PURIFICATION, SEROLOGY AND ELECTRON MICROSCOPY OF PEA ENATION MOSAIC VIRUS

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This is to certify that the

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ABSTRACT

PURIFICATION, SEROLOGY AND ELECTRON MICROSCOPY OF PEA ENATION MOSAIC VIRUS

by Antonio D. Bustrillos

The host range, physico-chemical properties, purification, serology and electron microscopy of pea enation mosaic virus (PEMV) were investigated. Isolates from Michigan (PEMV-2), Washington (PEMV-W), Oregon (PEMV-O), and New York (PEMV-NY) were employed in this study.

PEMV infected only leguminous plant species. Mung bean (Phaseolus aureus Roxb.) was found to be a new host plant of PEMV. All pea varieties and 55 pea introductions were susceptible to infection. A pea introduction from Iran showed some tolerance to the virus. A number of species of plants of 6 different families were tested in an effort to find a local lesion host, but none were found.

Dilution inactivation of PEMV occurred between 1:2000 to 1:3000. The virus was still infective after 10 minutes at 65°C but not at 68°C; it resisted inactivation in vitro for 4 days but lost its infectivity in air-dried diseased tissues after the third day. Infectivity of the virus in sap extracts buffered at pH 7-7.8 was maintained for about 12 days storage in the refrigerator (3°C).

The purification of PEMV was accomplished by low speed centrifugation of buffered extracts from frozen diseased tissues. The clarified extract was concentrated and further purified through 3 cycles of differential centrifugation of the appropriately buffered virus suspension. The purified virus preparation was infectious and electron micrographs revealed the presence of numerous apparently homogeneous nearly spherical particles, about 20 mμ in diameter. Such particles were absent from a similar healthy control preparation.

The purified PEMV preparation was antigenic and reacted serologically with its homologous antibody using Ouchterlony agar technique and microprecipitin tests. Serological cross-reaction of the four PEMV isolates showed that they were closely related. Strains of cucumber mosaic virus, southern bean mosaic virus, bean yellow mosaic virus, Maryland bean virus, and tobacco ringspot virus were not serologically related to PEMV. The presence of an antigenic non-infectious host protein in the virus preparation was detected by the Ouchterlony agar double-diffusion technique. The specific PEMV antigen-antibody reaction formed curved precipitation bands while the non-specific host-antibody reaction formed a separate straight precipitation band, hence, its identification was possible. PEMV in clarified extracts could be detected and identified serologically with the antiserum obtained.

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By

Antonio D. Bustrillos

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This thesis is

sincerely dedicated to

MY FAMILY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The application of serology and electron microscopy to the detection, identification and characterization of plant viruses can give precise results only if purified infectious preparations are employed. Such preparations permit a better study of the physical and chemical nature of the viruses and also yield more meaningful interpretations of serological and electron microscopic findings. A knowledge of the morphology and size of the virus particles obtained from electron micrographs of purified preparations facilitates their identification from ultra-thin sections of infected plant tissues. The usefulness of these preparations in qualitative and quantitative electron microscopy cannot be overemphasized (48, 68, 78, 92, 93, 94).

This investigation was initiated on the pea enation mosaic virus (PEMV), an unusual plant virus that causes tumor-like growths similar to sarcoma in animals, with a view to gaining possible insight into the nature of virus-caused abnormal growths. Earlier work (39) reported the isolation of high molecular weight proteins from healthy and PEMV-infected broad bean plants by ultracentrifugation. Except for some infectivity in the latter, both proteins had similar sedimentation constants. Limited investigation on the purification, serology and electron microscopy of PEMV is due

partly to the difficulty of obtaining infectious purified preparations, lack of adequate methods of assay and to some extent, on the instability of the PEMV. The present study attempted to explore these specific areas: tests were made on the host range in search for a local lesion assay plant and the physico-chemical properties of the virus were studied to determine the conditions affecting its stability.

REVIEW OF LITERATURE

The pea enation mosaic virus (PEMV), also known as Enation Pea Mosaic Virus (50), Pea Virus 1 (54), Pisum Virus 1 (72), and Marmor pisi (26), is a distinct legume virus which initiates enations on the infected pea plants, Pisum sativum L., The symptoms of PEMV disease are generally expressed by the formation of hyaline spots or translucent areas on the foliage, the production of enations or outgrowths of different sizes and developmental forms (86) on the abaxial side of the stipules and leaves, on pods and rarely on stems.

Occurrence of the disease has been observed in most of the pea growing areas of the United States (24, 28, 29, 36, 37, 42, 45, 55, 65, 73). The loss incurred on the crop from PEMV infection is manifested in (1) a general reduction of crop yield as a result of stunting and reduced productivity of the infected plants and (2) the unmarketability of the deformed pods produced.

Linford (36) first observed the occurrence of deformed pods on garden peas, the cause of which appeared similar to a pea virus causing enations and pod deformation reported by Snyder (74). PEMV disease was first described by Osborn (50), then by Pierce (54); Stubbs (83); Ainsworth (1); and others. A detailed description of the macroscopic symptoms and anatomical studies of PEMV-diseased pea at different

stages of infection was reported by Tsao (86).

Studies on the host range of PEMV were conducted by a number of investigators, Osborn (50) transmitted the virus to garden pea, sweet pea (Lathyrus odoratus L.), field pea (P. sativum var. arvense Poir.) and crimson clover (Trifolium incarnatum L.) using the pea aphid which was allowed to feed on PEMV-infected broad bean (Vicia faba L.). Pierce (54) obtained infection on soybean (Glycine soja (L.) Sieb. & Zucc.) and on the following pea varieties: Alderman, Alaska, Perfection, Wisconsin Early Sweet, Surprise, Progress, Green Admiral, Dwarf Telephone and Horal. Stubbs (83) observed PEMV infection on yellow sweet clover (Melilotus officinalis L. Lam.) and on ten more pea varieties, namely: Premium Gem, Improved Gradus, Laxton's Progress, Gradus, Horsford, Bruce, Icer, Canada Field Pea, Black Eyed Marrowfat and Mammoth Luscious Sugar. Alfalfa (Medicago sativa L.), alsike clover (Trifolium hybridum L.) and perennial pea (Lathyrus latifolius L.) were also found susceptible to PEMV but it was not transmitted to yellow sweet clover and broad bean (28). Ainsworth (1) observed PEMV infection on rough pea vine (Lathyrus hirsutus L.) and on other pea varieties in Great Britain. The susceptibility of crimson clover and sweet pea was confirmed by Chaudhuri (14). He was unable to obtain infection on vetch (Vicia sativa L.), tobacco (Nicotiana tabacum L.) and French bean (Phaseolus vulgaris L.). Bur clover (Medicago hispida Gaertn.) var. dulce and spotted medick

(M. arabica All.) were susceptible to PEMV whereas sugar beet (Beta vulgaris L.), smooth leaf mustard (Brassica juncia Coss.), cucumber (Cucumis sativus L.) and celery (Apium graveolens L.) were not susceptible to PEMV (69). Hagedorn and Walker (24) observed PEMV infection on most of the leguminous hosts reported earlier but found nonsusceptibility to virus infection in the following legumes: beans (Phaseolus vulgaris L.), cowpea (Vigna sinensis L.), white lupine (Lupinus alba L.), red clover (Trifolium pratense L.), white clover (T. repens L.), alsike clover (T. hybridum L.), alfalfa (Medicago sativa L.) and white sweet clover (Melilotus officinalis (L.) Lam.). Furthermore they obtained no infection on Nicotiana glutinosa L. and tomato (Lycopersicum esculentum L.). McEwen and Schroeder (42) transmitted the virus to white clover including Ladino (Trifolium repens L.) vetch, spring vetch (Vicia villosa L.), white sweet clover (Melilotus alba Dar.), and they confirmed PEMV infection on yellow sweet clover, rough pea vine, and on 6 alfalfa varieties: Grimm, Hairy Peruvian, Kansas Common, Narragansett, Ranger and Vernal. The same investigators further confirmed (43) transmission of PEMV to alsike clover. In the meantime, a considerable number of leguminous plants were reported susceptible to the virus. Studies conducted by Hagedorn (23) showed that PEMV would infect milk vetch (Astragalus rubyi L.), chick pea (Cicer arietinum L.), flat pod pea vine (Lathyrus cicera L.) Tangier pea

(L. tingitanus L.), chester clover (Trifolium glomeratum L.), Persian clover (T. resupinatum L.), subterranean clover (T. subterraneum L.), white lupine (Lupinus alba L.),blue lupine (L. angustifolius L.), wooly pod vetch (Vicia dascarpa L.) and Hungarian vetch (V. pannonica Crantz.).

Some physical properties in vitro of PEMV have been reported. The virus was inactivated by a temperature of 56°C with no infection after 10 minutes exposure at 58°C (54). Johnson and Jones (28) reported a wide thermal inactivation range of 40-80°C. Osborn (53) obtained infection at 64°C with no infection beyond 66°C. Ainsworth (1) indicated 55°C for the thermal death point of PEMV. A dilution end point of 1:3000 was reported by Stubbs (83). Osborn (53) found PEMV infectivity at dilutions of 1:1000 but not at 1:10,000. The virus has been reported to resist inactivation in vitro at 20-22°C for 3 days (54). In a separate similar investigation (53, 83), PEMV resisted inactivation for 4 days.

Early investigators reported that PEMV was difficult to transmit by mechanical means unless abrasive materials were employed. Stubbs (83) transmitted the virus by a rubbing method employing Norit or carborundum powder. He also transferred PEMV by insertion of macerated diseased tissue into slits made in the stem of suscept plants, however, the percentage of infection was low. The use of carborundum in sap transmission of the virus was further demonstrated by Osborn (53) who further noted that repeated serial transfers

of PEMV through broad bean by mechanical means resulted in the attenuation of the virus. Ainsworth (1) reported that the virus retained its virulence for more than two years through serial transfers mechanically in garden pea and sweet pea. The carborundum method was found superior to atomization of the virus suspension at a pressure of 28 lbs./ in.^{2} (23).

Insect transmission of PEMV and the virus-vector relationship was first studied by Osborn (49) and reported in his subsequent papers (50, 52, 53). The pea aphid, Macrosiphum pisi Kalt. and the potato aphid, M. solanifolii (Ashm.) = M. gei Koch, were observed vectors of PEMV. Furthermore, he reported that PEMV was a persistent virus requiring an incubation period of 12-18 hours in the vector before the aphid was able to transmit the virus. The green peach aphid, Myzus persicae (Sulz) was first reported a vector of PEMV by Chaudhuri (14). He noted that the pea aphid was the most efficient vector of the virus and that the aphid remains infective for a considerable period of time. Simons (69) showed that the ornate aphid, Myzus ornatus Laing, could transmit PEMV and he suggested that the aphid vectors probe the phloem in order to accomplish infection. Infection could be accomplished also by probing the parenchymatous tissues by the vector (43). Two more aphid vectors, Cerosipha gossypii (Clov.) and Acyrthosiphon

pisum Harris (A. onobrychis, B.D.F.) have been reported to transmit PEMV from diseased broad bean to healthy clover plants (25).

A search for resistance to PEMV in pea varieties was conducted by several investigators (64, 65, 73). The Geneva line No. 168 (PI No. 140295) was found to exhibit tolerance to the virus. Schroeder and Barton (65) reported that resistance of pea to PEMV was conditioned by a single dominant gene.

McWhorter and Cook (46) observed some strain differences in PEMV. Cross-protection studies have been conducted by Hagedorn and Walker (24). They obtained no protection when PEMV was tested against Bean Virus 2, Wisconsin pea streak, Red clover vein mosaic and Cucumber mosaic virus. Reciprocal tests also showed negative protection.

Some anatomical aspects of PEMV disease were reported by McWhorter (44) and McWhorter and Cook (46). They observed certain nuclear changes in the infected cells. The enations formed following PEMV infection arose from proliferated epidermal cells on veins of the lower surface of leaflets and stipules. The primary effect of PEMV (California isolate) on the pea plant (Perfected Wales var.) was the stimulation of cell division (hyperplasia) and the enlargement of the phloem parenchyma cells (hypertrophy) of the vascular bundles resulting in the differentiation of the abnormal phloem and necrosis of the mesophyll areas (86, 87). Furthermore, the

translucent areas were composed of hyperplastic and hypertrophic parenchyma cells containing degenerated chloroplasts arranged in compact fashion. In a similar study, Ruppel and Hagedorn (62) found that the giant enations observed on Perfected Wales following infection with the California isolate of PEMV were composed of highly organized and differentiated tissues comparable to healthy or normal ones. However, this type of enation was not reproduced in the subsequent inoculations from these.

The spread of PEMV in the field was reported reduced by controlling insect vectors. Parathion, demeton, and diazinon insecticidal sprays have been employed (19, 43, 84).

MATERIALS AND METHODS

PEMV isolates

Four pea enation mosaic virus (PEMV) isolates were employed in this study. The virus isolates from Washington (PEMV-W), Oregon (PEMV-O) and New York (PEMV-NY) were obtained from Dr. D. J. Hagedorn, Department of Plant Pathology, University of Wisconsin, Madison. The virus isolate from Michigan (PEMV-2) was from infected dried pea leaf tissues stored frozen over $CaCl_2$ for about 3-1/2 years in the refrigerator. Unless otherwise stated, the PEMV-W isolate was employed in the purification, serology and the identification of the virus by electron microscopy.

Source plant and biological assay

Garden pea variety Pacific Freezer was chosen as a suitable host plant for PEMV based on the uniformity of symptom expression, ease of infection and compactness of growth. Since no host plant responding to PEMV infection with local lesions was known, Pacific Freezer variety was also used as a systemic assay plant for virus activity. Virus infectivity assays were made by rubbing the carborundum-dusted leaves and stipules with polyurethane pads which had been saturated with the virus preparation to be tested.

Culture and method of inoculation

Five - 8 pea seeds lightly dusted with Arasan

(tetramethylthiuramdisulfide), to prevent pre-emergence damping off, were planted in 4-inch pots filled with greenhouse soil. The plants were given small amounts of complete fertilizer in solution a week after germination and once in two weeks thereafter. The seedlings to be inoculated were dusted lightly with carborundum powder, 400 to 600 mesh, to aid in the inoculation (59). Either frozen dried or recently infected leaves were macerated with a mortar and pestle with small amounts of neutral phosphate buffer added to the macerate to make a light thin paste or slightly liquid suspension. Polyurethane pads, 1/2" x 1" x 1", soaked lightly with the inoculum were employed. The inoculating pads caused no detectable injury on the inoculated plant tissues. Pea seedlings 10-14 days old were readily infected with PEMV. Infection of very young seedlings generally resulted in severe stunting due to retarded apical growth and leaf distortion. When older plants were used a low percentage of infection was observed and symptom expression was often delayed.

Determination of physico-chemical properties of PEMV

The methods described by Bos et al (9), slightly modified, were employed in the determination of some physico-chemical properties of PEMV. Leaves and stipules of Pacific Freezer peas, 12-14 days after inoculation, were used as source of PEMV. Where clarified extracts were

employed, the fresh infected tissues were ground in a mortar and the macerate was squeezed manually through 4 layers of cheese cloth.

(a) Dilution end-point. The following serial dilutions of the clarified juice with distilled water as diluent were prepared: 0, 1:1000, 1:2000, 1:3000, 1:4000, and 1:5000. These dilutions were used to inoculate carborundum-dusted leaves and stipules of healthy 12-day old pea seedlings. The tolerance to dilution of PEMV was recorded 24 days after inoculation.

(b) Inactivation in vitro. A clarified juice extract was stored in a stoppered Erlenmeyer flask and held at 20-22°C. A sample was drawn for infectivity test after 0, 6, 12 hrs., 1, 2, 3, 4, and 5 days storage. Disease symptoms were observed and recorded.

(c) Longevity of PEMV in dried tissue. Leaves of PEMV-infected plants were harvested and allowed to dry in loosely covered beaker (20-22°C) protected from direct sunlight. A sample was taken every day for 5 days. An inoculum with small amount of phosphate buffer pH 7.5 was prepared for mechanical inoculation on healthy pea plants. Virus survival in the dried tissue was measured in terms of infectivity.

(d) Thermal inactivation studies. Portions of 0.5 ml of the clarified extract were pipetted into micro-tubes and placed in a pre-set constant temperature water bath. After

10 minutes' exposure to the desired temperature they were withdrawn immediately and cooled in flowing cold tap water. Three samples exposed to the same temperature were pooled and tested for infectivity on healthy pea seedlings. The following temperatures were used: 50, 55, 58, 60, 63, 65, 68 and 70°C. The temperature at which loss of infectivity of PEMV occurred was recorded 25 days after inoculation.

(e) Effect of storage of virus juice extract buffered at different pH levels on virus infectivity. Three ml of the clarified juice was pipetted into test tubes containing 3 ml of 0.1 M phosphate buffer (K_2HPO_4-KH_2PO_4) at pH 5.0, 5.5, 6.0, 6.3, 6.5, 6.8, 7.0, 7.3, 7.5, 7.8 and 8.0. The inocula were stoppered and stored at 2-3°C. Inoculations were done at 4 days intervals for 12 days on healthy Pacific Freezer plants. Number of plants infected was recorded 3 weeks after inoculation.

Purification of PEMV

To the knowledge of the writer isolation and purification of PEMV has not been reported. Therefore known procedures of virus purification successfully employed with other plant viruses (81, 82) were tried on PEMV.

A milder method of virus purification employing techniques of alternate cycles of high and low speed centrifugation was developed for PEMV. The purification procedure is described in Results. Unless otherwise stated, low speed

centrifugations were done in a Servall Superspeed angle head centrifuge Type SS-1A. For high speed centrifugation work a Spinco Model L preparative ultracentrifuge with rotor no. 50 was employed.

Preparation of virus antigen and serum production

The methods described in the literature (41) for virus antigen preparation and serum production were slightly modified and adapted in this study.

(a) Experimental animals and preparation of normal serum. Rabbits weighing about 3-4 pounds were kept singly in suitable wire cages. Normal serum was obtained a week before the virus antigen preparation was administered.

(b) Preliminary work on antiserum production. The virus antigen preparations were obtained from clarified PEMV-infected extracts or from resuspensions of a virus pellet following one high speed centrifugation. These were prepared as follows: The filtered crude juice extracted from fresh leaf tissues of PEMV-infected peas was centrifuged at low speed (2590 g) for 15 minutes. The clarified virus suspension was emulsified with mineral oil adjuvant (63) containing 10:9:1 of virus, Bayol F, and Arlacel A respectively. Four - 6 ml of the emulsion was injected intramuscularly into the leg of the rabbit. Injections were administered once every 5-6 days for 4 weeks. After the third injection the rabbit was bled through the ear and an antiserum was obtained for antibody titre test.

A similar clarified virus suspension was subjected to one hour high speed centrifugation at 25,000 rpm (63,581 g) in a swinging bucket rotor of the ultracentrifuge. The resulting pellet was resuspended in 2-4 ml of 0.01 M neutral phosphate buffer-physiological saline solution and clarified by low speed centrifugation for 15 minutes. An emulsion of the virus-mineral oil adjuvant was prepared and administered to the rabbit intramuscularly. Antiserum was obtained after the third injection and tested serologically with a similar antigen preparation.

(c) Antiserum used in serological studies of PEMV. The virus antigen was prepared from a resuspension of the pellet obtained from the second cycle of differential centrifugation (Fig. 1). The pellet was taken up in 1-2 ml of 0.01 M neutral phosphate buffer-physiological saline (0.85%) solution. The virus resuspension from the third cycle of ultra-centrifugation was used in two instances for intravenous injection into the rabbit.

An emulsified preparation of the resuspended virus and Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan), 1:1 V/V, was injected into the thigh and calf muscles of the rabbit. Each leg received no more than 1 ml of the virus-adjuvant emulsion per injection. No attempt was made to determine the actual weight of the virus protein in any of the immunizing antigen preparations since virus yields were generally small. The injections were administered

4-6 days apart for about 6 weeks. When satisfactory titre was achieved the rabbit was sacrificed by bleeding from the heart completely with a 50 ml glass hypodermic syringe with a No. 18 needle. The blood collected in suitable glass containers was allowed to clot at room temperature for 2-3 hours. The serum layer was centrifuged once at low speed (1000-2000 rpm) for 10 minutes. The clear antisera containing the antibodies were stored in sealed vials in the freezer until needed.

Serological tests

The technique employed in the study of the serological properties of PEMV were essentially a modification of the precipitin test. The methods for the microprecipitin and the Ouchterlony agar double-diffusion tests described by Ball (3) were adopted with some modifications.

(a) Microprecipitin test. A titration reaction area was drawn in a checkerboard pattern with a wax pencil on a plastic petri dish. An appropriate dilution series of the antigen and of the antiserum was obtained in test tubes using physiological saline solution as diluent. A microdrop of the desired dilution of the antiserum was placed in the center of the reaction square and then layered with an equal volume of an appropriate dilution of the antigen. Physiological saline solution was added to the control series. To minimize the evaporation of the reaction drops during incubation and observation, the petri dish was flooded

carefully with mineral oil such that all the reaction drops were submerged completely. After 2 hours incubation at room temperature the dish was observed for any reaction under a binocular dissecting microscope. Incubation was carried further in the refrigerator overnight and observed again.

(b) Ouchterlony agar double-diffusion test. A 0.5% ion agar No. 2 (Consolidated Laboratories Inc., P.O. Box 234, Chicago Heights, Ill.) in physiological saline solution was prepared in 3-500 ml Erlenmeyer flasks and sterilized for 15 minutes in the autoclave. To insure sterile conditions aqueous stainless solution of merthiolate (1:10,000 final dilution) or sodium azide solution (1:5000 final dilution) were added to the agar. Ten ml of agar was poured into each sterile glass petri dish and allowed to solidify. Ten mm lengths were cut from a 12 mm diameter cellulose centrifuge tube and sterilized for a minute in a solution of merthiolate (1:10,000) or sodium azide (1:15,000) to serve as molds for the wells. The molds were pressed lightly on the thin layer of solidified agar following a predetermined arrangement drawn on paper underneath the petri dish. A small amount of liquid agar was layered around each mold to secure it. Then 15 ml more of agar was added to the petri dish and allowed to harden. The molds were then carefully removed from the agar plate, and the wells were carefully sealed with small amount of liquid agar to prevent the liquid reactants from spreading between the glass and the agar layer. A suitable

dilution of the antigen and the antiserum was pipetted into each designated well. The reaction dish was incubated at room temperature in a moist chamber. Formation of antigen-antiserum reaction bands was observed for two weeks.

The microprecipitin and Ouchterlony gel techniques were employed in the serological studies of the four PEMV isolates. Similarly, preparations of cucumber mosaic virus (CMV), southern bean mosaic virus (SBMV), Maryland bean virus (MBV), tobacco ringspot virus (TRSV), and bean yellow mosaic virus (BYMV) strains were serologically tested against the PEMV antiserum for virus relationship determination.

Electron microscopy

The homogeneity of the PEMV preparation was determined by electron microscopy (80, 82, 92) and the morphology and size of the virus particles were determined from the electron micrographs obtained. Following standard techniques in electron microscopy (21, 31) samples were obtained from the purification procedure and prepared for electron microscopy. Records of the examination were made on electron micrographs.

(a) Preparation of film-coated grids. Copper or silver Lecktromesh grids No. 200 were used as the supporting material for the thin films prepared from a 0.25% Formvar (polyvinylformal) solution in ethylene dichloride.

A clean glass slide was dipped into formvar solution, drained and dried at room temperature. The resulting thin

film layer was stripped from the glass slide over distilled water. The grids were placed on the floating film and tapped lightly to insure adhesion of the metal grids on the film. The film containing the grids was scooped out of the water, dried, and stored in petri dish until needed.

(b) Size calibration. A 0.01% polystyrene latex (PSL) suspension (Dow Chemical Company, Midland, Michigan) with an average particle size of 264 mμ ± 6 mμ was used as an internal standard. It was incorporated with the specimen prior to deposition on the film-coated grids.

(c) Specimen deposition. A microdrop of the prepared suspension of purified PEMV or healthy control preparation and PSL was placed on the filmed grid with a micropipette. The excess liquid was absorbed at the periphery of the drop with a strip of filter paper and the preparation was air dried in a petri dish. Deposition was also accomplished by spraying the specimen directly on the formvar-filmed grids (2). A commercial pyrex nebulizer, Vaponefrin No.166, was employed.

(d) Shadow-casting and electron microscopy examination. Tungsten as the shadowing metal was evaporated on the specimen at an angle of about 30° in a Kenney vacuum evaporator unit. The shadowed specimens were examined and electron micrographs were taken with an RCA EMU-2C electron microscope.

EXPERIMENTS AND RESULTS

Reaction of source plant to PEMV isolates

The PEMV-2 isolate was regularly infective on Pacific Freezer pea. The infected host plants developed typical symptoms of PEMV disease consisting of mottling, translucent or hyaline spots and enations in young and old leaves and stipules as well as enations on the pods. The symptoms exhibited by the virus isolates PEMV-O, PEMV-W, and PEMV-NY were similar to those of PEMV-2. The symptoms normally were observed about a week after inoculation.

Host range and search for local lesion host

A study on the host range in a search for a suitable local lesion assay plant for PEMV was conducted in the greenhouse. Twenty-three species of plants belonging to six families: Leguminoseae, Solanaceae, Chenopodiaceae, Amaranthaceae, Cucurbitaceae, and Compositae were used. The species tested and the results obtained are given in Table 1.

Susceptibility to PEMV was confined to the leguminous plants. These included all varieties of garden pea (*Pisum sativum* L.), two varieties of broad bean (*Vicia faba* L.), crimson clover (*Trifolium incarnatum* L.), subterranean clover (*T. subterraneum* L.), alfalfa (*Medicago sativa* L.), cowpea (*Vigna sinensis* Endl.), mung bean (*Phaseolus aureus* Roxb.), and soybean (*Glycine soja* (L.) Sieb. & Zucc.).

The 15 susceptible pea varieties were the following: Alderman, Alaska, Dwarf gray sugar, Dwarf telephone (Daisey), Dark skin, Early perfection, Green skin, Improved telephone, Little marvel, Mira green, Osaya endo (OSY 6212), Perfected Wales, Sugar pea (1842 and 2408), Thomas Laxton, and Wisconsin perfection.

The infected host plants showed mottling and faint or conspicious chlorotic spots on the leaves. The production of hyaline spots and enations on the leaves and stipules were frequently observed on the garden pea varieties. The infected mung bean and cowpea showed vain clearing and faint mottling of the leaves with no enations produced. In addition, a slight leaf distortion was observed in the former host plant. Although the PEMV-infected alfalfa appeared symptomless except for some occasional vein clearing, recovery of the virus was demonstrated.

The leguminous plants which were found not susceptible to PEMV were the following: white lupine (Lupinus alba L.), red clover (Trifolium pratense L.), white clover (T. repens L.), lima bean (Phaseolus limensis Macf.) and bean (Phaseolus vulgaris L.) var. Prince, Michelite, and Pinto.

The non-susceptible solanaceous plants were jimsonweed (Datura stramonium L. and D. tatula L.), tomato (Lycopersicum esculentum Mill.), and two species of tobacco: Nicotina tabacum L. var. Xanthi nc., Ky-57, Samsun, and Nicotiana glutinosa L.

No infection was obtained on the two species of Chenopodiaceae, Chenopodium paganum L. and C. amaranticolor Coste and Reyn. In addition, three other plant species from different families were found not susceptible to PEMV. They were: common globe amaranth (Gomphrena globosa L.), cucumber (Cucumis sativus L. var. National Pickling), and zinnia (Zinnia elegans Jacq.).

Back inoculations of the non-susceptible plants to healthy Pacific Freezer plants showed negative results. None of the susceptible plant species were found to be a local lesion host of PEMV.

Reaction of pea introductions to PEMV isolates

Fifty-five pea introductions, Table 2, were tested under greenhouse conditions for their reaction to inoculations with PEMV-0, PEMV-W, PEMV-NY, and PEMV-2 virus isolates. Three of the pea introductions, namely: Iran (PI-140295), Turkey (PI-175878), and Netherlands (PI-197989) were supposedly PEMV-resistant ones.

All of the pea introductions tested were to some extent susceptible to PEMV. The pea introduction from Iran showed some tolerance to virus infection. The young leaves of some successfully infected peas with PEMV-0 and PEMV-W showed faint mottling but no enations were observed 1-2 weeks after inoculation. Mottling was also expressed in fully expanded leaves but became inconspicuous later with no discernible symptom of the disease even in young terminal

Table 1. Results of host range study of PEMV.

Host Plant	Reaction to PEMV
Alfalfa (*Medicago sativa* L.)	+
Bean (*Phaseolus vulgaris* L.) var. Michelite	-
" Pinto	-
" Prince	-
Broad bean (*Vicia faba* L.)	
Broad improved long pod	+
McWhorter's selection, 1957 Lot	+
Chenopodium (*Chenopodium paganum* L.)	-
(*C. amaranticolor* Coste and Reyn.)	-
Common globe amaranth (*Gomphrena globosa* L.)	-
Cowpea (*Vigna sinensis* Endl.)	+
Crimson clover (*Trifolium incarnatum* L.)	+
Cucumber (*Cucumis sativus* L.) var. National Pickling	-
Garden pea (*Pisum sativum* L.)	+
Jimsonweed (*Datura stramonium* L.)	-
(*D. tatula* L.)	-
Lima bean (*Phaseolus limensis* Macf.)	-
Mung bean (*Phaseolus aureus* Roxb.)	+
Red clover (*Trifolium pratense* L.)	-
Soybean (*Glycine soja* (L.) Sieb. and Succ.)	+
Subterranean clover (*Trifolium subterranium* L.)	+
Tobacco (*Nicotiana tabacum* L.) var. Xanthi nc	-
" Ky-57	-
" Samsun	-
(*N. glutinosa* L.)	-
Tomato (*Lycopersicum esculentum* Mill.)	-
White clover (*Trifolium repens* L.)	-
White lupine (*Lupinus alba* L.)	-
Zinnia (*Zinnia elegans* Jacq.)	-

leaves. All the infected plants grew to maturity, produced pods, and in most respects were comparable to the accompanying uninoculated plants. A similar reaction was observed in most PEMV-2 inoculated peas except in one case where hyaline spots and blister-like enations appeared on the leaves and stipules much later, 3-4 weeks, after inoculation. Schroeder and Barton (65) observed a similar case which they described as the effect of an aberrant strain of PEMV.

Although yellowing and drying out of inoculated leaves which remain attached to the plant were observed in peas inoculated with PEMV-NY, no other recognizable symptoms of the virus disease were noticed throughout the whole period of observation. However, in a separate inoculation experiment of the virus isolate on the pea introduction from Iran, symptoms similar to those described in the earlier two virus isolates were obtained.

The pea introduction PI Nos. 175878 and 197989, from Turkey and Netherlands respectively exhibited a rather similar and fairly uniform reaction to PEMV inoculations. While some peas inoculated with the virus isolates showed either no detectible sign of virus infection or only mottling of leaves, other infected peas developed enation mosaic virus disease. Although the symptoms were generally mild some plants exhibited an appreciable stunting effect to virus infection. In most cases, normal-looking and slightly distorted pods with mild enations were produced.

Table 2. A list of pea (Pisum sativum L.) introductions* tested for their reaction to PEMV isolates.

Sample No.	PI Number	Origin
1	140295	Iran
2	175878	Turkey
3	197989	Netherlands
4	244092	Holland, Anthonie
5	244093	" Apollo
6	244094	" Artemes
7	244101	" Bon Amie
8	244110	" Ceroso
9	244121	" Crescent
10	244122	" Cupido
11	244124	" De Grace
12	244129	" Demi Main Debeve
13	244130	" Dutigdaagse
14	244137	" Engelsk Sabel
15	244144	" Fortuna
16	244145	" Fuga
17	244150	Goldkonigin
18	244151	Holland, Gaudraatje
19	244153	" Gryze Reuzen
20	244154	" Grote Slier
21	244158	" Halfstam
22	244161	Heraut

Table 2. Continued .....

Sample No.	PI Number	Origin	
23	244162	Holland,	Hoge-Late Groene
24	244163	"	Insulende
25	244165	"	Juni Sabel
26	244170	"	Konig der Carouley
27	244174	"	Kungsart
28	244175	"	Large Smelt Peul
29	244176	"	Large
30	244178	"	Lar Sabel
31	244189	"	Moerheimes Reuzen
32	244190	"	Monster
33	244191	"	Morgenster
34	244194	"	Namum
35	244196	"	Olympia
36	244199	"	Oscar
37	244201	"	Overload
38	244214	"	Rasperpeulen mixture
39	244215	"	Rembrandt
40	244217	"	Reuzenboter gekrenkt
41	244218	"	Reuzenboter rond
42	244225	"	Rubens
43	244235	"	Surpette Vert
44	244245	"	Superonaugetont
45	244246	"	Sylvester

Table 2. Continued .....

Sample No.	PI Number	Origin
46	244248	Holland, Tilly
47	244251	" Universales
48	244252	" Veense
49	244255	" Vermeer
50	244256	" Vesta
51	244257	" Vincent
52	244261	" Vroege Hendriks
53	244262	" Vroege Sinkepeul
54	244264	" Wilhelm Tell
55	244272	" Zwitzerse Reuzen

*Supplied by North Central Regional Plant Introduction Station, Ames, Iowa.

The other pea introductions inoculated with the PEMV isolates generally developed typical symptoms of enation mosaic virus disease. The symptoms were quite uniform though they varied somewhat in their severity. In most cases they exhibited a rather inconsistent symptom development to infection with the virus isolates.

Nineteen of the pea introductions, sample nos. 5, 7, 13, 14, 25, 26, 27, 28, 30, 31, 32, 34, 35, 43, 44, 46, 47,

49, and 52, showed severe PEMV infection resulting in a considerable size reduction. The following symptoms were observed: numerous hyaline spots and enations on leaves and stipules, numerous chlorotic spots or large blotches of chlorotic areas on the expanding leaves leading to severe distortion in apical leaves, sometimes top necrosis and the subsequent production of side shoots with typical enation symptoms on them, and the development of enations varying in size from small blister-like to leaf-like outgrowths on older leaves and stipules. Large leaf-like outgrowths, also referred to as lamina-like or the true enations (86, 87) were frequently observed on matured stipules, occasionally on the older leaves of pea introduction sample nos. 35, 38 and 44 infected with PEMV-0, PEMV-W and PEMV-2. The pods, if produced, were often distorted bearing a few crater-like irregular blisters or numerous about circular blisters or furrow-like enations. The former type of pod enation was observed in pea introduction sample nos. 26, 32 and 35 and the latter two were the common types. Pods of pea introduction sample No. 4 exhibited small nipple-like projections of papillae when infected with PEMV-NY and PEMV-2. A somewhat milder reaction to PEMV infection with no appreciable stunting effect was observed on pea introduction sample Nos. 4, 8, 14, 16, 20, 33, 37, 39, 40, and 47. The rest of the pea introductions showed an intermediate response to PEMV infection but as a rule they produced typical enation symptoms.

Physico-chemical properties of PEMV

(a) Tolerance to dilution. Variable results were obtained from two separate trials on the tolerance to dilution studies of the PEMV isolates (Table 3). Furthermore, the values were generally lower than those reported in the literature. The PEMV-0 was infective when diluted to 1:1000 but only slightly so at 1:2000. On the other hand PEMV-W was infective at 1:2000 dilution but not at 1:3000. A much lower dilution tolerance was found with PEMV-NY. At 1:1000 dilution no infectivity was demonstrated in one trial whereas slight infectivity occurred at 1:2000 in another trial. PEMV-2 was infectious at dilutions of 1:2000 and slightly so at 1:3000. From these results it appeared that PEMV has a dilution end point between 1:2000 - 1:3000.

(b) Inactivation in vitro. Virus infectivity was completely lost after 4 days aging of sap from infected plants at 20°-22°C.

(c) Longevity of PEMV in dried tissue. Leaves of Pacific Freezer pea infected with PEMV when allowed to dry in loosely covered beakers in the greenhouse (20°-22°C) lost its infectivity after the third day of drying.

(d) Thermal inactivation point of PEMV. PEMV-2 was infective after 10 minutes exposure to 55°C but not to 60°C. In two separate experiments employing PEMV-W the thermal inactivation temperature was between 63°-65°C. No virus activity was observed after 10 minutes exposure to 68°C. The values obtained were found higher than those for PEMV-2 isolate.

Table 3. Tolerance to dilution of the PEMV isolates.

Reciprocal of dilution	PEMV-0		PEMV-W		PEMV-NY		PEMV-2	
	1	2	1	2	1	2	1	2
0	3/5*	4/5	6/6	5/7	4/5	3/6	6/7	5/6
1000	1/6	2/5	3/5	2/5	2/5	0/5	3/5	3/6
2000	0/5	1/5	2/6	1/5	1/6	0/5	2/5	1/3**
3000	0/5	0/6	0/5	0/6	0/6	0/5	1/6	0/5
4000	0/5	0/6	0/5	0/5	0/5	0/6	0/5	0/5
5000	0/5	-	0/5	-	0/5	-	0/5	-

* No. of plants infected/no. of plants inoculated

** 2 inoculated plants died 5 days after inoculation, no PEMV infection detected.

\- not determined.

Effect of storage of virus-juice extracts buffered at different pH levels on virus infectivity

The infectivity of buffered PEMV-W juice extracts was generally higher at pH 7-8.0 than at lower levels in either freshly prepared inocula or when stored *in vitro* for 12 days at 2°-3°C (Table 4). Symptoms of the virus generally appeared earlier, within 4-6 days after inoculation, in plants mechanically inoculated with fresh inocula at pH 6.5-pH 7.5. Also more plants showed infection within a week from date of inoculation. The symptoms were a faint mottle and/or translucent spots in developing leaflets, stunting and chlorosis.

A reduction in infectivity of the buffered virus extracts occurred in all pH levels throughout the aging of 12 days. Plants inoculated with 4-day old inoculum stored at pH 5.0, 5.5, or 6.0 showed no symptoms to PEMV. This also occurred with 8-day old inoculum at pH 6.3 or 6.5 and with 12-day old inoculum at pH 6.8, 7.0 and 8.0. Additional tests with some preparations confirmed the complete loss of virus activity. The buffered inoculum at pH 7.3, 7.5, and 7.8 still retained some infectivity after 12 days.

Table 4. Effect of pH of buffered-virus juice extracts on virus infectivity and its relation to inactivation *in vitro* at 2-3°C.

Aging (days)	pH of buffered-virus juice extract										
	5.0	5.5	6.0	6.3	6.5	6.8	7.0	7.3	7.5	7.8	8.0
0	4/6*	5/7	5/6	4/5	6/8	6/7	7/8	6/6	5/5	6/6	5/5
4	0/5	0/7	0/6	1/5	2/8	2/5	5/7	6/8	5/6	5/7	3/4
8	-	0/5	0/5	0/5	0/8	1/8	2/6	3/7	4/7	3/5	3/8
12	-	-	0/5	0/6	0/5	0/8	0/7	1/7	1/6	1/8	0/5

*number of plants infected / number of plants inoculated.
-not determined.

The addition of a buffer solution, pH 7-7.8, to crude virus extracts at the rate of 1:1 V/V increased the survival of PEMV more than any of the other pH levels.

Isolation, clarification and purification of PEMV

(a) Preliminary investigation. PEMV-infected leaf tissues of Pacific Freezer pea, 10-18 days after inoculation, were used in the preliminary experiments on virus extraction, clarification and purification.

Freshly harvested tissues were macerated with K_2HPO_4 powder, 3% by weight, in a mortar or in Waring blendor. The crude juice extract was expressed by squeezing the macerate through cheesecloth. The resulting extract was infectious on the assay plant.

Various means of clarification of crude juice extracts were tried (82). To determine whether the treatments removed host components and other virus contaminants comparable virus-free plant tissue extracts were subjected to similar clarification procedure.

Low speed centrifugation of the buffered crude virus extracts eliminated most of the heavy plant debris. The incorporation of the K_2HPO_4 powder to the macerated tissues effected coagulation of the chloroplastic materials and other non-infectious fractions which upon centrifugation were thrown down in the green pellet. This was similarly observed in the clarification of other plant viruses (75, 76, 77). The addition of 0.1M phosphate buffer (K_2HPO_4-KH_2PO_4), pH 7.5, to the crude juice extracts, 1:2 V/V, prior to low speed centrifugation gave similar results. The buffer facilitated the sedimentation of the host contaminants with no apparent loss of virus activity in the resulting clarified suspension.

The pH of the buffer used falls within the range of pH 7.0-8.0 reported to yield infectious virus preparations (82). Addition of phosphate buffer at lower pH levels (below pH 6.8) to the crude juice gave much clearer supernatant liquids after centrifugation but loss of virus activity occurred. The pH of the crude juice extracts from PEMV-infected pea tissues ranges from 5.6-6.2 while these of comparable virus-free tissues ranges from 6.0-6.8.

In an attempt to improve the clarification method, the buffered crude sap was subjected to high speed centrifugation in the ultra-centrifuge (Swinging-bucket Type SW-25-1 rotor) for about 5 minutes at 25,000 rpm (63,581 g). The clear slightly green supernatant was not infectious, and most, if not all, of the virus was lost to the heavy dark green pellet. This was similarly observed in the clarification of tomato bushy stunt virus (93).

The green pigments which persisted in the low speed supernatant liquid were eliminated by adsorption with activated charcoal, Merck NF-powder 18351 (16, 17). It gave an almost colorless filtrate but no demonstrable virus activity. These results were similarly obtained with crude sap extracts.

The chloroform-butanol method of virus clarification (81) resulted in a clear light brown aqueous phase but the treatment reduced the infectivity of PEMV.

Buffered crude juice extracts from frozen infected tissues were as infectious as those obtained from recently harvested tissues (Table 5). Pre-treatment of the diseased tissues by freezing and the addition of phosphate powder or buffer facilitated the maceration and extraction of the crude virus juice.

Based on the preceding results and observation, freezing the infected tissues, use of appropriate buffer and low speed centrifugation were satisfactory for the clarification of PEMV.

The density gradient centrifugation method (10, 11) was tried for the purification of PEMV. Tubes layered with sucrose gradients and with the clarified virus suspension were centrifuged for 1 to 1½ hours at 25,000 rpm in the ultracentrifuge. A light brown upper band and a whitish lower band were obtained. The healthy control preparation run at the same time yielded similar bands indistinguishable in appearance and position in the gradient from those of the PEMV-infected preparation. The lower band from the gradient layered with the virus suspension was usually infective but infectivity was quite low or sometimes lacking. The comparable band in the healthy control preparation was never infectious. Electron microscopic examination of the infectious band revealed small masses of aggregated somewhat spherical virus-like particles. Microsomes were also present.

The differential centrifugation technique produced more satisfactory results than the density gradient centrifugation method. The virus preparation obtained was invariably infectious.

(b) The purification of PEMV. Based on the results and observation in the preliminary work pea enation mosaic virus was isolated and purified from the diseased tissue extracts following the procedure outlined in Figure 1. Leaves and stipules from PEMV-infected peas, 12-14 days after inoculation, and diseased green pods were harvested and weighed. The tissues were wrapped in aluminum foil and stored in the freezer 24-36 hours. The tissues with the phosphate powder added were macerated while frozen. When completely thawed, the crude juice was expressed through 4 layers of cheesecloth. The crude juice filtrate contained masses of chloroplastic materials and other aggregated denatured host components which tended to settle upon standing. The pulp residue was moistened with additional 0.1 M phosphate buffer pH 7.5 and re-extracted. Recovery of more virus from the pulp by re-extraction with phosphate buffer (4,57) was assumed but no infectivity test was conducted on the re-extracted juice. The two extracts were pooled and clarified by centrifugation for 15 minutes at 4,500 rpm (2,590 g). A clear slightly green to light brown supernatant liquid and a heavy green pellet with a whitish layer mostly starch were observed in the centrifuge tube. The clarified juice retained most of the virus activity present in the original crude sap (Table 5).

The clarified extract was frozen for 2 hours, allowed to thaw at room temperature, then centrifuged further at

6,500 rpm (5,400 g) for 15 minutes. Freezing the clarified suspension, then thawing and subjecting to a higher centrifugal speed removed some of the colored pigments and other denatured non-infectious host proteins with no apparent harmful effect on the infectivity of the virus.

The virus in the clarified juice was sedimented in the ultracentrifuge (-5°C) at 40,000 rpm (96,592 g) for 1 hour. Infectious pellets, light brown and somewhat glassy in appearance, were obtained. The non-infectious supernatant liquids were discarded and the pellets were then covered with 2 ml of buffer (0.01 M KCl, 0.005 M K_2HPO_4, 0.0005 M KH_2PO_4; pH 7.5) and allowed to resuspend overnight at 2-3°C. The resuspended pellets were pooled and then centrifuged for 15 minutes at 3,100 rpm (1,330 g). The clarified virus suspension was further subjected to two more cycles of differential centrifugation. Each of these consisted of a 1-hour high speed centrifugation (40,000 rpm), resuspension of the virus in buffer solution, then low speed centrifugation for 15 minutes at 3,100 rpm. A colorless gelatenous pellet was obtained from the final high speed centrifugation. The buffer resuspension of the virus pellet was infectious (Table 5).

Extracts from comparable healthy control plant tissues subjected to the same purification procedure sometimes gave variable results. Often no distinguishable pellets were observed from the second high speed centrifugation. In some trials, however, small amounts persisted

throughout the preliminary purification. Nevertheless, no infectivity was associated with the preparation. The final ultracentrifugation of the clarified healthy suspension invariably produced no pellet at all.

Purified virus suspension in glass distilled water was inactivated when stored frozen overnight. Virus inactivation during storage was minimized by using buffer solution pH 7.5 as the suspending medium. Freezing of purified virus aqueous suspension was reported deleterious to tobacco ringspot virus (75) and to bushy stunt virus (5).

Differential centrifugation technique has been reported satisfactory in the purification of the following plant viruses: cucumber mosaic 3 and 4 (33, 66, 82); potato yellow dwarf virus (8); potato virus X (38); southern bean mosaic (57); squash mosaic (35, 60); tobacco mosaic and strains (82); tobacco necrosis (6, 58); tobacco ringspot (75); tomato bushy stunt (76, 82); turnip yellow mosaic (15); and wild cucumber mosaic virus (70).

Serological properties of PEMV

(a) Microprecipitin reaction. In preliminary serological studies the antiserum was obtained from rabbits immunized with clarified virus extracts or with preparations obtained after one cycle of differential centrifugation. Results indicated the presence of antigenic host components which interfered in the detection and identification of a specific virus-antiserum reaction. Therefore the virus

Table 5. Relative infectivities of PEMV fractions obtained from three purification trials.

Stage of Purification	Fraction	Infectivity
2	Buffered crude juice extract, fresh infected tissue	12/15*
2	Buffered crude juice extract, frozen infected tissue	11/13
3	Clarified juice, first low-speed centrifugation	7/11
4	Clarified juice, second low-speed centrifugation	8/15
5	Supernatant of virus pellet, I	0/9
6	Resuspended virus pellet I in distilled water, clarified	1/10
6	Resuspended virus pellet I in buffer, clarified	9/14
8	Resuspended virus pellet II in buffer, clarified	7/13
10	Resuspended virus pellet III in buffer, clarified	6/15

*Total No. of plants infected / total no. of plants inoculated

antigen used for the immunization of the experimental animals was further purified and concentrated. The purified PEMV-W preparation, which consisted of more or less homogeneous spherical particles in the electron microscope and containing viral activity, was satisfactory for PEMV antiserum production in rabbits.

Figure 1. Purification procedure of pea enation mosaic virus (PEMV).

1. Infected tissue (pea, Pacific Freezer)

   freeze
   grind while frozen
   K_2HPO_4, 3% by weight
   thaw
   extract juice through cheesecloth

2. Crude juice ----------Pulp

   clarify - 4,500 rpm, 15 min.

3. Clarified juice ----------pellet*

   freeze, 2 hrs.
   thaw
   clarify - 6500 rpm, 15 min.

   PO_4 buffer pH 7.5
   regrind
   re-extract

   Crude juice - pulp*

4. Clarified juice ----------pellet*

   centrifuge - 40,000 rpm, 1 hr.

5. Pellet ----------supernatant liquid*

   resuspend in buffer pH 7.5 overnight in refrigerator
   shake tube gently
   clarify - 3100 rpm, 15 min.

6. Clarified suspension --------pellet*

   Centrifuge - 40,000 rpm, 1 hr.

7. Pellet ----------supernatant liquid*

   resuspend in buffer pH 7.5, 2-3 hrs.
   shake tube gently
   clarify - 3100 rpm, 15 min.

8. Clarified suspension ------pellet*

   centrifuge - 40,000 rpm, 1 hr.

9. Pellet ----------supernatant liquid*

   resuspend in buffer pH 7.5 or in glass distilled water - 2 hrs.
   clarify - 3100 rpm, 15 min.

10. Purified virus suspension - pellet*

*discard
PO_4 buffer (0.1M K_2HPO_4-KH_2PO_4) pH 7.5
Buffer pH 7.5 (0.01M KCl, 0.005M K_2HPO_4, 0.0005M KH_2PO_4

This antiserum gave a positive precipitin reaction when reacted with a similar preparation of PEMV. The characteristic precipitin reaction was indicated by a slightly fluffy white precipitate in the microdrop of virus-antiserum mixture. Formation of the precipitate at lower dilutions of the antigen was observed within two hours of incubation, at room temperature. When allowed to react further by incubating the titration dish in the refrigerator overnight, a virus titre of 1/1024 was observed (Table 6). The controls of the antigen and the antiserum, mixed with saline solution in neutral 0.01M phosphate buffer, showed no precipitation. In the microprecipitin test between the antiserum and a similar healthy preparation a non-specific reaction was observed after overnight incubation of the reaction dish. No characteristic precipitin reaction formed at any dilution of the healthy antigen and the PEMV antiserum (Table 7).

These results demonstrated the specificity of the antiserum to PEMV and also the presence of some antigenic host components in the antiserum preparation, but the non-specific and virus-specific precipitation reaction with the antiserum can easily be distinguished.

Tests were conducted with the normal serum of the rabbit and clarified extracts of PEMV-infected and non-infected leaves of Pacific Freezer pea. The crude juice

Table 6. Microprecipitin test between the purified PEMV-W preparation and its antiserum

Reciprocal of antiserum dilution	Reciprocal of antigen dilution									
	4	8	16	32	64	128	256	512	1024	AsC*
4	4+	4+	3+	3+	3+	2+	2+	+	+	-
8	4+	4+	3+	3+	2+	2+	+	+	+	-
16	3+	3+	2+	2+	2+	+	+	+	+	-
32	3+	2+	2+	+	+	+	+	-	-	-
AgC**	-	-	-	-	-	-	-	-	-	-

* Antiserum control
** Antigen control
\- No reaction
\+ Relative intensity precipitin reaction

Table 7. Microprecipitin test between PEMV-W antiserum and purified healthy control preparation.

Reciprocal of antiserum dilution	Reciprocal of antigen dilution									
	4	8	16	32	64	128	256	512	1024	AsC*
4	NS	NS	NS	NS	NS	NS	-	-	-	-
8	NS	NS	NS	NS	NS	-	-	-	-	-
16	NS	NS	NS	-	-	-	-	-	-	-
32	NS	NS	-	-	-	-	-	-	-	-
AgC**	-	-	-	-	-	-	-	-	-	-

* Antiserum control
** Antigen control
\- No reaction
NS Non-specific precipitation

obtained from the frozen tissue was diluted to 1/4 with neutral phosphate buffer and clarified by centrifugation for 15 minutes at 4,500 rpm. Neither clarified extracts from PEMV-infected nor healthy control leaves showed a specific precipitin reaction. A slightly milky greenish suspension was observed in first dilution but beyond which no precipitate was observed (Table 8).

Crude extracts of PEMV-W and PEMV-O were centrifuged for 5 minutes at 8,000 g in a Coleman Microcentrifuge (Cat. No. 6-810) to remove most of the cellular debris. A positive precipitin reaction was observed between the antiserum and the clarified PEMV-infected extracts. Preparations from healthy control plants gave no reaction (Table 9). This shows that the antiserum could be used to detect the presence of the virus in clarified extracts from diseased tissues.

Clarified extracts of other plant viruses were allowed to react with the antiserum to determine their serological relationship to PEMV. The following viruses were obtained from leaves of infected source plants: two strains of cucumber mosaic virus (CMV-2 and CMV-9) from tobacco, Nicotiana tabacum L. xanthi nc; a strain of southern bean mosaic virus (SBMV 15-2) from cowpea, Vigna sinensis L.; four strains of bean yellow mosaic virus (BYMV 61-35), (BYMV 61-36) pod distortion strain, (BYMV-81928), and (MBV-2) Maryland bean virus all of which were harvested from infected broad beans, Vicia faba L. var. minor, McWhorter strain;

Table 8. Microprecipitin test between normal rabbit serum and clarified extracts from PEMV-W infected and healthy control Pacific Freezer leaves.

Antigen dilution	Reciprocal of normal serum dilution									
1/4	4	8	16	32	64	128	256	512	1024	AgC*
Healthy preparation	NS	-	-	-	-	-	-	-	-	-
PEMV-W preparation	NS	-	-	-	-	-	-	-	-	-
AsC**	-	-	-	-	-	-	-	-	-	-

* Antigen control
** Antiserum control
NS Non-specific reaction

Table 9. Microprecipitin test between PEMV-W antiserum and clarified extracts from PEMV-infected and healthy control Pacific Freezer leaves.

Antigen dilution	Reciprocal of antiserum dilution									
1/4	4	8	16	32	64	128	256	512	1024	AgC*
Healthy preparation	NS	NS	NS	-	-	-	-	-	-	-
PEMV-W	3+	3+	2+	2+	2+	+	+	+	-	-
PEMV-O	3+	2+	2+	2+	+	+	±	-	-	-
AsC**	-	-	-	-	-	-	-	-	-	-

* Antigen control
** Antiserum control
NS Non-specific reaction
\+ Relative intensity of precipitin reaction

and three strains of tobacco ringspot virus (TRSV 60-21), (TRSV 60-22), and (TRSV-T) American type culture isolate AC-98 harvested from diseased tobacco, N. tabacum L. xanthi nc., leaves. The CMV strains were obtained from Dr. D. deZeeuw, the SBMV from Dr. H. H. Murakishi, and the rest of the legume viruses from Dr. A. Andersen, Department of Botany and Plant Pathology, Michigan State University.

Crude virus extracts were obtained from the infected source plants and clarified by centrifugation for 5 minutes at 8,000 g. Pre-chilled neutral phosphate buffer was used to form a serial twofold dilution of each clarified virus preparation. An antiserum dilution of 1/25 was made to react with the various virus dilutions. A similar preparation of PEMV-W and healthy control from P. Freezer pea was included in the test.

After 2 hours incubation at room temperature specific precipitation reaction was observed in the PEMV-W antigen-antiserum drops at lower dilutions. No reaction was noted at any dilution in the other viruses. Table 10 shows the results of the test after incubation overnight in the refrigerator. Only the clarified PEMV-W preparation gave a positive reaction to the antiserum. The CMV and TRSV strains showed no reaction to the antiserum while the SBMV, MBV, and BYMV strains exhibited trace amounts of non-specific precipitation reaction which resembled that of the healthy control preparation. The presence of normal host proteins in the

clarified preparations of SBMV, MBV, and BYMV strains having similar antigenic sites as those in the healthy control may account for the trace reactions.

Table 10. Microprecipitin reaction between the PEMV-W antiserum* and the clarified preparations of CMV, SBMV, MBV, BYMV and TRSV strains.

Antigen sample	Reciprocal of antigen dilution									
	4	8	16	32	64	128	256	512	1024	AsC**
PEMV-W	4+	3+	3+	2+	2+	+	+	+	-	-
CMV-2	-	-	-	-	-	-	-	-	-	-
CMV-9	-	-	-	-	-	-	-	-	-	-
SBMV-15-2	NS	-	-	-	-	-	-	-	-	-
MBV-2	NS	NS	-	-	-	-	-	-	-	-
BYMV-61-35	NS	NS	NS	-	-	-	-	-	-	-
BYMV-61-36	NS	NS	-	-	-	-	-	-	-	-
BYMV-81928	NS	NS	NS	-	-	-	-	-	-	-
TRSV-60-21	-	-	-	-	-	-	-	-	-	-
TRSV-60-22	-	-	-	-	-	-	-	-	-	-
TRSV-T	-	-	-	-	-	-	-	-	-	-
Healthy extract P. Freezer	NS	NS	NS	NS	-	-	-	-	-	-

* 1/25 dilution
** Antiserum control
NS Non-specific precipitation
- No reaction
+ Intensity of precipitin reaction

The CMV and TRSV strains which were obtained from a taxonomically different host plant from the other viruses gave no precipitation reaction to the antiserum. Results of the microprecipitin tests demonstrated PEMV as an individual virus entity and serologically unrelated to CMV, MBV, SBMV, and TRSV.

(b) Reaction of PEMV isolates and other plant viruses to the antiserum in agar gel diffusion test. Precipitation reaction bands were observed when the purified PEMV isolates and similar preparation of healthy control were allowed to react with the homologeous antiserum in agar double-diffusion wells (Figure 2). These bands, formed in the region of optimal proportion, were noticed 8-10 days after the reaction dishes were incubated in the moist chamber. All of the PEMV isolates produced two types of precipitation bands, a more or less straight and a slightly curved one, in the reaction area. The straight reaction bands, formed near the antiserum well, were usually observed earlier than the other. The reaction area between the healthy control and the antiserum showed only one precipitation band. It was similar in shape and position to those straight bands observed in the PEMV isolates. These bands were considered to represent a non-specific reaction between the normal host proteins and the antiserum. This type of reaction was also observed in the microprecipitin test.

The succeeding bands, slightly curved towards the antigen wells and differing slightly in intensity, were believed to represent a specific precipitin reaction between each of the PEMV isolates and the homologous antiserum. This type of reaction band was absent in healthy control preparation. These results were observed in reaction dishes containing ion agar treated with either merthiolate or sodium azide solutions as preservative. In the control reaction dish with no preservative added, similar results were indicated although the observation of the precipitation bands were at times obscured by bacterial contamination which appeared within a week of incubation in the moist chamber.

Results of the Ouchterlony agar double-diffusion test showed close relationship of PEMV-W with the other PEMV isolates and also confirmed those observed from the microprecipitin test. The agar gel technique was found to be a more sensitive test for the specificity of the antiserum and the homogeneity of the virus antigen preparation (18).

No precipitation bands were observed between the reservoirs of PEMV antiserum and the clarified extracts of CMV, SBMV, BYMV, and TRSV strains when sodium azide was in the agar (Figure 3). A clarified extract of PEMV-W showed two precipitation bands and one band in the healthy control. These results confirmed the microprecipitin test in showing no cross-reaction between the PEMV antiserum and the 10 other viruses tested. Extracts containing MBV and BYMV

strains obtained from broad beans tended to darken during incubation at room temperature.

A somewhat different result was obtained in the reaction dish treated with merthiolate solution. Clarified extracts of MBV, SBMV, CMV, and BYMV strains exhibited single reaction bands to PEMV antiserum similar to those of the healthy control (Figure 4). The CMV strains produced faint precipitation bands while those of MBV, SBMV, and BYMV strains were more distinct. The TRSV strains did not react with the PEMV antiserum.

Electron microscopy of PEMV

Attempts were made to examine PEMV-containing sap with the electron microscope. The exudate method (27); dip method (12, 13); and the spray technique (22) were tried with no positive results in the identification of likely virus particles.

Results of the electron microscopy of shadowed purified PEMV preparation showed that in gross appearance the PEMV particles were nearly spherical in shape as evidenced by approximately circular outline and their symmetrical shadows. A purified PEMV-W preparation in distilled water contained essentially monodispersed spherical particles of uniform size and morphology and generally free of microsomal contamination (Figure 5). A similar preparation from a phosphate buffer resuspension showed some kind of aggregation (Figure 6). The electron micrograph also disclosed the

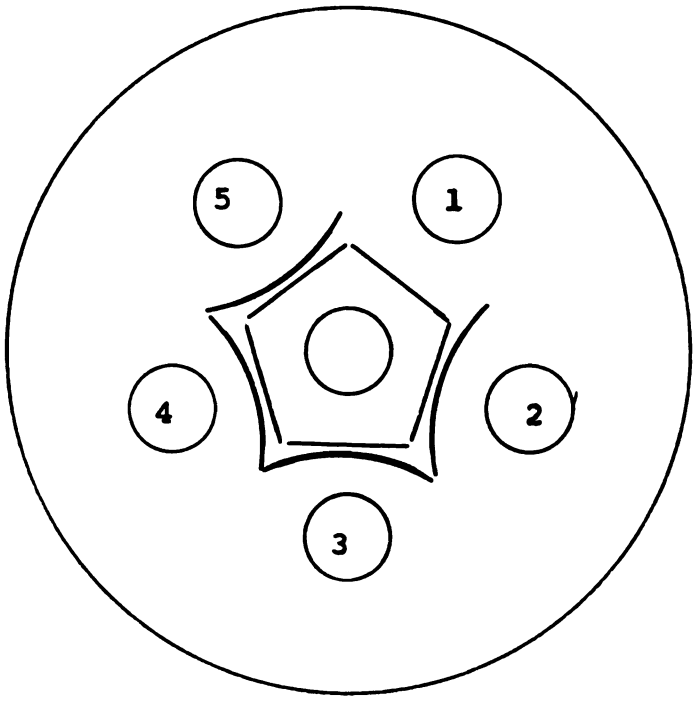

Figure 2. Precipitin reaction band patterns obtained in the Ouchterlony agar double-diffusion test between the PEMV-W antiserum and the purified preparations of healthy control and PEMV isolates. No. (1) healthy control, (2) PEMV-0, (3) PEMV-NY, (4) PEMV-W, (5) PEMV-2, center well-PEMV-antiserum. Curved precipitin lines are caused by the virus antigens and the straight lines by the antigenic host proteins. This was observed in a preparation of 0.5% ion agar with sodium azide (1:5000) preservative incubated for 12 days in a moist chamber.

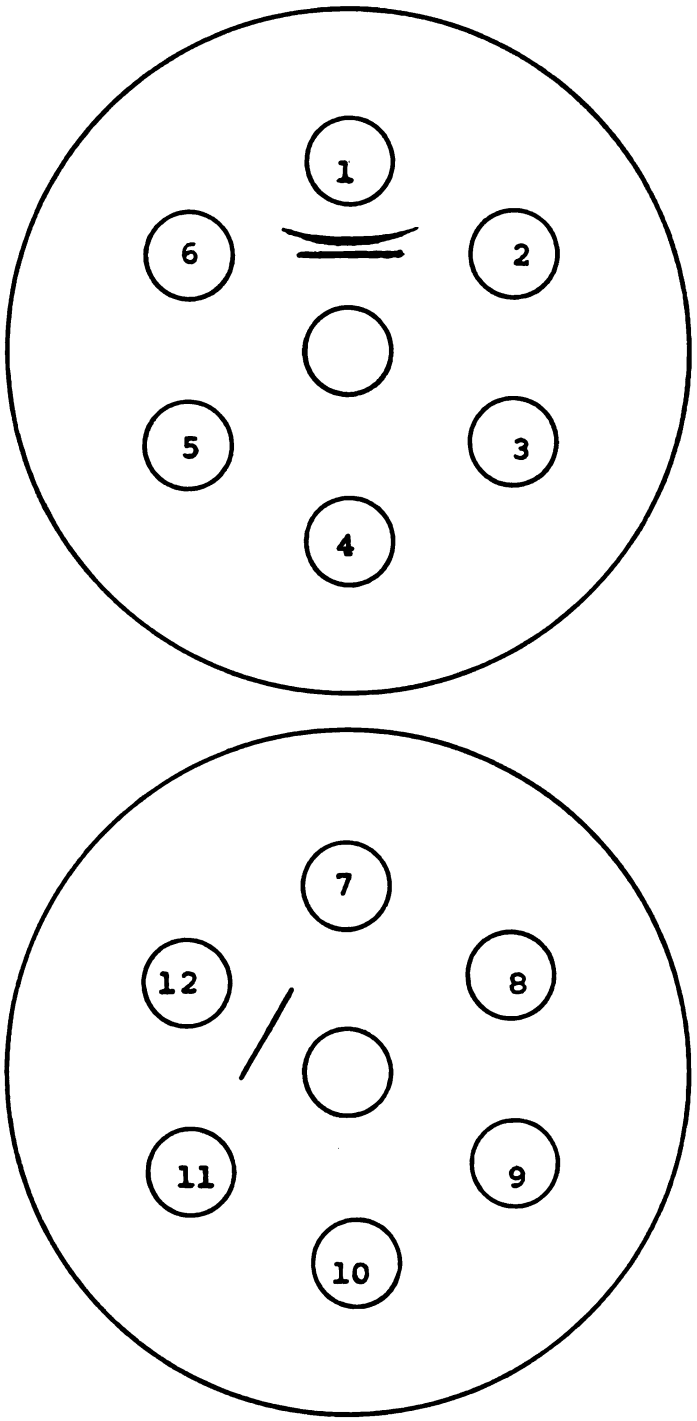

Figure 3. Ouchterlony agar double-diffusion test between the PEMV-W antiserum and other plant viruses observed in ion agar (0.5%) preparation with sodium azide (1:5000) preservative, 14 days incubation. Center wells contained the antiserum and the side wells (1) PEMV-W, (2) CMV-2, (3) CMV-9, (4) SBMV 15-2, (5) MBV-2, (6) BYMV 61-35, (7) BYMV 61-36, (8) BYMV 81928, (9) TRSV 60-21, (10) TRSV 60-22, (11) TRSV-T, and (12) Healthy control preparation from P. Freezer. Note that no precipitation bands are formed with the other viruses (Nos. 2-11).

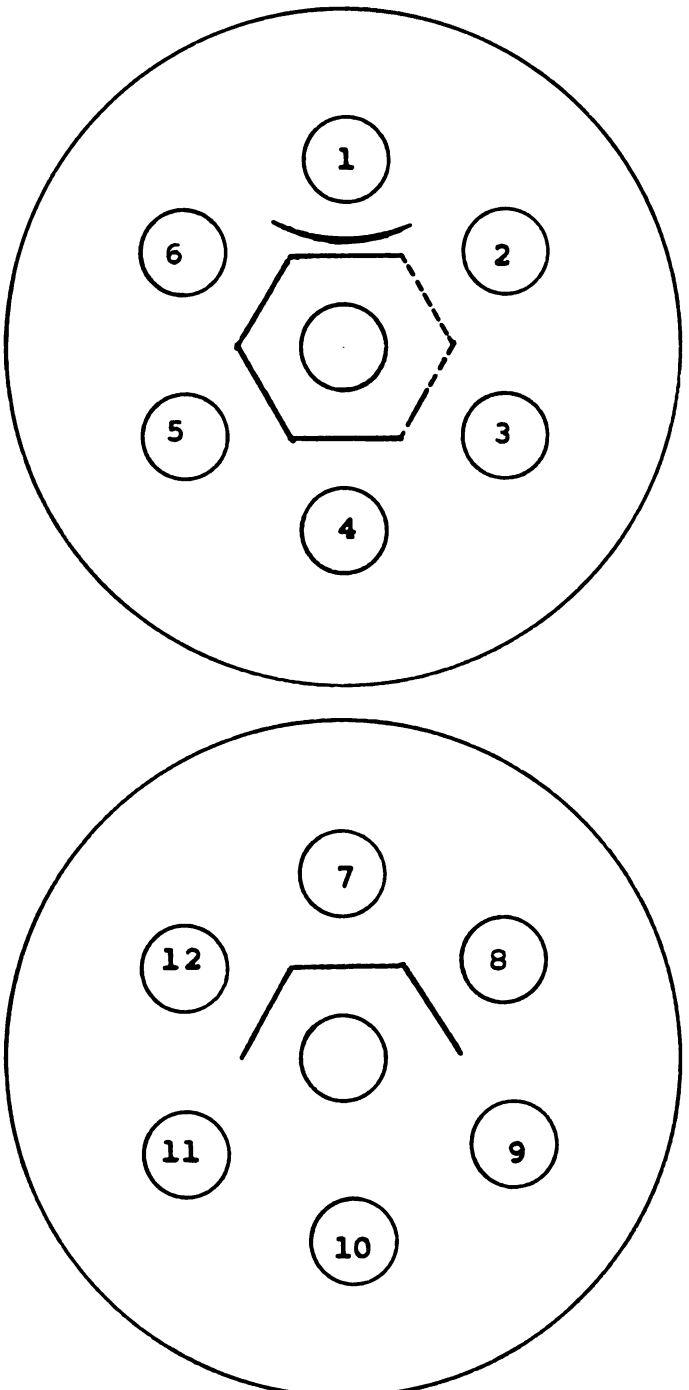

Figure 4. Ouchterlony agar double-diffusion test between the PEMV-W antiserum and other plant viruses observed in ion agar (0.5%) preparation with merthiolate (1:10,000) solution preservative, 14 days incubation. Center wells contain the antiserum and the side wells (1) PEMV-W, (2) CMV-2, (3) CMV-9, (4) SBMV 15-2, (5) MBV-2, (6) BYMV 61-35, (7) BYMV 61-36, (8) BYMV 81928, (9) TRSV 60-21, (10) TRSV 60-22, (11) TRSV-T, and (12) Healthy control preparation from P. Freezer. No precipitation bands are formed with the TRSV strains and the antiserum.

presence of a few smaller particulates of less uniform morphology and size. In general, the purification procedure developed for PEMV eliminated most if not all of the host contaminants. No comparable virus-like particles were observed in similar preparations of the healthy control (Figure 7 and 8). Occasional bits of small particles and aggregates, few in number could be observed. Although some of these particles were spherical, their sizes were less than 12 mμ. The existence of some host components of similar morphology but smaller in size corroborated the results obtained from the serological studies. These contaminants were believed to be normal host proteins (32). Healthy samples were generally free of these particles.

Numerous spherical particles were observed on a partially purified PEMV-W preparation (Figure 9). Aggregations of the virus particles are evident in this preparation. Individual virus particles could be observed along with some host contaminants in the preparation.

Earlier microscopic studies of PEMV-2 isolate prepared by the same purification procedure revealed the presence of two types of characteristic particles, spherical and rod-shaped ones (Figure 10). The former were of the same size and morphology as those observed in the PEMV-W preparations and therefore are believed to be the PEMV-2 particles. Obviously, the rod-shaped virus particles were contaminations. This preparation was infective, giving rise

to disease symptoms of PEMV. In some instances, however, leaf spotting was observed to be masked with milder type of mottling or mosaic on the leaves.

Another electron micrograph of PEMV-2 purified preparation shows long thread-like particles interspersed with the spherical PEMV particles (Figure 11). This purified preparation was obtained from frozen extracts of infected green pods of pea plants showing marked mottling of the leaves and small enations on the stipules. None of the rod-shaped particles were observed on the healthy control preparations.

PEMV particles, as compared with polystyrene spheres (264 mμ $\pm$ 6 mμ), had an average diameter of about 20 mμ.

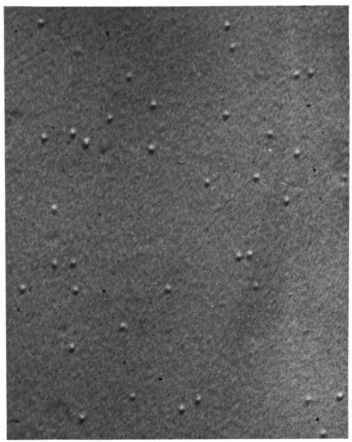

Figure 5. Electron micrograph of a purified PEMV-W from a distilled water suspension after the third ultra-centrifugation. The preparation was sprayed on formvar film coated grid and shadowed with tungsten (approximately X 57,000).

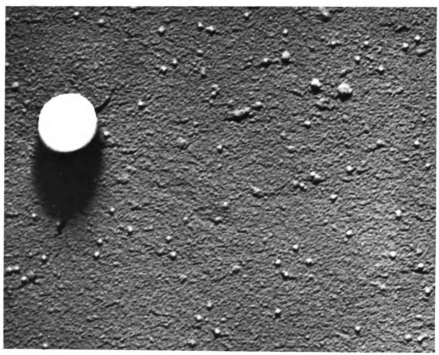

Figure 6. Electron micrograph of purified preparation from PEMV-W infected pea tissues. Air-dried from a droplet of virus suspension in phosphate buffer pH 7.5 on formvar film coated grid, shadowed with tungsten and examined in the electron microscope (RCS-EMU 2C). Large white sphere is a polystyrene latex particle which has a diameter of 264 mu $\pm$ 6 mu. (X 56,844).

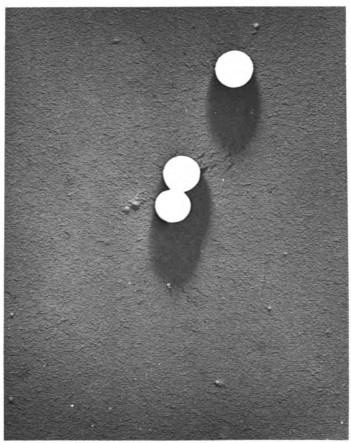

Figure 7. Electron micrograph of a purified preparation from healthy pea tissues, 3-cycles of differential centrifugation, obtained from the resuspended persistent pellet. Preparation in distilled water suspension, sprayed, air-dried and shadowed with tungsten. Polystyrene latex particles, 264 mu $\pm$ 6 mu (X 37,878).

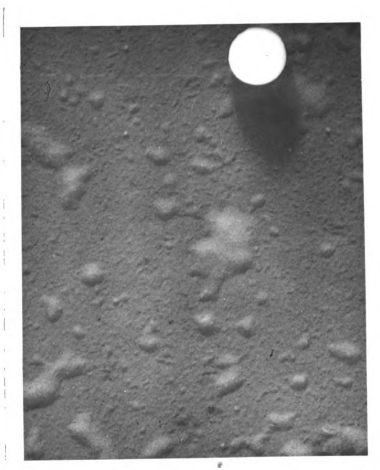

Figure 8. Electron micrograph of purified preparation from healthy pea tissues, 3-cycles of differential centrifugation. Preparation in phosphate buffer pH 7.5 suspension, sprayed, air-dried and shadowed with tungsten. Polystyrene latex particle, 264 mμ ± 6 mμ in diameter. (X 56,844)

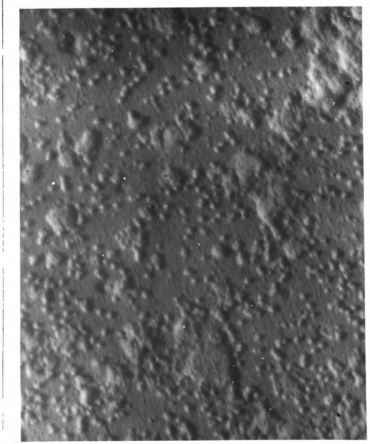

Figure 9. Electron micrograph of partially purified PEMV-W preparation resuspended in phosphate buffer pH 7.5 obtained from the second ultracentrifugation. An air-dried preparation and shadowed with tungsten (X 56,844).

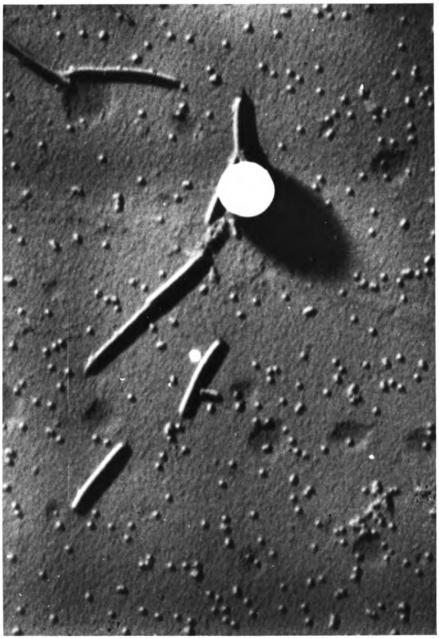

Figure 10. Electron micrograph of purified preparation from PEMV-2 infected pea leaves, 3 cycles of differential centrifugation, in distilled water suspension. Note the presence of some rod-shaped virus particles found a contaminant of the isolate, small spheres are PEMV particles. Polystyrene latex particle, 264 mu ± 6 mu in diameter, shadowed with tungsten. (X 56,844)

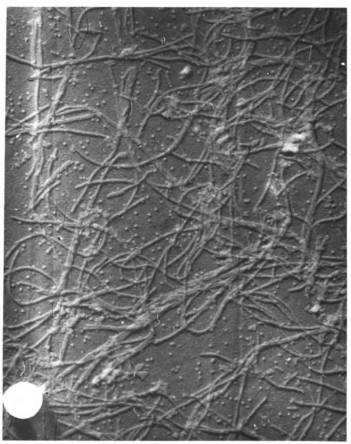

Figure 11. Electron micrograph of purified PEMV-2 infected pod preparation showing the presence of thread-like virus contaminant. Preparation was obtained after the third cycle of ultracentrifugation and resuspended in phosphate buffer pH 7.0, deposited as a microdrop on formvar film coated grid and shadowed with tungsten. Polystyrene latex particle has a diameter of 264 mu $\pm$ 6 mu (X 37,878).

DISCUSSION

The apparent limited range of PEMV infectivity to leguminous plant species (24, 42, 69) was further demonstrated in this study. Most alfalfa varieties were reported symptomless carriers of the virus (42) and this may be the reason why several investigators did not observe PEMV infection on the host plant. Results of the study on the susceptibility of alfalfa to virus inoculation agree with findings of other workers (42, 61). Although negative transmission of PEMV to broad bean has been reported (14, 69) most investigators agree on its susceptibility to the virus. This was also found true in the two varieties tested.

Induced virus infection observed on cowpea corroborated the observation made by Hagedorn and Walker (24). This plant has been reported to be not susceptible to PEMV (83). The susceptibility of subterranean clover confirmed earlier observations (24, 61). Chaudhuri (14) was not able to transmit the virus to this host plant. PEMV failed to infect red clover, white clover, lima bean, and beans variety Prince, Michelite and Pinto. Hagedorn and Walker (24) transmitted the virus to red and white clover while McEwen and Schroeder (42) found infection only on the latter. French bean variety Prince has been reported a non-host plant to the virus (14). Lima bean, Pinto and Michelite were not tried in host range studies by earlier investigators.

They, however, reported other bean varieties susceptible to PEMV.

The virus was not able to infect white lupine which is in agreement with the report by Stubbs (83). This was not found true by other investigators (23, 24).

All solanaceous plant species employed in this study were not susceptible to PEMV. Earlier attempts to infect tomato and varieties of Nicotiana tabacum L. and also of N. glutinosa L. were found unsuccessful (14, 24, 83). Cucumber has been reported not susceptible to PEMV infection (51, 69) and this was also true in this study. Plant species tested belonging to the family Compositae, Amaranthaceae, and Chenopodiaceae were observed non-host plants of PEMV. Except for sugar beets, Beta vulgaris L., of family Chenopodiaceae which has been reported not susceptible to PEMV (69), all the species of the three families mentioned earlier have apparently not been investigated prior to this work.

Discrepancies in the results obtained from host range studies of PEMV by several investigators may be attributed to several factors such as: low initial virus concentration in the inoculum, method of transmission employed, and age of the test plant or tissues inoculated.

No distinct difference could be detected between the four PEMV isolates as far as varietal reaction or symptom expression on the inoculated peas. It seemed however, that

the infected pea varieties and introductions showed severe symptoms to PEMV-0 infection similarly reported by Ruppel and Hagedorn (61). In some cases, however, the infected plants were less adversely affected compared to those of the other virus isolates. The reaction of the pea introduction from Iran (PI-140295) to PEMV infection resembled that of the resistant "Geneva" line, G-31 (86).

Tolerance to dilution of the PEMV isolates seemed to be influenced by the initial concentration of the virus in the infected sap extracts as shown by the variable dilution end points from the two trials of the same isolate. The samples used in the two separate trials were obtained from comparable infected peas but each sample came from a different batch of inoculated Pacific Freezer peas. A dilution between 1:2000 and 1:3000 was considered as the end point since most of the isolates lost its infectivity between this range. The value obtained agree with an earlier report (83). Inactivation of PEMV <u>in vitro</u> (20^{o}-22^{o}C) after 4 days was similarly observed by other investigators (53, 83). The longevity of PEMV in dried diseased leaf tissues stored at 20^{o}-22^{o}C for three days was not so great compared with other legume viruses. No report has been made other than this study. PEMV can tolerate heat inactivation to 55^{o}C (PEMV-2) and 63^{o}-65^{o}C (PEMV-W). The values obtained are within the range reported in earlier studies (1, 28, 53, 54).

The beneficial effect of phosphate buffers of pH 7-7.8 on PEMV activity in crude sap extracts stored 2^{o}-3^{o}C may have been to its neutralizing action on the inactivating substances present. Furthermore, at these pH levels the virus may be above its isoelectric point so that virus aggregation and precipitation in the sap was at minimum. Purified PEMV was more stable when resuspended in a weaker ionic concentration of buffer at elevated pH levels.

The non-specific reaction observed from similar preparations of PEMV-infected and healthy control demonstrated that an antigenic component of pea leaves cannot be separated completely from the virus by differential centrifugation alone. The difficulty of obtaining virus preparations free from an antigenic host component, identified as Fraction 1 protein, by ultracentrifugation technique has recently been reported (88). Non-specific reaction of SBMV, MBV, and BYMV strains with the PEMV antiserum in the microprecipitin test may indicate the presence of a common antigenic host protein (47, 67, 88, 90) with the Pacific Freezer pea. The CMV and TRSV strains which were extracted from tobacco host plants revealed no reaction to the specific PEMV antiserum. This observation may explain why antigenic host components are unlikely to be detected in serological cross-reaction with viruses prepared from unrelated hosts (40).

The Ouchterlony agar double-diffusion test disclosed two antigenic groups, indicated by a straight and a curved precipitation band, from the purified PEMV-infected preparation. The straight precipitation band, also formed in similar healthy control preparation and regarded as non-specific precipitin reaction, may represent residual smaller plant antigens (91). These host contaminants present as low as 1 percent in a purified virus preparation that appear homogenous in the electron microscope may still be revealed by the agar gel diffusion technique (47, 89). Results of the serological tests shows the possibility of ribosomal contamination possessing an antigenic property of the host plant in the apparently purified PEMV preparation.

The slightly curved precipitation band, interpreted as a specific virus antigen-antiserum reaction, was observed in all of the PEMV isolates. The precipitation band curves away from the antibody reservoir in the agar gel suggesting that the PEMV antigen has a heavier molecular weight than its antibody (34).

Precipitation bands were exhibited between the PEMV antiserum and CMV, MBV, SBMV, BYMV strains in the agar gel preserved in merthiolate. These bands were not of the characteristic type, i.e., curved band, so they were not the resultant serological reactions of each of the viruses with the prepared antiserum (89, 90). Furthermore, these precipitation bands may suggest a false type of precipitation

(95) since they were not observed in the sodium azide treated agar gel preparation.

Results of the serological studies show that the four PEMV isolates were closely related. The serological tests, however, reveal no information whether each isolate can be regarded as an individual strain (41).

The electron micrographs of purified PEMV preparation suggest the presence of characteristic virus particles with definite morphology and size. Although the majority of these particles appeared to be more like spheres than did the polyhedral-shaped tobacco ringspot virus (79, 81) slight irregularities in the outline of some particles could be observed. A pointed and flat-topped shadow and several intermediate symmetrical forms are suggested in some of them in the micrograph. Shadows of these kinds are formed only by angular objects but the image of the PEMV particles in the micrographs are not clear enough to permit an unequivocal inference. According to Kaesberg (30) the so called "spherical" viruses invariably assume hexagonal contours in clean, lightly-shadowed preparations.

In comparison with the size of other spherical plant viruses, PEMV is similar to tobacco necrosis, 20 mμ (72) and falls within the reported size range of tobacco ringspot virus, 13-26 mμ (7, 20, 71, 75, 79, 81, 85).

Ribosomes and other normal plant proteins were demonstrated to have a similar morphology and were about the

size of small spherical viruses (32, 56). The work reported herein showed that these components are smaller than PEMV.

SUMMARY AND CONCLUSIONS

The host range, physico-chemical properties, purification, serological properties and electron microscopy of pea enation mosaic virus (PEMV) were studied.

PEMV was limited in infectivity to leguminous plants and attempts to infect plant species of five other families failed. No suitable local lesion host for the virus could be found. Mung bean (Phaseolus aureus Roxb.) was found to be a new host of PEMV. All 55 pea introductions were experimentally infected but no distinct difference in their reaction to PEMV isolates were observed. A pea introduction from Iran (PI-140295) showed some tolerance to virus infection.

The tolerance of PEMV to dilution was fairly low (1:2000-1:3000) as compared to most other spherical plant viruses. It was slightly infectious when exposed to thermal inactivation at 65°C for 10 minutes but not at 68°C. It resisted inactivation in vitro for 4 days and was inactivated in air-dried infected tissues after the third day. Storage of infected crude sap extracts buffered at pH 7-7.8 in the refrigerator (2°-3°C) maintained the infectivity of the virus to about 12 days.

PEMV-W was concentrated and purified from buffered extracts of frozen infected tissues of garden pea (Pisum sativum L.) var. Pacific Freezer. Purification consisted

essentially of clarifying the extracts by low speed centrifugation, and concentration by 3 cycles of alternate high and low-speed centrifugation of appropriately buffered virus suspension. The resulting purified virus preparation was infectious and stimulated antibody production in the rabbit. Electron microscopic examination of shadowed air-dried PEMV suspension revealed monodispersed apparently homogenous and nearly spherical particles with an average diameter of about 20 mμ. Such particles were not observed in similar preparations from healthy control.

Microprecipitin and Ouchterlony agar double-diffusion tests showed that the virus preparation was antigenic and gave a positive precipitin reaction to its homologous antibody. Based on serological cross-reaction, PEMV-W isolate was demonstrated closely related to PEMV-O, PEMV-NY, and PEMV-2, but not to CMV nor to any of the following legume viruses: SBMV, MBV, BYMV, TRSV.

Some antigenic non-infectious host protein, possibly ribosomes, were detected in the purified PEMV preparation by the Ouchterlony technique. These contaminants are similar in morphology but smaller (12 mμ) than the PEMV particles. There was no difficulty in identifying the specific virus antigen-antibody reaction from the non-specific precipitation because the former produced a curved precipitation band while the latter formed a straight band.

Pea enation mosaic virus in extracts clarified by one low speed centrifugation could be detected and identified serologically employing the antiserum prepared from partially purified PEMV-W.

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