV. A brief study of 100 seedlings assayed on C. amaranticolor revealed a rate of seed transmission for AMV of between 3-12%.

Chapter Two

SEROLOGICAL STUDIES OF RCVMV AND PSV

INTRODUCTION

The specificity of antigen-antibody reactions has been successfully employed for various experimental purposes by plant virologists. Especially since 1960, serological assays for virus detection, for identifications, and for defining viral relationships have been developed and refined. It has been our purpose in this research to apply advanced serological techniques in elucidating carlavirus characteristics.

The serological distinctness between PSV and RCVMV was established previously (5); however, SDS-gel double diffusion procedures had not been applied definitively to carlaviruses prior to the present study. Use of SDS treated gels and PSV and RCVMV reactants was prerequisite to gel double diffusion precipitin reactions, since the long, slightly flexuous particles do not migrate in gels until at least partially degraded.

To provide maximum serological versatility antisera against both PSV and RCVMV were produced during these studies; although antisera to both viruses were available commercially. The antisera were used to gather qualitative and quantitative information in characterizing these viruses, and also in serologically detecting the viruses in

legume plants of the Pacific Northwest. In addition, the identities of various streak-inducing viruses collected and retained in storage over a 15-yr period were resolved by use of these antisera (Table 1-5).

A serological technique which has only recently been exploited by plant pathologists and which shows great potential for the detection of plant viruses is enzyme-linked immunosorbent assay (ELISA). ELISA can be over 1000x more sensitive than conventional serological procedures.

In this study, antibodies were purified and absorbed onto the plastic wells of a micro-ELISA plate. The wells with antibodies attached were then exposed to the test samples. Antigens were bound to homologous antibodies attached to the well. Following a rigorous washing, antibodies cross-linked by glutaraldehyde to alkaline phosphotase were placed in the well. Enzyme-linked antibodies become attached to the antigens and remained after further washing. Enzyme substrate, p-nitrophenyl phosphate (PNP) was added to the well and enzyme-linked antibodies produced yellow color more rapidly than in the control wells. The assay is considered by some to provide either qualitative or quantitative data (13). PNP reaction intensities were quantitated as A_{405} spectrophotometric determinations.

METHODS

Antibody production. Antibodies to PSV and RCVMV were produced by injection of purified virus into male, New Zealand-white rabbits. All serum collection was done via intracardial bleeding. Normal serum was

collected from each rabbit and the rabbits were injected with either intact or disrupted virus. The production of antiserum to intact virus began with a series of three intravenous injections of between 0.1 and 0.2 mg of virus. The injections were three to four days apart. One week after the last intravenous injection 1 to 2 mg/ml of purified virus was mixed in an equal volume of Freund's incomplete adjuvant and injected intramuscularly (IM) into the rabbit's hind legs. A 1.0 ml portion was also injected below the skin of the back. Rabbits were bled one week later. If the antibody titer was unsatisfactory, additional antigen was injected.

To compare the relative antigenicity of intact virus to disrupted virus, the following procedures were followed. Both PSV and RCVMV were disrupted by the addition of sodium dodecyl sulphate (SDS) (1.0%), and 2-mercaptoethanol (0.05%) followed by immersion in water for two minutes at 100 C. Between 1-2 mg of disrupted virus was injected both intramuscularly and subcutaneously as described for intact virus. Since is was not considered safe to inject the mixture of SDS and 2-ME directly in the rabbit's circulatory system, there were no intravenous injections of disrupted virus. Degraded RCVMV was injected IM into a rabbit followed by a second IM injection four weeks later. The rabbit was bled two weeks after the second injection. The injection schedule for the production of antiserum against degraded PSV was slightly modified from the schedule used for producing degraded RCVMV antiserum. SDS degraded PSV was injected three times at weekly intervals. Serum was collected one week following the final injection.

Antiserum preparation. After collection, blood was allowed to clot at room temperature for one hour. The clot was freed from the bleeding flasks and placed at 4 C overnight. Serum was gently withdrawn with a pipet and centrifuged at 5,000 x g for 10 min. The resulting serum was made 0.01% with sodium azide and stored either at 4 C or at -20 C.

Microprecipitin test. A titration grid pattern was drawn with a wax pencil on the inside bottom of a square plastic petri plate. Serial dilutions of both antiserum and antigen were made using phosphate buffered saline at pH 7.0 as diluent. Columns of each antigen dilution were placed in the squares, to fill the plate. Rows of equal drops of each antiserum were superimposed upon the grid pattern, added to antigen droplets and mixed by gentle tapping of the plate. Normal serum and buffer controls were added and the dish was covered and incubated in a dark moist chamber at room temperature for 12 hr. Microprecipitin reactions were examined and recorded with the aid of a diffused light from below the petri plate (3).

SDS Ouchterlony plates. SDS gel double-diffusion plates for immuno-diffusion tests were prepared from a mixture of 0.6% Ionagar, 0.2% SDS, 0.7% NaCl, 0.1% sodium azide and buffered with 9.0M Tris, pH 9.0 according to the methods of Tolin (60). Antibodies were generally applied undiluted or slightly diluted to gel matrices. Antigens were prepared by adding SDS to purified virus to a concentration of 1.0% or by homogenizing infected plant tissue with distilled water (1:1),

centrifugation at 5,000 x g and adding SDS to the supernatant fluid to a concentration of 1.0%. To facilitate viral disruption, SDS-treated antigens were immersed in boiling water for two min. SDS-treated antigen preparations were proved to be stable for months at either -20 C or 4 C as reported by Purciful et al. (51).

Various reaction matrices were produced by cutting wells into the agar with cork borers and removing the agar wells with gentle suction. Antibodies and antigens were added in appropriate configurations and allowed to diffuse toward each other for 12-24 hr in a closed moist chamber at 22 C. Precipitin bands were examined and recorded by diffuse light placed below the gel.

ELISA methodology. Antibodies were purified by ammonium sulphate precipitation (21). The antibodies were resuspended in ½xPBS. 1XPBS is 0.137 M NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na HPO4, and 2.7 mM KCl at pH 7.4 with .02% NaN_3 as preservative. Gamma-globulin was filtered through DE 22 cellulose pre-equilibrated in ½xPBS. The filtered gamma-globulin was adjusted to OD_{280} of approximately 1.4 which corresponds to 1.0 mg/ml and stored in silicone treated glass tubes at -20 C (12).

The purified gamma-gobulin was diluted in coating buffer (50 mM sodium carbonate, 0.2% NaN_3 at pH 9.6). Enzyme, alkaline phosphatase (Sigma Type VII), was added to antibodies at proportions of 2.0 mg enzyme to 1.0 mg globulin. Conjugation of enzyme to antibody was accomplished by adding fresh glutaraldehyde to a final concentration of 0.06%. The mixture was allowed to react for 4 hr at room temperature.

Glutaraldehyde was removed by dialysis against PBS, and bovine serum albumin was added to a concentration of about 5.0 mg/ml and stored at 4 C.

Two hundred microliters of purified gamma-globulin diluted to between 10-0.1 micrograms per ml with coating buffer were added to each well of a microtitre plate and held at 37 C for 4 hr. The wells were washed with PBS brought to a concentration of 0.05% of Tween-20 before receiving 200 microliters of test samples. Samples were prepared by extracting (infected pea tissue) or diluting (purified virus) in PBS-Tween buffer containing 2.0% polyvinylpyrrolidone and 0.2% egg albumin. Plates were then washed with PBS-Tween. Three hundred microliters of substrate, p-nitrophenyl phosphate, 0.6 mg/ml in 9.7% diethanolamine and 0.2% NaN_3 at OH 9.8, was added to each well and allowed to incubate for up to an hour at room temperature. Enzymatic reaction was stopped by the addition of 50 microliters of 3N NaOH. Results were assayed by visual reading and by optical density measurement at 405 nm light wave length.

RESULTS AND DISCUSSION

High quality antiserum to both RCVMV and PSV were produced. Of the antigens tested, the highest titers of antibodies were produced against intact virus. RCVMV antiserum produced visible microprecipitin reactions when diluted to 1/1024, and the PSV microprecipitin titer was 1/4096. Injection of disrupted virus in SDS and 2-mercaptoethanol resulted in relatively low titers for both RCVMV and PSV. Both antisera had titers of 32 against purified antigen in

microprecipitin tests (Table 2-1).

Antiserum against RCVMV and PSV produced precipitin bands against homologous antigens in 1% SDS agar Ouchterlony plates (Figures 2-1 and 2-2). Precipitin bands appeared within 12 hr. Normal serum produced no precipitin bands. Healthy sap produced no precipitin bands. Precipitin bands were produced against purified virus and against infected tissue extracts.

In order to establish sero-identity of the local virus isolates used in this thesis, type cultures of RCVMV isolate ATC and PSV isolate Wisconsin were tested for serological homology with the two virus antisera. In SDS-agar Ouchterlony plates RCVMV-ATC reacted to produce a conterminous band with RCVMV isolate WA-7641. Similarly PSV isolate Wisconsin and PSV isolate ID-3-2 appeared to be serologically indentical (Figures 2-1 and 2-2).

RCVMV antiserum did not react with PSV antigen in SDS Ouchterlony plates (Figure 2-1). In microprecipitin tests PSV isolate ID-3-2 also did not react with RCVMV antiserum. RCVMV antigen showed no reaction against PSV antiserum in SDS Ouchterlony Plates (Figure 2-2).

Red clover vein mosaic virus was successfully detected using the enzyme-linked immunosorbent assay. Virus concentrations of 10 ng/ml were detectable. With the concentration of the coating antiserum at 0.1 ug/ml, the relative sensitivities of the system at 800 and 3200 dilutions of the stock solutions of conjugated antiserum were studied (Figured 2-3). Virus was detectable at 50 ng at a conjugated antiserum dilution of 3200 and 10 ng when conjugated antiserum was diluted to only 800x. Significant differences in the sensitivity of the assay at these two

TABLE 2-1. Titer determinations of antibodies against RCVMV and PSV.

Antibodies	Titer	
	RCVMV WA-7641	PSV ID-3-2
RCVMV isolate WA-7641		
Intact	1024	0
Normal serum	2	-
RCVMV isolate WA-7641		
Disrupted[1]	32	-
Normal serum	1	-
PSV isolate ID-3-2		
Intact	-	4096
Normal serum	-	0
PSV isolate ID-3-2		
Disrupted [1]	-	32
Normal serum	-	0

1. Virus was disrupted by the addition of SDS and 2-mercaptoethanol to 1.0 and 1.25% (v/v) respectively, followed by immersion in boiling water for 1.0 min.

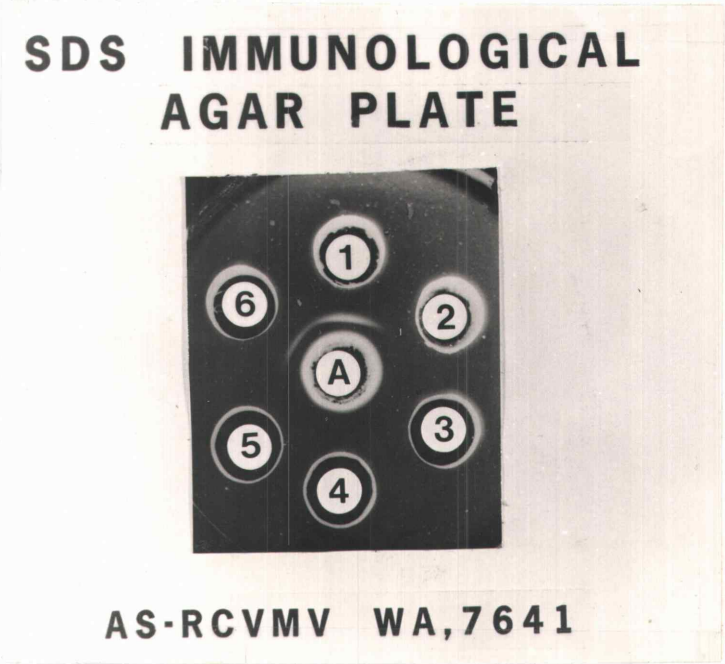


FIGURE 2-1. SDS-gel double diffusion plate (AS-RCVMV).

Center well contained antiserum against RCVMV isolate WA-7641.

Outside wells contained: 1. RCVMV isolate WA-7641, 2. Healthy plant extract, 3. PSV isolate ID-3-2, 4. PSV isolate Wisconsin, 5. Phosphate saline buffer, 6. RCVMV isolate ATC.

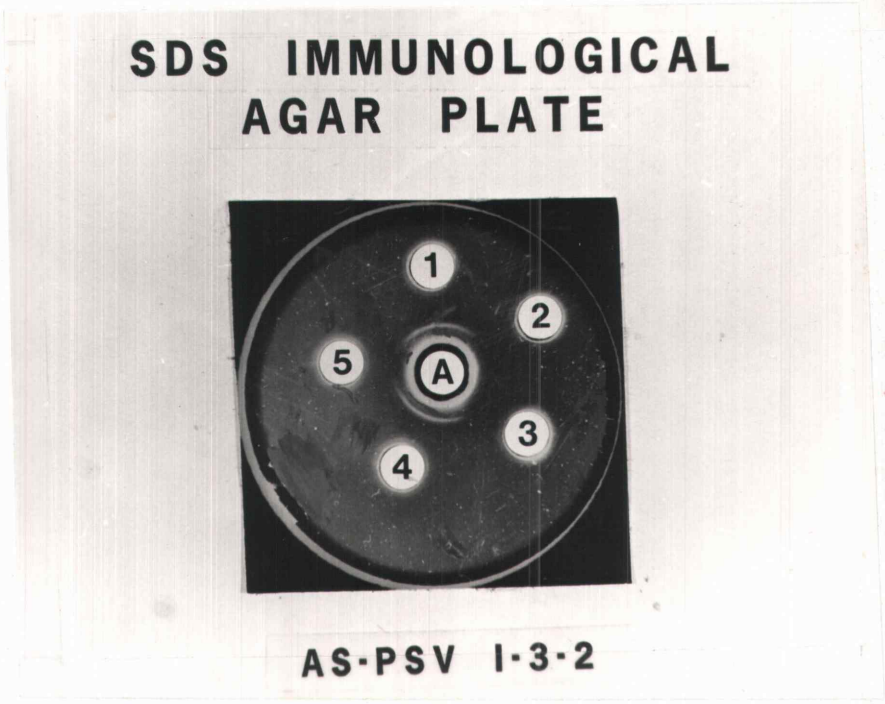


FIGURE 2-2. SDS-gel double diffusion plate (AS-PSV).

Center well contained antiserum against PSV isolate ID-3-2. Outside wells contained: 1. PSV isolate ID-3-2, 2. Healthy plant extract, 3. RCVMV isolate WA-7641, 4. PSV isolate Wisconsin, 5. RCVMV isolate ATC.

conjugate concentrations occurred at a virus concentration of 100 ng/ml. Above a 100 ng/ml of RCVMV, relative differences in optical density between reaction wells of equal virus concentration and of conjugated antiserum diluted to 800 and 3200x increased with increased virus concentration (Figure 2-3).

It has been reported that ELISA has some value as a quantitative assay (13). It appeared in my test that there was a linear relationship between optical density at A_{405} and virus concentration up to 500 ng/ml (Figure 2-3). With coating AS at 0.1 ug/ml and the conjugated AS at dilutions of 400 and 800X, the linearity of the ELISA system below 500 ng/ml was studied (Figure 2-4). There was linear relationship between virus concentration and optical density throughout the tested range of reactant concentration. Regression analyses of conjugated antiserum showed that over 98% of the variation in each set of data was accounted for by the line drawn, establishing unequivocal detection of virus at 25 ng/ml. Anomalous background pigmentation interferred with virus detection at concentrations at 10 ng/ml. A two-fold increase in conjugated antiserum failed to produce comparable increases in optical density at equivalent virus concentration.

To test the feasibility of detecting RCVMV within the body of an aphid feeding on either purified or infected plant tissue by ELISA, preliminary tests were conducted with known amounts of virus added to pea aphid extracts. The conjugated antiserum and coating antiserum were increased to maximize sensitivity to 1.2 ug and 400x dilution respectively.

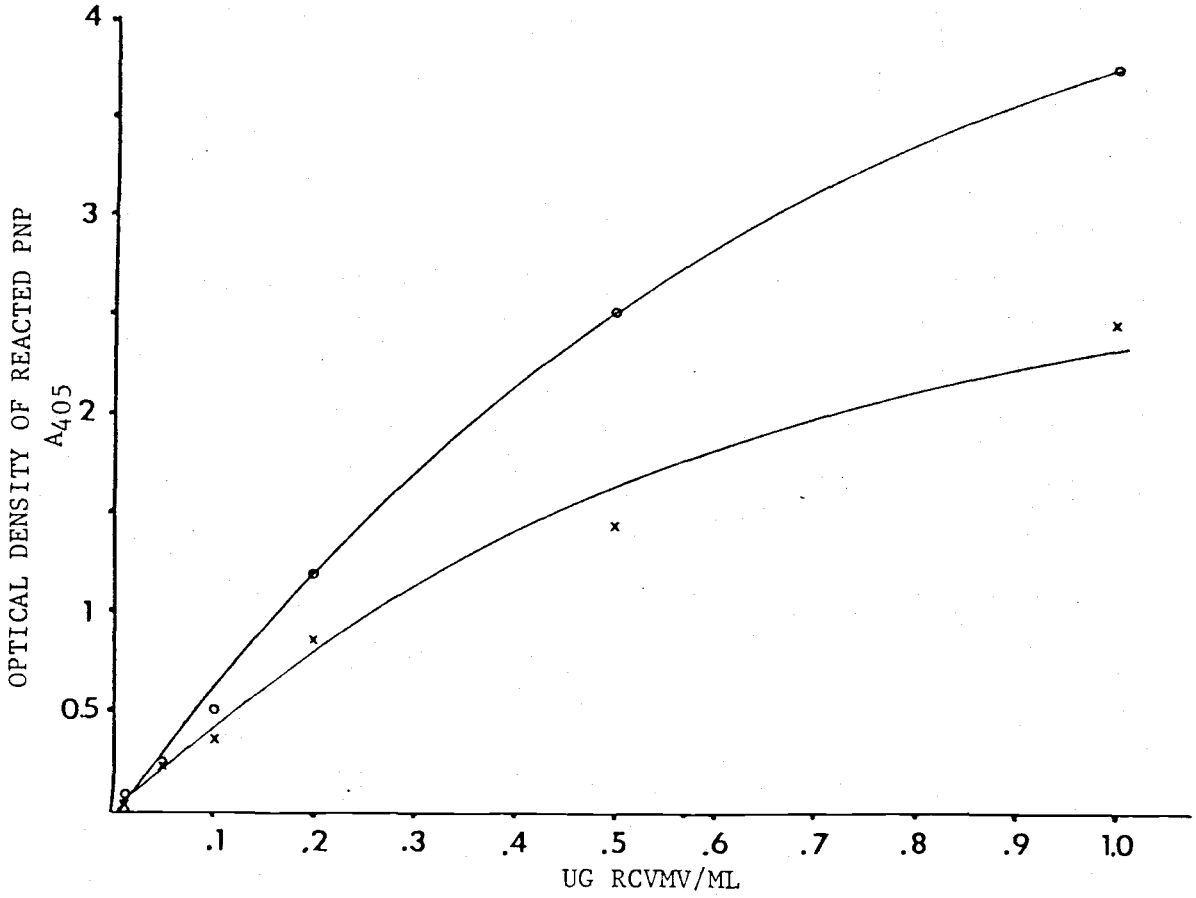


FIGURE 2-3. RCVMV detection using the enzyme-linked immunosorbent assay I.

Coating antiserum was diluted to 0.1 ug/ml. (o) Conjugated antiserum diluted to 800X stock (x) Conjugated antiserum diluted to 3200X stock. Each point represents the average of two trials.

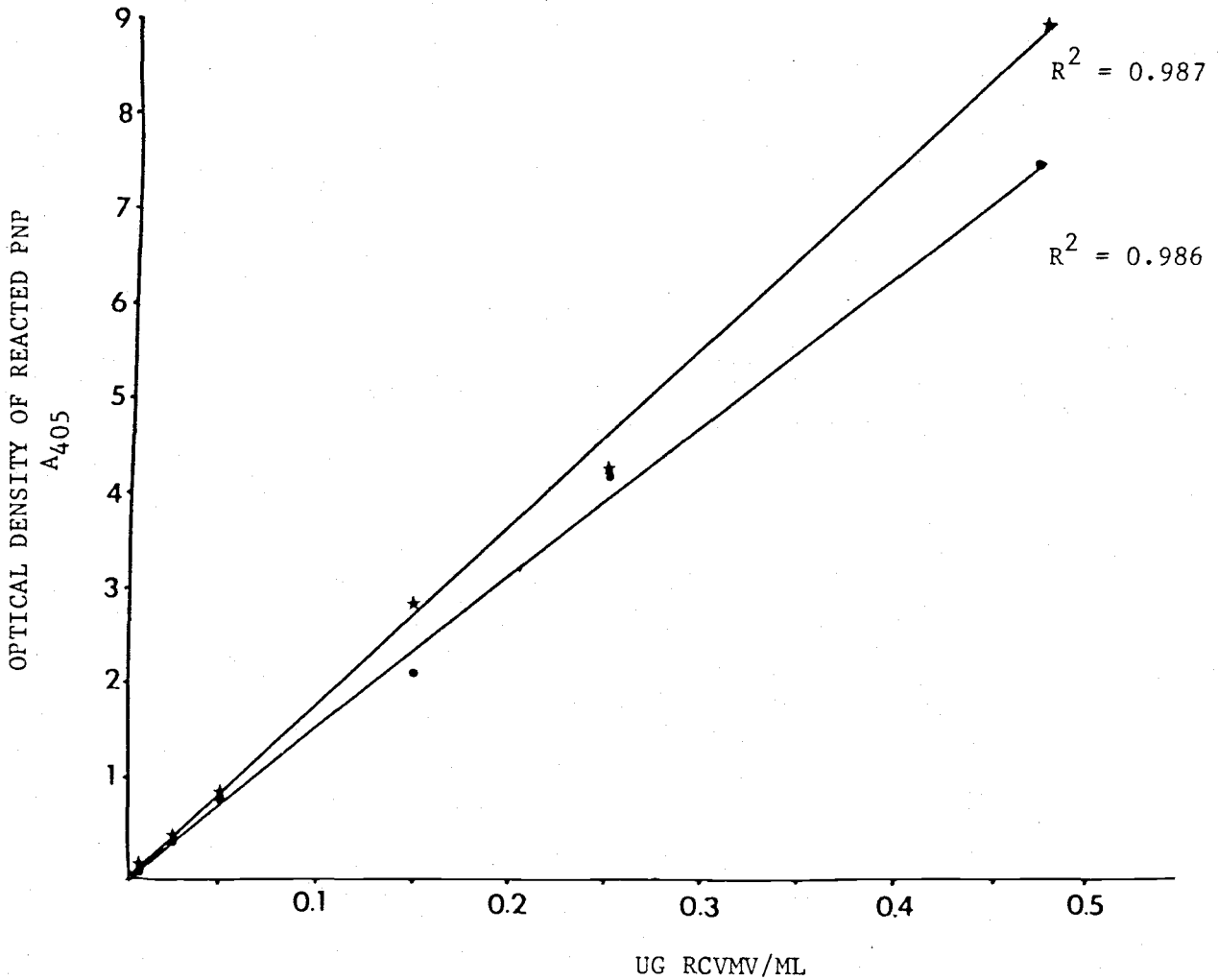


FIGURE 2-4. RCVMV detection using the enzyme linked-immunosorbent assay II.

Coating antiserum was diluted to 0.1 ug/ml. (★) Conjugated antiserum diluted to 400X stock. (•) conjugated antiserum diluted to 800X stock. Each point represents the average of two trials.

Results of these tests indicated that purified virus could be effectively detected down to 5.0 ng/ml. However background of aphid extracts was erractic and was considered to be too high to provide reliable detection of virus below 5.0 ng/ml. Based on results from cucumber mosaic virus in melon aphids by Gera et al. (22), typical RCVMV concentrations within aphids were assumed to be below 1.0 ng/ml. Therefore no further efforts were made to continue this line of investigation.

Chapter Three

PURIFICATION AND BIOCHEMICAL ANALYSIS OF LEGUME CARLAVIRUSES OF THE PACIFIC NORTHWEST

INTRODUCTION

Carlaviruses had been identified as important pathogens of legumes by 1960 (25, 27) and procedures for the purification of carlaviruses were developed in the late sixties (63, 54) . These procedures failed to yield significant quantities of purified virus and in 1977 Veerisetty reported an improved purification procedure for legume carlaviruses. We found Veerisetty's procedure unreliable and this chapter details experiments that enabled us to improve the yield and quality of purified viruses. Buffers, reducing agents, divalent cations and pH were among those parameters investigated.

It was essential that we obtain milligram quantities of relatively pure virus and one of the earliest objectives of our work was the development of a simple and reliable purification scheme for PSV and RCVMV. Such large amounts of virus were deemed necessary to answer important questions prerequisite to control strategies for these viruses in the Pacific Northwest. For example, high quality antiserum was needed to unequivocally identify and monitor Northwest isolates and production of that antiserum obviously required several milligrams of pure PSV and RCVMV.

Beyond the common virus characteristics used to establish isolate identity such as particle morphology, sero-identity, sap stability,

and host range we chose to investigate some of the biochemical properties of the Northwest isolates and compare these properties to type isolates, RCVMV isolate ATC and PSV isolate Wisconsin whose biochemical properties are well established (67). Comparisons of nucleic acid and coat protein electrophoretic mobilities were made, and each isolate was analyzed by ultraviolet spectrophotomertry.

Extinction coefficients ($E^{1\%}_{1\ cm,\ 260\ nm}$) for PSV isolate ID-3-2 and RCVMV isolate WA-7641 were determined. These values enabled us to better standarize our work by reliably converting viral suspensions of known A_{260} to milligrams per milliliter. In addition, the results of an amino acid analysis of RCVMV isolate ATC are reported.

METHODS

Virus purification. The most recently published purification procedure for legume carlaviruses was developed by Veeresetti (64). Briefly the procedure involved the trituration of frozen, infected pea tissue in 165 mM disodium phosphate, 18 mM trisodium phosphate, pH 9.0, containing 0.1% diethyldithiocarbamate and 0.5% mercaptoethanol or 0.1% sodium sulphite. The extract was pressed through cheesecloth, centrifuged at 12,000 g for 10 min and clarified with calcium phosphate. After centrifugation at low speed, the virus-containing supernatant fraction was precipitated by the addition of polyethylene glycol (PEG MW 6000) to 6%. The precipitated virus was resuspended in buffer (1/10 the concentration of the extraction buffer) containing 1% Triton X-100. Following low speed centrifugation the virus was concentrated by differential centrifugation through a layer of 30% sucrose containing buffer and 1% Triton X-100. The resulting pellet was resuspended in buffer with 1% Triton X-100 and further purified by sucrose density gradient centrifugation (10-40%) at 96,000 g for 2 hr. The resulting opalescent band of virus was removed by hypodermic needle and concentrated by a second differential centrifugation at 160,000 g for 50 min.

Modifications of the above procedure were investigated in order to improve the reliability of the purification, increase yield of purified virus, and to eliminate reagents and steps that appeared extraneous. Virus prepared under modified purification conditions were scrutinized for contaminating host material and for anomalous

changes in viral characteristics. Tests included centrifugation in linear sucrose density gradients, assaying for infectivity, electron microscopic analysis, and electrophoresis in polyacrylamide gels.

Sodium borate, pH 9.0 was recommended as an extraction buffer by Veerisetty (personal communication). The original procedure was modified to substitute 0.2 M borate with 0.01 M borate pH 9.0 as the resuspension buffer. In order to obtain the optimum pH for carlavirus purification, RCVMV, ATC was purified in borate buffer at pHs 8.0, 8.5, and 9.0.

The need for a calcium phosphate clarification was also treated. Simultaneous purifications with and without calcium phosphate were compared. Clarification tests were conducted on both PSV and RCVMV.

Tests to study the relative effectiveness of 0.1% sodium sulphite in comparison to mercaptoethanol (ME-2) (0.5%) with sodium diethyldithiocarbamate (DIECA) (0.1%) were conducted. Sodium sulphite and mercaptoethanol were evaluated as reducing agents for preventing the oxidation of sulphdral groups. DIECA which is commonly used as a chelator of copper ions, (23) was evaluated as a preventive to enzymatic oxidation of phenolic compounds commonly found in plant extracts. The relative effectiveness of the two reducing agents was tested using both phosphate-citrate and borate buffers and requirement for the presence of DIECA with the ME-2 was also investigated. RCVMV and PSV were purified simultaneously from infected pea tissue in the presence of sodium sulphite or DIECA and ME-2. RCVMV from infected pea tissue was purified in borate buffer with either 0.5% ME-2 and 0.1% DIECA or

0.5% ME-2 and yields were compared.

The optimum percentage of polyethylene glycol (PEG MW 6000) providing precipitation of carlavirus and the optimum yield were investigated. PSV isolate Wisconsin was purified in either borate or phosphate-citrate buffer, pH 9.0 with 0.1% sodium sulphite and 0.3% Dow antifoam B. The extracts were split into three equal parts. After a low speed centrifugation, the three extracts were made 4% (w/v) PEG, 4% PEG with 0.7% sodium chloride and 6% PEG respectively. PSV isolate ID-3-2 infected pea tissue was triturated in borate buffer, split into three equal fractions as above and PEG added to final concentrations of 4.0, 6.0, and 8%. The density of the viral bands after sucrose density gradient centrifugation was the main criteria for the estimation of yields.

The effect on yield of purified RCVMV by the divalent cation magnesium was also studied. RCVMV isolate WA-7641 was triturated in a borate extraction buffer and pressed through cheesecloth. Following a low speed centrifugation, the extract was divided into three equal portions and $MgCl_2$ added to 0, 1.0, 5.0 mM concentrations. The three extracts were kept at their respective Mg levels until the resuspension of the first high speed centrifugation, at which point each pellet was resuspended in a borate buffer with 1.0 mM $MgCl_2$. Virus yields were compared by band density in sucrose density gradients and viral purity was monitored by ultraviolet absorption spectral analysis.

Virus stability in the presence of $MgCl_2$ was investigated by dialyzing 2 ml of virus at a concentration of 1.0 mg/ml

(both PSV and RCVMV) against 0.01 M sodium borate pH 9.0. The effects of $MgCl_2$ added to 1.0 mM were studied. Virus loss due to aggregation and maintainance of viral stability were monitored by comparing UV absorption spectra before and after dialysis.

Electron microscopy. The particles of purified virus preparations were examined by means of a Phillips EM 300 electron microscope. Virus preparations were mixed with an equal volume of 3.5% glutaraldehyde and a drop placed on a formvar coated, carbon backed grid. After two min the solution was withdrawn by filter paper and the grid stained with 2.0% phosphotungstate at pH 7.0.

Infectivity. Purified virus in either phosphate-citrate or borate buffer were inoculated onto both pea (Pisum sativum) and bell bean (Vicia faba var minor) to assay infectivity. Plants were dusted with carborundum and virus suspensions of PSV isolates Wisconsin and ID-3-2 and RCVMV isolates WA-7641 and ATC were abraded across the leaf surface with a cotton swab.

Extinction coefficient. For the estimation of extinction coefficients, $E^{0.1\%}_{1\ cm,\ 260\ nm}$, PSV isolate ID-3-2 and RCVMV isolate WA-7641 were removed from sucrose density gradients after partial purification and subjected to an additional four cycles of high speed centrifugation. With each centrifugation, the pellet was resuspended in 10 ml of 0.1 M borate buffer pH 9.0 (in the case of PSV, 1.0 mM $MgCl_2$ was added with

the buffer). This series of centrifugations effectively removed all traces of sucrose. The virus pellets were resuspended after the fourth cycle in 0.5 ml buffer and the suspensions were low speed centrifuged 20 min at 12,100 g. The virus suspensions were then filtered through a glass fiber filter cut and fitted into a millipore filter holder. These procedures removed aggregates of virus and extraneous dust and bacteria. Samples of resuspension buffer was also centrifuged and filtered for use as virus-free controls.

Tare pans were made by cutting aluminum foil into one cm squares, and by pressing a 65 rotor tube cap into the foil square backed by foam rubber. The resulting pans were dusted with dry nitrogen gas and stored in lyophilyzing vials. The fabricating foil and finished pans were handled with gloves and tweezers. The pans were weighed to the nearest microgram (ug) on a Cahn electrobalance (Model 4100).

Filtered virus suspensions and buffer were pipeted into tared pans using a single Lang-Levy micropipet (0.1 ml) while the pans remained in position in lyophilizing vials. The vials containing virus- or buffer-filled pans were transferred to either a 90 C oven or 100^{o}C vaccum oven (30 in. mercury vaccum) and dried overnight. The vials were sealed and allowed to cool. Pans were removed and individually weighed to the nearest microgram. Total virus content was determined by subtracting the tare weights and the weights of the buffer samples from the weights of virus samples. Samples with obviously deviant readings were disregarded in the final computation of virus weights. Repeatable results were obtained with about 5% error between individual samples.

The calculation of $E^{1\%}_{1\ cm,\ 260\ nm}$ was based on the linear relationship between concentration and optical density (41). The formula $E = A_{260}/1.0$ mg/ml was used to calculate extinction coefficients. Samples of RCVMV isolate WA-7641 were weighed at two different concentrations. The average weight per optical density A_{260} was calculated and the extinction coefficient extrapolated for each concentration and averaged. The extinction coefficient of PSV isolate ID-3-2 was established by repeated weighing at both single concentrations and over a concentration range. By plotting A_{260} values versus micrograms virus, a regression line was fitted to predict the extinction coefficient of PSV.

Viral protein. Molecular weight estimations of viral protein were based on their electrophoretic mobility in SDS-polyacrylamide gels (PAGE) (14). Ethanol precipitated virus (approx. 1.0 mg) preparations were disrupted by suspension in 0.5 ml of 10 mM tris, 1.0 mM EDTA, 2.0% ME-2, 1.0% SDS and 5.0% sucrose at pH 8.0 and boiled for 3 min. PAGE was carried out on 10, 7.5 or 5.6% acrylamide with 3.8% bisacrylamide, N,N,N',N'-Tetramethylethylenediamine (TEMED) 0.15% ammonium persulphate, 1.0% SDS, 40 mM Tris, 20 mM sodium acetate and 2.0 mM EDTA at pH 7.4. Gels were poured into 5.0 nm i.d. silanized glass tubes and overlayed with 50 ul of 0.1% SDS, 0.15% ammonium persulphate, and 0.05% TEMED. Gels were allowed to set for one hr and then cured for 12 hr at 4 C. Gels were pre-electrophoresed for 30 min at 3-4 mA per gel in a running buffer of 1.0% SDS, 40 mM tris, 20 mM sodium acetate and 2.0 mM EDTA at pH 7.4.

Samples were layered onto each gel (5-10 ug/protein) and electrophoresed for approximately 3 hr. Gels were then fixed in 15% trichloracetic acid (TCA) for 2 hr and stained overnight in 1.2% coosmassie blue (10% TCA-33% methanol). Gels were destained in a mechanical destaining apparatus containing 10% TCA and 33% methanol for 8 hr and stored in 7.0% acetic acid. Gels were scanned at 546 nm wave length in an ISCO gel scanner model 1310.

Marker proteins used were bovine serum albumin, human gamma globulin (light and heavy chains), ovalbumin and alcohol dehydrogenase. These proteins were used to establish a regression line plotting Log_{10} molecular weight versus migration (cm)/gel length to be used for predicting the molecular weights of virus coat protein.

The amino acid composition of RCVMV, ATC was established. RCVMV, ATC was purified by standard procedures and dialyzed for two days against glass distilled water to remove divalent cations. The resulting virus precipitate was lyophilyzed and submitted for analysis to the laboratory of Dr. Robert Becker, Dept. of Biochem. at Oregon State University for amino acid analysis.

The nature of PSV isolate ID-3-2 protein complexity (PAGE band multiplicity) was investigated. Purified PSV (1.0 mg/ml) was divided into six 0.25 ml. fractions. Phenylmethylsulphonyl flouride (PMSF) 0.5 mM was added to three fractions to inhibit protease activity (15). Uninhibited and protease inhibited samples were treated differentially as a means of assessing viral protein complexity. Accordingly control samples were immediately frozen at $-20^{o}C$ and the experimental samples

were treated by incubation for 12 hr at 22 C or for 2 hr at 60 C. After treatment, virus was precipitated by the addition of 2 vol of ETOH and dried over nitrogen. The samples were then disrupted and electrophoresed on 7.5% SDS-acrylamide gels. Gels were scanned at 546 nm and the relative band densities recorded.

Viral nucleic acid. Purified carlaviruses were precipitated by the addition of two volumes of cold ethanol and storage at -20°C for 12 hr. Precipitated virus was centrifuged and the pellet disrupted in a phosphate buffer of 40 mM disodium phosphate 10 mM monosodium phosphate, 1.0 mM EDTA, 1.0% SDS and 1.0% 2-ME, pH 7.5 and by heating at 60°C for five min (67). Disrupted nucleoprotein was layered onto linear-log sucrose density gradients containing 0-32 mg sucrose/ml in neutral 0.5 x standard saline citrate buffer (1 x SSC is 15 mM trisodium citrate with 150 mM sodium chloride) (67). Gradients with samples were centrifuged at 39,000 rpm at 14°C for 5.5 hr in a SW 40 rotor and afterwards analyzed with an ISCO fractionator. For electrophoretic analysis of viral RNA, disrupted nucleoprotein was layered directly onto acrylamide gels.

The infectivity of PSV-RNA derived by this methodology was tested on pea and bell bean plants. Viral RNA collected from sucrose density gradients was made .06 m $MgCl_2$ and precipitated with two volumes of cold ethanol (55). The solution was stored overnight at -20°C and centrifuged at 18,000 g for 20 min. The pellet was dried over nitrogen and resuspended in buffer consisting of 35 mM dibasic

potassium phosphate and 50 mM glycine, pH 9.2. Samples assayed for infectivity contained 76 ug/ml of RNA. Bentonite was added to the RNA solution (100-200 ug/ml) (20) and the sample was inoculated by a gloved finger onto test plants dusted with carborundum.

The composition of viral nucleic acid was studied by electrophoresis through polyacrylamide gels (36) consisting of 2.2% acrylamide with 0.13% bisacrylamide, 0.07% TEMED, and 0.075% ammonium persulphate. Gels were formulated in a buffer of 36 mM tris, 34 mM sodium phosphate and 1.0 mM EDTA at pH 7.6, poured into 5.0 mm i.d. Plexiglas tubes were overlaid with water and allowed to set for 1.0 hr. The gels were pre-electrophoresed for 30 min at 6 mA/gel in running buffer of 36 mM tris, 34 mM disodium phosphate, 2.0% SDS and 1.0 mM EDTA at pH 7.6. After pre-electrophoresing the gels, RNA samples (10 ug/gel) were layered on and electrophoresed for 90 min at 5.0 mA/gel. Gels were removed from the Plexiglas tubes by gentle air pressure and soaked in 0.04 M sodium acetate pH 4.6 for 12 hr under agitation to remove SDS. The gels were stained in 0.02% methylene blue for 2 hr and destained for 12 hr in acetate buffer. The gels were scanned at 546 nm wave length in an ISCO gel scanner Model 1310.

RESULTS AND DISCUSSION

Preliminary virus purifications. In preliminary attempts to apply the methods of Veerisetty and Brakke (68) for carlavirus purification, virus yield was erratic. In some cases there appeared to be a total loss of virus, apparently due to a failure of the virus to resuspend

following the first high speed centrifugation. In other cases, virus resuspended normally at this point and produced normal banding in sucrose density gradients, but became irreversibly aggregated after the subsequent high speed centrifugation. Prescribed purification procedures involved the use of phosphate-citrate (165 mM disodium phosphate and 18 mM trisodium citrate) buffer containing either 0.1% sodium suplhite or 0.1% diethyldithiocarbamate (DIECA) and 0.5% 2-mercaptoethanol-2 (2-ME) as reducing agents. Virus yield and reliability of the method were significantly improved by the use of borate (0.2 M sodium borate) buffer, which proved most effective at pH 8.5-9.0.

Virus yields were equivalent in simultaneous purifications of both RCVMV and PSV, with and without calcium phosphate clarification and therefore calcium phosphate was concluded to be unnecessary and was omitted. Absorption spectra of the purified viruses also were unaffected by such clarification.

Conversely, the presence of sodium sulphite or mercaptoethanol (2-ME) as reducing agents was essential for carlavirus purification. 0.2 M sodium borate pH 8.5 with either 0.1% sodium sulphite or 0.5% 2-ME and 0.1% DIECA was an effective extraction medium for the purification of RCVMV. However, PSV yields were higher and more reliable with 2-ME than with sulphite. DIECA was also determined to be unnecessary for successful purification of either virus and was subsequently deleted from the extraction medium.

The effect of polyethlyene glycol concentration (PEG MW 6000) on yields of PSV was investigated. A 4.0% concentration failed to

precipitate significant amounts of either PSV isolate Wisconsin or ID-3-2. The addition of 0.7% sodium chloride to the 4.0% PEG increased the yield, but PEG concentrations of 6.0 to 8.0% resulted in optimal yields. The 6.0% concentration of PEG was adopted for the standardized purification procedure.

The effect of three levels of magnesium chloride 0, 1.0 and 5.0 mM, on yield of purified RCVMV isolate WA-7641 was studied. Equivalent viral bands in sucrose density gradients were produced at both levels of magnesium, but significantly less virus was produced in the absence of added magnesium. Evaluation of viral purity by relative ultraviolet absorption at 260 and 280 nm suggested the presence of extraneous host protein in the virus prepared with 5.0 mM of magnesium.

PSV aggregated when dialyzed against 0.01 M sodium borate pH 9.0 for 12 hr at 4 C, resulting in a greater than 50% loss of virus, as measured by A_{260}. The addition of magnesium chloride to 1.0 mM prevented aggregation duringg dialysis.

RCVMV was stable upon dialysis against 0.01 M sodium borate at pH 9.0 at 4 C. When the effect of 1.0 mM magnesium chloride was tested, however, it was found to induce a change in the ultraviolet absorption profile of purified RCVMV from $A_{260/280}$ of 1.24 to 1.33. It was assumed that the magnesium chloride had a destabilizing effect on the molecular structure of RCVMV.

On the basis of results from these experiments with $MgCl_2$, appropriate modifications were made in the final resuspension media

used for RCVMV (Figure 3-1).

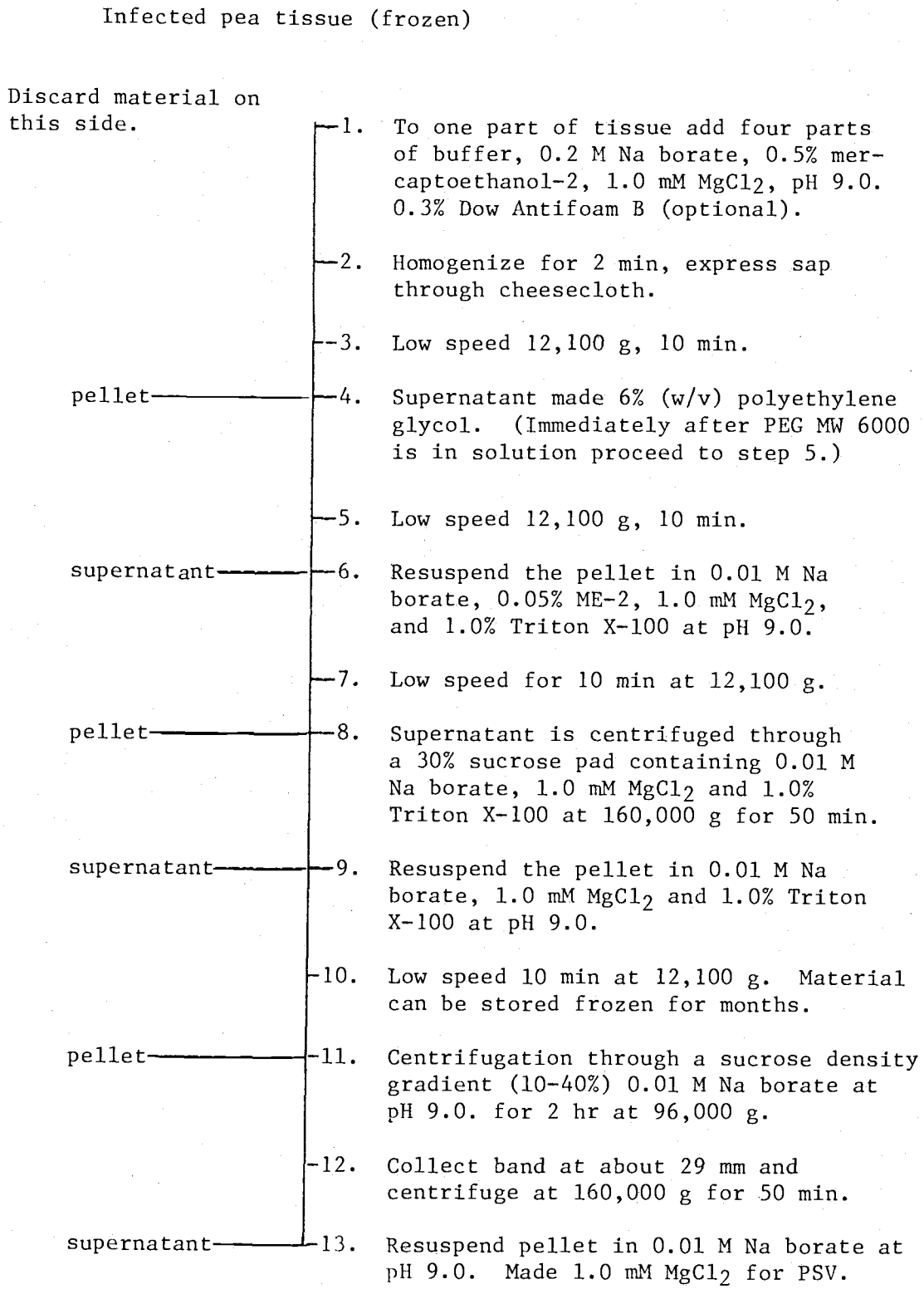
The final purification procedure for both PSV and RCVMV is diagrammed in Figure 3-1. When purified by this procedure PSV and RCVMV both migrated to single opalescent bands in sucrose density gradients Figure 3-2 and 3-3. Yields were somewhat variable among trials, but generally provided 3 to 70 ug of virus per gram of infected tissue.

Electron Microscopy. Electron microscopy of purified virus showed a background devoid of apparent host material (Figures 3-4 and 3-5). No significant differences were noted in modal length distribution, between negatively stained dip preparations and purified virus.

Infectivity. Purified virus preparations were highly infectious. RCVMV isolate WA-7641 was infectious to peas when diluted to 0.5 ug/ml and PSV isolate Wisconsin was infectious to peas when diluted to 5.5 ug/ml. Irrespective of optimal virus yield, all four isolates were infectious whether purified in P-C buffer or borate buffer. Symptoms induced in pea by purified viruses were consistently the same as those induced by crude-sap control inocula.

Ultraviolet absorption spectra. Ultraviolet absorption spectra of PSV and RCVMV were typical for filametous rods of approximately 5.0% nucleic acid. PSV isolates Wisconsin and ID-3-2 had similar spectra with 260/280 ratios of $1.15 \pm .02$. An absorption 'dip'

FIGURE 3-1. General purification scheme for pea streak and red clover vein mosaic viruses.



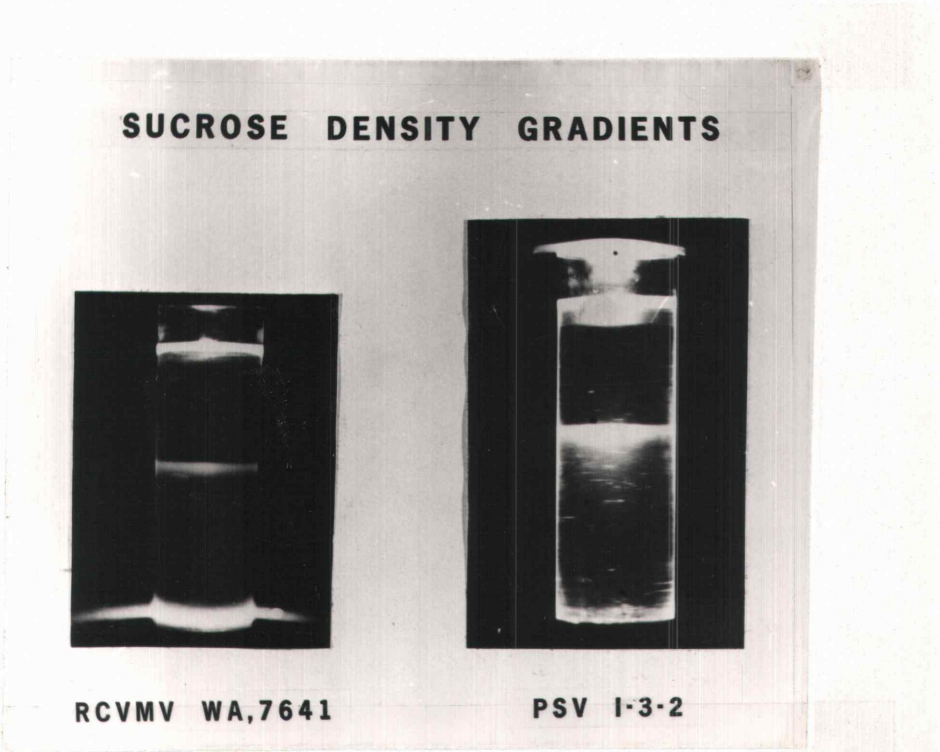


FIGURE 3-2. Linear sucrose density gradients (10-40%) containing RCVMV isolate WA-7641 (left) and PSV isolate ID-3-2 (right).

After centrifugation in a SW 27 rotor for two hours at 96,000 g, the virus bands had migrated to approximately 29 mm below the columns' miniscus.

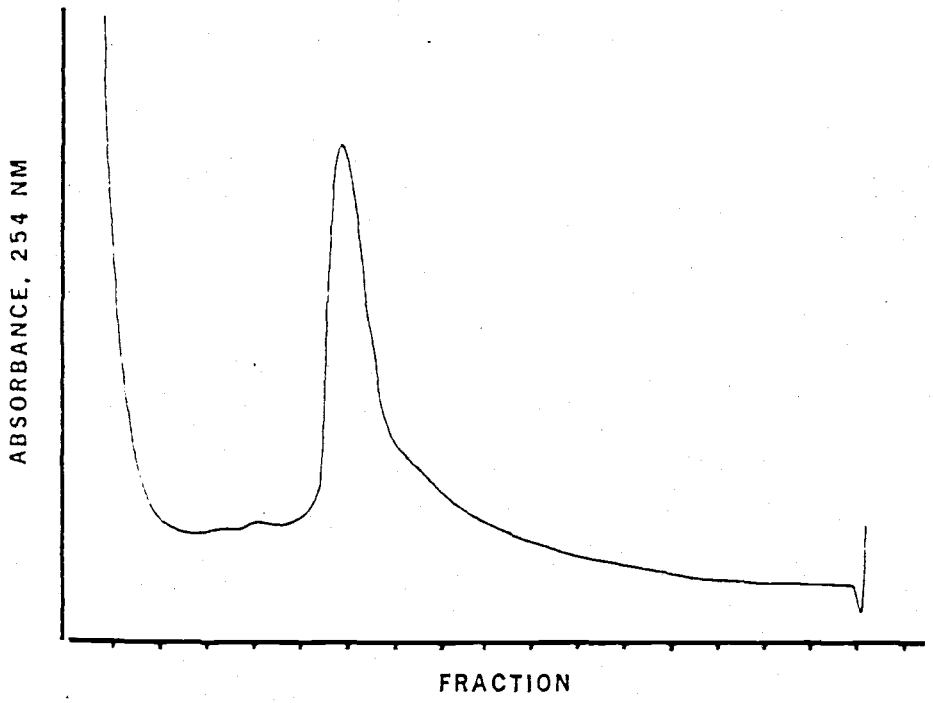


FIGURE 3-3. Photometric scanning pattern (A_{254}) of pea streak virus isolate ID-3-2 centrifuged through a linear sucrose density gradient (10-40%) for 2 hr at 96,000 g in a SW 27 rotor. Note the single major absorbance peak at fraction 6.

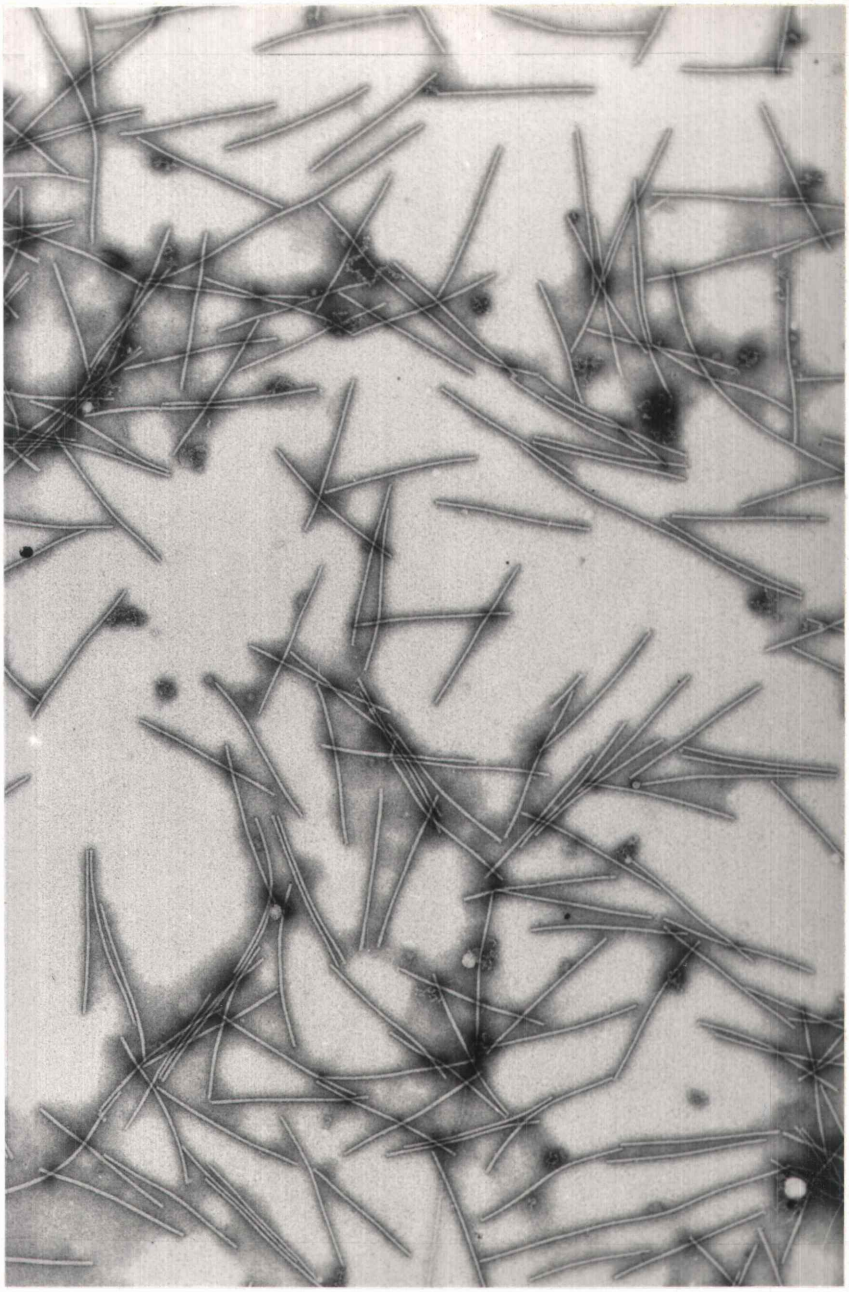


FIGURE 3-4. Purified red clover vein mosaic virus isolate WA-7641 negatively stained with phosphotungstate. 30,800X.

FIGURE 3-5. Purified pea streak virus isolate ID-3-2 negatively stained with phosphotungstate. 30,800X.

characteristically occurred at 247.0 nm wave length (Figure 3-6). RCVMV isolate WA-7641 had a 260/280 ratio of $1.24^{\pm}$ .02 and exhibited an absorption 'dip' at 246.5 nm wave length (Figure 3-7).

Low absorbance at 380-310 nm reflected a low degree of light scattering by the virus preparation. High light scatter, conversely, would have indicated a significant degree of particle size heterogeneity or aggregation. Characteristic absorption profiles were representative for nucleoprotein preparations; with specific absorption of nucleic acids at 260 nm wave length and peptide bonds of the protein coat strongly absorbing beyond 247 nm wave length.

Extinction coefficient. The extinction coefficient for RCVMV isolate WA-7641 was obtained by four separate weighings of a dried virus suspension at an optical density of 0.865 and by two weighings of virus at an optical density of 3.97. The average weight of the virus at each concentration was calculated and the expected optical density at 1.0 mg/ml extrapolated (Table 3-1). An extinction coefficient of approximately 2.0 was obtained for RCVMV isolate WA-7641.

The extinction coefficient of PSV isolate ID-3-2 was calculated from a line derived from regression analysis of six points ranging in optical density from 0.25 to 1.6 units/ml. Individual points represent the average of at least three separate weighings. The regressed line accounted for 97% of the total variation. The extinction coefficient of PSV isolate ID-3-2 was calculated to be approximately 2.2 (Figure 3-8).

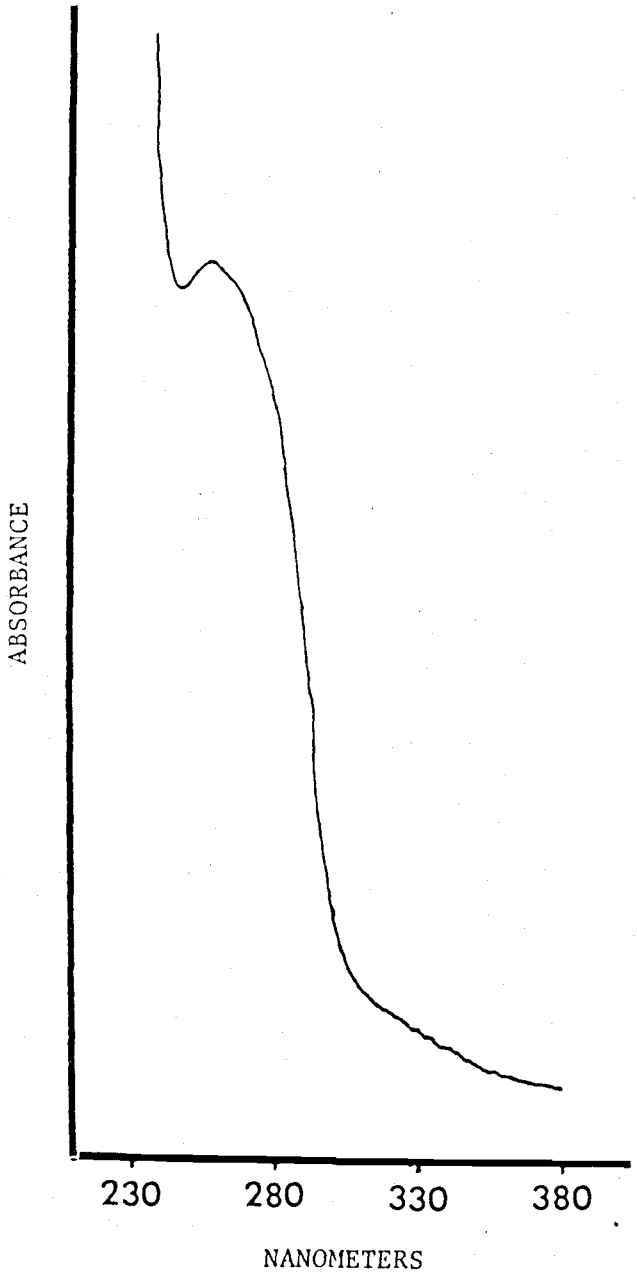


FIGURE 3-6. Ultraviolet absorption spectrum of purified pea streak virus isolate ID-3-2 in 0.01 M sodium borate, 1.0 mM $MgCl_2$ at pH 9.0.

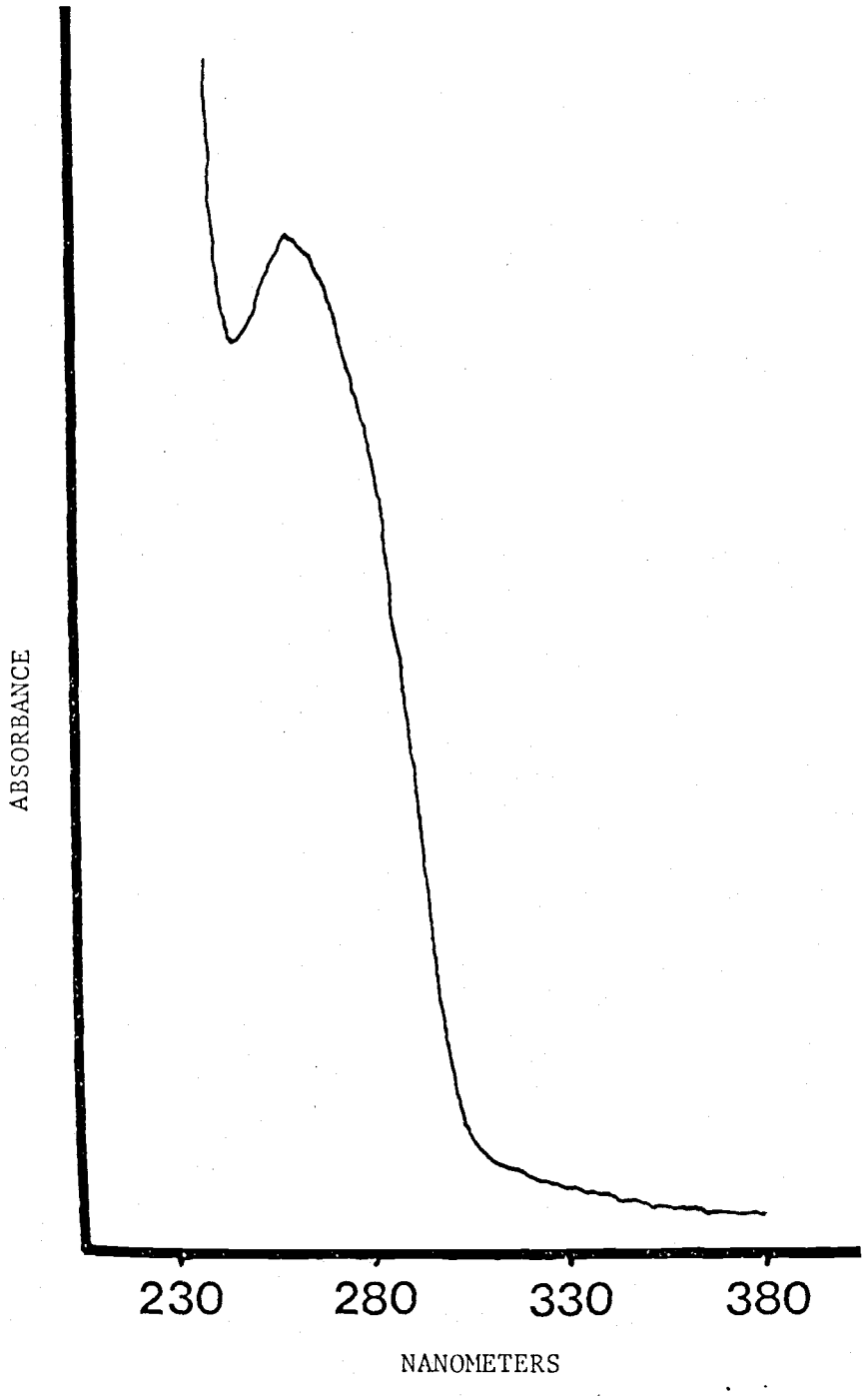


FIGURE 3-7. Ultraviolet absorption spectrum of purified red clover vein mosaic virus isolate WA-7641 in 10 mM sodium borate, pH 9.0.

TABLE 3-1. Data calculations for the extinction coefficient of RCVMV isolate WA-7641.

Optical density, A_{260}	ug virus/0.1 ml 1	2	3	X ug/ 0.1 ml	$E^{0.1\%}_{1.0cm,260nm}$
0.865	38	46	43	42.3	2.04
3.97	177.5	204.5		191.0	2.08

Note that $E^{0.1\%}_{1.0cm,260nm}$ is an expression of the optical density, A_{260nm} for a solution (virus suspension) containing 1.0 mg/ml of solute in a cuvette with a 1.0 cm path length.

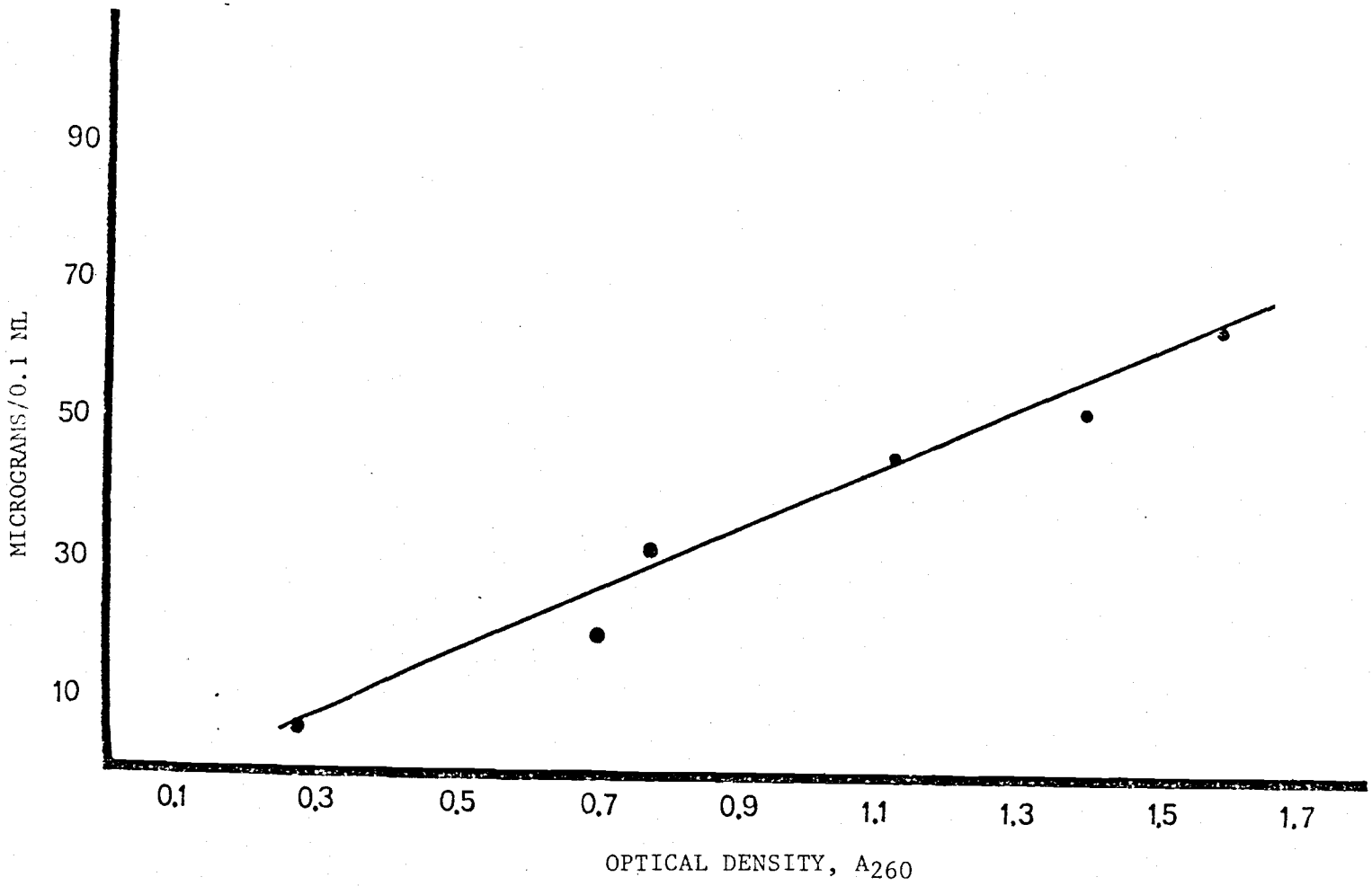


FIGURE 3-8. Regression line for prediction of the extinction coefficient, $E^{1\%}_{1cm,260nm}$ for pea streak virus isolate ID-3-2.

The extinction coefficients of RCVMV and PSV approximate the 2.1 extinction coefficient calculated by Wetter (70) for carnation latent virus, the type carlavirus. RCVMV gave repeatable results after drying at 90C for 12 hr at atmospheric pressure. PSV under the identical conditions failed to dry to a uniform weight and spurious, excessively low extinction coefficients were obtained until samples were processed under a vacuum (30" mercury vaccum) at 100C. This behavior suggests that PSV and RCVMV differ in their water affinity or water binding characteristics.

Protein coat analysis. Protein from RCVMV migrated as a single band in SDS PAGE (5.6%). The calculated weight of the subunit was estimated at 31,000 daltons (Figure 3-13). Figure 3-9 (bottom) is a tracing of the optical scan of a gel containing approximately 10 ug of RCVMV protein. Overloading the gel with 40 ug of protein caused an increase in band thickness and density but did not result in multiple bands. Figure 3-9 (top) is a gel scan tracing of RCVMV isolates WA-7641 and ATC protein applied in equal amounts and electrophoresed. The two proteins co-migrated indicating similar molecular weights. Figure 3-10 depicts the banding pattern of RCVMV with five protein standards from which the molecular weight was estimated.

PSV coat protein electrophoresed to a major band and was estimated by the same 5 protein markers to have a molecular weight of 29,000 daltons (Figure 3-11,top). One to three smaller protein bands 26,000-20,000 daltons consistantly appeared below the 29,000 MW band.

Figures 3-11, top and 3-12, bottom are optical scans of acrylamide gels (5.6%) containing approximately 10 ug of PSV isolates ID-3-2 and Wisconsin. The 24,000 molecular weight band could be resolved into two closely migrating proteins on higher percent acrylamide gels (10%), and third protein below the 24,000-dalton band was sometimes apparent on gels overloaded with PSV protein. Protein from both PSV isolates co-migrated when electrophoresed in the same gel (Figure 2-12, top), indicating that this protein species multiplicity reflects a PSV characteristic.

Figure 3-13 presents the regression line derived from the five protein standards (Figure 2-10, bottom) from which the molecular weights of both RCVMV and PSV were estimated. The regression line accounted for 97% of the variation among the points. As derived, this line predicted the molecular weight of the standards with 96% accuracy, relative to their accepted values.

Veerisetty and Brakke reported that PSV and RCVMV each had a protein subunit of 33,500 daltons (67). We were able to detect differences in molecular weights of PSV and RCVMV coat protein, for both the type isolates and Northwest isolates, of about 2,000 daltons. Figure 3-14 is the tracing of a gel scan (5.6% gel) containing equal amounts of RCVMV isolate WA-7641 and PSV isolate ID-3-2 protein. The separation of the bands clearly indicates a distinct difference in molecular weights of RCVMV and PSV proteins.

PSV isolate Wisconsin has been previously reported to exhibit multiple protein bands on SDS polyacrylamide gels by Veerisetty and

Brakke (67). These workers indicated that the bands below the slowest migrating band were inducible and that they were breakdown products of the intact subunit. Our work with PSV isolate ID-3-2 and two other isolates ID-3-1 and ID-3-3 confirmed the occurrence of a multiple banding pattern for Northwest isolates of PSV. Experiments designed to induce additional breakdown of the intact subunit were performed. The results of this work are summarized in Table 3-2.

Freshly prepared virus had a 3.4 to 1.0 ratio of 24,000 dalton band density to 29,000 dalton band density. After incubation at 60 C for 2 hr or 22 C for 12 hr, the ratio increased to 6.4 to 1.0 and to 5.8 to 1.0, respectively. Phenylmethyl sulphonyl flouride (PMSF) a potent protease inhibitor, stopped this change at 60 C for 2 hr, suggesting that the change was associated with progressive enzymatic breakdown of the 29,000 dalton coat protein to 24,000 daltons. Interestingly, the PMSF had only a slight inhibitory effect on the breakdown of the 29,000 MW protein when incubated for 12 hr at 22 C. This could indicate that there are two mechanisms inducing the breakdown of the labile PSV subunit. Since all preparations of PSV contained significant proportions of the smaller molecular weight proteins, it is possible that PSV coat protein consists of several different sized proteins *in vivo* and that the smaller molecular weight proteins, though inducible, are not simply artifacts of purifications.

FIGURE 3-9. Polyacrylamide-gel electrophoresis patterns of red clover vein mosaic virus coat protein.

Gels were electrophoresed for 3 hr at 3-4 mA/gel from left to right.

TOP: Equal amounts of both RCVMV isolates WA-7641 and ATC coat proteins comigrating; smaller peak (arrow) was considered to be a dimer and was eliminated upon reheating of the sample.

BOTTOM: Coat protein of RCVMV isolate WA-7641 migrating as a single band.

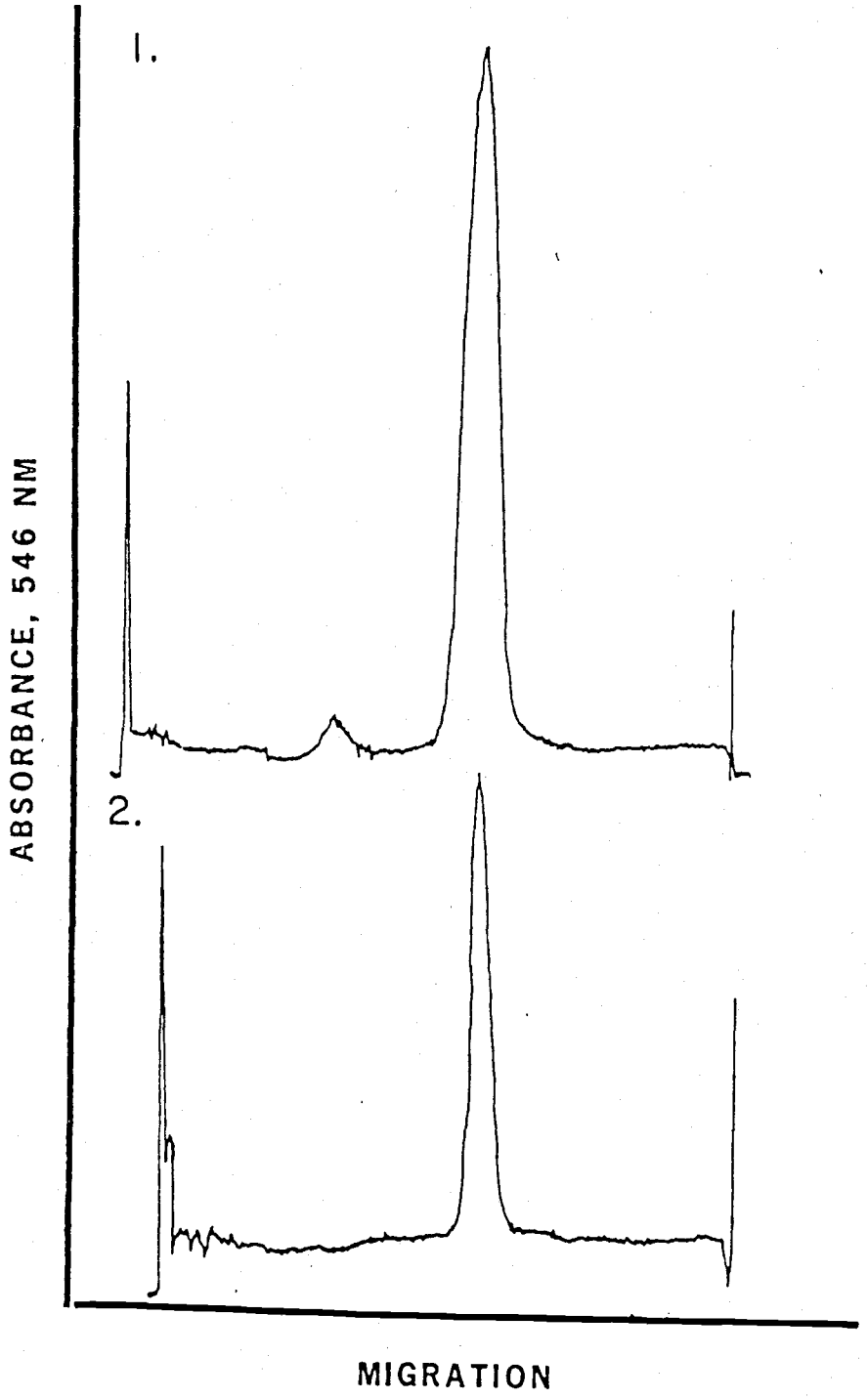


FIGURE 3-9.

FIGURE 3-10. Polyacrylamide-gel electrophoresis patterns of RCVMV and marker proteins.

TOP: RCVMV isolate WA-7641 coat protein and marker proteins.

BOTTOM: Protein markers.

a) bovine serum albumin, 68,000 daltons; b) human gamma-globulin heavy chain, 50,000 daltons; c) ovalbumin, 47,000 daltons; da) alcohol dehydrogenase, 37,000 daltons; dv) RCVMV isolate WA-7641, 31,000 daltons; e) human gamma-globulin light chain, 25,000 daltons.

Gels were electrophoresed for 3 hr at 3-4 mA/gel from left to right.

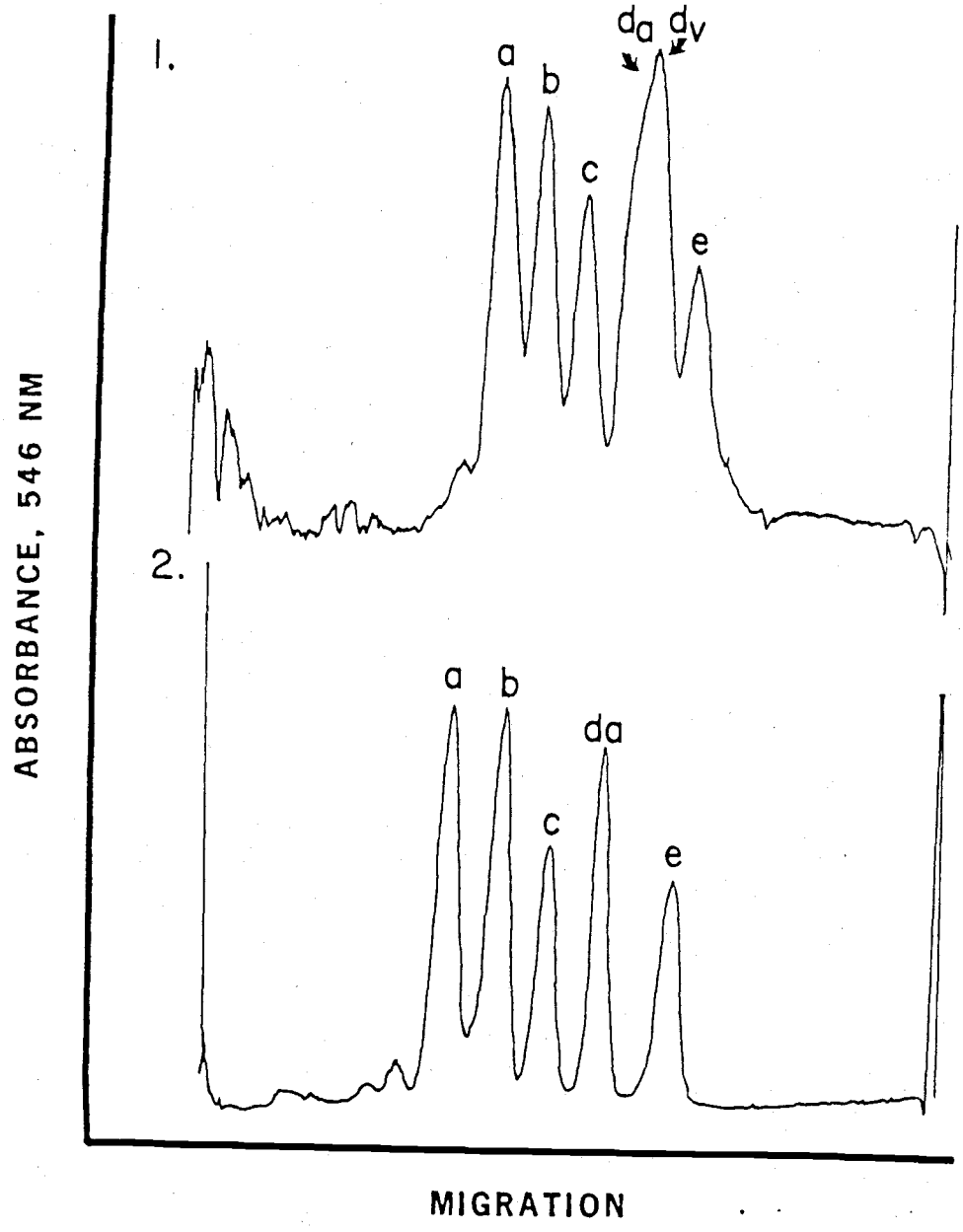


FIGURE 3-10.

FIGURE 3-11. Polyacrylamide-gel electrophoresis patterns of pea streak virus coat protein (TOP) and of marker proteins (BOTTOM).

TOP: pea streak virus isolate ID-3-2 coat protein a) 29,000 daltons b) 24,000 daltons.

BOTTOM: a) bovine serum albumin, 68,000 daltons; b) human gamma-globulin heavy chain, 50,000 daltons; c) ovalbumin 43,000 daltons; d) alcohol dehydrogenase, 37,000 daltons; e) PSV isolate ID-3-2, coat protein, 29,000 daltons; f) human gamma-globulin light chain 25,000 and PSV isolate ID-3-2, 24,000 daltons.

Gels were 5.6% acrylamide and were electrophoresed for 3 hr at 3-4 mA/gel. Protein migration was towards the right.

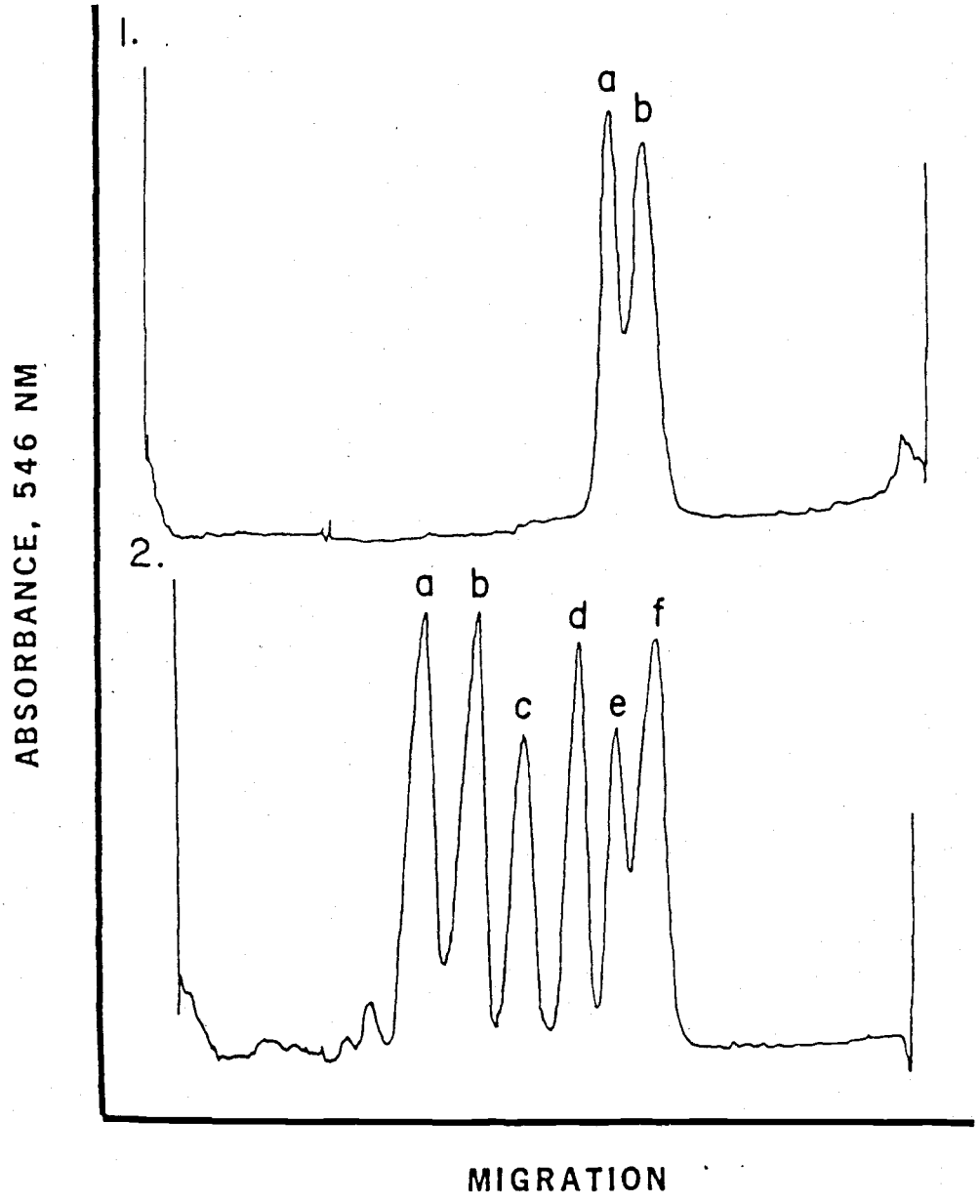


FIGURE 3-11.

FIGURE 3-12. Polyacrylamide-gel electrophoresis patterns of pea streak virus coat protein.

TOP: Pea streak virus isolates ID-3-2 and Wisconsin.

BOTTOM: Pea streak virus isolate Wisconsin.

Gels were 5.6% acrylamide and were electrophoresed for 3 hr at 3-4 mA/gel. Protein migration was toward the right.

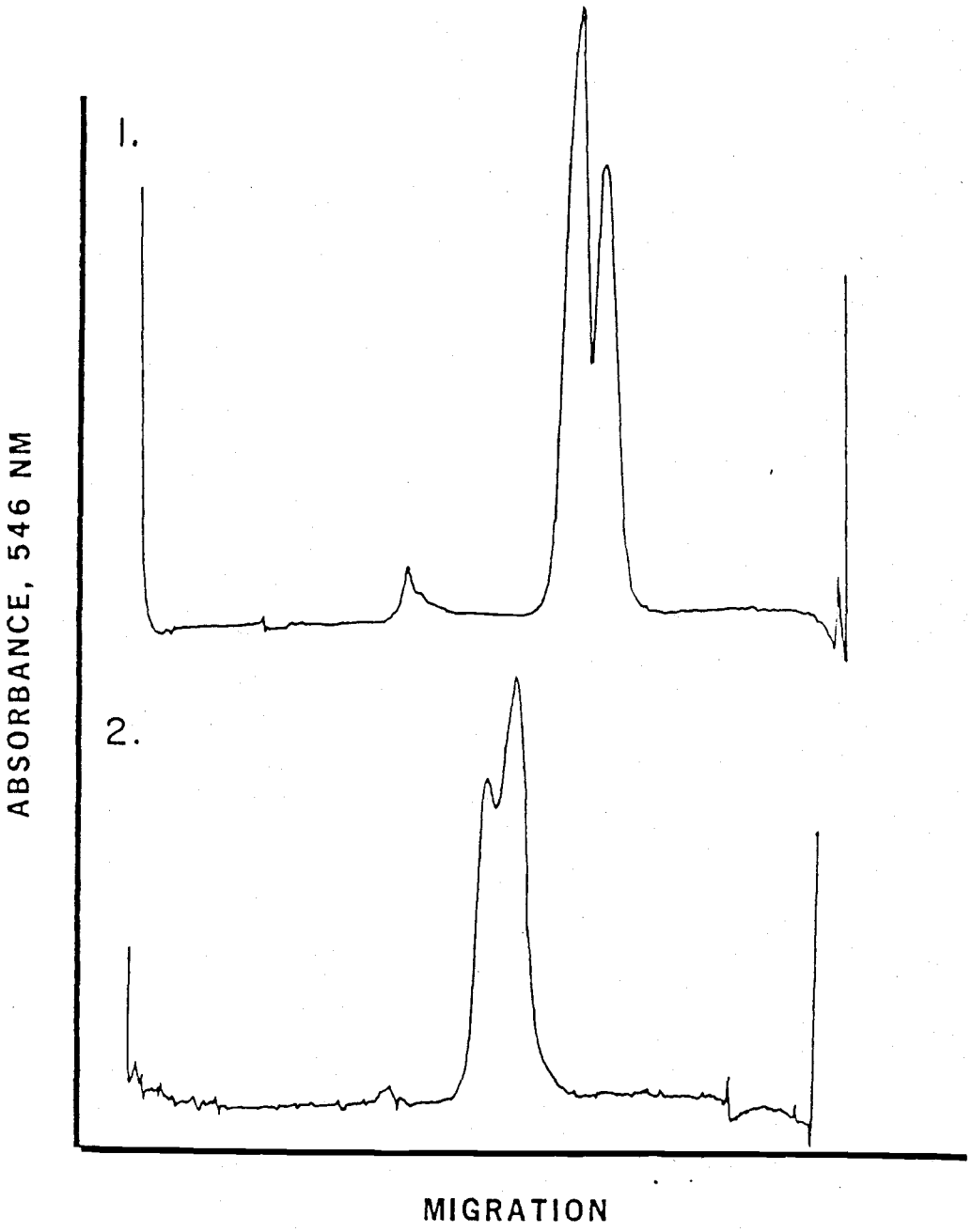


FIGURE 3-12.

FIGURE 3-13. Estimation of molecular weight of red clover vein mosaic and pea streak virus coat protein on 5.6% acrylamide gels, based on molecular weights of five marker proteins.

The line was obtained by plotting the log of molecular weight of marker proteins against their migration distances, divided by the total gel length. A) bovine serum albumin, 68,000 daltons; B) human gamma-globulin heavy chain, 50,000 daltons; C) ovalbumin, 43,000 daltons; D) alcohol dehydrogenase, 37,000 daltons; and E) human gamma-globulin light chain 25,000 daltons. RCVMV and PSV coat proteins were estimated at 31,000 and 29,000 daltons respectively.

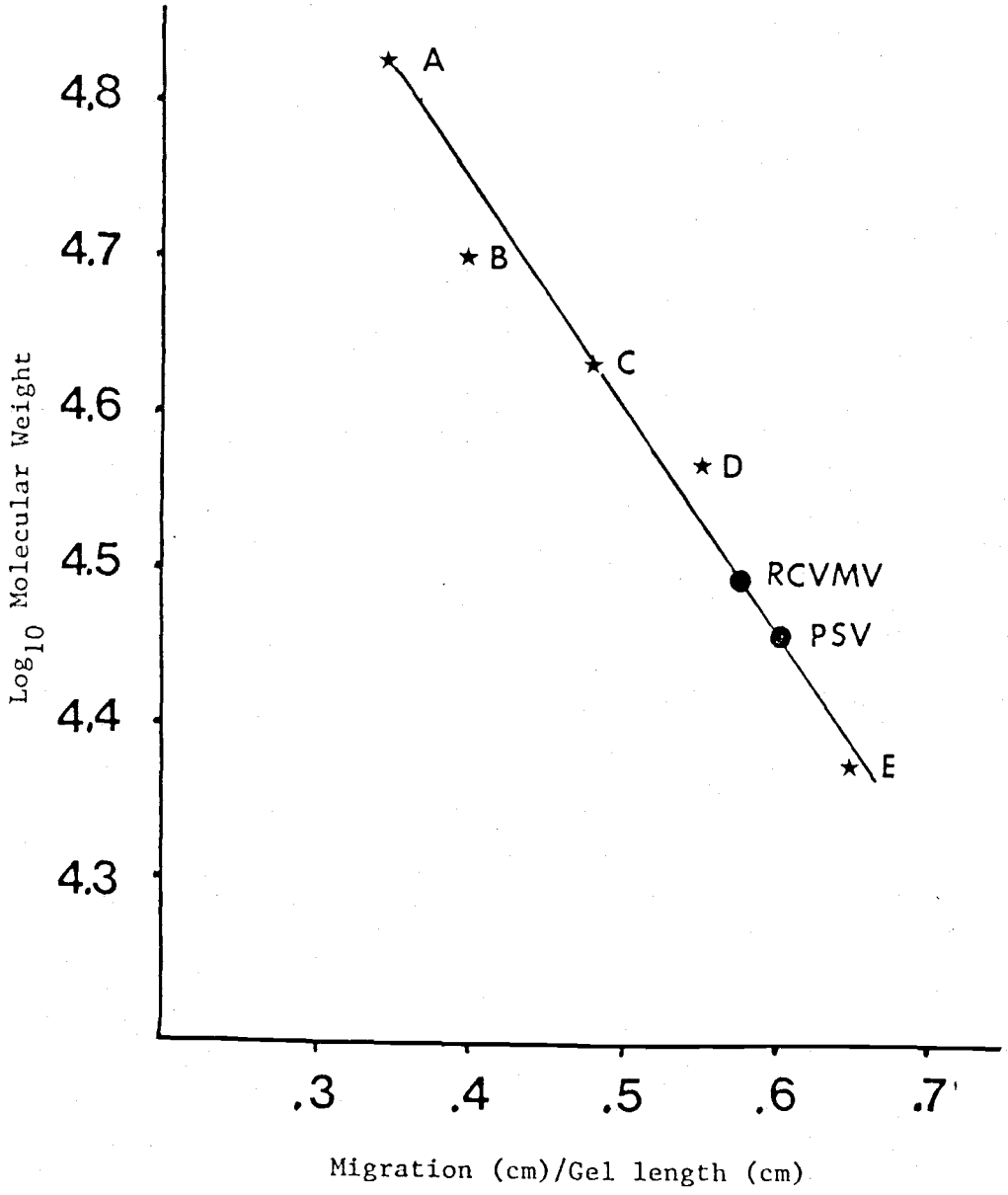

4.8
4.7
4.6
4.5
4.4
4.3
Log10 Molecular Weight
A
B
C
D
RCVMV
PSV
E
.3
.4
.5
.6
.7
Migration (cm)/Gel length (cm)

FIGURE 3-13.

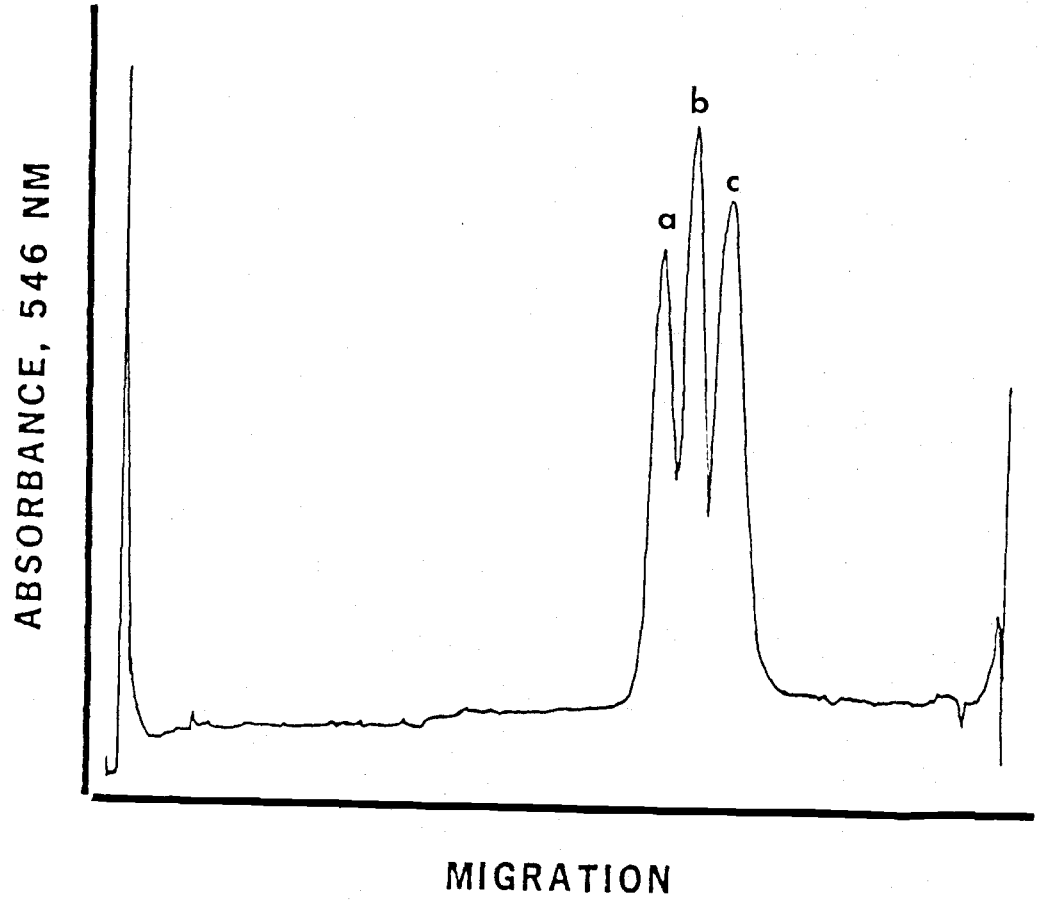


FIGURE 3-14. Polyacrylamide-gel electrophoresis pattern of red clover vein mosaic virus and pea streak virus coat protein.

Gels were 5.6% acrylamide and were electrophoresed for 3 hr at 3-4 mA/gel. Protein migration was towards the right.

a. RCVMV isolate WA-7641 coat protein, 31,000 daltons.
b. PSV isolate ID-3-2 coat protein, 29,000 daltons.
c. PSV isolate ID-3-2 coat protein, 24,000 daltons.

TABLE 3-2. Induced degradation[1] of pea streak virus coat protein.

Treatment	Proportion of 29,000 MW to 24,000 MW PSV coat protein moities.
Control (-20 C)	3.31:1.0
Control (-20 C) 0.5 mM PMSF[2]	3.49:1.0
2 hr at 60 C	6.42:1.0
2hr at 60 C 0.5 mM PMSF	3.42:1.0
12 hr at 22 C	5.76:1.0
12 hr at 22 C 0.5 mM PMSF	4.61:1.0

1. After treatment, viral protein was SDS-disrupted; 10 ug of disrupted protein was layered onto 7.5% polyacrylamide gels and electrophoresed; gels were stained, scanned on an ISCO gel scanner; banded protein moities were measured for relative densities.

2. Phenylmethylsulphonyl fluoride.

Amino acid analysis. The results of an amino acid analysis of RCVMV are given in Table 3-3. The amino acid residues occuring in greatest proportions were serine (12.3%) and glycine (11.9%). Plant virus isolectric points are generally below pH 7.0 (23). The relatively high proportion of Glx and Asx residues may indicate a high proportion of their respective acids and a subsequently negative electrical charge at physiological pHs. In addition, a substantial peak, eluted just before Lysine was noted, but was not identified.

Nucleic acid studies. Nucleic acid (NA) from PSV isolate ID-3-2 migrated to a single band in sucrose density gradients (Figure 3-15). NA was collected and concentrated by ethanol precipitation and resuspended in phosphate-glycine buffer pH 9.2. The ultraviolet absorption spectrum for PSV-RNA had a characteristic peak at 260 nm. The $A_{260/280}$ value ranged from 1.6-1.75 indicating that some protein remained attached to viral RNA despite the rather rigorous and well established procedures followed. Preparations of RNA examined in the electron microscope, was devoid of virus particles.

PSV-RNA (76 ug/ml) was highly infectious to both pea and bell bean with typical streaking symptoms being induced.

A single component was observed when RNAs from dissociated PSV, Wisconsin and ID-3-2 were electrophoresed in polyacrylamide gels. RNAs from either isolate were estimated to be about 3.5×10^6 daltons by comparison with mobility of two different sets of RNA standards (yeast and E. coli ribosomal RNA (Figure 3-16). Since the reported molecular

TABLE 3-3. Amino acid analysis for red clover vein mosaic virus type isolate, ATC.

AMINO ACID	A.A. residues per 1000 residues
Lysine	60.5
Histidine	16.7
Arginine	40.9
Aspartic acid-Asparagine, Asx	104.7
Threonine	47.5
Serine	122.8
Glutamic acid-Glutamine, Glx	112.3
Proline	42.3
Glycine	119.1
Alanine	93.7
Half Cystine	13.1
Methionine	16.4
Isoleucine	33.7
Valine	56.7
Leucine	55.7
Tyrosine	15.8
Phenylalanine	48.1

weight for PSV of $2.4x10^6$ daltons (67) is closer to the estimates of other filamentous virus of similar length, we concluded that our estimations were erroneous. The two sets of standards predicted the molecular weights of each other with an acceptable degree of accuracy.

Two alternative factors could have contributed to our apparently high estimates of PSV-RNA. First, the five min heating at 60 C may have failed to completely relax the secondary structure, with resultant impairment of electro-mobility. This explanation appears unlikely since the procedure and conditions (accepted as virological standards) have produced consistent results for other workers (43). Secondly, coat protein may have remained attached to PSV-RNA after disruptions, thus impeding electro-mobility. Credence to this possibility was provided by our subsequent discovery that coat protein remained associated with PSV-RNA through the processes of isopycnic centrifugation and alcohol precipitation.

RNA from dissociated RCVMV isolate WA-7641 migrated to a single band by polyacrylamide-gel electrophoresis (PAGE); however, RCVMV isolate ATC contained an anomalous second RNA species (12s) (Figure 6-3). This second smaller RNA species was reported by Veerisetty and Brakke (67) and chapter five contains more data related to the RNA of RCVMV. The $A_{260/280}$ values of RCVMV, ATC-RNA ranged from 1.76-1.88 indicating the presence of contaminating protein, though less than present in the PSV-RNA preparations.

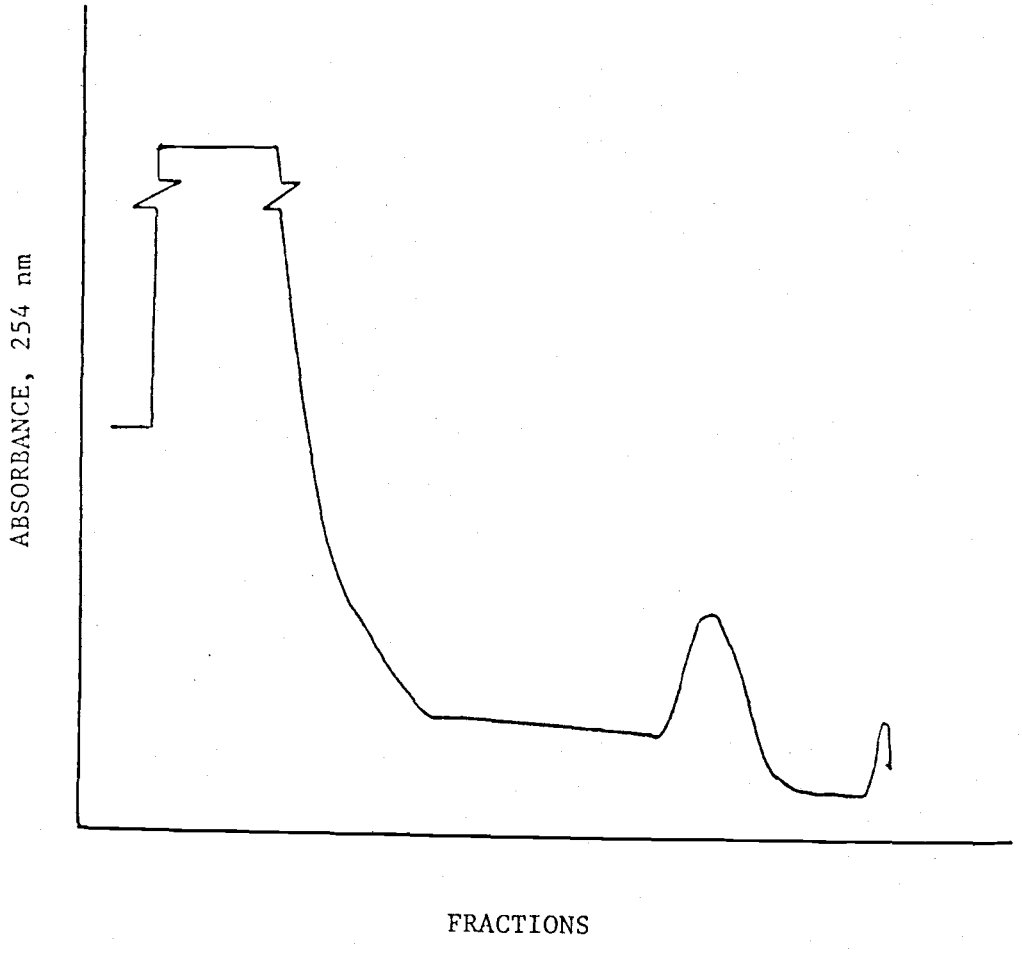


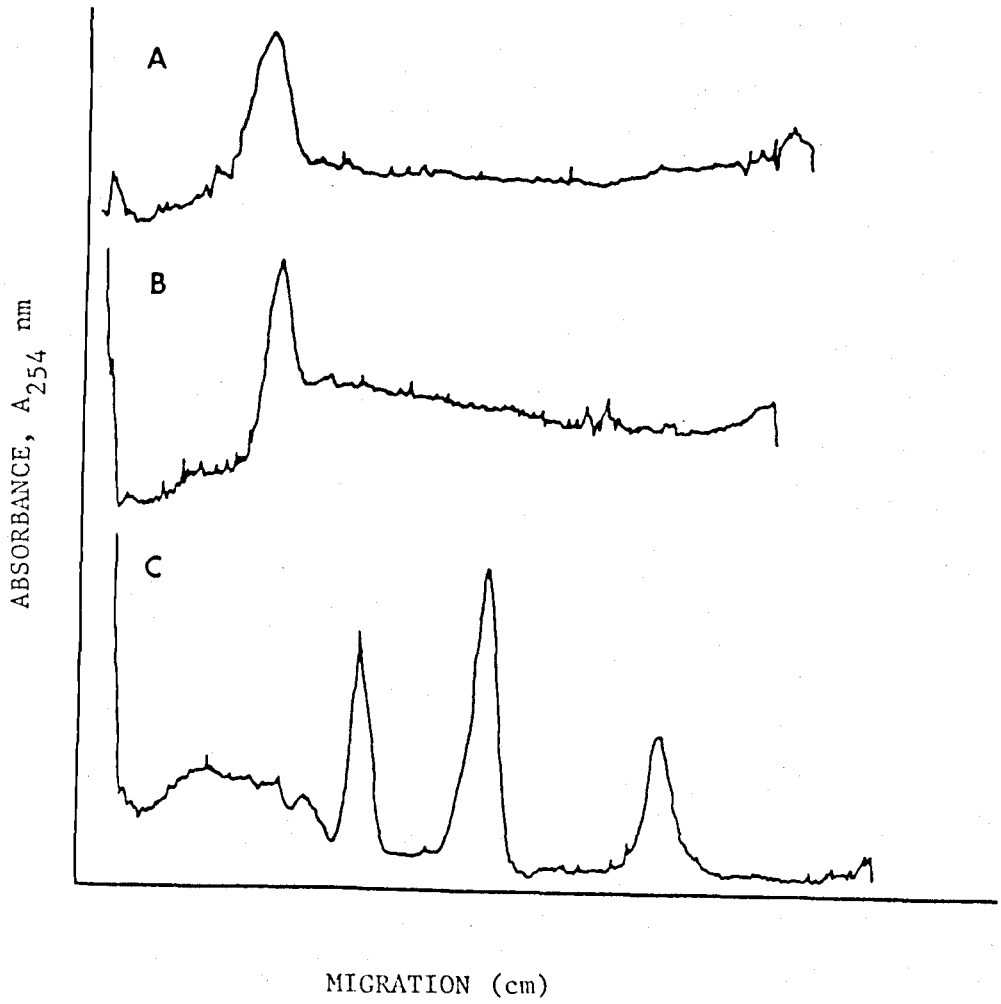
FIGURE 3-15. Scan tracing of pea streak virus RNA centrifuged through a sucrose density gradient.

Scan tracing of pea streak virus RNA centrifuged through a linear-log sucrose density gradient (0-32% 0.5XSSC pH 7.0) in a SW 40 rotor at 39,000 rpm for 5.5 hr.

FIGURE 3-16. Scan tracing of pea streak virus isolate ID-3-2 and Wisconsin RNA.

Gels were 2.2% acrylamide and were electrophoresed for 90 min at 5 mA/gel. Nucleic acid migration was towards the right.

A. Pea streak virus isolate ID-3-2 RNA.
B. Pea streak virus isolate Wisconsin RNA.
C. Yeast ribosomal RNA (1-r; 2.5, 1.3, 0.7×10^6 daltons) (61).


A
B
C
ABSORBANCE, A_{254} nm
MIGRATION (cm)

CHAPTER FOUR

THE TRANSMISSION OF TWO PURIFIED CARLAVIRUSES BY THE PEA APHID, ACRYTHOSIPHON PISUM

The Transmission of Two Purified Carlaviruses by the Pea Aphid, *Acrythosiphon pisum*

Kenneth A. Weber and Richard O. Hampton

Graduate research assistant and Research Plant Pathologist, U.S. Department of Agriculture, Science and Education Administration, Agriculture Research, respectively, Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331.

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ABSTRACT

WEBER, K. AND R. HAMPTON. 1979. The transmission of two purified Carlaviruses by the pea aphid, *Acrythosiphon pisum*.

Pea aphids [Acrythosiphon pisum (Harris)] were able to transmit purified pea streak (PSV) and red clover vein mosaic (RCVMV) viruses acquired through membranes. Purity of virus preparations was monitored by ultraviolet spectrophotometry and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Aphids were also able to transmit both viruses after feeding through a membrane on crude sap extracts from infected plants. Purified PSV was more readily aphid transmissible than purified RCVMV. There was no evidence that a second substance, acting as helper agent to facilitate aphid transmission, was present. This is the first report of a purified filamentous virus being transmitted aphids in the absence of accessory factors.

Only two plant viruses, alfalfa mosaic (5) and cucumber mosaic (6) have been demonstrated to retain stylet-borne aphid transmissibility after purification. Potyviruses (3) and caulimoviruses (4) lose aphid transmissibility upon purification due to separation from their respective helper agents. Others have speculated that the helper agent, an accessory factor produced as a result of infection, is involved in the aphid transmission of other plant virus groups (7). Our investigation deals with the aphid transmissibility of two carlaviruses, pea streak (PSV)and red clover vein mosaic (RCVMV)viruses, in the purified state. Evidence is presented that these filamentous viruses, after purification, were aphid transmissible in their characteristic non-persistent manner by the pea aphid, Acyrthosiphon pisum (Harris).

METHODS

Pea aphids used in this study were reared on bell bean (Vicia faba var. minor Peterm. Beck) at a constant temperature (20 C) and photoperiod (18 hr/dy). An Oregon colony collected from alfalfa in 1978 was used in all but three trials in which a Michigan clone (9) was used. In all cases, transmission trials were conducted with 5 to 7-day-old nymphs.

The experimental system of bell bean as the aphid-culture host, pea aphid vector, and pea virus-source and pea test plants has been extensively tested and used in our laboratory and found to be free of anomalous effects. To eliminate the possibility of seedborne or other extraneous viruses, aphids were routinely starved for 12hr after removal from the bell bean aphid culture plants (no viruses are known to be both seedborne in bell bean and aphid-transmitted in a persistent manner). Aphids cultured, starved, and tested before feeding on virus-source plants have not transmitted extraneous viruses.

Isolates of PSV and RCVMV were identified by host range, symptomology on pea (Pisum sativum L. cv. Cascade), particle morphology, and SDS-gel immunological reactions (8). For comparative purposes, we obtained Wisconsin pea streak virus (PSV-W) from Dr. D. J. Hagedorn and RCVMV-ATC from the American Type Culture Collection. All isolates were propagated in pea cv. Cascade. PSV isolate ID-3-2 was obtained

from an alfalfa stand in Idaho and RCVMV isolate WA-7641 originated in infected peas from Washington.

Virus extracts were prepared by vacuum infiltrating infected pea tissue with 0.018 M trisodium citrate, 0.165 M disodium phosphate and 0.15% diethyldithiocarbamate at pH 9.0. Tissue was ground in a mortar with sterile sand in extraction buffer at a ratio of 1:2 (w/v). Buffered extract was clarified by centrifugation for 10 min at 12,100 g and concentrated by the addition of polyethylene glycol, MW = 6000 (PEG), to 6% (w/v), centrifugation at 12,000 g for 10 min and resuspension of the resulting pellet with the extraction buffer to 1/5 original volume. Both the crude and concentrated extracts were highly infectious as determined by mechanical inoculation onto pea.

Virus purification. Viruses were purified from frozen pea tissue, essentially as described by Veerisetty and Brakke (11). Tissue was blended in phosphate-citrate buffer (1:4 w/v), clarified by low-speed centrifugation and precipitated virus was centrifuged at low speed and pellets were resuspended in buffer containing 1% Triton X-100, at 1/10 the molarity of the extraction buffer. Following a low speed centrifugation the virus-containing supernatant was concentration by 160,000 x g centrifugation through a layer of 30% sucrose containing buffer and 1% Triton X-100 in a 65 rotor (Beckman Instruments Inc.) for 50 min at 50,000 rpm. Virus pellets were resuspended in buffer containing 1% Triton X-100 and the suspension centrifuged at low speed. The resulting partially purified preparations were layered on top of linear sucrose density gradients (10-40%) and centrifuged at 24,000 rpm for 2 hr in a

SW 27 rotor (Beckman Instruments Inc.). Virus bands were collected and concentrated by a second differential centrifugation. Final virus pellets were resuspended in buffer without Triton X-100.

Relative concentrations of purified virus were estimated by UV absorption analysis on a model 25 spectrophotometer (Beckman Instruments Co.). A_{260} values were uncorrected for the negligible amounts of light scattering that occurred. Viral protein was analyzed electrophoretically on SDS polyacrylamide gels (5.6%) using the methods of Fairbanks (2). Marker proteins used to estimate molecular weights of viral coat protein were bovine serum albumin, ovalbumin, human gamma-globulin and alcohol dehydrogenase.

Aphid transmission tests. Aphids were starved for 12 hr before transmission tests. For plant-to-plant transmission studies, aphids were allowed 2-5 min acquisition access periods on source plants after which 3 to 10 aphids were transferred to each test plant. For solution-to-plant transmission studies, aphids were allowed 10-min feeding access to either solutions of purified virus or comparable non-infectious solutions sandwiched between two layers of stretched Parafilm 'M' (American Can Co.). For determinations of aphid transmissibility 5-10 aphids assuming characteristic feeding postures were transferred to each test plant. Feeding solutions were made 20% sucrose (w/v). Aphids were allowed inoculation periods on test plants of 8-12 hr. Determinations of transmission were based on test plant symptomatology. Virus content in plants with questionable symptoms was verified by electron microscopy or serology.

After completion of aphid access periods, virus solutions were removed from membranes and mechanically inoculated onto pea plants as experimental controls.

RESULTS

In preliminary plant-to-plant aphid transmission experiments, aphid clones (A. pisum) from Oregon and Michigan were determined under the conditions of our study to transmit both RCVMV and PSV. In these separate tests, rates of transmission from infected to healthy Cascade pea plants by 3 to 10 aphids per test plants were 5/10, 7/16, and 5/8 for RCVMV and 5/8, 3/18, and 3/16 for PSV. Aphids were also able to transmit both viruses when given access through membranes to non-concentrated or PEG-concentrated aqueous extracts from infected plants (Table 1). In four separate control tests, aphids which had fed upon non-infectious buffer-sucrose solutions failed to transmit RCVMV or PSV to test plants. Each test involved 40 aphids and 16 test plants.

Aphid transmission and characteristics of purified RCVMV. Aphid transmissibility of purified RCVMV was demonstrated at low levels over an A_{260} range of 0.1 to 5.7 O.D. (Table 1). Varying the number of aphids per test plant and the concentration of virus failed to increase transmission rates. Although only 4 to 50 plants fed upon by aphids after access to purified RCVMV became infected, each infection occurred in a separate trial, demonstrating reproducibility. The pathogenic integrity of purified RCVMV fed to aphids was indicated by typical, severe,

streak-like symptoms that rapidly developed in plants inoculated with preparations from feeding membranes at the conclusion of each test.

Purified RCVMV isolate WA-7641 exhibited a UV absorption spectrum typical of that for filamentous viruses. Within the 270 to 240 nm wave-length range, absorptions maxima and minima occurred at 260.0 nm and 246.5 nm, respectively. The 260/280 ratio of purified RCVMV was 1.24 $\pm$ 0.02. Purified WA-7641 was determined by electron microscopy to consist of particles with a modal length of 660 nm and to be free from discernible host material. Viral protein, prepared by sodium dodecyl sulfate (SDS) disruption of pure virus, migrated as a single band in SDS polyacrylamide gel electrophoresis. The molecular weight of RCVMV protein subunits was estimated to be 31,000 daltons based on measured electrophoretic mobilities of five protein standards. Protein from isolate WA-7641, mixed with equal amounts of disrupted protein from RCVMV-ATC, also migrated as a single band in gel electrophoresis. Purified RCVMV was highly antigenic. Antiserum prepared against WA-7641 produced a conterminous precipitin band between homologous antigen and RCVMV-ATC in SDS agar gels.

Aphid transmission and characteristics of purified PSV. Purified PSV isolate ID-3-2 was more readily aphid transmissible than was purified RCVMV (Table 1). There was an apparent relationship between transmission frequency and concentration of purified PSV. Aphids fed on purified virus suspensions with A_{260} values of 2.0 to 3.0 transmitted PSV to 18 of 40 test plants, whereas those fed on suspensions with A_{260} values

of 0.2 to 1.5 transmitted PSV to 9 of 40 test plants. Purified PSV fed to aphids was highly infectious, as evidenced by typical, severe streaking symptoms that rapidly developed in plants inoculated with preparations removed from feeding membranes.

Purified PSV isolate ID-3-2 exhibited a UV absorptions spectrum similar to that of RCVMV. Within the 270 to 240 nm wave-length range, absorptions maxima and minima occurred at 260.0 nm and 247.0 nm, respectively. The 260/280 ratio of purified virus was 1.15^{+}_{-} .02. Purified ID-3-2 consisted of particles with a modal length of 630 nm and were free from discernible host material. The protein of isolate ID-3-2 migrated in SDS polyacrylamide gels to a major band (29,000 daltons, based on electrophoretic standards) and two to three minor bands (26,000 to 20,000 daltons), in agreement with recently reported results (10) for a Wisconsin isolate of PSV. Minor bands occurred inconsistently in repeated tests, were inducible by incubation at 60 C, and are concluded to represent breakdown products of PSV protein. Purified PSV was highly antigenic and antiserum against isolate ID-3-2 produced a conterminous precipitin band between homologous antigen and PSV-W in SDS agar gels.

DISCUSSION

The data reported here demonstrate that PSV and RCVMV are both aphid transmissible after purification. Although no new or novel procedures were developed to exhaustively test for minute quantities of possible helper agent which could have remained attached to virus

TABLE 1. Aphid transmission of red clover vein mosaic and pea streak viruses, acquired by the pea aphid, Acyrthosiphon pisum, through membrane feedings[a].

Virus	Treatment	Exp No.	Aphids per test plant	Remarks	Transmission[b]
RCVMV WA-7641	Buffered[c] sap	1	10		2/10
		2	10		1/8
	PEG precip.[d]	1	10[e]	Conc. 5x	4/10
		2	10	Conc. 5x	1/8
	Purified	1	10[e]	A_{260}: 0.1	0/7
		2	10[e]	0.5	1/9
		3	8	1.0	1/10
		4	5	1.0	0/10
		5	5	3.8	1/6
		6	5	5.3	1/8
PSV ID-3-2	Buffered sap	1	10		1/8
	PEG precip.[d]	1	10	Conc. 5x	1/8
		2	10	Conc. 1.6x	4/8
	Purified	1	5	A_{260}: 0.2	0/8
		2	5	0.5	3/8
		3	5	1.0	1/8
		4	5	1.5	2/8
		5	5	1.5	3/8
		6	5	2.0	4/8
		7	5	2.5	3/8
		8	5	2.5	3/8
		9	5	3.0	3/8
		10	5	3.0	5/8

a. Comparable control experiments aphids failed to transmit RCVMV or PSV.

b. Numerator: No. of infected plants, demoninator: No. of test plants.

c. Pea tissue homogenized in 0.018 M Na_3 citrate, 0.165 M Na phosphate and 0.15% Na diethyldithiocarbamate pH 9.0 1:2 (w/v).

d. Sap concentrated by precipitation with polyethylene glycol (6000) and resuspension in extraction buffer.

e. Aphids used in these trials from East Lansing, Michigan, clones; Oregon colony used in other trials.

particles despite treatment with non-ionic detergent and isopycnic centrifugation, our procedures were essentially equivalent to those of previous workers who succeeded in separating helper agents from potyviruses (3) and caulimoviruses (4).

Purified cucumber mosaic virus, which is aphid transmissible either from infected plants or in the purified state, is not transmissible in the presence of infected sap (6). Our results demonstrate that both RCVMV and PSV are readily aphid transmitted when sap is present.

Aphids feeding on either infected pea tissue or sap extracts through membranes readily transmitted both PSV and RCVMV. After purification, however, RCVMV was less easily transmitted than PSV at equivalent optical densities. We considered the possibility that the multiple banding pattern of PSV coat protein represented factors that could influence aphid transmissibility, in light of recently published correlations between aphid transmissibility of pea enation mosaic virus with a second coat protein (1). However, since coat protein from all Northwest isolates and the Wisconsin isolate of PSV exhibited this multiple banding pattern it was not possible to test aphid transmissibility in the absence of these smaller proteins. Furthermore, these smaller proteins associated with PSV coat protein differed significantly from the helper agents reported in crude sap extracts from plants infected with potyviruses (3) and caulimoviruses (4) in that they persisted throughout the purification procedure which included treatment with non-ionic detergent and isopycnic centrifugation.

Based on an extinction coefficient of 2.0 (12) for both carlaviruses studied, the concentration of purified virus required for aphid transmission was estimated to be 0.1 mg/ml. Only moderate increases in aphid transmission rates resulted when virus concentrations were increased 2 to 3 mg/ml. Transmission efficiency achieved by workers using potyviruses (3) was much greater than ours. Because of the observed tendency for virus to precipitate upon ageing, we believe that low virus transmission rates obtained in this study are related to the limited virus particle stability provided by the extraction buffer. It therefore seems likely that use of an improved extraction medium could provide greater aphid transmission efficiency of purified virus.

ACKNOWLEDGEMENTS

We wish to than Patrick Barnett for his technical assistance in these studies.

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CHAPTER FIVE

AN INVESTIGATION OF PARTICLE DIVERSITY AND RNA DUALITY IN RED CLOVER VEIN MOSAIC VIRUS ISOLATES

An Investigation of Particle Diversity and RNA Duality in Red Clover Vein Mosaic Virus Isolates

By K. A. WEBER, V. VEERISETTY,* and R. O. HAMPTON

U.S. Department of Agriculture, Science and Education Administration, Agricultural Research, Department of Botany and Plant Pathology, Oregon State University, Corvallis Oregon 97331 and *Department of Plant Pathology, University of Missouri, Columbia Missouri 65201.

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SUMMARY

A subisolate of red clover vein mosaic virus originating from the American Type Culture Collection's reference isolate RCVMV (ATC PV/110 exhibited a multibanded profile in sucrose density gradient columns. The nature of the multiple bands was concluded to result from particle fragmentation. Bands contained virus particles whose length varied directly with band migration velocity, and infectivity assays established that short rods comprising upper bands were non-infectious. Protein from short and intact particles co-electrophoresed to a single

band on sodium dodecyl sulphate-polyacrylamide gels. RNA purified from this isolate consisted of two principle species, a small non-infectious (12-15S) RNA associated predominantly with the short particles of the upper bands and a large infectious (39 S) RNA from 660 nm particles of the lowest band. Particle lability could not be attributed to purification reagents; however, short particles and small RNA were inducible by prolonged freezing of infected tissue or by pelleting virus by high-speed centrifugation. A second subisolate derived from RCVMV (PV/110) and an isolate originating from the Pacific Northwest produced under the identical purification procedures single banded profiles in gradient columns and contained a single large RNA.

INTRODUCTION

Two strains of red clover vein mosaic virus, a 660 nm carlavirus, were reported to have multiple components of an unknown nature (Bos et al. 1971). Recently, Veerisetty and Brakke (1977) reported that a small (12-15S) RNA species was usually associated with purified RCVMV. In this paper we present evidence for the possible nature of both multiple components and small RNA species.

METHODS

Virus isolates and propagation hosts. Two isolates of red clover vein mosaic virus (RCVMV) were used in this study, RCVMV (ATC/PV 110) obtained from the American Type Culture Collection and RCVMV WA-7641 derived from a pea field in western Washington, USA. Subisolates of

RCVMV isolate ATC which upon purification produced multiple bands in sucrose density gradient columns were designated (MB) and those which weres single banded in identical gradients were designated (SB). The two isolates ATC and WA-7641 were conveniently separable by the inability of the latter to induce local lesions on Chenopodium amaranticolor Coste and Reyn. and on Gomphrena globosa L. In other tests including serological identity, particle morphology, sap stability, and symptomatology in pea, the isolates were identical. The viruses were propagated in greenhouses in pea (Pisum sativum L.) cvs. Cascade or Lincoln. Plants at the 1-2 leaf stage were mechanically inoculated by either infected plant extracts or purified virus mixed with 400 mesh Carborundum. All plant tissue above the soil was harvested when typical symptoms appeared about 14 days after inoculation. The tissue was either used immediately or frozen at -20 C.

Electron microscopy, infectivity assays and UV spectrophotometry. Virus preparations were examined with a Phillips EM 300 electron microscope. Samples were fixed in 1.75% glutaraldehyde, place on a formvar-carbon coated grid, and stained with 2.0% phosphotungstate. Grids prepared with samples taken directly from sucrose density gradients were floated on a 20% glycerin for 20 min to remove sucrose before treating with glutaraldehyde and stain.

For testing the infectivity of purified virus fractions, those containing absorbance peaks (A_{254}) were collected from sucrose density gradients. Virus suspensions were diluted to 2.5, 0.25 and 0.025 ug/ml of virus based on an extinction coefficient ($E^{1\%}_{1\ cm,\ 260\ nm}$) for RCVMV

of 2.0 (Weber, 1979). Each preparation was inoculated onto several half leaves of C. amaranticolor. The infectivity of each preparation was measured by counting the total number of local lesions induced. Inoculations of buffer containing 5% sucrose were included as controls.

Virus purification. We adopted the following modification of the purification procedure developed by Veerisetty and Brakke (1978). The infected pea tissue was triturated in 0.2 M sodium borate, 0.5% 2-mercaptoethanol (2-ME), 1.0 mM magnesium chloride and 0.3% Dow Antifoam B (Dow Chemical Co. Midland, Michigan) at pH 9.0 (1:4 w/v). The extract was passed through cheesecloth, centrifuged at low speed (12,000 x g for 10 min) and 6.0% w/v polyethylene glycol (PEG 6,000) was added to the virus supernatant. The resulting precipitate was centrifuged at low speed and the virus pellet resuspended in 10 mM sodium borate, 0.05% 2-ME, 1.0 mM magnesium chloride and 1.0% Triton X-100. After a low speed centrifugation, the resuspended virus was centrifuged through a 30% sucrose layer containing 10 mM borate, 1.0% Triton X-100, 0.05% 2-ME and 1.0 mM $MgCl_2$ at pH 9.0 at 160,000 x g for 50 min. in a 65 rotor (Beckman Instrument Co., Palo Alto, California). The pellet was resuspended in 10 mM borate, with Triton X-100 and $MgCl_2$. The resuspended virus was subjected to a low-speed centrifugation and layered onto a linear sucrose density gradient (10-40%) buffered with 10 mM borate at pH 9.0. The gradients were spun for 2 hr at 96,000 x g in a SW 27 rotor (Beckman) or for 70 min at 39,000 rpm in a SW 40 rotor (Beckman). The virus bands were collected by hypodermic needle or by an ISCO gradient fractionator (Instrumentations Specialties Co. Lincoln, Nebraska).

Preparation and analysis of RNA and protein. The virus preparations were centrifuged on linear sucrose density gradients in an SW 27 rotor, as previously described. Consecutive fractions of 2.5 ml each were collected, downward, starting from the meniscus and analyzed with an ISCO fractionation system. Two volumes of 97% ethanol with 0.15 M sodium acetate, pH 5.0, were added to each fraction. After storing overnight at -20 C, the ethanol precipitates were centrifuged at 15,000 rpm for 20 min and dried over nitrogen. Individual precipitates were resuspended in 0.5 ml of disruption buffer (0.04 M Na_2HPO_4, 0.01 M NaH_2PO_4, 1% 2-ME, 1.0 mM EDTA, pH 7.5), for both RNA and protein analysis. For RNA analysis, the samples were heated at 60 C for 5 min, and RNA was precipitated by ethanol, and resuspended in electrophoresis buffer (36 mM Tris, 34 mM NaH_2PO_4, 5 mM EDTA 0.2% SDS, 5% sucrose, pH 7.6) at 1 mg/ml. For infectivity assays of nucleic acid, disrupted virus was layered onto a linear-log sucrose density gradient and centrifuged for 6 hr at 39,000 in a SW 40 rotor. The columns were scanned with an ISCO fractionator and absorbance peaks (254 nm) collected. The nucleic acid containing peaks were again precipitated with ethanol and suspended in 35 mM NaH_2PO_4, and 50 mM glycine, pH 9.2 (30-70 ug/ml). Before inoculation onto bell bean (Vicia faba L. var. minor) bentonite was added (200 ug/ml) Fraenkel-Conrat et al. 1961). Protein samples were boiled for 2-3 min before electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) of virus RNA and protein. Viral RNA was electrophoresed in polyacrylamide gels according to the methods of Loening (1967). Samples of RNA (10-20 ug/gel) were electro-

phoresesed in 2.2% polyacrylamide gels for 90 min at 5 mA/gel. Gels containing RNA were soaked overnight in acetate buffer (.04 M sodium acetate, pH 4.6) and stained with .02% methylene blue in acetate (Peacock & Dingman, 1967). Viral protein (10 ug/gels) was electrophoresed on 5.0% SDS polyacrylamide disc gels at 8-9 mA/gel for 3-4 hr. PAGE of protein utilized the buffer, staining and destaining methods of Lesnaw and Reichmann (1969) as described by Powell (1976).

Linear log-sucrose density gradient centrifugation. Linear log gradients containing 0-325 mg sucrose/ml in neutral 0.5 x (standard saline-citrate buffer (SSC is 0.15 M NaCl, 0.015 M sodium citrate) were prepared according to Brakke and Van Pelt (1970). Gradients onto which RNA preparations had been layered were centrifuged at 5.5-6 hr at 39,000 rpm in an SW 40 rotor (Beckman). Centrifuged columns were scanned with an ISCO fractionation system.

Origin and induction of multiple bands. RCVMV reference isolate was (ATC PV/110) was inoculated on to pea cv. Cascade, purified, and scanned (A_{254}) in linear sucrose density gradients made. To study the stability of subisolates, serial transfers were made from pea every two weeks and the virus purified and gradient-scan profiles observed for changes.

RCVMV isolates were purified under modified conditions to examine the possibilities of eliminating the multiple banding pattern of ATC-MB or of reproducing a similar sucrose density gradient-scan profile for ATC-SB or WA-7641. Specifically, the modifications entailed the substitution of Na_2SO_3 (1.0%) for 2-ME, the omission of

1.0 mM $MgCl_2$ throughout and the substitution of 165 mM Na_2HPO_4 and 18 mM trisodium citrate with 1.0 mM $MgCl_2$ and 1.0% Na_2SO_3 for the standard extraction buffer (Veerisetty and Brakke, 1978).

To determine whether short particles associated with ATC-MB represented an artifact of purification or existed in vivo, infected pea extracts from various stages of purification were centrifuged through linear sucrose density gradients (10-40%) in an SW 49 rotor (Beckman). Fractions (1.2 ml) were collected with an ISCO fractionator and examined for short particles by electron microscopy. RCVMV subisolate ATC-MB infected pea was purified without freezing, after 1 hr at -20 C and after 1 wk at -20 C. Virus extracts sampled from the following stages of purification were examined: 1) after the first low speed centrifugation; 2) after precipitation by PEG; and 3) after high-speed centrifugation in a 65 rotor (Beckman). To compensate for variation in virus yields between purifications stages and trials, fractions were standardized according to optical density (A_{254}) of the lower most (viral) absorbance peak before microscopic examination.

Purified RCVMV, ATC-SB was subjected to freezing and repeated high-speed centrifugation at 160,000 x g for 50 min. The pellets were resuspended in the original volume of buffer and examined for excessive breakage as compared to non-frozen and non-centrifuged controls. Experimentally treated ATC-SB was also disrupted and analyzed for the presence of 12S RNA by polyacrylamide gel electrophoresis.

To determine if small RNA was present in vivo, total RNA was extracted fro RCVMV, ATC-MB infected pea tissue according to the methods of Pring (1971). Briefly, the tissue was homogenized in 0.5 M sodium borate, 10 mM KCl, 1.0 mM EDTA with the addition of SDS and phenol. After repeated phenol-SDS extractions, the aqueous phase was twice precipitated with ethanol and fractionated using 2.0 M LiCl. The LiCl-insoluble material was resuspended in neutral SCC and analyzed on linear-log sucrose density gradients and by PAGE.

RESULTS

Nature of the multiple banding pattern

A multiple banding pattern consisting of 5 absorbance peaks (A_{254}) occurred consistently in sucrose density gradients containing partially purified RCVMV subisolate ATC-MB. RCVMV subisolate ATC-SB and isolate WA-7641 were single-banded under identical purification procedures (Figure 1). Samples from the upper bands of ATC-MB preparations viewed by electron microscopy consisted of rods shorter than the 660 nm rods typical of RCVMV (Figure 2) and concentration by high-speed centrifugation followed by a second centrifugation through sucrose density gradient allowed sufficient separation of the peaks to develop a histogram for each (Figure 2). No virus-like particles were found in fractions containing peak 1. However, fractions containing peaks 2, 3, and 4 had particles with modal lengths of 79, 138 and 258 nm, respectively. The fraction containing peak 5 consisted of 660 nm rods, which corresponded to the modal length of RCVMV from leaf-dip preparations.

Particles shorter than 660 nm failed to infect either C. amaranticolor or pea, whereas the lower most band containing complete particles induced normal development of symptoms on test hosts.

The $A_{260/280}$ values of the combined upper four peaks of 1.55. After a second density gradient centrifugation the $A_{260/280}$ value of the first peak was above 2.0 and the three successive peaks had ratios of 1.44, 1.21, and 1.24. The lowest absorbance peak of ATC-MB and the single bands of ATC-SB and WA-7641 all had $A_{260/280}$ values of 1.24 ± 02.

PAGE of each density gradient band indicated that with the exception of peak 1, for which no protein was detected, that the upper peaks contained a single protein species which co-migrated with the coat protein of RCVMV, WA-7641 and the lower most peak of ATC-MB.

The sedimentation coefficients of the large and small RNAs of RCVMV reference isolate (PV/110) were determined in previous work (Veerisetty and Brakke, 1977) and PAGE analysis of nucleic acid from ATC-MB established that the predominant RNA from peak 5 was the 39S species and that a lesser amount of 12-15S RNA was also present. The combined peaks 2, 3, and 4 contained a predominance of small RNA and a lesser amount of large RNA (Figure 3). Preparations from peak 1 failed to produce a band in these gels and on the basis of its UV spectrum and our failure to detect a protein band, peak 1 was concluded to be degraded nucleic acid. In these tests nucleic acid had been derived from viral preparations consisting principally of 79 nm particles, corresponding to peak 2, Figure 3.

RNA derived from viral peaks 2, 3 and 4 was non-infectious upon

inoculation to bell bean, whereas RNA from peak 5 consisting predominantly of 39S RNA was highly infectious to bell bean. PAGE analysis of ATC-SB and WA-7641 demonstrated a single large RNA species and no evidence of the 12-15S RNA.

Origin and induction of multiple bands

Desicated pea tissue in which the RCVMV reference isolate (PV/110) was supplied to us was used on four separate occasions to establish RCVMV in the greenhouse during these studies. ATC-MB was derived in two of these occasions and ATC-SB was derived twice. ATC-SB was shown to be single-banded for 4 successive passages through pea, and ATC-MB remained multibanded through 8 successive transfers. However, the proportion of upper peaks in ATC-MB preparations varied considerably, relative to the lower most virus peak. No reversion of MB to SB occurred during the course of these studies. Isolate WA-7641 remained single banded throughout two years of successive transfers.

Modification of the purification procedure had no visible effect on the banding patterns of the RCVMV isolates. The substitution of sodium sulphite for mercaptoethanol or the use of phosphate-citrate in place of sodium borate buffer had no descernible effects on either banding patterns or yields. Likewise, the omission of $MgCl_2$ reduced virus yield but had no effect on the gradient banding profiles or proportions.

Preliminary particle length measurements from leaf dip preparations of RCVMV subisolates MB and SB indicated that each comprised a unimodal (660 nm) particle length distribution. However, as revealed

by electron microscopic examination of density gradient fractions in which variously treated infected-plant extracts had been centrifuged, two specific treatments were found to induce formation of short particles: prolonged freezing of tissue and high-speed centrifugation.

The following results illustrate this effect. ATC-MB infected fresh or briefly frozen plant extracts at purification stages 1 and 2, when centrifuged on sucrose density gradients, produced a distinct absorbance (A_{254}) peak, corresponding to peak 5 obtained during normal virus purification procedures. Electron microscopic examination of this peak revealed intact 660 nm particles, whereas fractions above this peak were virtually devoid of short virus-like particles. Conversely, virus-infected plant extracts subjected either to prolonged freezing or to high-speed centrifugation before analysis in density gradients produced copious quantities of short particles in those fractions above absorbance peak 5, suggesting that they were products of particle fragmentation induced by these treatments. Electron microscopic examination of sucrose density gradient fractions for the presence of short particle rather than direct examination of crude extracts avoided the possible confusion between short particles resulting from the purification and those resulting from the degradation during preparation of the electron microscope grid.

Lithium chloride insoluble fractions of nucleic acid from RCVMV infected pea tissue analyzed by PAGE and density gradient centrifugation were found to be devoid of non-host RNA. Failure to extract either 39S or 12-15S viral RNA species were interpreted to

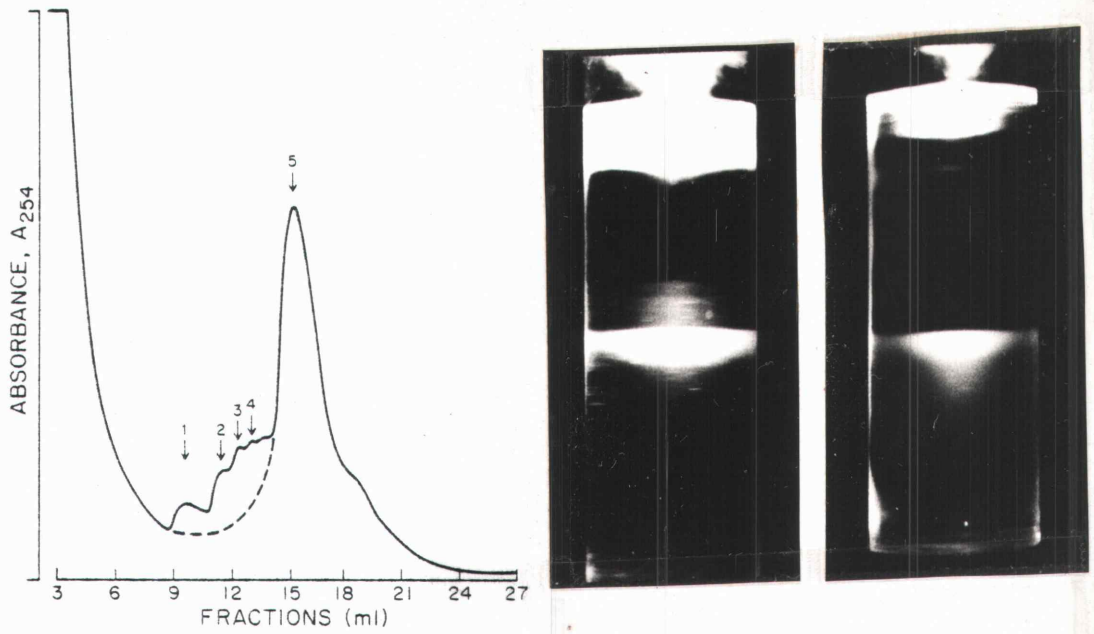

FIGURE 5-1. Photometric scanning patterns and photographs of centrifuged gradient columns containing RCVMV ATC (subisolates MB and SB) and WA-7641.

(Left) solid line-RCVMV ATC (MB) depicting 5 absorbance peaks; dashed line-RCVMV ATC (SB) also representative of WA-7641. (Middle) RCVMV ATC (MB). (Right) RCVMV WA-7641 also representative of (SB). Gradients were linear containing 10-40 mg of sucrose/ml in 10 mM sodium borate at pH 9.0. Gradients were centrifuged at 24,000 rpm at 4 C for 2 hr in an SW 27 rotor and monitored with an ISCO fractionator.

FIGURE 5-2. Particle length distribution and electron microscopy of the five absorbance peaks of RCVMV ATC subisolate MB after isopycnic centrifugation.

The modal length of each absorbance peak was: (1) no virus-like particles, (2) 79 nm, (3) 138 nm, (4) 258 nm, (5) 660 nm. Measurements for the determination of length were taken from negatively stained particles (phosphotungstate, pH 7.0) using TMV (modal length 300 nm) as an internal standard. Specimens on right were coated with platinum and paladium. Bar represents 500 nm.

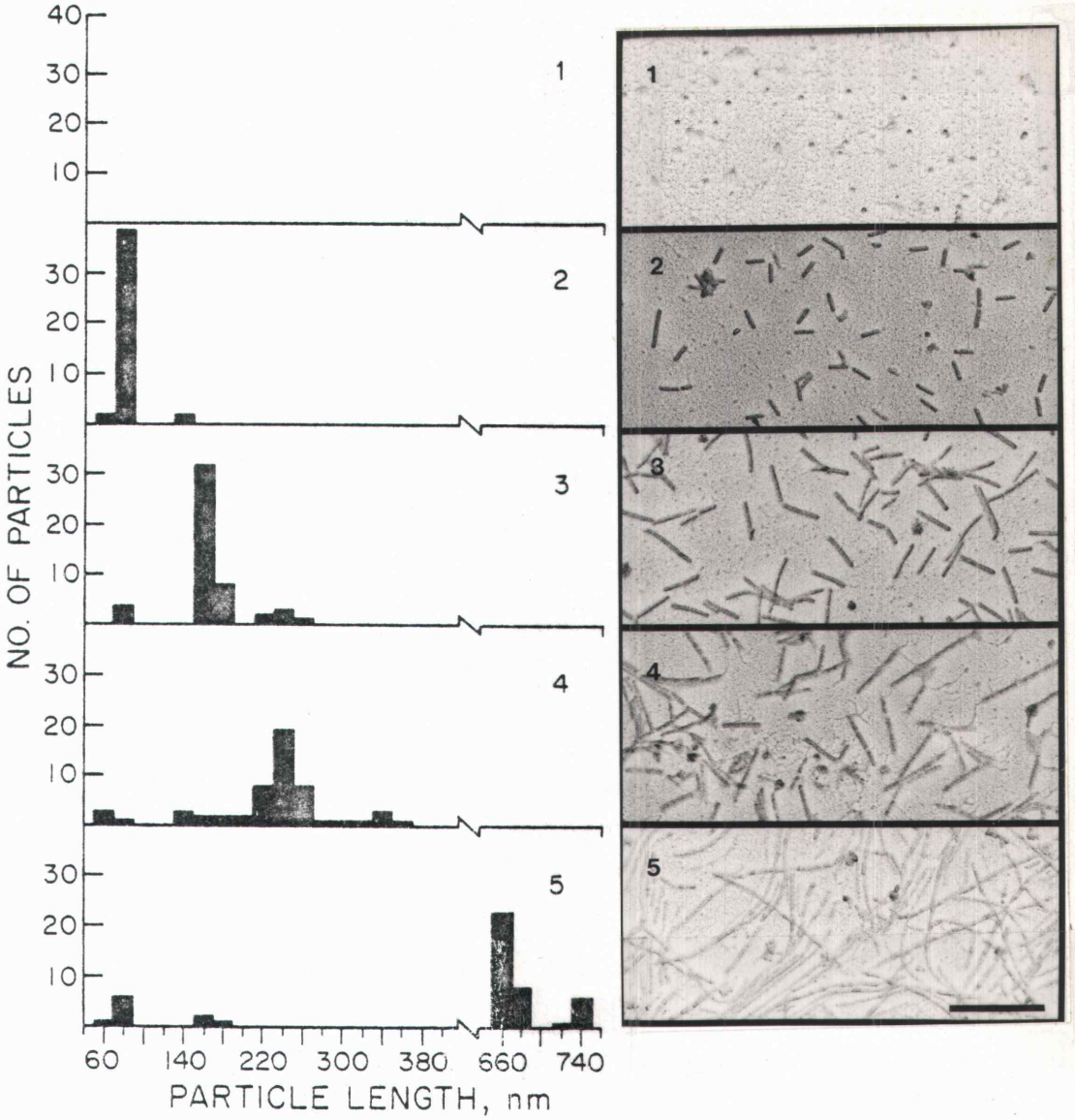
NO. OF PARTICLES
40
30
20
10
1
2
3
4
5
60
140
220
300
380
660
740
PARTICLE LENGTH, nm

FIGURE 5-2.

FIGURE 5-3. Photometric scanning patterns of centrifuged gradient columns containing RCVMV WA-7641 and ATC (MB); and polyacrylamide gel electrophoretic patterns of RNAs from RCVMV ATC subisolate SB and MB.

(Left) Top, RCVMV WA-7641; Bottom, RCVMV ATC (MB). Gradient columns were linear containing 10-40 mg of sucrose/ml in 0.01 M sodium borate $NaBO_3$ pH 9.0. Gradients were centrifuged at 39,000 rpm at 4 C for 70 min in an SW 40 rotor and monitored with an ISCO fractionator. The absorbance at the meniscus was due to Triton X-100 and ME in the buffer for partially purified virus.

(Right) A-RNA from RCVMV ATC (SB) also representative of WA-7641
(Middle) A-large RNA and B-small RNA from peaks 2, 3, and 4.
(Bottom) A-large RNA and B-small RNA from peak 5.
Electrophoresis was carried out in 2.2% gels at 6 mA/gel for 90 min. Gels were stained with methylene blue and scanned at 546 nm.

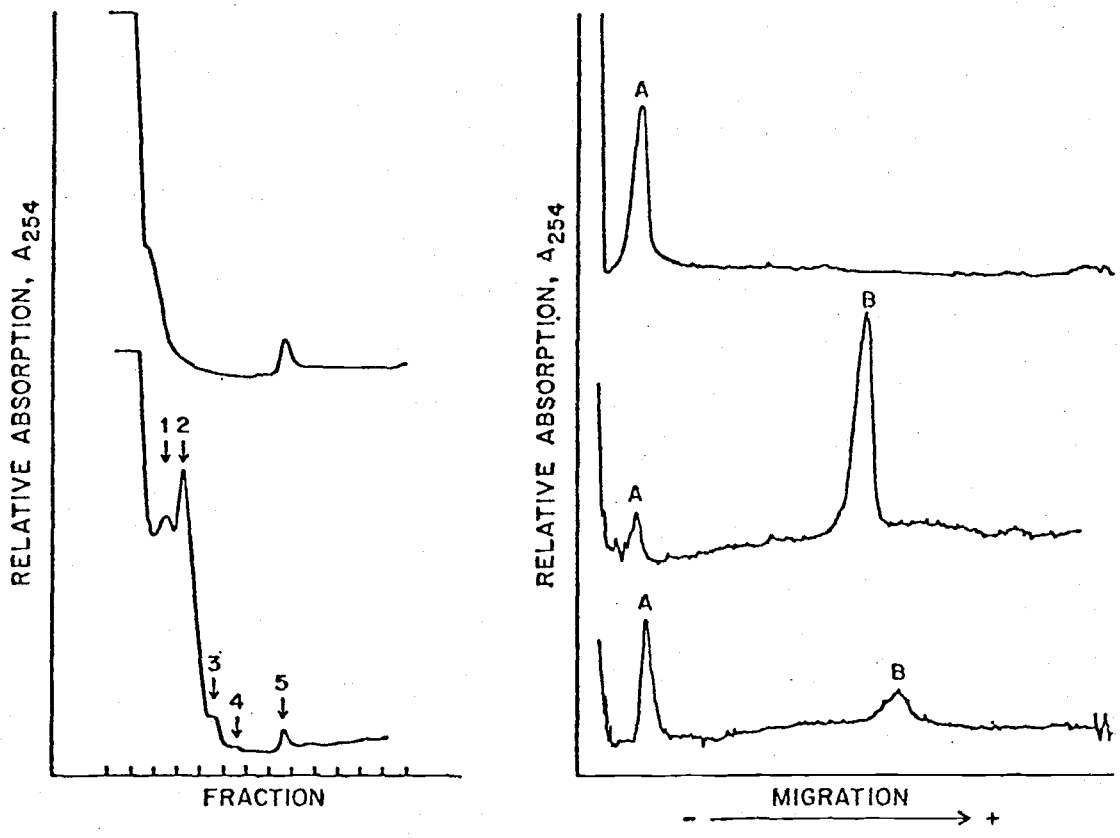
RELATIVE ABSORPTION, A_{254}
1
2
3
4
5
FRACTION
RELATIVE ABSORPTION, A_{254}
A
B
A
A
B
MIGRATION
-
+

FIGURE 5-3.

indicate that RCVMV was degraded or precipitated by exposure to phenol (Hill, et al., 1977). Transfer, ribosomal, and chloroplast RNAs were readily identifiable by published mobility values and profiles, in both healthy and infected pea tissues (Pring, 1971).

DISCUSSION

Two isolates of RCVMV, RK 31 and P42 were previously shown to produce multiple banding patterns in rate-zonal sucrose density gradient centrifugation (Bos et al., 1971). The present studies demonstrate the existance in RCVMV reference isolate ATC (PV/110) of two subisolates that were single banded (SB) and multiple banded (MB), respectively, under isopycnic centrifugation. The characteristics of these subisolates were stable under the conditions of our studies.

The results presented strongly suggest that the band multiplicity of RCVMV, subisolate ATC-MB, is due to degradation of virus particles and that this ATC reference isolate contains two subisolates, one sensitive and the other insensitive to breakage during purification. The circumstances by which one or the other became predominant in transfers from reference inoculum were not discerned. Limited evidence of subisolate stability, however, indicated that either subisolate can become durably predominant in the host plant.

When MB was predominant, 79 nm particles corresponding to those of density gradient absorbance peak 2, represented the greatest proportion of short particles. RNA from combined viral peaks 2, 3 and 4, centrifuged in analytical sucrose density gradients prior to assay

on bell bean, contained trace amounts of unreported RNA species. The two species had greater sedimentation coefficients than the small (12-15S) RNA and it was assumed that they originated from virus peaks 3 and 4 containing the 138 and 258 nm particles, respectively. The importance of characterizing these two species was considered minimal and efforts to study them were forgone.

Efforts to gain an understanding of the nature of ATC-MB particle lability was hampered by our failure, to induce reversions in either the SB or the MB isolate through selected purification modifications.

Our inability to extract RCVMV RNA from infected plant tissue was similar to the results of Hill et al. (1977) who reported that phenol failed to release RNA from Hydrangea ringspot virus. Pea tissue infected with pea streak virus, another carlavirus, also failed to yield viral RNA when processed by SDS-phenol extraction methods (Weber, unpublished). Coincident with the absence of 12-15S or 39S RCVMV RNA in SDS-phenol extracts from infected plants were very high yields of host single-stranded RNA.

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SUMMARY AND CONCLUSIONS

Two carlaviruses, pea streak and red clover vein mosaic, have been identified as principal streak-inducing pea diseases in the Pacific Northwest. Isolates collected from Oregon, Idaho and Washington were identified by comparison to U.S. type cultures of PSV and RCVMV. Research was directed toward characterization of these viruses and toward the development of new and significant information with the potential to facilitate the development of control stratagies.

Previous to this work it was known that PSV and RCVMV were distinct viruses, separable on the basis of serology, host range and reactions and particle morphology. Our work confirmed these findings and determined that the Northwest isolates differed from the U.S. type cultures only in minor characteristics and were serologically identical to the type isolates. No differentiation between Northwest and the type isolates of PSV and RCVMV on the basis of stability in sap or other biological properties was found; however, subtle differences in host reactions were determined.

In addition to comparing and studying the biological properties of legume carlaviruses, biochemical characteristics were studied. Previously published purification procedures were modified to significantly improve the yield and quality of both PSV and RCVMV. Northwestern isolates were indistinquishable from the type isolates in our tests which included electrophoretic mobility of protein subunits and RNA in acrylamide gels and comparisons of ultraviolet absorption spectra.

The extinction coefficients ($E^{1\%}_{260\ nm,\ 1\ cm}$) of PSV and RCVMV each were estimated at approximately 2.0, a figure which was close to that reported for carnation latent virus the type virus for the carlavirus group. Molecular weights of purified RCVMV and PSV coat proteins were estimated by SDS polyacrylamide gel electrophoresis to be 31,000 and 29,000 daltons respectively. Previously reported lability of PSV type culture protein was confirmed and determined to be characteristic also of Northwestern isolates of PSV. The RCVMV reference isolate (ATC PV/110) was found to contain a sub-isolate which typically produced multi-banded profiles during isopycnic centrifugation. Moreover, a small, non-infectious RNA species was typically associated with particle fragments of upper (light) bands. In contrast, wild type isolates consistently exhibited single bands during isopycnic centrifugation, suggesting that the sub-isolate from the reference isolate was unstable during normal purification procedures.

PSV and RCVMV were known to be aphid-borne in the non-persistent manner and Northwest isolates of both viruses were transmitted under laboratory conditions by pea aphids collected in Oregon. In addition to the determination of a potential vector, in perennial legumes were identified as inoculum reservoirs for these virses. Knowledge of a likely source and of a potential vector has increased our understanding of the ecological parameters affecting epiphytotic periodicity.

Beyond ecological considerations, experimentation designed to increase our understanding of the mechanism of non-persistent aphid transmission was conducted. Recently, it was hypothesized that a virus-induced component found in sap (helper agent) may be required for the transmission of carlaviruses by aphids as is required for the aphid transmission of caulimoviruses and potyviruses. Successful transmissions of both purified PSV and RCVMV are reported herein and provide the first definitive evidence indicating non-participation of a 'helper agent' in the aphid transmission of carlaviruses.

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